ABUNDANCE, MOLECULAR CHARACTERIZATION AND SYMBIOTIC POTENTIAL OF ENDOPHYTIC ROOT NODULATING RHIZOBIA COLONISING SOYBEAN IN SOILS OF KAKAMEGA COUNTY

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DECLARATION

This thesis is my original work prepared with no other than the indicated sources and support and has not been presented elsewhere for award of degree or any other award.

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of Masinde Muliro University of science and Technology a thesis entitled: **Abundance, Molecular Characterization and Symbiotic Potential of Endophytic Root Nodulating Rhizobia Colonizing Soybean in Soils of Kakamega County**.

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DEDICATION

This work is dedicated to my Aunt Sr. Maria Goretti Ingaso and my Wife Veronica Atsenga for their continual support and encouragement during my entire study period.

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ABSTRACT

Root nodulating bacteria are a diverse group of bacteria that may coexist with legumes and non-legumes in a symbiotic relationship. These bacteria have a wide range of symbiotic flexibility. Rhizobia inoculants have contributed to increase nitrogen fixation. The number of rhizobia in the soil impacts whether soybeans will respond to inoculation. This study aimed at identifying and selecting a potential root nodulating rhizobia of soybean plant which were tested in vermiculite in the greenhouse in the modified Leonard jars. The goal of the study was to characterize symbiotic rhizobia using 16S rRNA as a molecular tool, determine the abundance and symbiotic potential of indigenous rhizobia strains in soil samples collected from six locations in the three agro-ecological zones (Lower midland 1, Lower midland 2 and upper midland). Soil samples were obtained from 24 farms in six different sub-Counties: Kakamega South, Kakamega East in Isukha South, Kakamega Central, Kakamega North, Mumias East and Butere which has been used for soybean growing for more than two seasons. In order to determine the population size of rhizobia, the most probable number technique was applied. Quantitative data on concentration of discrete positive items was obtained through quantification of the concentration of viable rhizobia. The indigenous rhizobia counts were estimated using the most probable number enumeration approach, which revealed that 51.8 percent of the studied sites had high abundance. Each of the six regions had a different number of Rhizobia in the composite soil ranging from 7.3×10^1 to 1.2×10^5 cells g^- soil with confidence factor of 4.674404 at $p<0.95$. Deoxyribonucleic acid (DNA) was extracted from cultured bacteria using Qiagen DNA extraction kit and the 16S rDNA was amplified by 1492R and 27F primers followed by sequencing to establish their genetic diversity. Phylogenetic relationships of eight Kakamega isolates were analysed using their 16S rRNA gene sequences reflecting their heterogeneity as follows: *Rhizobium nepotum, Rhizobium skierniewisence, Rhizobium pisi, Rhizobium fabae, Rhizobium phaseoli, Rhizobium etli, Rhizobium azibense,* and *Pseudomonas glyciae.* The ANOVA method was used to determine the association between rhizobial abundance and symbiotic potential in relation to soil chemical conditions, and the means were compared using Turkey's honest significant difference test. Inoculation with bacterial isolates increased the number of nodules and the dry weight of the shoots significantly ($P<0.05$), however the weight of the seeds varied between groups. Inoculation by rhizobia promotes symbiotic competency for the generation of plant-growth-promoting rhizobacteria inoculants in Kakamega County soils, according to this study. Existence of varying population levels of rhizobia was demonstrated in soils from the region which were also influenced by different agro-ecological zones coupled by micro and macro nutrients. Based on this research, Mumias and Butere had a higher diversity of *Rhizobium fabae, Rhizobium phaseoli, Rhizobium pusence* and *Pseudomonas glyciae* per gram of soil, resulting in high shoot biomass and high yields.

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ABREVIATIONS AND ACRONYMS

16S rDNA	16 Subunit ribosomal deoxyribonucleic acid
16S rRNA	16 Subunit ribosomal ribonucleic acid
AEZs	Agro-ecological Zones
BLAST	Basic local alignment search tool
BNF	Biological nitrogen fixation.
Bp	Base pair
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetracetic acid
FAO	Food and Agriculture Organization
fD1	16S rDNA forward primer
LB	Lysogenic broth
MPN	Most Probable Number
N_2	Nitrogen gas
NDW	Nodule Dry Weight
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
PY	Peptone Yeast

RCBD	Randomized Complete Block Design
rD1	16S rDNA reverse primer
RNA	Ribonucleic acid
SDW	Shoot Dry Weight
SE	Symbiotic Efficiency
Taq	Thermus aquaticus
YMA	Yeast-mannitol agar

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Soybean (*Glycine max)* is dependent on root nodulating rhizobia for nitrogen fixation. Rhizobia are soil bacteria that can develop nitrogen-fixing symbioses with a variety of leguminous plants, increasing crop yields (Fall *et al*., 2003; Fox *et al*., 2007). The crop is grown by both commercial and subsistence farmers in numerous countries in Sub-Saharan Africa, including Kenya (Ouma *et al*.,2016). Vitamins and minerals have been enhanced in the young leaves and pods for human consumption and livestock feeding. Furthermore, soybeans have a high protein composition of roughly 40%, 18% fat, and 6.3% carbohydrates (Medic *et al*., 2014; Dukariya *et al*., 2020).

Soybean also has a high oil content (2-21%), making it one of the four most valuable crop legumes on the planet (Goyal *et al*., 2012; Sharma *et al*., 2014). Despite the fact that the crop can be intercropped with other plants, it is best cultivated in pure stands where it thrives (Biabani *et al.,* 2012; Metwally *et al.,* 2018). When intercropped with other plants, soybean has the potential of reducing a parasitic weed called *Striga hermonthica* especially when intercropped with sorghum and maize (Carsky *et al*., 2000). Root nodulating bacteria strains are crucial in soybean production because they must compete for improved yields in the rhizosphere (Tilak *et al*., 2006). Different soybean cultivars are currently effective in picking root nodulating rhizobia in the soil in their various ways, necessitating competitiveness for nodulation (Mungai *et al*., 2011). *Leguminosae* members make up the world's largest plant family, with over 18,000 species. Their

success can be attributed to their ability to form a nitrogen-fixing symbiosis (Wojciechowski M.F. 2003). However not all legumes produce nodules, for instance, most of the species in caesalpinoideae subfamily (cassia, caesalpina and Bauhinia) are an example of leguminosae that do not produce legumes. Soybean therefore may display various morphological and genotypic characteristics depending on the motif of the strain selection (Nguyen *et al*., 2020). This may be in the form of the strain ability to compete for nodule forming site, rhizosphere colonization ability in the period of crop development and the mode in which nodules are formed for a particular host (Zablotowicz *et al*., 1981). Most of these rhizobia isolates that nodulate soybean are mostly associated with slow growing root nodulating such as *Bradyrhizobia japonicum* although there are various known to be fast-growing rhizobia. Despite the fact that soybeans cultivated in Africa are good for crop productivity, they have poor levels of biological nitrogen fixing (Abaidoo *et al.,* 2007). However, nothing is known about the nitrogen-fixation capacity of various root nodulating rhizobia colonizing soybean in Kakamega County soils.

Research has revealed that there is losss of cultivated soybean varieties which negatively influence loss of genetic diversity of *rhizobium* species (Sadowsky, 2005). The latter problem is occasioned by conventional breeding programs associating with modern agricultural practices. Through shifting to a better yielding crop varieties adoption by farmers for better returns, soybean landraces may be lost (Carter 2004; Ainsworth *et al.,* 2012). In soils of Kakamega, however, little is known about the molecular characteristics of soybean root nodulating rhizobia. In this context, fascinating questions about the

evolution and ecology of root nodulating rhizobia arise (Doyle, 1998; Menna *et al*., 2009).

1.2 Statement of the Problem

Soybean (*Glycine max*) is an important human and livestock food with highly nutritive leaves, pods and high in oil content (protein≥40%) (Dukariya *et al*., 2020). Through symbiotic nitrogen fixation, the crop is also a significant legume for soil enhancement (Coskan, A. and Dogan, K 2011; Hungria *et al.,* 2015; Chemining`wa *et al*., 2012). Ninety five percent (95%) of the world populace are familiar with legume root nodules but only 26% consider them as beneficial (Kahindi *et al*., 1997; Garg, 2009; Hungria *et al.,* 2015). Genetic diversity of root nodulating rhizobia colonizing *Glycine max* in Africa has not been well studied, yet there is potential genetic loss in soybean varieties (Han *et al.*, 2020; Gitonga *et al*., 2021). The loss of this crop varieties may also affect *Rhizobia*, since the diversity of these beneficial bacteria varies between crop cultivars (Doumbia *et al*., 2013). Despite the fact that Kenyan soils may harbor a vast diversity of root nodulating rhizobial populations (Chemining`wa *et al*., 2011), information on diversity of rhizobia that nodulate soybean as well as their nitrogen fixing potential within Kakamega County is limited. Morphological characterization of soybean and their associated rhizobia may distinguish some features (Sharawy *et al.,* 2003; Chemining'wa *et al*., 2011), however, these morphological features are often less reliable which calls for molecular characterization. It is therefore imperative that the diversity of symbiotic rhizobia associated with soybean varieties in Kakamega County be determined using a molecular tool. Currently, sequencing methods such as next generation sequencing (Shendure *et al*., 2008) and Sanger sequencing are being proposed. This research

therefore focused on whether 16S rRNA as a molecular tool and using Sanger sequencing method could accurately characterize soybean nodulating rhizobia in soils of Kakamega County.

1.3 Justification of the Study

Soybean varieties are of high nutritional value for a vast majority of people in Africa (Odendo *et al*., 2011; Khojely *et al*., 2018). Furthermore, soybean is one of the earliest plants that have been domesticated by man (Krasova-wade *et al*., 2003; Thuita *et al*., 2012). Currently soybean production has been declining due to changing land use patterns, the use of inorganic fertilizers and other problems related to soil fertility (Trabaquini *et al.,* 2017). *Rhizobiaceae* members like *rhizobium* and *bradyrhizobium* forms root nodules in soybeans. Inside the nodules, the nitrogenase enzyme in rhizobium bacteroids converts nitrogen to ammonia (Pule-meulenberg *et al.,* 2010). This legume can fix a lot of nitrogen (Koskan *et al.,* 2011). Biofertilizer from biological nitrogen fixation (BNF) has the potential to boost global food production. Soybean crop is able to colonize nitrogen-deficient soils in Kenya especially in parts of Kakamega County by boosting their fertility thus making the soils conducive for crop production without necessarily using inorganic fertilizers (Rincon *et al*., 2008; Koskey *et al.,* 2017). However, there is diminutive information on their performace in nitrogen fixation in different soybean cultivars in Africa and Kenya in particular. Furthermore, limited research has been done on bacteria colonizing soybean varieties with the high nitrogen fixing potential yet this legume is considered as a poor man's meat. This was a research project aimed at characterizing root nodulating rhizobia of soybean crop in soils of Kakamega County. Soybean is one of the leguminous crops cultivated in Western part of Kenya. In recent

years, root nodulating rhizobia, which produces a considerable amount of fixed nitrogen in the soil, has gotten a lot of attention (Mabrouk *et al.,* 2018). Further research has revealed that there are rhizobia strains in Western Kenyan soils that have a high symbiotic potential when compared to commercial inoculants (Kawaka *et al*., 2014). Further research contacted on bean has revealed that there are rhizobia strains in Western Kenyan soils that have a high symbiotic potential when compared to commercial inoculants (Kawaka *et al*., 2014). Moreover, if the soil is inoculated by most efficient *rhizobial* strain, residual nitrogen which will be left will be more and even enough to provide nutrients to the incoming crops without application of synthetic nitrogenous fertilizers (Chemining'wa *et al*., 2012; Gitonga *et al.,* 2021). Determination of the most efficient Soybean cultivar in nitrogen fixation will be beneficial to farmers in Western Kenya since they will be able to maximise cropping system without necessarily incorporating synthetic fertilizers in the soil which is very expensive.

