PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF COWPEA RHIZOBIA IN WESTERN KENYA, EVALUATION OF THE SYMBIOTIC EFFICIENCIES OF STRAINS TOLERANT TO ABIOTIC STRESSES AND IDENTIFICATION OF THE SYMBIOTICALLY EFFICIENT STRAINS

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Phenotypic and Genotypic Characterization of Cowpea Rhizobia in Western Kenya, Evaluation of the Symbiotic Efficiencies of Strains Tolerant to Abiotic Stresses and Identification of Symbiotically Efficient Strains

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DECLARATION

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

Signature ……. Date …….

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DEDICATION

To my husband Wandera Ojanji for understanding and allowing me to be away from home at odd hours, my children Jesse, Juliana, Raymonds and Maria for all the times I was absent from home. I also dedicate this thesis to my parents Thomas Oguto and Philister Agola and siblings Wilkister, Winnie, Stanley and Humphrey.

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ABBREVIATIONS AND ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
BLAST	Basic Local Alignment Search Tool
BNF	Biological Nitrogen Fixation
BTB	Bromothymol Blue
CIAT	International Centre for Tropical Agriculture
CR	Congo Red
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
FAO	Food and Agricultural Organization of the United Nations
MEGA	Molecular Evolutionary Genetics Analysis
MIRCEN	Microbiological Resource Centre
N2	Nitrogen
NaCl	Sodium chloride
PCR	Polymerase Chain Reaction
PGPB	Plant Growth-promoting Bacteria
PPM	Parts per Million
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism

RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
Taq	*Thermus aquaticus*
TBSF	Tropical Soil Biology and Fertility
UPGMA	Unweighted Pair Group Mean Arithmetic
YEMA	Yeast Extract Mannitol Agar

ABSTRACT

Nitrogen is the most limiting nutrient for growth of most crop plants in sub-Saharan Africa and legume crops largely depend on fixed nitrogen from indigenous nitrogen-fixing bacteria. Providing nitrogen to a crop by application of synthetic nitrate fertilizers represents a significant cost both to the farmer and to the environment. The symbiotic characterization and identification of indigenous rhizobia is the basis for inoculants formulation for sustainable legume production in the face of climate change. The aim of this study was to evaluate the diversity of indigenous rhizobia responsible for cowpea nodulation in farmers' fields across three Counties of Western Kenya. The symbiotic performance of abiotic stress-tolerant rhizobia in the K80 cowpea variety was also assessed. Cowpea root nodules and soil samples were collected from agricultural farms in western Kenya and the farms had a history of growing cowpea for the last two years. Pure bacterial cultures from cowpea root nodules were isolated on Yeast Extract Mannitol Agar (YEMA). The isolates were characterized based on morphological characteristics, abiotic stress tolerance (salinity at 0, 3 and 5% with control at 0.01%, pH 4, 6, 8 and 10 with control at pH 7 and temperature at 20 ºC, 25, 30, 35, 40 and 45 ºC, with control at 28 ºC) and 48 isolates were subjected to ERIC-PCR fingerprinting. All the 54 isolates exhibited typical characteristics of *Rhizobium* species on YEMA, YEMA supplemented with Congo red and YEMA supplemented with Bromothymol blue. On YEMA the colonies appeared white, elevated, circular with smooth margins, mucoid, glistening and 1-5 mm in diameter. On CR-YEMA the colonies were white to pale pink, elevated, gummy and shiny with entire margins. A dendogram on phenotypic characterization grouped the isolates into cluster A and B at 42% dissimilarity. Cluster A isolates are low to moderate mucus producers while cluster B are high mucus producers. The results showed high genetic variability among the rhizobia isolates based on also ERIC-PCR fingerprinting. A dendogram on ERIC-PCR fingerprinting grouped the isolates at 53% similarity level into cluster A with 42 isolates along with the CIAT 899 reference strain with 2-15 amplified fragments ranging from 75-5000 bp while cluster B had only six isolates with 1-3 small fragments amplified, sizes ranging from 75-700 bp. Nine indigenous rhizobia isolates tolerant to extreme temperature, salinity and pH were tested for symbiotic efficiency in a glasshouse experiment Treatments with nitrogenous fertilizer KNO_3 (70 µg N mL^{-1}) without inoculation were positive control and treatments without inoculation and lacking chemical nitrogen served as negative control group. The rhizobia isolates efficiently nodulated cowpea and showed wide variations in their symbiotic efficiency (SE). Five isolates (M2bii, B6ii, B2i, V5ii and V11i) showed significantly higher SE ($p < 0.001$) of (114.6 - 179.6%) compared to the commercial inoculant (CIAT 899) with SE of 109.3% and nitrogen supplemented treatment (100%) which were the positive controls. Three isolates B6ii, V11i and V5ii with the highest symbiotic efficiencies of 179.6%, 143.35% and 127.3% respectively and three isolates M7b, B2ii and V9i exhibiting SE of 98.46%, 93.3% and 83.17% respectively lower than the positive controls were subjected to nifH symbiotic gene sequencing and confirmed their identities as *Bradyrhizobium* and *Rhizobium* species. These isolates have potential for inoculant formulation. The results indicate the existence of indigenous rhizobia isolates which can tolerate environmental stresses and are

symbiotically effective and thus can be developed into inoculants to improve the productivity of cowpea in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Nitrogen is a major mineral nutrient required for growth and productivity of crop plants and its deficiency both in the soil as well as in the crop adversely affects the growth and yield of crops (Gothwal *et al*., 2007). Nitrogen is essential in plant cells for synthesis of proteins, enzymes, chlorophyll, DNA, RNA and growth regulators thus essential for plant growth and production of food and feed. Its deficiency causes reduced growth, leaf yellowing, reduced branching and small trifoliate leaves in legumes (Simon *et al*., 2014; Russelle, 2008). Providing nitrogen to a crop by application of synthetic nitrate fertilizers is significantly costly to the farmer and harmful to the environment. World consumption of nitrogen fertilizer in 2014 was 113.1 million tons and was expected to be around 119.4 million tons in 2018. Africa accounted for 8 percent of world fertilizer consumption in 2018 (www.fao.org). Other than consumption of nonrenewable energy reserves which deplete natural resources, the environmental pollution through the escape of fertilizer nitrogen from the root zone to the underground water is high as the fertilizers are not used efficiently by crops (Sessistch, 2013). Despite the dramatic increase in the application of chemical fertilizers to crops in the last 50 years from 12 Tg/year to current 104 Tg/year there has been remarkable decrease in yield with persistence cycle of poverty among many farmers. The imbalanced application of chemical fertilizers can affect soil health hence application of beneficial Nitrogen-fixing microorganisms as a substitute to chemical nitrogen fertilizer in an integrated approach is a viable strategy (Onyango *et al.,* 2015*).*

The legume-rhizobial symbiosis which is the symbiotic association between the rhizobium and the leguminous plant has a large impact on productivity of legumes and plays a significant role in fixation of atmospheric nitrogen into plant usable forms of ammonia through its enzymatic reduction by nitrogen-fixing microbes such as rhizobia (Silva *et al*., 2012). This symbiosis can provide an inexpensive way to enhance soil fertility and improve crop production in developing countries. The root

nodule rhizobia approximately reduce 20 million tons of atmospheric nitrogen to ammonia which is 50 - 70% of the world biological nitrogen fixation (Simon *et al*., 2014). Encouraging the use of biological nitrogen fixation (BNF) serves a means to mitigate green-house gas emissions which leads to depletion of ozone layer. Rhizobia may also act as non-symbiotic plant growth promoting bacteria as in the case of economically important non-legume crops such as rice or wheat, which are the best studied examples that benefit from rhizobia as endophytes (Laranjo *et al*., 2014).

Cowpea (*Vigna unguiculata* L. Walp) is popular food grain legume playing a significant role in the economy of many African countries. The annual global production is estimated over 3 million tons. The crop is adapted to heat, drought and low nutrient environment (Mariam *et al*., 2015). It is primarily grown by small-scale farmers and represents one of the main subsistence crops because it can be used as grain with high protein content, green pods and green manure (Zilli *et al*., 2004). Small-scale farmers who are the major legume producers in Western Kenya rarely apply fertilizers during legume production due to their low income (Laranjo *et al*., 2014). Hence, the crop is largely dependent on fixed nitrogen from native nitrogen fixers. The most limiting factors to productivity of cowpea are low nutrients availability, especially P and N in agricultural soils. However, cowpea is a promiscuous plant and can establish symbiosis with several species (Moreira, 2006), receiving most of its nitrogen from biological fixation. Symbiotic promiscuity is beneficial when legumes associate effectively with different rhizobia species allowing their adaptation to different environments. *Bradyrhizobium genus* has been shown to be the predominant microsymbiont of cowpea in agricultural soils. Several studies on the diversity of the nitrogen-fixing leguminosae-associated nodulating bacteria have used cowpea as the trap plant species (Amanda *et al*., 2012).

The survival of rhizobia in nature depends on the genetic and physiological traits of the bacteria in addition to the environmental conditions (Agrawal *et al*., 2012). Edaphic factors and prevailing environmental conditions such as soil pH, phosphorous level, temperature, salinity and soil moisture content have been reported to determine the diversity and distribution of native rhizobia populations. In the

Rhizobium-legume symbiosis, the process of N_2 fixation is strongly related to the physiological state of the host legume. A competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors (such as salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases and grazing) impose limitations on the vigor of the host plant. Temperature affects root hair infection, bacteroid differentiation, nodule structure, and the functioning of the legume root nodule (Simon *et al*., 2014). In addition, the relative activity of the rhizobia is altered by temperature, so that a bacterium that is highly effective at one temperature is less active at different temperatures (Monica *et al*., 2013). The favourable temperature range for root hair development and large number of infection is 15 to 20˚C. This is supported by the study done by Benny *et al*., 2015 which demonstrated that host genotype mainly determines rhizobial diversity although other ecological factors such as soil acidity and site elevation were positively correlated with genetic variation of Mesorhizobia and site elevation respectively showing a relationship between the host and environmental factors in the distribution of rhizobia.

Kipngetich et al., (2024) reported that cowpea production in Western Kenya has not been stable over the last eight years at an average of 0.6 Mg ha^{-1} against the potential yield of 2.5 Mg ha^{-1}. Cowpea can fix atmospheric nitrogen in symbiotic relationship with rhizobia which makes it very important in cropping systems. In efficient nutrient management importantly phosphorous and nitrogen, cowpea is capable of fixing and availing 60-70 kg ha-1 of nitrogen to succeeding crops grown in rotation with it from 240 kg ha-1 of atmospheric nitrogen. When cowpea is rotated with cereals, it can fix up to 88 kg N ha-1 and enhance nitrogen usage efficiency of the next cereal crop to 28% from 20% in continuous cereal monoculture. When cowpea is associated with nitrogen fixing rhizobia, its net nitrogen contribution to soil could increase to 150 kg ha-1. Incorporating cowpea in strategies for crop production could result in provision of up to 337 kg N ha-1 from biological nitrogen fixation. The potential for a strong symbiotic relationship with rhizobia and fixation of nitrogen could be a source of supply of nitrogen for the production systems of crops that is

eco-friendly to the soil systems and to the environment and attractive economically (Kabede et al., 2022).

Knowledge of the morphological and genetic diversity of the native rhizobia in Western Kenya will help develop ecological strategies to ensure survival in the prevailing conditions. Increased interest in the utilization of symbiotic N fixing bacteria as biofertilizers in agriculture has prompted studies of their diversity. The objective of this study was to determine the diversity of rhizobia populations from agricultural soils of Western Kenya using phenotypic and molecular markers and evaluate the symbiotic efficiencies of isolates tolerant to extreme environmental conditions.

1.2 Problem Statement

Nitrogen is the most limiting nutrient for growth of most crop plants. Providing nitrogen to a crop by application of synthetic nitrate fertilizers represents a significant cost to the farmer and harm to the environment. Small-scale farmers who are the major legume producers in Western Kenya rarely apply fertilizers during legume production due to their low income. In the period 1925-2015, Kenya Soil Health Consortium, in collaboration with other partners reported that soil fertility and soil health deteriorated in Western Kenya resulting in low crop yields. To maintain increase in crop productivity, there is need to apply moderately higher quantities of fertilizer although commercial farm inputs are expensive for most farmers to access. On average, the poverty index of 51% in Western Kenya implies that affordability of fertilizer is a major concern for many small scale farmers. This has potentially lowered the fertilizer application rates predisposing farming households to land degradation and food insecurity hitches. In Kakamega County for instance, the fertilizer subsidy program was aimed at achieving increased application rate of fertilizer from the usage of 37 kg/ha to 100 kg/ha in 2014, but this was not achieved as subsidies did not reach targeted farmers.

