NITROGEN FIXING POTENTIAL OF INDIGENOUS BRADYRHIZOBIA NODULATING SOYBEAN VARIETIES GROWN IN EMBU AND THARAKANITHI COUNTIES IN EASTERN KENYA

SIMON MBURU WAMBUI
(BSc. Microbiology)

156/28268/2014

A THESIS SUBMITTED IN PARTIAL FULFILLMENT 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.
Simon Mburu Wambui
156/28268/2014
Signature Date: 29/11/2016

APPROVAL BY THE SUPERVISORS
This thesis has been submitted for examination with our approval as University supervisors.

Dr Ezekiel Mugendi Njeru
Department of Microbiology
Kenyatta University
Signature Date: 29/11/2016

Dr. Jacinta M. Kimiti
School of Environment and Natural Resources Management
South Eastern Kenya University
Signature Date: 29/11/16
DEDICATION

I dedicate this work to the Almighty God for making the entire process a reality. I also dedicate it to my loving family for the support and patience during the study.
ACKNOWLEDGEMENT

I would like to extend my sincere gratitude to my supervisors Dr. Ezekiel Mugendi Njeru and Dr. Jacinta M. Kimiti for the excellent supervisory role and the insightful advice they gave to me during the study. I acknowledge Dr. John Maingi and Dr. Omwoyo Ombori for their relentless support throughout the course of this research. I acknowledge all the technicians in the Department of Microbiology, Kenyatta University for the encouragement and support they provided me during my research. I would like to appreciate Regional Universities Forum for Capacity Building in Agriculture (RUFORUM), 5th Graduate Research Grant (GRG), for scholarship and financing this research work.

Special thanks goes to Mr. Morris Muthini, for his technical support and guidance. I express my heartfelt gratitude to Pastor Antony Njeru and his entire staff from AIC Mufu Children’s home who gave support and made my work easier during field work. I acknowledge all smallholder farmers from Embu and Tharaka-Nithi Counties who participated in the field study for their kind support. I am pleased to thank my mum, Zipporah Wambui, for her support during the period of my study. Finally, special thanks go to my classmates and specifically Gilbert Koskey, James Muhunyu and Marjorie Oruru for the teamwork and encouragement.
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# ABBREVIATIONS AND ACRONYMS

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<tr>
<td>AAS</td>
<td>Atomic Absorption Spectrophotometer</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<td>BNF</td>
<td>Biological Nitrogen Fixation</td>
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<tr>
<td>BTB</td>
<td>Bromothymol blue</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>FAO</td>
<td>Food and Agricultural Organization</td>
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<td>GLM</td>
<td>General Linear Models</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IITA</td>
<td>International Institute of Tropical Agriculture</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal transcribed spacer</td>
</tr>
<tr>
<td>LSD</td>
<td>Least Significant Difference</td>
</tr>
<tr>
<td>MEGA</td>
<td>Molecular Evolutionary Genetics Analysis</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NaOCl</td>
<td>Sodium hypochlorite</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NP</td>
<td>Non-promiscuous</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RCBD</td>
<td>Randomized Complete Block Design</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SSA</td>
<td>Sub-Saharan Africa</td>
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<tr>
<td>YEMA</td>
<td>Yeast Extract Mannitol Agar</td>
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Nitrogen deficiency in most African soils is the main factor limiting crop productivity. Grain legumes such as soybean (*Glycine max* (L.) Merril) can form a symbiotic association with soil root nodule bacteria (rhizobia) and the process can provide sufficient nitrogen for crop production. Continuous cultivation without nutrient replenishment, leaching and poor soil fertility are the major limitations for soybean production by smallholder farmers in Embu and Tharaka-Nithi Counties. The objectives of this study were to assess the soybean varieties grown by smallholder farmers from Embu and Tharaka-Nithi Counties, to determine the diversity of indigenous bradyrhizobia nodulating soybean varieties and to determine symbiotic effectiveness of bradyrhizobia isolates on biological nitrogen fixation. DNA fingerprinting was carried out using 16S rRNA gene for polymerase chain reaction (PCR) products based on restriction digest using *Hae*III, *Msp*I and *Eco* RI enzymes. The symbiotic effectiveness of isolates was carried out both in greenhouse and field conditions. A split plot arrangement in a randomized complete block design (RCBD) replicated three times was used. During the study, four soybean varieties namely Gazelle, Nyala, Namsoy, and Mausoy were recorded where Gazelle was most abundant with 85% of smallholder farmers surveyed. Thirty-nine (39) isolates were obtained from trapping experiments and placed into nine groups based on their morphological characteristics. Analysis of molecular variance (AMOVA) on genetic data based on restriction digest revealed significant (*p* < 0.015) variation within populations and not among the zones or populations. Based on principal coordinate analysis (PCA), there was sympatric speciation of indigenous rhizobia isolates. EUMZ rhizobia isolates had the highest genetic diversity estimates (*H* = 0.419) compared to other agroecological zones according to Shannon's Information Index I (*H*). Authentication experiment showed that most of the isolates were bradyrhizobia due to their ability to infect soybeans. The test isolates had varied ability to infect and fix nitrogen. The best performing indigenous isolates (RI9 ad RI4) in greenhouse outperformed commercial bradyrhizobia (Bio-fix) in terms of symbiotic effectiveness with 119.17 %, 142.35 %, and 101.01 %, respectively, when compared to nitrogen controls. Results from the field experiment indicated that indigenous isolates were competitive where they improved significantly soybean shoot biomass (*p* < 0.001). Different zones were found to have a significant influence on yield after use of inoculants (*p* = 0.001). The indigenous isolate RI9 scored highest grain yield of 823 kg ha⁻¹ when compared to other treatments. The effective indigenous bradyrhizobia therefore showed the potential of being sources of inocula for soybean smallholder farmers.
CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Nitrogen fixation through symbiotic association between soil microorganisms and leguminous plants convert atmospheric free nitrogen into forms that are fully or partially utilizable by host plant (Sikora et al., 1997; Aung et al., 2013). The process is a low cost and sustainable source of nitrogen compared to inorganic fertilizers in small farming systems in Africa (Lesueur et al., 2012). The method is highly underutilized due to insufficient information on its management, mechanism and biological background. To overcome this setback, a vital strategy of identifying elite indigenous rhizobia strains with superior symbiotic abilities and environmentally adaptable can be utilized. This will result in increased nitrogen content in plants, improved crop yield and income to smallholder farmers.

Soybean (Glycine max (L.) Merril) is one of the most important sources of food to man and provide animal feeds among the grain legumes. It is an oilseed crop whose oil content is much higher compared to other oilseeds such as peanuts and sunflower (Jaiswal et al., 2011). The protein composition of soybean is about 40 %, oil content 21 % and carbohydrates is approximately 34 % (Hungria et al., 2013). In the world, soybean is relied upon for the supply of oil and fats (Kaizzi et al., 2012). According to Food and Agricultural Organization FAO (2010), countries characterized by rapidly growing
populations, soybean is considered as a crop that can reduce the shortage of national food and oil apart from enhancing nutritional value. Many households depend on soybean as a source of food and when in abundance it is sold to generate income (Sikora et al., 1997).

Soybean yield can be increased through inoculation with bradyrhizobia which improve nitrogen fixation (Ampomah et al., 2008). However, if the inoculum cannot form an effective symbiosis with soybeans or cannot compete well with indigenous rhizobia that nodulate soybeans, the efficiency of inoculum is said to be poor (Clement et al., 2015). To counter this problem, it is vital to assess indigenous rhizobia that nodulate soybeans in terms of genetic diversity, distribution and dominance, compatibility with local soybean cultivars, and environmental factors associated with rhizobia strains in soil (Thuita et al., 2011).

Growth of soybean in Embu and Tharaka-Nithi Counties by smallholder farmers has not been sustainable in the past one decade (Njeru et al., 2013). The region is characterized by low economic growth and reduced soil fertility that is contributed by conventional farming (Lesueur et al., 2012). Despite the significance of soybean cultivation in Kenya, little is known about the genetic diversity of indigenous rhizobia which nodulate soybeans and their potential to improve soil fertility (Thuita et al., 2011). In addition, smallholder farmers in Sub-Saharan Africa (SSA) are resource poor and cannot afford expensive chemical fertilizers to improve nitrogen content for increased soybean yield. To ensure environmental stability and high food production, it is crucial to manage
sustainable soil fertility with minimal application of chemical fertilizers. Currently, there is a challenge of overcoming the high demand for food by increasing population compared to soybean productivity (FAO/IAEA, 2014).

The cost of chemical fertilizers is continuously increasing making it unaffordable to smallholder farmers. Due to the adverse impacts of chemical fertilizers in the environment, farmers are forced to explore other sources of plant nutrients (Lin et al., 2012). Nitrogen fixation on this regard has drawn much attention as viable and a potential substitute for fertilizers (Sharma et al., 2012). The leguminous plants such as soybean maximize the symbiotic association with nitrogen-fixing bacteria to obtain nitrogen (Thuita et al., 2011). However, rhizobia strains are host-specific with soybean plant. This triggers the need to use specific rhizobia inoculants for successful nodulation. To avoid the need for chemical fertilizers and commercial inoculants, International Institute of Tropical Agriculture (IITA) developed promiscuous soybean genotypes for Africa that would nodulate with a wide diversity naturally occurring Bradyrhizobia (Risal et al., 2010).

Despite the availability of commercial inoculants, improved soybean production has not been achieved due to their ineffectiveness in the soil (Kaizzi et al., 2012). The presence of high competitive indigenous strains in the soil that are poor nitrogen fixers also limits the effectiveness of commercial inoculants (Rahmani et al., 2011; Wasike et al., 2009). Rhizobia are host specific bacteria and poor selection of inoculants has resulted to low nitrogen fixation (Waswa, 2013). In some cases, the inoculated rhizobia fail to nodulate
entirely due to unfavorable environmental stress such as drought, high salinity, waterlogged soil, and high acidity (Thuita et al., 2011). There is also a risk of declining soil fertility and depletion of water resources which implies that there is a need to intensify soybean production (Ashworth et al., 2014). The high population in Embu and Tharaka-Nithi Counties region limits the expansion of agricultural land. It is vivid that increased soybean productivity will require improvement in yields rather than increasing planting field.

To overcome the challenge of using commercial inoculants, indigenous rhizobia with high adaptive capabilities to local environment and that can withstand competition and adverse climatic conditions will be required for inoculation (FAO/IAEA, 2014). Genetic identification and molecular characterization of indigenous rhizobia strains is also vital for the isolation of most effective in biological nitrogen fixation. In addition, soils that have not been grown soybean initially lack rhizobia nitrogen-fixing strains such as *Bradyrhizobium* hence the need for inoculation (Lesueur et al., 2012).

**1.2 Problem statement**

Soybean production is rapidly increasing in Embu and Tharaka-Nithi Counties and has been chosen by the government, International Institute of Tropical Agriculture (IITA) and other organizations to fight food shortage in SSA (FAO, 2010). This is because the quality of protein and oils from soybean is higher compared to other grains (Ashworth et al., 2014). However, soybean production has been hindered by low soil fertility, lack of required knowledge on cultivation and lack of awareness of soybean broad nutritional
value. In Kenya, the production of soybean compared to other Sub-Saharan Africa countries is very low and it is estimated to be 480 kg/ha while that of Uganda is 1,110 kg/ha despite being in the same agro-ecological zone (FAO, 2010). Kenya produces about 2,000 metric tons annually while the demand is at 150,000 to 200,000 tonnes per year (FAO, 2015). This therefore requires that soybean is imported to fulfill domestic demand.

Availability of highly effective indigenous rhizobia in Kenyan soils is not fully exploited considering the fact that commercial inoculants still contain exotic strains from foreign countries such as United States of America (Mugabo et al., 2014). In Embu and Tharaka-Nithi Counties, soils have been characterized with low levels of soil fertility and hence require use of costly chemical fertilizers or commercial inoculums that have failed due to competition and adverse climatic conditions (Waswa, 2013). In addition, indigenous Bradyrhizobium are uncharacterized and their nitrogen fixing potential is unknown in Embu and Tharaka-Nithi Counties.

1.3 Justification

Studies have shown that soybean varieties in association with indigenous rhizobia species have the potential of improving soil fertility through biological nitrogen fixation (Thuita et al., 2011). Currently soybeans grown in Embu and Tharaka-Nithi Counties are not inoculated with bradyrhizobia while chemical fertilizers are used by a few of the smallholder farmers (Risal et al., 2010). The potential of indigenous rhizobia species in association with different leguminous plants have been demonstrated in Rwanda and
proved to be efficient method of increasing soil fertility (Mugabo et al., 2014). Unfortunately, limited information is available on this subject.

Increased soybean production will contribute extensively towards poverty reduction, improving food security, health, and nutrition and increased nitrogen content in farms. Soybean grown by smallholder farmers will improve the well-being of farmers as it will provide food and could be marketed to generate income for the family. In addition, the soybean production has agronomic benefits in the smallholder farms by complimenting other plants nutrition such as cereals, root and tubers (FAO/IAEA, 2014). The soybean plants could utilize production niche that are underexploited enhancing their productivity. The plant control erosion due to its fast growth rate where the plant forms a protective soil cover. The rapid growth rate of soybean plant also breaks the disease, weeds, and pest cycles hence increasing farm productive and reduced management cost. It is therefore paramount to replenish soil fertility problems in Embu and Tharaka-Nithi Counties by developing suitable bio-inoculants in the pursuit of food security.

1.4 Research questions

i. Which soybean varieties are grown in Embu and Tharaka-Nithi Counties by smallholder famers?

ii. Are indigenous bradyrhizobia from Embu and Tharaka-Nithi Counties morphologically and genetically diverse?
iii. What is the effect of indigenous bradyrhizobia isolates inoculation on nitrogen fixation and nodulation of soybean varieties grown in Embu and Tharaka-Nithi Counties?

1.5 Research hypotheses

i. There are different soybean varieties that are grown by smallholder farmers in Embu and Tharaka-Nithi Counties.

ii. Indigenous bradyrhizobia from Embu and Tharaka-Nithi Counties are morphologically and genetically diverse.

iii. Inoculation with indigenous bradyrhizobia isolates increases nitrogen fixation and nodulation in soybean varieties grown in Embu and Tharaka-Nithi Counties.

1.6 Objectives

1.6.1 General objective

To determine diversity, effectiveness and impact of indigenous bradyrhizobia isolates on nodulation and nitrogen fixation on soybean varieties grown in Embu and Tharaka-Nithi Counties.

1.6.2 Specific objectives

i. To document soybean varieties grown by smallholder farmers in Embu and Tharaka-Nithi Counties.
ii. To determine the diversity of indigenous bradyrhizobia isolates that nodulate soybean varieties in Embu and Tharaka-Nithi Counties.

iii. To determine the effectiveness and potential of indigenous bradyrhizobia isolates in symbiotic nitrogen fixation and nodulation with soybeans grown in Embu and Tharaka-Nithi Counties.

1.7 The significance of the study

Rhizobia diversity in soils is high and unexploited (Gronemeyer et al., 2014), finding from the study can be utilized for better land management and improvement of legume production by smallholder farmers. Indigenous rhizobia species with high nitrogen fixing capacity isolated will be used to produce inoculums that are highly adaptive to local environment and effective in biological nitrogen fixation (BNF). Therefore, finding from the present study will generate more knowledge to agricultural agents, farmers and policy makers on the use of inoculums for soybean production. Smallholder farmers from Embu and Tharaka-Nithi Counties could also use the results of this study to enhance sustainable farming and management systems on soil fertility. Identified high yielding soybean genotypes with high symbiotic efficiency with bradyrhizobia can be utilized by smallholder farmers to increase the yield and reduce production cost. In addition, use of effective bradyrhizobia inoculums will results in reduced use of chemical fertilizers whereas increased soybean production will lead to improved food security and increased income for smallholder farmers.
CHAPTER TWO

LITERATURE REVIEW

2.1 Importance of soil microorganisms

In Sub-Saharan Africa (SSA), there is continuous decline of soil fertility in rural farms which is an essential impediment to food production in agriculture. According to FAO (2011), Kenya is food insecure which is the case for most SSA countries. The factors behind food insecurity include redundant farming systems, unreliable rainfall, cost of farm inputs and increasing population (Labeyrie et al., 2014). Nutrient deficiency in soil has increased over time due to the use of limited nutrient inputs among smallholder farmers and continuous cultivation (Mateus et al., 2014). Although nitrogen fertilizers are known to improve yield, to resource-poor farmers they are beyond reach due to their high cost. This, however, has contributed to food insecurity in Africa (Kumba et al., 2015). According to FAO/IAEA-(2014) replenishment of soil fertility in SSA is a critical process for the alleviation of poverty.

Soil fertility problems to smallholder farmers can be mitigated through the use of organic resources and specifically from nitrogen-fixing legumes (Loureiro et al., 2007). For instance, most organic matters are decomposed by soil microorganism which enables nutrients recycling in soil. Mycorrhizal fungi which are among the soil microorganisms increase mineral nutrients such as phosphorus availability to plants hence promoting growth. Nitrogen gas from soil atmosphere is made available to plants by nitrogen fixing bacteria by transformation into nitrogenous compounds which can be taken by roots for growth (Mateus et al., 2014). Therefore, nitrogen fixing bacteria and other soil
microorganisms improve plant growth and soil fertility status. These microorganisms are currently receiving high attention since they are considered as biofertilizers in agriculture (Labeyrie et al., 2014).

2.2 Soybean

Soybean is an oilseed crop whose oil content is much higher compared to other oilseeds such as peanuts and sunflower (Labeyrie et al., 2014). The protein composition of soybean is about 40%, oil content 21% and carbohydrates make approximate 34% (Perrineau et al., 2011; Thuita et al., 2011). In the world, soybean is relied upon for the supply of oil and fats. For countries characterized by rapidly growing populations, soybean is considered as a crop that can reduce the shortage of national food and oil apart from enhancing nutritional value. Moreover, within sustainable systems, there is need to improve the yield of soybean to counter community high food demand (Yan et al., 2014). In the near future, there is a risk of declining soil fertility and depletion of water resources which implies that there is a need to intensify soybean production (Florentino et al., 2010).

Soybean is sub-tropical crop and is currently grown worldwide although it originated from Southern Asia (Kaizzi et al., 2012). It is a leguminous plant that forms a symbiotic association with rhizobia forming roots nodules that convert atmospheric nitrogen into forms that are utilizable by plants (Udvardi and Poole, 2013). This property of soybean reduces the need to apply nitrogen fertilizers to enhance growth. To increase soybean production in tropical Africa with the affordable cost to smallholder farmers, IITA has
developed soybean varieties which are promiscuous and nodulate freely with bradyrhizobia strains. Promiscuous genotypes nodulate effectively with native rhizobia unlike non-promiscuous that requires specific strains of rhizobia (Javaid and Mahmood, 2010).

Soybean belongs to grain legumes. It is a bushy annual plant with a hard stem and produces many leaves (Labeyrie et al., 2014). At the root tip, there is apical meristem whose function is for early root growth. The cells of meristems are capable of continuous division forming undifferentiated root cells (Chang, 2014). However, the soybean growth habit is divided into two, the indeterminate and determinate. The determinate type is known to have a high growth rate and mature very faster as compared with indeterminate. They produce fewer leaves despite the high production of beans. On the other hand, indeterminate growth flowering is from the top to bottom (descendent) and they produce few bean seeds and leaves (Wu et al., 2011).