1.4. General Objective

To characterize root nodulating rhizobia of soybean in soils of Kakamega County, Western Kenya.

1.4.1 Specific Objectives

i) To determine the abundance of root nodulating rhizobia colonizing soybean from selected agroecological zones of Western Kenya.

ii) To determine the Genetic diversity of root nodulating rhizobia that infect *Glycine max*.

iii) To determine the symbiotic potential of root nodulating bacteria.

1.5 Hypotheses

H_{01}: Abundance of native soybean rhizobia is expected to vary in Kakamega County, depending on the site of collection and respective soil physiochemical characteristics.

$H_{02:}$ There will be similarity in bootstrap value during phylogenetic analysis of clustered isolates.

$H_{02:}$ Success of symbiotic efficiency will depend on inoculation ability of the most competitive strain of rhizobia.

CHAPTER TWO

LITERATURE REVIEW

2.1 World Production of Soybean

Soybean is grown throughout the world, with the United States being the largest producer (Vieira *et al.,* 2021). Soybean cultivation is estimated at 121.53 million hectares yielding to 2.76 tons per hectare (Milanovic *et al.,* 2020). America is the leading producer, followed by Asia, Europe and Africa (Terzic *et al.*, 2018). The plant is believed to have emerged in Eastern China in 11th Century BC as a domesticated plant (Thuita *et al*., 2012). Because of the strong demand for livestock and industrial use, soybean production in Africa has quickly gained popularity in recent years (Engelbrecht *et al.,* 2020). Production of this crop in Sub-Saharan Africa has increased exponentially from 13,000 T planted at 20,000 ha in 1970's to about 2,300,000 T planted at 1,500,000 ha in 2016 (Khojely *et al*., 2018). According to FAOSTAT, (2008), production of soybean in SSA has been spreading steadily but unevenly. Soybean is widely distributed in SSA nations such as Senegal, Uganda, Nigeria, Ethiopia, and Kenya, where IITA and soybean breeders have released over 195 soybean varieties. Soybean yields in Western Kenya are currently 0.6 tons per hectare every season, compared to a potential of 3 to 3.6 tons per hectare per season (Omondi, J. O. (2013).

2.2 Rhizobia Interaction with Soybean in the Nitrogen Fixation Process

Despite the crops morphological characteristics expressed in seed proteins, seed color, plant type, seed size, and pod type, the genetic diversity of rhizobia inhabiting soybean

has been confined, particularly among human produced soybean (Sharawy *et al.*, 2003; Bala, A., & Giller, K. E. 2006). Currently, much efforts is made by many researchers for BNF by rhizobial bacteria in order to offer innovative and sustainable solutions to small scale farmers. Many poor farmers do not have access to fertilizers leading to sustainability of rhizobium inoculum being an important aspect in saving money for their essentials (Thuita *et al*., 2018).

It is estimated that the leguminous crop fixes approximately 20 to 100 kg of nitrogen per hectare (Rondon *et al*., 2007). However, biological fixation do not account for all N_2 fixation. Lightning accounts for 10% of global supply of fixed nitrogen; the fertilizer industry contributes a significant amount of chemically fixed nitrogen, estimated at 25%, but biological processes account for roughly 60% (Herridge *et al.*, 2008). Natural biological fixation (NBF) by far can play a key role in restoring misused land to productivity. Legumes have a huge potential for contributing fixed nitrogen to soil ecosystems (Cleveland *et al*., 1999; Gruber *et al*., 2008). Soybean nodulation in a given field is said to be induced by different rhizobia strains (Sargent *et al*., 1987; Kouyate *et al*., 2014). This relationship is associated with lectin which are located in root hairs, the site of rhizobial entry for many legumes. One possibility is those lectins acts as glue that attach the rhizobia to a site on root hair providing a source point for attachment (Bhagwat *et al.*, 1982, Hirsch M Ann, 1999). Nevertheless, the nodules are devoid of bacteria presumably if defective lectin occupies root hair tips (Gage, D. J. (2004). As it stands, the lectin may not merely glue but works its wonders transmitting signals that hold the rhizobia and the plant in close contact until plant interact leading to nodules development (Endre *et al*., 2002; Peix *et al*., 2015). This association generates half of the total 175

million tons of BNF (Adiquzel *et al*., 2010). Root nodulating rhizobia bacteria invade legume roots, producing phytohormones and siderophores (Kaushal, M 2017). Soybean is a peculiar plant in that, unlike other legumes, it does not nodulate when first planted in the field, especially in African soils (Thuita *et al.,* 2012). There are approximately 700 genera and 1,300 species of legumes a portion of only 20% shown to have ability to fix N_2 (Rashid *et al.,* 2015). Legumes have attracted a lot of attention as a result of their well-known ability to fix nitrogen. Legumes nodules and their symbiotic partner, rhizobium, can withstand low photosynthetic water stress, salinity, soil nitrate, temperature, heavy metals, and biocides (Carrasco *et al*., 2005).

2.3 Biological Nitrogen Fixation

Under normal field circumstances, soybean fixes around 200 kg of nitrogen per hectare per year (Chemining'wa *et al*., 2011). The progression of nitrogen fixation is the result of intensive communication between plant and bacterium. This communication is often very specific since one bacterial strain can only nodulate one plant species with exception of a few which can nodulate more than one species (Williems 2006; Ampomah *et al*., 2008; Thuita *et al*., 2012). Nitrogen fixation period is said to be optimal ranging between 28 days and 35 days after infection (Zahran, 2001). Synthesis of majority of enzymes, proteins, chlorophyll and nucleic acids requires nitrogen as the prime element (Ahmad *et al*., 2012). The bacteroids create the enzyme nitrogenase that converts N_2 (nitrogen) to NH_3 (ammonia), which subsequently binds to a plant-supplied molecule to form amino acids (Andrew *et al*.,2009; Hirsch M Ann, 1999). Overall equation can be summarized as follows:

$$N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16ADP + 16Pi$$

The amino acids eventually leave the nodule and go to other areas of the plant, where they undergo more systematic modifications to become proteins. To support their nitrogen-fixing activity, the bacteroids require a lot of energy, which is provided by plant sugars created by the photosynthetic process.

2.4 Factors Affecting Rhizobia Symbiosis

Biological nitrogen fixation which is led by root nodulating bacteria is affected by several factors ranging from biotic to abiotic (Gebremedhin, W. (2018). These factors may influence rhizobial diversity in the soil or at the same time affecting growth of the host plant (Niste *et al.,* 2013).

2.4.1 Temperature

When dealing with subtropical plants, temperature limits the ability of legume root nodulating bacteria to grow (Lu *et al.,* 2017). In the temperate region, soybean production requires suitable environmental conditions to allow proper and adequate root nodulating rhizobia association. Temperature influences legume root nodulating symbiosis (Giller, 2001; Marsh *et al*., 2006; Bansal *et al*., 2014). Temperature also affects the host plant's growth, either directly by suppressing the microsymbiont growth or indirectly by regulating the microsymbiont growth (Hashem *et al*., 1998; Hungria and Vargas, 2000). Extreme temperature may lead to reduced rhizobial survival and consequently disrupting molecular signal change between legume host and plant and rhizobia. Required temperature for BNF in tropical legumes as stated by Zahran (2001), ranges between 27^0 C and 40^0 C whereas the growth of rhizobia requires temperature between 32^0 C and 47^0

C depending on the specie and strains. However, despite the effects of high or low temperature on soybean, variability exists among the root nodulating rhizobia trains and genotypic exhibition of the crop especially at varying temperature conditions (Marsh *et al*., 2006). *Glycine max*, on the other hand, is said to be a drought-resistant crop that can thrive in a wider range of environmental circumstances than any other legume, yet it does not withstand overly wet conditions (Du *et al.,* 2009; FAO 2010).

2.4.2 Strain of the root nodulating Bacteria

A wide diversity of legumes are able to be infected and nodulate symbiotically by *Bradyrhizobium* and rhizobia (Mason-Boivin *et al.,* 2009). *Rhizobium* species include *Mesorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium,* and *Sinorhizobium* (Hayat *et al.,* 2010). It is documented that a small number of host genotypes can be infected and nodulated (Denarie *et al.,* 1992). Only highly effective nitrogen-fixing rhizobial strains should be used in inoculants acquired from reputable sources. For effective nodulation to occur, there should be a close relationship between the legume and *rhizobium* strain (Nana *et al.,* 2015). Free-living rhizobacteria, which live outside plant cells, and symbiotic bacteria, which live inside plant cells and are sometimes referred to as endophytic bacteria (Kaur *et al.,* 2016). Rhizobacteria that promote plant growth are free-living bacteria that aid plant growth (Ashrafuzzaman et al., 2009; Hayat *et al.,* 2010). Plant growth promoting rhizobacteria (PGPR) are also used as phytostimulants in the production of hormones such as cytokine, gibberellins and auxins (Ouf *et al.,* 2023). Plant can also be protected from infection by phytopathogenic organisms by PGPRs. Plant growth-promoting rhizobacteria are required to increase agricultural output by enhancing soil fertility, boosting plant nutrient uptake, and

lowering the environmental impact of chemical fertilizers in the soil (Yadegari *et al.*,2008).

Both free-living and symbiotic bacteria are vital in food metabolism, disease biocontrol within a niche, and plant matter breakdown (Otsyula *et al.,* 2004; Lugtenberg and Faina 2009). Bacteria community in the rhizosphere is important in the growth of soybean in terms of nitrogen fixation and at the same time bacterial community can be hindered by too much acidity or aluminum content in the soil. Different soil types with different soil parameters such as moisture content, nutrient limitation and pH harbors different bacterial communities totally independent of plant species (Zahran, 2001; Katerji *et al.,* 2003). Both rhizosphere and endophytic bacteria are affected by various parameters. For example, according to Sessitsch *et al* (2002), newly imported *bacterium* strains in a different region frequently fail to compete with the well-adapted indigenous population. An efficient inoculum strain must be available for competition and survival against indigeneous soil bacteria. Commercialization of these bacteria also faces challenges such as poor bacterial establishment on seed and roots, inconsistency of field test results and non-persistence of seed before its planted (Backer *et al.,* 2018).