Hence, the crop is largely dependent on fixed nitrogen from native nitrogen fixers. The survival of rhizobia in nature basically depends on the genetic and physiological traits of the bacteria in addition to the environmental conditions. Edaphic factors and

prevailing environmental conditions such as soil pH, phosphorous level, temperature, salinity and soil moisture content have been reported to determine the diversity and distribution of native rhizobia populations. Knowledge of the morphological and genetic diversity of the native rhizobia in Western Kenya will help develop ecological strategies to ensure survival in the prevailing conditions.

1.3 Justification

Nitrogen is a major mineral nutrient required for the growth and productivity of crop plants and its deficiency both in the soil as well as in the crop adversely affects the growth and yield of the crop. It is well known that biological N fixation is one way of converting elemental inert atmospheric nitrogen into plant usable form as organic compounds through its enzymatic reduction by N-fixing microbes such as rhizobia (Gothwal *et al*., 2007). Since chemical fertilizers are expensive and its imbalanced application can affect soil health including waterway pollution, chemical burn to crops, increased air pollution, acidification of the soil and mineral depletion of the soil, application of beneficial N-fixing microorganisms as a substitute to chemical N fertilizer in an integrated approach is a viable strategy (Onyango *et al.,* 2015*)*. Interest in utilizing rhizobia as biofertilizers in agriculture has prompted studies of their diversity and the description of a large number of rhizobial species. In addition to contributing to the knowledge of soil biodiversity and increasing the utility of rhizobial collections, assessments of rhizobial genetic diversity are important in developing long-term strategies to increase the role of biological N2 fixation in improving agricultural productivity.

It is well known that legume crop in Western Kenya is largely dependent on fixed nitrogen from native nitrogen fixers. In most cases, native nitrogen fixers (*Rhizobia* and *Bradyrhizobia* species) are competitive to inoculants (e.g. *Bradyrhizobium* sp. (*Vigna*) and this nitrogen fixing bacteria is a product for cowpeas, peanut and mung beans) but may not be the most efficient strain and possibly incompatible to the host plant. Therefore, relying on native nitrogen fixers without prior information on its efficiency and compatibility with host legume leads to low crop production (Simon *et al*., 2014). The purpose of this study is to characterize and determine genetic

diversity of legume nodulating rhizobia in Western Kenya soil and to identify strains that efficiently nodulate legumes. The findings of this study will provide basis for selecting superior strains for manufacturing commercial inoculants for small-scale legume farmers in Western Kenya, thus improve soil fertility and increase legume production (Mariam, 2015).

1.4 Null Hypotheses

i. There is no morphological difference between rhizobia isolates from Western Kenya
ii. The rhizobia isolates from Western Kenya are not tolerant to abiotic stress
iii. The rhizobia isolates from Western Kenya are not genetically diverse
iv. The selected abiotic stress tolerant rhizobia isolates from Western Kenya are not symbiotically efficient
v. The symbiotically efficient rhizobia isolates from Western Kenya are not different

1.5 Objectives

1.5.1 Broad Objective

To characterize phenotypic and genetic diversity of cowpea rhizobia populations from Western Kenya agricultural soils.

1.5.2 Specific Objectives

i. To characterize cowpea rhizobia from Western Kenya agricultural soils using morphological markers.
ii. To evaluate the tolerance of the cowpea rhizobia isolates from Western Kenya to abiotic stresses (acidity, salinity and temperature).
iii. To determine genetic diversity of cowpea rhizobia isolates from Western Kenya agricultural soils.
iv. To determine the symbiotic efficiencies of selected cowpea rhizobia isolates from Western Kenya tolerant to abiotic stresses.

v. To identify the symbiotically effective cowpea rhizobia isolates from Western Kenya using molecular techniques

CHAPTER TWO

LITERATURE REVIEW

2.1 Rhizobia and its Importance

Rhizobium is a nonspore-forming rod-shaped, motile, aerobic, gram-negative soil bacterium able to colonize in the rhizospheric region of leguminous plants and symbiotically fixes atmospheric nitrogen (Laranjo *et al.*, 2014). Rhizobia are physiologically and genetically diverse group of bacteria that produce nodules on the roots and scarcely on the stem of legume hosts plants, in which the bacteria reduce atmospheric nitrogen into ammonia and nitrates (Ali *et al*., 2009). The symbiotic relationship between legumes and rhizobia bacteria is very critical for provisional of nitrogen needed by the plants leading to reduced need for chemical fertilizer application. Nitrogen fixation is determined by the physiological status of the host and the microsymbiont environment fitness and effectiveness and also the interaction between the host and the microsymbiont (Rodrigues *et al*., 2006). Rhizobia can live in close association with the plant roots as saprophytes or endophytes (rhizobacteria) (Mohammadi & Sohrabi, 2012). Depending on nitrogen fixing ability, rhizobia are classified as *Rhizobia* (fast growing) and *Bradyrhizobium* (slow growing). The growth of *Rhizobium and Bradyrhizobium* observable in Yeast extract Mannitol Agar (YMA) takes 3 - 5 and 6 – 8 days, respectively. The process by which rhizobia inhabits rhizosphere, infect the legumes roots and facilitates nitrogen fixation results in improved plant development and yield. In recent years, biological nitrogen fixation has earned attention as an alternative for chemical fertilizer since it is an environmental friendly farm approach and cost less to the farmers. It also curbs nitrate pollution of groundwater. The efficiency of nitrogen fixation by rhizobia corresponds to soil fertility level. Soil acidity leads to reduced effectiveness of rhizobia strain nitrogen fixation activity (Simon *et al*., 2014).

2.2 Current Genera and Species of Rhizobia Nitrogen-Fixing Bacteria

Rhizobia is a generic name a group of Gram negative bacteria that belong to the Phyllum *Protobacteria* that form nodules on the roots and in some cases on the stems of their host plants and fix nitrogen in symbiosis with their legume host plants. The Rhizobium genus was the first to be described among this bacteria and the reason why the name has been frequently used for the nitrogen-fixing bacteria in legumes. Currently these groups of bacteria are distributed in 18 genera in the families: *Rhizobiacea (Rhizobium, Ensifer (syn. Sinorhizobium), Allorhizobium, Pararhizobium, Neorhizobium (Shinella)), Phyllobacteriaceaceae (Mesorhizobium, Aminobacter, phyllobacterium), Brucellaceae (Ochrobactrum), Methylobacteriaceae (Methylobacterium, Microvirga), Bradyrhizobiaceae (Bradyrhizobium), Xanthrobacteraceae (Azorhizobium), Hyphomicrobiaceae (Devosia) and Burkholderiaceae (Paraburkholderia, Cupriavidus*). The genus Rhizobium having 112 species is the largest genus of rhizobia (Lindstron, 2019).

Due to advanced analytical techniques, the taxonomy of rhizobia is still in flux. *Allorhizobium undicola* is known to induce nodulation on *Neptunia prostrate* an indigenous aquatic legume found in humid tropics and commonly used for human consumption and green manure preparation. Azorhizobium induces nodulation on aquatic legume *Sesbania rostrata*. They have a unique feature of growing with N_2 as the only nitrogen source whereby *Azorhizobium caulinodans* is the only species for genus Azorhizobium. *Bradyrhizobium* grows slowly and commonly associated with symbiotic relationship with soybean and rarely with peanut, lupine, and cowpea. *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* establishes a symbiotic relationship with soybean while *Bradyrhizobium lupini* is associated with lupine. *Mesorhizobium* is a fast grower compared to *Bradyrhizobium*, but a slow grower when compared with *Sinorhizobium* and *Rhizobium*. They nodulate chickpea, trefoils and various legume species (Russelle, 2008). Rhizobium establishes a symbiotic relationship with peas, vetches, lentils, beans and clovers. *Rhizobium japonicum* nodulates soybean, cowpea and siratro. *Rhizobium leguminosarum* can facilitate nodulations in vetch while *Rhizobium tropici* establishes a symbiotic relationship with beans and other legumes, *Rhizobium etli* nodulates alfalfa and beans, *Rhizobium*

gallicum can nodulate bean, *Rhizobium giardinii* leads to nodulation in leuceana, *Rhizobium galegae* facilitates nodulation in galega, and Rhizobium species NGR234 facilitates nodulation in 112 legume genera and nonlegume *Parasponia andersonii*. *Sinorhizobium meliloti* nodulates alfalfa, medics, and sweetclover. *Sinorhizobium fredii* nodulates soybean; *Sinorhizobium terangae* and *Sinorhizobium saheli* facilitates nodulation in of the roots of Acacia, Sesbania, *Neptunia prostrata* and *Leucaena leucocephala* (Sylvia *et al*., 2005).

2.3 Legume-Rhizobia Symbiosis

Legumes are believed to have emerged since 59 - 60 Million years ago (Ma) early in the Tertiary period and its subfamilies to have evolved between 56 - 50 Ma and can survive even in poor soils where there is not enough nitrogen to support other types of plants. However, rhizobia are thought to be present before the legumes for nodulation because *Sinorhizobium-Bradyrhizobium* split was reported about 500 Ma. Bacteria of the genus *Bradyrhizobium, Rhizobium Sinorhizobium* and others induce nitrogen-fixing nodules on the roots of legumes such as peas, beans, cow peas and soybeans.

Symbiosis process is in such a way that rhizobia provides a continuous source of utilizable nitrogen to host plant interchangeably with energy and nutrients requirement because plants are not capable of using nitrogen right away or the one stored in the soil as organic matter except in form of ammonium and nitrates (Simon *et al*., 2014). The interaction between symbiotic bacteria and their root nodules can be analyzed through proteins (proteomics) generated by partners in their exchange of signals and through symbiosome growth. The study results by Mastronunzio and Benson, (2010) identified a number of proteins facilitating development and functioning of rhizobial symbiosis. The pathogen-responsive proteins produced by various abiotic and biotic stimuli safeguard plants from pathogenic attack by viruses, bacteria, fungi, and unfavourable environmental factors. Inadequate nitrogen supply in the soil, leads to legumes discharge of flavonoids which indicates to rhizobia that the plant desires symbiotic bacteria (Ndakidemi & Dakora, 2003). The rhizobia releases nodulation factor which stimulates legume plant root hairs to be deformed.

Nitrogen fixation is a dynamic and high energy demanding process. The pathway for the biological reduction of inert nitrogen (N_2) into the reactive compound NH3 (ammonia) under micro-aerobic conditions is as follows:

$$N2 + 8H+ + 8e- + 16Mg\text{-}ATP \rightarrow 2NH3 + H2 + 16Mg\text{-}ADP + 16\ P$$

The rhizobia reduces atmospheric nitrogen to form ammonia that is utilizable for nucleotides and amino acids synthesis while the bacteria gets sugars from the plant host establishing a symbiotic relationship (Glick, 2003).

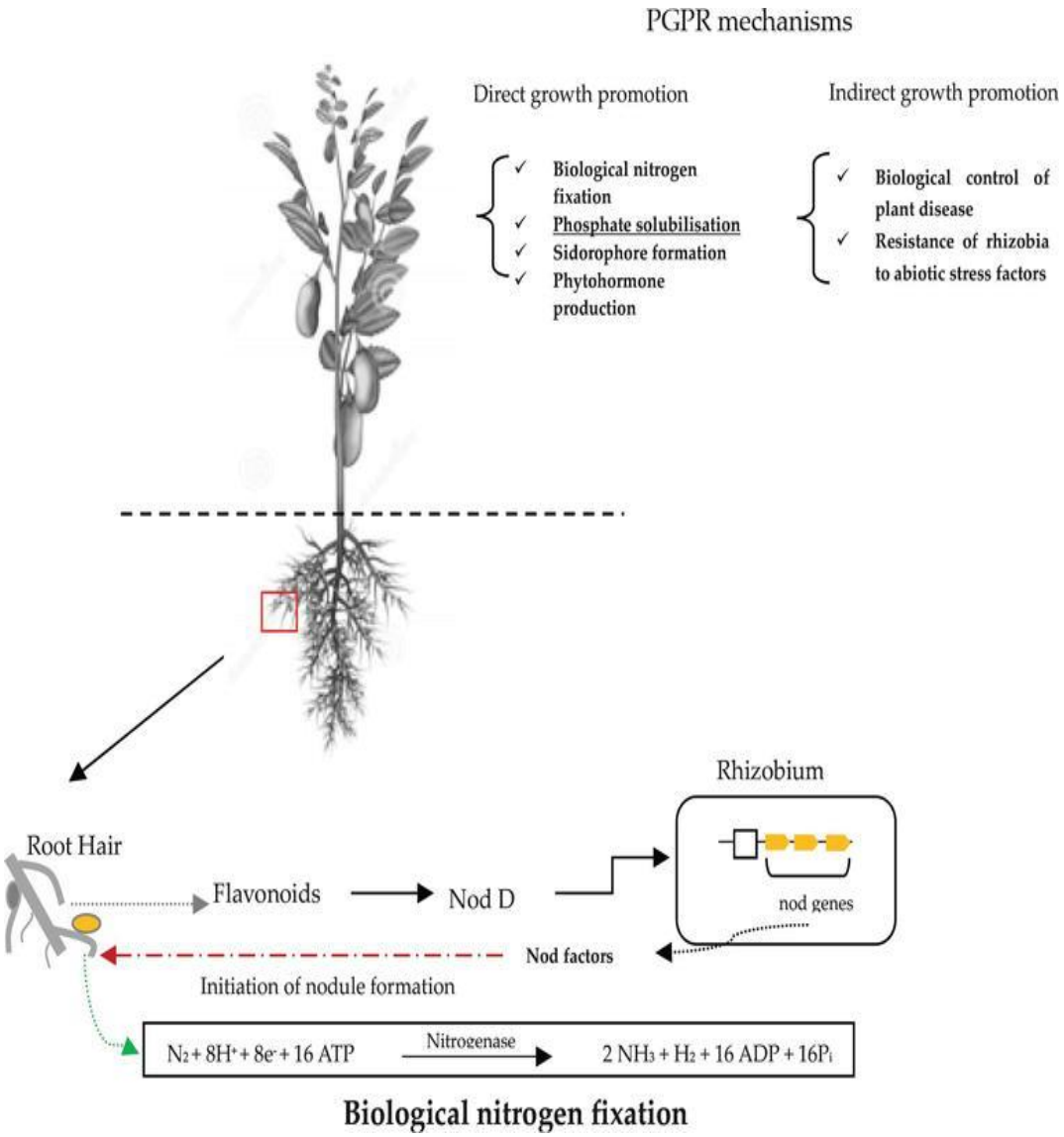

Figure 2.1: Schematic Overview of the Nodulation Process and Biological Nitrogen Fixation

