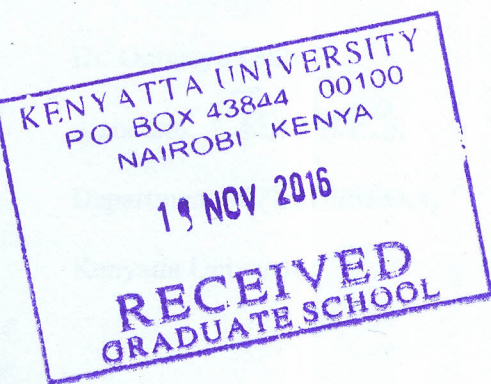


**SYMBIOTIC EFFICIENCY AND DIVERSITY OF NATIVE RHIZOBIA
ISOLATED FROM CLIMBING BEANS (*Phaseolus vulgaris* L.) IN EMBU AND
THARAKA NITHI COUNTIES, KENYA**

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I56/26830/2013**

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



NOVEMBER, 2016

DECLARATION

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

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Date ... 17/11/2016

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DEDICATION

I dedicate this work to my late grandfather Mr. Alexander Turieny, my parents Andrew Keter and Sarah Keter and my siblings Daisy Keter, Erick Koskey, Ronald Langat, Mercy Keter and Davies Langat who have been supportive all through my educational life.

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ABBREVIATIONS AND ACRONYMS

AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
ARDRA	Amplified rDNA Restriction Analysis
ASL	Above Sea Level
BNF	Biological Nitrogen Fixation
BTB	Bromothymol Blue
CR	Congo Red
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
GDP	Gross Domestic Product
HSD	Honestly Significant Difference
ITS	Internally Transcribed Spacer Genes
MAC	Mid-Altitude Climbers
PCA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen Ions
SE	Symbiotic Efficiency
SSA	Sub-Saharan Africa
TBE	Tris-Borate-EDTA
YEMA	Yeast Extract Mannitol Agar

ABSTRACT

Kenya is the eighth highest producer of common beans (*Phaseolus vulgaris* L.) worldwide with a production of 529,265 tons annually. Climbing beans are highly productive, and can produce yields 2 to 3 times higher than bush beans and is therefore a promising option to increase bean yields in Kenya. However, climbing bean production is greatly affected by the decline in soil fertility, particularly low nitrogen levels in the soil. Replenishment of soil nitrogen using effective rhizobia inoculants presents a sustainable solution to climbing bean production. The objectives of this study were to assess climbing bean varieties grown by smallholder farmers in Eastern Kenya, to determine the genetic diversity of native rhizobia strains that nodulate climbing beans and to determine nodulation and symbiotic nitrogen fixation efficiencies of native rhizobia strains isolated from climbing beans grown in Eastern Kenya. Questionnaires were used to obtain information on climbing bean varieties grown in Eastern Kenya. The experiments involved laboratory analysis, greenhouse and on farm activities. A complete randomized design and a randomized complete block design were used in the greenhouse and field experiments respectively. Five climbing bean varieties were identified in Embu and Tharaka Nithi Counties of Eastern Kenya; Gatune, Raila, Mama safi, Kithiga and Muviki, of which Gatune was the most preferred variety. In regards to bean diversity, Embu Lower Midland zone had the highest Shannon-Wiener diversity index (H') of 2.01, while Embu Upper Midland zones had the lowest diversity index (H') of 1.83. From the laboratory analysis, 41 native rhizobia isolates were isolated from the root nodules of mid-altitude climbing beans (MAC 13 and MAC 64) and placed into 9 groups based on their morphological characteristics. Rhizobia diversity was determined based on restriction digestion of PCR amplified 16S rRNA genes using *Msp* I, *EcoR* I and *Hae* III enzymes. Analysis of molecular variance based on restriction digestion of 16S rRNA genes showed that there was a 100 % genetic variation within population and no variation (0 %) among the four rhizobia populations and across the two regions (0 %). Nine representative native rhizobia isolates were tested in the greenhouse for their symbiotic nitrogen fixation efficiency on MAC 13 and MAC 64 climbing bean varieties. Four native isolates ELM3, ELM5, ELM8 and ELM4 with symbiotic efficiency (SE) of 123.72%, 99.21%, 98.24% and 96.75%, respectively, compared favorably with the commercial rhizobia inoculant (Biofix-CIAT 899) (SE of 95.21%). The best native rhizobia isolate (ELM3) was evaluated in the field experiment using MAC 13 and MAC 64 climbing beans. The mean nodule number of climbing beans differed significantly ($p < 0.0001$) among the test isolates. Climbing beans inoculated with test isolate ELM3 recorded a higher mean nodule number (85.58 plant⁻¹) compared to the Biofix-CIAT 899 (76.13 plant⁻¹) and non-inoculated control plants (52.08 plant⁻¹). There was a significant effect of rhizobia isolates on shoot dry weight ($p < 0.0001$) and total bean seed yield ($p < 0.0001$). Climbing beans inoculated with native isolate ELM3 produced the highest mean seed yield of 4,397.75 kg/ha, indicating 89 % increase over non-inoculated control which yielded 2,334.81 kg/ha. This study demonstrated the presence of diverse native rhizobia isolates that are potentially superior over the commercial inoculant (Biofix-CIAT 899) bean inoculants. However, these isolates need to be identified and tested further in different geographical locations to determine their efficiency and stability on bean production.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Agriculture plays a key role in Kenya's economy accounting for about 29.3 % Gross Domestic Product (GDP) and about three-quarter of Kenya's population depend on agriculture for food and income (FAO *et al.*, 2014). Kenya's annual production share of common beans is at 3.2 % and is ranked position 8 worldwide with 529,265 tons, though, this is about a half of Tanzania's annual production 1,113,541 tons (FAOSTAT, 2015). Climbing bean just like other common beans (*Phaseolus vulgaris* L.) form the main source of protein especially for the low-level income populations and ranks second after maize as a staple food crop. Its consumption and contribution to human nutrition is relatively high as it adds folic acid, vitamin, lysine and insoluble fiber to the diet (Broughton *et al.*, 2003).

In the recent years, Kenya has been facing severe food insecurity and this has been attributed to several factors including frequent droughts, the declining soil fertility and decreasing agriculturally productive lands due to increasing human population (Muthuri, 2013). The growing human population in Eastern Kenya has led to fragmentation and intensive use of agriculturally prolific lands leading to exhaustion of available soil nutrients. Each household is estimated to have an average agriculturally productive farm of about 1.2 hectares (Gichangi *et al.*, 2012). To counter this trend, various measures including use of organic manure, mineral fertilizers and biofertilizers have been applied to replenish soil fertility. However, the use of inorganic fertilizers has drawn a number of reactions due to the negative effects

on soil biodiversity, high cost and other environmental concerns (Jansa *et al.*, 2011; Hester and Harrison, 2012; Mutuma *et al.*, 2014). Moreover, the high cost of inorganic fertilizers impedes their affordability by smallholder farmers. Thus, there is a need to adopt environmentally friendly technologies that are sustainable and affordable to smallholder farmers.

Climbing bean (*Phaseolus vulgaris* L.) is one of the legumes that is widely cultivated in tropical regions including Sub-Saharan Africa (Ramaekers *et al.*, 2013). Climbing bean is a short-season legume with most varieties maturing between 65 to 110 days after emergence. This legume is advantageous over other bean cultivars due to their high yields, large biomass production and biological nitrogen fixation which enhance soil fertility (Gichangi *et al.*, 2012). Moreover, climbing beans can be used in intercropping and crop rotation systems and thus adding value to smallholder farmers (Gicharu *et al.*, 2013).

International Centre for Tropical Agriculture (CIAT) released improved climbing bean varieties in 1984 to Rwanda which later spread to the rest of East African countries. Six of the new varieties were later introduced to Kenya Agricultural Research Institute (KARI) Embu in 1995. The varieties were exposed to participatory testing on-farm and on the station. However, adoption of these new varieties by farmers went slowly mainly due to marketability and seed unavailability problems that farmers faced (Gicharu *et al.*, 2013). This prompted the introduction of mid-altitude climbers from CIAT to KARI Embu in 2002. They were designed to be disease and heat tolerant and to mature early (Blair *et al.*, 2006). After successful field

trials, the Ministry of Agriculture in 2008 released 3 new varieties (MAC 13, MAC 34 and MAC 64) to the farmers.

In Kenya, climbing beans are mainly grown in highland areas where population density is high and land is limiting (Mmbaga *et al.*, 2014). Like other legumes, climbing bean establishes a symbiotic association with rhizobia that lead to nitrogen fixation, which can increase crop yields (Buruchara *et al.*, 2011). This legume is nodulated by diverse genera and species of fast growing rhizobia, including *Rhizobium tropici*, *Rhizobium etli*, *Rhizobium phaseoli*, *Rhizobium leguminosarum*, *Rhizobium gallicum* and *Sinorhizobium meliloti* (Adhikari *et al.*, 2013). The productivity of climbing beans greatly depend on nitrogen content available in the soil, but most often, nitrogen is found in low quantities (Hester and Harrison, 2012). In East African region, the production of climbing beans is mainly affected by biotic factors such as pests and diseases and abiotic factors which include low soil fertility, particularly low levels of nitrogen and phosphorous in agricultural lands. Several mid-altitude climbers that were introduced in the highlands region of East Africa by CIAT have significantly increased the bean annual production share of East Africa (Beebe *et al.*, 2012).

The Eastern Kenya region is characterized by a rapidly growing human population with over 1000 people per km² and this has resulted in land fragmentation, intensive farming and the decline in soil fertility which has contributed to decreasing crop yields in the area (Mugwe *et al.*, 2009). Biofertilizers mainly from rhizobia bacteria have been in use in legume production to enhance yields and soil fertility (Buruchara

et al., 2011). Rhizobia are nitrogen fixing bacteria that are either free-living or form symbiotic association with the roots of legumes (Martínez-Romero, 2003). Biological nitrogen fixation (BNF) presents an alternative farming system that is eco-friendly, resilient to climate change, enhance soil biodiversity, and soil structure management (Adhikari *et al.*, 2013). BNF, thus, is of much significance in highly populated areas such as Eastern Kenya where there is great land pressure and intense agricultural land usage (Ramaekers *et al.*, 2013).

Rhizobia when used as biofertilizers, have been reported to increase the availability of nutrients to the legumes through biological nitrogen fixation process. They may also increase the availability of soil nutrients through solubilization of phosphates and other bound minerals. Research by Chabot *et al.* (1998) indicated that *Rhizobium leguminosarum* bv. *phaseoli* solubilize phosphates in the rhizosphere of common beans. Rhizobia native to African soils have been found to nodulate and fix nitrogen with climbing beans, a process that is important in increasing yield and enriching soil fertility. However, this symbiotic interaction is not always effective depending on the type of rhizobia strain interacting with the host plant. Native strains adapt well to the local environmental conditions and in most cases, they have been reported to be more effective compared to rhizobia strains introduced in to the new environment (Gicharu *et al.*, 2013). The inability of introduced inoculants to compete well with native rhizobia population due to negative microbial interactions impedes their use (Martínez-Romero, 2003). Native rhizobia strains have a competitive edge over imported inoculants. They have the ability to establish themselves and persist for a

longer period of time in the soil and are sustainable over a longer period of time (Kawaka *et al.*, 2014).

CIAT has been on the forefront in the development of improved climbing beans through genetic breeding. With the current decline in soil fertility and increase in global warming, emphasis still remains on developing climbing bean varieties that are tolerant to low soil fertility, heat and that are promiscuous for *Rhizobium* strains native to East African soils (Ramaekers *et al.*, 2011).

1.2 Problem statement and justification

The increasing food insecurity in Kenya is attributed to the declining agricultural productivity caused by soil infertility, pests, diseases, environmental and economic factors (FAO, 2012). In Kenya, production of climbing beans is mainly carried out by smallholder farmers who in most cases have difficulty in accessing financial resources in order to optimize their bean production. They also experience challenges in acquiring high yielding certified bean seeds such as MAC 13 and MAC 64 varieties, thus opt to use local seeds which are often low yielding and prone to pests and diseases (Katungi *et al.*, 2011). The ever-increasing human population in Eastern Kenya has led to fragmentation of agriculturally productive lands. In order to meet high food demand, farmers intensify their land use for crop production, a factor which has contributed to the decline in soil fertility and in particularly the nitrogen levels (Akibode and Maredia, 2011).

The use of inorganic fertilizers in crop production has drawn a number of reactions due to the negative effects on soil biodiversity, high cost and other environmental concerns (Jansa *et al.*, 2011). Research has also shown that most farmers apply an inadequate amount of nitrogen fertilizers to their beans instead of recommended standards due to the high cost of the fertilizers (Gikonyo *et al.*, 2014; Mutuma *et al.*, 2014). This means that beans will have to depend on biological nitrogen fixation from nitrogen fixing bacteria found in the soil in order to acquire the much-needed nitrogen for growth and development.

There are diverse strains of rhizobia that nodulate and symbiotically fix nitrogen with climbing beans; however, they greatly vary from one geographical area to another. Therefore, the use of specific native rhizobia strains well adapted to the soil and with high symbiotic N fixation efficiencies is significant in increasing the productivity of climbing beans (Adhikari *et al.*, 2013; Kawaka *et al.*, 2014). In addition, the inaccessibility to native rhizobia strains effective in nodulation and nitrogen fixation potential is a big challenge for smallholder farmers. Native rhizobia populations thrive in soil for a longer period of time, therefore, farmers can be relieved of frequent fertilizer application and thus lowering the cost of bean production (Mathu *et al.*, 2012).

The information on genetic diversity and symbiotic efficiency of native rhizobia that nodulate with mid-altitude climber (MAC) beans in Eastern Kenya is limited, as most of the studies focus on biological nitrogen fixation of rhizobia on bush beans, and traditional climbing bean varieties and their yield performance in different cropping

systems. Therefore, this study aimed to determine rhizobia diversity in Eastern Kenya and provide data on their symbiotic efficiency with a view of developing a suitable bio-inoculant. This study also identified and provided information on the local bean varieties grown in Eastern Kenya.

1.3 Research questions

- i) Which climbing bean varieties are grown in upper and lower midland agro-ecological zones of Eastern Kenya?
- ii) Are native rhizobia strains that nodulate with climbing beans grown in Eastern Kenya genetically diverse?
- iii) Which native rhizobia strains are effective in nodulation and symbiotic nitrogen fixation with climbing beans grown in Eastern Kenya?

1.4 Research hypotheses

- i) Different varieties of climbing beans are grown in upper and lower midland agro-ecological zones of Embu and Tharaka Nithi Counties in Eastern Kenya.
- ii) The native rhizobia strains that nodulate climbing beans in Embu and Tharaka Nithi Counties of Eastern Kenya are genetically diverse.
- iii) Native rhizobia strains have different efficiency in nodulation and symbiotic nitrogen fixation with climbing beans grown in Embu and Tharaka Nithi Counties of Eastern Kenya.

1.5 Objectives

1.5.1 General objective

To identify the climbing bean varieties, and determine the genetic diversity and efficiency of native rhizobia in nodulation and symbiotic nitrogen fixation on climbing beans grown in Embu and Tharaka Nithi Counties of Eastern Kenya.

1.5.2 Specific objectives

- i) To identify climbing bean varieties grown in upper and lower midland agro-ecological zones of Embu and Tharaka Nithi Counties of Eastern Kenya.
- ii) To determine the genetic diversity of native rhizobia strains that nodulate climbing beans in Embu and Tharaka Nithi Counties of Eastern Kenya.
- iii) To determine nodulation and symbiotic nitrogen fixation efficiency of native rhizobia strains isolated from climbing beans grown in Embu and Tharaka Nithi Counties of Eastern Kenya.

1.6 Significance of the study

The information on climbing bean varieties grown in Eastern Kenya will help in improving bean productivity through use of high yielding varieties that are well-adapted to the region. High productivity of climbing beans will translate to a higher income for smallholder farmers, hence, will contribute to poverty reduction and enhance food security. The genetic variation among the native rhizobia isolates will indicate the diverse nature of rhizobia in Eastern Kenya and this will help in selecting elite rhizobia strains for bean production. Effective native rhizobia strains with higher nodulation and symbiotic nitrogen fixation efficiencies can be used for bio-inoculant

development that can be availed to smallholder farmers in Eastern Kenya. The use of native rhizobia inoculants will not only reduce the reliance on inorganic fertilizers but also increase yields of climbing beans in the region.

CHAPTER TWO

LITERATURE REVIEW

2.1 General traits of climbing beans

Climbing beans (*Phaseolus vulgaris* L.) form an important component of traditional crop farming practised in several parts of the world (Ramaekers *et al.*, 2013). They are either grown solely or intercropped with other crops such as maize and bananas, which provide support for their vertical growth. The most apparent trait key to climbing bean is the climbing ability. The climbing bean cultivars are known to have a higher yield potential compared to bush beans (Ramaekers, 2011). The vertical growth associated with these cultivars gives them an edge over bush bean cultivars, as it increases the surface area for pod formation. In sole cropping, climbing beans acquire support from stakes put in place by the farmers. Trellising system offers an alternative method of staking and involves the use of erected wires or strings to provide support to the plant. This method is more economical and discourages deforestation.

2.2 Climbing bean production in Kenya

Climbing beans in Kenya are mainly grown in Western, Eastern and Central highlands of Kenya (Beebe *et al.*, 2012). The world leading countries in production of dry beans are India, Brazil and Myanmar and in Africa, the top producers are Tanzania, Uganda and Kenya (FAOSTAT, 2015). According to FAO *et al.* (2014), Kenya's annual bean production is much below the country's annual demands. Due to land fragmentation caused by high population pressure in highly potential regions,

farmers are unable to produce beans in sufficient amounts that can feed the Kenyan population of over 38.6 million (FAO, 2012).

In Kenya, the adoption of high yielding climbing bean varieties such as mid altitude climbers MAC 13, MAC 34 and MAC 64 varieties has been quite slow among farmers due to poor availability of the certified seeds (Ramaekers *et al.*, 2013). A survey conducted in Kenya by FAO reported that most of the regions in the country experience low bean yields due to numerous factors including non-use of high yielding varieties, pest and disease infestation and decline in soil fertility (FAO, 2012). Climbing beans are considered to have more advantages over bush type cultivars which include; high grain yields of up to 5 tons/ha, diverse utilization as human and animal feed, enhanced biological nitrogen fixation (BNF) and large biomass which is the basis for improvement of soil fertility (Monyo and Laxmipathi, 2014).

Climbing beans require different management practices unlike bush beans. They need proper staking, a practice which is more laborious and climbers are often infested by pests such as aphids and birds due to their sweet taste (Gichangi *et al.*, 2012). Previous research by Ramaekers *et al.* (2013) revealed that high labour requirements, pest infestation, unavailability of staking materials and reducing soil fertility are some of the major constraints affecting adoption of climbing beans by smallholder farmers in Kenya. According to Ramaekers *et al.* (2013), about 11 % of the farmers in Kenya have adopted the cultivation of climbing beans while about 90 % of the farmers are aware of climbing beans.

2.3 Traditional and new climbing bean varieties

Some of the traditional climbing bean varieties that have been grown in East Africa include G2333 (Umubano), G685 (Vuninkingi), Flora, G20797 (Gisenyi) and G3323 (Puebla) (Ramaekers *et al.*, 2013). In Kenya, most cultivated climbing bean varieties are reported to do well in medium and high altitude regions, a similar agro-ecological adaptation to the wild climbing beans from which they were derived from in Andes and Central America (Buruchara *et al.*, 2011).

In the 1990s, CIAT designed mid-altitude climbers (MAC) to have improved features such as yields, heat tolerance and pest and disease resistance. In addition, they mature earlier compared to the traditional climbers (Blair *et al.*, 2006). MAC 13 commonly referred to as Kenya safi, produces large, speckled sugar white grains and takes between 80 and 85 days to mature. MAC 34 also known as Kenya tamu, has large red mottled grains and matures within 90 to 105 days while MAC 64 (Kenya mavuno) produces medium sized red mottled grains and also takes between 90 to 105 days to mature.

In Kenya, the new climbing bean varieties, which have been successfully adopted by farmers, include MAC 13, MAC 34 and MAC 64. These MAC varieties were developed in order to boost bean production in the region. MAC varieties can grow up to a height of 4 meters and can produce up to 4-5 tonnes of grains per hectare. They do well in highland areas that have adequate rainfall of above 1000 mm annually but in dry conditions, they can be grown under irrigation.

Multiple stress resilient bean varieties which were released in Kenya between 1998 and 2008 by the Pan-Africa Bean Research Alliance (PABRA) include KATB1, KATX56 and KATX69 (Buruchara *et al.*, 2011). Other new bean varieties, which are not necessarily climbers include; the large dull red kidney (M18), large speckled sugar red (E7), red mottled Kenya umoja (AFR 708), dull red Kenya wonder (L41), speckled sugar brown Miezi mbili (E2), red mottled new Rosecoco (E80) and the large red kidney Kabete super variety (Ramaekers *et al.*, 2013).

2.4 Legume-rhizobia symbiosis

2.4.1 Rhizobia taxonomy

Rhizobia are phylogenetically heterogenous and taxonomically diverse as it comprise of the alpha group which forms the majority of the rhizobia species and the beta group, which interacts with *Mimosa* genus (Saikia *et al.*, 2007). Classification order of rhizobia includes: class *Alphaproteobacteria*; family *Rhizobiaceae*, and order *Rhizobiales* (Valdés-Ramírez, 1995). There are six genera of rhizobia that are widely distributed in the soil which include; *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium*, *Allorhizobium*, *Mesorhizobium* and *Rhizobium*. They belong to the alpha sub-class of the Proteobacteria (Laguerre *et al.*, 2001). Rhizobia from different genera have different nitrogen fixing potentials and their ability to infect and competitively occupy nodules differ significantly (Laguerre *et al.*, 2001; Kabahuma, 2013). Climbing bean is a promiscuous legume and forms symbiotic interactions with a diverse species of rhizobia (Muthini *et al.* 2014).

2.4.2 Use of rhizobia in biological nitrogen fixation

Biological nitrogen fixation (BNF) is of great importance in reduction of atmospheric nitrogen to ammonia, providing the earth's ecosystem with about 200 million tons of nitrogen per year (Broughton *et al.*, 2003). It is estimated that about 80 % to 90 % of nitrogen available to plants in natural ecosystems originates from BNF (Saikia *et al.*, 2007).

Climbing beans like other legumes, form a symbiotic association with bacteria of *Rhizobium* genus, a process that is important in replenishment of lost soil fertility (Morad *et al.*, 2013). The processes of infection may occur in the root hairs or between the root epidermal cells. The interaction of the bacteria with the root cells initiates nodulation. The rhizobia interact with the roots of climbing beans to form nitrogen-fixing nodules through a complex process aided by two nitrogenase enzymes that are encoded by *nif* genes. Another protein significant in nodulation is the leg-haemoglobin that acts to maintain a high oxygen flux at low concentration (Bohloul *et al.*, 1992). The nitrogenase enzymes take part in reduction of atmospheric nitrogen into ammonium anions which are further converted to nitrogenous forms that can be easily assimilated by beans (Broughton *et al.*, 2003). BNF process requires a lot of energy and its success heavily relies on the availability of phosphorous (Shamseldin *et al.*, 2012).

Rhizobia-legume host specificity is highly suggested to be a factor of rhizobia nodulation (*nod*) genes (Valdés-Ramírez, 1995). The *nod* genes are switched on by plant root-cell exudates such as flavones, isoflavones and other related compounds.