2.4.3 Acidity and Salinity of the Soil

Research done in the past has shown that soils having high salt content and high pH (greater than 6.8) content are not good for legume production (Phang *et al.,* 2008). Salinity of the soil is associated with excess sodium chloride at approximately 40mM and condition reduces crop yield by hindering plant growth, senescence and eventually plant death (Wekesa *et al.,* 2022; Shahzad *et al.,* 2017). High soil alkalinity or high soil salinity will lower the rate of nodulation (Phang *et al.,* 2008). Due to the presence of

magnesium, sulphate, and chlorides, soil pH of about 8 has significant alkalinity, but low pH is generally associated with increased Al and Mn toxicity and reduced Ca availability (Giller, 2001). Low pH affects the survival of active rhizobium strains since majority of many stains grow well in moderately (6.8) acidic conditions (Kannaiyan, 2002).

2.4.4 Nitrogen and Phosphorus

Uptake of nitrogen and phosphorus by plants greatly depend on microbial activities (Tairo, E.V and Ndakidemi, P.A, 2014). P-supplementation can enhance plant growth by increasing the efficient of BNF (Mitran *et al.*, 2018). Addition of excessive ammonium, nitrates and phosphates in the soils have high impact on symbiotic relationship between rhizobia and legume (Kawaka *et al.*, 2014). Microorganisms influence P availability to plant through the process of mineralization and immobilization (Jadhav, R.N. 2013). According to Gage (2004), nitrogen fertilizer will stymie or postpone the symbiotic process by inhibiting root hair infection, nodule start, and growth progression. On the other hand, P deficiency will slow down N fixation because phosphorus is vital in the process of nodulation (Mitran *et al.*, 2018). When phosphorus is adequately supplied so does the increase in nodulation process and increases symbiotic dinitrogen fixation through stimulation of host plant growth (Tairo *et al.*, 2013; Tairo, E.V and Ndakidemi, P.A, 2014).

2.5 Quantification of Nitrogen Fixation Amount

In order to determine its efficacy and symbiotic potential, the amount of nitrogen fixed by rhizobia must be quantified. Different studies have shown different methods in quantification of amount of nitrogen fixed by different leguminous crops. The nitrogen-15 isotope dilution method, for example, was used to determine how much nitrogen was

fixed by lentils inoculated with *Rhizobium leguminosarum* (Hafeez *et al.*, 2000). The method produces precise estimates of nitrogen fixed percentage while at the same time presents other possible nitrogen sources from the soil and fertilizer (Rennie, 1986). Another quantification method is described by Boddey *et al* (2000). This approach is based on soil nitrogen variability, which takes advantage of naturally occurring changes in nitrogen composition between plant nitrogen and atmospheric nitrogen.

2.6 Molecular Methods of Studying Rhizobial Diversity

Molecular diversity of rhizobia is the genotypic characterization resulting to variations within species. Molecular methods reveal differences in genotypes (Nievas *et al.*, 2012). Information obtained from studying diversity of *rhizobia* helps in decision making for the purpose of conservation activities (Kennedy, A.C. 1999). Studying diversity helps the researcher in knowing the rates of genetic divergence among the organisms (Mondini *et al.*, 2009).

2.6.1 Diversity of Rhizobia Based on 16S rRNA Gene Sequencing

The diversity of the 16S rRNA gene plays a critical role in determining rhizobia's phylogenetic relationships and species identification (Menna *et al.,* 2009). 16S rRNA gene sequencing is a macromolecular method of evaluating phylogenetic relationships. Gene sequence has been majorly used to study bacterial phylogeny for several reasons which include first the occurrence of 16S rRNA in bacteria exists as either an operon or multigene family. Second, there is no indication that the function of the 16S rRNA gene has changed with time. In addition, for the purpose of informatics, 1,500 bp of 16S rRNA gene can be used (Janda and Abbott. 2007). Finally, study of 16S rRNA offers good

distant relationships, has high information content and has high conservative nature (Lane *et al.* 1985; Martiny *et al.,* 2013). The sequence of the 23S or large subunit ribosomal RNA gene has been studied in addition to 16S rRNA. The 23S rRNA gene, on the other hand, has not been widely used to estimate genetic relationships among the *rhizobiaceae*, but there are a few notable differences that could be useful for classification and identification (Laguerre *et al.,* 2001).

Despite genetic differences within the gene, the identities of 16S rRNA sequences are described to be largely conserved among bacteria, allowing strains in a given species to be entirely differentiated (Hennecke *et al.,* 1985). Wasike *et al* (2009), found that all indigenous strains of *bradyrhizobium* infecting promiscuous soybean in Kenya belonged to the genus *bradyrhizobium* using 16S rRNA gene sequencing.

2.6.2 Whole Genome Sequencing

This is a molecular approach in which the full chromosomal DNA is sequenced at the same time. Unlike other sequences, whole genome sequencing majors on chromosomal DNA, mitochondrial DNA and chloroplast DNA in higher organisms. This method is cost effective and convenient in identifying and addressing microbiological questions (Didelot *et al.,* 2012).

2.6.3 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a novel molecular fingerprinting technique applied to nucleic acids especially DNA. The method works by digesting the total genomic DNA by two restriction enzymes (Blears *et al.,*1998). In addition, application of this method has been widely used to

assess diversity, phylogeny, various ecological studies and development of high-resolution genetic maps (Maughan *et al.,* 1996; Savelkoul *et al.,* 1999).

2.6.4 Restriction Fragment Length Polymorphism (RFLP)

This method works by visualizing DNA fragments that have been cut by restriction endonuclease on electrophoretic gel (Aoki *et al.,* 2010). Sequence polymorphism (Variations of DNA fragments) are affected by target restriction enzymes. Mutations can be detected by RFLP to reveal the rearrangements in the genome. The approach has been used to find markers needed for strain identification, determining the level of genetic diversity, and estimating relationships between strains (Demezas *et al.,* 1991).

2.7 *Nif* and *Nod* Genes

Nitrogen fixation genes and nitrogen nodulation genes in rhizobia are tightly linked and more often located at transmissible elements commonly called plasmids (Laguere *et al.* 2001). Symbiosis genes can spread at a given frequency by conjugation scattered among many chromosomes and plasmid. Unlike *nif* genes, *nod* genes cannot be expressed in the free-living cells. Plant inducers are required for transcription, and phenolic chemicals secreted by the host plant activate them in this situation (Arora, 2013). Nod genes determine synthesis of nod factors which is a nodulation signal molecule (Laguere *et al.* 2001). The host plant can harbor different rhizobia having dissimilar nod genes which produce different nod factors. According to research, phylogenetic trees based on nod genes cannot be compared to those based on 16S rDNA sequences. Nonetheless, the range of nod trees will be related to the variety of host plants. Nod factor is vital in determining host specificity (Giraud *et al.*,2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

Research was done in the green house and in the laboratory located at an altitude of 1585 meters within Masinde Muliro University of Science and Technology (MMUST) in Kakamega county, western Kenya.

3.2 Soil Sampling Sites.

There were six sampling areas within Kakamega County where soil samples from the fields planted with soybean was collected. Soil collection was based on agro-ecological conditions and prevalence of soybean cultivation. These sites were chosen basing on low, medium and upper midland ago-ecological zones. Areas were mapped and the altitude and coordinates were acquired using a GPS equipment as shown in Fig 1; Kakamega south ($N00.2839^0$, $E034.72255^0$), Butere ($N00.16896^0$, $E034.49766^0$), Kakamega Central (N00.28751, $E034.76546^0$), ($N00.28752^0$, E034.76547, N00.29178, E034.73947). Kakamega East (N00.29178, E034.73947, N00.21563, E034.76547, N00.21365, E034.77162). Kakamega North (N00.424070, E034.887390, N00.429220, E034.900790, N00.426200, E034.91863) and Mumias (N00.2636, E034.44457, N00.26426, E034.44666, N00.26231, E034.44499).

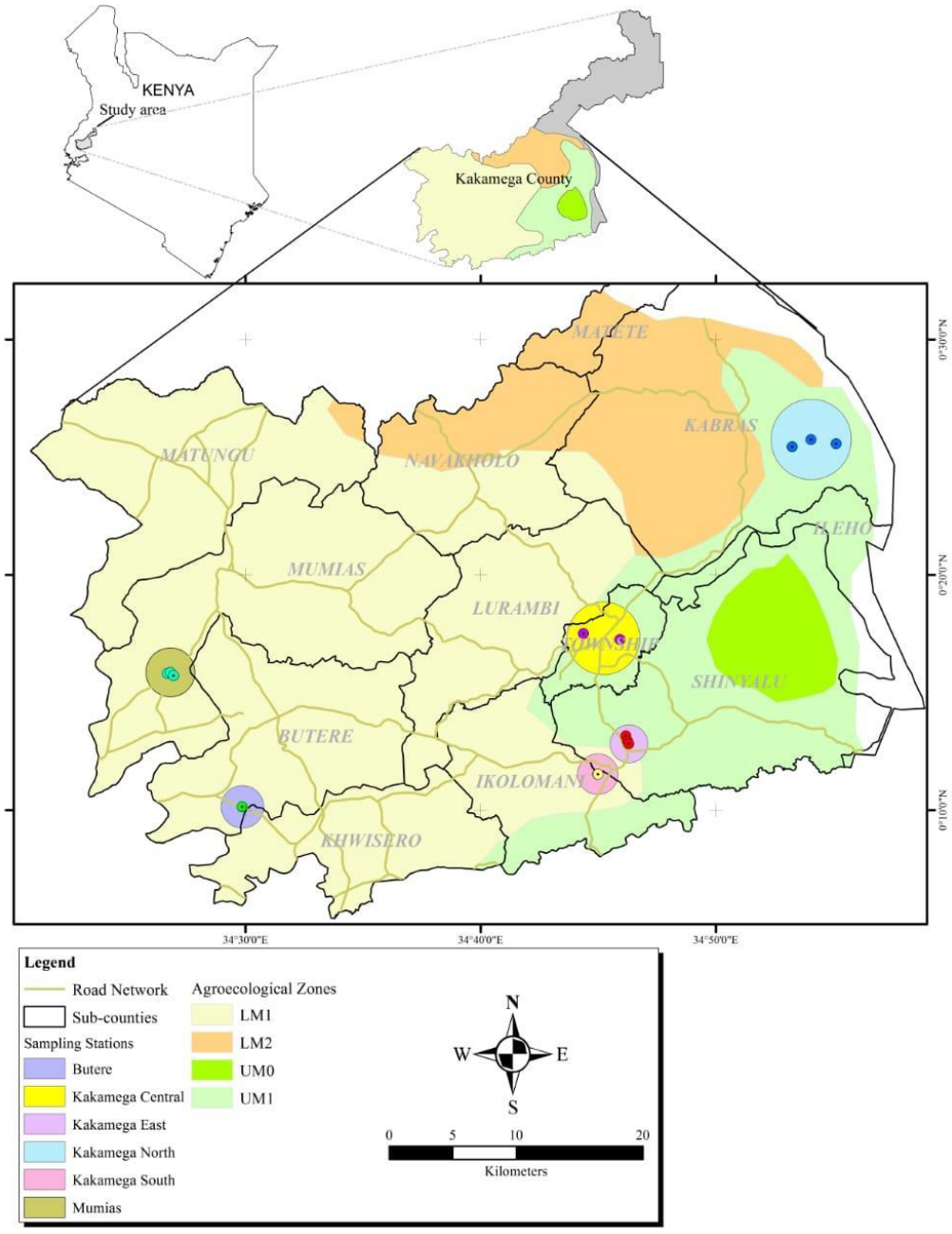

Figure 1: A map of Kakamega county showing AEZs where soil samling was done. LM1-Lower Midland, UM-Upper midlnd.