After planting, the seedlings emerge within 5-15 days. The cotyledons unfold after 3-12 days followed by unfolding of the first trifoliolate leaf. Flowering can take approximate within 25-75 days depending on temperature day length and cultivar (Zimmerer, 2013). Soybean flowers are self-pollinated. The number of pods varies from plant to plant that range from few to hundreds. The soybean takes 65 to more than 145 days to mature for harvesting after sowing (Kaizzi et al., 2012).
2.3 Rhizobia taxonomy

Rhizobia are free-living and aerobic soil bacteria that are resident in soils. They are non-spore formers, Gram negative and rod-shaped bacteria (Nur, 2014). According to Zhang et al. (2014), rhizobia have 60 species that belong to 13 different genera that include: *Rhizobium*, *Allorhizobium*, *Azorhizobium*, *Methylobacterium*, *Devosia*, *Burkholdera*, *Bradyrhizobium*, *Allorhizobia*, *Mesorhizobium*, *Phyllobacterium*, *Cupriavidus*, *Ochrobactrum* and *Sinorhizobium*. Rhizobia being soil bacteria have the ability to infect and induce the formation of root nodules in leguminous plants. Rhizobia strains which are associated with soybeans are *Bradyrhizobium*. Bradyrhizobium belongs to the order Rhizobiales and class Bradyrhizobiaceae. The genus *Bradyrhizobium* has nine species and strains that nodulate soybeans are *B. elkanii*, *B. japonicum*, *B. huanghuaihaiense*, *B. diazoefficiens* and *B. liaoningeense* which are slow growers while Sinorhizobia species: *S. fredii* and *S. soyaee* are fast-grower (Althabegoiti et al., 2008). Rhizobia diversity in the soil is abundant which provides a large natural sources of germplasm to strain selection with desirable characteristics.

Rhizobia have diverse metabolic capabilities and hence they can utilize alcohols, sugars, some acids and other carbon compounds as a source of energy (Vauclare et al., 2013). Rhizobia growth media such as Yeast extract mannitol agar (YEMA) requires the provision of these nutrients and vitamins to enhance growth although some strains can provide growth factors by themselves. Rhizobia cells penetrate the leguminous roots that are susceptible during infection where they increase in number resulting in the colonization of root hairs. Embryonic nodules form after colonization when the
Bradyrhizobium penetrates the root cortex (Udvardi and Poole, 2013). The nodules are, therefore, able to fix free atmospheric nitrogen into forms that can be utilized by legumes.

2.4 Bradyrhizobia identification

Major rhizobia strains that form nodules with soybean during nitrogen fixation are Bradyrhizobium japonicum, Sinorhizobia fredii and Bradyrhizobium elkanii (Zhang et al., 2014). Rhizobia that nodulate legumes are present in different regions where their genetic diversities depict climatic and geographical differences and also the diversity of host soybean varieties. However, B. japonicum is the most effective strain on nitrogen fixation with soybean especially in SSA and can make the best inoculants to increase the yield of soybean (Yao et al., 2014).

According to Sharma et al. (2012), most rhizobia strains produce a sticky substance that is gum-like with varied composition. In YEMA, individual colonies are translucent, dome-shaped, viscid and slimy in entire margins (Appunu et al., 2008). However, molecular methods are more definitive in the identification of rhizobia strains. Study of bradyrhizobia diversity and identification can be studied using several molecular and phenotypic approaches. Phylogeny inference identification methods include 16S rDNA RFLP, random amplified polymorphic DNA (RAPD) and use of specific primers for gene sequencing of 16S ribosomal RNA (Atieno et al., 2012; Shiro et al., 2013). In the recent studies, high variations in the sequence of ITS region have proved to be more informative for Bradyrhizobium taxonomic evaluation (Shiro et al., 2013). The ITS region between 16S-23S rRNA genes provides greater resolution compared with 16S rRNA gene and it is
therefore, an important tool to the study of indigenous bradyrhizobia genetic diversity as demonstrated by Yan et al. (2014). Nur (2014) points out that, indigenous bradyrhizobia out-compete commercial inoculants more frequently and therefore, calls for the analysis of indigenous bradyrhizobia genetic diversity. In addition, analysis of field distribution of rhizobia strains is vital in improving the understanding of rhizobia ecology and methods of inoculation under different field conditions (Yan et al., 2014).

2.5 Biological nitrogen fixation

Biological nitrogen fixation (BNF) is the process in which symbiotic bacteria convert atmospheric nitrogen into forms utilizable by plants (Mutuma et al., 2014). Many farms are in need of nitrogen from BNF for effective crop production. The outcome of intense farming has been loss of soil nutrients through runoff, removal of farm produce, leaching, and removal of crop residue (Bizarro et al., 2011). As a result of soil nutrients losses, Kenya has been on the list of being chronically starving nation with the need for food aid (FAO, 2010). Moreover, it's unfortunate that nitrogen removal from the soil far exceeds nitrogen input via biological nitrogen fixation and fertilizers in farming systems applied in Kenya (Woomer, 2012).

The association between bradyrhizobia and leguminous soybean plant forms a distinctive system in the interaction. The interaction results in the formation of nodules on the roots of the host soybean (Bizarro et al., 2011). In the relationship, the rhizobia provide a constant supply of nitrogen to the host plant which as a result receives phosphate and nutrients nourishment (Kaizzi et al., 2012). The leguminous plant as well provides
favorable conditions for growth and metabolism. Rhizobia proliferate in the rhizosphere because they are soil saprophytes and they are specific to legumes (Nur, 2014). An infection thread where rhizobia penetrate into the cortex of the host soybean (Vauclare et al., 2013). The infected cells usually increase in size to form a structure surrounded by healthy cells as a source of nitrogen.

In the soil, rhizobia are associated with the process of denitrification which is a vital process in supplying ATP during environmental stress conditions as exemplified by root flooding which usually results to hypoxia (Appunu et al., 2011). Denitrification activities generate ATP for rhizobia survival under these conditions and also in maintaining the function of nodules. Under low oxygen conditions, rhizobia can grow using nitrate as the electron acceptor in the process of denitrification to support respiration where nitrite (NO₂) or nitrate (NO₃) is reduced to nitrogen (N₂). Periplasmic or membrane bound nitrate-reductase to reduce nitrate to nitrite while the reduction of nitrite to nitric oxide (NO) is catalyzed by nitrite reductase (Bizarro et al., 2011). Nitrous oxide is formed from reduction of nitric oxide by the action of nitric oxide reductases which is further reduced to nitrogen (N₂) by the enzyme nitrous oxide reductase. During the process, 24 molecules of ATP are released for every nitrate molecule reduced which act as an energy source for some rhizobia species during environmental stress conditions (Dhami and Prasad, 2009).

The levels of biological nitrogen fixation are affected by variation of cultivars in many grain legumes. As reported by Furseth et al. (2012), community of soybean-nodulating bradyrhizobia also varies due to differences in soil pH, soil texture and salinity among
other differences in the fields. Guimarães et al. (2012) have shown that particular combinations of cultivar and strain in some crops are efficient in nitrogen fixation. The scarcity of information about biological nitrogen fixation (BNF), the cost of inoculants, availability and cost of inoculants have been the main factor hindering BNF technology adaptation in the region. BNF directly contributes to productivity (Parr, 2014).

Soils without the history of soybeans growth usually lack bradyrhizobia strains especially if they have more than five years without being used (Sarr et al., 2011). Most soils are having no prior history of legumes, therefore, requires inoculation during first planting. However, to get maximum benefit through inoculation, it is necessary to follow carefully and correctly guidelines on inoculation where inoculant should carry quality and effective bacterial cells. Bio-inoculants have recorded erratic performances under field conditions with legumes biological nitrogen fixation making inoculation among most effective technology of improving productivity and yield of grain legumes (Vauclare et al., 2013).

2.6 Importance of biological nitrogen fixation (BNF)

The atmosphere contains mixture of gasses that are nearly homogenous where nitrogen is the most abundant gas with 78.1 % (Jaiswal et al., 2011). It has been confirmed that about 96 % of plants nitrogen have been derived from the atmosphere (Lin et al., 2012). During biological nitrogen fixation (BNF) the nitrogen from the atmosphere is converted into nitrogen forms that are utilizable by plants. The process of BNF involves enzyme
dinitrogenase which catalyzes the reaction by splitting nitrogen triple-bond of the inert atmospheric gas to organic ammonia molecule (Loureiro et al., 2007).

In agriculture, BNF is considered as a sustainable renewable resource by reducing the application of inorganic nitrogen fertilizers and hence increasing economic returns to farmers (Mwenda, 2010; Zhang et al., 2011). Additionally, it contributes to globally understanding of soil microorganisms’ biodiversity, to the importance of indigenous rhizobia and to the designing of strategies that can act as a long-term supply of legume-fixed nitrogen to agriculture. According to Wasike et al. (2009) legumes, BNF contributes greatly to environmentally friendly and economically viable agriculture. About 90% of plants’ utilisable nitrogen is estimated to originate from BNF (Udvardi and Poole, 2013). The main source soil nitrogen replenishment is considered to be BNF and provides ecologically sound and economically attractive means of replacing external nitrogen input (Suzuki et al., 2008). Shiro et al. (2013) pointed out that agricultural systems in Sub-Saharan Africa have in recent years changed to prevent environmental degradation but improve its quality. In regard to this, inoculants consisting of diazotrophic bacteria can prevent environmental degradation and also be used as an alternative nitrogen source in place of nitrogen fertilizers (Parr, 2014).

During soybean production, adequate nitrogen (N) supply is required for maximum grain yield (Vauclare et al., 2013). Legumes including soybeans have the potential of acquiring most of their nitrogen requirement through a symbiotic association with rhizobia. Successful symbiosis is ensured through inoculation of seeds by introducing effective
rhizobia which as a results enhances nitrogen fixation. Many tropical countries have shown a positive remarkable response after inoculation of soybeans with bradyrhizobia (Dhami and Prasad, 2009). Chianu et al. (2009) reported that experiments conducted in Nigeria with soybean cultivars like Bossier recorded up to 100 % increase in yield after inoculation with indigenous bradyrhizobia. A study carried out in Zimbabwe demonstrated the contribution of indigenous strains in increasing soybean yield (Zingore et al., 2011).

Inoculation of soybeans with bradyrhizobia enriches soil nitrogen economy which improves other crops especially in an intercrop system (Sarr et al., 2011). A number of authors recommend intercrop of soybeans with crops such as maize to allow maximum utilization of nitrogen that is obtained during symbiosis (Nur, 2014). Dhami and Prasad (2009) demonstrated how crop rotation improves the yield of other crops after rotation with soybean inoculated with bradyrhizobia. However, this farming system requires consistency in inoculation and use of quality bradyrhizobia strains (Chianu et al., 2009).

2.7 Factors affecting biological nitrogen fixation (BNF)

Environmental factors affect biological nitrogen fixation especially effective nodulation, reducing rhizobia survival and population in the soil. Solomon et al. (2012) described phosphorus (P) as a major mineral nutrient in legumes and it variation significantly affect the yield. Effective N fixation is constrained by the deficiency of phosphorus as it affects the process of nodulation (Sarr et al., 2011). Phosphorus in the nodules is involved in high rates of energy transfer hence is highly required. The nodules become more
abundant when there is a high supply of phosphorus while during phosphorus shortage conditions results to poor nitrogen fixation (Saeki et al., 2005). Additionally, phosphorus is a key functional and structural component of the plant where its efficiency cause effects on plant metabolism and its morphology. Decreased phosphorus supply has an effect on nitrogen fixation which is reflected on the reduced shoot dry matter, shoot and root phosphorus content and leaf area (Pule-Meulenberg et al., 2011).

Water stress or drought is a change of plant water status which may cause a temporary or durable change in the functioning of the plant. Drought is considered as a vital environmental factor that results in yield loss of crops (Meghvansi et al., 2005; Ansari et al., 2014). Legume yield including that of soybeans is limited due to inhibition of biological nitrogen fixation by drought commonly in regions characterized as arid or semi-arid (Achonga et al., 2015). Water deficiency affects biological nitrogen fixation through carbon shortage, limitation of oxygen and nitrogen metabolism regulation (Appunu et al., 2008). Drying soil declines nitrogen fixation which results to inadequate nitrogen for the formation of protein hence reduced yields. A number of studies have also demonstrated that fixation of nitrogen by leguminous plants is reduced by drought stress (Dhami and Prasad, 2010; Aung et al., 2013; Mutuma et al., 2014).

Soil salinity and pH affect nitrogen fixation according to Ormeño-Orrillo et al. (2006). Salinized soil affects plants by inhibiting growth due to Cl and Na ions. When Na ions are elevated they inhibits other nutrients uptake such as K, Zn, Cu, and Fe which interfere directly with root plasma membrane transporters (Perrineau et al., 2011). There is little
nitrogen in salinized soils and hence not suitable for most plants cultivation and especially legumes that require higher nitrogen for metabolism processes. However, most legume plants are known to be sensitive to salinity even at low levels (Pule-Meulenberg et al., 2011). According to Risal et al. (2010) soil microorganisms encounter detrimental effect from rising salt concentrations in soil due to osmotic stress or toxicity (Saeki et al., 2005).

Rhizobia specifically are sensitive to salinity at both high and moderate levels which affect the symbiotic process. A pH between six and seven is considered optimal for rhizobia species. A pH greater than 8 or less than 5 is considered significantly out of this range and has adverse effects to biological nitrogen fixation by upsetting communication process during root hair infection by rhizobia thereby inhibiting the development of nodule (Sharma et al., 2010). Additionally, the amount of the nitrogen fixed is affected by soil pH and for example amount of fixed nitrogen is reduced up to 30% in a very low pH of 4 (Solomon et al., 2012).

2.8 Effectiveness and potential of bradyrhizobia under different field conditions

Bradyrhizobia is host specific and effective nodulation of grain legumes mostly rely on compatible and specific strains in the soil for a given legume (Meghvansi et al., 2005). Bradyrhizobia and legume symbiosis performance are usually affected by environmental stresses and several soil factors such as pH which may hinder nodulation and biological nitrogen fixation (Graham and Vance, 2003; Jaiswal et al., 2011). Bradyrhizobial
persistence is affected by acidity in soil and rhizosphere of plants and has been a common phenomenon in tropical areas (Meghvansi et al., 2005). However, most indigenous bradyrhizobia have evolved adaptive strategies known as acid inducible tolerance mechanism to overcome damage due to high acidity in the soil (Ansari et al., 2014).

The adaptive strategies by indigenous bradyrhizobia have made them to be highly competitive in field conditions when compared to commercial inoculants. However, harsh environmental conditions have resulted in a low number of indigenous bradyrhizobia which reduce the effectiveness of biological nitrogen fixation. For optimal nodulation and valuable nitrogen fixation, sufficient numbers of bradyrhizobia are required (Chianu et al., 2009; Singh et al., 2011). Efficient and specific bradyrhizobia populations are required during inoculation as a valuable and proven strategy to improve the productivity of soybeans and other grain legumes.

2.9 Methods for estimating biological nitrogen fixation

Accurate measures to determine symbiotic nitrogen fixation in legumes are vital in establishing the contribution of bradyrhizobia and improving fixation efficiency (Furseth et al., 2012). There are several methods to quantify biological nitrogen fixation. The site and the type of experiment determines the choice of a particular method (Solomon et al., 2012). The methods includes: Xylem sap analysis, acetylene reduction assay (ARA), N isotope methods and total nitrogen difference (TND) (Perrineau et al., 2011). The methods have their own limitations, however, total nitrogen difference have known
limitations hence and can be avoided to reduce their effect on symbiotic efficiency calculations (Singh et al., 2011).

Nitrogen difference and nitrogen balance method involves growing a non-nitrogen fixing plant (control) and adjacent nitrogen-fixing crop and comparing the difference in the tissue N (Chianu et al., 2009; Muthuri et al., 2014). The method is easy to carry out under field conditions and inexpensive. The alternative method is to determine nitrogen difference where nitrogen in the soil and both test legume and control crops are taken into account (Perrineau et al., 2011; Muthuri et al., 2014). The soil nitrogen component enables nitrogen transformation over the growing season and nitrogen can be assessed by assuming the nitrogen transformation between the test plants is equal (Meghvansi et al., 2005).
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites

This study was carried out in Embu and Tharaka-Nithi Counties in Eastern Kenya (Figure 3.1). Embu County is located at the foot of Mt. Kenya with an elevation of 1100-1500 meters above sea level. The area receives an annual precipitation ranging from 600-1800 mm bimodally, where over 55% of the rain falls in the long season (October to February). Temperatures are moderate ranging from 12 °C to 26 °C. Fields experiments were carried out in two agroecological zones in Embu County namely, Embu Upper Midland Zone (EUMZ) (1500-2000 m asl) and Embu Lower Midland zone (ELMZ) (1000-1500 m asl).

Tharaka-Nithi is on the South-Eastern side of Mt. Kenya with an elevation of 1500 m above sea level (Njeru et al., 2013). During cold season the average temperature is 11 °C while in the hot season it is 25 °C. The area receives low rainfall that range between 200-800 mm per year, bimodally distributed (Mwenda, 2010). Short rains occur between October and February while long rains falls from March to June. Field experiments in the region were also divided into two agroecological zones, Tharaka-Nithi Upper Midland Zone (TUMZ) (1500-2000 m asl) and Tharaka-Nithi Lower Midland zone (TLMZ) (1000-1500 m asl).
3.2 Assessment of soybean varieties grown by smallholder farmers

3.2.1 Questionnaires administration in the selected sites

A structured questionnaire (Appendix II) was administered face-to-face to individual smallholder farmers in form of interviews through random household visits. The interviews were carried out over a period of six days in the month of May 2015. The survey coincided with mid growing seasons of long rains that start from March to August where information from farmers was compared with crops in the farms. A total of 60 households were interviewed in the selected study sites. The sample size was determined following formula from Snedecor and Cochrane (1977).
\[ n = \frac{4pq}{(L)^2} \]

Where; \( n \) = sample size, \( p \) = proportion in the target population, \( q = 1-p \), and \( L \) = accepted error (5 %). During this study, the target population was the number of soybean producers in each Zone which was obtained from ministry of agriculture under County Agricultural Extension Officer. In Embu, the target population was 12844 soybean farmers. The total soybean farmers in upper and lower midland zones was 258 farmers. The calculated sample was; \( P = \frac{12844}{258} = 0.0201 \), \( q = 1-0.0201 = 0.9671 \), \( L^2 = (0.05)^2 = 0.0025 \)

\[ n = \frac{4 \times 0.0201 \times 0.9671}{0.0025} = 31.5 = 32 \text{ smallholder farmers} \]

In Tharaka-Nithi, the target population was 9671 smallholder soybean farmers while 170 farmers in the upper and lower midlands zone. The calculated sample size was; \( p = \frac{9671}{170} = 0.0176 \), \( q = 1-0.0176 = 0.9824 \), \( L^2 = (0.05)^2 = 0.0025 \)

\[ n = \frac{4 \times 0.0176 \times 0.9824}{0.0025} = 27.66 = 28 \text{ smallholder farmers} \]

### 3.3 Determination of indigenous bradyrhizobia diversity

#### 3.3.1 Soil sampling

Soil sampling was carried out across and diagonally in 20 points in every selected two farms in each agroecological zones before the rains. Soil samples were collected in the upper part (5-20 cm) of the soil. The soil sampled from each farm were mixed thoroughly to make a homogenous composite sample. A sub-sample (1 kg) of the composite samples
per farm was packed independently to avoid cross contamination. The samples were then transported to Kenyatta University for analysis.