2.4 Host Specificity and Symbiotic Effectiveness

Host specificity describes the ability of a given rhizobia species to induce nodulation in specific legumes. Use of efficacious and superior exotic rhizobia strains inoculants in several environments has not shown successful results due to many reasons such as non-competitiveness and ineffectiveness of rhizobia strains (Simon *et al*., 2014). Host specificity gives rise to perfect match between rhizobia and legume leading to effective nodulation (deep red inside) hence enhanced nitrogen fixation. In cross inoculation without perfect match, ineffective nodulation (green or white inside) or nodules are not formed and nitrogen fixation fails to occur (Gwata *et al*., 2002).

The symbiotic interaction and host specificity results from specific exudates (flavonoids) produced by the roots and also from Nod factor composition. The flavonoids produced from hosts intensify bacterial recognition enhancing the opportunity for host plant selectivity inducing symbiotic gene expression in the specific rhizobium strain (Simon *et al*., 2014). The specific signal by flavonoid in various rhizobial strains is to resolve bacteria to take part in regulator NodD transcriptions. The host plants genes are regulated after the formation of lipo-chitin oligosaccharide (LCO) "nod factors" to recognize a particular legume host. The bacterial nod factors are formed by fatty acids and 4 to 5 β1 – 4 linked N-acetyl glucosamine units (a chitin backbone). The nod factors differ in relation to the length of sugar backbones, fatty acids and decorations (methylation, sulphation and glycosylation) of both reducing and non-reducing backbone ends and saturation of the acyl unit (Simms & Taylor, 2002). The rhizobia nod factors vary and their host plant selectivity is attributed with the specificity level towards interaction and creates chances for plant partner choice. The cell wall of *rhizobium* are composed of numerous polysaccharide which creates chances for host plant partners choices to employ ''lock and key'' cascade which dictates the level of bacterial and host plant specificity (Simms & Taylor, 2002). Studies indicate that different rhizobia genera can infest same host plant species while some host plants species can be infected only by strictly one specific genus.

Cowpea is nodulated by rhizobia isolated from legumes like Bambara ground nuts and soybean. Some of the rhizobia nodulating these legumes are not isolated from the cowpea. This phenomenon is important in recommending and developing effective legume inoculants to facilitate nitrogen fixation in regards to environmental changes (Simon *et al*., 2014). The strain 042B is reported in China to have high symbiotic efficiency compared to *Bradyrhizobium japonicum* which indicated early nodule formation with better nitrogenase activity. Thus, rhizobia strains identification in local farmers' farms on specific hosts and their nitrogen fixation effectiveness analysis and cross inoculation efficiency is vital for improved legume production.

Abera *et. al*, (2018) reported that 100% and 80% of the soil samples from different sampling sites induced nodulation on cowpea and soybean varieties, respectively; indicating that the soils harbor compatible nitrogen fixing bacteria for both crops. This possibly indicate that rhizobia may originally evolve from cowpeas that cross nodulate the recently introduced soybean crop. Several studies in the past have showed that cowpea is one of the most promiscuous legume hosts in the so called 'cowpea' miscellany group of rhizobia.

Studies by Abera *et al*., (2018) also demonstrated the ability of 15 selected rhizobial isolates to form nodules and effectively fix nitrogen which was determined on other cross-nodulating hosts, of which 12 isolates (80%) nodulated either or all cowpea, mung bean hosts and pigeon pea. The data showed that 75% and 58% of these isolates displayed effective and very effective symbiosis with their heterologous cowpea and pigeon pea hosts, respectively. However, half of them formed slightly effective and effective nodules on mung bean. All soybean isolates tested nodulated cowpea with 58% being moderately effective to very effective and about 82% nodulated pigeon pea, 36% of which were effective, but all failed to form nodules on peanut (Musiyiwa *et al.* (2005). Studies by Appunu *et al.* (2009) also reported that all rhizobia isolated from soybean nodulated cowpea, 67% nodulated mung bean and 22% formed nodules with pigeon pea and peanut. Abera *et al*., (2018) also demonstrated that two Bradyrhizobium strains isolated from soybean could nodulate

peanut growing in the same ecological niche that may have phylogenetic connection with each other.

The majority of the described indigenous isolates effectively nodulating common bean roots were identified as *Rhizobium etli*, *R. leguminosarum*, *R. leguminosarum* sv. *phaseoli* and *R. tropici*, whereas potent diazotrophs symbionts of soybean and cowpea were identified as *Bradyrhizobium elkanii* and *B. japonicum*. Additionally, some strains of *B. yuamingense* were found to be effective as inoculates for cowpea along with several unidentified isolates effective as seed inoculants of cowpea, common bean and soybean (Abera *et al*., 2018).

2.5 Nitrogen Fixation and Crop Productivity

Legumes optimum productivity is usually restricted by level of nitrogen fixed in different environments within favourable water supply and proper climatic conditions. Soil fertility is improved by restoring and improving the symbiotic rhizobia (Gothwal, 2007). The biofertilizers prepared by Rhizobia are inexpensive and the best way to achieve legume supply to the rapidly expanding population. When the legumes are cut back or ploughed to the ground they release the fixed nitrogen fixed in the root nodules to the soil for use by the following crop. The prokaryotes approximately contribute 0 to 60 Kg/ha/year or 175 million metric tons per year and about 70% of total nitrogen fixed to the earth; other organisms, such as heterotrophs or autotrophs known as “free fixers” contribute the remaining balance (Simon *et al*., 2014).

Biological nitrogen fixation provides approximately 30 to 300 million tons for marine ecosystems, 100 million tons of nitrogen for terrestrial ecosystems and 20 million tons from chemical fixation as a result of atmospheric phenomena. After the World War II, the use of nitrogenous fertilizers has increased in production of cereal and other crops. This has led to numerous negative effects like water pollution and eutrophication as a result of leaching (Jalali *et al.*, 2022). To reduce these negative effects, use of nitrogen-fixing rhizobia have a significant value in host plant since it ensures they facilitate nitrogen supply, adaptation to various environmental stresses and defense against pathogens and pests as well.

Botha *et al*., (2004) concluded that CB 1809 strain in soybeans was 60% effective isolate analyzed in effective nitrogen fixation in Morgenzon and Bergville, South Africa. Small scale farmers in Zimbabwe regard nitrogen fixing rhizobia inoculants to have unique properties since they lead to high growth rate with dark green colour legume plants in relation to the untreated plants. Farmers in Africa produce legumes in spite of the low necessary micro- and macronutrients in the soil. There are insufficient data regarding the connection linking nitrogen fixation and crop productivity records for various farming systems utilized in Africa. Determination of native strains efficient in nitrogen fixation will bring a low cost solution for increasing legume production if well made use of by the greater number of smallholder farmers in Africa (Simon *et al*., 2014).

A study in India on molecular and phenotypic characterization of chickpea rhizobia reported that higher yield in chickpea depends on the site and not on inoculation with high quality rhizobial strains. This can be attributed to the fact that the naturally occurring rhizobial populations are better adapted and more competently involved in nodulation in different geographical areas than the inoculant strains. Hence, to improve the rhizobial inoculant benefits to the chickpea, there is a great need to establish characteristics similar to the indigenous rhizobia population in the field. Chickpea rhizobia nitrogen fixation effectiveness and efficiency has been established in different countries including Tunisia, Portugal and Morocco (Rai *et al*., 2012).

Cowpea can fix atmospheric nitrogen through biological nitrogen fixation, a symbiotic association between legume host and soil-dwelling bacteria known as rhizobia, which makes it an important component of cropping systems. With efficient nutrient management, especially nitrogen and phosphorus, cowpea can fix about 240 kg ha−1 of atmospheric nitrogen and make about 60–70 kg ha−1 nitrogen available for succeeding crops grown in rotation with it. Cowpea can fix up to 88 kg N ha−1 and increase the nitrogen use efficiency of the succeeding cereal crop from 20% in continuous cereal monoculture to 28% when cereals are rotated with cowpea. Cowpea's net contribution of nitrogen to soil could be up to 150 kg ha−1 particularly when associated with N-fixing rhizobia. Incorporating cowpea into crop production strategies could provide up to 337 kg N ha−1 from nitrogen fixation. This potential

for a strong symbiotic association with rhizobia and nitrogen fixation could be a source of nitrogen supply for the crop production systems, which is eco-friendly to the environment and soil system and economically attractive (Kabede *et. al*., 2022).

2.6 Factors Influencing Legume, Rhizobia Population and Nitrogen Fixation

Different environmental conditions limit the cultivation and action of the N_2-fixing plants. The principle of limiting factors is the fact that "the level of crop production cannot be higher than that allowed by the maximum limiting factor". *Rhizobium*-legume symbiotic relationship, a N_2-fixingsystem, the technique for N_2 fixation is firmly associated with the host plant physiological condition. Thus, a persistent and competitive legume nodulating strain is not anticipated to display full competence in relation to fixation of nitrogen if inhibiting parameters (e.g., mineral toxicity, unfavorable soil pH, salinity, temperature extremes, nutrient deficiency, excessive or insufficient soil moisture, inadequate photosynthesis, plant diseases and pests and grazing) inflict hindrance on the host legume plant (Sidhu *et al*., 2020).

High soil temperatures in tropical and subtropical areas are a major problem for biological nitrogen fixation of legume crops. Temperature influences nodule structure, hair root infection, differentiation of bacteroid and the root nodule functioning of the leguminous plants (Simon, *et al.,* 2014). The ideal temperature for optimal nodule operation in beans (*Phaseolus vulgaris*) ranges from 20˚C - 25˚C (Poustini *et al*., 2005). The optimal temperature range is 25 - 31˚C for rhizobia in culture media but some strains secluded from dry and hot Sahel Savannah environment can grow competently at 40 ˚C. Furthermore, the relative rhizobia action dependent on temperature, thereby a bacterium can be extremely active at a given temperature and less effective at a different temperature (Monica *et al*., 2013). Desirable proposed temperature for large number of infection and root hair development range between 15 - 20˚C. Low temperatures limits for indigenous crops to temperate zone is 2 ºC and for tropical species at 10 ˚C (Simon *et al*., 2014). High (not extreme) soil temperatures delays nodulation or restrict the subsurface region of legume yield.

Soil acidity is an important issue affecting agricultural crop production in numerous areas all over the world and inhibits production of legumes. Soil acidity, aluminum and manganese toxicity in addition to calcium insufficiency severely affect plant growth particularly when they rely on symbiotic N_2 fixation which is efficient at neutral or moderately acidic soils (Simon *et al*., 2014). Researchers have reported that most legume species are unsuccessful in nodulating at below pH 5.0 since they cannot withstand acidic conditions. The fast-growing rhizobia strains are considered less acid pH tolerant than the slow growing strains especially the *Bradyrhizobium*. Some fast – growing strains like the *R tropici* and *R. loti*, are highly acid tolerant. Various up to date reports supports that there is existence of fast growing acid-tolerant strains, because both fast and slow-growing *Vigna unguiculata* strains are tolerant to pH values as low as 4.0. The pH tolerance among *Rhizobium* and *Bradyrhizobium* strains is not clear, but various workers indicate that the cytoplasmic pH of acid-tolerant strains is less likely affected by the external acidity (Lei *et al.,* 2011).