Some of these compounds strongly inhibit the expression of *nod* genes. The common *nod* genes, the *nod* ABC, are present in all rhizobia (Valdés-Ramírez, 1995). They are involved in induction of nodules in leguminous plants. The *nod* D regulates other *nod* complex genes. It is also thought to be responsible for rhizobia-legume promiscuity. For instance, rhizobia having restricted host range are thought to have a single copy of *nod* D gene (Valdés-Ramírez, 1995). It is of great interest to bring together compatible rhizobia-legume strains mainly when introducing crops into new areas.

2.4.3 Legume-rhizobia inoculation: potentials and limitations

Legume inoculation is a widespread and established agricultural practice that has beneficial effects on nitrogen fixation, soil fertility and legume yield (Laguerre *et al.*, 2001). Inoculation provides a means of transferring elite or selected rhizobia strains isolated in the laboratory to the legumes grown in the field. Transfer can be done by applying the rhizobia inoculant directly on to the soil or by coating the legume seeds at the time of planting. In the absence of a specific rhizobia in the soil, inoculation enhances nodulation, legume growth, nitrogen fixation and legume yield (Rahmani *et al.*, 2011). Through BNF, legume inoculation indirectly contributes to crop productivity through enhancement of soil fertility.

The government of Kenya has encouraged farmers to use alternative methods such as biofertilizers and farmyard manure to complement inorganic fertilizers. Bean inoculation with *Rhizobium* strains, has been reported to successfully enhance legume productivity in Iran (Morad, 2013; AkramJafari *et al.*, 2014; Rahman *et al.*, 2014). In

Kenya, rhizobia inoculation has been reported to successfully enhance nodulation and bean production (Kimani *et al.*, 2007; Gicharu *et al.*, 2013; Onyango *et al.*, 2015).

Although bean inoculation is considered the most successful in the field of applied soil microbiology, it has some major limitations. One of the major setback that has been frequently reported is the failure of the applied inoculum to effect nodulation. This is often caused by the competition by native soil bacteria that often out-compete the inoculant strain. The elite strain therefore are unable to occupy a larger proportion of the root nodule (Meghvansi *et al.*, 2010). Other factors that negatively affect BNF in legume production include poor management practices, low soil pH and low soil nutrition, water stress, pests and diseases. Reduced water supply in the soil affects the survival of rhizobia and consequently interrupts the nodulation process. Tropical soils often tend to be acidic and are deficient of phosphorous and calcium. This may adversely affect nodulation in non-acid tolerant rhizobia species (Mwangi *et al.*, 2011).

2.4.4 Symbiotic efficiency of rhizobia on legumes

Symbiotic nitrogen fixation (SNF) is a process in which certain microorganisms convert atmospheric nitrogen into ammonia, a form of nitrogen readily available for uptake by the plants (Stajković *et al.*, 2011). Authentication of rhizobia to determine their symbiotic efficiency is usually carried out in a greenhouse under bacteriologically controlled conditions (Maingi *et al.*, 2001; Liu, 2014). Total nitrogen accumulation in bean legumes is one of the best parameters that indicates the amount of nitrogen fixed under controlled conditions (Anglade *et al.*, 2015).

2.4.5 Quantification of biological nitrogen fixation (BNF)

The quantification of nitrogen fixed during BNF is useful in determining the efficiency of a particular symbiotic association between rhizobia strain and the host legume (Peoples *et al.*, 2002). A precise method of measuring nitrogen fixation is essential for assessing symbiotic nitrogen fixation capability in legumes, however, these remain remarkably elusive. This is largely contributed to the fact that differentiating nitrogen gained by the plant from the atmosphere and nitrogen absorbed from the soil is difficult (Giller, 2001). The common methods used in quantifying BNF include; acetylene reduction assay (ARA), the total nitrogen difference (TND), ureide (Xylem-solute) method and ¹⁵N-labelled isotope (Ramaekers, 2011). Although some methods are more accurate than others are, each one has its own shortcomings.

2.4.5.1 Total nitrogen difference (TND)

In this method, the total nitrogen of nitrogen fixing plant is compared with that of non-nitrogen fixing plant, commonly referred to as a reference or a control plant (Peoples *et al.*, 2002). The assumption is that the nitrogen fixing plant obtains its nitrogen from the soil and the atmospheric air. On the other hand, the non-nitrogen fixing crop has only one source of nitrogen, that is, soil mineral nitrogen. Therefore, the difference between the two is estimated to be the amount of nitrogen derived from the atmospheric air (Broughton *et al.*, 2003). This method is cheaper and simple, as it does not require specialized equipment. It is also a more reliable method especially in soil with low nitrogen content (Giller, 2001). However, the method may lead to underestimation or overestimation of nitrogen content fixed from the air especially

that, the two plants may not necessarily have a similar rooting system to use the soil nitrogen (Ramaekers, 2011).

2.4.5.2 Acetylene reduction assay (ARA) technique

It is a widely used method in quantification of nitrogen fixed by plants during BNF. The basic principle behind this method is the ability of nitrogenase enzyme to reduce acetylene to ethylene. It measures the rate of acetylene reduction to ethylene. The amount of ethylene produced is used to estimate the amount of nitrogen derived from the atmosphere by multiplying it by a certain conversion ratio (Peoples *et al.*, 2002). The ARA technique is simple and relatively cheap. It is highly sensitive and can be used to detect nitrogenase enzyme activity. However, the method is limited by the fact that it only measures the nitrogenase activity for the duration of the assay (Giller, 2001). Hence, many such measurements should be taken in order to reflect and account for diurnal or seasonal variations that affect nitrogenase enzyme activity. Its validity is also questionable due to the use of conversion ratio. Intrinsic inhibition of acetylene conversion to ethylene also affects the results (Ramaekers, 2011).

2.4.5.3 ^{15}N abundance and enrichment techniques

This technique comprises ^{15}N natural abundance and ^{15}N enrichment methods. This method is used to quantify the amount of nitrogen that is accumulated by plants through biological nitrogen fixation (BNF). Its principle is that the concentration of ^{15}N in the air differs from that for plants (N-obtained from soil) (Peoples *et al.*, 2002). Therefore, amount of nitrogen fixed is calculated from the difference of ^{15}N of the nitrogen fixing plants and that of non-fixing plant (Broughton *et al.*, 2003). When

using this technique, it is important to determine the ^{15}N abundance of the nitrogen of the legume under study, the ^{15}N abundance of nitrogen derived from both soil and that from the air via BNF. The mean of the ^{15}N natural abundance of the sampled plants can be calculated using a mass balance as described by Peoples *et al.* (2002). This method is more accurate but is very expensive and requires specialized equipment (Giller, 2001).

2.4.6 Rhizobia *nifH* gene expression and nitrogenase activity

The *nifH* gene encodes for an iron nitrogenase protein that is important in catalyzing nitrogen fixation (Ribeiro *et al.*, 2013). This gene can be detected in rhizobia using PCR methods such as PCR-RFLP, PCR-Denaturing gradient gel electrophoresis (DGGE) or 16S rRNA gene sequences (Liu, 2014). In order to quantify nitrogen fixation potential of rhizobia, several studies have used *nifH* gene and has revealed significant variation in the community structure and nitrogen fixing potential of different strains of rhizobia (Laguerre *et al.*, 2001; Liu, 2014). Based on quantitative PCR techniques, *nifH* gene has also been used to study abundance and diversity of rhizobia in different geographical zones (Young *et al.* 1991). The expression this gene by rhizobia is as a result of low oxygen and nitrogen concentrations within the root environment of the legume (Adhikari *et al.*, 2013). In some rhizobia, the *nifH* genes are located on symbiosis plasmids and are regulated at the transcriptional level (Young *et al.*, 1991). The rhizobia phylogenies derived from *nifH* genes have been reported to closely follow those of 16S rRNA genes (Ribeiro *et al.*, 2013). The use of *nifH* genes is advantageous as it has the largest number of available rhizobial

sequences that can be used for comparison and making conclusion (Laguerre *et al.*, 2001).

2.5 Factors affecting biological nitrogen fixation

The interactions between rhizobia and the legume host are complicated by edaphic, climatic, and biotic factors. These factors limit the biological nitrogen fixation (BNF).

2.5.1 Edaphic factors

These factors relate to the soil and include; drought, excessive soil moisture, soil acidity, phosphorus deficiency, excess soil nitrogen and the deficiency of Calcium, Molybdenum, Cobalt and Boron minerals (Abubakari and Abubakari, 2015). Waterlogged soils prevents proper development of root hairs and nodulation sites. It also interferes with the diffusion of oxygen in the root system of the legumes (Beebe *et al.*, 2012). Water stress during drought condition reduces the number of viable rhizobia in the soils, thus inhibiting nodulation and nitrogen fixation. Prolonged drought has been reported to promote the decay of nodules (Anglade *et al.*, 2015). A soil pH range of 6.0 to 7.0 is considered optimum for rhizobia growth and any pH value less than the optimum range can adversely affect the symbiotic relationship between rhizobia and the legume host (Faoro *et al.*, 2010). Low soil pH and related problems of manganese toxicity and calcium and aluminium deficiency are known to adversely affect nodulation, nitrogen fixation, plant growth and development (Andrade *et al.*, 2002a). However, research has shown that rhizobia that are acid-tolerant naturally exist in the soil and therefore they can be identified and used in inoculation of legumes particularly in areas where soil is acidic to enhance crop yield

(Adnan *et al.*, 2014). *Rhizobium leguminosarum* biovar *trifolii* has been reported to work well in BNF with red clover grown in soils with pH of less than 5.0 (Blanco *et al.*, 2010).

In Sub-Saharan Africa, phosphorous (P) deficiency is common in soil. Low P-soils leads to reduced nodulation, nitrogen fixation and diminished plant growth and yield (Ndakidemi *et al.*, 2014). The role of arbuscular mycorrhiza fungi (AMF) in enhancing plant P uptake and its synergistic effect with rhizobia on nodulation and nitrogen fixation has been reported (Nyaga *et al.*, 2014). Other beneficial P-solubilizing microorganisms such as *Bacillus*, *Aspergillus* and *Penicillium* have been used particularly in areas with soils which are acidic and have low-P content (Ramaekers, 2011). Excessive mineral nitrogen content in the soil has been reported to inhibit the infection process by *Rhizobium* species and hence inhibiting nitrogen fixation (Gicharu *et al.*, 2013). This probably occurs due to the impairment of recognition process caused by the nitrates and also as a result of diversion of photosynthates towards the assimilation of nitrates (Rondon *et al.*, 2007).

2.5.2 Climatic factors

Temperature and light are two important determinants affecting BNF. Extreme temperature is known to affect adversely nitrogen fixation. It interrupts the enzymatic process involved during BNF (Öğütçü *et al.*, 2008). The availability of light regulates the process of photosynthesis, which consequently affect BNF. This has been successfully demonstrated by diurnal variations in nitrogenase activity (Shamseldin and Dietrich, 2004). Relatively high-root temperature has been demonstrated to affect

rhizobia infection, nitrogen fixation potential and legume growth (Faoro *et al.*, 2010). It appears to have a strong effect on rhizobia strain and cultivar interactions. For example, the optimum root temperature for clover and pea is 30 °C and 35 to 40 °C for soybean and cowpea while 25 to 30 °C for common bean (Mohammadi *et al.*, 2012).

2.5.3 Biotic factors

The absence of the required novel rhizobia species in the soil is one of the major constraints in the process of BNF. Insect pests, nematodes, birds and diseases such as viruses, bacteria and fungi, are known to affect plant physiology and thus interfering with nodule development and functions (Mmbaga *et al.*, 2014). If specific elite rhizobia are present in low numbers or entirely absent in the soil, it is necessary to introduce them via inoculation in order to enhance plant nodulation and nitrogen fixation. If the rhizobia present in the soil are able to colonize and nodulate legumes and are not effective in terms of nitrogen fixation, then it becomes a barrier to the successful exploitation of rhizobia inoculants (Shamseldin and Dietrich, 2004). Therefore, introduced rhizobia inoculants must be more aggressive and competitive nodulators compared to the native strains.

2.6 Rhizobia diversity

The diversity of rhizobia vary significantly from one geographical region to another with exchangeable acidity and soil pH being the major factors (Anyango, *et al.*, 1995; Aserse *et al.*, 2012). According to the study carried out by Anyango *et al.* (1995) in Kenya, the distribution of rhizobia nodulating *Phaseolus vulgaris* differ with soil pH,

with *Rhizobium tropici* dominating in acidic soil. *Rhizobium tropici* is an acid-tolerant *Rhizobium* species described to date (Hernandez-Lucas *et al.*, 1995) hence its dominance in acidic soil. Rhizobia diversity and abundance has also been attributed to differences in chemical and physical properties between soils, history of nitrogen fertilization and land management (Andrade *et al.*, 2002). A large rhizobia diversity has been found in the tropics, therefore, there is a need to exploit the diversity maximally in order to come up with a true assessment of the biodiversity within the tropics (Pongslip, 2012).

Rhizobia could be native inhabitants or introduced and are either free living or form symbiotic association with host plants. Methods for rhizobia identification and enumeration have been developed, however, numbers can be underestimated due to inconsistencies caused by soil factors and choice of trapping host plant (Somasegaran and Hoben, 1994). Rhizobia strains have been characterized based on traditional methods such as morphological, physiological and biochemical methods. However, these methods often fail to identify strains within a species, hence, the development of serological and molecular methods (Berrada *et al.*, 2012). Morphologically, rhizobia exhibiting white, creamy, milky, gummy, soft gummy or watery colonies with convex, domed or raised elevation have been reported (Gachande and Khansole, 2011; Kawaka *et al.*, 2014; Muthini *et al.*, 2014). Rhizobia can be categorised as slow, intermediate or fast growers. *Rhizobium* species, which are often associated with climbing beans are known to be fast growers and acidify YEMA containing BTB indicator (Berrada *et al.*, 2012).

Several methods have been designed to gather data that assesses rhizobia diversity based on phenotypic and genotypic features. They include morpho-cultural, biochemical and molecular methods. In molecular techniques, the use of polymerase chain reaction (PCR) method to create highly distinct patterns that are distinguished in agarose gels has made it possible to identify a number of rhizobia strains (Laguerre *et al.*, 1996; Diouf *et al.*, 2000). Other methods such as amplified ribosomal DNA restriction analysis (ARDRA), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) have been used to evaluate the diversity of rhizobia (Gachande and Khansole, 2011).

In addition, the 16S rRNA and 16S-23S rRNA genes have been sequenced to determine the taxonomic position of different rhizobia strains (Rahmani *et al.*, 2011; Adhikari *et al.*, 2013). Several rhizobia specific and universal primers have been used for amplification and sequencing. In recent times, 16S-23S rRNA ITS region is getting consideration among researchers as phylogenetic marker for species and sub-species delineation among rhizobia. The high sequence variation of the ITS region allows discrimination between closely related species (Kwon *et al.*, 2005). According to the PCR-RFLP analysis of 16S-23S ITS region of common bean nodulating rhizobia isolated by Rahmani *et al.* (2011) in Iran, it was reported that the rhizobia isolated in the study exhibited high genetic diversity and contained 43 ITS genotypes that were clustered into 10 groups at the similarity of 64 %.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Description of the study sites

The study involved field, greenhouse and laboratory experiments. Field study was carried out in selected farms in upper and lower zones of Embu and Tharaka Nithi Counties in Eastern Kenya (Figure 3.1) during the long rainy season from March to June 2015 and short rainy season from October to December 2015. Greenhouse and laboratory experiments were carried out at the Department of Microbiology, Kenyatta University. In the field study, two farms from each County were selected based on history of crop cultivation and previous use of commercial rhizobia inoculant. The field sites were selected based on agro-climatic conditions and prevalence of climbing bean (*Phaseolus vulgaris* L.) cultivation. The selected farms were demarcated before carrying out any experiment. Climbing bean varieties (Kenya safi - MAC 13 and Kenya mavuno - MAC 64) were sourced from Simlaw Seeds Company Limited (Licensed dealers of Kenya Seed Company) and were used as test plants. These plants were preferred over the local bean varieties as they are pure line breeds and have high potential yields.

In Embu County, the field study sites were located in Runyenjes sub-county, situated at the foot of Mt. Kenya at 0.53° S, 37.45° E within an elevation of 1100 to 1500 m above sea level. The area is located within a high potential agro-ecological zone and with an average annual precipitation ranging from 1000 to 1500 mm and a mean annual temperature ranging between 12 °C to 30 °C (Nyaga *et al.*, 2014). In Tharaka Nithi County, the field study was carried out in parts of Chuka (Figure 3.1), which is

located on the South-Eastern side of Mt. Kenya at 0.30° S, 38.06° E and lies within an elevation of 600 m to 1500 m above sea level. The area is largely semi-arid and receives a bimodal rainfall averaging between 800 mm and 1200 mm annually and an annual mean temperature of 25°C (Jaetzold *et al.*, 2006).

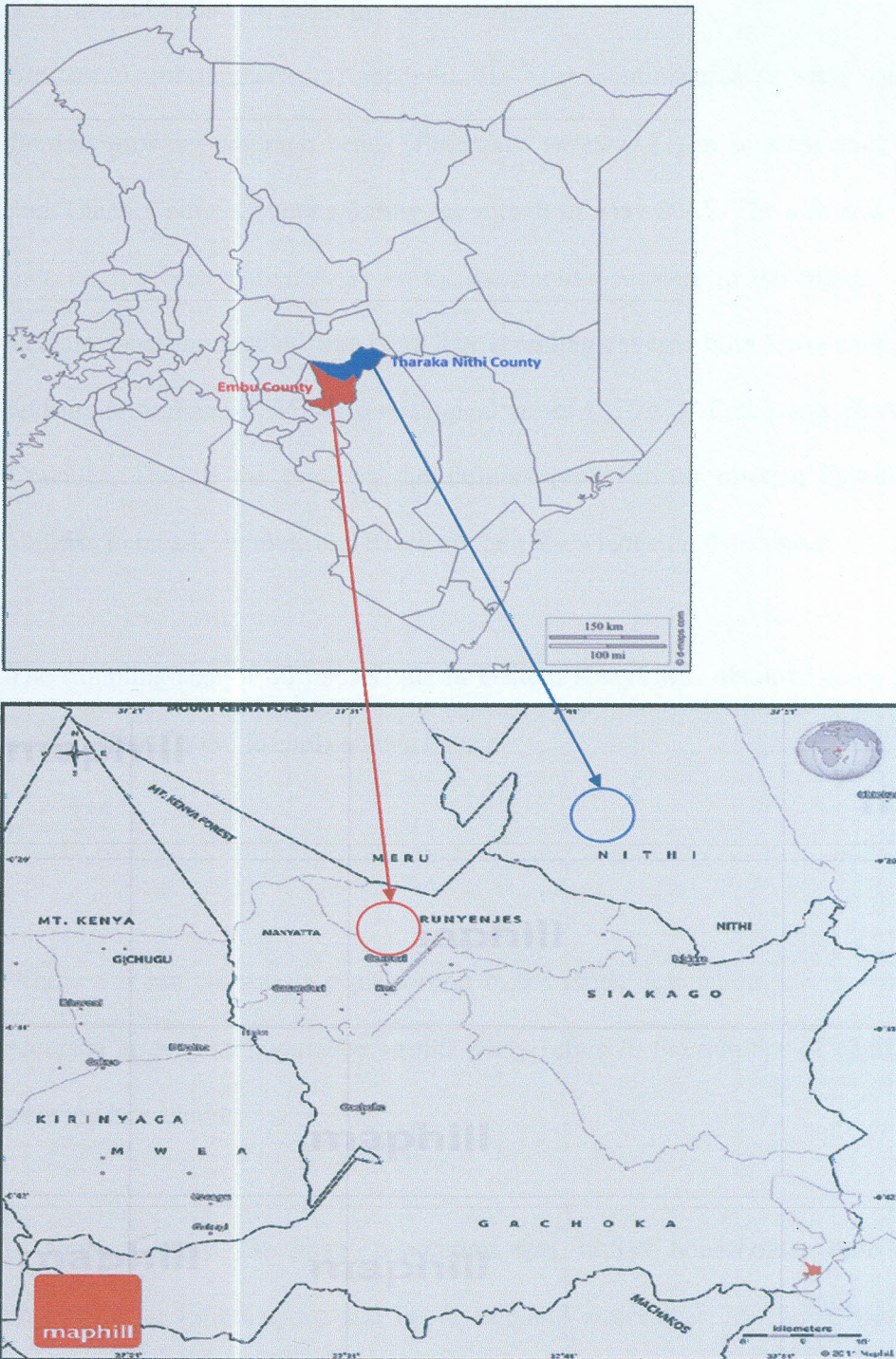


Figure 3.1: The map of Kenya showing field study sites in Embu and Tharaka Nithi Counties. Source: Google map, 2015.

3.2 Data collection on climbing bean varieties

Structured questionnaires (Appendix II) were administered to sixty smallholder farmers growing common beans (*Phaseolus vulgaris* L.) in selected parts of Embu and Tharaka Nithi Counties during the month of May 2015. The aim was to collect data on the bean varieties grown by smallholder farmers in the region. A simple random sampling method was used. The sampling covered both lower midland (LM) and upper midland (UM) agro-ecological zones (AEZ) of Embu and Tharaka Nithi Counties. During the process, the farmers provided information based on their abilities to recall, estimate and based on their knowledge on the subject.

The sampling size of 60 households in Eastern Kenya was obtained using Snedecor and Cochran (1989) formula shown below.

$$n = \frac{4pq}{(L)^2}$$

Where n = sample size, p = proportion in the target population, $q = 1 - p$ and L = accepted error. The population in this study refers to the number of climbing bean growing households.

In Embu County, the target population was 12,844 households while the total population in Embu upper and lower midland zones was 259 households (Embu County Agricultural Extension Department). Therefore; $p = 259/12844 = 0.02017$ while $q = 0.97983$ and L is the allowed error margin of 5%.

$$n = \frac{4 \times 0.02017 \times 0.97983}{(0.05)^2}$$

n = 32 households

For Tharaka Nithi County, the target population was 9,671 households and the total population in Tharaka Nithi upper and lower midland zones was 170 households (Tharaka Nithi County Agricultural Extension Department). Therefore; $p = 170/9671 = 0.01758$ while $q = 0.98242$ and L is the allowed error margin of 5%.

$$n = \frac{4 \times 0.01758 \times 0.98242}{(0.05)^2}$$

n = 28 households

3.3 Soil sampling and analysis

Soil sampling was carried out in all the selected farms in both counties before the onset of the long rains (March, 2015). The soil was sampled across and diagonally from 20 points in each farm at a depth of 15 – 20 cm using a hand shovel. The shovel was sterilized before sampling each point using 5 % sodium hypochlorite solution, then rinsed three times with sterile water and dried using a sterile cloth. The 20 soil samples collected from each farm were thoroughly mixed to form a composite sample and a kilogram of a homogenous composite soil sample was made per farm and packed into sterile bags for laboratory analysis. Soil samples were air-dried in the laboratory and sieved through a 2 mm diameter sieve for physical, chemical and microbial analysis.

3.3.1 Soil pH analysis

The soil pH was determined using a glass electrode pH meter (Adwa AD1000 – Hungary) in 1:2.5 soil to water suspension (Okalebo *et al.*, 2002). Ten grams of the sieved soil sample was weighed and put in a clean 100 ml conical flask and 25 ml of distilled water added. The samples were then shaken thoroughly, left to stand for 10 minutes and the pH reading was taken using a pH meter. The electrode of the pH meter was rinsed thoroughly with distilled water before measuring the next sample.