3.3.2 Chemical and physical analysis of the soils collected from study sites

Collected soil samples were analyzed for both chemical and physical properties including pH, texture, total nitrogen, available phosphorus, organic carbon, Cation exchange capacity and percentage content of clay, sand and silt.

3.3.2.1 Soil pH

Soil pH was determined using a pH meter in a prepared soil-water suspension ratio of 1:2.5. In a 250 ml conical flask, 20 g of soil sample from each farm was weighed and mixed thoroughly with 50 ml of sterile distilled water. The mixture of soil and water was allowed to stand for 20 minutes before measuring pH. The pH meter was calibrated using buffers of pH 7.00 and 4.00. The pH values were recorded after immersing the pH meter electrode into soil suspension (Perrineau et al., 2011).

3.3.2.2 Soil organic carbon

Walkley-Black combustion method was applied to determine organic carbon as described by Ashworth et al. (2014). The process involved the use of sulphuric acid and potassium dichromate (Himedia) mixture for wet combustion of soil organic matter. Dichromate which was in excess after the reaction was titrated against ferrous sulphate (FeSO₄) (Himedia). Air-dried soil was finely grounded and 1 g was weighed and transferred into a
sterile 250 ml Erlenmeyer flask containing 2 ml of sterile distilled water. To the flask, 10 ml of 0.17 M potassium dichromate was added and swirled to form a solution that was uniform. To the resulting solution, 20 ml of concentrated sulphuric acid was added. The digestion of the solution was allowed to take place for 30 minutes at 150 °C and then allowed to cool before 100 ml of distilled water was added. The solution was mixed well where 1 ml of diphenylamine indicator and 10 ml of ortho-phosphoric acid was added. Reference sample and a blank were added to the mixture. The solution was titrated by adding ferrous sulphate drop-wise up to an end point when the solution turned from purple color to dark green (Mason et al., 2013). The volume of ferrous iron used was recorded and used to calculate organic carbon as follows:

\[
C (\%) \text{ in the soil} = \frac{\text{Molarity of FeSO}_4 \text{ solution} \times (V_{\text{Blank}} - V_{\text{Sample}}) \times 0.39}{\text{Weight of dry soil sample (g)}}
\]

Where;

\[V_{\text{Sample}} = \text{H}_2\text{SO}_4 \text{ volume (ml) used to titrate the sample}\]

\[V_{\text{Blank}} = \text{H}_2\text{SO}_4 \text{ volume used to titrate the blank}\]

3.3.2.3 Total Nitrogen

Total nitrogen was determined using distillation and digestion. Concentrated sulphuric acid (Himedia) digested soils to raise boiling temperature which resulted to conversion of organic nitrogen into ammonium nitrogen (Ansari et al., 2014). Steam distillation was
used to obtain ammonium-N from digest while using NaOH to raise the pH. Saturated H$_3$BO$_3$ (Himedia) was used to collect distillate and titrated using diluted sulphuric acid to pH 5.0 (Ashworth et al., 2014).

3.3.2.4 Extractable phosphorus P and potassium K

Available phosphorus and potassium (K) were determined according to Mehlich-3 (M-3) procedures (Furseth et al., 2012). Dry soil samples were grounded and 3 g of each soil was passed through a sieve of 2 mm size into Erlenmeyer flasks. Thirty (30) ml extraction solution of M-3 was added at the ratio of 10:1 (solution:soil). The mixture was shaken at 120 oscillations min$^{-1}$ using rotating shaker. Whatman filter paper was used to filter the suspension into plastic vials and solution was analyzed using Atomic Absorption Spectrophotometer (AAS) (Furseth et al., 2012).

3.3.3 Isolation and enumeration of soil bacteria from the soil samples

3.3.3.1 Dilution of soil samples

The sampled soils were mixed thoroughly to make a homogenous composite soil. Ten grams of the mixture was diluted with 100 ml of sterile distilled water which was taken as $10^{-1}$ dilution factor. After mixing thoroughly, 1 ml of $10^{-1}$ dilution was transferred to 9 ml sterile distilled water in screw capped test tubes using sterile pipettes which resulted to $10^{-2}$ dilution (Chianu et al., 2009). In the same way, a series of up to $10^6$ dilutions were prepared under aseptic conditions.
3.3.3.2 Inoculation

After dilution, inoculation was carried out using spread plate technique: 1 ml of aliquot from the suspensions of the test tube(s) labeled: $10^{-3}$ and $10^{-4}$ were inoculated onto nutrient agar and incubated at 37 °C for 24 hours for the estimation of general bacterial population. Dilutions $10^{-1}$ and $10^{-2}$ were inoculated on Yeast Extract Mannitol Agar (YEMA) with Congo red and incubated at 28 °C for 4 days for the estimation of rhizobia population: A $10^{-1}$ dilution sample was inoculated on Potato Dextrose Agar (PDA) and incubated at room temperature for 3 days for the estimation of fungal population; Another $10^{-1}$ dilution sample was inoculated on M1 media and incubated at 30 °C for 2 days for the estimation of actinomycetes population. After inoculation and incubation, colonies that grew were counted using an electric colony counter and percentage populations tabulated.

3.3.4 Field trapping of bradyrhizobia

The farms used for soil sampling were prepared for planting in the month of March 2015 prior to the onset of the long rains. Three soybean varieties (SB8, SB24 and SB126) were planted in the four agroecological zones (TUMZ, TLMZ, EUMZ and ELMZ) during the short rain season (April to August). Four plants from each plot were sampled for nodule analysis at the onset of flowering stage. Nodules were detached from the roots after washing carefully with sterile distilled water and then wrapped with absorbent tissue paper to dry. Four nodules from each plant pink in color were selected for nodule analysis.
3.3.5 Isolation of indigenous bradyrhizobia

3.3.5.1 YEMA media for isolation
Bradyrhizobia from soybean root nodules were grown in Yeast extract mannitol agar (YEMA) media which was prepared according to Ashworth et al. (2014). The media comprised of Yeast extract (0.5 g) (Oxide), mannitol (10.2 g) (Oxide), K$_2$HP0$_4$ (0.5 g) (Oxide), MgSO$_4$·7H$_2$O (0.1 g), NaCl (0.2 g) and agar (15.0 g) (Oxide). The components were dissolved using distilled water and the volume adjusted to one litre. pH meter was used to adjust the pH to 6.8 using either 1.0 M sodium hydroxide (NaOH) or 1.0 M hydrochloric acid (HCl). The medium was sterilized by autoclaving for 15 minutes at 121 °C and a pressure of 15 psi.

3.3.5.2 Isolation of bradyrhizobia and culture preservation
Nodules from trap experiment were placed in sterile distilled water for one hour to imbibe water. They were then rinsed with distilled water and dipped in 95 % ethanol for 6 seconds to reduce the surface tension. The nodules were then sterilized using 1 % sodium hypochlorite (NaOCl) (Oxide) for 5-10 minutes and then rinsed with sterile distilled water in six changes (Thuita et al., 2011). Crushing of nodules was carried out using a blunt-tipped pair of flame-sterilized forceps. A loopful of the nodule suspension was streaked on a petri dish containing YEMA media supplemented with Congo red and incubated at 25 °C in the dark. After an incubation period of 72 hours, single colonies were identified and checked for purity by sub-culturing on YEMA (Mungai and Karubi, 2011). The stock cultures were stored on slants in McCartney screwed cap bottles. The slants in McCartney bottles were prepared by dispensing plain YEMA to about half of the
bottle volume. The bottles were allowed to cool after sterilization before transferring the pure isolates. Screw caps for the bottles with stock cultures were tightened and refrigerated at 4 °C. Sub-culturing of the isolates was carried out after every three months to maintain their viability.

3.3.6 Identification of bradyrhizobia isolates

3.3.6.1 Morphological characterization of the indigenous isolates

Gram staining reaction was carried out as described by Zhang et al. (2014). Isolates were streaked on YEMA supplemented with Congo red dye. The Congo red media was used to test whether the isolates could absorb the stain in the dark. Bradyrhizobia species do not absorb or they absorb little Congo red in the dark. To establish whether the isolates were alkaline producers or acid producers, the bradyrhizobia isolates were streaked on Bromothymol blue (BTB) media. Cultural characteristics of the isolates from different agro-ecological zones were established through presumptive tests. The colony morphology characteristic that were considered included form, color, margin, transparency, mucosity and acid or alkaline reaction (Thuita et al., 2011).

3.3.7 DNA fingerprinting for bradyrhizobia isolates

3.3.7.1 DNA extraction

The bradyrhizobia isolates (39 isolates) prior to DNA extraction were cultured on YEMA for 5 days at 28 °C. Pure single colonies of indigenous bradyrhizobia isolates were picked with sterile wire loop, washed at in a 100 μl TE (pH 7.5) (Appunu et al., 2008). Three
hundred microliters of the lysis buffer (0.12 M Na₂HPO₄ [pH 8.0], 1% sodium dodecyl sulfate [SDS], 0.1 mg of proteinase K per ml) (Biolab) was added to the washed cells and vortexed for 30 seconds followed by incubation at 65 °C for 15 minutes (Atieno et al., 2012). Three hundred microliters of chloroform isoamyl alcohol (Biolab) was added and mixture inverted several times until a milky solution was formed to remove cell debris and protein. The samples were centrifuged at 13,000 rpm for 10 minutes and the supernatant was transferred into a sterile 1.5 ml appendorf tube. DNA was precipitated using 300 μl of 100 % ethanol (Himedia) and incubated for 30 minutes at -20 °C. The samples were centrifuged at 13, 000 rpm for 10 minutes and the supernatant was discarded to remain with DNA pellet. The DNA pellet was air dried at room temperature and re-dissolved in 30 μl of deionized water (Shiro et al., 2013). The extracted total DNA samples from the bradyrhizobia isolates were detected using gel electrophoresis. Each DNA sample (5 μl) was loaded on 1 % agarose gel containing Cyber green in 0.5 X TBE buffer (Biolab) and was run at 80 V for 50 minutes (Sharma et al., 2012).

3.3.7.2 PCR amplification of 16S rRNA gene

In each PCR reaction, the total volume of 25 μl contained 2.5 μl of 10 x PCR buffer, 0.5 mM dNTPs, 0.75 μl of Taq DNA polymerase, 0.4 μl of 10 μM primer Y1, 0.4 μl of 10 μM primer Y3, 1.5 mmol·L⁻¹ MgCl₂, and 15 μl deionized water (Biolab). The DNA template (4 μl) was then added to the PCR reaction mix. The sequence of the forward primer Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') corresponding to positions 20-43 while the reverse primer (Y3) 5'-TACCTTGTTACGACTTCAACCCCAGTC-3' corresponding to positions 1482-1507 for
16S rDNA sequence of *Escherichia coli* (Zhang et al., 2014). Thermo-cycler (Techne, UK model) was used to carry out the reactions. The PCR amplification conditions comprised of initial denaturation for 2 minutes at 95 °C, followed by 37 cycles (denaturation at 94 °C for 40 seconds, annealing for 30 seconds at 55 °C, extension for 2 minutes at 94 °C and final elongation at 72 °C for 10 minutes the amplified DNA was then held at 4 °C (Ashworth et al., 2014).

3.3.7.3 Agarose gel electrophoresis

Amplified PCR products (5 μl) were stained using SYBR green (Biolab) and separated by gel electrophoresis (1.4 % w/v) in Tris-Acetat-EDTA (0.5XTAE) (Biolab) and run at 80 V for 50 minutes. Molecular marker (100 bp DNA ladder) was used to estimate the molecular size of the bands. The bands were then visualized with UV trans-illuminator lamp after gel electrophoresis and photographed (Perrineau et al., 2011).

3.3.7.4 16S rRNA gene (16S rDNA) restriction fragment analysis

Restriction fragment analysis for PCR products (5 μl each) was carried out according to methods described by Zhang et al. (2014). Restriction enzymes *HaeIII, MspI,* and *EcoRI* (Biolab) 10 μl each were used separately according to manufacturers’ instructions. The digested fragments were stained using SYBR green (0.4 μl) and separated at 80 V for 50 minutes in agarose gel electrophoresis (1.4 % w/v) in Tris-Acetat-EDTA (0.5XTAE) buffer. Fragment sizes were estimated using a 100 kb DNA ladder while SYBRE green was used for staining. UV trans-illuminator lamp was used to visualize restriction
products. The analysis of restriction fragments was carried out as described by Zhang et al. (2014)

3.4 Determination of effectiveness and potential of indigenous bradyrhizobia isolates in symbiotic nitrogen fixation and nodulation with soybean varieties

3.4.1 Authentication of the bradyrhizobia isolates

Authentication of representative bradyrhizobia isolates from field trapping experiment was carried out to confirm their ability to nodulate soybean plants in a greenhouse conditions. Three soybean varieties (SB8, SB24 and SB126) obtained from Kenya Seeds were used as the test plants and were planted in Leonard jar assemblies. The Leonard jars were sterilized in 3 % sodium hypochlorite (NaOCl) and rinsed in five changes of sterilized distilled water. After drying the jars were decontaminated by swabbing using 70 % ethanol. The rooting medium was vermiculite which was soaked in tap water for 3 days and then washed in running water to remove the nutrients. The vermiculite was rinsed with distilled water and autoclaved at 121 °C for 15 minutes and at pressure of 15 psi. The sterile vermiculite was transferred to the jars and covered with aluminum foil swabbed with 70 % ethanol. To remove any possible contaminants, the assemblies were steamed for 10 minutes in an autoclave.

Soybeans with uniform size, shape and colour were surface sterilized in 3 % NaOCl followed by rinsing in five changes of sterile distilled water. The seeds were pre-germinated in damp sterile vermiculite contained in kilner jars at 28 °C for three days.
Three seedlings were transplanted to the Leonard jars and later thinned into one. After eight days, the plants were inoculated with representative isolates selected from each group based on morphological characteristics. The bradyrhizobia isolates were cultured in YEMA broth media and 1 ml of each isolate was inoculated after three days. The treatments were arranged in a complete randomized design with four replicates. Negative controls consisted of uninoculated seedlings while positive control was a commercial inoculant. The plants were supplied with nitrogen free growth media weekly. Growth media comprised of full-strength plant growth solution and was applied to the plants throughout the experiment. For positive nitrogen control treatment, 0.05 % KNO$_3$ was added to give a nitrogen concentration of 70 ppm. Depending on the treatment, stock solutions were separately prepared and mixed at the required rate as described by Ashworth et al. (2014) and Mason et al. (2013) (Appendix I). Negative controls comprised of non-inoculated N-free plants and were supplied with sterile distilled water weekly. The pH of the solution was adjusted using 1.0 N NaOH or 1.0 N HCl to 6.8. Each stock solution before the application was sterilized for 15 minutes at 121 °C and a pressure of 15 psi.

Plants were harvested after 40 days and were washed gently with tap water to remove the vermiculite. The roots were checked for ineffective or effective nodulation by presence or absence of nodules (Adhikari et al., 2012). The nodules were removed from plants, enumerated and dried at 28 °C to a constant dry weight before their biomass was recorded.
3.4.2 Determination of symbiotic efficiency of the indigenous isolates

Isolates were tested to determine their effectiveness on nodule formation and their compatibility with the three soybean genotypes (SB8, SB24, and SB126) under bacteriologically controlled environment. The study design was a randomized complete block design (CRD) replicated four times (Jaiswal et al., 2011). The soybean varieties were the main plots while bradyrhizobia isolates (RI1 to RI9), a consortium (ICT), commercial inoculants (Bio-fix) (BT), consortium + commercial inoculant (BICT) and a negative control (UT) made the sub-plots. The bradyrhizobia isolates (RI1-RI9) were selected as representative isolates in group based in morphological characteristics. Leonard jar assemblies were filled with rooting medium vermiculite and nitrogen-free growth media and they were autoclaved for 15 minutes at 121 °C and pressure of 15 psi.

Soybean seeds with uniform size, shape, and color were surface-sterilized using 70 % ethanol and NaOCl with 0.25 % chlorine. Kilner jars with sterile vermiculite were used to pre-germinate seeds. Vermiculite was moistened with distilled water and covered with aluminum foil before sterilization by autoclaving to remove possible contaminant. The vermiculite was cooled overnight and using a sterile spatula, sterilized soybean seeds were planted. The seeds were incubated for three days at 28 °C when uniform early germination was achieved. After the incubation period, seedlings whose radicle size was about 1-2 cm were considered for transplanting to Leonard jar assemblies. Two seedlings were aseptically transplanted into Leonard jar assemblies with their radicle facing downwards in the rooting medium. Before inoculation the plants were thinned to one
plant per Leonard jar assembly. Each of the seedling was inoculated with 0.1 ml of the broth media cultured for three days (Jaiswal et al., 2011).

After 45 days, soybean plants were uprooted carefully from the vermiculite while ensuring no nodules were left. The nodules were removed from plants, enumerated and dried at 28 °C to a constant dry weight. Shoot and root materials were separated and dried at 70 °C in the Oven until a constant dried weight was achieved. Dry weight of shoots, roots and nodules were recorded. Bradyrhizobia isolates which did not nodulate soybeans were considered non-infective and hence dropped for further consideration.

3.4.3 Field experiments

3.4.3.1 Land preparation and Seeds planting

In each of the County, the upper and lower zones that were used in the first season during the trapping experiment were used for the field experiments. The field sites were cleared off the prevalent weeds before planting using hand hole after demarcation. The size of each plot measured 3 m by 3 m. Furrows were made 45 cm apart which made the planting rows, while planting was carried out at a spacing of 20 cm and a depth of 12 cm (Adhikari et al., 2012). Non-inoculated row was used to separate each plot to reduce cross contamination. The study was set up as a split plot arrangement in a randomized complete block design (RCBD) replicated three times (Jaiswal et al., 2011). Two soybean varieties (SB8 and SB126) were used as the main plots while bradyrhizobia best isolate (RI9) from greenhouse experiment, a consortium (ICT), commercial inoculant (Bio-fix)
(BT), consortium + commercial inoculant (BICT) and a negative control (UT) were the sub-plots.

3.4.3.2 Inoculation

Filter mud was used as a carrier material for inoculants. The inoculant from the filter mud was applied at the rate of 10 g per kg of soybean seeds while 16% of gum arabic sticker was applied as an adhesive to help inoculant carrier material to stick onto the seeds (Furseth et al., 2012). To increase chances of germination, clean and undamaged seeds of soybean, with high viability were selected for planting. The seeds were first coated with the gum arabic solution and swirled until all the seeds were uniformly wet. The inoculant was added, and the container swirled gently until all the seeds were uniformly coated. Before planting, the seeds were air-dried under a shade to enhance adhesion of the inoculant. Three seeds were sown per every hole. After germination, thinning of seedlings was carried out when they attained at least two pairs of true leaves. One healthy and uniformly growing seedling was left to maturity (Jaiswal et al., 2011).