Researchers have reported the detrimental effect of salt on endurance and the growth of rhizobia. The legume salinity reaction relies on plant growth stage, climatic conditions and soil characteristics. Salt and osmotic stress influences the first stages of rhizobial- legume interaction and the formation of nodules than it affects rhizobia. The formation of root hair on plants is highly salt sensitive than rhizobia cells (Simon *et al*., 2014). The *Rhizobium leguminosarum* in beans (*Phaseolus vulgaris*) permits up to 350 mM NaCl concentration in liquified culture medium and the *Vigna Unguiculata* can grow up to 450 mM NaCl concentration. Although the *Rhizobium* and *Bradyrhizobium* which are root colonizing nodule bacteria are salt tolerant compared to their legume hosts, their salt tolerance also varies. To initiate a fruitful legume-rhizobia symbiotic association in salty environments, effective salt tolerant indigenous rhizobia strains should be secluded from salty soils (Hawkins, 2022).

2.7 Characterization of Rhizobia

Presently, rhizobial characterization addresses a combined analysis of phenotypical, physiological, and different molecular tools to evaluate the similarities and

differences among rhizobial species. Due to this, phenotypic and growth characteristics of rhizobia have been used to identify new groups of rhizobia. For example, phenotypic and growth characteristics were the parameters used to characterize the rhizobial population in different ecosystems. These evaluation methods have the advantage of being fast, allowing a preliminary analysis of diversity and providing valuable information for their grouping and identification (Kabede *et. al.,* 2022).

However, advanced molecular techniques, like repetitive DNA sequence (BOX element)-based PCR (BOX-PCR), ERIC-PCR, REP-PCR and 16S rRNA gene sequencing, are highly recommended since their results offer more precise identification and evaluation terms for their genetic diversity (Amanda *et al*., 2012).

2.7.1 Morphological/Phenotypic Characterization

Phenotypic characteristerization of rhizobia could also reveal a wide variability within and among rhizobial species, which in turn relates to variability at the DNA level. Studies have described that phenotypic evaluation of diverse collections of rhizobial isolates could be a method for discovering potentially superior rhizobial strains. The evaluation of rhizobia diversity based on the phenotypic characteristics involves the study of parameters such as the time taken for the bacteria to form colonies in culture, color, shape, colony diameter, acid and alkali production, and mucus production among others. These characteristics are necessary for generating a resource from which highly adapted and efficient rhizobial strains can be selected for inoculant production and preliminary information about their genetic diversity. Rhizobia nodulating cowpea have specific variations in their growth culture and phenotypical characteristics, which are used for identification and diversity studies. Previously, the rhizobia that nodulate cowpea have been routinely considered as belonging to the miscellaneous group ‘cowpea,’ that is, *Bradyrhizobium* spp. comprising a large number of slow-growing strains. This has led to a fallacy about their diversity as studies have revealed the existence of both slow- and fast-growing rhizobial isolates in the root nodule of cowpea (Kabede *et al*., 2022). These

phenotypic methods of analysis of cultural properties of microorganisms have the advantage of being fast and allowing a preliminary analysis of diversity.

2.7.2 Genetic Characterization

The taxonomy of root nodulation bacteria for example rhizobia traditionally have been based on phenotypic characters such as cross inoculation group but this approach has been widely criticized. It has been reasoned that a reclassification of the genus rhizobium should be carried out on the basis of variation in the chromosomal genome, and not on rhizobial plant host specificity or other phenotypic characterization that are mainly encoded by plasmid-borne genes. In the recent years, there have been many advances in molecular biology which have provided powerful tools that are PCR-based. The difference in the fragment profile of different genotype can be used as genetic marker for variety or genotypic identification, genome mapping and gene tagging. In the past decade, ribosomal RNA based detection and identification methods have become routine techniques used in the disciplines of microbiology. Comparison of 16S r-RNA genes has allowed differentiation between rhizobium species. Diversity in 16S rRNA genes can be estimated by restriction fragment length polymorphism (RFLP) analysis of 16S r-RNA sequences amplified by polymerase chain reaction. At times PCR based genomic finger printing methods provide more fine taxonomic resolution than 16S r-RNA sequencing (Rashid *et. al*, 2009).

Molecular techniques have been widely used for typing and assessing the genetic diversity of microorganisms. The techniques based on PCR fingerprinting have been employed to examine genotypic diversity of rhizobia population and to discriminate among rhizobia strains (Neelawan, 2012). The sensitive and accurate availability of PCR-based genotyping is closely related to bacterial strains facilitating greater consideration of the detection of higher rhizobial diversity (El-Fiki, 2006). Although phenotypic methods play a significant role in identification, molecular methods are more reliable and authenticated for identification and to study genetic diversity of bacterial isolates. Major molecular techniques include restriction fragment length

polymorphism (RFLP), amplified fragment length polymorphism (AFLP), single sequence repeats (SSR) and 16S-rRNA gene sequencing.

2.7.2.1 ERIC-PCR Fingerprinting of Rhizobia

Rhizobia are very diverse taxonomically, hence there is need for efficient methods to facilitate strain classification based on genotypic characterization may be by displaying properties like, better nitrogen-fixation capacity. Molecular techniques assist in developments of better microbial characterization including distinguishing genera, species and strains. The polymerase chain reaction (PCR) employment of primers corresponds to consensus repetitive sequences scattered in the eubacteria genome, such as Enterobacterial Repetitive Intergenic Consensus (ERIC) and enterobacterial repetitive sequences (REP) can create highly characteristic patterns when resolved in agarose gels, providing well separation on strain level. ERIC sequences are highly protected among rhizobia genomes and they facilitate selection and categorization of different rhizobia strains in population works and evaluation of the environmental effect in defined populations (Hatice *et al*., 2009). ERIC and Repetitive element (REP) PCR sequences have successfully been used to differentiate bacterial strains from diverse species (Lindsay and Sharp, 2006). They have demonstrated the importance of DNA fingerprinting by PCR using REP and ERIC primers (ERIC-PCR and REP-PCR) for the identification and classification of strains of various *Rhizobium* species. These methods have been used to characterize several rhizobial strains

2.7.2.2 Sequencing (Based on 16S rRNA, nodC Genes)

The nodule formation by rhizobia symbiotic relationship with the host plant corresponds to the presence of flavonoids which stimulates expression of the bacterial nodulation genes (*nod* genes). The *nod* genes encode for the enzymes involved in the biosynthesis of the Nod factors which acts as the determinants of the host specificity and result in nitrogen-fixing nodules in the root. The *nodABC* genes are characterized by common *nod* genes, which are essential for nodule development. The NodC is homologous to chitin synthase and acts as an *N*-

acylglucosaminyltransferase, which is involved in the formation of the Nod factor backbone (Bao *et al*., 2009).

The *nodC* partial sequence can be involved in identification of rhizobia to at least the species level. The 16S rRNA sequences have been used in classic molecular phylogenetic studies of rhizobia. However, it is important to use other molecules in combination to this, because contrasting molecular phylogenies are sometimes noticed as a result of widespread lateral transfer in rhizobia. Further work concerning molecular phylogenetics involving the rhizobia may require not only the identification of 16S rRNA molecules but other characteristics like the common genes encoding nodulation like *nodC*. Generally, the degree of conservation is significantly involved in resolving the problems associated with phylogenetic. The 16S rRNA sequences, which are incomplete sequence in description, cannot be used to identify rhizobia at the species level due to the molecule high degree of conservation. Moreover, the 16S rRNA molecule phylogeny of the rhizobia does not correspond with that of the legume host plants. Hence, the 16S rRNA information alone might not be sufficient to identify and classify the rhizobia. Furthermore, our data also suggest that phylogenetic analysis of common nodulation genes may be important in assessing phylogenetic relationships in host plants (Bao *et al.,* 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

Soil samples and K80 cowpea plants for the study were collected from agricultural farms from Vihiga (0.038N, 34.71E), Kakamega (0.33N, 34.71E) and Busia (0.46N, 34.10E) Counties in Western parts of Kenya (Figure 3.1). The sites were selected based on prevalence of cowpea cultivation. The soil characteristics for the sampling sites were sandy clay-loam for Mumias and Busia counties (Kabeney *et al.*, 2015) and sandy clay for soil in Vihiga County. The K80 variety was used in the study because it was the most commonly grown variety in the three Counties. The samples were transported at 4 °C to the Soil Science Laboratories, University of Nairobi and stored in refrigerator at 4 °C. The physico-chemical analysis of soil was done at the Soil chemistry laboratory, morphological and physiological characterization was done at Microbiology Resource Center (Soil Science Laboratories-University of Nairobi), Molecular characterization was carried out at the Biochemistry Laboratories (University of Nairobi) and symbiotic efficiency experiment was done in glass house, Biochemistry Laboratories, University of Nairobi.

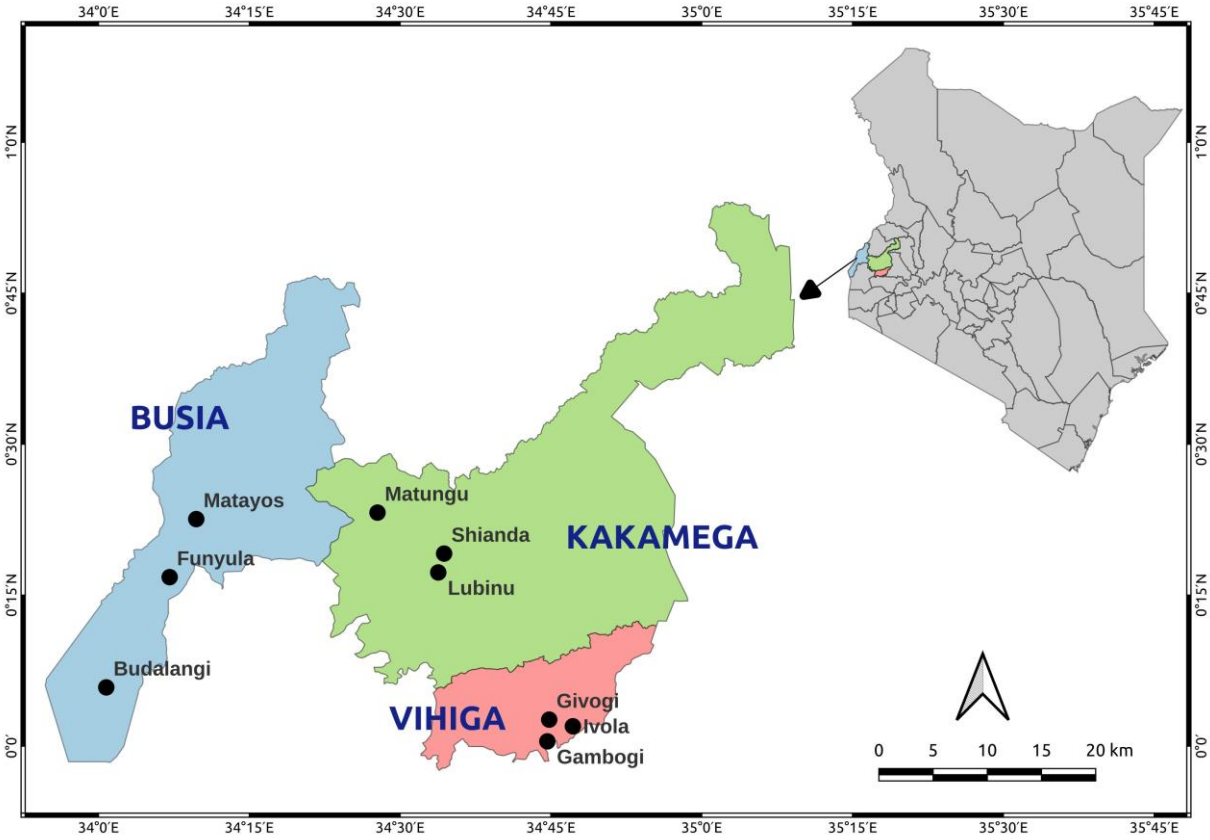

Figure 3.1: Map Showing Sample Collection Sites

Table 3.1: Sample Collection Sites and their Locations

ID	County	Site	Latitude	Longitude
1	Vihiga	Gambogi	0.00784792	34.7442048
2	Vihiga	Ivola	0.03283944	34.7865195
3	Vihiga	Givogi	0.04382671	34.74726
4	Busia	Matayos	0.37595214	34.1621445
5	Busia	Funyula	0.27983377	34.118042
6	Busia	Budalangi	0.09722142	34.0129439
7	Kakamega	Matungu	0.38670509	34.4626227
8	Kakamega	Lubinu	0.28757311	34.5633174
9	Kakamega	Shianda	0.31883789	34.5730305

3.1.1 Study Design

The selection of soil collection areas was based on the status of cowpea production. The cowpea farming history of the region was known making the selection of farms easy. Nodules were collected from agricultural farms and the selection criteria of the farms were based on history of having cultivated cowpea the last two years without rhizobial inoculations. Three farms from each County were sampled for soil and K80 cowpea nodules. The soil samples were collected by random sampling method. From each field, five soil samples were collected in a zigzag pattern. For nodules, five dark green looking cowpea plants were randomly collected in a zigzag pattern from each field. The zigzag pattern involved collection of sample at four corners and one sample at the center of the field. Experiments for soil physico-chemical analysis, abiotic stress tolerance and symbiotic efficiency were done in triplicates.