3.3 Experimental Design

The treatments comprised of soybean "trap" host plants and one inoculation source which had 6 dilution levels (from 10^{-1} to 10^{-6}). The treatments had two positive and two negative controls. Negative controls were soybeans grown in vermiculite without being inoculated, while positive controls were the two treatments using commercial strain. Experimental layout was in the form of CRBD with a factorial arrangement and replicated three times. A total of 112 growth pouches spaced 20 cm apart were arranged in each of the three blocks. The blocks were spaced 1m apart.

3.4 Determination of Native Rhizobia in the Soil

3.4.1 Soil Sampling

A sterile shovel was used to clean the surface material and a total of 20 soil cores was extracted from each farm at a depth of 20 cm using a sterile shovel and fully mixed into a composite sample. To achieve a homogeneous sample, 15 grams of soil were obtained at 10-meter intervals within the field of interest, in a Z pattern. The collected soil was homogenized into a composite soil, which was put into khaki bags and sent to the lab in an ice box. The soil was divided into two sections: one for bacterial quantification and the other for chemical and physical examination. The later was air dried in the laboratory before being taken for analysis. The numbers of *rhizobia* present in Kakamega County soils which nodulated *Glycine max* was estimated by the most probable-number (MPN), plant infection technique, based on Sutton, (2010) protocol. Soil samples collected was analyzed for physio-chemical characteristics at the Department of land resource management and agricultural technology, University of Nairobi, College of Agriculture

and Veterinary Sciences. In the greenhouse, one milliliter of each dilution was inoculated onto a three-day-old seedling growing in vermiculite. Dilutions ranged from 10^{-1} to 10^{-6} in three soil replication samples collected at the sites. Diluted soil was used to inoculate the seedlings in the greenhouse jars.

The sample size was made up of a total of 121 Leonard jars, which were placed in brown khaki bags on the greenhouse shelves in a complete randomized block design (CRBD).

3.4.2 Seed Treatment

KALRO-Njoro provided pure gazelle soybean seeds, which were used as a trapping host. The seeds were pre-tested for germination to ensure synchronization, and a 99% germination rate was achieved (Chemining'wa *et al.,* 2012). The seeds were then soaked in 95% ethanol for 5 minutes to remove waxy compounds from the cortex and lower surface tension before being surface sterilized with 3% sodium hypochlorite. After rinsing with distilled water five times, the surface was soaked in 0.2 percent acidified mercuric chloride for five minutes to thoroughly sterilize it. Seeds were then rinsed five times in sterile distilled water, placed in petri dishes with moist cotton wool, wrapped in parafilm, and placed in a germination chamber. After three days, two germinated seeds were transplanted into Leonard jars with pH 6.8 sterilized vermiculite, and they were reduced to leave one plant per jar after seven days.

3.4.3 Rhizobia Trapping Experiment

A sterile, nitrogen-free plant nutrient solution was used to water the seedlings. The solution included macro- and micro-nutrients, iron salts, and vitamins in g/l, with the pH moderated at 6.8 with 1M NaOH or 1M HCL. Stock solutions used consisted 0.1$CaCl_2$, 0.12$MgSO_4.7H_2O$, 0.1KH_2PO_4, 0.15$Na_2HPO_4.2H_2O$, 0.005 Ferric Citrate. 1.0 mL trace elements consisted of 2.86 H_3BO_3, 2.03$MnSO_4.7H_2O$, 0.22 $ZnSO_4.7H_2O$, 0.08$CuSO_4.5H_2O$ and 0.14$NaMoO_2.2H_2O$ in g/l (Wekesa *et al.,* 2017). After six weeks, the plants were removed, analyzed, and assessed based on whether or not nodules were present. The presence of one or more nodules was declared positive, whereas the absence of nodules was deemed negative (Kawaka *et al.,* 2014). Based on this observations, estimation of quantitative bacteria or bacterial enumeration was determined using MPN technique (Woomer *et al.,* 1988; Ciafardini and Marotta, 1989)

3.5 Genetic Diversity of Root Nodulating Rhizobia that Infect Soybean.

3.5.1 Nodule Sampling and Bacterial Isolation

At early poding and late flowering stage, plants in the fields were carefully uprooted and transferred to the laboratory. Nodules from the roots were carefully removed and placed on petridishes. They were surface sterilized in 3% NaOCl and 70% ethanol for 3-5 minutes respectively. The nodules were then rinsed in distilled water, crushed on sterilized petridishes with a blunt pathogen-free forceps, and a loop of crushed suspension was streaked over the media. The culture media for putative rhizobia contained 5g/l peptone from casein, 3g/l yeast extract, 1.5g/l agar, 1 ml calcium chloride 0.7M of 10ml/l (5g/l), and was supplemented with nalidixic acid before being incubated

in the dark at 28^0 C. Restreaking on new media was done after 3 days of observable development to obtain particular colonies. Plating on Lysogeny Broth (LB) and peptone yeast (PY)s was used where the former was used as a negative control. After 2 days, specific colonies from the set of cultures were selected, scooped using a sterile inoculation loop, and placed in a 2ml tube with 1ml sterile water. At 28^0 C, isolates were cultivated on trypton-yeast agar plates.

3.5.2 Isolation of Genomic DNA.

Bacterial cultures were pipetted into 2mL microtubes and spined at 20,000x g for 5 minutes in a centrifuge. Total DNA extraction was done using QIAmp, Qiagen quick-start protocol, DNeasy Plant Mini Kit according to manufacturer's protocol.

3.5.3 Amplification and Sequencing of the 16S rRNA Gene

Amplification and sequencing of a 16S rRNA gene was done according to the method described by Rincon *et al* (2008) as indicated in Table 3.1. The quality of DNA was checked using Nano drop spectrophotometric from pacBio. PCR amplification was carried out in a 50 uL volume mixing the template DNA (5 ng/uL) with polymerase reaction buffer (100mM Tri-HCL,15 mM $MgCl_2$,500mM KCl at pH of 8.3). Twenty-five (25) uM of each dATP, dGTP, dCTP and dTTP. Temperature which was used for DNA amplification was 94^0 C for 5 min at denaturation step followed by 35 cycles of 94^0C for 40 seconds, 72^0 C for 1.5 mins and then 72^0 C for 2 mins which was followed by extension step of 72^0C for 7 mins. This reaction of PCR was done in a thermal cycler express instrument (Table 1).

A negative control containing 1uL of H_2O was included in every PCR run to avoid contamination. DNA molecules obtained were subjected to real time (SMRT) sequel sequencing technology from pacBio (Pacific Biosciences, Menlo Park, CA, USA) where long-read data-sets of rhizobial isolates were concentrated for purity (Eid *et al*., 2009). PCR, sequencing and sequence analysis of the 16S was performed at Macrogen Europe (Netherlands).

Table 1: Amplification of 16S rRNA gene

Primer	**Target Gene**	**Sequence (5'-3')**	**Length**	**PCR Condition**
27F	16S rRNA	AGAGTTTGATCMTGGCTCAG	20	94^0C for 5 mins; 35 cycles, 94^0C for 40 Secs, 72^0C for 1 min and final extension of 72^0C for 7 mins.
1492R	16S rRNA	TACGGYTACCTTGTTACGACTT	22	

3.6 Determination of Symbiotic Potential

3.6.1 Authentication of the Isolates and Symbiotic Potential

Pure isolates (K. East 3, K. Central 3, Mumias East 3, K. South 2, Butere 2, Butere 1, Mumias 2, K. South 1, K. North 3, Butere 3, Mumias 3, K. North 3, K. North 2, Mumias 3 and K. South 3) each 1ml was re-inoculated on five-day soybean plantlets growing in vermiculite and irrigated with nitrogen free nutrient solution in Leonard jars. Watering with nitrogen free media was done on the test plants. Experiment comprised of soybean gazelle variety planted in vermiculite without inoculation which served as negative control and two treatments applied with 1ml of commercial strain on each Leonard jar for

positive controls. After 35 days the plants were assessed for the nodule number and average nodules. To establish their weight, nodules were hand-selected, placed in khaki bags for each plant, and oven dried for 72 hours at 60^0C. Symbiotic potential of representative *rhizobia* isolates was determined based on the method described by Somasegaran and Hoben (1994). Gazelle seeds were sterilized and pre-germinated as described in section 3.4.2 and nitrogen free media prepared as described in section 3.4.3 which was used for the irrigation of the plants in Leonard jars. Pre-germinated seeds were then inoculated with identified rhizobial strains identified from the sequences. Plants were assed in terms of performance under different treatments based on the root nodule, plant shoot weight, pod number and number of seeds.

3.7 Statistical Analysis

Abundance data was subjected to most probable number Enumeration (MPNEs) and results analysed using analysis of variance (ANOVA). All figures from abundance and symbiotic potential were drawn separately by matplotilib version 3.1.3 in python 3.7.6. ANOVA and Turkeys posthoc was performed with stats model version 0.11.0 in python 3.7.6.

3.8 Molecular Phylogenetic analysis of Rhizobia

Molecular phylogenetic analysis was done using the contigs containing the forward and reverse nucleotides. Paired end reads were joined together to reconstruct about 1400 base pair long contigs in Bioedit v7.2.6 (Hall 1999) and nucleotide alignments generated by ClustalW multiple alignment v1.4 (Thompson *et al.*, 1994). Phylogenetic analysis of the 16 rRNA gene was done by editing the sequences on bioedit and chromas lite programs.

Using the Maximum Likelihood approach and the Tamura-Nei model, the evolutionary history was inferred (Tamura *et al.,* 1993). The sequences were checked for quality using nano drop spectrophotometric from pacBio and then sent to BLAST for comparison with other sequences. Phylogenetic trees were constructed using neighboring joining method (Krasova Wade *et al.,* 2003). Evolutionary analyses were conducted in MEGA7 (Kumar *et al.,* 2016).

CHAPTER FOUR

RESULTS

4.1 Soil Characteristics

Chemical analysis of the soil samples revealed significant differences in soil pH, phosphorus, organic carbon, nitrogen, potassium, and aluminum. Soil collected from Mumias was sandy clay with 0.85% organic carbon. The pH of Kakamega East was 5.43, with a slightly high aluminum content of 0.95 cmol/kg, resulting in low nodulation among the six regions. The soil pH at Kakamega South was 6.06, and the organic carbon content was 1.90% (Table 2).

Kakamega south for instance had high amount of calcium (6.75cmol/kg) across the six regions. Results indicated wide range in variations amongst the strains in soil pH. This was observed that extreme pH reduced survival and growth of rhizobia and therefore poor plant growth. Survival of rhizobia in the soil are highly affected by the combined effect of acidity and aluminum. Kakamega central sub county had aluminum content of 1.07 cmol/Kg followed by Kakamega North. Soil textural class showed variations based on the region of collection. Kakamega central, Kakamega North and Butere had sandy loam while the remaining three sub counties had textural class of sandy clay.