3.4.3.3 Nodule assessment

Four plants at mid-flowering (50% flowering) were sampled randomly from each plot for nodule analysis. The uprooting of the whole plant was carried out carefully to obtain roots with intact nodules. Water was gently used to wash off the soil adhering on the root system over a sieve. Nodule from each sampled plant were detached carefully from the roots and their number per plant recorded. Nodule dry weight was determined by drying
the nodules at 70 °C to a constant dry weight. The average dry weight was given in g
plant\(^{-1}\) (Muthini et al., 2014).

### 3.4.3.4 Shoot %Nitrogen, available Phosphorus and Potassium content

At mid-flowering, uprooted plants samples were dried to a constant weight at 70 °C. The
material was ground into fine particles that can pass a 1 mm sieve. The Kjeldahl method
was applied to determine the concentration of nitrogen as described by Vauclare et al.
(2013). Available phosphorus (P) and potassium (K) were extracted by Mehlich-3
procedure and analyzed using Atomic Absorption Spectrophotometer (AAS) (Furseth et
al., 2012).

### 3.4.3.5 Harvesting

All the plants were harvested after reaching physiological maturity, 120 days after
planting. The pods for each plant were detached and their number recorded. From the
pods, the seeds were removed and their number per plant recorded. Stover dry weight for
each plot and number of plants per plot were also recorded. Grain dry weight per plant
after threshing and seed dry weight for 100 seeds for each treatment were also recorded.

### 3.5 Data analyses

Data on the number of nodules per plant, nodule weight, shoot and root dry weight and
total nitrogen were analyzed using analysis of variance (ANOVA) using GLM (General
Linear Models) procedure. Tukey’s Honest Significant Difference (HSD) test was used at
p< 0.05 (Bintu, 2014) to separate the means. Morphological data cluster analysis for the isolates was carried out using Euclidean distance similarity procedures (Shiro et al., 2013; Yao et al., 2014). Jaccard similarity (1908) was used to analyze data from PCR products. Gene Alex software (version 6.5) was used to analyze ARDRA restriction patterns to determine the genetic diversity of the bradyrhizobia isolates. UPGMA (Unweight pair group method with the arithmetic average) clustering method was used to convert similarity matrix values into dendrogram (Yao et al., 2014).

Soybean diversity index, abundance and evenness for all the zones was calculated using Shannon-Weiner index and Simpson diversity index as follows:

i. Shannon-Weiner index \( (H') = -\sum (Pi) \ln (Pi) \)

Where; \( Pi = ni/N, \)

\( Ni = \) number of individuals of species
\( N = \) total number of individuals of all species in a sample.

ii. Simpson diversity index \( (D) = \sum n(n - 1)/N(N-1) \)

Where; \( N = \) total number of individuals in the sample of all species
\( n = \) is the total number of individuals of species
CHAPTER FOUR

RESULTS

4.1 Documentation of soybean varieties grown by smallholder farmers

4.1.1 Survey of soybean varieties

Four different varieties of soybeans were recorded in the study sites (Table 4.1). The varieties were Gazelle, Nyala, Namsoy, and Mausoy where all were local varieties. Many farmers preferred Gazelle soybean genotype which was reported by 71% of the households interviewed while the second most preferred soybean genotype was Nyala (12%). The two varieties, Gazelle and Nyala, were distributed in all the selected agroecological zones. (Table 4.1). Namsoy and Mausoy varieties were not very common in the region and hence had low frequencies among the soybean grown by smallholder farmers (Table 4.1). Mausoy variety was only in the lower midland zones (ELMZ and TLMZ).

Table 4.1: Percentage distribution of soybean varieties in the selected agroecological zones in Embu and Tharaka-Nithi Counties

<table>
<thead>
<tr>
<th>Soybean varieties (%)</th>
<th>Nyalla</th>
<th>Gazell</th>
<th>Namsoy</th>
<th>Mausoy</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUMZ</td>
<td>13</td>
<td>75</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>ELMZ</td>
<td>9</td>
<td>73</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>TUMZ</td>
<td>20</td>
<td>70</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>TLMZ</td>
<td>8</td>
<td>67</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Overall (All zones)</td>
<td>12</td>
<td>71</td>
<td>10</td>
<td>7</td>
</tr>
</tbody>
</table>

TUMZ; Tharaka-Nithi Upper Midlands zone, TLMZ; Tharaka-Nithi Lower Midlands zone, EUMZ; Embu Upper Midlands zone, ELMZ; Embu Lower Midlands zone.
Among the smallholder farmers interviewed, the average area under soybean cultivation was 0.25 acres per household. TUMZ had the largest average farm size of between 0.25-0.5 acres per household. Other zones recorded small average farm sizes (<0.25 acres household⁻¹) and had a characteristic of farm fragmentation and intercropping of soybean with other crops such as maize. TLMZ and TUMZ had highest average soybean yield of between 90-180 kg year⁻¹ per household (Table 4.2). Households in EUMZ and ELMZ recorded average soybean yield of <90 kg year⁻¹. The average income per household surveyed due to soybean farming was <2700 Ksh year⁻¹. The highest soybean income was recorded from households at TLMZ. All the zones reported average poor soil fertility except TUMZ that had borderline soil fertility (Table 4.2).

<table>
<thead>
<tr>
<th>Agro-ecological Zones</th>
<th>Cultivated area (Acres household⁻¹)</th>
<th>Yield (kg year⁻¹)</th>
<th>Average Income (Ksh year⁻¹)</th>
<th>Average level of soil fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUMZ</td>
<td>&lt;0.25</td>
<td>&lt;90</td>
<td>&lt;2700</td>
<td>Poor</td>
</tr>
<tr>
<td>ELMZ</td>
<td>&lt;0.25</td>
<td>&lt;90</td>
<td>&lt;2700</td>
<td>Poor</td>
</tr>
<tr>
<td>TUMZ</td>
<td>0.25-0.5</td>
<td>90-180</td>
<td>&lt;2700</td>
<td>Border-line</td>
</tr>
<tr>
<td>TLMZ</td>
<td>&lt;0.25</td>
<td>90-180</td>
<td>2701-5400</td>
<td>Poor</td>
</tr>
</tbody>
</table>

TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; ELMZ, Embu Lower Midland Zone.

4.1.2 Soybean diversity

The results showed that soybean diversity was relatively low according to Shannon Weiner diversity index ($H'$). The highest $H'$ (1.81) was recorded at TLMZ followed by TUMZ with $H'$ of 0.95. In Embu, the two agroecological zones EUMZ and ELMZ had
lowest $H'$ of 0.85 and 0.86, respectively (Table 4.3). Soybean evenness was reported highest at TUMZ with 0.86 followed by TLMZ with evenness of 0.81. EUMZ and ELMZ had the least soybean evenness of 0.78 and 0.79 respectively (Table 4.3).

**Table 4.3: Diversity indices of soybean varieties grown in Embu and Tharaka-Nithi Counties**

<table>
<thead>
<tr>
<th></th>
<th>EUMZ</th>
<th>ELMZ</th>
<th>TUMZ</th>
<th>TLMZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individuals</td>
<td>9</td>
<td>11</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Dominance</td>
<td>0.36</td>
<td>0.49</td>
<td>0.44</td>
<td>0.35</td>
</tr>
<tr>
<td>Simpson I-D</td>
<td>0.49</td>
<td>0.51</td>
<td>0.56</td>
<td>0.65</td>
</tr>
<tr>
<td>Shannon H</td>
<td>0.85</td>
<td>0.86</td>
<td>0.95</td>
<td>1.81</td>
</tr>
<tr>
<td>Evenness e^H/S</td>
<td>0.78</td>
<td>0.79</td>
<td>0.86</td>
<td>0.81</td>
</tr>
</tbody>
</table>

TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; ELMZ, Embu Lower Midland Zone.

**4.2 Determination of the diversity of indigenous bradyrhizobia isolates**

**4.2.1 Soil characteristics of experimental sites**

The soils from the study sites were characteristically acidic. The pH ranged from 4.52 to 6.31. The soil from TUMZ had a relatively lower pH value compared to the rest of sites (Table 4.4). TLMZ and ELMZ soil had also low pH of 5.42 and 5.01 respectively, while soil sampled from EUMZ was slightly acidic with a pH of 6.31. The soil %N ranged from 0.25 % in soils from TLMZ to 0.42 % in soils from EUMZ (Table 4.4). Available phosphorus ranged from 17.51 ppm in soils obtained from ELMZ to 27.00 ppm in soils from EUMZ. The soils from TUMZ and TLMZ had available P of 26.50 ppm and 21.00
ppm respectively. The concentrations of exchangeable potassium ions (K⁺) in soils ranged from 0.40 cmol.kg⁻¹ in soils from ELMZ to 1.50 cmol.kg⁻¹ in soils from TLMZ. The percentage organic carbon from the soils ranged from 2.49 % in TLMZ to 3.29 % in EUMZ. The clay content in the soils ranged from 21 % to 53 % and soil from EUMZ had the highest clay content. The soil texture from the farms was either clay, sandy clay loam or sandy clay (Table 4.4).

Table 4.4: Characteristics of soil from the experimental sites

<table>
<thead>
<tr>
<th>Properties</th>
<th>TUMZ</th>
<th>TLMZ</th>
<th>EUMZ</th>
<th>ELMZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ph</td>
<td>4.52</td>
<td>5.42</td>
<td>6.31</td>
<td>5.01</td>
</tr>
<tr>
<td>OC (%)</td>
<td>2.98</td>
<td>2.49</td>
<td>3.29</td>
<td>3.06</td>
</tr>
<tr>
<td>N (%)</td>
<td>0.26</td>
<td>0.42</td>
<td>0.25</td>
<td>0.28</td>
</tr>
<tr>
<td>cmol.kg⁻¹ K</td>
<td>0.70</td>
<td>1.50</td>
<td>1.00</td>
<td>0.40</td>
</tr>
<tr>
<td>P (ppm)</td>
<td>26.50</td>
<td>21.00</td>
<td>27.00</td>
<td>17.51</td>
</tr>
<tr>
<td>Sand (%)</td>
<td>51</td>
<td>57</td>
<td>45</td>
<td>47</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>35</td>
<td>21</td>
<td>53</td>
<td>41</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>14</td>
<td>22</td>
<td>39</td>
<td>12</td>
</tr>
<tr>
<td>Texture class</td>
<td>Sandy clay</td>
<td>Sandy clay</td>
<td>clay Clay</td>
<td>Sandy clay</td>
</tr>
</tbody>
</table>

OC, Organic carbon; N, Nitrogen; K, Potassium; TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; ELMZ, Embu Lower Midland Zone.

4.2.2 Soil microbial population

Soil bacteria population from the soil samples ranged from $4.0 \times 10^3$ colony forming units (cfus) to over $2.80 \times 10^4$ CFU (Table 4.5). EUMZ recorded highest bacterial population ($2.8 \times 10^4$ CFU) while ELMZ had lowest (Table 4.5). The indigenous rhizobial population in soil was $6.1 \times 10^2$, $4.7 \times 10^2$, $5.5 \times 10^2$, and $6.0 \times 10^2$ per gram of
soil from TUMZ, TLMZ, EUMZ, and ELMZ respectively. Actinomycetes were recorded to be highest in soils from EUMZ with a population of $6.4 \times 10^3$ in one gram of soil while TLMZ had the lowest ($3.1 \times 10^3$). The fungal population was notably high for soil samples collected from TLMZ $1.29 \times 10^3$ while TUMZ recorded the lowest colony forming units of $6.4 \times 10^2$ (Table 4.5).

Table 4.5: Average microbial populations in soil samples collected from Embu and Tharaka-Nithi trial fields

<table>
<thead>
<tr>
<th>Zone</th>
<th>Rhizobia population CFU</th>
<th>Bacteria population (NA) CFU</th>
<th>Actinomycetes pop (M1 media) Colony forming units (CFU)</th>
<th>Fungal pop (PDA) CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUMZ</td>
<td>$6.0 \times 10^2$</td>
<td>$2.80 \times 10^3$</td>
<td>$6.4 \times 10^3$</td>
<td>$8.1 \times 10^2$</td>
</tr>
<tr>
<td>ELMZ</td>
<td>$5.5 \times 10^2$</td>
<td>$4.0 \times 10^3$</td>
<td>$4.6 \times 10^3$</td>
<td>$1.13 \times 10^3$</td>
</tr>
<tr>
<td>TUMZ</td>
<td>$4.7 \times 10^2$</td>
<td>$4.8 \times 10^3$</td>
<td>$3.7 \times 10^3$</td>
<td>$6.4 \times 10^2$</td>
</tr>
<tr>
<td>TLMZ</td>
<td>$6.1 \times 10^2$</td>
<td>$5.2 \times 10^3$</td>
<td>$3.1 \times 10^3$</td>
<td>$1.29 \times 10^3$</td>
</tr>
</tbody>
</table>

TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; ELMZ, Embu Lower Midland Zone. CFU, Colony forming units.

4.2.3 Morphological characterization of isolates

During the study, 39 pure bradyrhizobia isolates were obtained from the root nodules of three soybean varieties grown during field trapping experiment in Embu and Tharaka-Nithi Counties. TLMZ and EUMZ recorded highest number of isolates with 13 and 12 isolates respectively while TUMZ and ELMZ had 8 and 6 isolates respectively. Soybean SB126 had highest number of isolates with 16 isolates followed by SB8 with 12 isolates while SB24 recorded 11 isolates. The majority (80%) of the isolates had a characteristic of fast-growing bradyrhizobia since they turned YEMA media supplemented with BTB dye from deep green to yellow (Table 4.6). The observation indicated production of
acidic substances which diffused into the media. Twenty percent (20\%) of the isolates turned BTB medium from deep green to blue which is a typical characteristic of slow growing bradyrhizobia due to production of alkaline substances in the medium. In addition, all the isolates absorbed little or no Congo red (Plate 4.1).

During Gram staining reaction, the isolates obtained were Gram negative and rod-shaped. Some isolates had larger colonies with a diameter ranging between 2-5 mm and they showed production of copious extracellular polysaccharides (EPS) on the growth medium. The morphological features of the isolates had slight variations (Table 4.6). The 39 isolates obtained were grouped into nine groups based on their morphological characteristics. The most abundant was morphotype IX accounting for 40\% of the total isolates while morphotype IV followed with 35\%. Morphotypes III and VI were the rarest accounting for about 3\% each (Table 4.6). The reference strain USDA 110 grouped with majority of isolates in morphotype IX while USDA 136 was in morphotype VII.
### Table 4.6: Colony characteristics of the isolated bradyrhizobia isolates

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Isolate</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congo red Absorption</td>
<td>Crna</td>
<td>Crna</td>
<td>Crna</td>
<td>Crna</td>
<td>Crna</td>
<td>Crna</td>
<td>Crna</td>
<td>Crna</td>
<td>Crna</td>
<td>Crna</td>
</tr>
<tr>
<td>BTB Reaction</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Margin</td>
<td>S</td>
<td>S</td>
<td>Sc</td>
<td>Sc</td>
<td>Sc</td>
<td>Sc</td>
<td>Sc</td>
<td>Sc</td>
<td>Sc</td>
<td>Sc</td>
</tr>
<tr>
<td>Color</td>
<td>Cw</td>
<td>Mw</td>
<td>Ww</td>
<td>Mw</td>
<td>Ww</td>
<td>Mw</td>
<td>Ww</td>
<td>Mw</td>
<td>Mw</td>
<td>Mw</td>
</tr>
<tr>
<td>Elevation</td>
<td>Rs</td>
<td>Dmd</td>
<td>Cvx</td>
<td>Rs</td>
<td>Cvx</td>
<td>Rs</td>
<td>Cvx</td>
<td>Cvx</td>
<td>Cvx</td>
<td>Cvx</td>
</tr>
<tr>
<td>Gram Stain</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Transparency</td>
<td>O</td>
<td>O</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>Size (mm)</td>
<td>0.5</td>
<td>1</td>
<td>3.5</td>
<td>5</td>
<td>0.5</td>
<td>1.5</td>
<td>0.5</td>
<td>1.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Colony shape</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>Texture</td>
<td>Sg</td>
<td>G</td>
<td>Sg</td>
<td>Sg</td>
<td>Sg</td>
<td>Sg</td>
<td>Sg</td>
<td>Fg</td>
<td>Sg</td>
<td>Sg</td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>22</td>
<td>5</td>
<td>3</td>
<td>13</td>
<td>5</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

**Key:** Crna, congo red non-absorbing; Y, yellow; S, smooth; Sc, smooth clear; Cw, creamy white; Mw, milky white; Ww, watery white; W, white; Rs, raised; Cvx, convex; Dmd, domed; -ve, gram negative; O, opaque; T, translucent; C, circular; Sg, soft gummy; G, gummy; Fg, firm gummy.

### Plate 4.1: Indigenous bradyrhizobia isolates from field trapping using three soybean varieties (SB8, SB24 and SB126). A and B shows some of the isolates on plain YEMA; C, First growing bradyrhizobia on YEMA media supplemented with Bromothymol-blue; D, Bradyrhizobia isolate on YEMA supplemented with Congo red; E, Actinomycetes in Mj media; F, Bacteria population in Nutrient Agar (NA).
The isolates were clustered into two main phenotypic clusters (Cluster A and cluster B) (Figure 4.1). Phenotypic cluster B had majority of the isolates which were grouped in to two sub-clusters (2 and 3). Sub-cluster 2 represented isolates in group V and group VII which were morphologically close with a bootstrap support of 50%. USDA 136 was also in sub-cluster 2 presented in group VII. Phenotypic cluster 3 had majority of the isolates presenting five groups of bradyrhizobia isolates including reference strain USDA 110 which was in group IX. Phenotypic cluster A had two groups (group I and group II) that clustered together with a bootstrap support of 39% (Figure 4.1).

Figure 4.1: Dendogram on morphological diversity of bradyrhizobia isolates from soil trapping in Embu and Tharaka-Nithi Counties. The percentage bootstrap support for 1000 iterations are shown at the nodes of the dendrogram.
4.2.4 DNA extraction

Genomic DNA was extracted successfully from all the 39 indigenous bradyrhizobia isolates and from two reference strains (USDA 110 and USDA 136) (Plate 4.2).

Plate 4.2: Agarose gel electrophoresis of genomic DNA of representative bradyrhizobia isolates run in 1 % agarose gel; lane M, 1kb plus DNA ladder; lanes 1-17 had bradyrhizobia isolates (1, TL1; 2, EU5; 3, TL2; 4, EL1; 5, TL3; 6, EL2; 7, EU1; 8, EU2; 9, TL4; 10, EL3; 11, EU3; 12 TL5; 13, EU4; 14, TU1; 15, EU5; 16, EU6; 17, USDA 110; 18, USDA 136).