3.1.2 Sample Collection

Soil samples were collected from the farmers' fields in a zigzag pattern at a depth of 20 cm from soil surface. The 15 soil samples collected from the three fields in a County, were thoroughly mixed together to obtain a homogeneous soil sample on a sampling field basis and 2 kg composite sample was collected and packed separately in sterile plastic bags then labeled. Out of the 15 cowpea plants collected from each County, twenty pinkish-red looking nodules were collected. The nodules were wrapped in cotton wool then placed in plastic containers containing silica gel. The containers were labeled with the name of the county. The soil samples and nodules were placed in cool boxes containing ice packs at 4 °C. *Rhizobium tropici* (CIAT 899), the reference strain was acquired from the microbiological Resource Centre (MIRCEN), University of Nairobi and was used in the experiments as one of the positive controls

3.2 Soil Physico-Chemical Characteristics Analysis

The composite soil samples for physico-chemical analysis from each county were air dried, and crushed by use of a mortar and pestle before sieving with a 0.5 cm sieve to separate grass or plant residues. Five hundred grams of processed soil samples at 2

mm and 5 mm was carried to the Soil Science Laboratories, University of Nairobi for physico-chemical analysis.

The pH of the suspension was measured using Model 290-MK2 of the Pye-Unican pH meter as per the Okalebo *et al*., 2002 protocol. Soil texture was analysed by Hydrometer (Bouyoucos) method (1962).The soil samples organic carbon content was established by Walkley-Black method as described by Nelson and Sommers (1982). The exchangeable potassium in soil samples was determined using ammonium acetate as reported by Ingram and Anderson (1993). The plant and soil total nitrogen was determined using macro-Kjedahl method (Anderson & Ingram, 1989). Phosphorus in soil samples was analysed based on the calorimetric method (The Bray 2 method). These experiments were done in triplicates.

3.3 Isolation of Rhizobia

The rhizobia were isolated from intact root nodules as described by Somesagaren and Hoben, (1994). Flowing tap water was used to clean the intact root nodules collected to remove attached soil particles. Clean root nodules were surface sterilized for 1 minute using 70% ethanol before they were passed for 4 minutes in a 3% v/v solution of sodium hypochlorite. The nodules were then rinsed using sterilized distilled water five times (Somesagaren & Hoben, 1994).

The nodules were squashed in 100 µl distilled water using a pair of sterilized blunt-tipped forceps. Nodules suspensions were inoculated on yeast extract Mannitol agar (YEMA) and the plates were incubated at 28 °C in dark for 10 days. A single colony was sub-cultured following the growth on fresh YEMA medium by streaking.

3.4 Morphological Characterization of the Isolates

3.4.1 Colony Morphology

The rhizobia isolates were characterized based on colony morphology as described by Anaylem *et al*. (2018). YEMA media was used to sub culture isolated colonies and plates were incubated at 28 °C for approximately 3 – 10 days. The colonial morphology was evaluated and the following characteristics noted: colour (white or

cream), elevation (raised or convex), size (0.5-5 mm), margins (smooth/entire or irregular), form (circular, lobate) and mucus production (The intensity of production) (Anaylem *et al*., 2018).

3.5 Biochemical Tests of the Isolates

3.5.1 Gram Staining

Gram staining reaction was performed as outlined by Hoben and Somasegaran (1994). Using a sterile wire loop, a drop of sterile water was placed on a well labelled slide. The loop was sterilized, cooled and a tiny sample of the bacterial colony was emulsified in a drop of sterile water on a slide to create an emulsion. The smear was allowed to air dry. The slide was passed through a flame with the smear side up to fix the bacterial cells. The slide was placed on a staining rack and gently flooded with crystal violet for 1 minute. The slide was rinsed with tap water. The smear was de-colourized with acetone drop by drop until the acetone ran clear. The slide was rinsed with water then flooded with safranin for 45 seconds. The slide was tilted and gently rinsed with tap water using a wash bottle which was then blotted then examined under a light microscope with oil immersion.

3.5.2 Ability to Absorb Congo Red Dye

The isolated colony was streaked on a YEMA plate supplemented with 0.0025% Congo red (CR). The plate was incubated at 28 °C for 3 - 10 days and typical colonies of rhizobia absorbed no or little Congo red dye (Vincent 1970).

3.5.3 Bromothymol Blue (BTB) Reaction

The isolates were streaked on YEMA plate supplemented with 0.002% (w/v) bromothymol blue (BTB) to determine their ability to produce acid or base. The plates were incubated at 28 °C in the dark for 7 days and color change of the medium was observed after every 24 hours of incubation. The rhizobia isolates were characterized based on their ability to change the colour of the media. Acid or alkaline producer colonies turned media yellow or blue respectively (Vincent, 1970).

3.6 Physiological Characterization of the Isolates

54 isolates were physiologically characterized by evaluating their tolerance to different pH, temperature and salt concentration levels. The experiments were done in triplicates.

3.6.1 pH Tolerance

The rhizobia isolates were streaked onto YEMA media at pH 4, 6, 8 and 10, pH having been adjusted with 0.1 M HCl and 0.1 M NaOH for acid and alkaline pH respectively. All plates were incubated at 28 °C for 10 days along with inoculated control at pH 7. Growth rate was evaluated on a scale of 1-5 described by Anaylem *et. al*., (2018), where 1 = no growth, 2 = 1 - 20 colonies, 3 = 21 - 40 colonies, 4 = 41 - 60 colonies and 5 = more than 60 colonies.

3.6.2 Temperature Tolerance

Rhizobia isolates tolerance to temperature was evaluated on YEMA plates following incubation for 10 days at 20, 25, 30, 35, 40 and 45 °C. Control was incubated at 28 °C. The growth rate was measured using a scale of 1-5 as described by Rai *et al*., (2012), where 1 = no growth, 2 = 1 - 20 colonies, 3 = 21 - 40 colonies, 4 = 41 - 60 colonies and 5 = more than 60 colonies.

3.6.3 Salt Tolerance

Salt tolerance was tested by evaluation of growth of isolated rhizobia on YEMA media containing 0 - 5% (w/v) NaCl. The control plate was supplemented with 0.01% (w/v) concentration NaCl. Rhizobia isolates were incubated at 28 °C for 10 days. The growth rate was measured on a scale of 1-5 as described by Rai *et al*., (2012), where 1 = no growth, 2 = 1 - 20 colonies, 3 = 21 - 40 colonies, 4 = 41 - 60 colonies and 5 = more than 60 colonies.

3.7 Molecular Characterization

3.7.1 DNA Isolation

Genomic DNA was extracted from 48 isolates (six isolates were contaminated and were therefore excluded from this study) including CIAT 899 as the control using a cetyltrimethyl ammonium bromide (CTAB) protocol as described by Berrada *et al*., (2012). Each of the rhizobia isolate was grown in 5 ml yeast extract mannitol (YEM) broth on a shaker at 200 rpm until an OD_{600} nm of 0.8 was obtained. The rhizobia suspension was centrifuged at 13800 rpm for 15 minutes to pellet the cells. The supernatant was discarded and the pellet suspended in 540 µl of the DNA extraction buffer (5 ml 1M Tris Cl pH 8,10 ml of 1M EDTA, 2.5 ml of 5 M NaCl) with addition of 20 µl of(50 µg/ml)proteinase K. The volume was adjusted to 50 ml with sterile distilled water. 60µl of 10% SDS was added and the samples incubated at 65 ºC for 30 minutes. After incubation, 100 µl of 5 M NaCl and 80 µl of 2% cetyltrimethylammonium bromide (CTAB) were added and mixed thoroughly, then the cell lysate was incubated at 65 ºC for 10 minutes. The mixture was then cooled for 10 minutes at room temperature, followed by addition of 1 µl of RNAse to each sample and incubated at 37 ºC for ten minutes. The mixture was again cooled at room temperature for 5 minutes then centrifuged at 14000 rpm (MIKRO 200R Microliter Centrifuge- Hettich, Tuttlingen, Germany) for ten minutes resulting in a multi-layer solution. The upper supernatant from the heterogeneous mixture was pipetted into a sterile and labelled eppendorf tube. Then an equal volume of chloroform: Isoamyl alcohol (24:1) was added to the supernatant, gently mixed ,and allowed to settle for 5 minutes at room temperature before centrifuging for 10 minutes at 14000 rpm. The resulting supernatant was pipetted into a sterile labelled eppendorf tube. This step was replicated and an equal volume of ice - cold isopropanol was added to the final supernatant and precipitated overnight at -20 °C.

The precipitated solution was centrifuged at 14000 rpm for 10 minutes, the supernatant was discarded and the step repeated using 70% ethanol. The resulting pellet was then air dried by inversion of eppendorf tube on a paper towel for 1 hr at room temperature. The pellet was then re-suspended into 50 ul nuclease free water

and stored at -20 °C. The quality of the isolated DNA was confirmed using gel electrophoresis method (Vijay Kumari, 2012).

The DNA quantity and quality was determined by horizontal electrophoresis. Agarose gel was prepared in Tris borate-EDTA (TBE) buffer and the samples were loaded at 1:5 ratio of 6× loading dye. I Kb standard was loaded as a control and for estimation of the size of fragments. Gels were run on a Bio-rad gel electrophoresis system at 80 volts/cm for 45 minutes, Ethidium bromide was incorporated for visualization on UV transilluminator and photographed using a digital camera (Nikon).

3.7.2 The Enterobacterial Repetitive Intergenic Consensus- PCR (ERIC-PCR)

The enterobacterial repetitive intergenic consensus (ERIC) primers were employed in the amplification of the intergenic repeat sequences of the genomic DNA (Berrada *et al., 2012*; Irum *et al*., 2009). The primers of 5'-ATG TAA GCT CCT GGG GAT TCA C-3' (F) and 5'-AAG TAA GTG ACT GGG GTG AGC G-3' (R) were applied (Ranjbar *et al*., 2017). The sequences were amplified in a reaction volume of 20 µl comprising 4 µl of PCR pre mix, 1µl of each primer, 2 µl genomic DNA and 12 µl nuclease free water. Bio-rad thermal cycler was employed in amplification. The thermal cycler was programmed using the following program: 5 minutes at 94 ºC, 30 seconds with 35 cycles at 94 ºC, 1 minute at 44 ºC, 1 minute at 72 ºC and 8 minutes at 72 ºC final extensions. The amplicons were confirmed by use of ethidium bromide (1 mg/ml) stained, 2% (w/v) agarose gel electrophoresis before visualization on ultraviolet (UV) transilluminator.

3.8 Evaluation of Symbiotic Effectiveness

The 9 isolates demonstrating unique physiological characteristics were involved in the symbiotic efficacy studies. The Rhizobia isolates M2bii, M7b, V4, V11i and B6ii showed tolerance to low pH, V5ii and B2ii showed high temperature tolerance, V9i was highly tolerant to increased salinity and B2i was tolerant low pH and high temperature conditions. Pure 1ml of 72-hour culture was inoculated on cowpea K80 roots planted on plastic pots containing sterilized sand to authenticate the isolate as

root nodulating bacteria. Alcohol (98% v/v) was used to sterilize cowpea seeds for 30 seconds followed by treatment with 2% sodium hypochlorite for 2 minutes. The seeds were then washed thoroughly six times using sterile distilled water followed by I hour immersion in sterile distilled water. The seeds were placed in petri dishes containing moistened sterile cotton wool before placing them in appropriate growth chamber for 48 hours at 28 °C (Amanda *et al*., 2012), after which they were transferred to pots in three replicates set out in a complete randomized design (CRD). Treatments with nitrogenous fertilizer KNO_3 (70 µg N mL^{-1}) without rhizobia inoculation and treatment with *CIAT 899* were positive control and treatments without inoculation and lacking chemical nitrogen served as negative control group. The solution was applied to the plants that served as the positive control group weekly (Somasegaran & Hoben, 1994). After every 2 days plants were supplied with one litre of water and N- free nutrient solution applied on weekly basis (Aynalem *et al*., 2018). The experimental model was performed in a 12-hour photoperiod (light) green house. The plants were uprooted and root nodule evaluated after 50 days of growth and nodule number (NN) was recorded. The plants were dried in the oven for 48 hours at 70 ºC and dry matter of the shoots, roots and nodules were measured. The shoot nitrogen content of the plants was analyzed by Kjedahl method (Anderson & Ingram, 1993). Symbiotic efficiency (SE) was assessed as follows:

> SE = (Plant nitrogen content present in inoculated pots / Plant nitrogen content in nitrogen applied control) x 100. (Kawaka *et al*., 2014).