Table 2: Soil Properties collected from identified locations and analysed at University of Nairobi.

Region	pH	%N (ppm)	%O C (ppm)	P (ppm)	K cmol/kg	Ca cmol/kg	Mg cmol/kg	Al cmol/kg	Zn (ppm)	Textural Class
K. Central	5.58	0.49	1.39	25.00	0.63	2.83	1.09	1.07	5.43	Sandy loam
K. East	5.53	0.37	1.82	22.50	0.63	2.50	1.20	0.95	5.35	Sandy clay
K. South	6.06	0.37	1.90	37.50	1.70	6.75	2.41	0.70	6.29	Sandy Clay
K.North	5.80	0.43	1.70	25.00	0.77	3.42	1.22	1.03	6.32	Sandy loam
Mumias East	5.30	0.19	0.85	25.50	0.72	3.45	1.30	0.52	5.45	Sandy Clay
Butere	5.27	0.18	1.37	24.50	0.65	3.00	1.34	0.94	5.30	Sandy loam

4.2 Abundance of Root Nodulating Rhizobia Colonizing Soybean

In comparison to Kakamega East and Kakamega South, which had low nodule mass per plant, Mumias East and Butere had a large population of bacteria per gram of soil, resulting in high shoot biomass and thus the best plant performance and production. For instance, at 95% confidence, the number of bacteria (MPN) were 120000 which ranged from 25672 to 560929 while in Kakamega East the number of bacteria were found to be low at 74 which ranged from 16 to 346 per gram of soil (Table 3).

Table 3: Composite soil analysis for abundance from six sub-counties of study (Kakamega South, Kakamega East in Isukha South, Kakamega Central, Kakamega North, Mumias East and Butere)

Region	Confidence factor	MPN	P-Value (P<0.95)
K. South	4.674404	919	197-4292
K. East	4.674404	74	16-346
K. Central	4.674404	2755	589-12876
K. North	4.674404	2091	447-9772
Mumias	4.674404	120000	25672-560929
Butere	4.674404	2755	197-4295

The presence of nodules per plant indicated positive samples, whereas plants with no nodules earned a negative score as indicated in table 4.

Table 4 : Total number of nodule number count indicated by a positive and a negative score per plant.

Sr.No	Region	No. of Samples	Positive (+)	Negative (-)
1	Kakamega South	18	8	10
2	Kakamega East	18	5	13
3	Kakamega Central	18	10	8
4	Kakamega North	18	10	8
5	Mumias East	18	14	4
6	Butere	18	11	7
	Total	**108**	**58**	**50**

Out of a sample size of 108 plants planted in Leonard jars containing vermiculite, 58 were positive while 50 were negative. Mumias east had a total of fourteen positive samples which were the highest with low negative score of four. Kakamega east had the lowest positive score of five and high negative score of thirteen (Table 4). Mumias East, for example, which is in the low medium zone, had the highest population of bacteria per gram of soil, as well as the highest nodule number, shoot dry weight, and population of soybean rhizobia that had 1.2×10^5 cells per gram of soil. Isolates significantly increased symbiotic potential when the host plant interacted with rhizobial isolates. Mumias East had the highest number of isolates (Figure 2).

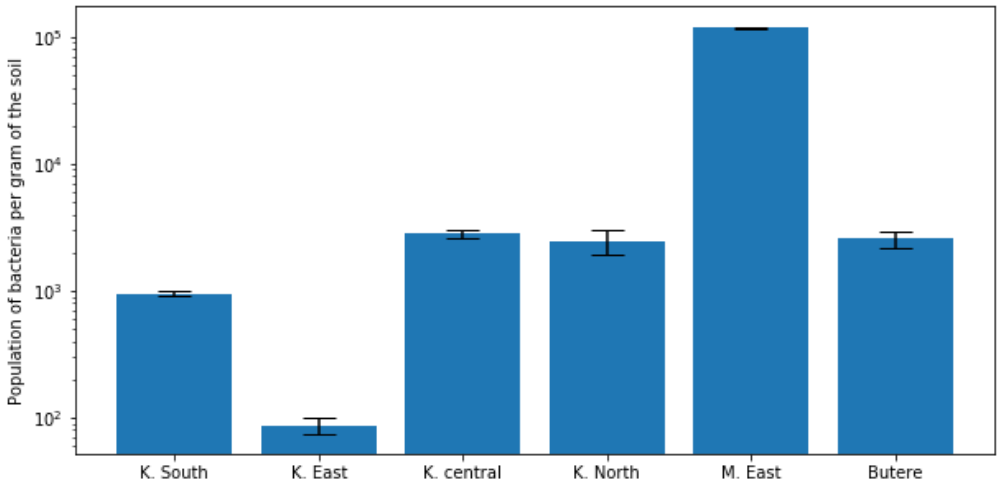

Figure 2: Enumerated Most Probable Numbers of bacteria population acquired from the soil sampled from the six regions.

4.3 Authentication

When inoculated on 4-day soybean seedlings in Leonard jars, all isolates enhanced the number of nodules (Figure 3 A). Un-inoculated plants, on the other hand, showed no nodule formation (Figure 3 B). After two days of incubation at 28^0 C in the dark, verification of bacteria on YEM revealed small colorless colonies. The presence of rhizobia was indicated by colorless colonies.

Figure 3: Sample soybean plants, A1 has nodules while B1 has no nodules

4.4 Molecular Characterization of Root Nodulating Rhizobia

4.4.1 Nanodrop (spectrophotometric) Results

Downstream DNA samples from the pacBio revealed high concentration of DNA quality. Mumias 2 and Butere samples indicated a good A 260/280 ratios that were manifested with no presence of organic contaminants (Table 5).

Table 5: Nanodrop concentration of DNA samples.

Sample	Concentration (µg/µL)	260/280	260/230
B1	480	1.72	1.71
M3	510	1.74	1.87
M2	1213	1.89	1.68
S4	719	1.66	1.78
E1	280	1.83	1.91
N2	490	1.81	1.83
C1	1120	1.87	1.74
S1	379	1.61	1.59

4.4.2 Sequence Analysis and Diversity of 16S rRNA Genes

Figure 4 depicts the tree with the highest log likelihood (-2403.68). Next to the branches is a percentage of trees in which the linked taxa clustered together. The initial tree(s) for the heuristic search were automatically generated by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with the optimal log likelihood value. Branch lengths are measured in the number of substitutions per site, and the tree is depicted to scale. A total of eight *rhizobia* sequences were examined where the 1st+2nd+3rd+Noncoding codon locations were included. All positions with gaps or missing data were removed. There was a total of 652 positions in the final dataset. *Escherichia coli* with accession number JO1859.1 was used in this research as an outgroup. Three *Agrobacterium pusense,* Four *Pseudomonas* species, *Pseudomonas koreensis,* five *rhizobium* species, *Rhizobium chutanense, Agrobacterium pusense* and *Agrobacterium tumefaciens* were used in this research as reference strains.

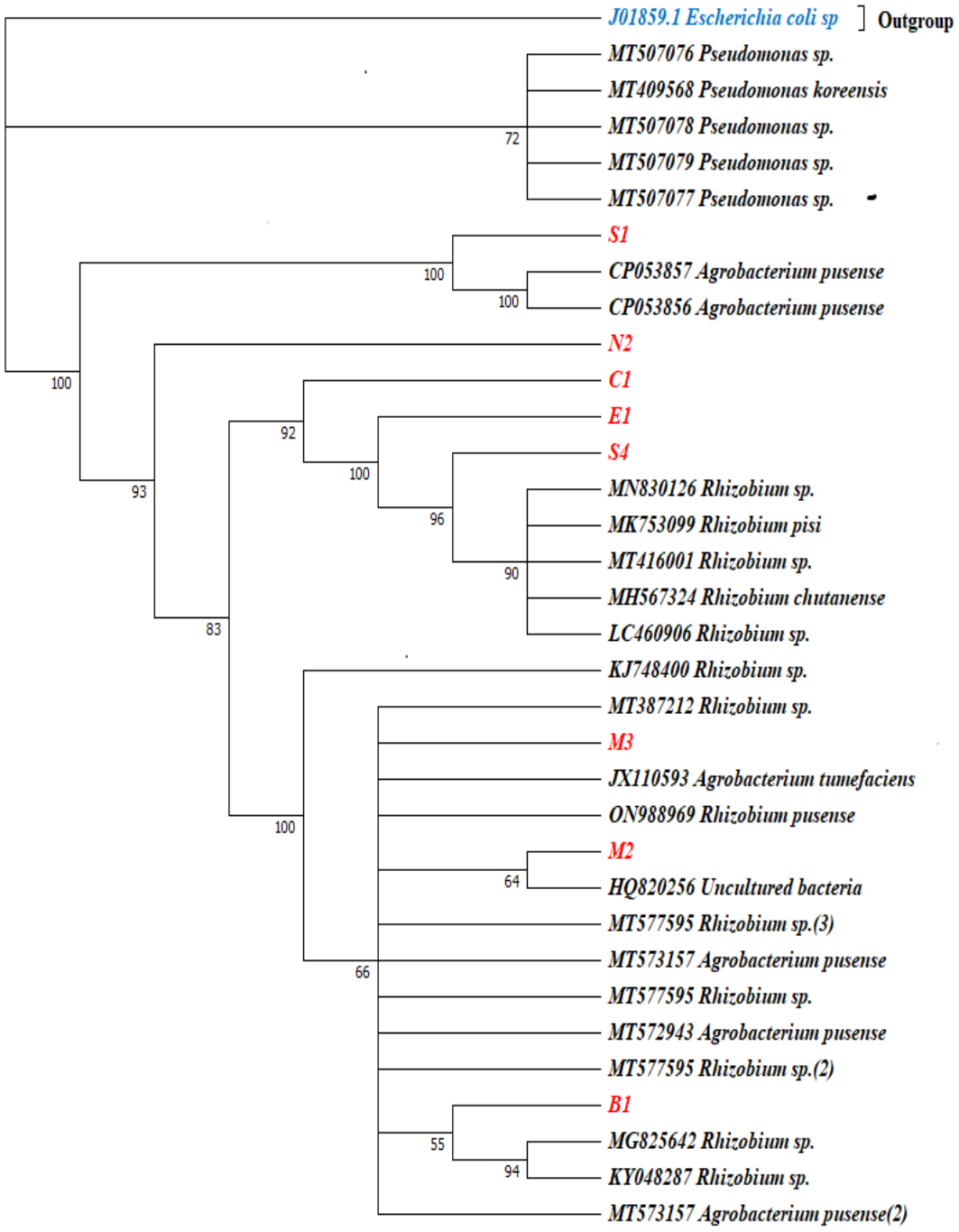

Figure 4: Evolutionary relationships of root nodulating bacteria isolates from Kakamega County. This tree was constructed using the MEGA 7 software with Tamura and Neil (TN93) gama distribution (+G) with invariant sites (+1) model. Number of branches are bootstraps percentage (%) at >50%. The scale bar=5 %(0.05).