4.2.5 PCR amplification and restriction analysis of 16S rRNA

The PCR amplification of 39 bradyrhizobia isolates and two reference strains of soybeans produced a single band of 1500 base pair in size using Y1 and Y3 primers (Plate 4.3).

Plate 4.3: PCR amplification products of the 16S rDNA for representative bradyrhizobia isolates on 1.4 % agarose gel. Lane M, 1kb plus DNA ladder used as a molecular marker; lanes 1-17 had bradyrhizobia isolates (1, TL1; 2, EU5; 3, TL2; 4, EL1; 5, TL3; 6, EL2; 7, EU1; 8, EU2; 9, TL4; 10, EL3; 11, EU3; 12 TL5; 13, EU4; 14, TU1; 15, USDA 110; 16, USDA 136; 17, negative control).
16S rRNA gene amplicons of the bradyrhizobia isolates from restriction digestion with enzymes *Hae*III, *Msp*I and *Eco*RI had different banding patterns. *Hae*III and *Msp*I produced most diverse polymorphic band patterns. *Hae*III resulted to different restriction patterns and the size of the restriction fragments obtained ranged from 100 base pairs to 600 base pairs (Plate 4.4).

![Plate 4.4](image)

**Plate 4.4:** Restriction fragments for rhizobia isolates obtained after digestion with the *Hae*III enzyme on 1.4 % agarose gel. Lane M, 100 kb DNA ladder used as a molecular marker; lanes 1-17 had bradyrhizobia isolates (1, TL1; 2, EU5; 3, TL2; 4, EL1; 5, TL3; 6, EL2; 7, EU1; 8, EU2; 9, TL4; 10, EL3; 11, EU3; 12 TL5; 13, EU4; 14, USDA110; 15, USDA136; 16, negative control).

Digestion using *Msp*I enzyme resulted to different restriction patterns and the range of fragments ranged from 100 base pairs to 1000 base pairs (Plate 4.5).

![Plate 4.5](image)
Plate 4.5: Restriction fragments for rhizobia isolates obtained after digestion with the MspI enzyme on 1.4% agarose gel and stained with SYBR green. Lane M, 100 kb DNA ladder used as a molecular marker; lanes 1-17 had bradyrhizobia isolates (1, TL1; 2, EU5; 3, TL2; 4, EL1; 5, TL3; 6, EL2; 7, EU1; 8, EU2; 9, TL4; 10, EL3; 11, EU3; 12 TL5; 13, EU4; 14, USDA 110; 15, USDA 136; 16, negative control).

The digestion with the EcoRI enzyme resulted also to different restriction patterns (Plate 4.6). The length of the obtained restriction fragments varied between 600 and 1200 base pairs. The sample EU5 in lane 3 PCR product did not form restriction bands (Plate 4.6).

Plate 4.6: Restriction fragments for rhizobia isolates obtained after digestion with the Eco RI enzyme on 1.4% agarose gel. Lane M, 100 kb DNA ladder used as a molecular marker; lanes 1-17 had bradyrhizobia isolates (1, TL1; 2, EU5; 3, TL2; 4, EL1; 5, TL3; 6, EL2; 7, EU1; 8, EU2; 9, TL4; 10, EL3; 11, EU3; 12 TL5; 13, EU4; 14, TU1; 15, EU5; 16, USDA110; 17, USDA136; 18, negative control.

The highest genetic variation (99%) was within populations and not among the regions or among the populations according to the analysis of molecular variance (AMOVA) (Table 4.7).
Table 4.7: Analysis of molecular variance (AMOVA) for 39 bradyrhizobia isolates based on restriction digest of 16S rRNA using HaeIII, MspI and EcoRI enzymes

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>Ss</th>
<th>MS</th>
<th>Est. Var.</th>
<th>%</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among Regions</td>
<td>1</td>
<td>2.93</td>
<td>2.93</td>
<td>0.03</td>
<td>1</td>
<td>0.108</td>
</tr>
<tr>
<td>Among Pops</td>
<td>2</td>
<td>4.70</td>
<td>2.35</td>
<td>0.00</td>
<td>0</td>
<td>0.815</td>
</tr>
<tr>
<td>Within Pops</td>
<td>42</td>
<td>139.19</td>
<td>3.31</td>
<td>3.31</td>
<td>99</td>
<td>0.015</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>146.83</td>
<td>3.35</td>
<td></td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Df, degrees of freedom; Ss, sum of squares; MS, mean square; Est. Var, estimated variance; Pops, populations; % Mol var, percentage molecular variance.

Principle coordinate analysis (PCA) of the 39 indigenous rhizobia isolates from the four agroecological zones showed some differentiation. However, isolates were widely distributed in all the zones and appear overlapping in all the four quadrants (Figure 4.2). Isolates from EUMZ were the most widely distributed appearing in all the quadrants while isolates from ELMZ were the least distributed (Figure 4.2).
Figure 4.2: Principal coordinate analyses (PCA) of indigenous bradyrhizobia isolates based on restriction banding patterns. The percentage variations for the first two coordinates (1) 25.2 %, (2) 17.6 %.

The highest genetic distance (0.039) based on pairwise Nei unbiased genetic assessment was between the population isolates from TUMZ and ELMZ. The lowest Nei unbiased genetic distance (0.012) was between rhizobia isolates populations from TUMZ and TLMZ and also between population isolates from TLMZ and EUMZ (Table 4.8). The four indigenous bradyrhizobia populations were clustered into two closely related groups using Nei unbiased genetic distance matrix (Figure 4.4). Bradyrhizobia populations from TUMZ and TLMZ were closely related with a bootstrap value of 80 % while bradyrhizobia isolates from EUMZ and ELMZ population were also closely related and clustered together with a bootstrap value of 80 % (Figure 4.3). bradyrhizobia across all the zones were also related and clustered together with a bootstrap support of 100 %.
Table 4.8: Nei Unbiased Genetic Distance for Pairwise Population Matrix of rhizobia populations trapped from TUMZ, TLMZ, EUMZ and ELMZ from Embu and Tharaka-Nithi Counties

<table>
<thead>
<tr>
<th></th>
<th>TUMZ</th>
<th>TLMZ</th>
<th>EUMZ</th>
<th>ELMZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUMZ</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLMZ</td>
<td>0.012</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EUMZ</td>
<td>0.024</td>
<td>0.012</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>ELMZ</td>
<td>0.039</td>
<td>0.036</td>
<td>0.021</td>
<td>0.000</td>
</tr>
</tbody>
</table>

TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; ELMZ, Embu Lower Midland Zone.

Figure 4.3: Neighbour joining dendrogram showing genetic relationship between agroecological zones of rhizobia populations based on Euclidean similarity index and Nei Unbiased genetic distance. Percentage bootstrap support for 1000 iterations are indicated at the nodes of the dendrogram. TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; ELMZ, Embu Lower Midland Zone.
Indigenous bradyrhizobia populations from EUMZ and TUMZ had the highest mean number of alleles (Na = 1.600) as compared with other populations while ELMZ had bradyrhizobia populations with lowest mean number of different alleles (Table 4.9). The rhizobia populations were very diverse according to Shannon’s information mean diversity index estimate H (I) where EUMZ and TLMZ showed highest diversity estimates of H = 0.419 and H = 0.404 respectively. The lowest genetic diversity (H = 0.393) was recorded from ELMZ (Table 4.9). The highest percentage of polymorphic loci (% P) (80 %) was recorded on rhizobia populations from TUMZ and EUMZ while rhizobia populations from ELMZ had the lowest number of polymorphic loci (70 %). The mean expected heterozygosity (He) for the indigenous bradyrhizobia population ranged from to 0.263 to 0.283 (Table 4.9).

<table>
<thead>
<tr>
<th>Pop</th>
<th>Na</th>
<th>Ne</th>
<th>H</th>
<th>He</th>
<th>UHe</th>
<th>%P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUMZ</td>
<td>1.600±0.18</td>
<td>1.437±0.08</td>
<td>0.398±0.057</td>
<td>0.263±0.041</td>
<td>0.278±0.044</td>
<td>80</td>
</tr>
<tr>
<td>TLMZ</td>
<td>1.500±0.21</td>
<td>1.451±0.08</td>
<td>0.404±0.059</td>
<td>0.270±0.041</td>
<td>0.279±0.043</td>
<td>75</td>
</tr>
<tr>
<td>EUMZ</td>
<td>1.600±0.18</td>
<td>1.496±0.09</td>
<td>0.419±0.061</td>
<td>0.283±0.045</td>
<td>0.293±0.046</td>
<td>80</td>
</tr>
<tr>
<td>ELMZ</td>
<td>1.400±0.21</td>
<td>1.439±0.08</td>
<td>0.393±0.061</td>
<td>0.263±0.042</td>
<td>0.284±0.045</td>
<td>70</td>
</tr>
</tbody>
</table>

TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; ELMZ, Embu Lower Midland Zone.

Phylogenetic analysis inferred by neighbor joining method and Jaccard similarity index clustered indigenous bradyrhizobia isolates into three main clusters (cluster A, B and C).
Cluster A had two main sub-clusters namely A (i) and A (ii) which comprised most of the indigenous isolates. Cluster B was the second largest and comprised two main sub-clusters B (i) and B (ii). Cluster C had the lowest number of indigenous isolates. Isolate coded EU8 clustered together with reference strain USDA136 while isolate EU4 clustered together with USDA110 an indication they are closely related.
Figure 4.4: Phylogenetic relationship of 39 indigenous bradyrhizobia isolates and two reference strains (USDA110 and USDA136) based on combined HaeIII, MspI and EcoRI restriction patterns of amplified 16S rRNA gene inferred using Neighbor-Joining method. The percentage bootstrap support for 1000 iterations in which the associated taxa clustered together are shown next to the branches. Only bootstrap values ≥ 40 % are shown.
4.3 Effectiveness and N-fixing potential of indigenous bradyrhizobia isolates

4.3.1 Authentication of isolates in the greenhouse

During authentication, out of nine (9) representative isolates used, seven (7) nodulated with the three soybean genotypes used while two of the bradyrhizobia isolates failed to nodulate in the greenhouse (Table 4.10). Bradyrhizobia isolate RI9 recorded highest nodulation with the three soybean genotypes with a mean nodule number of 8. Bradyrhizobia isolates RI1 and RI2 did not infect the host plants and hence no nodulation was observed. Other isolates had significant variation on nodulation with the soybean varieties (Table 4.10). BT, the commercial inoculant was authenticated alongside the isolates which also nodulated effectively. The positive control (NT) which involved nitrogen treatment without bradyrhizobia inoculation and a negative control (UT) did not form nodules with soybeans during authentication (Table 4.10).

4.3.2 Symbiotic effectiveness of the isolates in the greenhouse experiment

Inoculation of bradyrhizobial isolates showed significant (p < 0.001) enhancement of shoot biomass of the three soybean genotypes tested. Plants inoculated with isolate RI9 (EUMZ) had the highest shoot dry weight (SDW) of 0.58 g plant⁻¹ compared to the positive control treatment NT (0.43 g plant⁻¹). Seeds inoculated with isolate RI1 led to the plants with the lowest SDW of 0.14 g plant⁻¹ although it was above the negative control which had an average of 0.09 g plant⁻¹. Nonetheless, soybean genotype did not have significant (p < 0.475) effect on shoot dry weight per plant with 0.33 g, 0.35 g, and 0.31 g
Inoculation increased the number of nodules (NN) significantly \( (p < 0.001) \) among the tested soybean genotypes (SB8, SB24 and SB126) under greenhouse conditions. SB126 achieved the highest mean nodule numbers (3.18) per plant followed by SB8 (2.55 NN plant\(^{-1}\)) while SB24 had the least mean nodule numbers (0.36 NN plant\(^{-1}\)). Inoculation with indigenous bradyrhizobia significantly \( (p = 0.001) \) increased nodule number (NN) per plant. The mean nodule number due to inoculation ranged from 0.33 to 8.25 nodules which demonstrated the different ability of indigenous bradyrhizobia isolates to infect host soybean plants. Two of the indigenous isolate (RI4 and R19) outperformed commercial isolate (BT12) in nodule numbers with an average of 5.91 NN plant\(^{-1}\) and 8.25 NN plant\(^{-1}\) respectively. The consortium ICT had 1.92 NN plant\(^{-1}\) which was lower than the commercial BT (3.83 NN plant\(^{-1}\)). Uninoculated treatments both negative and nitrogen treatment did not nodulate with the three soybean varieties (Table 4.10). There was no significant \( (p = 0.123) \) interaction between soybean varieties and the isolates on mean nodule number.

Inoculation of plants with bradyrhizobia significantly enhanced nodule dry weight per plant from the greenhouse (Table 4.10). Plants obtained after inoculation with isolate R19 recorded highest mean nodule dry weight of 19.28 mg followed by consortium with 8.73 mg. The lowest mean nodule dry weight (0.37 mg plant\(^{-1}\)) was recorded in plants...
obtained after inoculating with isolate RI6 while those inoculated with the commercial inoculant (BT) recorded 2.17 mg plant$^{-1}$. Soybean genotype SB8 relatively achieved higher nodule dry weight which was significant when compared to genotypes SB24 and SB126 (Table 4.10). Inoculation had no significance effect ($p = 0.278$) on root dry weight. In addition, soybean varieties had no significant ($p = 0.071$) influence on root dry weight. There was significant interaction ($p = 0.866$) recorded between variety and isolates (Table 4.10).

Soybean varieties had a significant ($p = 0.005$) enhancement on symbiotic effectiveness. The SB126 variety recorded highest symbiotic effectiveness of 98.70 % compared to SB8 and SB24 which had symbiotic effectiveness of 74.06 % and 64.51 % respectively. Among the tested indigenous isolates, there was significant variation in symbiotic potential where some exhibited superior symbiotic performance ($p<0.001$). Isolate RI9 (EUMZ) and RI4 (EUMZ) had the highest symbiotic effectiveness of 142.35 % and 119.17 % respectively. Isolates RI3, RI5, RI6 RI7 and RI8 had low symbiotic effectiveness below that of nitrogen control NT (100 % SE). Consortium ICT was also below positive control in terms of symbiotic effectiveness with 84.45 % SE. Variety × isolates had insignificant effect on symbiotic effectiveness (Table 4.10).
Table 4.10: Average shoot dry weight, root dry weight, nodule number, nodule dry weight and symbiotic effectiveness of soybean plants from the greenhouse

<table>
<thead>
<tr>
<th>Isolates</th>
<th>SDW (g)</th>
<th>RDW (g)</th>
<th>NN</th>
<th>NDW (mg)</th>
<th>SE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI1</td>
<td>0.14±0.02de</td>
<td>0.12±0.02a</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RI2</td>
<td>0.15±0.02de</td>
<td>0.16±0.03a</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RI3</td>
<td>0.42±0.07abc</td>
<td>0.20±0.05a</td>
<td>2.08±0.65bc</td>
<td>1.43±0.49b</td>
<td>99.82±20.19ab</td>
</tr>
<tr>
<td>RI4</td>
<td>0.48±0.06ab</td>
<td>0.18±0.02a</td>
<td>3.00±0.94bc</td>
<td>4.85±1.56b</td>
<td>119.17±19.68b</td>
</tr>
<tr>
<td>RI5</td>
<td>0.32±0.05bcde</td>
<td>0.14±0.03a</td>
<td>2.08±1.16bc</td>
<td>1.16±0.91b</td>
<td>81.25±13.30bcde</td>
</tr>
<tr>
<td>RI6</td>
<td>0.28±0.03bcde</td>
<td>0.13±0.02a</td>
<td>0.42±0.26c</td>
<td>0.37±0.26b</td>
<td>67.36±12.49bcdef</td>
</tr>
<tr>
<td>RI7</td>
<td>0.19±0.05cde</td>
<td>0.21±0.08a</td>
<td>0.33±0.25c</td>
<td>0.39±0.35b</td>
<td>39.62±8.17cdef</td>
</tr>
<tr>
<td>RI8</td>
<td>0.35±0.04abcd</td>
<td>0.11±0.02a</td>
<td>5.91±2.49ab</td>
<td>2.21±1.26b</td>
<td>84.84±13.35abcd</td>
</tr>
<tr>
<td>RI9</td>
<td>0.58±0.08a</td>
<td>0.19±0.04a</td>
<td>8.25±1.77a</td>
<td>19.28±6.10a</td>
<td>142.35±22.60a</td>
</tr>
<tr>
<td>ICT</td>
<td>0.33±0.04bcde</td>
<td>0.15±0.03a</td>
<td>1.92±1.21bc</td>
<td>8.73±8.00ab</td>
<td>84.45±12.92abcd</td>
</tr>
<tr>
<td>BT</td>
<td>0.43±0.06abc</td>
<td>0.12±0.02a</td>
<td>3.83±1.34abc</td>
<td>2.17±0.84b</td>
<td>101.01±13.13ab</td>
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<tr>
<td>NT</td>
<td>0.43±0.06abc</td>
<td>0.12±0.02a</td>
<td>0</td>
<td>0</td>
<td>100.00±14.52ab</td>
</tr>
<tr>
<td>BICT</td>
<td>0.39±0.04abc</td>
<td>0.12±0.02a</td>
<td>0.58±0.26c</td>
<td>0.38±0.17b</td>
<td>94.45±11.42abc</td>
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<tr>
<td>UT</td>
<td>0.09±0.03e</td>
<td>0.09±0.02a</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variety</th>
<th>SDW (g)</th>
<th>RDW (g)</th>
<th>NN</th>
<th>NDW (mg)</th>
<th>SE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB8</td>
<td>0.33±0.03a</td>
<td>0.16±0.02a</td>
<td>2.55±0.61a</td>
<td>5.02±2.27a</td>
<td>74.06±6.19b</td>
</tr>
<tr>
<td>SB24</td>
<td>0.35±0.03a</td>
<td>0.17±0.02a</td>
<td>0.36±0.18b</td>
<td>0.84±0.61b</td>
<td>64.51±5.00b</td>
</tr>
<tr>
<td>SB126</td>
<td>0.31±0.03a</td>
<td>0.15±0.02a</td>
<td>3.18±0.70a</td>
<td>2.99±0.90ab</td>
<td>98.70±10.10a</td>
</tr>
</tbody>
</table>

P values of the main isolates and their interaction

| Isolates | 0.001 | 0.278 | 0.001 | 0.004 | 0.001 |
| Variety  | 0.475 | 0.071 | 0.001 | 0.102 | 0.005 |
| Variety  | 0.809 | 0.482 | 0.123 | 0.866 | 0.715 |

Key: Values within the same column without common letters differ significantly according to Tukey's HSD p<0.05. Key: SDW, Shoot dry weight; RDW, Root dry weight; NN, Nodule number; NDW, Nodule dry weight; SE, Symbiotic effectiveness. RI1 to RI9 represent bradyrhizobia isolates tested in the greenhouse; ICT, Consortium; BT, Commercial inoculant; NT, Nitrogen treatment (positive control); BICT, Consortium + commercial; UT, uninoculated (Negative control).
Plate 4.7: Effect of inoculation on nodulation of soybeans in the greenhouse. A, Soybean plants one week after emergence of seedlings; B, Roots without nodules from non-infective isolate (RI2); C, Root with nodules due to RI9 treatment; D, Comparison of nitrogen fixing plant (Dark green leaves) and a negative control (green-yellow leaves).