3.3.2 Soil texture analysis

Soil texture was determined using Bouyoucos Hydrometer method (Kissel and Sonon, 2008). A 50 g of sieved and dried soil was weighed into a 500 ml beaker and saturated with distilled water. Ten millilitres of 10 % Calgon solution was added before transferring the suspension to the dispersing cup. The suspension was topped up with 300 ml tap water and thoroughly stirred for 2 minutes using an electric stirrer and transferred into a measuring cylinder. The graduated cylinder was covered tightly using a fitting rubber band and inverted ten times to allow the suspension to mix well. The time was noted and 2 to 3 drops of amyl alcohol were added in order to remove froth. After 20 seconds, the hydrometer was carefully placed into the column. After 40 seconds, the hydrometer and thermometer readings were recorded. The cylinder was covered tightly again with a fitting rubber band and the cylinder was inverted 10 times to allow the suspension to mix. The cylinder was then left to stand for 2 hours after which the hydrometer and thermometer readings were taken. The percentage (%) sand, clay and silt were then calculated to determine the soil textural class.

3.3.3 Soil available phosphorous determination

The available phosphorous (P) was determined using Bray 1 method (Carter *et al.*, 2008) where 2.5 g soil sample was weighed into 250 ml plastic beaker and 25 ml of the Bray P1 extracting solution (ammonium fluoride, NH_4F) was added and shaken thoroughly for 5 minutes. The extracts from the suspension were filtered into 50 ml clean bottles using Whatman No. 42 filter paper. The blanks and standards used were prepared in the Bray P1 extracting solution. The phosphorous content was then analysed by the colorimetric method and P-content in parts per million (ppm) was determined (Kissel and Sonon, 2008).

3.3.4 Soil total nitrogen analysis

Kjeldahl digestion method was used to determine the total nitrogen (Okalebo *et al.*, 2002). A half a gram (0.5 g) sieved dried soil was weighed into a 250 ml Kjeldahl flask. A selenium tablet, which is a digestion catalyst, was added followed by 5 ml concentrated sulphuric acid (H_2SO_4) acid. The digestion was left to continue until the digest became clear. The flask was then cooled before transferring the digest content into a 100 ml volumetric flask with distilled water. A 5 ml of 40 % sodium hydroxide (NaOH) solution was added to a 5 ml aliquot of the digest and the mixture was then distilled. The distillate was collected in 5 ml of 2 % boric acid (H_2O_3). Three drops of a mixture of methyl red and methylene blue indicators were added to the distillate in a 50 ml conical flask and titrated against 0.01 M HCl acid. The percentage nitrogen was calculated as; $\text{N (\%)} = \text{Titre} \times \text{HCl Molarity} \times \text{Extract Volume} \times 0.014 / \text{aliquot volume} \times \text{soil weight}$ (where 0.014 is milliequivalent weight of nitrogen).

3.3.5 Soil organic carbon analysis

The wet combustion method of Walkley and Black colorimetric method was used to determine the soil organic carbon (Okalebo *et al.*, 2002). Half a gram (0.5 g) of air-dried sieved soil sample was weighed into a conical flask where 10 ml of potassium dichromate and 20 ml of sulphuric acid (H₂SO₄) solutions were added and gently swirled to mix well. The content was digested at 150 °C for 30 minutes to ensure efficient combustion and afterwards, 200 ml of distilled water was added. Ten millilitres of orthophosphoric acid and barium phenylalanine sulphate indicator was then added to the solution before titrating against ferrous ammonium sulphate solution. The percentage carbon (% C) was calculated from the titre value as follows; % SOC = (10 – XN) x 0.003 x 1.33 x100 / W, where X = titre value of ferrous ammonium sulphate, N = molarity of ferrous ammonium sulphate, W = weight of soil sample, 0.003 = milliequivalent weight of carbon (g) and 1.33 = correction factor (f) assuming more than 75 % recovery of organic carbon by wet combustion.

3.4 Rhizobia field trapping experiment

Field trapping of native rhizobia was carried out in all the selected farms in Embu and Tharaka Nithi Counties during the long rainy season (March to August 2015). The field farms were prepared by hand harrowing to a fine tilth. Bean seeds (MAC 13 and MAC 64 climbing bean varieties) were procured from Simlaw Seeds Company, Nairobi. Two Seeds of each climbing bean variety were selected and planted in each farm at a recommended spacing of 75 by 30 cm. Triple superphosphate (TSP) fertilizers (46.0 P₂O₅) were applied at recommended rates of 50 kg ha⁻¹ in order to supplement the much-needed phosphorus. Thirty five days after emergence, 10 plants

from each farm were randomly sampled and harvested by uprooting for nodule analysis. The root nodules showing pink colouration from each bean plant were selected, removed and packed in a sterile sampling vial containing cotton wool and desiccated silica gel and transported to the laboratory where they were air-dried for storage and rhizobia isolation.

3.5 Rhizobia isolation and characterization in the laboratory

Laboratory experiments involved isolation and characterization of rhizobia extracted from root nodules of climbing beans (MAC 13 and MAC 64) that had been trapped in the field. This was carried out in the Department of Microbiology laboratory, Kenyatta University.

3.5.1 Isolation of rhizobia from root nodules

Five dried nodules selected in section 3.4 were thoroughly washed, and rinsed using distilled water. Clean dry nodules were then immersed in sterile distilled water for one hour to allow rehydration. The rehydrated nodules were surface sterilized in 1 % sodium hypochlorite (NaOCl) solution for 3 minutes, rinsed in 7 changes of sterile distilled water and then crushed with a sterilized glass rod (Muthini *et al.*, 2014). A loop-full of the resulting suspension was streaked on the surface of a petri dish containing Yeast Extract Mannitol Agar (YEMA) supplemented with Congo red (0.00125 mg/kg) and incubated in the dark at 28 °C for 3 - 5 days (Vincent, 1970). Emerging single colonies, which were typical of rhizobia species were sub-cultured by repeated streaking on Yeast extract mannitol agar (YEMA), and YEMA containing bromothymol blue (YEMA-BTB) (0.00125 mg/kg) plates.

3.5.2 Preservation and maintenance of the isolates

All the pure isolates were stored on YEMA agar slants in screw-capped McCartney bottles where they were preserved at 4 °C as stock cultures. The rhizobia isolates were sub-cultured every three months to ensure that the isolates retain their viability. All the isolates were also stored at -20 °C in 25 % glycerol- Yeast extract mannitol broth (YEMB).

3.5.3 Presumptive screening of pure isolates

Using the standard microbiological techniques described by Somasegaran and Hoben (1994), all the isolates were characterized for selected morphological parameters such as colony size, shape, border, elevation, colour, mucosity, transparency and capacity to produce exo-polysaccharide gum (Liu, 2014; Muthini *et al.*, 2014). Other presumptive tests that were carried out included Gram staining where young pure isolates (3 – 4 days old) cultured on YEMA were smeared on clean microscope slides. The wet smears were air-dried, heat fixed and then Gram stained as described by Beck *et al.* (1993). The prepared slides were observed under oil immersion on a compound light microscope. The production of acid or alkali was determined in YEMA medium containing bromothymol blue (YEMA-BTB). The plates were incubated at 28 °C for 3 – 5 days in the dark. Based on the differences in the observed morpho-cultural features, the isolates were placed into different groups.

3.5.4 Molecular characterization of rhizobia isolates

Polymerase chain reaction (PCR) technique using amplified ribosomal DNA restriction analysis (ARDRA) was used to study the molecular diversity of 41 selected

rhizobia isolates obtained during the study (Young *et al.*, 1991). Young pure colonies of different rhizobia isolates and standard reference *Rhizobium* strains (*R. etli* bv. *Phaseoli* USDA 2667, *R. tropici* CIAT 899 and *R. leguminosarum* bv. *Phaseoli* strain 446) cultured in YEMA and incubated at 28 °C in the dark for 2 days were used for PCR process (Packer *et al.*, 2013). The three reference *Rhizobium* strains were obtained from the Microbiological Resource Centre (MIRCEN), University of Nairobi.

3.5.4.1 Genomic DNA extraction of rhizobia isolates from Eastern Kenya

The extraction of total genomic DNA of rhizobia isolates was carried out following modification of a method by Young *et al.* (1991). The young rhizobia cultures were resuspended in eppendorf tubes containing 400 µl of normal saline, vortexed for 20 seconds and centrifuged at 13,000 rpm for 10 minutes. The supernatant was poured out carefully leaving the cell pellets. The cell pellets were repeatedly washed five times with normal saline to remove the slimy exopolysaccharide (EPS). The cell pellets were then harvested and resuspended in 400 µl of cetyltrimethylammonium bromide (CTAB) lysis buffer, vortexed and incubated in a water bath at 65 °C for 2 hours. The tubes were intermittently inverted every 20 minutes during the incubation period. DNA was extracted by adding equal volumes of phenol: chloroform: isoamyl alcohol solution (25:24:1), and centrifuged at 13,000 rpm for 10 minutes at 4 °C. The supernatant was transferred carefully to a sterile eppendorf tube where an equal volume (400 µl) of absolute ethanol was added and incubated at 4 °C for 10 minutes to allow precipitation. The precipitated DNA was centrifuged at 13,000 rpm for 8 minutes and the supernatant was discarded. The DNA pellet was air-dried for 40

minutes and dissolved in 40 μ l of DNase free water. The DNA was stored at -20 °C (Berrada *et al.*, 2012).

3.5.4.2 The PCR amplification of 16Sr DNA

The PCR amplification was carried out in 30 μ l reaction with 25.05 μ l sterile PCR water, 3.0 μ l buffer (Biolabs), 0.6 μ l dNTP (10 mM), 0.3 μ l of each Y1 and Y3 primers (10 μ M), 0.6 μ l 5 % Tween 20, 0.15 μ l *Taq* DNA polymerase (Biolabs) and 1.5 μ l of DNA template. The sequences of the primers used for PCR amplification of the 16S rRNA gene were: Y1 forward primer (5'-TGGCTCAGAACGAACGCTGGCGGC-3') that corresponds to positions 20 – 43 and Y3 reverse primer (5'-TACCTTGTTACGACTTCACCCCAGTC-3') corresponding to positions 1482 – 1507 for 16S rDNA sequence of *Escherichia coli* (Young *et al.*, 1991). A negative control without DNA was used. The DNA was amplified in a Techgene Thermal Cycler (Techne) programmed to run as follows; initial DNA denaturation at 94 °C for 2 min; 35 cycles (denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s and extension at 72 °C for 2 min). Final extension was carried out at 72 °C for 5 minutes. Amplified PCR products were visualized by gel electrophoresis on a 1 % agarose gel. The stain used was SYBR-Green (Biolabs). The DNA ladder of 1 Kb (Biolabs) was used to estimate the band sizes. The gel was run in Tris-Borate Ethylenediaminetetraacetic acid (0.5X TBE) buffer at 80 V for 50 min and was visualized using a UV trans-illuminator and photographed using a digital camera.

3.5.4.3 Restriction fragment analysis of the 16Sr DNA

PCR products of the isolates and reference strains were digested using *Hae* III, *Eco*R I and *Msp* I restriction enzymes (Biolabs) (Laguerre *et al.*, 1996). A master-mix (10.0 μ l) containing 3.8 μ l PCR water, 1.0 μ l reaction buffer, 0.2 μ l restriction enzyme and 5.0 μ l PCR amplicons were digested for 1 hour at 37 °C. The restriction digests (fragments) were then stained using SYBR-Green and separated on a 2 % agarose gel. The gel was run at 80 V for 50 min and was visualized using a UV trans-illuminator and photographed using a digital camera. The different band patterns were noted and the frequency of similar patterns was scored. A 100 bp DNA ladder (Biolabs) was used to estimate the fragment sizes.

3.6 Authentication and determination of symbiotic efficiency of native rhizobia isolates in the greenhouse

The greenhouse experiments were carried out to establish the symbiotic efficiency of the native rhizobia isolates and to authenticate the isolates, obtained from climbing beans grown at the experimental site during field trapping. From each group, pure isolate (a representative) that conformed to the morpho-cultural characteristics of rhizobia were selected for plant nodulation test in the greenhouse at the Department of Microbiology, Kenyatta University. The experiment was carried out following the standard procedures described by Somasegaran and Hoben (1994) between May and August 2015. The test plants were MAC 13 (Kenya safi) and MAC 64 (Kenya mavuno) climbing beans varieties.

3.6.1 Rooting medium and Leonard jar assembly

Sterilized vermiculite was used as the rooting media. This vermiculite was thoroughly washed for 3 days by constantly stirring and changing water four times a day. In the final rinse, distilled water was used and its pH was adjusted to 6.8. Water was drained off before autoclaving the medium. The sterile vermiculite medium was packed into sterile Leonard jar apparatus and then covered with aluminium foil to minimize contamination. The Leonard jar assembly, which consisted of a modified 250 ml cup, a 500 ml jar and a wick, were sterilized using 5 % sodium hypochlorite (NaOCl) solution.

3.6.2 Plant nutrient growth medium

Sterile nitrogen-free plant nutrient stock solution, which was prepared as described by Somasegaran and Hoben (1994) were used during the experiment. The use of nitrogen free medium ensured that the only nitrogen available for the test plants was from BNF. Five stock solutions were prepared as shown in appendix I. For each litre of full strength solution, 0.5 ml was added from each of the 5 stock solutions. The pH of the solution was adjusted to 6.8 using 1 N HCl or 1 N NaOH solutions and autoclaved at 121 °C for 15 min.

3.6.3 Plant seed sterilization and pre-germination

Quality seeds of uniform size and shape of the test plants were selected and surface sterilized for 30 seconds using 70 % alcohol, followed by 3 % sodium hypochlorite solution for 3 minutes and finally rinsed in 7 changes of sterile distilled water. The seeds were then placed in sterile distilled water for one hour to allow imbibition.

Twenty seeds were placed on each petri-plate containing water agar (10 % agar in distilled water) and pre-germinated aseptically in the dark at 28 °C for 3 days. Two seedlings with radicles of length 1 – 2 cm were transplanted into the sterilized Leonard jar assemblies.

3.6.4 Planting of seedlings in Leonard jars

A pair of flame-sterilized forceps was used to prepare two holes in the vermiculite in each jar. The seedlings selected for planting were picked up with sterile forceps and placed in each hole with the radicle pointing downwards. The holes were covered by moving a small lump of the vermiculite until it completely covered the rooting system of the pre-germinated seeds. The two seedlings transplanted were later thinned into one per jar just before inoculation. Eight days after transplanting, the base of the seedlings was inoculated with 1 ml (10^9 cfu/ml) of the representative rhizobia isolates cultured in YEMA broth for three days. Each of the two climbing bean varieties was inoculated with the isolates obtained from the climbing bean root nodules (section 3.5.1). The nitrogen controls were left non-inoculated but instead, was supplied with sterile 1.0 M KNO_3 solution (nitrogen source). The material control was also left non-inoculated and was treated only with nitrogen-free nutrient solution just like the other treatments. These controls were purposely put in place in order to cross check for any contamination.

The experiment was laid out in a completely randomized design (CRD) with four replicates per treatment. Treatments included the representative native rhizobia isolates (ELM1, TUM2, ELM3, ELM4, ELM5, EUM6, EUM7, ELM8 and ELM9),

native rhizobia consortium (TC) (a mixture of all native isolates), non-inoculated (nitrogen treated and nitrogen-free) controls (TN), commercial rhizobia inoculants (Biofix-CIAT 899 - CIAT 899) (TB) and a mixture of native consortium (combination of all native rhizobia ELM1 to ELM9) and the commercial rhizobia inoculant (TCB). Non-inoculated seedlings served as negative control (TUC) while seedlings inoculated with the commercial inoculants (Biofix-CIAT 899) (TB) served as the positive control. During growth, the plants were constantly supplied with the nitrogen-free nutrient solution after every three days.

3.6.5 Plant harvesting and data collection

Plant harvesting was carried out at mid-flowering stage (40 days after emergence) when nodulation was peaking. Harvesting involved emptying the rooting medium out of the Leonard jar cup. The shoots were then separated from the roots by cutting at the root collar. The roots were then placed on a coarse sieve where they were washed using a gentle stream of tap water. All the root nodules were detached and counted for each plant.

The nodulated and non-nodulated plants were recorded. Apart from the nodule number (NN) and nodule dry weight (NDW), other parameters considered included shoot (SDW) and root (RDW) dry weights. The presence of a single nodule formed on the plant roots was a positive indication and confirmation that the isolates were rhizobia (Morad, 2013).

3.6.6 Dry weight and relative symbiotic efficiency determination

The shoot and root dry weights were determined by drying to a constant weight in an oven at 70 °C (Kimani *et al.*, 2007). The nodules were air-dried to a constant weight in the laboratory. The dry weights were measured using a high-precision digital MRC Lab weighing balance (Model ASB-220-C2-V2, Israel). The relative symbiotic efficiency (SE) was determined following the formula used by Gibson (1987); by dividing shoot dry weight (SDW) of inoculated plants over SDW of non-inoculated control plants supplemented with nitrogen (1.0 M KNO₃) and then converted into percentages. The SE (%) values were rated as : >80 % = highly effective, 51 – 80 % = effective, 35 – 50 % = lowly effective and <35 % = ineffective (Lalande *et al.*, 1990).

3.7 Chemical analysis of plant shoots

The plant shoots were analysed for nitrogen, phosphorous and potassium content.

3.7.1 Shoot nitrogen analysis

The total nitrogen in bean shoot samples was determined using Kjeldahl method (Okalebo *et al.*, 2002). A sample of 0.3 g of oven dried, grounded and sieved (<0.25 mm) plant shoot material was weighed into a dry 50 ml volumetric digestion tube and 2.5 ml mixture of Salicylic acid dissolved in sulphuric acid-selenium was added to each tube and the reagent blanks for each sample. The prepared samples were digested at 110 °C for one hour, then left to cool to 40 °C before carefully adding three 1 ml portions of hydrogen peroxides. The temperatures were then raised to 330 °C to ensure complete plant tissue digestion. The digestion continued until the solution turned colourless, then it was left to cool to 40 °C before adding 25 ml of distilled water to dissolve the remaining sediment. From the digest, Total N was

determined through distillation, whereby ammonia was liberated from the solution by steam distillation in the presence of excess sodium hydroxide. The distillate was collected in an Erlenmeyer flask containing excess boric acid-indicator solution. The collected distillate was titrated using N/70 hydrochloric acid until a pink end-point was reached. The volume of the acid used was recorded and used to determine the total N using the formula:

$$\% N = (a-b) \times 0.2 \times V \times 100/1000 \times W \times Z$$

Where; a: Volume of the titre HCl for the blank, b: Volume of the titre HCl for the sample, V: Final volume of the digestion, W: weight of the sample taken and Z: an aliquot of the solution taken for analysis.

3.7.2 Shoot phosphorus analysis

The amount of phosphorus in bean shoot samples was determined using ammonium hepta-molybdate vanadate method (Kissel and Sonon, 2008). A sample of 0.25 g of oven dried, milled plant tissue was weighed into digestion tubes and ashed in the muffle furnace and was then allowed to cool. A volume of 0.5 ml mixture of digestion acids and hydrogen peroxide was added and left to evaporate to dryness. Blank samples were prepared for the same but without plant samples. Twenty-five millilitre (25 ml) of 0.05 N HCl was added to re-dissolve the digested sample and left for 5 hours after cooling. Two millilitre (2 ml) of the digested sample was pipetted to 25 ml volumetric flask and 5 ml of complexation reagent (Vanadomolybdophosphoric acid; LabChem) was added to the top mark level. The standards and sample absorbance was measured at 400 nm wavelength using a UV-Vis spectrophotometer (Halo-DB 20, Dynamica) from which the concentration of P in ppm was determined.

3.7.3 Shoot potassium analysis

The concentration of potassium in bean shoot samples was determined using atomic absorption spectrophotometry (AAS) method (Carter *et al.*, 2008). A sample of 0.25 g of oven dried, milled plant tissue was weighed into digestion tubes, ashed and sieved (0.25 mm) in the muffle furnace and was then allowed to cool. A volume of 0.5 ml mixture of digestion acids and hydrogen peroxide was added and left to evaporate to dryness. Blank samples were prepared for the same but without plant samples. Twenty-five millilitre (25 ml) of 0.05 N HCl was added to re-dissolve the digested sample and left for 5 hours after cooling. One millilitre (1 ml) of the solution was pipetted into 50 ml volumetric flask where 5 ml of strontium chloride was added and topped to the mark. The absorbance of the standard and the sample solutions was measured at wave length of 766.5 nm using AAS (Model 3300, Thermo Scientific) and the concentration of K in plant sample was determined and expressed in ppm.

3.8 Effectiveness of rhizobia inoculation on climbing beans in the field

3.8.1 Field experimental design and treatments

The experiments were laid out in a randomised complete block design (RCBD) with three replications. Two climbing bean varieties (MAC 13 and MAC 64) were used. In each block, the treatments included the best native rhizobia isolate (ELM3), native consortium (TC) (a mixture of all native isolates ELM1, TUM2, ELM3, ELM4, ELM5, EUM6, EUM7, ELM8 and ELM9), negative control (non-inoculated) (TUC), commercial inoculant – Biofix-CIAT 899 (TB) and a mixture of the native consortium and commercial inoculant (TCB). Five treatments were therefore represented in each of the 10 plots per block and hence the whole experimental layout constituted a total

of 30 plots. The plant spacing used was 75 cm by 30 cm within the rows. Each plot measured 3 m x 2.1 m and a spacing of 1 m between the plots was left to minimize inter-plot interference.

3.8.2 Land preparation and planting

The land was hand harrowed to a fine tilth before the first rains during the month of September 2015. The climbing bean seeds were purchased from Simlaw Seeds Company in Nairobi. A sterile filter mud was used as a carrier material for the rhizobia inoculants and inoculum was applied at recommended rates of 100 g of the inoculum per 15 kg of the seeds. The Biofix-CIAT 899 commercial inoculum for beans was procured from MEA Company Limited (Nairobi) and administered as per the manufacturer's instructions (100 g of inoculant per 15 kg of seeds). The seeds were planted on October 20th, 2015, just after the first rains. Only two climbing bean seeds of high quality were selected for planting. The seedlings were thinned from two to one per hole after emergence. Seeds requiring rhizobia inoculation were prepared by coating with a filter mud containing respective inoculants using 4 % gum Arabica (supplied with the inoculum). The gum Arabica helps the filter mud to stick on to the wet bean seeds. The negative control plots were left uninoculated and were planted before plots requiring inoculation in order to avoid cross contamination. Triple superphosphate (TSP) fertilizer was applied at recommended rates (50 kg ha⁻¹) in order to supplement the much-needed phosphorus. Weeding was carried out in three phases; 2nd, 5th and 8th week after germination respectively.

3.8.3 Plant growth and harvest

During mid-flowering stage (50 % flowering), 3 plants from each plot were selected randomly and uprooted for nodulation, shoot and root dry weight assessment. The sampled plants were dug up, uprooted carefully, and repeatedly washed with water to remove soil particles adhering to the roots. The root nodules from each bean plant were removed, packed in a sterile sampling vial containing cotton wool and desiccated silica gel and transported to the laboratory. Nodules were detached, counted and placed in well-labelled zip-lock bags and transported to the laboratory. The sampled plants were cut into roots and shoots and placed in well-labelled zip-lock bags for transportation to the laboratory where they were dried in an oven to a constant weight at 70 °C. The dry weights of nodules, shoots and roots were taken using MRC Precision balance (Model ASB-220-C2-V2, Israel) and recorded. The plant shoots were also analysed for nitrogen, phosphorous and potassium content following techniques outlined in section 3.7. During the final harvesting (at full physiological maturity – 100 days), the following yield parameters per plant were assessed; stover dry weight, number of pods, the weight of 100 seeds and total seed dry weight. Seed yield per hectare was extrapolated from the total seed yield per plot.