3.8.1 nifH Gene Sequencing and Phylogenetic Analysis

The products of PCR – amplified *nifH* gene for three isolates B6ii, V11i and V5ii with the highest symbiotic efficiencies of 179.6%, 143.35% and 127.3% respectively and three isolates M7b, B2ii and V9i exhibiting lower SE of 98.46%, 93.3% and 83.17% respectively lower than the positive controls were sequenced using DNA sequencer (ABI model 377, Applied Biosystems) at Kenyatta national hospital according to the instructions by the manufacturer. The nifH primer pair [PolF (TGCGAYCCSAARGCBGACTC)/ PolR (ATSGCCATCATYTCRCCGGA)] (Poly *et al*., 2001). Bio-Rad thermal cycler was used for amplification using the following

program: 5 minutes at 94 ºC, 45 seconds 30 cycles at 94 ºC, 1 minute at 62 ºC, 1.5 minutes at 72 ºC and a final extension of 5 minutes at 72 ºC. The PCR products quality was determined using gel electrophoresis and visualized on transillumination. PCR product was sequenced using this nifH primer pair. The obtained sequences were tested for integrity, pairwise aligned, and were edited using Bioedit software package before subjection to BLAST. The generated sequences were unanimously matched with those at National Center for Biotechnology Information (NCBI). Uninamous *nifH* gene sequences were compared online using CLUSTAL-W MEGA version 7.0 was used to generate phylogenetic trees (Kumar *et al*., 2016). The nifH sequences obtained in the study were submitted in the GenBank.3.9 Data analysis

The data from morphological and biochemical characterization used to generate a dendogram using 20 SPSS statistical program (Anaylem *et al*., 2018).

Each experiment for physiological characterization for 54 isolates was carried out in triplicates and the data was statistically analyzed. Dendrograms were constructed using 20 SPSS statistical program (Anaylem *et al*., 2018).

The amplified fragments sizes were determined by comparing with a 1 Kb standard. The fingerprints score was recorded as binary data: 0 = band absent and 1 = band present. Experiments were done in duplicates. The neighbor joining method with the Kimura -2- parameter model was applied in construction of dendrograms which were bootstrapped using the software programs in the MEGA 3.1 package. Bootstrap 100 replications was used to test the reliability of the branches. (Silva *et. al.,* 2012).

The nodule number (NN), root dry matter (RDM), shoot dry matter (SDM), nodule dry matter (NDM), shoot nitrogen content (SNC) and symbiotic efficiency (SE) means were subject to the Turkey's test for comparison, at significant level of ($p<0.01$) using the Gene stat Version 15 program. The means significance was tested for using the least significance difference means of (LSD) at $p < 0.05$ (Asei *et al.,* 2015; Camille *et al*., 2014). The obtained sequences were tested for integrity, pairwise aligned, and were edited using Bioedit software package before subjection to BLAST. Uninamous *nifH* gene sequences were compared online using

CLUSTAL-W (Gaby and Buckley, 2014). MEGA version 7.0 was used to generate phylogenetic trees.

CHAPTER FOUR

RESULTS

4.1 Soil Physico-Chemical Characteristics

4.1.1 Physico-Chemical Characteristics of Soils from the Study Sites

The physico-chemical properties of soils varied across farmers' fields in the three study sites (Table 4.1). The soil pH values were generally acidic and ranged from 4.9 to 6.7. The nitrogen levels in all the study sites were very low measuring 0.13%, 0.17% and 0.11% for Mumias, Busia and Vihiga, respectively. Potassium levels were high with a range of 1.3 - 1.7 $Cmolkg^{-1}$. Organic carbon was moderately low ranging from 1.09 to 1.71%. Available phosphorous levels were considerably low, with Vihiga and Busia Counties recording the lowest (8.7 ppm) and highest (9.5 ppm) levels, respectively. The textural classes of the study sites were sandy clay-loam for Mumias and Vihiga and sandy clay for Busia.

Table 4.1: Chemical Characteristics of the Soil Samples Collected from Three Study Sites in Western Kenya

Soil Properties	Mumias	Busia	Vihiga
Latitude	0.33N	0.46N	0.04N
Longitude	34.71E	34.10E	34.71E
No of isolates	28	17	11
pH (1:2.5 water ratio)	5.9	6.7	4.9
Total nitrogen (%)	0.13	0.17	0.11
Organic carbon (%)	1.71	1.87	1.09
Potassium (Cmol/Kg)	26	30	34
Phosphorous (ppm)	8.99	9.5	8.7
Sand (%)	65	55	64
Silt (%)	10	4	3
Clay (%)	25	41	33
Texture class	Sandy clay- loam	Sandy clay	Sandy clay-loam

Cmol/Kg and ppm represents Centimol per kilogram and parts per million, respectively.

4.2 Phenotypic Characterization of Rhizobia Isolates

A total of 54 isolates of rhizobia were obtained from root nodules of cowpea from three Counties in Western Kenya. The highest number of isolates (28) was from Mumias in Kakamega followed by 17 from Busia and 11 from Vihiga County. All the isolates were similar in appearance on YEMA media (Plate. 4.1). The colonies appeared white, elevated, circular with smooth margin, mucoid, glistening and 1 – 5 mm in diameter after 10 days of culture on YEMA media (Table 4.2). Samples from Vihiga County did not grow well on YEMA and this led to less number of isolates unlike from the Busia and Mumias.

Upon growth on CR-YEMA, the rhizobia colonies appeared white or pale pink, elevated, gummy and shiny with entire margins. On BTB-YEMA plates, both fast and slow growers formed circular and raised colonies. Different isolates gave different reactions on this media classifying the isolates into three groups. Out of the 54 rhizobia isolates, 28 (51.6%) turned the medium from green to blue (Plate 4.1) and were classified as alkaline producers. Twenty two (40.7%) of the rhizobia isolates turned the media yellow and were classified as acid producers, while four (7.4%) had a neutral reaction. Colony characteristics and dye absorbance ability of these isolates were summarized in Table 4.2.

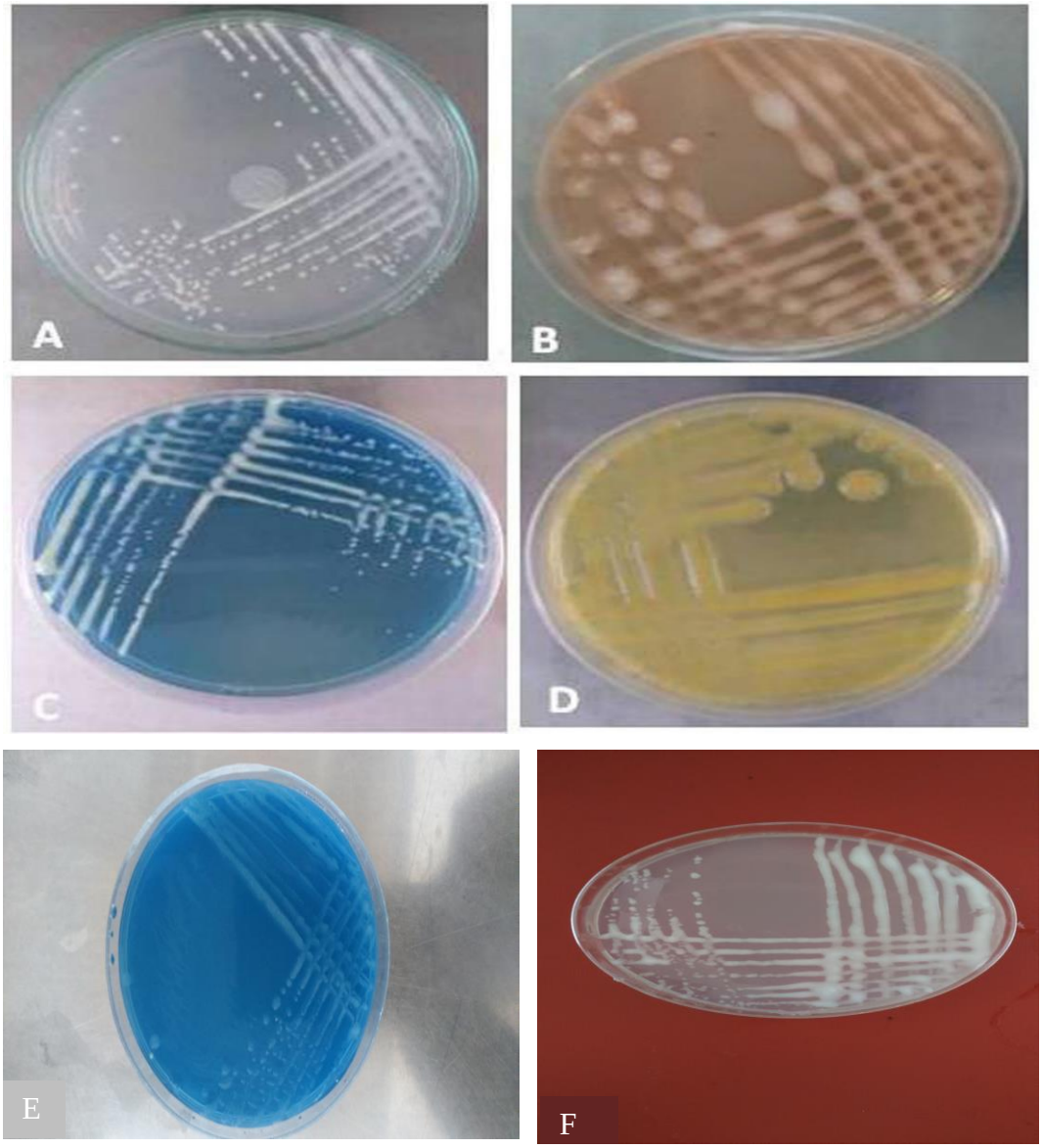

Plate 4.1: Morphological and Biochemical Characteristics of Rhizobia Isolates Obtained from Nodules Collected from Cowpea Plants in the Field

(A) Rhizobia isolate on YEMA medium; (B) rhizobia isolate on YEMA-CR; (C) alkaline producer rhizobia isolate on YEMA-BTB (D) acid producer rhizobia isolate on YEMA-BTB; (E) Neutral isolate on YEMA-BTB medium; (F) CIAT 899 isolate on YEMA.

Table 4.2: Origin, Morphological Characteristics and Biochemical Reactions of the Rhizobia Isolates

Origin	Isolate	Colony size (mm)	Colony appearance	Colour	Colony structure	Margin	EPS production	Colony color on CR-YEMA	BTB reaction
Mumias	M1ai	1.5	Shiny	White	Circular raised	Smooth	+	Pale pink	Neutral
	M1aii	3.0	Dull	White	Punctate raised	Smooth	+++	Pale pink	Neutral
	M1bi	1.0	Shiny	White	Circular raised	Smooth	++	Pale pink	Neutral
	M1bii	2.5	Shiny	White	Circular raised	Smooth	+	White	Neutral
	M2a	3.0	Shiny	White	Circular raised	Smooth	++	Pale pink	Acidic
	M2bi	2.5	Shiny	White	Circular raised	Smooth	++	White	Acidic
	M2bii	4.0	Shiny	White	Circular raised	Smooth	+	Pale pink	Acidic
	M2biii	1.5	Dull	White	Punctate raised	Smooth	+	Pale pink	Acidic
	M3ai	2.0	Shiny	White	Circular raised	Smooth	+++	Pale pink	Acidic
	M3aii	3.5	Shiny	White	Circular raised	Smooth	++++	White	Acidic
	M3bi	1.5	Shiny	White	Punctate raised	Smooth	++++	Pale pink	Acidic
	M3bii	3.0	Shiny	White	Circular raised	Smooth	+++	Pale pink	Acidic
	M4ai	2.0	Shiny	White	Circular convex	Smooth	+++	Pale pink	Acidic
	M4aii	3.5	Shiny	White	Circular raised	Smooth	++++	Pale pink	Acidic
	M4bi	2.5	Shiny	White	Circular raised	Smooth	+++	Pale pink	Alkaline
	M4bii	4.0	Shiny	White	Circular raised	Smooth	++	Pale pink	Alkaline
	M6a	2.5	Shiny	White	Circular raised	Smooth	+++	Pale pink	Acidic
	M6b	3.0	Shiny	White	Circular raised	Smooth	++	White	Alkaline
	M7a	2.0	Dull	White	Circular convex	Smooth	++	White	Acidic
	M7b	1.0	Shiny	White	Punctate raised	Smooth	+++	Pale pink	Alkaline
	M9	4.0	Shiny	White	Circular convex	Smooth	+++	White	Alkaline
	M10	3.0	Shiny	White	Circular raised	Smooth	++	White	Alkaline
	M11	1.5	Shiny	White	Circular raised	Smooth	+++	Pale pink	Alkaline
	M12	4.0	Shiny	White	Circular raised	Smooth	++	Pale pink	Alkaline
	M13i	3.5	Shiny	White	Circular raised	Smooth	++	Pale pink	Alkaline
	M13ii	2.0	Shiny	White	Circular raised	Smooth	+	Pale pink	Alkaline