4.3.2 Soybean shoots %N, P and K amount during greenhouse experiment

Soybean varieties in the greenhouse experiment had no significant (p = 0.911) effect on shoot %N but SB24 shoots had the highest %N of 1.81 %. Inoculation of plants with isolates had significant (p < 0.001) enhancement on shoot %N (Table 4.11). Plants inoculated with the positive control (NT) had the highest shoot %N followed by RI9 with 3.29 % and 2.58 % respectively. The isolate Rl1 and negative control UT scored the
lowest shoot %N of 0.88 % and 0.57 % respectively. Soybean shoots %N were not significantly ($p = 0.675$) influenced by of inoculation $\times$ soybean varieties.

Soybean varieties did not differ significantly ($P = 0.896$) in shoot available phosphorus. The shoots of SB8 had the highest amount of the available P (3633.45 ppm) while SB24 recorded the least amount of the available P (3525.57 ppm). Inoculation had a significant ($p < 0.001$) effect on soybean shoot available P. Inoculated soybean plants achieved slightly higher shoots available P compared to uninoculated plants. Inoculation of plants with isolates RI5 and RI3 let to plants with the highest amount of the available P compared with those inoculated with RI1 which recorded lowest amount (Table 4.11). Insignificant ($p = 0.362$) interaction effect of inoculation $\times$ soybean variety on amount of available P was observed.

Soybean varieties had no significant ($P = 0.801$) enhancement on soybean shoot sodium (K) content. SB126 variety was the highest on shoot K content (6188.33 ppm) followed by SB8 variety (6151.43 ppm) while SB24 recorded the lowest amount of shoot K (5970.71 ppm). There was a significant ($p < 0.001$) effect on shoot K due to inoculation with bradyrhizobia (Table 4.11). RI9 recorded significantly highest amount of K (10166.67 ppm) in the shoots which followed by RI12 (9760.00 ppm). Plants inoculated with commercial inoculant (BT) scored relatively low shoot K (4533.33 ppm) compared to most of the isolates while the negative control UT (2851.11 ppm) had the lowest amount of K (Table 4.11).
Table 4.11: Soybean shoot total N, Phosphorus (P), and Potassium (K) from the greenhouse experiment

<table>
<thead>
<tr>
<th>Treatments</th>
<th>%N</th>
<th>P (ppm)</th>
<th>K (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Varieties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB8</td>
<td>1.78±0.15a</td>
<td>3633.45±191.06a</td>
<td>6151.43±407.90a</td>
</tr>
<tr>
<td>SB24</td>
<td>1.81±0.14a</td>
<td>3525.57±194.35a</td>
<td>5970.71±348.81a</td>
</tr>
<tr>
<td>SB126</td>
<td>1.75±0.15a</td>
<td>3556.48±182.20a</td>
<td>6188.33±387.74a</td>
</tr>
<tr>
<td><strong>Isolates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RI1</td>
<td>0.88±0.12cd</td>
<td>2072.22±200.37d</td>
<td>4011.11±402.23d</td>
</tr>
<tr>
<td>RI2</td>
<td>2.04±0.28b</td>
<td>3088.89±296.81c</td>
<td>6666.67±519.88c</td>
</tr>
<tr>
<td>RI3</td>
<td>1.65±0.10bcd</td>
<td>4452.22±190.90b</td>
<td>7192.22±688.06bc</td>
</tr>
<tr>
<td>RI4</td>
<td>2.47±0.23ab</td>
<td>3968.89±461.35bc</td>
<td>6253.33±629.60c</td>
</tr>
<tr>
<td>RI5</td>
<td>2.35±0.19ab</td>
<td>5381.11±472.82a</td>
<td>7837.78±316.44ab</td>
</tr>
<tr>
<td>RI6</td>
<td>0.67±0.14d</td>
<td>3089.00±420.13c</td>
<td>4661.11±546.76d</td>
</tr>
<tr>
<td>RI7</td>
<td>1.71±0.18bcd</td>
<td>3160.56±277.46c</td>
<td>5216.67±184.47d</td>
</tr>
<tr>
<td>RI8</td>
<td>1.46±0.24bcd</td>
<td>3838.22±359.69bc</td>
<td>4788.89±368.63d</td>
</tr>
<tr>
<td>RI9</td>
<td>2.58±0.19ab</td>
<td>4498.33±356.69b</td>
<td>10166.67±319.29a</td>
</tr>
<tr>
<td>ICT</td>
<td>1.83±0.13bc</td>
<td>3641.67±268.92bc</td>
<td>5866.67±736.17c</td>
</tr>
<tr>
<td>BT</td>
<td>1.56±0.36bcd</td>
<td>2455.67±177.82cd</td>
<td>4533.33±516.67d</td>
</tr>
<tr>
<td>NT</td>
<td>3.29±0.24a</td>
<td>3373.11±269.26c</td>
<td>9760.00±224.16ab</td>
</tr>
<tr>
<td>BICT</td>
<td>1.90±0.20bc</td>
<td>3384.67±231.75c</td>
<td>5643.33±335.37c</td>
</tr>
<tr>
<td>UT</td>
<td>0.57±0.13d</td>
<td>3601.11±253.86c</td>
<td>2851.11±384.94e</td>
</tr>
</tbody>
</table>

P-values of the main factors and their interactions

<table>
<thead>
<tr>
<th></th>
<th>Variety</th>
<th>Isolates</th>
<th>Variety × isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variety</td>
<td>0.911</td>
<td>0.896</td>
<td>0.801</td>
</tr>
<tr>
<td>Isolates</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Variety × isolates</td>
<td>0.675</td>
<td>0.362</td>
<td>0.344</td>
</tr>
</tbody>
</table>

**Key:** Values within the same column without common letters differ significantly according to Tukey’s HSD at p<0.05. RI1 to RI9 represent bradyrhizobia isolates tested in the greenhouse; ICT, Consortium; BT, Commercial inoculant; NT, Nitrogen treatment (positive control); BICT, Consortium + bio fix; UT, uninoculated (Negative control).
4.3.3 Field experiment

4.3.3.1 Shoot biomass due to application of inoculants

There was a significant difference ($p < 0.001$) on shoot dry weight in terms of Zones. Tharaka-Nithi Upper Midland Zone (EUMZ) and Tharaka-Nithi Lower Midland Zones showed highest overall shoot dry weight (Table 4.12). Agroecological zone ELMZ recorded the lowest shoot dry weight of 5.49 g plant$^{-1}$. The two soybean varieties used had no significant variation in shoot dry weight ($p = 0.919$). Soybean SB 8 had highest mean shoot dry weight (7.27 g plant$^{-1}$) compared to SB 126 which recorded 7.25 g plant$^{-1}$.

The average shoot dry weight was significantly influenced by inoculation (Table 4.12). The indigenous isolate RI9 and commercial inoculant BT had highest average shoot dry weight of 8.44 g plant$^{-1}$ and 7.47 g plant$^{-1}$ respectively. The mixture of commercial inoculant and consortium (BICT) also increased shoot dry weight and scored an average weight of 8.47 g plant$^{-1}$. The un-inoculated control (UT) recorded the lowest shoot dry weight in both soybean varieties SB8 and SB126 (Plate 4.8). In regards to shoot dry weight, there was no interaction between the inoculant and the two soybean varieties (Table 4.12). However, there was strong interaction of varieties × zones where SB8 performed better in specific zones compared to SB126 ($p = 0.007$).
Table 4.12: Average shoot dry weight (SDW g plant\(^{-1}\)), root dry weight (RDW g plant\(^{-1}\)), nodule number (NN) and nodule dry weight (NDW g plant\(^{-1}\)) of soybean plants from the field experiment

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SDW (g plant(^{-1}))</th>
<th>RDW (g plant(^{-1}))</th>
<th>NDW (g plant(^{-1}))</th>
<th>NN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUMZ</td>
<td>6.34±0.53b</td>
<td>1.32±0.15b</td>
<td>0.28±0.04a</td>
<td>16.00±3.11a</td>
</tr>
<tr>
<td>TLMZ</td>
<td>10.64±0.89a</td>
<td>3.01±0.25a</td>
<td>0.51±0.12a</td>
<td>22.76±6.61a</td>
</tr>
<tr>
<td>EUMZ</td>
<td>6.26±0.59b</td>
<td>1.98±0.13b</td>
<td>0.27±0.04a</td>
<td>15.16±3.58a</td>
</tr>
<tr>
<td>ELMZ</td>
<td>5.49±0.54b</td>
<td>1.32±0.15c</td>
<td>0.28±0.04a</td>
<td>17.30±0.96a</td>
</tr>
<tr>
<td><strong>Variety</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB8</td>
<td>7.27±0.58a</td>
<td>2.24±0.43a</td>
<td>0.31±0.04a</td>
<td>14.35±2.23a</td>
</tr>
<tr>
<td>SB126</td>
<td>7.25±0.58a</td>
<td>2.08±0.14a</td>
<td>0.37±0.06a</td>
<td>21.26±3.65a</td>
</tr>
<tr>
<td><strong>Isolates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RI9 (EUMZ)</td>
<td>8.44±0.63a</td>
<td>2.23±0.21a</td>
<td>0.50±0.12a</td>
<td>27.91±7.45a</td>
</tr>
<tr>
<td>ICT</td>
<td>7.47±0.58a</td>
<td>2.23±0.19a</td>
<td>0.24±0.05b</td>
<td>13.95±3.09c</td>
</tr>
<tr>
<td>BT</td>
<td>8.47±1.26a</td>
<td>2.30±0.20a</td>
<td>0.52±0.07a</td>
<td>25.75±4.70a</td>
</tr>
<tr>
<td>BICT</td>
<td>6.47±1.26b</td>
<td>2.28±0.30a</td>
<td>0.36±0.07b</td>
<td>17.45±3.51b</td>
</tr>
<tr>
<td>UT</td>
<td>4.39±0.43c</td>
<td>1.87±0.24a</td>
<td>0.05±0.02c</td>
<td>3.95±2.09d</td>
</tr>
</tbody>
</table>

P values of the main treatments and their interaction

| Zones     | 0.001 | 0.001 | 0.028 | 0.550 |
| Variety   | 0.919 | 0.340 | 0.372 | 0.092 |
| Isolates  | 0.002 | 0.494 | 0.001 | 0.003 |
| Zone × Variety | 0.007 | 0.332 | 0.118 | 0.119 |
| Zone × Isolates | 0.286 | 0.015 | 0.655 | 0.721 |
| Variety × Isolates | 0.681 | 0.605 | 0.301 | 0.631 |
| Zone × Variety × Isolates | 0.882 | 0.381 | 0.061 | 0.355 |

Key: Values within the same column followed by the same letter are not significantly different according to Tukey’s HSD at p<0.05. SDW, Shoot dry weight; RDW, Root dry weight; NN, Nodule number; NDW, Nodule dry weight; RI9, Indigenous isolate; ICT, Indigenous consortium; BT, Bio fix; BICT, Indigenous consortium + bio fix; UT, Uninoculated (control). TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; ELMZ, Embu Lower Midland Zone.
4.3.3.2 Nodule number

There was effective root nodulation in all the inoculated and uninoculated soybean plants although there was a significant variation in the number of nodules per plant ($p < 0.001$). Treatment RI9 recorded the highest mean nodule number per plant (27.91 NN plant$^{-1}$) followed by commercial inoculum BT (25.75 NN plant$^{-1}$) (Table 4.12). The uninoculated treatment (UT) had the lowest (3.95 NN plant$^{-1}$). The inoculated treatments formed more nodules compared to control on the two soybean genotypes. However, there were no significant ($p = 0.092$) differences in numbers of nodule per plant as influenced by two soybean varieties though SB126 had a higher number compared to SB8 with mean nodule number of 21.26 and 14.35 respectively (Table 4.12). Nodule number per plant had insignificant ($p=0.631$) interaction between the test inoculants used and the soybean varieties. There was also no significant interaction between agroecological zones and the
isolates and also the interaction between zones and the variety on nodule number \( (p = 0.721 \text{ and } p = 0.119) \) respectively.

### 4.3.3.3 Nodule dry weight (NDW)

Among the soybean genotypes, there was no significance difference \( (p = 0.372) \) in terms of nodule dry weight although SB126 scored highest nodule dry weight of 0.37 g plant\(^{-1}\) while SB126 had 0.31 g plant\(^{-1}\). The use of inoculants increased NDW per plant significantly as the inoculated soybean plants had high nodule dry weight compared to uninoculated control (Table 4.12). The commercial inoculant (BT) (0.52 g plant\(^{-1}\)) produced highest NDW compared to the best isolate RI9 (0.50 g plant\(^{-1}\)) inoculant although not statistically significant. The mixture of indigenous consortium and commercial inoculant (BICT), had an average nodule dry weight of 0.36 g plant\(^{-1}\). Results obtained showed that there was insignificant \( (p = 0.301) \) interaction effect of bradyrhizobia inoculants and soybean varieties in regard to nodule dry weight per plant (Table 4.12).

### 4.3.3.4 Number of pods

The number of pods per plant at farm level in different zones was significantly different with some regions performing better compared to others (Table 4.13). The average number of pods per plant ranged from 33.67 to 83.00 pods plant\(^{-1}\) for ELMZ and EUMZ respectively. The two soybean varieties had no significant variation in the number of pods per plant \( (p = 0.259) \). SB8 produced 53.43 pods plant\(^{-1}\) while SB126 produced 56.93
pods plant\(^{-1}\). Application of bradyrhizobia inoculants had a significant effect (\(p = 0.001\)) on the number of pods per plant (Table 4.13). Plants from the seeds inoculated with indigenous isolate RI9 recorded the highest mean number of pods (68.50 pods plant\(^{-1}\)) followed by commercial inoculant BT and consortium ICT with 59.45 pods plant\(^{-1}\) and 56.54 pods plant\(^{-1}\) respectively. The uninoculated control had the lowest number of pods per plant (Table 4.13). Bradyrhizobia inoculants interaction of soybean varieties was not statistically significant (\(p = 0.820\)) in terms pods number per plant. However, the interaction between zones and the test isolates differed significantly (\(p = 0.009\)) on pod numbers. Embu Upper Midland Zone recorded highest pods number plant\(^{-1}\) (Table 4.13).

4.3.3.5 Grain yield

EUMZ and TLMZ recorded highest grain yield of 1313.77 kg ha\(^{-1}\) and 1097.58 kg ha\(^{-1}\) respectively and differed significantly from that of ELMZ and TUMZ (Table 4.13). The soybean varieties varied significantly with a \(p < 0.001\) in regards to yield. The variety SB8 recorded the higher yield 967.88 kg ha\(^{-1}\) compared to SB126 with 500.70 kg ha\(^{-1}\). Grain yield of soybean varieties varied significantly as a result of inoculation using bradyrhizobia isolates (Table 4.13). The commercial inoculant BT recorded highest grain yield of 823.55 kg ha\(^{-1}\) although not statistically difference with indigenous isolate RI9 with 823 kg ha\(^{-1}\). The zone \(\times\) variety interaction was significant (\(p < 0.001\)) on grain yield where some zones showed higher grain yield on SB8 variety compared to SB126 (Figure 4.5) Similarly, Zone\(\times\)isolates interaction differed significantly (\(p = 0.002\)) on grain yield per hectare. This observation was evident on EUMZ where grain yield was strongly
enhanced by inoculation compared to other agroecological zones (Figure 4.5). In addition, the mean grain yield obtained from soybean varieties was affected by the zone \times variety interaction (p < 0.001).

**Figure 4.5:** Interactive effects of inoculation with indigenous bradyrhizobia isolates and soybean varieties on grain yield. Bars followed by the same letter are not significantly different using Turkey’s HSD test at P<0.05. A, TUMZ; B, TLMZ; C, EUMZ; D, ELMZ; RI9, Indigenous isolate; ICT, Indigenous consortium; BT, commercial inoculant; BICT, Indigenous consortium + commercial inoculant; UT, Uninoculated (control), TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; and ELMZ, Embu Lower Midland Zone.

### 4.3.3.6 Stover dry weight

Agroecological zones had a significant (p < 0.001) variation in stover mean dry weight. The stover dry weight of plants in EUMZ was the highest (2451.75 kg ha\(^{-1}\)) followed by
TLMZ with 2150.00 kg ha\(^{-1}\) while those in ELMZ were the lowest at 669.75 kg ha\(^{-1}\) (Table 4.13). Result showed that different bradyrhizobia inoculants increased significantly stover dry weight of the soybeans (\(p < 0.001\)). The application of commercial inoculant BT led to the highest stover dry weight of 2038.55 kg ha\(^{-1}\), although it was not statistically different from that of indigenous isolate RI9, consortium ICT and BICT with 1957.25 kg ha\(^{-1}\), 1567.71 kg ha\(^{-1}\) and 1864.57 kg ha\(^{-1}\) respectively. The un-inoculated treatment (UT) recorded the lowest stover dry weight (table 4.13). Variety had no significant effect on stover dry weight although SB8 showed higher stover dry weight of 1782.33 kg ha\(^{-1}\) compared to SB126 (1583.75 kg ha\(^{-1}\)).