3.9 Data analyses

The data generated using a questionnaire was analysed using descriptive statistical tools of SPSS software (version 22) for Windows. The greenhouse and field data on symbiotic efficiency, shoot nutrient content, plant root and shoot dry weight, number of nodules and pods, stover and nodule dry weights were subjected to analysis of variance (ANOVA) using Statistical Analysis Software (SAS) version 9.1. The means

were separated using Tukey's HSD test at 5 % level of significance (Steel *et al.*, 1997). The correlation analysis (coefficient of determination, R^2) was used to determine the relationship and magnitude of the association between the studied parameters. The data on rhizobia genetic diversity based on the different band patterns formed after PCR restriction were coded in binary form and analyzed as described by Silva *et al.* (2012) using Gene Alex Software version 6.5. Euclidean distance similarity, Nei's unbiased genetic distance and single linkage (nearest neighbor) methods available in PAST program (version 1.92) and Darwin software (version 6), were used to construct dendrograms (Hammer *et al.*, 2001). Shannon-Wiener diversity index (H) was used to determine climbing bean and rhizobia diversity in Embu and Tharaka Nithi Counties of Eastern Kenya.

CHAPTER FOUR

RESULTS

4.1 Climbing bean production

4.1.1 Production status in Eastern Kenya

The average cultivated farm size owned by households under climbing beans in Embu and Tharaka Nithi Counties ranged between <0.25 acres to 0.50 acres (Table 4.1). Households in Tharaka Nithi lower midland (TLM) agro-ecological zone had the largest average farm size (0.25 – 0.50 acres household⁻¹) under climbing bean cultivation. Households in Embu upper midlands (EUM) recorded the lowest average yield of less than 90 kg year⁻¹ household⁻¹. Households in TLM had the highest average yield (1881 – 270 kg year⁻¹ household⁻¹) and the highest average income (Ksh. 5401-8100 year⁻¹ household⁻¹). Households interviewed in EUM and ELM reported average poor soil fertility in their farms. The soil fertility status of the farms reported by households in TUM and TLM agro-ecological zones were neither fertile nor poor (border-line) (Table 4.1).

Table 4.1: Average climbing bean production status per household in upper and lower midland agro-ecological zones of Eastern Kenya

Agro-ecological zones	Cultivated area (Acres household ⁻¹)	Yield (kg year ⁻¹)	Average income (Ksh year ⁻¹)	Average level of soil fertility
EUM	<0.25	<90	<2700	Poor
ELM	<0.25	90-180	2701-5400	Poor
TUM	<0.25	90-180	2701-5400	Border-line
TLM	0.25-0.50	181-270	5401-8100	Border-line

ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

4.1.2 Bean varieties grown in Eastern Kenya

In the study areas, there were eight commonly cultivated bean types, which included climbing and non-climbing bush-type varieties (Table 4.2). The climbing bean varieties consisted of Gatune, Kithiga, Raila, Mama-safi and Muviki while non-climbing bean varieties included Maasai, Nduriandu and Kamucere. Out of the 8 varieties, the most common bean variety cultivated by farmers interviewed was Maasai with a frequency distribution of 37 while the least cultivated variety was Kamucere with a frequency of 8. Among the climbing bean varieties grown, Gatune and Raila were the most common varieties grown by farmers in the region with distribution frequencies of 31 and 30 respectively. For non-climbers, Maasai and Nduriandu were the most preferred cultivars with frequency distributions of 37 and 36 respectively. The least cultivated climbing bean variety was Muviki with a frequency distribution of 13 (Table 4.2).

Table 4.2: Distribution of different common bean varieties grown in selected sites of Eastern Kenya

Bean Variety	Tharaka Nithi County		Embu County		Total Distribution	
	TUM	TLM	EUM	ELM	Freq.	%
Maasai	14	9	11	3	37	19
Nduriandu	7	8	10	11	36	18
Gatune*	8	8	5	10	31	16
Raila*	8	11	5	6	30	15
Mama safi*	3	6	8	9	26	13
Kithiga*	3	4	4	3	14	7
Muviki*	2	4	5	2	13	7
Kamucere	1	3	1	3	8	4

ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland; *, Climbing bean varieties.

4.1.3 Bean species diversity across upper and lower midland zones of Eastern Kenya

The components of bean diversity consisted of species richness and their relative abundance (species evenness) across the four agro-ecological zones (AEZ). The results indicated that Embu Lower Midland (ELM) zone had the highest bean Shannon Wiener diversity index (H) of 2.01, followed by Tharaka-Nithi Upper Midland (TUM) zone with a H of 1.94 (Table 4.3). However, Tharaka-Nithi Lower Midland (TLM) zone and Embu Upper Midland (EUM) zones had the lowest H of 1.90 and 1.83, respectively.

Table 4.3: Diversity indices of bean species grown in selected study sites of Eastern Kenya

Region	Embu County		Tharaka Nithi County	
	EUM	ELM	TUM	TLM
Taxa_S	8	8	8	8
Individuals	46	53	49	47
Dominance_D	0.19	0.14	0.16	0.17
Simpson_1-D	0.81	0.86	0.84	0.83
Shannon_H	1.83	2.01	1.94	1.90
Evenness_e ^{H/S}	0.78	0.92	0.88	0.84

ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

4.2 Soil properties of the selected farms in Eastern Kenya

The soil physical and chemical properties varied across the farms in the two counties.

The soil was characteristically acidic with pH ranging from 4.27 to 6.02 (Table 4.4).

The soil from farms located in upper midland AEZ (EUM and TUM) was relatively more acidic compared to farms in lower midland zones (ELM and TLM). This was moderately acidic for bean production and rhizobia to biologically fix nitrogen. Soil

from farm ELM, located in Embu lower midland AEZ, had the highest pH (6.02), organic carbon content (3.42 %) and available phosphorus (32.15 ppm). Soil from farm EUM recorded the highest total nitrogen content (0.31 %); however, it had the lowest potassium content of 0.70 cmol kg⁻¹. The potassium content from all farms ranged from 0.70 to 1.80 cmol kg⁻¹. Soil texture was classified as sandy clay for farms in upper midland zones (EUM and TUM), sandy clay loam for ELM and clay for TLM farm (Table 4.4).

Table 4.4: Soil characteristics (before planting) of experimental study sites compared with the critical values for East African soils

Soil sample	pH	% O.C	% Total N	K (cmol/kg)	Available P (ppm)	% Sand	% Clay	% Silt	Texture Class
EUM	4.27	2.63	0.31	0.70	16.52	47	47	6	Sandy clay Sandy clay
ELM	6.02	3.42	0.22	0.85	32.15	54	27	19	loam
TUM	5.31	3.29	0.25	1.00	27.00	49	45	6	Sandy clay
TLM	5.85	3.33	0.20	1.80	25.10	45	49	6	Clay
*Critical value	5.50	3.00	0.25	0.22	15				

*Okalebo *et al.* (2002). ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland; O.C, Organic Carbon; N, Nitrogen; K, Potassium; P, Phosphorous.

4.3 Morphological characteristics of the isolates

In this study, 41 pure isolates were obtained from the root nodules of climbing beans (MAC 13 and MAC 64) grown in selected farms in Eastern Kenya. The isolates were placed into 9 different groups based on their differences on morphological features (Table 4.5). All isolates and reference strains were identified as Gram negative rods which did not absorb Congo red when they were grown and incubated in the dark on YEMA-CR medium (Plate 4.1A). On streaking on YEMA-BTB medium, all isolates

tested were acid producers and turned BTB indicator from deep green to yellow (Plate 4.1B) after 3 to 5 days incubation in the dark. The colony diameter of the isolates ranged from 0.5 mm to 5.0 mm within 3 to 5 days of incubation (Table 4.5). The colony colour varied with milky white, cream white, cream yellow and watery colonies being observed which were either opaque or translucent with either firm gummy or smooth mucoid texture (Plate 4.1C). All isolates had an entire colony margin, however, in terms of elevation, the colonies varied consistently with convex and raised elevations being observed on YEMA media (Plate 4.1D). Isolates in group (iii) carried the highest percentage of the total number of isolates with 28.89 %. Based on similarity in morpho-cultural features, *Rhizobium tropici* CIAT 899 and *Rhizobium etli* USDA 2667 were placed under group (iii) together with other native isolates. *Rhizobium leguminosarum* strain 446 was placed in group (v) (Table 4.5).

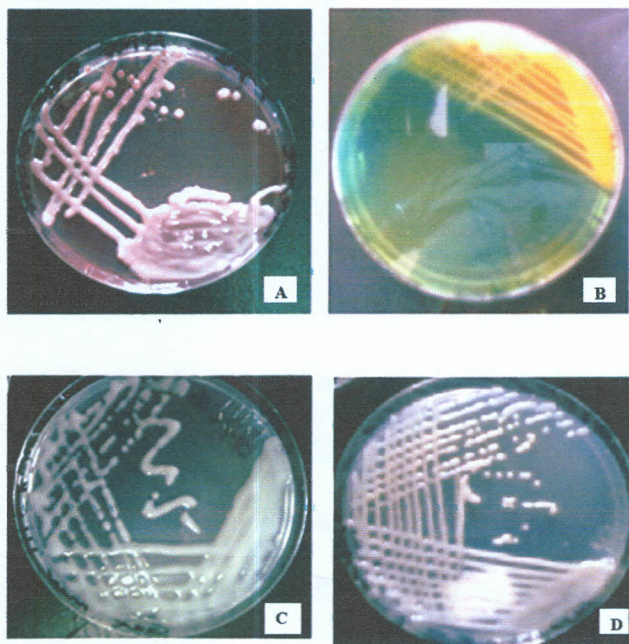


Plate 4.1: Morphological characteristics of rhizobia isolates from field trapping. A, Native rhizobia isolate EUM7 on YEMA with Congo red dye. B, Acidic reaction of native rhizobia isolate ELM3 on YEMA with BTB. C, Growth of native rhizobia isolate ELM5 on YEMA media. D, native rhizobia isolate TUM2 on YEMA media.

Table 4.5: Morphological characteristics of the rhizobia isolates from field trapping

Characteristic	Isolate grouping								
	i	ii	iii	iv	v	vi	vii	vii	ix
Congo red absorption	Na	Na	Na	Na	Na	Na	Na	Na	Na
BTB reaction	Y	Y	Y	Y	Y	Y	Y	Y	Y
Gram Reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Cell Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Elevation	Cvx	Cvx	Cvx	Cvx	Cvx	Cvx	Cvx	Raised	Cvx
Margin	Ent	Ent	Ent	Ent	Ent	Ent	Ent	Ent	Ent
Colony nature	Dull	Dull	Sny	Sny	Dull	Sny	Sny	Sny	Sny
Colouration	Cy	Cw	Mw	Mw	Mw	Mw	W	Mw	Mw
Transparency	Op	Trl	Trl	Op	Op	Trl	Trl	Trl	Op
Colony Diam. (mm)	1.5	3.5	1.0	3.0	0.5	3.5	5.0	1.0	1.0
Colony texture	Fg	Fg	Sm	Sm	Fg	Sm	Sm	Fg	Sm
EPS production	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No
Percentage % (x/41)	4.44	2.22	28.89	6.67	8.89	20	17.78	4.44	6.67

Na, Non-absorbing; Y, Yellow on BTB; -ve, Negative; Cvx, convex elevation; Ent, Entire margin; Sny, Shiny; Cy, Cream yellow; Cw, Cream white; Mw, milky white; W, watery; Op, Opaque; Trl, Translucent; Fg, firm gummy; Sm, Soft mucoid; Diam, colony diameter.

4.4 Genetic diversity of native rhizobia isolates from Eastern Kenya

Genomic DNA (Plate 4.2) was extracted from 41 native isolates obtained from root nodules of climbing beans in Eastern Kenya. The PCR amplification of the 16S rDNA produced a single band of approximately 1500 bp (Plate 4.3).

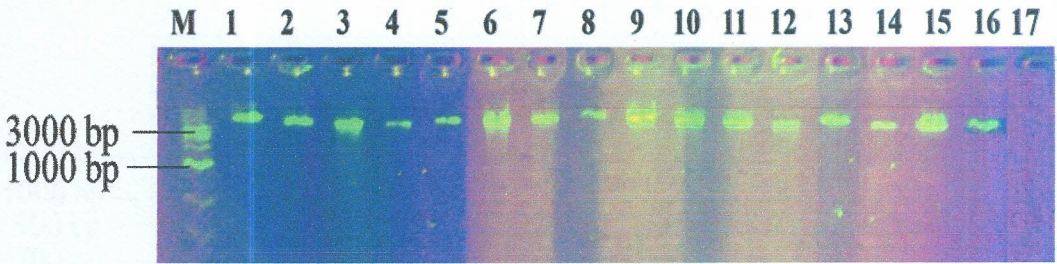


Plate 4.2: Gel electrophoresis of genomic DNA of the selected isolates in 1 % agarose gel. Lane M, 1 kb DNA ladder (Biolabs) used as a molecular marker; Lanes 1 – 13, genomic DNA of native rhizobia isolates (ELM1, TUM2, ELM3, ELM4, ELM5, EUM6, EUM7, ELM8, ELM9, TLM10, TUM11, TLM12, EUM13); Lane 14, CIAT 899; Lane 15, USDA 2667; Lane 16, strain 446; Lane 17, Negative control.

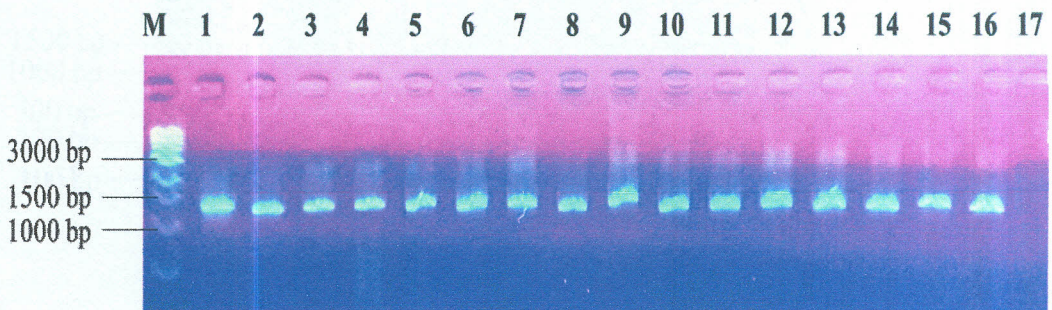


Plate 4.3: PCR amplified 16S rDNA of the isolates in 1 % agarose gel. Lane M, 1 kb DNA ladder (Biolabs); Lanes 1 – 13, genomic DNA of selected native rhizobia isolates (ELM1, TUM2, ELM3, ELM4, ELM5, EUM6, EUM7, ELM8, ELM9, TLM10, TUM11, TLM12, EUM13); Lane 14, CIAT 899; Lane 15, USDA 2667; Lane 16, strain 446; Lane 17, Negative control.

The analysis of genetic relatedness of the native isolates from Eastern Kenya was carried out by using amplified rDNA restriction analysis (ARDRA). Restriction enzymes *EcoR* I, *Hae* III and *Msp* I successfully restricted the PCR amplified 16S rDNA (Plate 4.4 A, B and C). Based on the number, pattern and size of bands formed after restriction, it was possible to cluster the isolates.

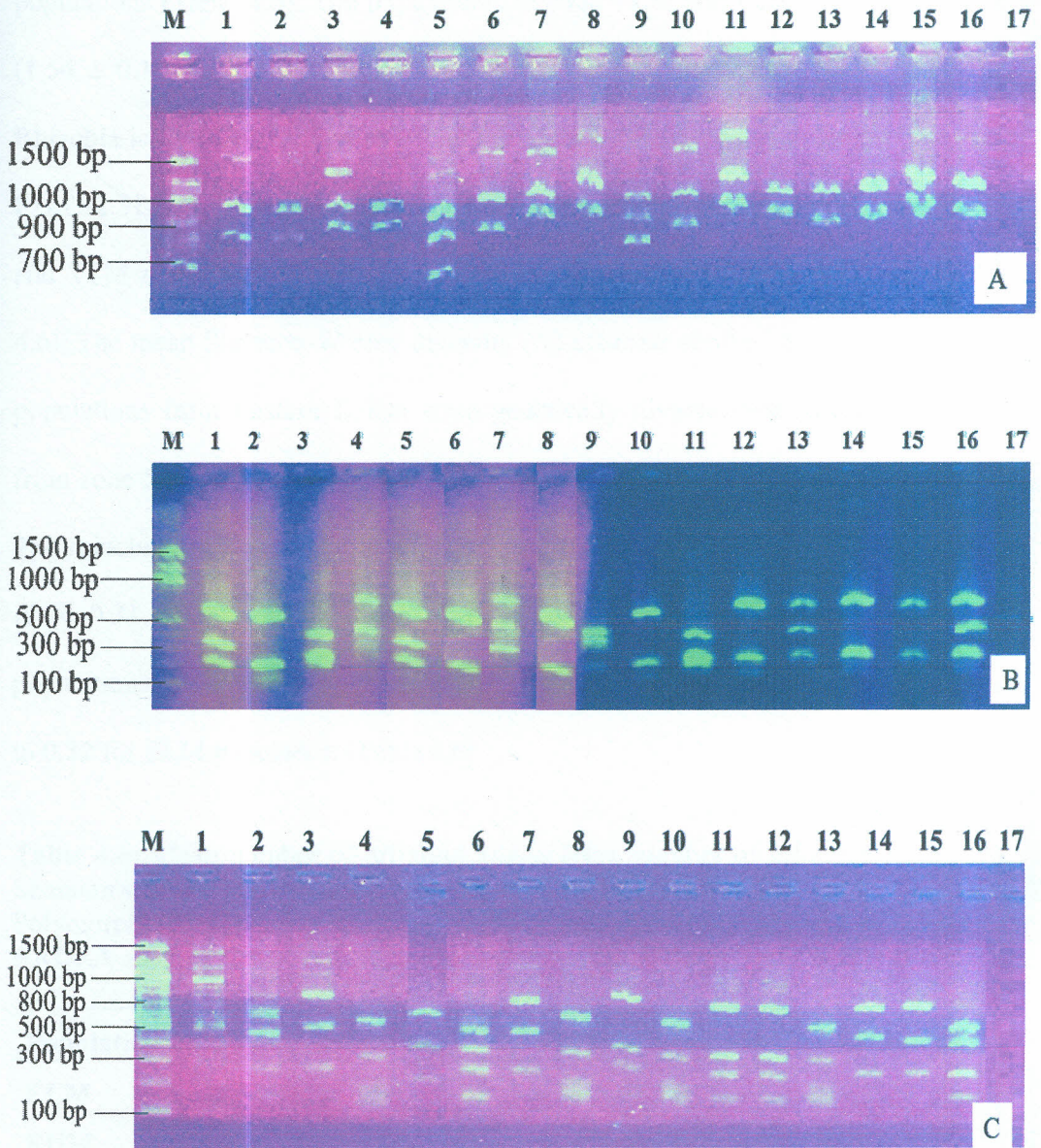


Plate 4.4: Gel electrophoresis of the restriction digestion products of 16S rDNA of selected native rhizobia isolates in 2 % agarose gel. Lane M, 100 bp DNA ladder (Biolabs); Lanes 1 – 13, genomic DNA of native rhizobia isolates (ELM1, TUM2, ELM3, ELM4, ELM5, EUM6, EUM7, ELM8, ELM9, TLM10, TUM11, TLM12, EUM13); Lane 14, CIAT 899; Lane 15, USDA 2667; Lane 16, strain 446; Lane 17, Negative control. A, Restriction digestion with *EcoR* I; B, Restriction digestion with *Hae* III; C, Restriction digestion with *Msp* I.

Rhizobia population from zone ELM had the highest average number of different alleles ($N_a = 1.92 \pm 0.08$) and effective alleles ($N_e = 1.55 \pm 0.10$) compared to other

populations (Table 4.6). The populations in zone TLM and EUM had the lowest N_a (1.54 ± 0.18) while population from zone TLM had the lowest N_e (1.37 ± 0.08). Rhizobia isolates from ELM zone had the highest percentage of polymorphic loci (% P) of 92.31 % followed by isolates in TUM zone (69.23 %). Isolates from zones EUM and TLM recorded the lowest number of polymorphic loci both at 61.54 % (Table 4.6). The mean Shannon-Wiener diversity (H) estimate showed that the four rhizobia populations from Eastern Kenya were genetically diverse with rhizobia population from zone ELM having the highest genetic diversity estimate of $H = 0.47$ (Table 4.6). The rhizobia population from zone TLM had the lowest genetic diversity estimate of $H = 0.31$. The average expected heterozygosity (H_e) varied among the four populations. Heterozygosity ranged from 0.21 for rhizobia population from zone TLM to 0.32 for ELM population (Table 4.6).

Table 4.6: Mean number of different alleles (N_a), number of effective alleles (N_e), Shannon's Information Index $I(H)$, expected Heterozygosity (H_e) and percentage of Polymorphic Loci (% P) of native rhizobia populations from Eastern Kenya based on ARDRA analyses

Population	N_a	N_e	$I(H)$	H_e	% P
ELM	1.92 ± 0.08	1.55 ± 0.10	0.47 ± 0.07	0.32 ± 0.05	92.31
EUM	1.54 ± 0.18	1.39 ± 0.11	0.33 ± 0.08	0.22 ± 0.06	61.54
TLM	1.54 ± 0.18	1.37 ± 0.12	0.31 ± 0.08	0.21 ± 0.06	61.54
TUM	1.62 ± 0.18	1.41 ± 0.10	0.37 ± 0.08	0.25 ± 0.05	69.23

ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

Analysis of molecular variance (AMOVA) for the four populations in Eastern Kenya showed that there was a 100 % genetic variation within populations (within a test

agroecological zone) but not among populations (0 %) or among the two regions (Counties) (0 %) (Table 4.7).

Table 4.7: Analysis of molecular variance (AMOVA) for 41 rhizobia isolates for the four populations from Eastern Kenya based on restriction digestion of 16S rDNA

Source	Df	SS	MS	Est. Var.	% Mol Var.	P-value
Among Regions	1	1.010	1.010	0.005	0	0.412
Among Pops	2	1.812	0.906	0.000	0	0.870
Within Pops	49	81.442	1.662	1.662	100	0.0406
Total	52	84.264		1.667	100	

Df, Degrees of freedom; SS, Sum of squares; MS, Mean Square; Est. Var, Estimated variance; % Mol. Var, Percentage molecular variance; Pops, Populations.

The principle coordinate analysis (PCA) of 41 native isolates from the four zones of Eastern Kenya and 3 rhizobia reference strains showed considerable differentiation. Isolates from TUM zone were the most distributed and appeared in all the four quadrants, while isolates from ELM zone were the least distributed (Figure 4.1).