4.4.3 Identification of Sequences

Sequences were subjected to BLAST and aligned giving an evolutionary relationship of the taxa during phylogenetic analysis. Eight strains of rhizobia were produced (Table 6). *Rhizobium nepotum, Rhizobium skierniewisence, Rhizobium pisi, Rhizobium fabae, Rhizobium phaseoli, Rhizobium etli, Rhizobium azibense,* and *Pseudomonas glyciae.*

Table 6: Gene data bank identities of root nodulating rhizobia from *Glycine max*

NCBI Identification	**Sample Name**	**Region of collection**
Rhizobium skierniewisence	B1	Mumias and Butere
Rhizobium nepotum	M3	Kakamega Central
Rhizobium phaseoli	M2	Mumias
Rhizobium pisi	S4	Kakamega south and Mumias
Rhizobium fabae	E1	Kakamega East and Butere
Rhizobium azibense	N2	Kakamega North
Rhizobium etli	C1	Kakamega Central
Pseudomonas glyciae	S1	Kakamega Central and Mumias

Mumias had the highest diversity of root nodulating rhizobia which included *Rhizobium pisi, Rhizobium fabae, Rhizobium phaseoli, Rhizobium aegyptiacum, Rhizobium azibense, Rhizobium pusence* and *Rhizobium etli.* Phylogenetic tree identities show that bacteria from the same cluster are genetically related, whereas bacteria from separate clusters are not.

4.4.4 Gene Bank Results during BLAST

Table 4.6: Sequence Identities and their accession numbers

Organism	Accession Number	Percentage Identity (%)	Base Pair
Rhizobium skierniewisence	NR118559.1	97.68	1626
Rhizobium nepotum	NR117203.1	97.68	1626
Rhizobium phaseoli	CP020896.1	99.59	5277
Rhizobium pisi	NR115253.1	99.59	1762
Rhizobium fabae	ON988969.1	99.80	1873
Rhizobium azibense	NR133841.1	98.58	1611
Rhizobium etli	NRO74499.2	99.70	1572
Pseudomonas glyciae	NR179889.1	99.80	1520

4.4 Symbiotic Potential in Nitrogen Fixation of Soybean.

Growth and symbiotic traits under greenhouse condition were noticed. Plants inoculated with *Rhizobium pisi, Rhizobium Fabae, rhizobium phaseoli, rhizobium aegyptiacum* and *rhizobium pusence* manifested various symbiotic traits such as nodule number and nodule dry weight per plant at higher numbers. Above ground biomass of the soybean inoculated with inoculum from Mumias was significantly higher compared to soybean inoculated with Kakamega North and Kakamega south inoculum. This higher growth might be caused by superior symbiotic nitrogen fixing activity in the roots of the soybean.

Data on nodule number, nodule dry weight, shoot dry weight, number of pods per plant, and seed dry weight was analyzed using ANOVA method. An analysis of variance demonstrated a link between rhizobia inoculation and plant growth in terms of symbiotic performance (Table 7). Using the Pearson correlation co-efficient and controlling for the treatment (inoculum), all possible correlations among traits were checked for. Plants that were inoculated with bacterial isolates performed considerably better in terms of yield and biomass.

Table 7: Averages on Number of Nodules (NN), Nodule Dry Weight (NDW), Shoot Dry eight (SDW), Number of Pods (NP), Weight of Pods (WP), Number of Seeds (NS) and Weight of seeds (WS) in grams.

Region	**NN**	**NDW**	**SDW**	**NP**	**WP**	**NS**	**WS**
Kakamega South	22	0.13	23.1	9	8.67	44	7.51
Kakamega East	12	0.06	25.9	27	9.29	62	5.07
Kakamega Central	34	0.91	28.2	20	7.80	53	4.67
Kakamega North	29	0.79	20.0	35	11.70	86	7.55
Mumias East	74	2.61	21.5	33	10.66	55	7.82
Butere	61	2.94	28.7	55	14.43	94	11.38

Analysis of variance revealed that the effect of rhizobium inoculation treatments, abundance and their interaction on nodule number, nodule dry weight and shoot dry weight were significant. In Kakamega County, interactions between rhizobia isolates were observed to distinguish between various environments depending on ecological zones.

Interaction between the host plant and the rhizobial isolates showed variations across the six sub-counties. Positive control having commercial strain of rhizobia had the highest shoot dry weight. Inoculum from Butere and Kakamega Central were the best when analysis was done on shoot dry weight. Soybean inoculated with commercial innoculant had the highest shoot dry weight as indicated in Figure 5. Higher shoot dry weight was seen in the plants treated with rhizobia than in the negative controls. compared to other isolates that indicated significant difference from the isolates from Kakamega central. These finding suggest that nodule number plays a significant role in determining the weight that a shoot weighs. (Figure 5).

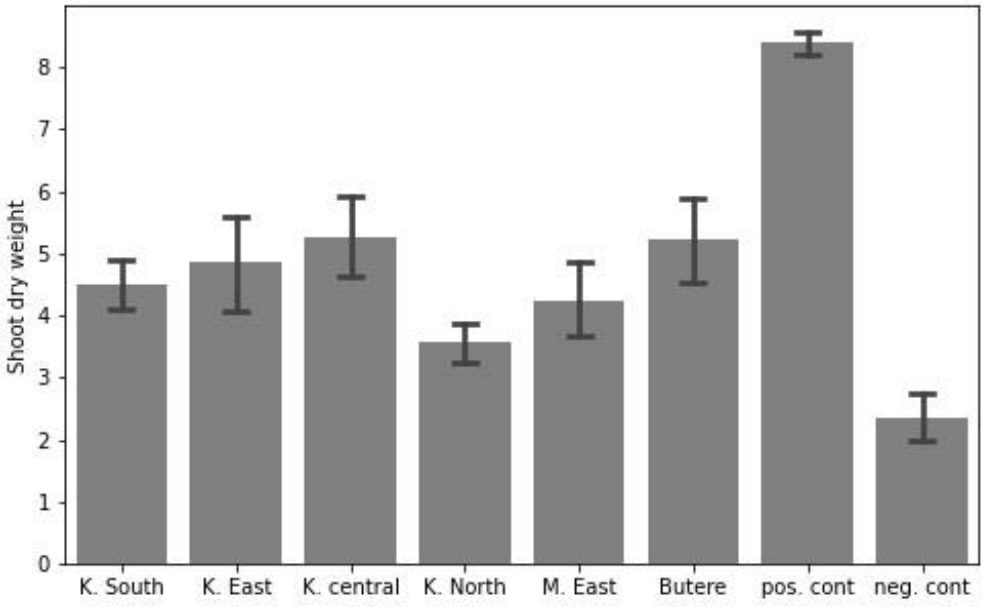

Figure 5: Interaction between the host plant and the rhizobia isolates as observed on shoot dry weight.

Soils from Butere had significantly ($P \leq 0.05$) higher number of pods per plant than all other sites as indicated in Figure 6. When isolates from Butere was inoculated on gazelle plants, the number of pods were higher at maturity followed by plants that were inoculated with inoculum from Mumias East. Kakamega South sub-county inoculum produced the least number of pods per plant. Means of shoot dry weight when inoculated with bacteria isolates was significantly higher compared with uninoculated ($p<0.05$) as indicated in Figure 6.

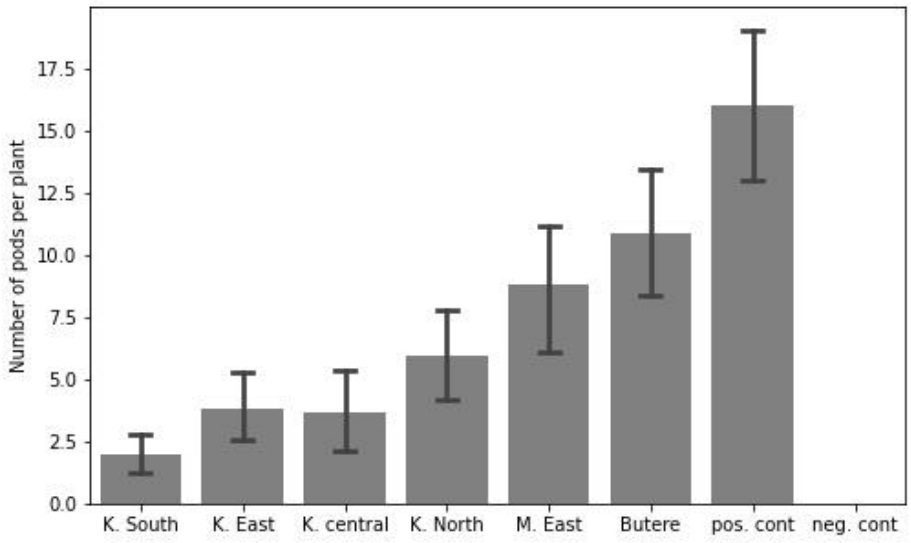

Figure 6: Impact of rhizobia on number of pods produced by the host plant.

Further, there was high number of pods per plant (Figure 7) in all inoculated plants which correlated with the weight of the pods of plants and seed number during the interaction between the host plant and the rhizobial isolates. Plants inoculated with commercial stain as positive controls produced higher number of pod weight and weight of the seeds per plant (Figure 8 and Figure 9).

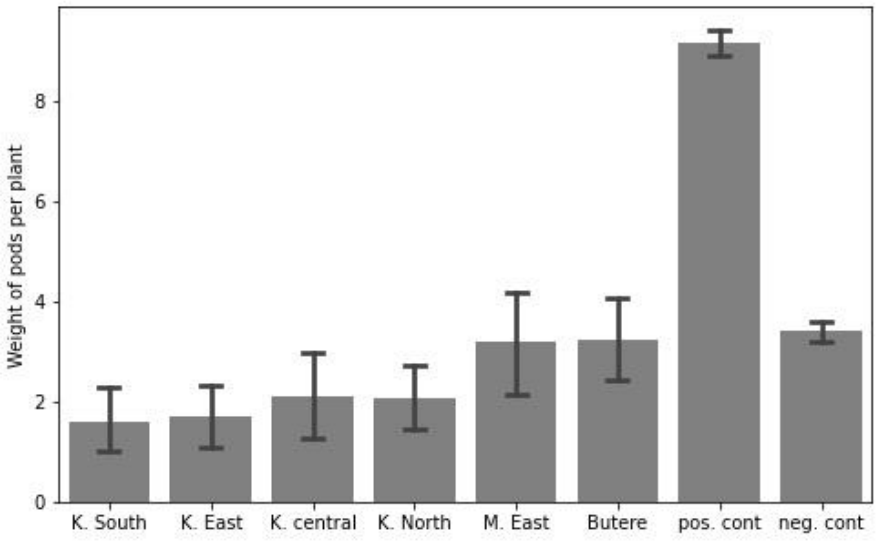

Figure 7: Effect of inoculation with bacteria on weight of the pods.