4.3.3.7 Dry weight for 100 seeds

Inoculation of soybean seeds using indigenous isolates showed insignificant (\(p = 0.191\)) effect in 100 seed weight (Table 4.8). The 100-seed dry weight due to inoculants ranged from 14.39 g recorded for the control (UT) to 16.05 g recorded for the commercial isolate RI9 (Table 4.13). Similarly, varieties had no significant (\(p = 0.060\)) effect on dry weight of 100 seeds. SB8 variety recorded the higher 100-seed mean dry weight of 16.16 g compared to SB126 which had 14.38 g. Agroecological zones did not have a significant (\(p = 0.139\)) effect on 100 seeds dry weight. The 100 mean seed dry weight based on agroecological zones ranged from 14.32 g to 16.48 g. There was no significant interaction between the variety and bradyrhizobia inoculants on the dry weight of 100 seeds (Table 4.13).
Table 4.13: Mean values for 100-seed weight, grain yield, number of pods per plant and stover dry weight

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight 100 seeds (g)</th>
<th>Grain yield kg/ha</th>
<th>No of pods per plant</th>
<th>Stover dry weight kg/ha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zones</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUMZ</td>
<td>14.84±0.42ab</td>
<td>319.75±18.30b</td>
<td>44.70±4.23c</td>
<td>1410.83±49.3b</td>
</tr>
<tr>
<td>TLMZ</td>
<td>15.44±0.69ab</td>
<td>1097.58±37.32a</td>
<td>59.37±3.99b</td>
<td>2150.00±52.41a</td>
</tr>
<tr>
<td>EUMZ</td>
<td>16.48±0.46a</td>
<td>1312.77±64.38a</td>
<td>83.00±4.77a</td>
<td>2451.75±79.49a</td>
</tr>
<tr>
<td>ELMZ</td>
<td>14.32±0.56b</td>
<td>207.23±17.16b</td>
<td>33.67±3.26c</td>
<td>669.75±32.53c</td>
</tr>
<tr>
<td>Variety</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB8</td>
<td>16.16±0.431a</td>
<td>967.88±44.79a</td>
<td>53.43±3.83a</td>
<td>1782.33±58.84a</td>
</tr>
<tr>
<td>SB126</td>
<td>14.38±0.314a</td>
<td>500.70±21.35b</td>
<td>56.93±3.65a</td>
<td>1583.75±47.63a</td>
</tr>
<tr>
<td>Isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RI9 (EUMZ)</td>
<td>15.12±0.79a</td>
<td>770.73±50.59ab</td>
<td>68.50±5.47a</td>
<td>1957.25±86.72a</td>
</tr>
<tr>
<td>ICT</td>
<td>14.44±0.66a</td>
<td>668.03±59.30ab</td>
<td>56.54±6.92a</td>
<td>1567.71±87.00a</td>
</tr>
<tr>
<td>BT</td>
<td>16.05±0.51a</td>
<td>823.55±75.12a</td>
<td>59.45±5.39a</td>
<td>2038.55±92.34a</td>
</tr>
<tr>
<td>BICT</td>
<td>15.82±0.76a</td>
<td>899.48±60.62a</td>
<td>59.50±5.76a</td>
<td>1864.57±81.06a</td>
</tr>
<tr>
<td>UT</td>
<td>14.39±0.49a</td>
<td>509.47±40.71b</td>
<td>31.91±2.69b</td>
<td>987.08±42.63b</td>
</tr>
</tbody>
</table>

P values of the main treatments and their interaction

| Zones     | 0.139                | 0.001              | 0.001                | 0.001                   |
| Isolates  | 0.191                | 0.018              | 0.001                | 0.001                   |
| Variety   | 0.060                | 0.001              | 0.259                | 0.088                   |
| Isolates  | 0.083                | 0.002              | 0.009                | 0.112                   |
| Zone      |                      |                    |                      |                         |
| Variety   | 0.396                | 0.001              | 0.067                | 0.137                   |
| Isolates  | 0.111                | 0.269              | 0.820                | 0.422                   |
| Variety   | 0.141                | 0.007              | 0.053                | 0.227                   |

Key: Values within the same column followed by the same letter are not significantly different according to Tukey’s HSD at p≤0.05. RI9, Indigenous isolate; ICT, Indigenous consortium; BT, Bio fix; BICT, Indigenous consortium + bio fix; UT, Uninoculated (control), TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; and ELMZ, Embu Lower Midland Zone.
4.3.3.8 Shoot total nitrogen

Zones differed significantly (p < 0.001) on shoot N among the two soybean tested. Zones that were experimented, TLMZ recorded highest shoot N of 2.96 % while TUMZ had 2.91 % being the second best (Table 4.14). However, ELMZ scored lowest shoot N (1.98 %). The two soybean varieties had significant (p=0.002) variation in shoot total N. Soybean SB8 recorded 2.817 % shoot total N while SB126 had 2.547 % (Table 4.14). Soybean seed inoculation with indigenous bradyrhizobia isolate RI9 recorded highest accumulation of shoot total N (2.93 %) followed by commercial inoculant BT with 2.77 %. The uninoculated control (UT) had the lowest shoot total N of 2.39 % (Table 4.14). The use of consortium-biofix BICT led to plants with high shoot N (2.73 %) and was found to be competitive with the commercial inoculant. The increase in shoot total N was affected by the bradyrhizobia inoculant treatments due to significant (p < 0.001) variety × inoculants interaction. Similarly, there was a significant (p < 0.001) interaction between inoculant × zones (Figure. 4.6).
Figure 4.6: Interactive effects of inoculation with indigenous bradyrhizobia isolates and soybean varieties on grain yield. Bars followed by the same letter are not significantly different according to Turkey’s HSD test at P<0.05. A, TUMZ; B, TLMZ; C, EUMZ; D, ELMZ; RI9, Indigenous isolate; ICT, Indigenous consortium; BT, commercial inoculant; BICT, Indigenous consortium + commercial inoculant; UT, Uninoculated (control), TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; and ELMZ, Embu Lower Midland Zone.

There was evident significant difference (p < 0.001) between agroecological zones in terms of shoot available P. The highest value was recorded in TUMZ (3767.67 ppm) followed by TLMZ (3694.03). The lowest value was recorded in ELMZ (2589.03 ppm) which was statistically different from other agroecological zones. Soybean variety SB126 differed significantly from SB8 in terms of shoot available phosphorus (Table 4.14). SB126 had 3517.98 ppm available phosphorus while SB8 variety had 3209.50 ppm. Bradyrhizobia + commercial inoculant (BICT) and indigenous isolate RI9 resulted to
high increase in shoot P accumulation in soybean plants which differed significantly (p<0.001) from other treatments (Table 4.14). Uninoculated control UT was lowest in regards to accumulation in the shoot P (2898.71 ppm)

Table 4.14: Soybean shoot total (%N), Phosphorus (P), and Potassium (K)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%N</th>
<th>P (ppm)</th>
<th>K (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUMZ</td>
<td>2.91±0.12a</td>
<td>3767.80±119.31a</td>
<td>7471.67±463.04b</td>
</tr>
<tr>
<td>TLMZ</td>
<td>2.96±0.11a</td>
<td>3694.30±209.39a</td>
<td>8199.67±302.73ab</td>
</tr>
<tr>
<td>EUMZ</td>
<td>2.88±0.12a</td>
<td>3403.83±157.29a</td>
<td>8667.73±368.54a</td>
</tr>
<tr>
<td>ELMZ</td>
<td>1.98±0.12b</td>
<td>2589.03±116.76b</td>
<td>5222.33±323.45c</td>
</tr>
<tr>
<td>Variety</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB8</td>
<td>2.82±0.10a</td>
<td>3209.50±120.09b</td>
<td>7090.87±352.97b</td>
</tr>
<tr>
<td>SB126</td>
<td>2.55±0.08b</td>
<td>3517.98±126.13a</td>
<td>7689.87±256.80a</td>
</tr>
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<td>8005.83±424.64a</td>
</tr>
<tr>
<td>ICT</td>
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<td>3023.17±207.94bc</td>
<td>6647.92±649.02b</td>
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<tr>
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<td>3383.92±142.57ab</td>
<td>8110.42±401.38a</td>
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<tr>
<td>BICT</td>
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<td>3726.88±187.14a</td>
<td>8245.08±407.36a</td>
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<td>UT</td>
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<td>2898.71±214.68c</td>
<td>5942.50±356.18b</td>
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P values of the main treatments and their interaction

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<td>Isolates × Variety × Farm</td>
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<td>0.062</td>
<td>0.011</td>
</tr>
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</table>

Key: Values within the same column followed by the same letter are not significantly different according to Tukey’s HSD at p<0.05. RI9, Indigenous isolate; ICT, Indigenous consortium; BT, commercial inoculant; BICT, Indigenous consortium + bio fix; UT, Uninoculated (control), TUMZ, Tharaka-Nithi Upper Midland Zone; TLMZ, Tharaka-Nithi Lower Midland Zone; EUMZ, Embu Upper Midland Zone; and ELMZ, Embu Lower Midland Zone.
Correlation analysis showed that nodule dry weight (NDW) had positive and significant correlation with field grain yield \((R^2 = 0.675, p = 0.001)\). There was a strong positive correlation between soybean grain yield and amount of shoot nitrogen accumulated due to inoculation with bradyrhizobia \((R^2 = 0.511, P < 0.001)\). This correlation is an indication that increase total N in shoots due to inoculation resulted in an increase in grain yield (Figure 4.8). The amount of shoot total N accumulated after inoculation differed significantly which is similar with grain yields where there was high variation among the treatments. Similarly, nodule dry weight had positive and significant \((R^2 = 0.688, P < 0.001)\) correlation with shoot dry weight. The increase in nodule dry weight resulted in the increase of shoot biomass due to inoculation (Figure 4.8).

![Graphs showing correlations between variables](image)

**Figure 4.2:** Correlations due to inoculation with different bradyrhizobia isolates between; A= B=, C, Grain yield (Kg/ha) and % shoot N and D, Shoot dry weight (SDW) and nodule dry weight (NDW)
CHAPTER FIVE

DISCUSSION

5.1 Soybean varieties grown by smallholder farmers in Embu and Tharaka-Nithi Counties

During the study, four local soybean varieties were recorded from the 60 households that were surveyed. This soybean species diversity was high considering that there were no exotic varieties that were documented. It also shows that the region is a hub of conservation of indigenous species. The different soybean varieties serve as a source of food to the households. Guimarães et al. (2012) recorded three soybean varieties from Western parts of Kenya where smallholder farmers depend on grain legumes and cereals. Soybean diversification, especially for local varieties which are adapted to the environment, are preferred because of their survival during drought or change of weather patterns (Sarr et al., 2011). The highest soybean diversity H' and species richness was recorded in ELMZ and TLMZ where farmers cited experience of food scarcity. The high diversity in the two zones serves as a way of addressing the problem of food during drought. In contrary, upper midland zones had a preference to particular varieties and hence recorded low soybean diversity. Upper midland zones have different climatic conditions which may not favor some varieties resulting to low diversity.

Despite the presence of different soybean varieties, yield per household per year was not adequate, an indication that farmers may be relying on supplies from other regions. More than half of the farms were documented to have poor soil fertility which may be the
reason for low soybean productivity. Low soil fertility can contribute up to 60% loss of legume production (Perrineau et al., 2011). According to Mateus et al. (2014), inorganic fertilizers are commonly used by most of the households as farm inputs for crop growing to supplement soil infertility problem. However, Muthuri et al. (2014) reported that excessive use of inorganic fertilizer adds acidity to the soil which hinders nitrogen fixation. Therefore there is a need to devise ways to improve soil fertility and reduce acidity for sustainable agriculture.

Gazelle and Nyala varieties were the most preferred soybeans by the households of the selected zones. The two soybean varieties are local cultivars and are said to be tolerant to environmental conditions of the region and resistance to crop diseases (Njeru et al., 2013). In addition, Appunu et al. (2014) pointed that farmers may prefer particular soybean variety due to taste preferences, access to seeds and marketability. However, Mausoy variety was the least preferred and was present in ELMZ and TLMZ. The variety is suitable for low altitude regions which may be the reason why it was present at the Lower Midland Zones (Serafim et al., 2013).

5.2 Soil biological and physicochemical characteristics

The soil pH of the study sites was acidic and varied from one site to another. The soil samples characteristically recorded low pH which could be due to continual cultivation and extensive application of inorganic fertilizers Muthuri et al., 2014). Low pH results to the high solubility of Fe$^{2+}$, Mn$^{2+}$, and Al$^{3+}$ in the soil where they accumulate to toxic
levels for soil microorganism (Wasike et al., 2009). Additionally, excess toxicity due to
the accumulation of ions of Mn, Fe, and Al results to stopping or slowing root growth of
legume plants (Aung et al., 2013). Legumes require neutral or slightly acidic soil pH for
maximum nitrogen fixation. However, some bradyrhizobia strain are tolerant to acidic
soil and can compete effectively with soil microorganisms. The soils also had high total
N in all the zones since they had more than 0.20% N. Use of inorganic nitrogen fertilizers
by farmers could have contributed to the observed high soil %N. According to Aryal and
Prasad (2008), both organic and inorganic farm inputs used to replenish soil fertility
increases soil N.

Phosphorus was relatively low across all the agroecological zones which may be due to
continued cultivation with nutrient replenishment in the soil. Clement et al. (2015)
reported that standard range of P on field soils should be between 2650 ppm and 3100
ppm. P is also a critical element in the soil for it enhances nodule formation and
biological nitrogen fixation in the grain legume. Lin et al. (2012) reported the decrease of
soybean yield due to variation in levels of phosphorus. Phosphorus functions as an energy
source in plants and hence influence the development of nodules. Most of the soils from
the study sites were deficient of phosphorus limiting nitrogen fixation and farmers are left
with the choice of using fertilizers to supplement the soil that is costly to smallholder
farmers (Kaizzi et al., 2012).
The soils from the study sites were acidic while rhizobia requires neutral pH to grow effectively. Legumes also require neutral or slightly acidic soil with pH 6 or 7 for effective symbiotic association with rhizobia. Althabegoiti et al. (2008) reported the effect of exchangeable acidity and soil pH on rhizobial type’s dominance which depends on tolerance to acidity which is evident in the current study.

In some zones, the rhizobia population was high compared to other areas. This may be explained by the fact that some rhizobia strains may gain tolerance to low pH hence they are able to survive and nodulate in acidic soils (Zhang et al., 2011). Low soil pH disrupts communication signals between the rhizobia and leguminous plants which may results to poor nodulation (Pule-Meulenberg et al., 2011). Other factors such as high fungal and actinomycetes population which act antagonistically against rhizobia growth may be a possibility for the low population. Rhizobia compete with other microbes in the soil which may affect them positively or negatively (Dhami and Prasad, 2010).

5.3 Morphological characteristics of bradyrhizobia

From the current study, 39 isolates were obtained from the root nodules of soybeans used as trap cultures. The isolates were consequently grouped into 9 morphotypes based on their morphological characteristics. This was a high diversity of bradyrhizobia isolates from the region which shows that the soils from the study sites harbors a wide range of bradyrhizobia species. Use of nodules trapped directly from the field allows for isolation of diverse bradyrhizobia available in the soil which may be the reason for the different
morphotypes obtained. Zhang et al. (2014) isolated 58 isolates of bradyrhizobia in which after morphological characterization the isolates grouped into 6 groups of bradyrhizobia isolates.

The isolates obtained had typical colony characteristics of bradyrhizobia. BTB reaction showed that most of the isolates had moderate slow growth and produced alkaline substance where media turned blue. Some bradyrhizobia are slow grower where they take up to 5 days to grow in YEMA media (Kaizzi et al., 2012). Atieno et al. (2012) reported that soybean bradyrhizobia are slow growers and alkaline producers hence BTB media take a characteristic blue color. Additionally, typical bradyrhizobial colonies when they were incubated in the dark they showed little or no absorption of Congo Red. Similar findings have been reported by Risal et al. (2010). Bradyrhizobia are differentiated from other bacteria such Agrobacterium by non-absorption of Congo red in the dark (Ormeño-Orrillo et al., 2006). The isolate morphological properties recorded were similar to those recorded earlier for bradyrizobia (Achonga et al., 2015). All the isolates were gram negative. According to Adhikari et al. (2012), strains of bradyrhizobia are Gram negative rods with glistening, small circular and whitish colonies which concurs with the findings from the current study.

Some isolates had larger colonies and this may be due to production of copious extracellular polysaccharides (EPS). Bradyrhizobia are known to produce surface polysaccharides (lipopolysaccharides and exopolysaccharides) that are believed to
restrain defense mechanism of the host (Wasike et al., 2009; Thuita et al., 2011; Zhang et al., 2011). Most of the soil samples analysis tested acidic as indicated and production of polysaccharides may reflect the adaptation to acid soil of Embu and Tharaka-Nithi Counties. Suzuki et al. (2008) reported that bradyrhizobia isolates have increased tendency to produce mucus as an adaptation to acidic soils.

5.4 Genetic diversity of indigenous bradyrhizobia isolates

Amplification of the target 16S rRNA gene resulted to a single band of 1.5 kb in size. This band size obtained correspond to the expected size due to the use of primers specific to bradyrhizobia as reported by Sharma et al. (2012) and Zhang et al. (2014). The restriction of the 16S rRNA gene with different enzymes resulted to different fragment patterns. The finding indicates that there is high variation among the indigenous isolates. This shows that the soils from the study zones harbor bradyrhizobia populations of high diversity bradyrhizobia species. This finding concurs with those reported in other parts of the world as reported by Atieno et al. (2012) and Shiro et al. (2013). Restriction digestion of 16S rDNA of the 39 bradyrhizobia isolates using HaeIII enzyme resulted to different fragment patterns. However, five of the isolates were not restricted which may be attributed to the lack of enzyme recognition sites in the gene sequence (Nur, 2014). According to Yan et al. (2014), lack of these enzyme recognition sites does not mean the isolates are different from others in the same group. Mutations and horizontal gene transfer result to the change of genetic makeup of the isolates which cause lack of specific recognition sites (Risal et al., 2010).
Most of the genetic variation of the isolates was within populations based on analysis of molecular variance (AMOVA). There was significantly high level of variation partitioned within bradyrhizobia populations. According to Ormeño-Orrillo et al. (2006), this is an indication that there are limited physical barriers to the flow of the gene and bradyrhizobia populations from study zones are not well structured. Risal et al. (2010) and Vauclare et al. (2013) recorded high variation of bradyrhizobia isolates within populations and a small variation across different regions. PCA analysis of the bradyrhizobia isolates showed similar genetic differentiation patterns as AMOVA where bradyrhizobia isolates were not corresponding with geographical location. Ansari et al. (2014) documented that PCA analysis of cow pea bradyrhizobia isolates were independent of the regions they were isolated which is similar with the findings from this study.

Indigenous bradyrhizobia isolates that were evaluated for their genetic diversity using 16S rDNA showed a narrow Nei unbiased genetic distance. Use of soybeans as the trap cultures could contribute to this observation where only specific strains of bradyrhizobia can have effective nodulation. In addition, this observation could also be due to the targeted 16S rRNA gene which might be affected by horizontal gene transfer and also due to genetic recombination among the soil bradyrhizobia (Zhang et al., 2014). Appunu et al. (2008), reported similar findings where bradyrhizobia populations isolated from different agroecological zones in Ethiopia had close genetic distance.
Cluster analysis of the 16S rRNA based on restriction digestion showed that the bradyrhizobia isolates were highly diverse. This diversity may be contributed by the trapping of the bradyrhizobia from their natural environment (Yan et al., 2014). Isolates from various zones were clustered together in the Neighbor-Joining method hence isolates did not correlate with their geographical zones of origin. Similar findings have been reported for indigenous rhizobia in *Phaseolus vulgaris* L. from various agroecological zones in Nepal (Risal et al., 2010). Most of the indigenous bradyrhizobia isolates were not clustered together with the reference strains USDA 110 and USDA 136. Few of the isolates were clustered closely to the reference strains but they were not identified to species level based on restriction digestion of 6S rRNA gene.

5.5 Authentication of the isolates in the greenhouse experiment

During authentication, seven isolates nodulated effectively with soybean genotypes used. Although there was variation on the level of nodulation, presence of nodules is a complete justification that the isolate used were the bradyrhizobia. Bradyrhizobia isolates should have ability to nodulate with soybean plants (Kwon et al., 2005). However, isolates RII and RI2 did not nodulate the test soybean varieties and hence not considered in tests for symbiotic effectiveness. The variation on nodulation may be due to lack of compatibility genes responsible for nodulation of the isolates. Mwenda (2010) reported that some bradyrhizobia isolates lacked functional genes for infecting legumes and are common in soil rhizosphere which have been grown legumes before. On the other hand, high number of nodules does not guarantee that all nodules are active and this is because some nodules may form but lack effective nitrogen fixation (Risal et al., 2010).
In regard to this, Delić et al. (2010) had reported that some nodules may undergo early senescence while others may never be active causing variation on nitrogen fixation.