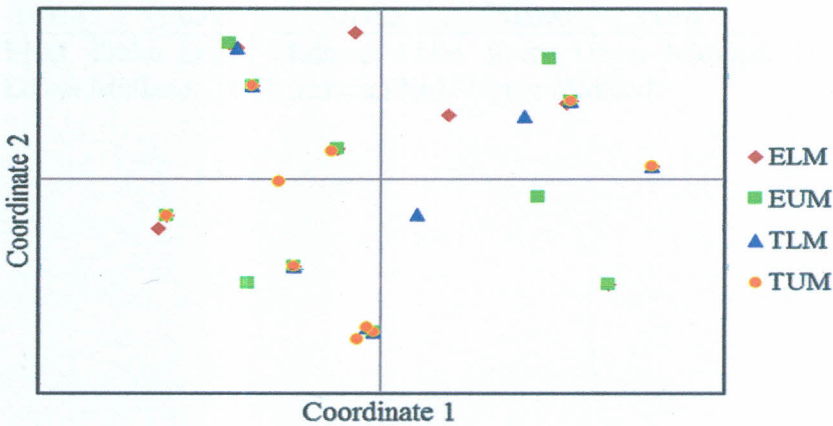


Figure 4.1: Principle coordinate analyses (PCA) of 41 native rhizobia isolates from Eastern Kenya based on ARDRA restriction patterns. Percentage variation explained by the first 2 coordinates; 1, 33.82 %; 2, 20.74 %. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

The Pairwise Population Matrix of Nei unbiased genetic distance of four rhizobia populations in Eastern Kenya showed that the highest genetic distance (0.037) was between the isolates from EUM and TUM zones, while the lowest genetic distance (0.004) was observed between isolates from EUM and TLM zones (Table 4.8). Based on Nei unbiased genetic distance matrix and Euclidian similarity index, the neighbor joining dendrogram clustered rhizobia populations from Eastern Kenya into 2 main groups (Figure 4.2). Rhizobia populations from zones ELM and TUM clustered together with a bootstrap value of 85 % while rhizobia populations from zones EUM and TLM clustered together with a bootstrap value of 82 % (Figure 4.2).

Table 4.8: Pairwise Population Matrix of Nei Unbiased Genetic Distance of four rhizobia populations from Eastern Kenya based on ARDRA restriction patterns

ELM	EUM	TLM	TUM	
0.000				ELM
0.028	0.000			EUM
0.023	0.004	0.000		TLM
0.034	0.037	0.029	0.000	TUM

ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

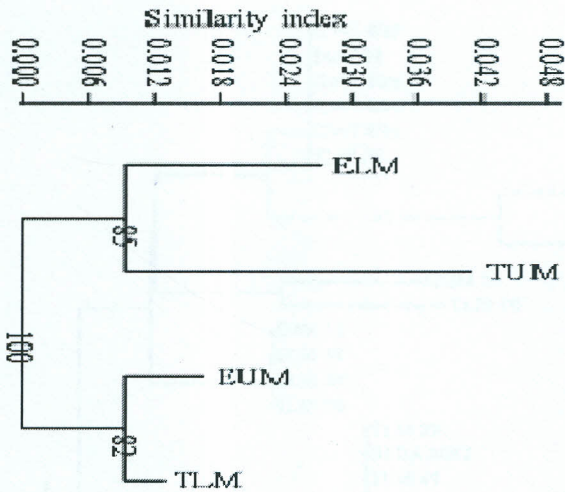


Figure 4.2: A neighbour joining dendrogram based on Nei's 1978 unbiased genetic distance and Euclidian similarity index showing the genetic distance of four rhizobia populations from Eastern Kenya. Numbers shown at the nodes of the dendrogram indicate the percentage bootstrap support for 1000 iterations. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

Based on the genetic distance after amplified rDNA restriction analyses and Euclidian similarity index, the phylogenetic tree clustered native rhizobia isolates into three main clusters (I, II and III) (Figure 4.3). Cluster I, which comprised of majority of the isolates. Cluster II was the second largest, while cluster III had only one isolate ELM5, which was completely different from the rest. The reference strain *Rhizobium tropici* CIAT 899 which was placed in cluster I, clustered together with native rhizobia isolates ELM33 and TLM32, indicating their close genetic relationship. Native isolates TUM26, TLM28 and EUM23 clustered together despite originating from different agroecological zones of Eastern Kenya. *Rhizobium etli* USDA 2667 strain was also placed in cluster I and was grouped together with TLM27 and TUM 41 isolates (Figure 4.3). The native isolate EUM7 clustered closely to *Rhizobium leguminosarum* strain 446 in cluster II but in a separate sub-branch.

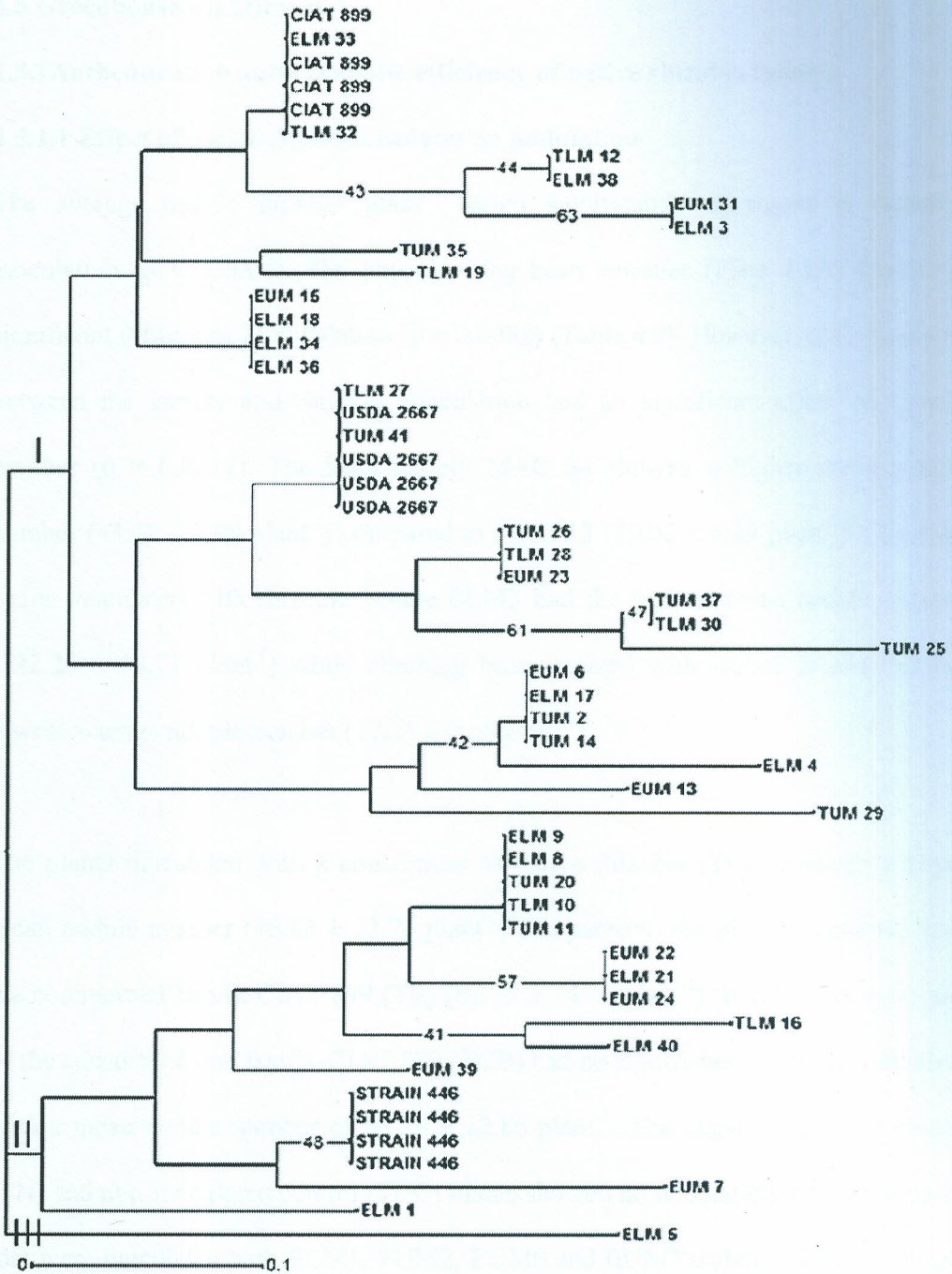


Figure 4.3: Phylogenetic relationship of 41 native rhizobia isolates from Eastern Kenya and 3 reference rhizobia strains (CIAT 899, USDA 2667 and strain 446) inferred using the Neighbor-Joining method. Numbers shown at the nodes of the dendrogram indicate the percentage bootstrap support for 1000 iterations. Only bootstrap values $\geq 40\%$ are shown. Scale bar indicates number of substitutions per site. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

4.5 Greenhouse experiment

4.5.1 Authentication and symbiotic efficiency of native rhizobia isolates

4.5.1.1 Effect of native rhizobia isolates on nodulation

The average nodule number plant⁻¹ varied significantly in regard to rhizobia inoculation ($p < 0.0001$). The two climbing bean varieties (Plate 4.5A) showed a significant difference in nodulation ($p = 0.0082$) (Table 4.9). However, the interaction between the variety and rhizobia inoculation had no significant effect on nodule number ($p = 0.0817$). The bean variety MAC 64 showed a higher mean nodule number (43.41 ± 7.08 plant⁻¹) compared to MAC 13 (29.05 ± 5.49 plant⁻¹). Climbing beans inoculated with rhizobia isolate ELM3 had the highest mean nodule number (122.25 ± 13.71 plant⁻¹) while climbing beans treated with isolate ELM4 had the lowest average nodule number (12.25 ± 5.68 plant⁻¹).

The plants inoculated with a consortium of native rhizobia (TC) produced a lower mean nodule number (46.63 ± 12.76 plant⁻¹) compared to the plants inoculated with the commercial Biofix-CIAT 899 (TB) (63.75 ± 14.76 plant⁻¹). In addition, a mixture of the consortium and Biofix-CIAT 899 (TCB) had no significant effect on nodulation with a mean nodule number of 52.25 ± 12.86 plant⁻¹. The negative nitrogen-treated (TN) and non-inoculated control (TUC) plants showed no nodulation. Climbing beans that were inoculated with ELM1, TUM2, EUM6 and EUM7 isolates did not nodulate (Table 4.9). The nodules of both climbing bean varieties occurred mainly on the lateral roots with a few on the taproots (Plate. 4.5B). Majority of the nodules were pink in colour (Plate. 4.5C) with a few appearing dull, green and whitish.

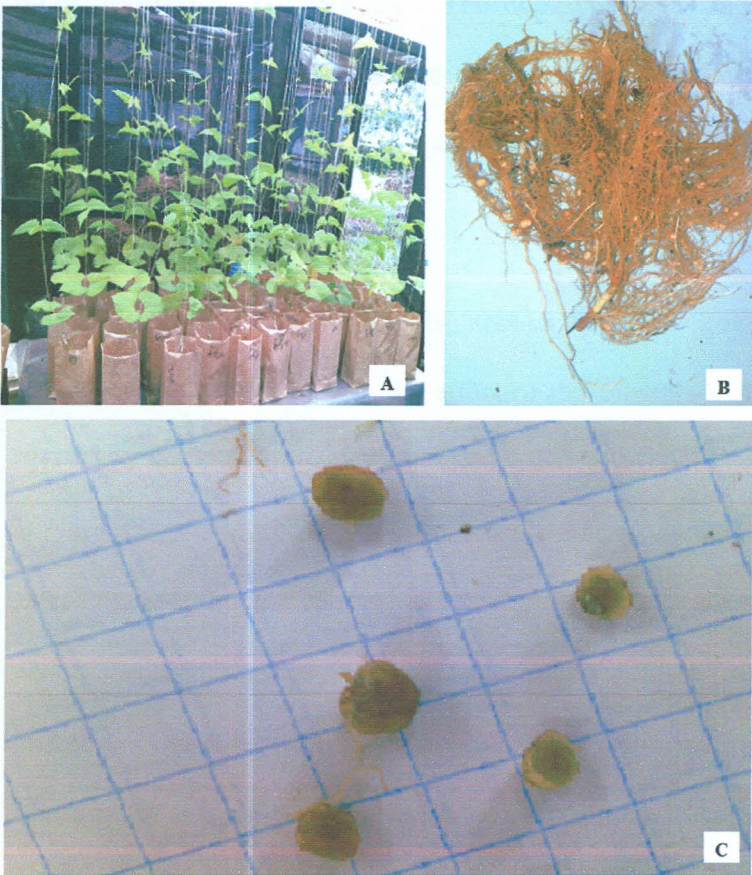


Plate 4.5: Authentication experiment in the greenhouse. **A**, Greenhouse experiment showing 3 weeks old MAC 13 climbing beans variety. **B**, Root nodules of MAC 64 climbing bean inoculated with ELM3 native rhizobia isolate. **C**, Dissected root nodules of MAC 64 climbing bean inoculated with native rhizobia isolate ELM3 showing pink centred coloration.

4.5.1.2 Effect of native rhizobia isolates on nodule dry weight

The nodule dry weight plant⁻¹ (NDW) significantly differed between the two climbing bean varieties ($p = 0.0147$) and across rhizobia inoculated plants ($p < 0.0001$) (Table 4.9). There was no significant interaction between bean variety and rhizobia inoculants on NDW ($p = 0.1574$). The climbing bean variety MAC 64 had a higher mean NDW of 0.05 ± 0.01 g plant⁻¹ compared to MAC 13 with 0.03 ± 0.01 g plant⁻¹. Climbing beans inoculated with isolate ELM3 had the highest mean NDW of 0.15 ± 0.02 g plant⁻¹ while climbing beans treated with isolate ELM4 had the lowest average

NDW of $0.02 \pm 0.01 \text{ g plant}^{-1}$. The climbing beans inoculated with commercial Biofix-CIAT 899 (TB) produced a higher mean NDW ($0.08 \pm 0.02 \text{ g plant}^{-1}$) compared to the plants inoculated with the consortium of native rhizobia (TC) ($0.05 \pm 0.02 \text{ g plant}^{-1}$) (Table 4.9).

4.5.1.3 Effect of native rhizobia isolates on shoot dry weight (SDW)

Based on two-way ANOVA, the effect of the rhizobia isolates on shoot dry weight (SDW) of the climbing bean was significant ($p < 0.0001$) (Table 4.9). The effect of climbing bean variety on SDW was also found to be significant ($p = 0.0106$) with MAC 13 recording a higher mean SDW ($0.96 \pm 0.03 \text{ g plant}^{-1}$) compared to MAC 64 which recorded a mean SDW of $0.87 \pm 0.03 \text{ g plant}^{-1}$. There was no significant interaction effect between bean variety and rhizobia isolates on SDW ($p = 0.9119$). The highest mean SDW was recorded in climbing beans inoculated with rhizobia isolate ELM3 ($1.25 \pm 0.07 \text{ g plant}^{-1}$), followed by beans supplemented with nitrogen (TN) with $1.01 \pm 0.06 \text{ g plant}^{-1}$ and the lowest mean SDW was recorded in climbing beans inoculated with isolate ELM1 which had $0.67 \pm 0.06 \text{ g plant}^{-1}$. The climbing beans inoculated with Biofix-CIAT 899 (TB) had a SDW of $0.96 \pm 0.07 \text{ g plant}^{-1}$ and it performed almost equally with the treatment consisting of a mixture of a consortium of the isolates and the Biofix-CIAT 899 inoculum (TCB) ($\text{SDW} = 0.97 \pm 0.04 \text{ g plant}^{-1}$) (Table 4.9).

4.5.1.4 Effect of rhizobia isolates on root dry weight (RDW)

The effect of rhizobia isolates on root dry weight (RDW) plant^{-1} was significant ($p = 0.0011$) (Table 4.9). However, the effect of bean variety on RDW plant^{-1} was not significant ($p = 0.2815$). There was no significant interaction effect between the

rhizobia isolates and bean variety on RDW ($p = 0.2961$). The climbing beans supplemented with nitrogen (TN) had the highest RDW of $0.58 \pm 0.06 \text{ g plant}^{-1}$, followed closely by beans inoculated with isolate ELM3 with $0.57 \pm 0.02 \text{ g plant}^{-1}$ (Table 4.9). The negative controls (TUC) had the lowest RDW ($0.30 \pm 0.02 \text{ g plant}^{-1}$).

Table 4.9: Effect of rhizobia isolate treatments, bean variety, and their interaction on shoot dry weight, root dry weight, nodule number and nodule dry weight in the greenhouse experiment

Treatments	Shoot dry weight (SDW) g plant^{-1}	Root dry weight (RDW) g plant^{-1}	Nodule dry weight (NDW) g plant^{-1}	Nodule number (NN) plant^{-1}
Test isolates				
ELM1	$0.67 \pm 0.06\text{d}$	$0.42 \pm 0.03\text{ab}$	0	0
TUM2	$0.84 \pm 0.06\text{bcd}$	$0.50 \pm 0.06\text{ab}$	0	0
ELM3	$1.25 \pm 0.07\text{a}$	$0.57 \pm 0.02\text{a}$	$0.15 \pm 0.02\text{a}$	$122.25 \pm 13.71\text{a}$
ELM4	$0.98 \pm 0.09\text{abc}$	$0.51 \pm 0.06\text{ab}$	$0.02 \pm 0.01\text{cd}$	$12.25 \pm 5.68\text{cd}$
ELM5	$1.00 \pm 0.05\text{abc}$	$0.55 \pm 0.04\text{a}$	$0.07 \pm 0.02\text{bc}$	$63.50 \pm 14.76\text{b}$
EUM6	$0.85 \pm 0.06\text{bcd}$	$0.44 \pm 0.03\text{ab}$	0	0
EUM7	$0.79 \pm 0.06\text{bcd}$	$0.51 \pm 0.07\text{ab}$	0	0
ELM8	$0.99 \pm 0.06\text{abc}$	$0.39 \pm 0.04\text{ab}$	$0.10 \pm 0.02\text{ab}$	$77.63 \pm 17.54\text{ab}$
ELM9	$0.92 \pm 0.03\text{bcd}$	$0.43 \pm 0.03\text{ab}$	$0.08 \pm 0.02\text{b}$	$69.00 \pm 16.11\text{b}$
TC	$0.87 \pm 0.06\text{bcd}$	$0.39 \pm 0.04\text{ab}$	$0.05 \pm 0.02\text{bcd}$	$46.63 \pm 12.76\text{bcd}$
TB	$0.96 \pm 0.07\text{abc}$	$0.47 \pm 0.06\text{ab}$	$0.08 \pm 0.02\text{b}$	$63.75 \pm 15.93\text{b}$
TN	$1.01 \pm 0.06\text{ab}$	$0.58 \pm 0.06\text{a}$	0	0
TCB	$0.97 \pm 0.04\text{abc}$	$0.49 \pm 0.05\text{ab}$	$0.06 \pm 0.02\text{bc}$	$52.25 \pm 12.86\text{bc}$
TUC	$0.71 \pm 0.03\text{cd}$	$0.30 \pm 0.02\text{b}$	0	0
Variety				
MAC 13	$0.96 \pm 0.03\text{a}$	$0.48 \pm 0.02\text{a}$	$0.03 \pm 0.01\text{b}$	$29.05 \pm 5.49\text{b}$
MAC 64	$0.87 \pm 0.03\text{b}$	$0.46 \pm 0.02\text{a}$	$0.05 \pm 0.01\text{a}$	$43.41 \pm 7.08\text{a}$
P. values of the main factors and their interactions				
Test isolates	<0.0001	0.0011	<0.0001	<0.0001
Variety	0.0106	0.2815	0.0147	0.0082
Variety x Test isolates	0.9119	0.2961	0.1574	0.0817

Means followed by same lower case letter(s) within the same column are not significantly different at $p < 0.05$ according to Tukey's HSD test. ELM1 to ELM9, Native rhizobia isolates; TC, Consortium of native rhizobia; TB, Commercial inoculant (Biofix-CIAT 899); TN, Negative control with nitrogen treatment; TCB, Biofix-CIAT 899 combined with consortium; TUC, Negative control without nitrogen; MAC, Mid altitude climbers. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

4.5.1.5 Symbiotic efficiency of native rhizobia isolates

Generally, the native rhizobia isolates that showed positive nodulation with MAC 13 and MAC 64 climbing beans recorded a high symbiotic efficiency (SE above 80 %) (Figure 4.4). There was no significant difference on SE between the two bean varieties ($p = 0.1871$). There was a significant effect of the rhizobia isolates on symbiotic efficiency ($p < 0.0001$). Four native isolates ELM3, ELM5, ELM8 and ELM4 with SE of 123.72%, 99.21%, 98.24% and 96.75%, respectively, compared favorably with the commercial rhizobia inoculant (Biofix-CIAT 899) (SE of 95.21%). Climbing beans inoculated with isolate ELM3, which had the highest mean SDW and the highest average nodule number, produced the highest SE of 123.72 %. The climbing beans inoculated with a consortium of the native isolates (TC) had the lowest SE of 86.17 %. There was no significant difference in SE of climbing beans inoculated with native isolates ELM4, ELM5, ELM8, Biofix-CIAT 899 (TB), and a mixture of native consortium and Biofix-CIAT 899 (TCB) (Figure 4.4). Native isolates ELM1, TUM2, EUM6 and EUM7 were not tested for their SE because they did not form nodules with climbing beans.

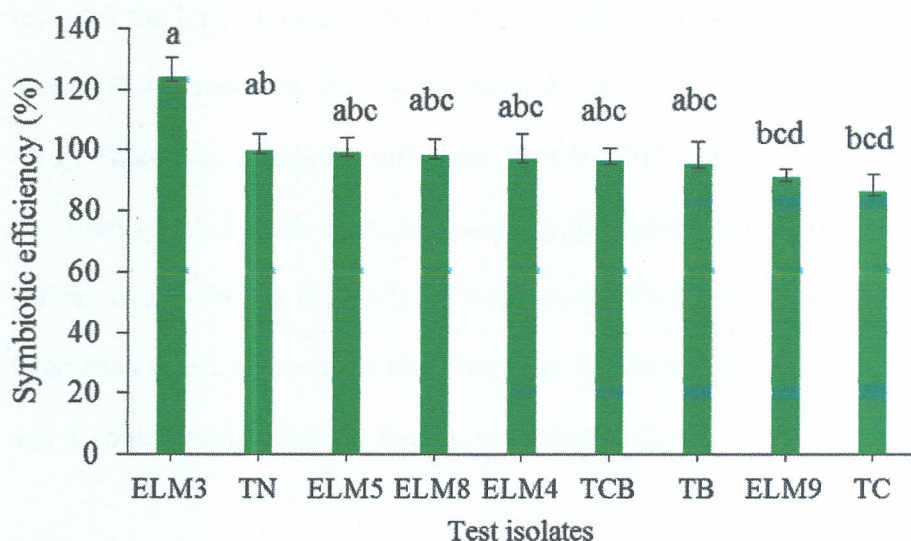


Figure 4.4: Symbiotic efficiencies of native rhizobia isolates from Eastern Kenya. ELM3, ELM4, ELM5, ELM8 and ELM9, Native rhizobia isolates which nodulated; TC, Consortium of native rhizobia; TB, Commercial inoculant (Biofix-CIAT 899); TN, Negative control with nitrogen treatment; TCB, Biofix-CIAT 899 combined with consortium.

4.5.1.6 Effect of inoculation on total nitrogen (%N), phosphorous (P) and potassium (K)

There was a significant difference ($p < 0.0001$) in percentage total nitrogen (%N) accumulated by climbing beans inoculated with rhizobia isolates over non-inoculated controls (Table 4.10). Nitrogen treated (TN) climbing beans recorded the highest %N (3.71 ± 0.09 %) accumulated in the shoots, followed by beans inoculated with test isolate ELM3 (3.46 ± 0.11 %). Non-inoculated plants without nitrogen application (TUC) had the least %N of 0.73 ± 0.02 %. There was no significant difference on %N and P content between the two bean varieties ($p = 0.5464$ and $p = 0.6389$ respectively) (Table 4.10).