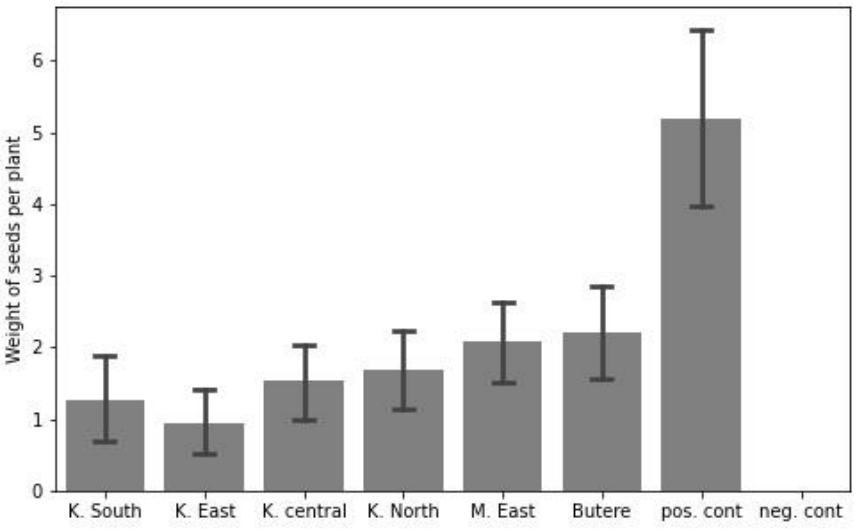

Figure 8: Effect of inoculation with bacteria on weight of seeds of soybean.

The treatments differed significantly from each other ($P \leq 0.05$) in both sites. Application of bacterial isolates increased grain yields in both sites. However, commercial strain produced the highest grain yield.

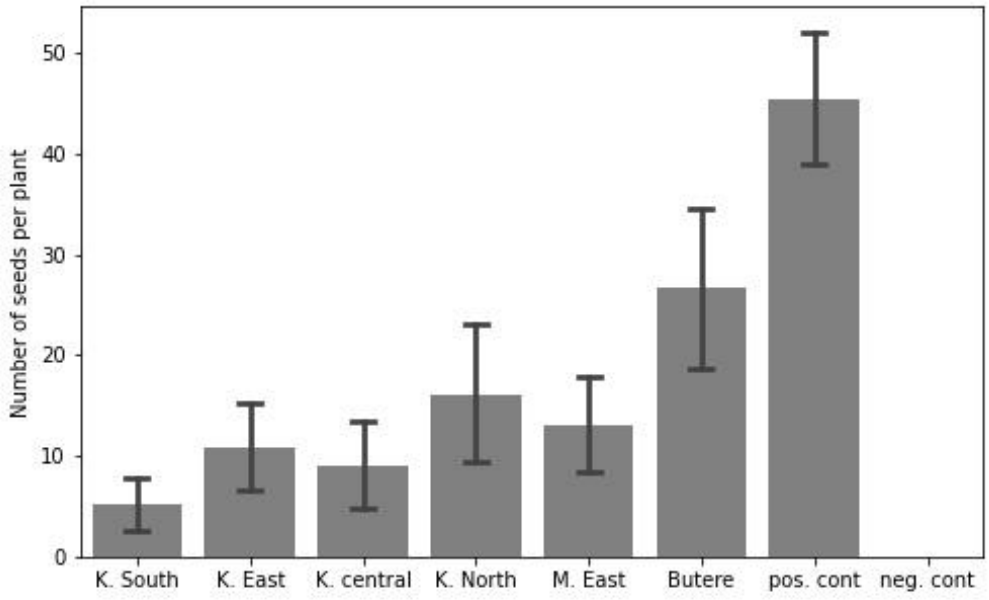

Figure 9: Effect of inoculation with bacteria on quantity of seeds of soybean.

CHAPTER FIVE

DISCUSSION

5.1 Discussion

The goal of this study was to check the availability (abundance) of rhizobia in soils of Kakamega County and then to characterize these symbiotic root nodulating rhizobia that colonize soybean crop appropriately using a molecular tool. There was a strong correlation between the trials conducted during this study in the greenhouse conditions, particularly with regard to the abundance, molecular characterization, and potential for symbiosis between the host plant and root-nodulating rhizobia. This suggests that one should evaluate results by considering all attributes collectively and giving close attention to the relationships between each of these characteristics in each experiment. A large portion of microbial diversity among studies may be attributed to the diverse agroecological conditions used in each experiment. Studies that have been published on nitrogen fixed by plants only take into account the amount of nitrogen in the shoot tissue, not the root tissue. Indigenous population of rhizobia nodulating *Glycine max* ranged from 7.4×10^1 to 1.2×10^5 per gram of soil across the regions where the soils were sampled. This clearly indicates that soybean rhizobia are sometimes specific for soybean. Research done by Ansari *et al.,* (2014) on cross nodulation suggested that soybean rhizobia did not nodulate mungbean but nodulated cowpea. In the presence of rhizobial population as lower as 7.4×10^1 cells per gram of soil led to low significant occupancy within the nodules. Mumias had high population which might be attributed to the legume wide spread in the cropping system and edaphic associated factors while low

microbial population was witnessed in soils of Kakamega south. Fluctuations in both biotic and abiotic factors greatly influences metabolic activity of N-fixation bacteria (Ahammed, G.J.,and Li, X, 2023). Chemining'wa *et al.,* (2012) in their research on effectiveness of indigenous pea rhizobia concluded that soils with history of legume tend to have high active and total nodule number, results that supports this research findings. Some soybean had very low nodule production even though inoculation was done especially isolates from Kakamega south formed very low nodules on the trap plant. Averagely, the number of nodules ranged from 1-18 per plant. Subsequently, bacterial isolates from the region when inoculated on the host plant produced higher yields and high plant biomass. For instance, interaction of *Rhizobium fabae* with the host plant had significantly high results. However, inoculation did not have any significant effect on shoot biomass but effect was noticed on nodule number and nodule dry weight. In this study, inoculated plants resulted to nodulation and high shoot dry matter which indicates that nitrogen was fixed when compared with uninoculated control plants.

The study showed that different agro-ecological zones have different soil nutrients coupled with biotic and abiotic factors which may influence availability of root nodulating bacteria and nodulation process and hence positively or negatively affecting nitrogen fixation and symbiotic performance (Thuita *et al.,* 2012; Tobita *et al.,* 2013; Lindstrom *et al.,* 2020). Albareda *et al.,* (2006) found out that variability of rhizobia population is attributed to the plant rhizosphere which could include colonization resulting to symbiotic and endophytic relationship. Different factors such as high level of soil soil pH of about 8 is usually linked with high aluminum levels (>2.0 cmol/Kg) thus affecting rhizobial survival. When the soil chemical analysis of the study was done,

the pH ranged from 5.43 to 6.52, a condition that is favorable for rhizobial survival. Soil pH did not vary greatly across the sampling sites regardless of agro-ecological zones. The number of bacteria isolates obtained per soybean varied due to different level of nodulation at the field sites and success of bacterial isolation.

High shoot dry matter may have been influenced by availability of phosphorus (Jemo *et al.,* 2006). Plant shoot weight, pod number, nodule number and number of seeds per pod differed when bacterial strains were inoculated as compared with uninoculated plants under greenhouse condition (Abbasi *et al.,* 2010).

Root nodulating rhizobia colonizing *Glycine max* varied from one strain to the other in their specificity. Various studies have also revealed differences in specificity and rhizobia inoculation from one genus to the next. During serial dilution of the inoculum that was carried out on vermiculite, the most concentrated inoculum led to formation of nodules hence the presence of rhizobia while the least concentrated inoculum produced no nodules. Inappropriate inoculation in terms of perfect match will result to ineffective nodule or no nodule formation hence, limiting nitrogen fixation (Simms, E.L., and Taylor, D.L. 2002; Musiyiwa *et al.,* 2005). Mumias had high population of nitrogen fixing bacteria manifested in different diversity as indicated by phylogenetic analysis.

Although soybean is well known to be inoculated by *Bradyrhizobium japonicum* strains (Ansari *et al.,* 2014), all of the isolates from soybean nodules in this study were in the genus *Rhizobium.* Ansari *et al* (2014), researching on diversity of soybean rhizobia in central India reported that soybean nodules are largely dominated by slow growing *Bradyrhizobia*. In contrast to what had previously been discovered, Tian *et al* (2008),

reported that soybean nodules are dominated by *Sinorhizobium* and *Bradyrhizobium* depending on the soil conditions in spite of no gene specifically shared by the two strains for the purpose of symbiosis. 16S rRNA was considered because the sequences are described to be highly conserved and present in large and diverse nitrogen fixing bacteria. 16SrRNA gene sequences is useful when assigning specie to a genera but it is limited in indicating relationships between close species. Previous research on taxonomy and diversity of rhizobia have been done alongside with 16S rRNA gene due to its limitations. (Ansari *et al.,* 2014; Martens *et al.,* 2008). 16S rRNA sequencing indicated that a diverse range of rhizobia could form effective nodule on soybean in soils of Kakamega. This was a reason why 16S rRNA was selected for the purpose of rhizobial identification for the study as compared with other methods of gene sequencing (El-Akhal *et al.,* 2008).

5.2 Conclusion

1. From the study, 53.7% were positive (+ve) samples indicating occurrence of nodules as compared to negative samples that comprised of 46.3%. Soil from Mumias responded well to inoculation while soils from Kakamega east did poorly with certain isolates.

2. Genetic diversity of rhizobia nodulating soybean was indicated across all regions of study, Soils from Mumias East region having higher diversity.

3. Occurrence of varying population levels of rhizobia has been demonstrated in soils from the region which were also influenced by micro and macro-nutrients. According to this research, Butere and Mumias had a large population of bacteria

per gram of soil, resulting in high shoot biomass, high yields, and a wide range of rhizobia.

5.3 Recommendations

1. This study recommends the need to compare rhizobial communities in soil, with soybean nodulating rhizobial communities and the potential of rhizobia of wild relatives to further clarify ecological interactions among host plant, rhizobia and environment factors.

2. It is recommended that effective strains from Butere-Mumias should be evaluated and verified for their potential genetic diversity and stability in N-fixation. This research excluded determination of nitrogen content using Djedal method which I strongly recommend the method to be used in the future study.

3. For inoculation to be effective, the soils of Kakamega East and Kakamega Central requires pH adjustments and addition of phosphorus. High level of aluminum seems to hinder survival of rhizobia within the soil.