Origin	Isolate	Colony size (mm)	Colony appearance	Colour	Colony structure	Margin	EPS production	Colony color on CR-YEMA	BTB reaction
	M14i	3.0	Shiny	White	Circular raised	Smooth	++	White	Acidic
	M14ii	2.0	Shiny	White	Circular convex	Smooth	+	White	Alkaline
Busia	B1i	1.0	Shiny	White	Circular convex	Smmoth	+++	Pale pink	Alkaline
	B1ii	3.0	Shiny	White	Circular convex	Smooth	+++	Pale pink	Alkaline
	B2i	1.0	Shiny	White	Circular convex	Smooth	+++	Pale pink	Acidic
	B2ii	2.0	Shiny	White	Circular raised	Smooth	+	Pale pink	Alkaline
	B2iii	3.0	Shiny	White	Circular raised	Smooth	+	Pale pink	Alkaline
	B3	1.0	Shiny	White	Circular convex	Smooth	+++	Pale pink	Alkaline
	B4i	1.0	Shiny	White	Circular convex	Smooth	++++	Pale pink	Alkaline
	B4ii	3.0	Shiny	White	Circular convex	Smooth	+++	Pale pink	Alkaline
	B5	3.0	Shiny	White	Circular convex	Smooth	+++	Pale pink	Alkaline
	B6i	2.0	Shiny	White	Circular convex	Smooth	+++	Pale pink	Alkaline
	B6ii	3.0	Shiny	White	Circular convex	Smooth	++++	Pale pink	Alkaline
	B7	2.0	Dull	White	Circular convex	Smooth	++	Pale pink	Alkaline
	B8i	1.0	Shiny	White	Circular convex	Smooth	++	Pale pink	Alkaline
	B8ii	2.0	Shiny	White	Circular convex	Smooth	+++	Pale pink	Alkaline
	B9	3.0	Shiny	White	Circular convex	Smooth	+++	Pale pink	Alkaline
	B10	2.0	Shiny	White	Circular raised	Smooth	+++	Pale pink	Alkaline
	B11	2.0	Dull	White	Circular raised	Smooth	+	Pale pink	Alkaline
Vihiga	V2	2.5	Shiny	White	Circular raised	Smooth	+++	Pale pink	Acidic
	V4	3.0	Dull	White	Circular flat	Smooth	++	White	Alkaline
	V5i	1.0	Shiny	White	Circular convex	Smooth	++	White	Acidic
	V5ii	2.0	Shiny	White	Circular convex	Smooth	++	Pale pink	Acidic
	V7	3.0	Shiny	White	Circular raised	Smooth	+++	White	Acidic
	V9	3.0	Shiny	White	Circular raised	Smooth	+++	Pale pink	Acidic
	V10	2.0	Shiny	White	Circular raised	Smooth	++	White	Acidic
	V11i	1.0	Shiny	White	Circular convex	Smooth	++	White	Acidic
	V11ii	3.0	Shiny	White	Circular raised	Smooth	+++	Pale pink	Acidic

Colony structure represents the form/shape and elevation of the colony, EPS, Exopolysaccharide/Mucus; +, low; ++, moderate; +++, high; and ++++, very high. BTB reaction: Alkaline - Blue, Acidic - Yellow and Neutral - Green

The dendogram obtained from the computer numerical analysis of phenotypic traits of the 54 cowpea nodulating rhizobia grouped the isolates into two clusters at 42% dissimilarity level. Cluster A diverged further at 32% disimilarity into sub cluster I and II. Sub cluster I had 19 isolates and M13ii isolate diverged into sub cluster II (Figure 4.1). Cluster B diverged at 28% disimilarity level into sub cluster I with 25 isolates and sub cluster II having 9 isolates. The clustering of the isolates was random and the most distinguishing characteristic is that cluster A isolates are low to moderate mucus producers while cluster B are high mucus producers.

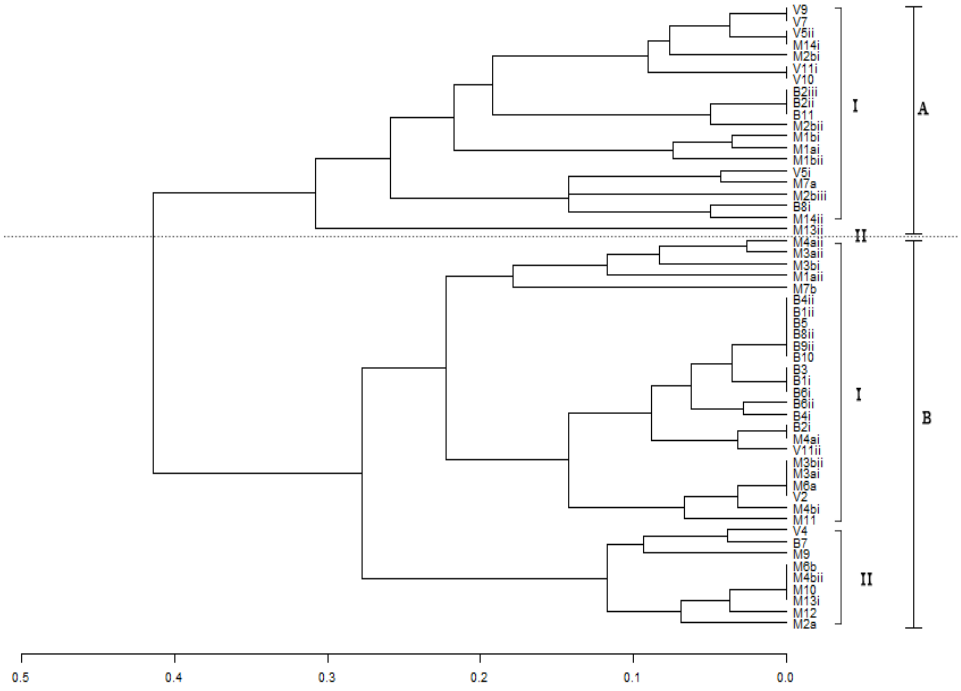

Figure 4.1: A Dendogram Highlighting Phenotypic Dissimilarity among the Rhizobia Isolates Extracted from the Cowpea Nodules on YEMA

4.3 Physiological Characterization

4.3.1 Diversity of Rhizobia Isolates Based on Tolerance to pH

The dendogram obtained from the computer numerical analysis of the 54 isolates based on pH tolerance placed the isolates into 2 distinctive clusters, cluster A and B at 57% dissimilarity level. Isolates in cluster A at 45% dissimilarity level were grouped into sub cluster I and II. Majority of the isolates (36) were placed in sub cluster I and 13 isolates in sub cluster II. Cluster B diverged at 18% dissimilarity level into sub clusters I and II. Sub cluster I had 4 isolates B8ii, B8i V7 and M7b while M7a was placed into sub cluster II alone (Figure 4.2). M7a thrived in acidic to slightly acidic conditions (pH 4 – 6) only, unlike the rest of the isolates which grew in neutral and alkaline conditions. Cluster A isolates (49) exhibited low growth in low pH while cluster B isolates (5) exhibited optimal growth in low pH conditions. The clustering was random and not geographical based.

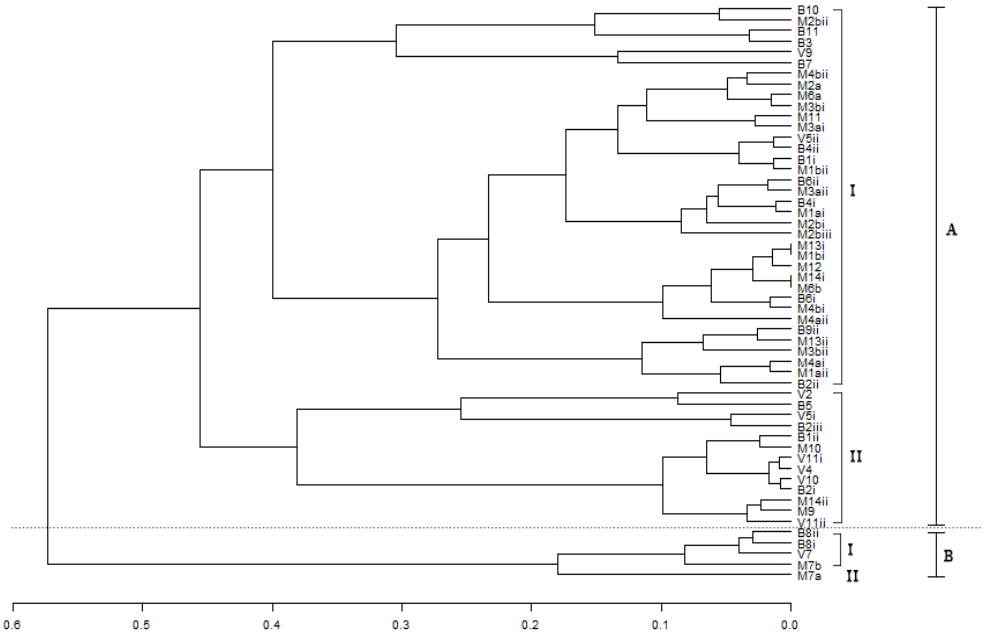

Figure 4.2: Dendogram Highlighting Dissimilarity among the Rhizobia on Media at Different pH

4.3.2 Variation of Rhizobia Isolates Based on their Tolerance to Temperature

The dendogram generated on tolerance of the isolates to different temperature levels clustered the isolates into A and B at 46% dissimilarity level. The isolates in cluster A diverged into sub clusters I and II at 39% dissimilarity level placing 30 isolates in sub cluster I and 4 isolates in sub cluster II. Cluster B diverged at a dissimilarity level of 20% into sub clusters I and II. 10 isolates falling into each sub cluster (Figure 4.3).

There are groups of isolates e.g. B8ii, B6i and B10, B3 and B7, B6ii and B9ii, B11 and M4bii, which showed 0% dissimilarity indicating 100% similarity of these isolates in their tolerance to temperature.

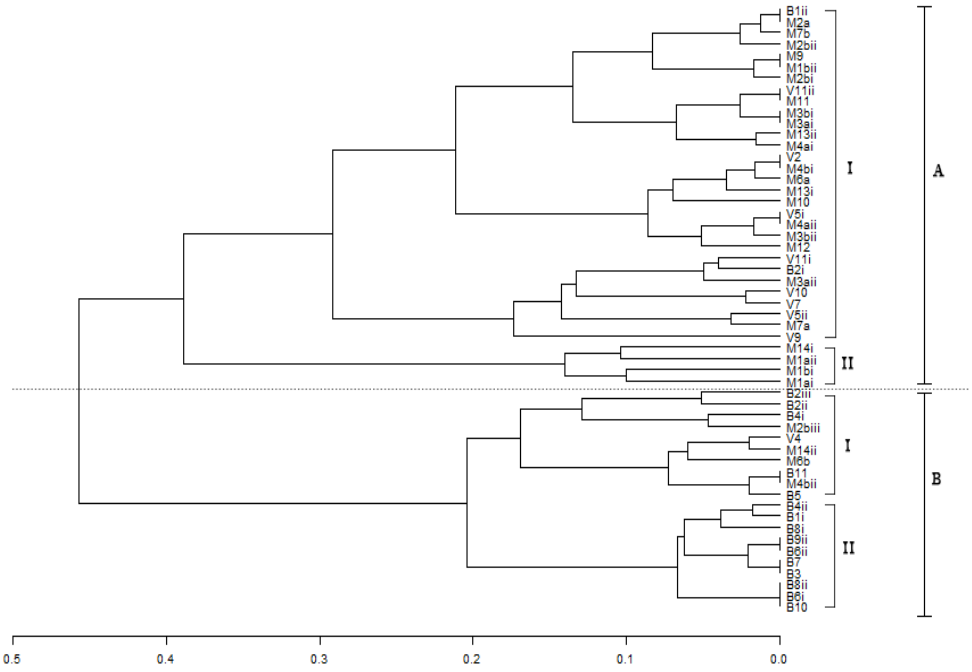

Figure 4.3: Dendogram Showing Dissimilarity among the Rhizobial Isolates on Growth at Different Temperature Conditions

4.3.3 Variation of Rhizobia Isolates Based on Tolerance to Salinity

The dendogram generated based on growth of the isolates in different sodium chloride concentrations grouped the isolates into cluster A and B at 65% dissimilarity level, 34 isolates falling into cluster A and 20 in cluster B (Figure 4.4). Cluster A further diverged at dissimilarity level of 39% into sub cluster I comprising of 27 isolates and sub cluster II having 7 isolates. M13ii diverged on its own due to its ability to grow both in absence and presence of up to 5% NaCl concentration. Cluster B grouped the isolates into sub clusters I and II at a dissimilarity level of 26%. Sub cluster I comprised of 12 isolates and sub cluster II of cluster B had 8 isolates which grew in 0.01-3% NaCl concentrations.