Inoculation of climbing beans significantly ($p < 0.0001$ for P and K) affected P and K content accumulated in the shoots. Climbing beans inoculated with test isolate TUM2

recorded the highest shoot P ($4,026.25 \pm 194.50$ ppm) while beans inoculated with isolate ELM3 recorded the highest shoot K ($9,125 \pm 184.36$ ppm) content (Table 4.10). There was significant difference ($p < 0.0001$) in K accumulated between the two bean varieties, with MAC 64 recording the highest K ($7,160.71 \pm 208.62$ ppm) content over MAC 13 ($6,473.21 \pm 243.14$ ppm). However, there was no significant interaction effect between the climbing bean variety and rhizobia isolates on %N, P and K content accumulated in the shoots (Table 4.10).

Table 4.10: Effect of inoculation on shoot total nitrogen (%N), phosphorous (P) and potassium (K) contents from the greenhouse experiment

Treatments	Total nitrogen (%N)	Phosphorous (P) ppm	Potassium (K) ppm
Test isolate			
ELM1	1.63 ± 0.06f	2587.50 ± 333.29cde	5625.00 ± 205.94def
TUM2	1.89 ± 0.11def	4026.25 ± 194.50a	8000.00 ± 353.55ab
ELM3	3.46 ± 0.11ab	3543.75 ± 184.36ab	9125.00 ± 409.16a
ELM4	2.56 ± 0.20cd	3109.38 ± 208.51bcd	6000.00 ± 694.37def
ELM5	3.01 ± 0.08bc	3234.38 ± 431.97bc	8000.00 ± 517.55ab
EUM6	1.60 ± 0.30f	2495.00 ± 101.08de	5125.00 ± 156.70f
EUM7	1.84 ± 0.16ef	2549.38 ± 412.64de	6375.00 ± 540.75cdef
ELM8	2.56 ± 0.09cd	3488.75 ± 513.93ab	7625.00 ± 279.51bc
ELM9	2.46 ± 0.10cde	3979.88 ± 116.28a	6625.00 ± 279.51bcde
TC	2.44 ± 0.12cd	2623.75 ± 200.32cde	5250.00 ± 389.60ef
TB	2.83 ± 0.07bc	3476.00 ± 308.06ab	6750.00 ± 574.77bcd
TN	3.71 ± 0.09a	2436.63 ± 110.69e	6750.00 ± 779.19ab
TCB	2.11 ± 0.14df	1771.88 ± 8.44f	6937.50 ± 519.16bcd
TUC	0.73 ± 0.02g	3087.50 ± 394.18bcde	6000.00 ± 566.95def
Variety			
MAC 13	2.37 ± 0.12a	3012.36 ± 159.67a	6473.21 ± 243.14b
MAC 64	2.32 ± 0.11a	3046.21 ± 99.73a	7160.71 ± 208.62a
P. values of the main factors and their interactions			
Test isolates	<0.0001	<0.0001	<0.0001
Variety	0.5464	0.6389	<0.0001
Variety x Test isolates	0.8609	0.5102	0.6231

Means followed by same lower case letter(s) within the same column are not significantly different at $p < 0.05$ according to Tukey's HSD test. ELM1 to ELM9, Native rhizobia isolates; TC, Consortium of native rhizobia; TB, Commercial inoculant (Biofix-CIAT 899); TN, Negative control with nitrogen treatment; TCB, Biofix-CIAT 899 combined with consortium; TUC, Negative control without nitrogen; MAC, Mid altitude climbers. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

4.5.1.7 Correlation analysis between parameters of nitrogen fixation in the greenhouse experiment

Correlation analysis showed a significant relationship between nodule dry weight and shoot dry weight of climbing beans ($R^2 = 0.5763$, $p = 0.029$) (Figure 4.5). Similarly, there was a significant relationship between nodule dry weight and shoot nitrogen concentration ($R^2 = 0.631$, $p = 0.021$) (Figure 4.6).

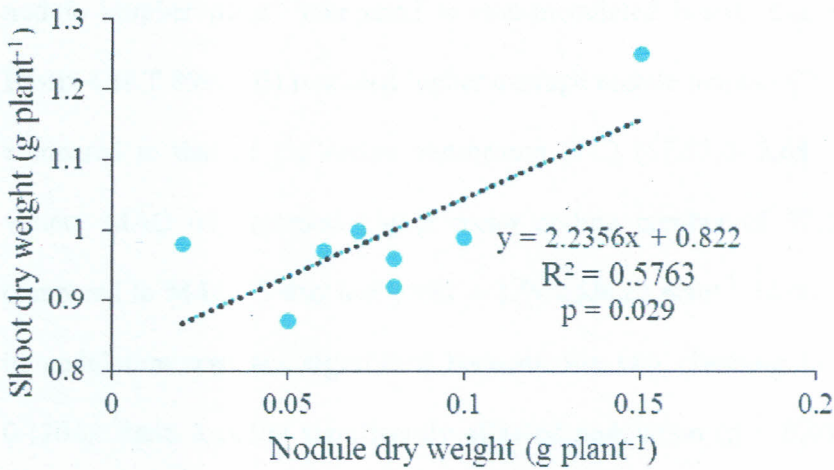


Figure 4.5: Correlation analysis between shoot dry weight and nodule dry weight.

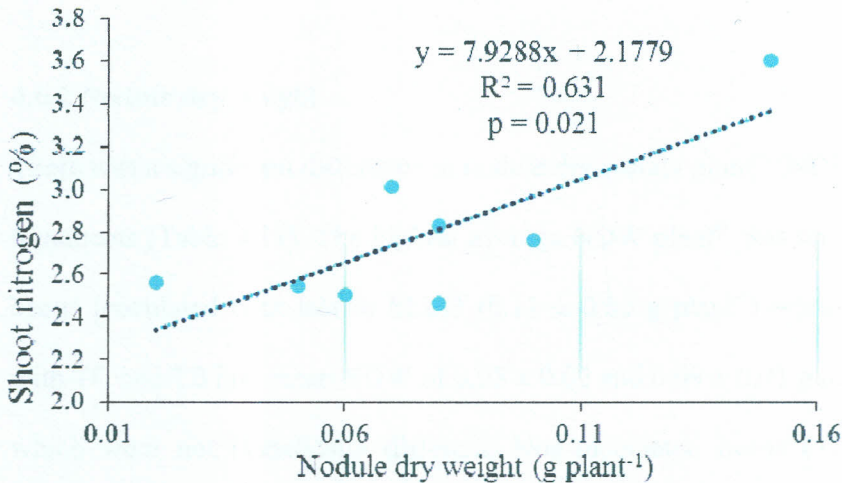


Figure 4.6: Correlation analysis between nodule dry weight and shoot nitrogen.

4.6 Effect of rhizobia inoculation in the field

4.6.1 Nodulation

At mid-flowering, results on nodulation showed that climbing beans inoculated with ELM3 (best native isolate) had the highest average number of nodules (85.58 ± 3.74 plant⁻¹) while non-inoculated beans TUC (negative control) had the lowest average nodule number of 52.08 ± 3.33 plant⁻¹ (Table 4.11). Rhizobia inoculation significantly affected nodulation ($p < 0.0001$) since inoculated beans produced a higher mean nodule number plant⁻¹ compared to non-inoculated beans. Beans inoculated with Biofix-CIAT 899 (TB) recorded higher average nodule number (76.13 ± 3.77 plant⁻¹) compared to that of the native consortium (TC) (67.63 ± 3.68 plant⁻¹). The bean variety MAC 64 recorded higher mean nodule number of 72.62 ± 2.77 plant⁻¹ compared to MAC 13 that had 68.82 ± 2.59 nodules plant⁻¹. However, the difference in nodulation was not significant between the two climbing bean varieties ($p = 0.1204$). Farm location significantly affected nodulation ($p < 0.0001$) (Table 4.11). Farm ELM had the highest average nodule number of 88.07 ± 2.94 plant⁻¹ while farm EUM had the lowest mean nodule number with 51.13 ± 2.36 plant⁻¹ (Table 4.11).

4.6.2 Nodule dry weight

There was a significant difference in nodule dry weight plant⁻¹ (NDW) among the five treatments (Table 4.11). The highest average NDW plant⁻¹ was recorded by climbing beans inoculated with isolate ELM3 (0.11 ± 0.06 g plant⁻¹) while beans inoculated with TC and TB had mean NDW of 0.08 ± 0.02 and 0.09 ± 0.01 g plant⁻¹ respectively, which were not statistically different. Non-inoculated beans (TUC) recorded the lowest mean NDW of 0.06 ± 0.01 g plant⁻¹. There was also a significant difference in

NDW plant⁻¹ between the two varieties ($p = 0.015$) whereby MAC 64 bean variety recorded a mean NDW of 0.09 ± 0.01 g plant⁻¹ while MAC 13 had a mean NDW of 0.08 ± 0.01 g plant⁻¹. Farm location had significant effect on NDW of climbing beans ($p < 0.0001$). Farm ELM had the highest average NDW of 0.11 ± 0.01 g plant⁻¹ while farm EUM had the lowest mean NDW of 0.05 ± 0.01 g plant⁻¹ (Table 4.11).

4.6.3 Shoot dry weight

In the field, inoculation of climbing beans with different rhizobia isolates increased SDW. This is evident by the high SDW plant⁻¹ recorded by the inoculated plants as compared to the non-inoculated control TUC (Table 4.11). Significant differences ($p < 0.0001$) were observed on SDW among the five treatments. The plants inoculated with the best native isolate (ELM3) showed the highest mean SDW of 11.90 ± 0.80 g plant⁻¹. The effect of climbing bean inoculation with Biofix-CIAT 899 (TB), native consortium (TC) and as well as the combination of the consortium + Biofix-CIAT 899 (TCB) on SDW did not differ significantly with values of 10.56 ± 0.73 , 9.58 ± 0.76 and 9.92 ± 0.78 g plant⁻¹ respectively. Although MAC 64 bean variety recorded a relatively higher SDW of 10.08 ± 0.49 g plant⁻¹ compared to MAC 13 (9.63 ± 0.49 g plant⁻¹), the difference was statistically insignificant ($p = 0.0946$). Farm location affected significantly ($p < 0.0001$) the SDW plant⁻¹ of the climbing beans. Farm ELM, located in Embu lower midland AEZ recorded the highest mean SDW (13.66 ± 0.49 g plant⁻¹) while farm EUM recorded the lowest mean SDW of 4.79 ± 0.23 g plant⁻¹ (Table 4.11).

4.6.4 Root dry weight

There was a significant difference ($p = 0.006$) (Table 4.11) observed on RDW between inoculated and non-inoculated plants. Although climbing beans inoculated with the native rhizobia (ELM3) had a higher mean RDW ($3.53 \pm 0.36 \text{ g plant}^{-1}$) compared to other treatments, there was no significant difference observed in beans inoculated with isolates TB, TC and TCB. Non-inoculated climbing beans (TUC) however, had the lowest mean RDW of $2.49 \pm 0.16 \text{ g plant}^{-1}$. There was a significant difference ($p = 0.003$) in RDW of the two climbing bean varieties, where MAC 64 had higher mean RDW of $3.27 \pm 0.17 \text{ g plant}^{-1}$ compared to MAC 13 which had a mean RDW of $2.74 \pm 0.14 \text{ g plant}^{-1}$. Farm location influenced significantly ($p < 0.0001$) the mean RDW of climbing beans. Among the four farms, where climbing beans were grown, TLM farm had the highest average RDW of $3.66 \pm 0.25 \text{ g plant}^{-1}$ while the lowest mean RDW was recorded in plants grown in farm EUM, $1.97 \pm 0.19 \text{ g plant}^{-1}$ (Table 4.11).

Table 4.11: Effect of rhizobia isolates, bean variety, farm location, and their interactions on shoot dry weight, root dry weight, nodule number and nodule dry weight in the field experiment

Treatments	Nodule number (plant ⁻¹)	Nodule dry weight (g plant ⁻¹)	Shoot dry weight (g plant ⁻¹)	Root dry weight (g plant ⁻¹)
Inoculant				
ELM3	85.58±3.74a	0.11±0.06a	11.90±0.80a	3.53±0.36a
TC	67.63±3.68b	0.08±0.02b	9.58±0.76b	2.96±0.25ab
TB	76.13±3.77ab	0.09±0.01b	10.56±0.73b	2.91±0.18ab
TCB	72.17±3.70b	0.09±0.02ab	9.92±0.78b	3.15±0.25ab
TUC	52.08±3.33c	0.06±0.01c	7.34±0.54c	2.49±0.16b
Variety				
MAC 13	68.82±2.59a	0.08±0.01a	9.63±0.49a	2.74±0.14b
MAC 64	72.62±2.77a	0.09±0.01b	10.08±0.49a	3.27±0.17a
Farm				
EUM	51.13±2.36c	0.05±0.01c	4.79±0.23c	1.97±0.19c
ELM	88.07±2.94a	0.11±0.01a	13.66±0.49a	3.39±0.18ab
TUM	73.03±3.52b	0.09±0.02b	10.87±0.35b	3.02±0.15b
TLM	70.63±2.94b	0.09±0.01b	10.13±0.38b	3.66±0.25a
P. values of the main factors and their interactions				
Variety	0.1204	0.015	0.0946	0.003
Farm	<0.0001	<0.0001	<0.0001	<0.0001
Inoculant	<0.0001	<0.0001	<0.0001	0.006
Variety x Farm	0.4129	0.0686	0.9933	0.0511
Variety x Inoculant	0.8706	0.4976	0.9669	0.9607
Farm x Inoculant	0.9993	0.0504	0.2166	0.5980
Farm x Inoculant				
*Variety	0.9975	0.4137	0.8269	0.0694

Means followed by same lower case letter(s) within the same column are not significantly different at $p < 0.05$ according to Tukey's HSD test. ELM3, Test native rhizobia isolate; TC, Consortium of native rhizobia; TB, Commercial inoculant (Biofix-CIAT 899); TCB, Biofix-CIAT 899 combined with consortium; TUC, Negative control (Non-inoculated); MAC, Mid altitude climbers. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

4.6.5 Pod number

Inoculation of climbing beans significantly affected the average number of pods produced by the plants ($p < 0.0001$) (Table 4.12). Climbing beans inoculated with

native isolate ELM3 produced the highest mean pod number of $29.75 \pm 1.19 \text{ plant}^{-1}$ while non-inoculated climbing beans had the lowest average pod number of $16.44 \pm 0.74 \text{ plant}^{-1}$. Bean variety had a significant effect on pod number ($p = 0.001$) and variety MAC 64 produced a higher mean number of pods of $24.01 \pm 1.93 \text{ plant}^{-1}$ compared to MAC 13 ($21.79 \pm 0.79 \text{ plant}^{-1}$). Farm location affected significantly the number of pods formed per plant ($p < 0.0001$), whereby climbing beans grown in farm ELM had the highest average pod number of $28.10 \pm 1.49 \text{ plant}^{-1}$ while beans grown in farm EUM had the lowest mean pod number of $19.93 \pm 1.01 \text{ plant}^{-1}$.

4.6.6 Stover dry weight

Inoculation of climbing beans significantly enhanced their stover dry weight (STW) ($p < 0.0001$) (Table 4.12). Climbing beans treated with native isolate ELM3 had the highest STW ($35.14 \pm 1.56 \text{ g plant}^{-1}$) while non-inoculated TUC plants recorded the lowest STW of $21.76 \pm 1.58 \text{ g plant}^{-1}$. There was no significant difference in STW of plants inoculated with isolates TC and TCB, which recorded 25.09 ± 1.46 and $26.41 \pm 1.51 \text{ g plant}^{-1}$ respectively. The STW between the two climbing bean varieties differed significantly ($p < 0.0001$). MAC 64 bean variety had higher mean STW of $30.17 \pm 1.16 \text{ g plant}^{-1}$ compared to MAC 13, which had an average STW of $25.26 \pm 0.96 \text{ g plant}^{-1}$. Farm location significantly affected the bean STW per plant ($p < 0.0001$), whereby beans grown in farm ELM and EUM recorded the highest ($34.23 \pm 0.94 \text{ g plant}^{-1}$) and the lowest ($20.34 \pm 1.01 \text{ g plant}^{-1}$) mean STW respectively (Table 4.12).

4.6.7 Seed yields

There was a significant difference in the mean total seed yield ($p = 0.0017$) between the two climbing bean varieties (Table 4.12). Variety MAC 64 had higher mean seed yield of $3,495.23 \pm 148.45$ kg/ha compared to MAC 13, which had an average seed yield of $3,170.98 \pm 125.36$ kg/ha. Inoculation of climbing beans had a significant effect ($p < 0.0001$) on seed yield per plant. Inoculated climbing beans recorded a higher mean grain yield over non-inoculated plants. Beans treated with the test isolate ELM3 produced the highest seed yield of $4,397.75 \pm 185.01$ kg/ha while non-inoculated beans recorded the lowest seed yield of $2,324.81 \pm 165.68$ kg/ha. Farm location also affected significantly ($p < 0.0001$) the seed yield with climbing beans grown in farm ELM recording the highest yield of $4,173.05 \pm 225.82$ kg/ha. Climbing beans grown in farms TUM and EUM, both of which are located in upper midland AEZ, produced the least seed yield of $3,244.32 \pm 143.96$ kg/ha and $2,777.81 \pm 151.72$ kg/ha respectively. A significant interaction effect was observed between farm location and bean variety ($p = 0.0008$) on total seed yield (Table 4.12). MAC 64 bean variety recorded the highest seed yield ($4,691.26$ Kg/Ha) in ELM farm while MAC 13 produced the lowest seed yield ($2,644.05$ Kg/Ha) in EUM farm (Figure 4.7).

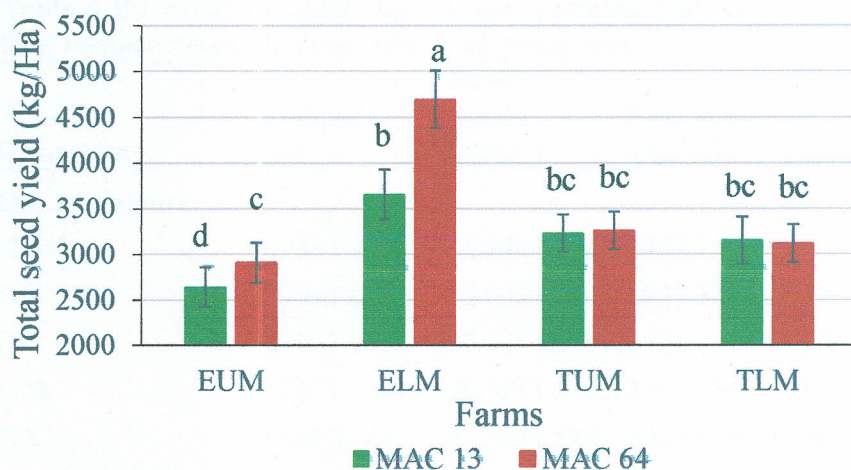


Figure 4.7: Interactive effects of bean variety with farm location on total seed yield of climbing beans. Bars followed by the same letter are not significantly different according to Turkey's HSD test at $P \leq 0.05$. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

4.6.8 100 seeds weight

The effect of inoculation on 100 seeds weight of climbing beans in all the five treatments was statistically significant ($p < 0.0001$) (Table 4.12). Non-inoculated beans had an average 100 seeds weight of $70.55 \pm 0.50 \text{ g plant}^{-1}$, which was statistically similar to that of climbing beans inoculated with native consortium TC ($70.47 \pm 0.76 \text{ g plant}^{-1}$). Climbing beans inoculated with Biofix-CIAT 899 TB had the highest average 100 seeds weight of $74.42 \pm 0.59 \text{ g plant}^{-1}$. There was no significant difference in 100 seeds weight between the two climbing bean varieties ($p = 0.8038$). Farm location affected significantly ($p < 0.0001$) the 100 seeds weight of climbing beans. Beans grown in farms TLM and ELM recorded the highest mean weight of 100 seeds ($73.98 \pm 0.34 \text{ g plant}^{-1}$ and $73.71 \pm 0.52 \text{ g plant}^{-1}$ respectively) while the lowest mean weight of 100 seeds was recorded in farm EUM with $69.37 \pm 0.71 \text{ g plant}^{-1}$ (Table 4.12).

Table 4.12: Effect of isolate inoculation, climbing bean variety, farm location and their interactions on climbing bean yield parameters

Treatments	No of pods (plant ⁻¹)	Stover weight (plant ⁻¹)	Seed yield (kg/ha)	100 seeds weight (plant ⁻¹)
Test inoculants				
ELM3	29.75±1.19a	35.14±1.56a	4397.75±185.01a	73.79±0.46ab
TC	20.79±1.05c	25.09±1.46c	2941.32±165.68c	70.47±0.76c
TB	24.78±1.25b	30.20±1.38b	3698.79±200.37b	74.42±0.59a
TCB	22.74±1.03bc	26.41±1.51c	3302.85±167.51bc	72.36±0.64b
TUC	16.44±0.74d	21.76±1.58d	2324.81±109.24d	70.55±0.50c
Variety				
MAC 13	21.79±0.79b	25.26±0.96b	3170.98±125.36b	72.37±0.41a
MAC 64	24.01±1.93a	30.17±1.16a	3495.23±148.45a	72.27±0.45a
Farm				
EUM	19.93±1.01c	20.34±1.01d	2777.81±151.72c	69.37±0.71c
ELM	28.10±1.49a	34.23±0.94a	4173.05±225.82a	73.71±0.52a
TUM	22.40±0.93b	25.57±1.16c	3244.32±143.96b	72.21±0.40b
TLM	21.18±1.06b	30.73±1.73b	3137.24±161.09bc	73.98±0.34a
P. values of main treatments and their interactions				
Variety	0.001	<0.0001	0.0017	0.8038
Farm	<0.0001	<0.0001	<0.0001	<0.0001
Test inoculant	<0.0001	<0.0001	<0.0001	<0.0001
Variety x Farm	0.0522	0.1020	0.0008	0.0589
Variety x inoculant	0.5273	0.8936	0.4172	0.6914
Farm x inoculant	0.0967	0.9303	0.0535	0.0614
Farm x inoculant x Variety	0.0585	0.9903	0.0681	0.0508

Means followed by same lower case letter(s) within the same column are not significantly different at $p < 0.05$ according to Tukey's HSD test. ELM3, Test native rhizobia isolate; TC, Consortium of native rhizobia; TB, Commercial inoculant (Biofix-CIAT 899); TCB, Biofix-CIAT 899 combined with consortium; TUC, Negative control (Non-inoculated); MAC, Mid altitude climbers.

4.6.9 Total nitrogen (%N), phosphorous (P) and potassium (K) shoot content in the field experiment

The total nitrogen (% N) shoot content of climbing beans were significantly ($p < 0.0001$) affected by rhizobia inoculation as observed in both climbing bean varieties (Table 4.13). The highest % N (3.342 ± 0.115 %) was found in climbing beans inoculated with test isolate ELM3. The non-inoculated control plants recorded the lowest % N (1.766 ± 0.134 %). There was a significant difference ($p = 0.001$) in % N accumulated by the two climbing bean varieties, where MAC 64 had a higher mean %N of 2.667 ± 0.089 % compared to MAC 13 which had a mean %N of 2.499 ± 0.105 %. Farm location influenced significantly ($p < 0.0001$) the mean % N of shoots. Among the four farms, climbing beans grown in farm ELM and EUM recorded higher mean % N (2.844 ± 1.49 % and 2.790 ± 1.01 % respectively) compared to farms TLM and TUM which recorded a mean % N of 2.419 ± 1.06 % and 2.280 ± 0.93 % respectively. There was a significant interaction effect between farm location and rhizobia inoculants ($p = 0.0102$) on % N of shoots (Table 4.13). Climbing beans inoculated with native isolate ELM3 accumulated a higher % shoot N in all the farms (Figure 4.8).