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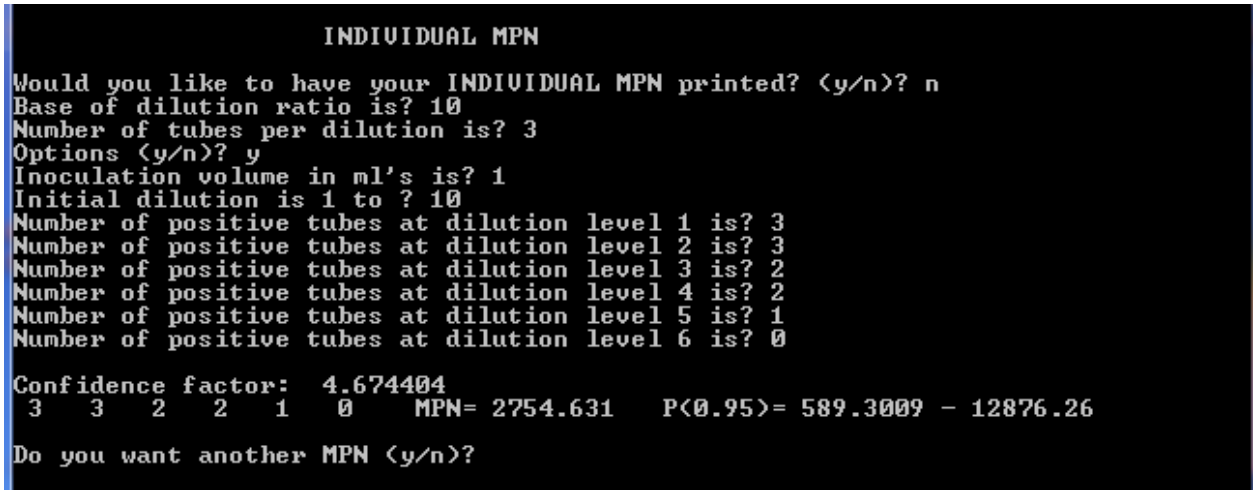
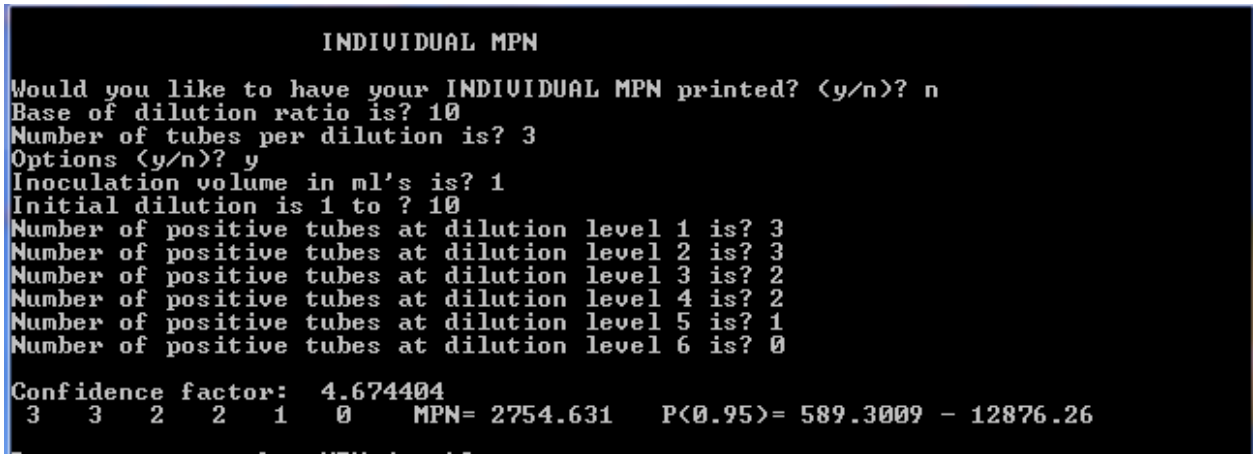
APPENDICES

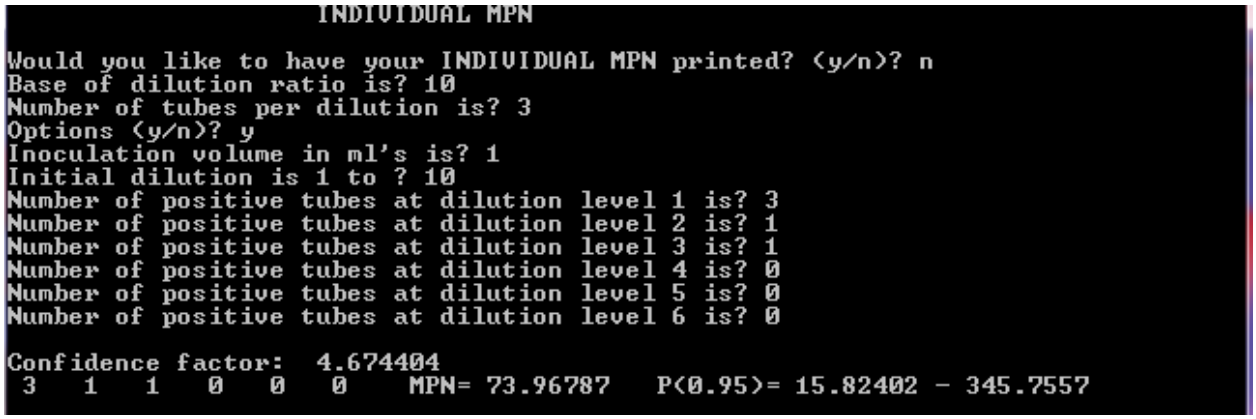
Appendix 1: MPN enumeration of root nodulating bacteria from soils of Butere.



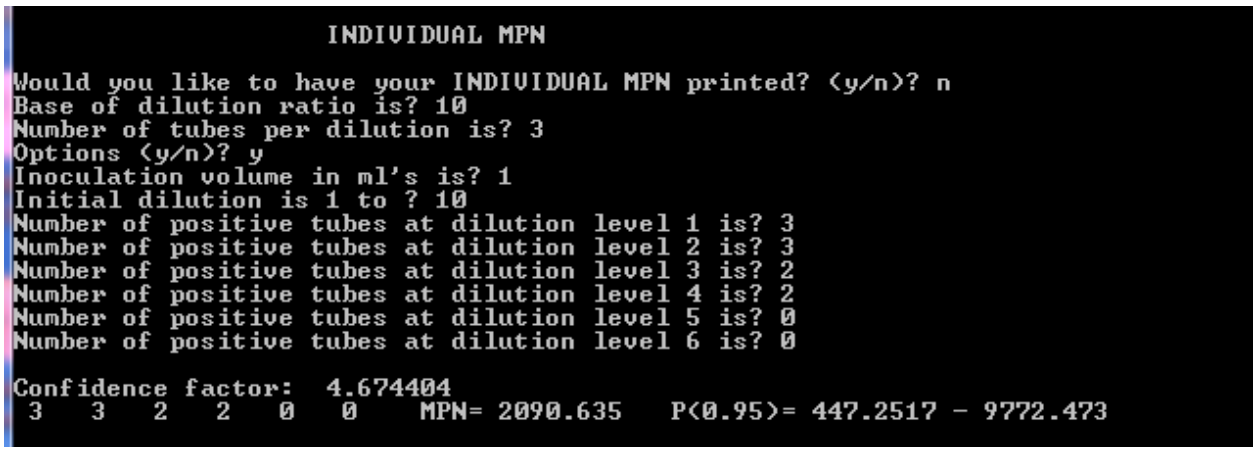
Appendix 2: MPN enumeration of root nodulating bacteria from soils of Kakamega Central



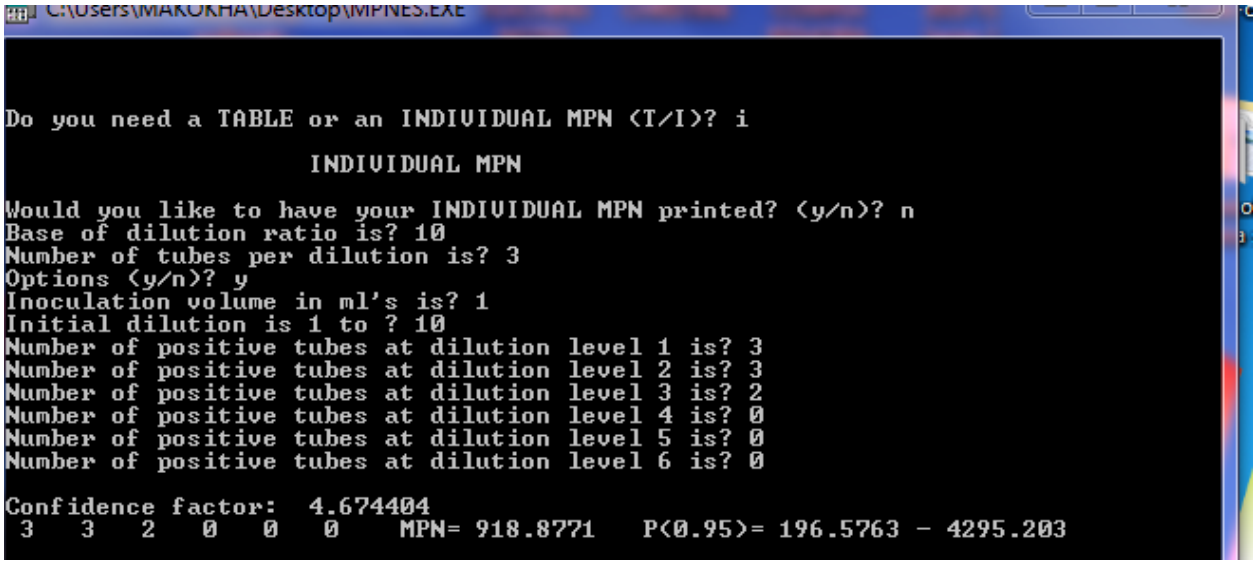
Appendix 3: MPN enumeration of root nodulating rhizobia from soils of Kakamega East



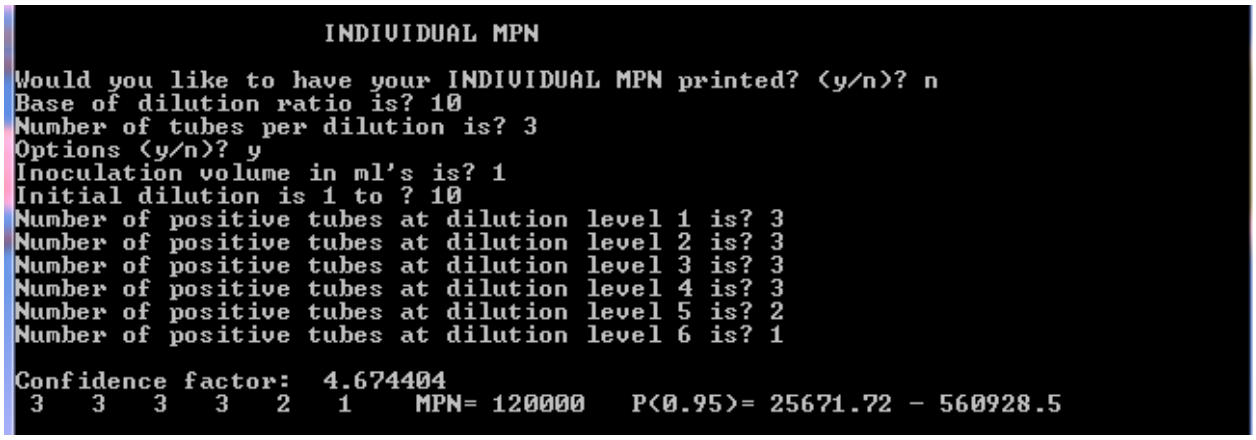
Appendix 4: MPN enumeration of root nodulating rhizobia from soils of Kakamega North



Appendix 5: MPN enumeration of bacteria from soil of Kakamega South



Appendix 6: MPN enumeration of bacteria from soil of Mumias



Appendix 7: Consensus sequences from isolates

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Appendix 8: Pregermination of soybean seeds

Appendix 9: Sequence Alignment