5.6 Effectiveness of indigenous bradyrhizobia isolates in the greenhouse experiment

Greenhouse experiment indicated that inoculation of the three soybean genotypes (SB8, SB24, and SB126) with bradyrhizobia improved shoot dry weight. Bradyrhizobia inoculation enhances biological nitrogen fixation which results to improvement of shoot dry weight. This observation was similar to that reported by other studies (Saeki et al., 2005; Rahmani et al., 2011; Parr, 2014). Muthuri et al. (2014) demonstrated that there is variation in nitrogen fixing efficiency and can vary from one isolate to another. Lin et al. (2012) reported that inoculation of soybean genotypes with bradyrhizobia isolates significantly increased shoot dry weight after using sterile sand in a controlled environment. High shoot dry weight after bradyrhizobia inoculation compared with controls is an indication of improved nitrogen fixation.

There was variation in the performance of the tested isolates on soybean varieties. Inoculated SB8 and SB126 soybean genotypes responded better in their symbiotic efficiency compared with SB24 genotype. Soybean genotype SB24 may not be compatible with most of the indigenous isolates hence low nodulation which indicates the need for varieties selection to the farmers. Javaid and Mahmood (2010) during their study of nitrogen fixation of soybeans with strains of Bradyrhizobia japonicum reported variations in dry matter and nodule nitrogen content among the soybean varieties. The
genomic variations of the bacteria or the host plant or both that regulate symbiosis and compatibility may attribute to the differences observed in the study (Jaiswal et al., 2011). There was a clear effect of inoculation on nodule number and nodule dry weight. Inoculation also had effect on the three soybean genotypes on nodulation which suggest that it is vital or beneficial for smallholder farmers to use soybean seeds dressed with inoculants (Kaizzi et al., 2012).

Indigenous bradyrhizobia inoculation significantly increased shoots %N in soybean plants from the greenhouse experiment compared to uninoculated control. The accumulation of nitrogen in soybean shoots may be due to presence of available nitrogen from effective biological nitrogen fixation. Aung et al. (2013) and Sikora et al. (1997) reported an increase in nitrogen uptake by soybean plants after inoculation in a sterilized soil. Some isolates recorded low to moderate nitrogen accumulation in soybean leaves. This may be explained by the difference in isolates in their ability to fix nitrogen with soybean varieties under study. Ampomah et al. (2008) reported variability in performance of bradyrhizobia strains in accumulation of nitrogen in soybean shoots.

There was evident significant increase of available shoot phosphorus due to inoculation of soybeans with indigenous bradyrhizobia isolates in the greenhouse. Effective bradyrhizobia isolates usually induce increased number of root hairs which favors nutrient uptake and may account for the increased phosphorus uptake (Rahmani et al., 2011). Labeyrie et al. (2014) reported a significant increase of nutrients due to
inoculation of cowpea plants with bradyrhizobia compared with uninoculated plants. In addition, some bradyrhizobia isolates have greater potential of solubilizing precipitated phosphorus which enhance nutrients uptake (Kumba et al., 2015).

5.7 Field experiment

5.7.1 Shoot biomass

There was a variation in shoot dry weight in different agroecological zones. Biological nitrogen fixation and plant growth depend on soil conditions such as pH and soil reaction with microorganisms. Farm management practices that create soil condition that favor bradyrhizobia survival and persistence have shown to increase shoot dry matter (Dhami and Prasad, 2009). Soils from different zones have a complex matrix to control and manipulate hence the inoculants effectiveness depends on the nature of the soil (Florentino et al., 2010). Physical and chemical properties were also different hence adaptability of bradyrhizobia to the various condition may contribute to differences observed. Guimarães et al. (2012) stated that some soil conditions in a given region may compromise nodulation competitiveness of the inoculants.

The two soybean varieties SB8 and SB126 did not differ significantly in shoot dry weight. The ability of the soybeans to nodulate freely with different strains of bradyrhizobia may as well account for the insignificant interaction between the test varieties and the treatments. Yao et al. (2014) recorded similar results after testing six cultivars of soybean with five strains of B. japonicum for symbiotic efficiency.
Both the indigenous isolate and the commercial inoculants were competitive which may be associated with bradyrhizobia ability to nodulation with different soybean varieties. The accumulation of above ground biomass was different possibly due to the difference in nitrogen-fixing potential among the isolates. According to Soea et al. (2013), legume performance may vary depending on the symbiotic effectiveness of the bradyrhizobial strains. Pule-Meulenberg et al. (2011), reported similar results where bradyrhizobial strains had a significant effect in soybean shoot dry weight.

The variation in shoot dry weight between the test inoculants may be due to incompatibility with soybean genotypes under study. Zhang et al. (2014) found that there are variations on soybean response to inoculation between the cultivars. The consortium was lower in performance on shoot dry weight. This observation may be accounted by competition of isolates where some act antagonistically against others to ensure their survival. Appunu et al. (2011) reported instances where rhizobial mixture failed to nodulate effectively with legumes planted in Ethiopian plains. In addition, variations in shoot dry weight also may be attributed to the difference on genomic constitutions of bacteria or the host plant or both which take part in symbiosis (Soea et al., 2013).

The results showed that inoculation had influence on all soybean plants planted in all agroecological zones except the varieties grown in ELMZ due to variety × zone interaction. The results suggest that difference in soil physical-chemical properties may affect inoculation causing variations in the zones. This imply that soil status is improved
where there is effective inoculation. Sarr et al. (2011) and Mateus et al. (2014) had similar results where soybean inoculated with bradyrhizobia increased significantly above ground biomass on some soybean varieties in specific sites studied.

5.7.2 Nodule assessment

All the inoculated treatments of soybean plants increased nodulation compared to uninoculated control. Inoculation of soybeans especially with indigenous bradyrhizobia which are adapted and compatible to the local condition may account for the high nodulation compared to commercial inoculant (Soea et al., 2013). This effective nodulation depict the importance of inoculation. The uninoculated treatment (UT) of soybean plants had less number of root nodules which may be due to lack sufficient bradyrhizobia cells in the soil for effective nodulation. According to Kwon et al. (2005), inoculation with indigenous bradyrhizobia strains increases nodulation significantly. The indigenous isolate may as well be able to compete favorably in the field condition hence outperforming commercial isolates. Serafim et al. (2013) reported 200% nodule number increment as a result of inoculating soybeans with *B. japonicum* which was higher than the current study.

Similarly, nodule dry weight increased with increase in nodule numbers per plant. During inoculation, effective bradyrhizobia infect more root hairs which enhance nodulation where a large number of nodules contributes to high nodule dry weight. The efficiency of nodules and the host species in nitrogen fixation may attribute to the difference observed.
Suzuki *et al.* (2008) reported increase of nodule dry weight from 29-116% over control after soybeans were inoculated with bradyrhizobia under field conditions. There was no variation on nodulation among the two soybean varieties. The varieties were promiscuous hence nodulate freely with different bradyrhizobia although nodulation does not assure effective nitrogen fixation.

### 5.7.3 Pod number per plant

The number of pods per plant usually determines the number of seeds or grain yield. Soybeans treated with inoculants significantly increased pod number compared to uninoculated. This observation shows that application of inoculants results to increased number of pods in soybeans. Pods development requires N which is made available through biological nitrogen fixation (Risal *et al.*, 2010). Zhang *et al.* (2014) reported that there are variations on soybean response to inoculation and effective nitrogen fixation resulting to the difference in pods yield. Although the difference between pods number among the varieties was not significant, SB126 had the highest number pods per plant. The two varieties (SB8 and SB126) were both promiscuous hence able to nodulate with different strains of bradyrhizobia which may account for the insignificant variation observed among the soybean varieties.

In addition, the number of pods were directly linked with grain yield where higher pods number resulted to higher grain yield. Serafim *et al.* (2013) reported that the number of pods plant\(^1\) on soybean TGx-1937-1F influenced grain yield, wherein higher number of pods resulted to high yield, while the fewer the pods number the lower the yield. In
addition, inoculation resulted to high pods number plant\(^{-1}\) at EUMZ and TLMZ due to inoculation × zone interactive effect. Bradyrhizobia inoculation responded effectively in these two zones which may be supported by relatively favorable soil properties to inoculation. Pule-Meulenberg et al. (2011), reported that effective inoculation results to increased number of pods and grain yield on both promiscuous and non-promiscuous soybeans.

5.7.4 Stover dry weight

The inoculated soybean plants significantly increased stover dry weight compared to the uninoculated. The increased stover dry weight indicates that inoculants had a greater influence on stover dry weight. Inoculation enhances biological nitrogen fixation which improves above ground biomass. Additionally, inoculation improves crop performance on yield parameters obviously because of sufficient nitrogen in root nodules via biological nitrogen fixation (Loureiro et al., 2007). SB8 variety had higher stover dry weight compared to SB126, but the difference was not significant. The promiscuous soybeans are known to produce higher above ground biomass which is important for it improves soil organic matter (Zhang et al., 2011). SB126 was a late maturing variety which gives time to build more biomass thus accounting for the higher stover dry weight. According to (Sharma et al., 2010) soybean genotypes mature differently depending on their genetic makeup.
5.7.5 100-seed dry weight

Seeds dry weight as a yield component is vital since it reflects on the magnitude of seed development which ultimately determine the final yield of the soybean crop. SB8 recorded highest 100-seeds dry weight compared to SB126 although the difference was not significant. In addition, bradyrhizobia inoculation indicated positive response on 100-seeds dry weight especially for III and BT. This observation could be due to contribution of effective nitrogen fixation which supply N, a major constituents of biological compounds such as amino acids which play a role in seed development (Guimarães et al., 2012). Likewise, Kaizzi et al. (2012) reported that inoculation of soybean seeds increased 100-seeds dry weight.

5.7.5 Soybean shoots %N

Farms in terms of agroecological zones affected shoot %N accumulation which may depend on soil fertility. Extensive application of nitrogen fertilizers may raise nitrogen content in the soil which as a results affect the amount of nitrogen uptake in plants. According to Perrineau et al. (2011), use of nitrogen fertilizers and other farm nutrients inputs cause significant variations on shoot %N on legumes. Soybean varieties under study had varied accumulation of shoot %N, which may be due effectiveness in nitrogen fixation after inoculation and growth nature of each soybean variety. SB8 variety which scored highest shoot biomass was best on nitrogen uptake. While studying the activity of nitrogen assimilation by enzymes and nitrogen fixation, Li et al. (2011) reported variations on bradyrhizobial strains performance with different soybean varieties in terms
of shoot nitrogen content. This variation may be attributed by compatibility of inoculants with soybean cultivars (Risal et al., 2010).

Inoculation of soybeans with isolates resulted in significant accumulation of shoot total N. High total nitrogen accumulation due to inoculants may be because of presence of high nitrogen derived from effective symbiotic nitrogen fixation. Barcellos (2009) reported that soybeans inoculated with bradyrhizobia increased nitrogen uptake significantly in sterilized soil in the greenhouse. However, some isolates recorded low to moderate accumulation of nitrogen content in shoots which may be attributed by the difference in symbiotic effectiveness among the test isolates (Hungria et al., 2013). According to Delić et al. (2010), inoculant application in legumes increased uptake nitrogen.

5.7.6 Phosphorus

Indigenous isolates had the highest accumulation of shoot P in soybean plants. This high available P in shoots is an indication that indigenous isolates were able to solubilize P components effectively. According to Serafim et al. (2013), some rhizobia isolates have the potential of solubilizing precipitated available P and increasing the formation of lateral roots and root hairs. Sharma et al. (2010) sited significant increase in uptake of phosphorus to bradyrhizobia inoculation to soybean plants in sterilized sand. Phosphorus is vital during symbiosis between rhizobia and legumes where it enhances nodulation (Thuita et al., 2011). Successful nitrogen fixation requires phosphorus as a source of
energy which is one of the macronutrients necessary for the growth of grain legumes. Bradyrhizobia utilize energy from phosphorus during infection process with root hair cells (Kwon et al., 2005). Phosphorus compounds in plants are essential for transferring and storing energy in the form of ATP from photosynthesis. Soea et al. (2013) further indicated that phosphorus act as an enzyme component and have an important role in energy metabolism.
CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

After the analysis, the following conclusions were made:

i. During field surveys, four local soybean varieties were identified (Nyala, Gazelle, Namsoy and Mausoy). The varieties had varied distribution in the four agroecological zones where Gazelle and Nyala were the most preferred local soybeans varieties.

ii. In the present study, 39 bradyrhizobia isolates were obtained from field trapping experiment and based on morphological characteristics, the isolates were diverse.

iii. Cluster analysis on morphological and molecular characteristics showed high diversity of indigenous bradyrhizobia isolates that were obtained from field trapping experiments an indication that the soils from the study sites harbors diverse strains of bradyrhizobia.

iv. Indigenous bradyrhizobia isolate RI9 had a significant difference on symbiotic effectiveness and performed better compared to a commercial isolate BT12 an indication that indigenous bradyrhizobia had a higher potential.

6.2 Recommendations

i. Different local soybean varieties were identified and there was no currently advance varieties such as Tropical Glycine cross (TGX) such as SB8 and SB126
hence it is recommended for farmers to try currently advanced varieties that nodulate freely with different strains of bradyrhizobia.

ii. Indigenous bradyrhizobia isolates had high genetic variation within populations and symbiotic effectiveness demonstrated that the region is a hub for diverse bradyrhizobia isolates with superior qualities that can be used for soybean inoculation.

iii. The effective bradyrhizobia isolate RI9 is recommended to soybean smallholder farmers for inoculation in order to improve the yield. The isolate should also be tried in other regions of Kenya to test their effectiveness in order to be considered for inoculant production.
REFERENCES


## APPENDICES

**Appendix I:** Nitrogen-free solution according to Broughton and Dilworth (1971)

<table>
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<th>Stock solution</th>
<th>Element</th>
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<th>Form</th>
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<th>Gram/L</th>
<th>Molarity of stock solution</th>
</tr>
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<td>CaCl₂·2H₂O</td>
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<td>294</td>
<td>2.0</td>
</tr>
<tr>
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<td>P</td>
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<tr>
<td>4</td>
<td>B</td>
<td>2</td>
<td>H₃BO₄</td>
<td>61.84</td>
<td>0.247</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>0.5</td>
<td>ZnSO₄·7H₂O</td>
<td>287.56</td>
<td>0.288</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td>0.2</td>
<td>CuSO₄·5H₂O</td>
<td>249.69</td>
<td>0.100</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Co</td>
<td>0.1</td>
<td>CoSO₄·7H₂O</td>
<td>281.12</td>
<td>0.056</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Mo</td>
<td>0.1</td>
<td>Na₂MoO₄·2H₂O</td>
<td>241.98</td>
<td>0.048</td>
<td>0.0002</td>
</tr>
</tbody>
</table>
Appendix II: Questionnaire

I am Simon Mburu, student at Kenyatta University taking a Master of Science degree in Microbiology and I am taking a research to determine soybean varieties grown by smallholder farmers from Embu and Tharaka-Nithi Counties. This questionnaire is designed to solicit for your contribution on soybean varieties grown by smallholder farmers. All the information gathered will be treated with utmost confidentiality and will be used for academic purpose only.

Your assistance and participation will be highly appreciated. Thank you in advance.

SECTION A: BACKGROUND INFORMATION

Questionnaire No.  

Date of interview:  

County:  

Sub-county:  

Location:  

Sub-location:  

Village:  

Household code:  

Name of respondent:  

Code:  

Code:  

Code:  

Code:  

Code:  

Code:  

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 B1. Which crops types do you grow?

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Legumes</td>
<td></td>
</tr>
<tr>
<td>Cereals</td>
<td></td>
</tr>
<tr>
<td>Tubers</td>
<td></td>
</tr>
<tr>
<td>Others (specify)</td>
<td></td>
</tr>
</tbody>
</table>

Table B2. Legume information

<table>
<thead>
<tr>
<th>Field ID</th>
<th>Legumes grown</th>
<th>Planted area (Acres)</th>
<th>Yield (kg)/year</th>
<th>Income (KES)/ year</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Common beans</td>
<td>1. &lt; 0.25</td>
<td>1. &lt; 90</td>
<td>1. &lt; 2700</td>
</tr>
<tr>
<td>2.</td>
<td>Soya beans</td>
<td>2. 0.25- 0.5</td>
<td>2. 901-180</td>
<td>2. 2701-5400</td>
</tr>
<tr>
<td>3.</td>
<td>Pigeon peas (Cajun cajan)</td>
<td>3. 0.5-1</td>
<td>3. 181-270</td>
<td>3. 5401-8100</td>
</tr>
<tr>
<td>4.</td>
<td>Cowpea (Vigna unguiculata)</td>
<td>4. &gt; 1</td>
<td>4. 271-360</td>
<td>4. 8100-10800</td>
</tr>
<tr>
<td>5.</td>
<td>Green grams (Vigna radiata)</td>
<td></td>
<td>5. 361-450</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Others (specify)</td>
<td></td>
<td>6. &gt;450</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE B3. Information on soya beans varieties grown

<table>
<thead>
<tr>
<th>SNo.</th>
<th>Soya beans variety</th>
<th>Planted area (Acres)</th>
<th>Cropping practice</th>
<th>Yield(kg)/ year</th>
<th>Income (KES)/ year</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mixed</td>
<td>1. &lt; 0.25</td>
<td>1. Mixed</td>
<td>1. &lt;90</td>
<td>1. &lt;2700</td>
</tr>
<tr>
<td>2</td>
<td>Sole</td>
<td>2. 0.25-0.5</td>
<td>2. Sole</td>
<td>2. 901-180</td>
<td>2. 2701-5400</td>
</tr>
<tr>
<td>3</td>
<td>0.5-1</td>
<td>3. 181-270</td>
<td>3. 181-270</td>
<td>3. 5401-8100</td>
<td>3. 5401-8100</td>
</tr>
<tr>
<td>4</td>
<td>&gt;1</td>
<td>4. 271-360</td>
<td>4. 271-360</td>
<td>4. 8100-10800</td>
<td>4. 8100-10800</td>
</tr>
<tr>
<td>5</td>
<td>&gt;450</td>
<td>5. 361-450</td>
<td>5. 361-450</td>
<td>5. 8100-10800</td>
<td>5. 8100-10800</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6. &gt;450</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### SECTION C. SOIL FERTILITY INFORMATION

#### TABLE C1. Information on soil fertility

<table>
<thead>
<tr>
<th>Code</th>
<th>Soil fertility Status</th>
<th>Farm Input</th>
<th>Soil Erosion Problem</th>
<th>Soil Conservation structures present</th>
<th>Conservation structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Fertile</td>
<td>1). Farmyard manure</td>
<td>1). Yes</td>
<td>1). Yes</td>
<td>1). Bench terrace</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>3). Compost</td>
<td></td>
<td></td>
<td>3). Bench terrace with trees</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4). Crop residues</td>
<td></td>
<td></td>
<td>4). Bench terrace with grass and trees</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5). None</td>
<td></td>
<td></td>
<td>5). Check dam</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6). Bio-fertilizers</td>
<td></td>
<td></td>
<td>6). Cut off drain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7). Others (specify)</td>
<td></td>
<td></td>
<td>7). Other (specify)</td>
</tr>
</tbody>
</table>

1.

2.
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 [ ]