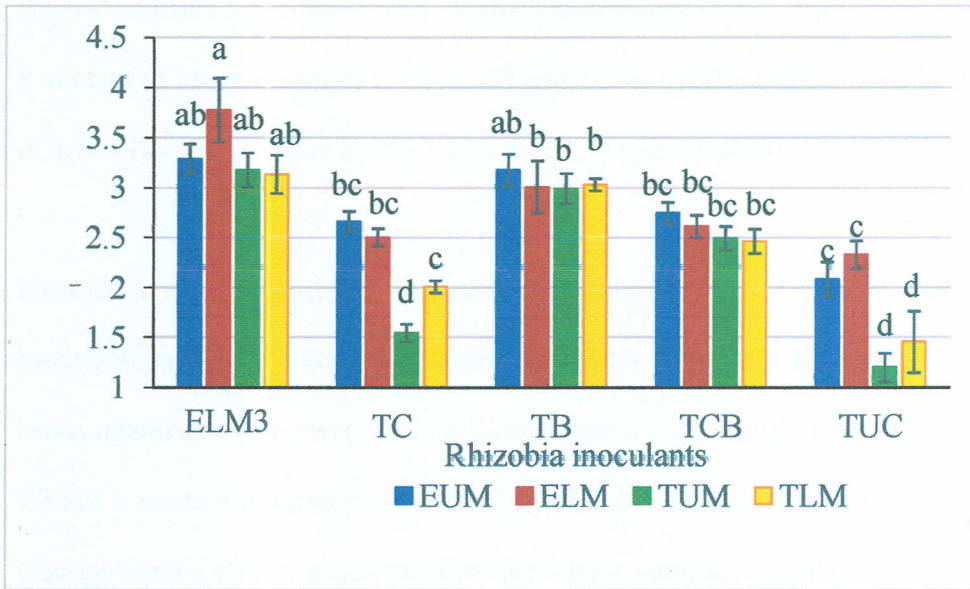


Figure 4.8: Interactive effects of farm location with rhizobia inoculants on % shoot N of climbing beans. Bars followed by the same letter are not significantly different according to Turkey's HSD test at $P \leq 0.05$. ELM3, Test native rhizobia isolate; TC, Consortium of native rhizobia; TB, Commercial inoculant (Biofix-CIAT 899); TCB, Biofix-CIAT 899 combined with native consortium; TUC, Negative control (Non-inoculated); ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

The shoot phosphorous (P) and potassium (K) contents varied significantly ($p < 0.0001$) in the field and remarkably, non-inoculated beans recorded the highest shoot P content ($3,608.60 \pm 192.49$ ppm) (Table 4.13). The shoot P content of beans inoculated with isolates ELM3 and TCB did not differ significantly. Climbing beans inoculated with the native consortium TC recorded the lowest P content ($2,609.20 \pm 256.97$ ppm). There was a significant difference ($p < 0.0001$) in shoot P content of the two bean varieties, whereby MAC 64 had a higher mean shoot P content of $3,528.62 \pm 147.61$ ppm compared to MAC 13 bean variety, which had a mean P content of $2,721.0 \pm 116.85$ ppm. Farm location influenced significantly ($p = 0.0211$) the mean P content of shoots. Climbing beans grown in farm EUM recorded the highest mean

shoot P content ($3,335.80 \pm 205.31$ ppm). There was no significant difference in shoot P content of beans grown in farms TUM and TLM, which recorded a mean P content of $3,105.70 \pm 228.51$ ppm and $3,102.10 \pm 159.10$ ppm respectively (Table 4.13).

Inoculation of climbing beans with test isolates significantly ($p < 0.0001$) affected the shoot potassium (K) content of the plants. However, the shoot K content of climbing beans inoculated with native isolate ELM3, native consortium TC, Biofix-CIAT 899 TB and a mixture of consortium + Biofix-CIAT 899 TCB did not differ significantly. Non-inoculated control plants recorded the lowest shoot K content ($5,733.30 \pm 360.64$ ppm). There was no significant difference ($p = 0.6406$) in shoot K content of the two climbing bean varieties. Farm location influenced significantly ($p = 0.003$) the mean K content of the shoots. Climbing beans grown in farm EUM recorded the highest mean shoot K content ($7,773.30 \pm 272.71$ ppm). Lower shoot K content of $6,770 \pm 433.38$ ppm and $6,550 \pm 326.66$ ppm respectively was recorded for climbing beans grown in farm ELM and TUM (Table 4.13).

Table 4.13: Effect of inoculation on shoot total nitrogen (% N), phosphorous (P) and potassium (K) contents from the field experiment

Treatments	%N	P (ppm)	K (ppm)
Test inoculants			
ELM3	3.342±0.115a	3018.10±241.10b	7254.20±366.48a
TC	2.179±0.099d	2609.20±256.97c	6991.70±333.02a
TB	3.051±0.081b	3268.80±193.23ab	8016.70±315.58a
TCB	2.579±0.057c	3120.00±202.54b	7033.30±404.94ba
TUC	1.766±0.134e	3608.60±192.49a	5733.30±360.64b
Variety			
MAC 13	2.499±0.105b	2721.30±116.85b	7061.70±258.26a
MAC 64	2.667±0.089a	3528.62±147.61a	6950.00±226.14a
Farm			
EUM	2.790±1.01a	3335.80±205.31a	7773.30±272.71a
ELM	2.844±1.49a	2956.30±210.85b	6770.00±433.38b
TUM	2.280±0.93b	3105.70±228.51ab	6550.00±326.66b
TLM	2.419±1.06b	3102.10±159.10ab	6930.00±285.36ab
P. values			
Variety	0.001	<0.0001	0.6406
Farm	<0.0001	0.0211	0.003
Test inoculant	<0.0001	<0.0001	<0.0001
Variety x Farm	0.0522	<0.0609	0.0516
Variety x Test inoculant	0.7194	<0.0713	0.0596
Farm x Test inoculant	0.0102	<0.0507	<0.101
Farm x Test inoculant x Variety	0.0635	0.1021	0.0137

Means followed by same lower case letter(s) within the same column are not significantly different at $p < 0.05$ according to Tukey's HSD test. ELM3, Test native rhizobia isolate; TC, Consortium of native rhizobia; TB, Commercial inoculant (Biofix-CIAT 899); TCB, Biofix-CIAT 899 combined with consortium; TUC, Negative control (Non-inoculated); MAC, Mid altitude climbers.

4.6.10 Correlation analysis between parameters of nitrogen fixation in the field experiment

Correlation analysis showed a significant relationship between nodule dry weight and shoot dry weight ($R^2 = 0.7895$, $p = 0.044$) (Figure 4.9). There was a strong

relationship between nodule dry weight and total seed yield ($R^2 = 0.8792$, $p = 0.012$) (Figure 4.10). Similarly, there was a significant and strong association between % shoot nitrogen and total seed yield ($R^2 = 0.9704$) at $p = 0.002$ (Figure 4.11).

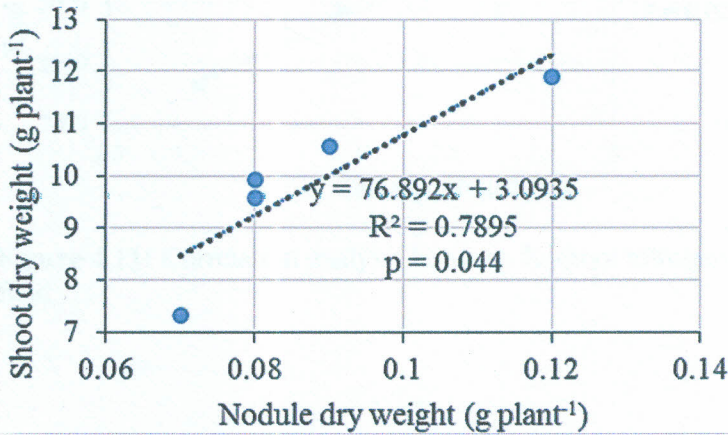


Figure 4.9: Correlation analysis between nodule dry weight and shoot dry weight in the field.

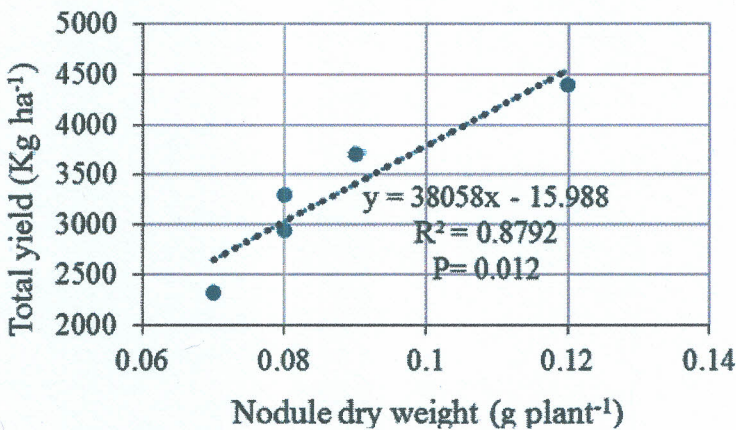


Figure 4.10: Correlation analysis between nodule dry weight and total seed yields in the field.

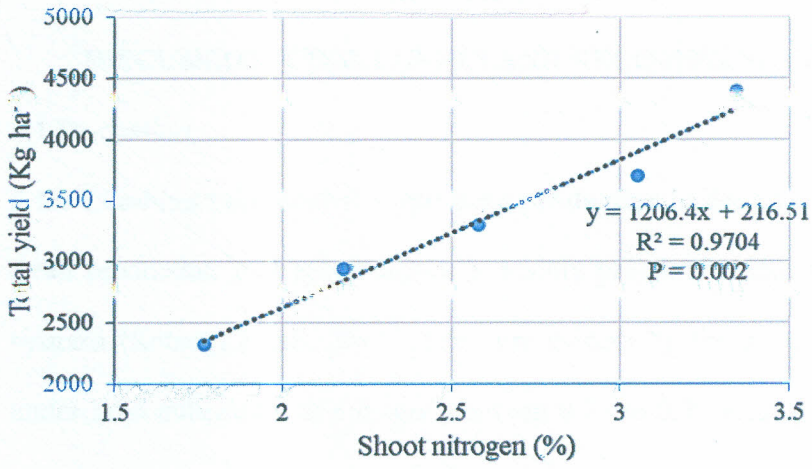


Figure 4.11: Correlation analysis between % shoot nitrogen and total seed yield in the field.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Climbing bean varieties and bean production in Eastern Kenya

Bean production in Eastern Kenya is mainly practiced under smallholder farming systems (Katungi *et al.*, 2010). This was evident by the small average farm sizes under bean cultivation that ranged between 0.25 to 0.50 acres per household (Table 4.1). In respect to climbing bean annual yields and income, it was evident that the harvest and income were low. The low values reported (Table 4.1) are considerable since most of the farmers are smallholder land holdings and mainly use their produce for home consumption (Ramaekers *et al.*, 2013). The poor soil fertility status of the farms and other economic factors might have limited their production and earnings. Katungi *et al.* (2010) reported that land shortage, pests and diseases among other economic factors pose a serious obstacle to bean production among smallholder farmers in Eastern Kenya.

Findings from the present study identified eight local common bean varieties of which three varieties were bush beans while five were climbing beans (Table 4.2). Maasai and Gatune bean varieties were evidently the most preferred bush bean and climbing bean varieties respectively grown by farmers in Eastern Kenya. According to the study carried out by Katungi *et al.* (2010), farmers often prefer bean varieties that have high yields and are resistant to drought, pests and diseases. On the other hand, Kamucere was the least preferred bush bean variety and was only grown by a few farmers (Table 4.2). Kamucere is one of the new bean varieties introduced recently

into the area (Ramaekers *et al.*, 2013). According to Figliuolo and Cerbino (2014), farmers avoid those varieties which are not compatible with the local conditions and this might explain the low percentage of some bean varieties in the study sites. Additionally, according to Sibhatu *et al.* (2015), non-cultivation of a species by farmers is driven by a range of factors including lack of seeds, marketability, taste preferences and lack of sufficient land.

The high bean diversity (Table 4.3) recorded in Eastern Kenya indicates the suitability and production potential of the region for bean cultivation. According to Ramaekers *et al.* (2013), farmers in Eastern and Central highlands of Kenya grow climbing beans due to their high yielding capacity, sweet taste and their suitability for only a small land area. The TLM zone, which recorded the highest bean diversity and richness, had the highest percentage of smallholder farmers growing different varieties of common beans (Table 4.3). On the contrary, farmers had more interest in some specific bean varieties such as Maasai in TUM region and this resulted in low bean diversity recorded in the area. The high diversity of beans in lower midlands zones compared to upper midlands could be due to differences in soil and climatic conditions. Ramaekers *et al.* (2013) explained that pH and temperature decreases, and rainfall increases with increase in altitude. Hence, at higher altitudes (mainly UM zones), soils are more acidic and the climate is cooler with more rainfall mainly as heavy showers. Such conditions limits bean productivity especially during flowering and pod formation stages (Broughton *et al.*, 2003).

5.1.2 Soil characterization

The pH of soils across the study farms varied especially in respect to the agro-ecological zonation, where farms in upper midlands zones recorded lower soil pH compared to those in lower midland zones (Table 4.4). This could be due to high mineralization levels and loss of exchangeable bases (Ca, K, and Mg) through leaching. The low soil pH below 5.0 and high nitrogen amounts above the critical value 2.0 % described by (Okalebo *et al.*, 2002) suppresses nodulation and nitrogen fixation and hence does not favour the production of legumes (Okalebo *et al.*, 2002). The low soil pH in EUM farm results in phosphorous being fixed by the clay soil fractions rendering them unavailable to the climbing beans (Muthini *et al.*, 2014). Legumes such as beans require a slightly acidic or neutral soil for growth especially when the crops depend on biological nitrogen fixation for acquiring nitrogen. Martínez-Romero (2003) reported that high soil nitrogen inhibits the formation of nodules, bacteria root colonization and nitrogen fixation in common beans.

The high phosphorous and organic content in most of the study farms (Table 4.4) may have been as a result of farmer's cultivation practices that may involve use of farm yard organic manure and phosphate fertilizers (Nyaga *et al.* 2014). Phosphorous is an essential component of adenosine triphosphate (ATP) and about 16 ATP is required for the formation of one mole of ammonia (NH_3) from free nitrogen during biological nitrogen fixation process (Shamseldin *et al.*, 2012). The study region is located near Mt. Kenya forest and this could explain the high organic carbon recorded in the farms (Table 4.4). Farms with higher organic carbon, phosphorous and nitrogen content slightly above the critical values can be considered as moderately fertile and suitable

for legume production (Okalebo *et al.*, 2002). The potassium content from all the farms was above the critical value ($0.22 \text{ cmol kg}^{-1}$) described by Okalebo *et al.* (2002). Moderate levels of available K have been reported in soils of the volcanic hills and across Mt. Kenya basement in Eastern Kenya (Katungi *et al.*, 2010).

The soil analysis showed that the texture was classified as sandy clay loam, sandy clay and clay soil, hence aerated soils that are ideal for the growth of aerobic rhizobia cells. In the present study, the sandy clay loam soil texture of farm ELM, which promotes soil drainage and infiltration, gave the best climbing bean yield harvest. Light textured soil are beneficial for the survival and proliferation of soil bacteria especially root-nodule rhizobia (Okalebo *et al.*, 2002). Kawaka *et al.* (2014) reported a higher rhizobia population in Kisumu farms that had sandy loam textured soil compared to clay-textured soils in Masinde Muliro University of Science and Technology (MMUST) farms.

5.1.3 Morphological characteristics of native rhizobia isolates

The morpho-cultural characteristics of the isolates observed (Plate 4.1), Gram staining results and growth on YEMA-CR and YEMA-BTB media under dark incubation, preliminary confirmed the standard morpho-cultural characteristics of *Rhizobium* species as described by Vincent (1970), Beck *et al.* (1993) and Somasegaran and Hoben (1994). The Gram-negative rods observed and poor absorption of congo red dye of the isolates on YEMA-CR further suggested that the isolates could have been rhizobia. Their growth within 3 – 5 days and the colour change of YEMA-BTB from deep green to yellow (Table 4.5) suggested that all isolates were fast growers and

could probably fall under the genus *Rhizobium*. The results of this study are similar to the findings from other studies on morphological characterization of rhizobia nodulating common beans (Berrada *et al.*, 2012; Kawaka *et al.*, 2014; Muthini *et al.*, 2014). Changes in temperatures, pH and soil salinity are among the main factors restricting symbiotic nitrogen fixation in legume-rhizobia symbiosis (Berrada *et al.*, 2012). The production of exopolysaccharides (EPS) by most of the rhizobia isolates in this study indicate their versatility to withstand desiccation due to high temperatures, change in soil pH and salinity. Soils in agroecological zones surrounding Mt. Kenya region are known to be slightly acidic due to excessive precipitation and soil erosion (Nyaga *et al.*, 2014). Therefore, rhizobia strains native to Mt. Kenya and its environs are expected to have survival adaptations to stressful soil conditions. The isolates exhibiting a wide adaptation to environmental stresses could be able to circumvent limiting factors and maintain a higher capacity for nitrogen fixation, thus, may be considered suitable candidates for inoculum development (Kawaka *et al.*, 2014).

5.1.4 Genetic diversity of native rhizobia isolates from Eastern Kenya

The genetic diversity of forty one native rhizobia isolates obtained from the root nodules of MAC 13 and MAC 64 climbing bean varieties grown in Eastern Kenya was evaluated based on amplified 16S rDNA restriction analyses (Plate 4.3). The 3 restriction enzymes *EcoR* I, *Hae* III and *Msp* I showed highly polymorphic and distinct DNA fragment patterns (Plate 4.4) indicating the divergence of the native rhizobia isolates in Eastern Kenya. The mean Shannon-Wiener diversity (H) estimate of rhizobia populations from Eastern Kenya based on genetic distance of ARDRA pattern analysis showed that the isolates were genetically diverse with rhizobia

population from ELM zone having the highest genetic diversity estimate of $H = 0.47$ and TLM zone recording the lowest diversity estimate of $H = 0.31$ (Table 4.6). Rhizobia diversity variations due to the differences in agroecological zones or sites have been reported in Kenya. Wasike *et al.* (2009) reported a relatively higher diversity of indigenous bradyrhizobia in Bungoma ($H = 1.9$) compared to Mitunguu ($H = 1.7$) as a result of agroecological differences between the two sites. Other factors such as cropping history, land use and host genotype have been attributed to the variation of rhizobia diversity in different parts of Central highlands of Kenya (Mwenda *et al.*, 2011).

The Pairwise Population Matrix of Nei unbiased genetic distance of rhizobia populations in Eastern Kenya showed a narrow range (0.004 to 0.037) (Table 4.8). Similar findings have been reported by Ismail *et al.* (2013) who worked on *P. vulgaris* rhizobia populations from different sites in Egypt. The narrow genetic distance could possibly be as a result of the conserved nature of the 16S rRNA gene, which could not discriminate between closely related rhizobia species (Berrada *et al.*, 2012). The 16S rDNA region despite having variable genetic regions that can be used to efficiently define the genetic diversity of a population, it has limitations to identify species especially when ARDRA is used alone as a tool for genetic diversity determination (Silva *et al.*, 2012).

The use of ARDRA as a finger printing tool in this study may also have contributed to the narrowed genetic diversity observed. It has been reported that ARDRA has a poor resolving power (Berrada *et al.*, 2012) and thus other molecular finger printing tools

such as sequencing and phylogenetic analysis could be adopted. Dai *et al.* (2012) noted that the phylogenetic analyses of bacteria using 16S rDNA alone may not clearly show a distinctive relationship within and among the bacteria populations involved. In addition, horizontal gene transfer and genetic recombination could have possibly contributed to the limited diversity (Silva *et al.*, 2012) of rhizobia in Eastern Kenya.

The analyses of molecular variance (AMOVA) based on amplified 16S rDNA restrictions showed that 100 % of the genetic variation of native rhizobia isolates was within and not among the four populations (0 %) or across the two regions (0 %) in Eastern Kenya (Table 4.7). Based on ARDRA fingerprints, there was low level of genetic differentiation of climbing bean rhizobia and this could suggest that the rhizobia population within the region is weakly structured. This could be due to the absence of physical barriers to limit gene flow (Muthini *et al.*, 2014). The circulation of climbing bean seeds within the region by farmers (Ramaekers *et al.*, 2013) could have also contributed to the rhizobia genetic conservation in Eastern Kenya. In contrast to the findings of this study, Rashid (2013) reported high variability of rhizobia isolates in different geographical regions in Europe.

However, the true diversity of rhizobia in the region should be studied using other molecular tools such as sequencing that has a higher resolving power. The principal coordinate analysis (PCA) (Figure 4.1) also showed low level of genetic differentiation of native rhizobia isolates showing congruent results with the dendrogram (Figure 4.2). The grouping and distribution patterns of the native isolates did not correspond to the agroecological zones. Similarly, Ismail *et al.* (2013),

reported a PCA analysis of rhizobia isolates that did not correspond to the geographical locations in Egypt.

Based on evolutionary relationship of 41 isolates inferred using Neighbour-Joining method, some of the native isolates clustered closely with the 3 reference rhizobia strains used in the study. The phylogenetic tree (Figure 4.3) showed genetic variation among the native rhizobia isolates as evident by the different clusters and sub-clusters. However, there was generally a low bootstrap support (Figure 4.3) for the clustered isolates and this depicts the main weakness of using ARDRA alone in studying genetic diversity (Ismail *et al.*, 2013).

The main cluster I had *R. tropici* CIAT 899 and *R. etli* USDA 2667 clustering with most of the native rhizobia isolates. The closest native isolates to *R. tropici* CIAT 899 were ELM 33 and TLM 32 while native isolates TLM 27 and TUM 41 clustered together with *R. etli* USDA 2667. The native isolate EUM 7 clustered closely to *R. leguminosarum* strain 446 but in a different sub-branch (Figure 4.3). These indicate the close genetic relationship between some of the rhizobia isolates to *R. tropici* CIAT 899, *R. etli* USDA 2667 and *R. leguminosarum* strain 446. Other studies in Kenya have shown the dominance of these *Rhizobium* strains in Kenyan soil (Anyango *et al.*, 1995; Mwenda *et al.*, 2011; Onyango *et al.*, 2015). Native isolates TUM 26, TLM 28 and EUM 23 clustered together (Figure 4.3) despite originating from different agroecological zones of Eastern Kenya showing 100 % similarity based on ARDRA analysis. This indicates the close genetic distance between some of the rhizobia isolates native to different agroecological zones of Eastern Kenya. There is also a

possibility of such isolates having originated from the same genetic background (Mwenda *et al.*, 2011). However, this may not be conclusive unless other molecular tools used in studying genetic diversity such as sequencing and phylogenetic analyses are employed (Silva *et al.*, 2012). The distinct cluster III had only one isolate ELM 5 (Figure 4.3) which did not cluster with any reference strain or other native isolates. This indicates the diverse nature of the rhizobia isolates in Eastern Kenya.

5.1.5 Nodulation and symbiotic nitrogen fixation efficiency of native rhizobia isolates in the greenhouse experiments

The climbing beans inoculated with rhizobia in the greenhouse experiment recorded higher values for all the measured parameters compared to the non-inoculated controls. Between the two bean varieties, MAC 64 showed superior nodulation over MAC 13 (Table 4.9). There was a significant difference in nodulation ($p = 0.0082$) between the two climbing bean varieties under controlled greenhouse conditions (Table 4.9). This supports observations made by Gicharu *et al.* (2013) who noted the differences in nodulation among the three climbing bean cultivars; (G59/1-2, NG224-4 and Cargamanto) grown under controlled conditions in the greenhouse. The pink colouration of most of the nodules (Plate 4.5C) indicated the presence of leghaemoglobin, a factor necessary for effective nitrogen fixation (Farid and Navabi, 2015). Such nodules are known to contain actively express *nifH* genes responsible for synthesis of nitrogenase enzymes (Rondon *et al.*, 2007). The MAC 64 bean variety had the highest NDW compared to MAC 13, despite having a fewer average number of nodules (Table 4.9). This showed that MAC 64 formed larger and heavier nodules compared to MAC 13.

Some of the native isolates did not cause nodulation in the greenhouse despite being extracted from the climbing bean nodules. This could be attributed to the loss of viability of some of the rhizobia isolates during culturing and preservation in the laboratory. This could also be as a result of non-effective, deleterious isolates (Njeru *et al.*, 2013; Muthini *et al.*, 2014). Previous work carried out by Chemining'wa *et al.* (2011) suggested that inoculation does not always enhance nodulation. The non-inoculated controls did not show any nodulation demonstrating that the experimental set up and maintenance of plants was carried out under aseptic conditions (Mungai and Karubiu, 2011).

Nodulated plants had higher SDW compared to non-nodulated plants (Table 4.9). This suggests that nodulation could have improved the plant biomass. These results imply that those plants that were able to form effective nodules accumulated higher biomass (Gicharu *et al.*, 2013) compared to those that did not nodulate. Some of the native isolates such as ELM3 and ELM8 showed superior nodulation compared to the commercial inoculant Biofix-CIAT 899 (Table 4.9). This indicates that there could be native rhizobia strains that compete better for nodulation compared to the commercially available strains. This supports the findings reported by Onyango *et al.* (2015) who found that native strains showed better competence for nodule occupancy in Bambara groundnuts compared to the market available commercial strain USDA 110 under controlled greenhouse conditions.

The plant shoot dry weight was used during the study to estimate nitrogen-fixing efficiency of the isolates. This method is easy to use and is relatively cheap. It is the

most appropriate method for use in soils with low nitrogen content (Rondon *et al.*, 2007). The greenhouse results showed that at mid-flowering, rhizobia inoculation had influence on shoot dry weight (Table 4.9). This is in agreement with the studies carried out by Kawaka *et al.* (2014) who reported higher SDW of the inoculated plants compared to non-inoculated controls. The findings from the present study are in agreement with what Giri and Joshi (2010) reported, when they inoculated chickpea with *Rhizobium* as biofertilizer in which the nodule number, root and shoot biomass was significant.

Symbiotic efficiency (SE) differed significantly among the isolates ($p < 0.0001$) in the greenhouse (Figure 4.4). This conforms to the findings reported by Mwangi *et al.* (2011) who tested a hundred isolates for SE with siratro plants. In this study, all the isolates that nodulated had an SE above 85 % with the highest having 123.72 % (ELM3) and the lowest being TC with 86.17 % (Figure 4.4). These results are in parallel with those of Kawaka *et al.* (2014) who reported SE ranging between 67 % and 170 % by common bean nodulating native rhizobia. Mungai and Karubiu (2011) found that native rhizobia isolate nodulating common beans from Njoro had a higher symbiotic efficiency compared to the commonly used commercial inoculants (Biofix-CIAT 899 and USDA 9030). Based on the rating used by Lalande *et al.* (1990), it was evident that all the native isolates were highly effective (> 80 %) in nitrogen fixing efficiencies. The consortium of native isolates had the lowest SE (86.17 %) (Table 4.9), inferring that the combination of several native isolates could not give a better synergistic effect that would enhance BNF (Meghvansi *et al.*, 2010).

5.1.6 Efficiency of native rhizobia isolates in the field experiments

5.1.6.1 Effect of native rhizobia isolates on nodule number, nodule, shoot and root dry weights

Inoculated plants showed higher mean nodule number and nodule dry weight compared to the non-inoculated controls in field experiments (Table 4.11). This shows that the nitrogen fixing potential of soil rhizobia in the farms was relatively poor. Inoculation with isolate ELM3 led to the highest average number of nodules and nodule dry weight as compared to the commercial inoculant, Biofix-CIAT 899 (Table 4.11). This indicated that native rhizobia strains were more competitive and efficient in nodulation in the field compared to the commercial inoculants. These results supports the findings reported Kawaka *et al.* (2014) and Tena *et al.* (2016), who noted that native rhizobia that exist in the field, often out-compete the commercial inoculant rhizobia strains. The establishment and persistence of an introduced rhizobia strain often decrease with increase in population density of the native soil rhizobia strains (Nkot *et al.*, 2015). There was no significant differences in nodule number between the two climbing bean varieties (Table 4.11). This shows that the bean varieties used had no preference for certain rhizobia and this provides an agronomic advantage in the field.

The inoculation of climbing beans in the field significantly enhanced shoot and root dry weights (Table 4.11). This is evidenced by the high SDW and RDW recorded by the plants inoculated with rhizobia isolates as compared to the non-inoculated controls. Climbing beans inoculated with ELM3 native rhizobia isolate had the highest SDW and RDW compared to the other isolates including the commercial

inoculant, Biofix-CIAT 899. This showed that isolate ELM3 was able to form highly effective nodules with efficient nitrogen fixation capabilities and, therefore, the plants accumulated higher shoot and root biomass. The superior ability of native rhizobia isolate ELM3 in the study indicates the presence of more effective elite rhizobia strains in the sampled farms. The presence of highly effective strains of rhizobia with high nitrogen fixation capabilities in Kenyan soils has been reported by Anyango *et al.* (1995) and Kawaka *et al.* (2014), indicating the potential of native rhizobia isolates in bean production.

Native consortium of rhizobia did not give better results compared to individual isolates (Table 4.11) and this shows the antagonism when several isolates are mixed together leading to negative interaction. The effect of Biofix-CIAT 899, native consortium and as well as the combination of the consortium + Biofix-CIAT 899 inoculation on SDW did not differ significantly (Table 4.11). This indicates that there was no need to diversify the rhizobia strain in the field experiments. However, this contradicts to the findings by Gicharu *et al.* (2013), who reported a high total dry matter yields on climbing beans inoculated with a mixture of three native rhizobia strains. The SDW of the two climbing bean varieties had no significant difference and this may be attributed to their parental similarities in their genotypic make up.

5.1.6.2 Effect of native rhizobia isolates on stover dry weight, pod number, total seed yield and 100-seed dry weight

Inoculation of bean plants with rhizobia significantly increased the average number of pods produced by the plants (Table 4.12). Climbing beans inoculated with isolate

ELM3 produced the highest mean pod number (Table 4.12) compared to the multi-strain inoculated and non-inoculated beans. This could be attributed to the higher nitrogen fixation caused by the isolates during BNF. Thuita *et al.* (2011) reported a higher vegetative growth and pod number on a promiscuous soybean variety TGx1740-2F after inoculation with rhizobia strains. These results are also supported by Morad *et al.* (2013) who reported that inoculated beans had significantly higher seed yield, pod number per plant and biologic dry matter over control and chemical nitrogen fertilizer treatments.

Rhizobia inoculation of climbing beans significantly enhanced bean stover dry weight (STW) over non-inoculated ones (Table 4.12). This could be attributed to the influence of increased nitrogen fixation by the isolates, which increased the vegetative growth of plants and consequently increasing plant biomass and height. Bhuiyan *et al.* (2008) also found significantly higher STW of mungbean due to the application of *Bradyrhizobium* inoculation; therefore supports the present findings. The highest STW recorded by MAC 64 bean variety could be attributed to its enhanced vegetative growth that increased leaf density.

Climbing bean variety had a significant effect on pod number and seed yield but the interaction between bean variety, and test isolates had no significant effect (Table 4.12). Rhizobia inoculation significantly increased pod number and hence the seed yields. Beans inoculated with isolate ELM3 had the highest seed yield compared to multi-strain inoculated and non-inoculated plants. Beans inoculated with isolate ELM3 produced the highest nodule number, SDW and pod number per plant, which

resulted in higher seed yield (Table 4.12). This finding is in agreement with Patra *et al.* (2012) who reported that soybean inoculation with rhizobia strains significantly increased seed harvest yields. However, this contrasts the findings by Kimani *et al.* (2007) who reported that the multi-strain rhizobia inoculated beans had higher yields compared to those inoculated with a single strain and non-inoculated control beans. The plants inoculated with a combination of the Biofix-CIAT 899 and native consortium led to relatively lower seed yield compared to the Biofix-CIAT 899 alone but relatively higher compared to the native consortium when used alone (Table 4.12). This suggests that there is rhizobia strain-host specificity in bean-rhizobia symbiosis. Martínez-Romero (2003) explained that inoculation with a diverse rhizobia population does not necessarily translate to a higher legume grain yield due to inefficiency of rhizobia to cause infection, occupy nodules and effect nitrogen fixation efficiently.

The field study showed significant interactive effects between bean variety and farm location on seed yield (Figure 4.7), indicating that the bean varieties responded differently in different farms located in different agroecological zones. The effect of rhizobia inoculation on growth and yield parameters of climbing beans differed in different farms located in different agroecological zones. Farm (ELM) located in Embu lower midland agroecological zone produced the highest seed yields and this could be attributed to various factors including soil fertility, efficiency of rhizobia and climatic factors (Farid and Navabi, 2015). Monyo and Laxmipathi (2014) in a research work carried out on soybean and common bean reported that certain legume varieties are well adapted for production in lower midland agroecological zones in

Malawi and thus this may explain the variation in growth and yield parameters of climbing beans across the four agroecological zones of Eastern Kenya.

Similarly, Farid and Navabi (2015) in a study on symbiotic nitrogen fixation on common beans, reported a significant interaction of host genotype with rhizobia strain and location and thus locally adapted grain legume cultivars should be considered to improve symbiotic nitrogen fixation. The interactive effects between farm location and rhizobia inoculants ($p = 0.0102$) (Figure 4.8) on % N of shoots indicates that different rhizobia inoculants responded differently in different farm locations in relations to nitrogen accumulation in the shoots of climbing beans. These results are in agreement with the findings of Monyo and Laxmipathi (2014) who reported that there are varietal variations in legume production across all the major agroecological regions of Uganda.

Climbing beans inoculated with isolate ELM3 produced the heaviest 100-seed weight. This could suggest that part of the nitrogen fixed by inoculated plants was stored in the seeds. Partitioning of nitrogen to the seeds by certain cultivars of legumes has been reported. The interaction effect of variety and rhizobia inoculation was not significant in respect of 100-seed weight. This might be due to the similar response of the two varieties to rhizobia isolates. These results are in line with the findings of Elsheikh and Elzidany (1997) who reported an increased grain yield and 100-seed weight of the faba beans when they were inoculated over the control beans. In addition, they found that *Rhizobium* inoculation significantly increased the total nitrogen of faba beans.

5.1.6.3 Effect of native rhizobia isolates on climbing bean shoot nitrogen, phosphorous and potassium content

The high nitrogen content in climbing beans, which had been grown in farm ELM (Table 4.13), may be as a result of effective nodulation and nitrogen fixation of rhizobia strains. Nitrogen fixation in legumes has been reported to be sensitive to water stress and thus can be affected by severity and timing of precipitation (Abbasi *et al.*, 2010). In this study, the large differences in %N during field experiments may be attributed to the water stress that occurred before early flowering stage which affected nitrogen fixation. The higher % N in shoots of bean variety MAC 64 (Table 4.13) showed that this bean variety is able to accumulate higher amounts of N per tissue weight. Therefore, legume inoculation with highly infective and effective native strains of rhizobia, well adapted to the field conditions, is necessary to achieve maximum nitrogen fixation rates and thus maximize total productivity and profitability.

Phosphorous (P) is an essential nutrient that drives BNF and, therefore, P deficient soils could result into low BNF despite the high abundance of native rhizobia strains (Broughton *et al.*, 2003). Ndakidemi *et al.* (2014) reported that the higher the P content in the soil, the higher the nitrogen fixation rate in legumes. Potassium plays various roles at different stages of photosynthesis process such as ATP synthesis, enzyme activation, CO₂ uptake and ion balance (Mmbaga *et al.*, 2014). Potassium deficiency in soil has been reported as a BNF limiting factor, which may influence nodule number and yields of common beans (Farid and Navabi, 2015). The significant interaction between farm location and rhizobia inoculants on climbing bean shoot N

content in the field indicates the differences in suitability and adaptability of the rhizobia isolates to different agroecological zones (Farid and Navabi, 2015).

5.1.7 Correlation analysis between different climbing bean parameters

The significant correlation between nodule dry weight and shoot dry weight in both greenhouse (Figure 4.5) and field experiments (Figure 4.9) confirmed the dependence of shoot biomass on nodulation (Kawaka *et al.*, 2014). The significant relationship between nodule dry weight and nitrogen accumulation (%N) in the experiments (Figure 4.6) confirms the assertion made by Delić *et al.* (2010) that there is a direct relationship between nodulation and nitrogen accumulation in legumes. Results from the present study concurs with the findings of Unkovich *et al.* (2010) who reported that there is a strong positive correlation between biomass production and nitrogen accumulated by rhizobia-inoculated lentils and peas. These findings, therefore, support the use of such parameters as measures of nitrogen fixation potential of rhizobia strains (Patra *et al.*, 2012). Nitrogen is an essential component of chlorophyll that plays a big role in the formation of carbohydrates during photosynthesis (Ramaekers *et al.*, 2013). In the present study, the significant relationship between climbing bean seed yields and %N in shoots (Figure 4.11) further emphasizes the importance of nitrogen in legume production.

5.2 Conclusion

- i) Gatune, Raila, Mama safi, Kithiga and Muviki were the climbing bean varieties identified in upper and lower midland agroecological zones of Eastern Kenya.

- ii) Based on morphological characteristics, 41 native rhizobia isolates were obtained from the root nodules of mid-altitude climbing (MAC) beans and were placed into 9 groups.
- iii) Cluster analysis based on 16S rDNA ARDRA restriction grouped native rhizobia isolates into 3 main clusters and showed a considerable genetic variation within the populations and not among populations or across the regions.
- iv) Representative native rhizobia isolates had significant differences ($p < 0.0001$) in terms of symbiotic efficiencies in the greenhouse experiments and native isolate ELM3 had higher symbiotic efficiency in comparison with the Biofix-CIAT 899 commercial strain.
- v) Native isolate ELM3 was confirmed after field experiment as the most effective and suitable isolate that have a commercial potential for use in climbing bean production in Eastern Kenya.

5.3 Recommendations

- i) The high bean diversity recorded in Eastern Kenya indicates the suitability and production potential of the region for bean cultivation and thus high yielding climbing bean varieties such as MAC 64 should be adopted.
- ii) The use of effective native rhizobia isolates as bio-fertilizer inoculants enhances bean nodulation, plant biomass and grain yields and thus, should be employed by smallholder farmers as an alternative and cheap sustainable agricultural practice in farming.

- iii) Native isolate ELM3 that showed higher symbiotic efficiency in the greenhouse and higher seed yield in the field experiments compared to Biofix-CIAT 899 commercial inoculant should be identified and recommended as a rhizobia bean inoculant.
- iv) Further molecular studies using either full or partial gene sequences of bacterial genome need to be carried out to establish the characterization and diversity of native rhizobia isolates up to the species and strain levels.
- v) Further research focusing on unearthing the vast diversity of native rhizobia from other parts of the country that have not been studied should be done.
- vi) There is a need to carry out further field trials across the extended geographical areas in Kenya using MAC 13 and MAC 64 climbing beans as the host plants to ascertain the performance of the native rhizobia isolates.

- iii) Native isolate ELM3 that showed higher symbiotic efficiency in the greenhouse and higher seed yield in the field experiments compared to Biofix-CIAT 899 commercial inoculant should be identified and recommended as a rhizobia bean inoculant.
- iv) Further molecular studies using either full or partial gene sequences of bacterial genome need to be carried out to establish the characterization and diversity of native rhizobia isolates up to the species and strain levels.
- v) Further research focusing on unearthing the vast diversity of native rhizobia from other parts of the country that have not been studied should be done.
- vi) There is a need to carry out further field trials across the extended geographical areas in Kenya using MAC 13 and MAC 64 climbing beans as the host plants to ascertain the performance of the native rhizobia isolates.

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APPENDICES

Appendix I: Nitrogen-free nutrient solution

Preparation of nitrogen-free plant nutrient solution (Broughton and Dilworth, 1971)

Solution	Formulation	Quantity (g L ⁻¹)
Stock (i)	CaCl ₂ .2H ₂ O	294.10
Stock (ii)	KH ₂ PO ₄	136.10
Stock (iii)	MgSO ₄ .7H ₂ O	123.30
	K ₂ SO ₄	87.00
	MnSO ₄ .H ₂ O	0.34
Stock (iv)	H ₃ BO ₃	0.247
	ZnSO ₄ .7H ₂ O	0.288
	CuSO ₄ .5H ₂ O	0.100
	Na ₂ MoO ₄ .2H ₂ O	0.048
	CoSO ₄ .7H ₂ O	0.056
Stock (v)	Fe Citrate	5.40

Appendix II: Questionnaire used to collect data from climbing beans farmers in Eastern Kenya

Introduction:

I am Gilbert Koskey, a Master of Science student from the Department of Microbiology, Kenyatta University. I am carrying out a research study on local common bean varieties grown in Embu and Tharaka Nithi Counties of Eastern Kenya. Kindly fill the questionnaire form correctly to the best of your knowledge and ability. The information provided will be used only for academic and research purpose and will be kept confidential. Thank you.

Questionnaire No.

Date of interview: ^{DD} ^{MM} ^{YYYY}

Name of respondent

County: Code

Sub-county: Code

Location: Code

Sub-location: Code

Village: Code

Household code:

SECTION A: FAMILY INFORMATION

Table A.1. Personal information for members of the household currently resident:
Fill where appropriate.

Total resident H/H Members _____

1(A) What is the general condition of housing in the HH? Specify condition for up to five houses:

I D	Name	Age (Group)	Marital status	Sex	Relationship to H/H head	Highest education Level	Main occupation
		1-≤20 yrs 2- 21-35 3- 36-45 4- 46-55 5- 56-65 6-≥66 yrs	1- Single 2- Married 3- Widowed 4- Divorced 5- Separated	Male-1 Female- 2	1- Head 2- wife/Husband 3- Son/daughter 4- mother/father 5-other	1-None 2- Primary 3- Secondary 4- University 5-Other	1-Farming/ Livestock keeping 2-Business 3-Employed 4- Other 5-None
1							
2							
3							
4							

Table A.2 Housing conditions within the Household

House No	Wall	Floor	Roof
	1- Earth mud 2- Bricks/Stones 3- Iron sheets 4- Wood slabs 5-Others	1 Earth/mud 2- Cement	1-Thatch 2-Iron sheets 3-Tiles 4-Other
1			
2			
3			

SECTION B: FARM INFORMATION

Farm owned and managed by household

Which year did you settle in this farm?

How many fields do you operate?

Table B.1. Please provide fields information as specified in the table below for the current season

Field ID	Total area (Acres)	Cultivated area (Acres)	Tenure System 1-surveyed 2-Lease 3-unsurveyed 4-Communal 5- Other	Ownership 1-Own 2-Rent	Rent in Land cost 1.<5000 2.5001-7000 3.7001-9000 4.>9000	Rent out land income 1.<5000 2.5001-7000 3.7001-9000 4.>9000

Table B2. Type of crops grown

	Yes	No
Legumes		
Cereals		
Tubers		
Others (specify)		

Table B3. Cereal information

Field ID	Cereals grown 1. Maize 2. Finger millet 3. Sorghum 4. Pearl millet 5. Other (specify)	Planted area (Acres) 1. < 0.25 2. 0.25- 0.5 3. 0.5-1 4. >1	Yield (kg) 1. <90 2. 901-180 3. 181-270 4. 271-360 5. 361-450 6. >450	Income (KES)/ year 1. <1,800 2. 1801-3600 3. 3601-5400 4. 5401-72000 5. >72000

Table B 4. Legume information

Field ID	Legumes grown 1. Common beans 2. Soya beans 3. Pigeon peas (<i>Cajanus cajan</i>) 4. Cowpea (<i>Vigna unguiculata</i>) 5. Green grams (<i>Vigna radiata</i>) 6. Others (specify)	Planted area (Acres) 1. < 0.25 2. 0.25- 0.5 3. 0.5-1 4. > 1	Yield (kg)/year 1. <90 2. 901-180 3. 181-270 4. 271-360 5. 361-450 6. >450	Income (KES)/ year 1. <2700 2. 2701-5400 3. 5401-8100 4. 8100-10800 5. >10800

TABLE B 5. Information on bean varieties grown

SNo.	Beans variety 1. 2. 3. 4. 5. 6.	Climbing 1. Yes 2. No	Planted area (Acres) 1. < 0.25 2. 0.25- 0.5 3. 0.5-1 4. > 1	Cropping practice 1. Mixed 2. Sole	Yield (kg)/ year 1. <90 2. 901-180 3. 181-270 4. 271-360 5. 361-450 6. >450	Income (KES)/ year 1. <2700 2. 2701- 5400 3. 5401- 8100 4. 8100- 10800 8. >10800
1.						
2.						
3.						
4.						

SECTION C: LIVESTOCK PRODUCTION

TABLE C1. Information on livestock kept

SNo	Livestock keeping 1). Yes 2). No	Livestock types kept 1) Cattle 2) Goats 3) Sheep 4) Rabbit 5) Poultry 6) Donkey 7) Other (specify)	Method of livestock keeping 1) Free- range 2) Tethering	Income (KES/Year)
				1.>3000 2.3001-5000 3.5001-8000 4.8001-11000 5.11001-14000 6. 17001-20000 7. > 20000

SECTION D. SOIL FERTILITY INFORMATION

TABLE D1. Information on soil fertility

Code	Soil fertility Status 1). Fertile 2). Border- line, (neither fertile nor poor) 3). Poor	Farm Input 1). Farmyard manure 2). Inorganic Fertilizers 3). Compost 4). Crop residues 5). None 6). Bio-fertilizers 7). Others (specify)	Soil Erosion Problem 1. Yes 2. No	Soil Conservation structures present 1).Yes 2). No	Conservation structures 1). Bench terrace 2). Bench terrace with grass 3).Bench terrace with trees 4). Bench terrace with grass and trees 5). Check dam 6).Cut off drain 7). Other (specify)
1.					
2.					
3.					
4.					

In case you use fertilizer in your farm, is fertilizer available in local market when required?

1-Yes 2-No

In case you have soil erosion problems and you do not have terraces, give reasons why you don't have them?

Where do you get your manure?

1-Own 2-Buy 3-Neighbour Other (specify) _____

Would you like to try other fertility improvement methods? 1-Yes 2-No

SECTION E: PROBLEMS IN PRODUCTION

TABLE 1D – Weeds, pests and diseases

Field ID	Weeds in your farm	Weed control method	Pests problems	Disease problems
	1-Thangari 2-Igoka 3-Mucegee (black jack) 4-Mbiu (kivi) <i>Cyperus rotundus</i> 5-Other (specify)	1-Hoe/panga weeding 2-Plough-weeding 3-Use of herbicide 4-Burning 5-Carrying out of farm 6-Other (specify)	1-Squirrels 2-Birds 3-Monkeys 4-Weevils 5-Aphids 6-Cartapilars 7-Other (specify)	1-Mbaa (Powdery mildew) 2-Rust 3-Other (specify)

SECTION F: FOOD SECURITY

Do you have food shortage during the dry seasons? 1-Yes 2-No

What is your main source of food during dry seasons?

1-Reserve (own) 2-Purchase 3-Relief

4-Neighbours 5-Other (specify)

End. Thank you.