34 isolates in cluster A grew in 0-5% NaCl concentration while cluster B isolates (20) grew only in the absence and presence of very low (0.01%) sodium chloride. 5 groups of isolates B2i and M3bii, M3bii and M1aii, B7, B4 and B11, B1, M1ai, B4i, B8i, B8ii and B10 and B2ii, B1i, B3, B6i, B6ii and B9i showed 0% dissimilarity indicating 100% similarity in their tolerance to NaCl

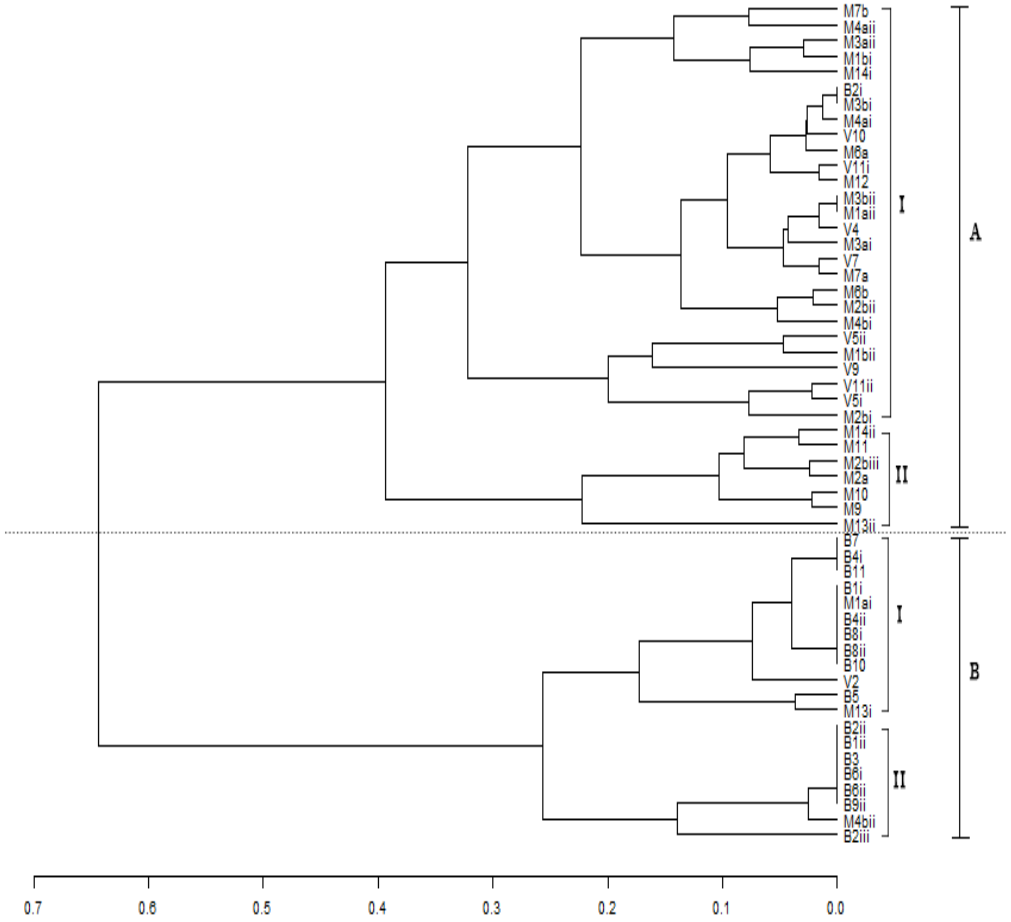

Figure 4.4: Dendogram Highlighting Dissimilarity among Rhizobial Isolates in Growth in YEMA Supplemented with Different Concentrations of NaCl

4.4 Analysis of ERIC-PCR Fingerprinting

Forty eight rhizobia isolates were amplified using ERIC primers and produced distinct and reproducible banding patterns (Fig. 4.5). The sizes of the amplified DNA bands ranged from 75 - 5000 bp and the total number of fragments was 1 - 12 depending on the isolate while the reference strain had 15 bands. The ERIC-PCR fingerprints showed that all the rhizobia isolates exhibited unique band profiles, indicating that they are different isolates. ERIC-PCR fingerprinting data for 48 isolates was used to generate the dendogram but only 14 isolates along with isolate

15 (reference control (CIAT 899)) were used in the ERIC-PCR electrophoresed gel (figure 4.5) .

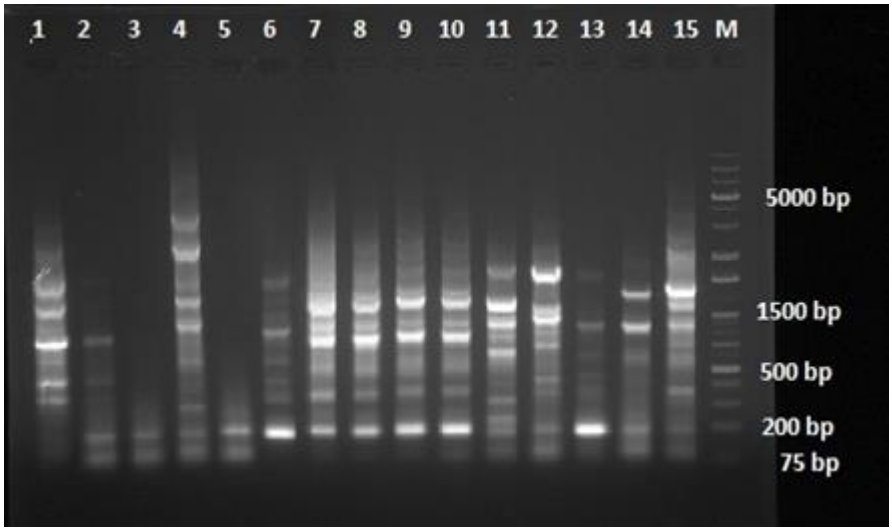

Figure 4.5: ERIC-PCR Amplification Profile of Different Rhizobia Isolates Separated on 2% Agarose Gel

Lanes 1 - 15 shows amplification profile of genomic DNA from rhizobia isolates V11i, V5i, V5ii, B7, B6i, B6ii, B2iii, M14ii, M3bi, M1bii, M7b, M6a, M11i, M13ii and CIAT 899 respectively. M shows 1 kb molecular marker.

The UPGMA dendrogram constructed from the banding patterns grouped the rhizobia isolates into two clusters A and B diverging at 53% similarity level (Fig. 4.6). Forty two isolates clustered in A along with the reference strain had 2-15 number of fragments amplified whose size ranged from 75-5000 bp while cluster B had only six isolates with 1-3 small fragments amplified, sizes ranging from 75-700 bp. All the six isolates in cluster B had a common 75 bp fragment. The clusters and sub-clusters were heterogeneous, and consisted of isolates from all the three Counties. The isolates in the dendrogram were not grouped based on the geographical regions of collection or tolerance to abiotic stresses, the grouping was random. Three pairs of isolates B2iii and V5ii, M3aii and V9 and V2 and V11ii displayed 100% genetic similarity in ERIC-PCR profiling. Isolates B2iii and V5ii had 3 fragments amplified of sizes 350bp, 200bp and 100bp, Isolates M3aii and V9 had four fragments amplified by the ERIC primer of size 900 bp, 300 bp, 250 bp and

100 bp while V2 and V11ii were genetically similar having only one fragment amplified of 75 bp.

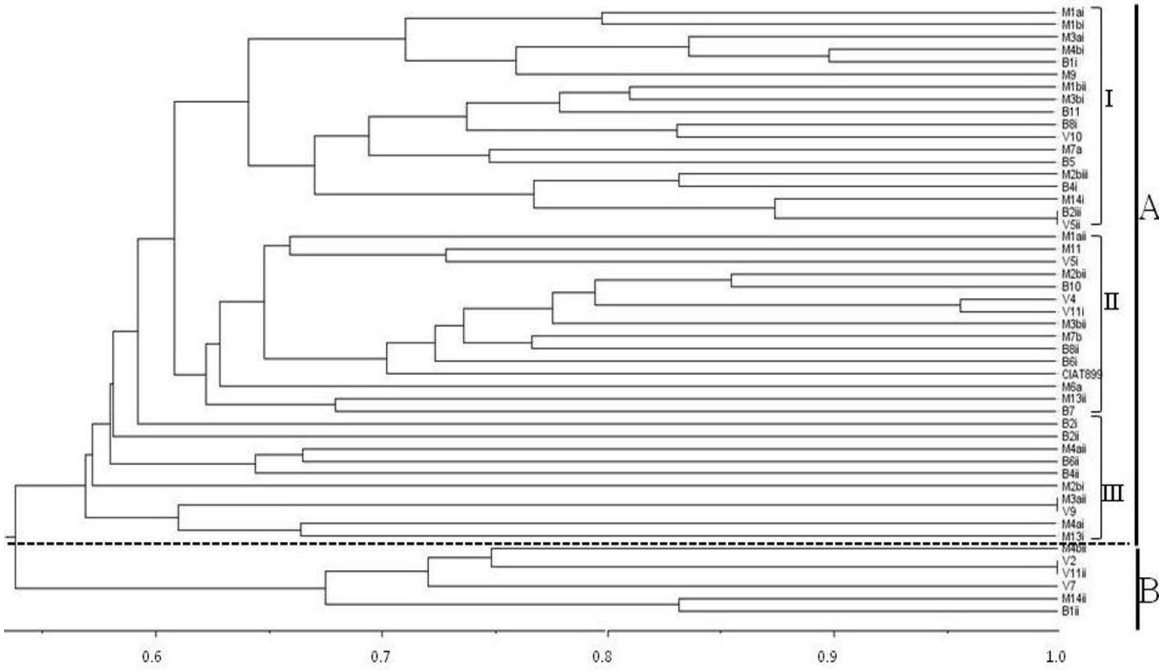

Figure 4.6: A Dendogram of ERIC-PCR Fingerprints of Rhizobium Isolates Nodulating Cowpea Variety K80 in Soils from Western Kenya and the Reference Strain CIAT 899 Constructed Using NTSYPC Software Package Version 2.10

4.5 Evaluation of Symbiotic Efficiency of Rhizobia Isolates Tolerant to Abiotic Stress

Upon re-inoculation of the host plant, all the nine isolates plus the reference strain CIAT 899 initiated nodulation and were authenticated as root nodulating bacteria. The 9 isolates showed variation in host plant nodulation with scores ranging from 2 to 94 nodules per plant. The means of the nodule number (NN), nodule dry matter (NDM), root dry matter (RDM), shoot dry matter (SDM), total nitrogen content (SNC) and the Symbiotic efficiency (SE) were compared by Turkey's test at 5% probability ($p<0.01$) and it was found that inoculation of the cowpea plant with rhizobia strains increased NN and NDM (Table 4.3). Highly significant ($P<0.001$)

differences were observed on NN, NDM, RDM, SDM, SNC and SE among the isolates tested.

Inoculation of cowpea by rhizobia strains was highly significant ($p<0.001$) in the increase of nodule number per plant. In this experiment all the nine isolates showed variation in the host plant nodulation with mean scores 1.67-94 nodules per plant (Table 4.3). Cowpea plant inoculated with B6ii, V4 and V11i produced higher number of nodules 94, 63.67 and 45 respectively compared with the plant inoculated with the reference strain CIAT 899 which produced 36 nodules. Six rhizobia isolates produced lower number of nodules in comparison to the reference strain.

Inoculation of the host plant with rhizobia isolate was highly significant ($p<0.001$) in the outcome of the nodule dry matter. All the rhizobia isolates increased the nodule dry matter significantly except B2ii in comparison to the reference strain. The mean nodule dry matter ranged from 0.073 to 0.2803 mg per plant. V4 inoculated plants recorded the highest NDM while B2ii the lowest NDM. CIAT 899 treatment produced second lowest NDM of 0.083 mg per plant.

The interaction between the rhizobia isolates and the cowpea plant was highly significant ($p<0.001$), and highly influenced the outcome of RDM (Table 4.3). Increased RDM means were observed in plants inoculated with rhizobia isolates compared to the reference strain which ranged from 0.215 to 1.93 mg per plant. Nitrogen supplemented plants showed the highest RDM means of 1.9297 mg per plant and lowest means of 0.215 mg per plant were observed in non-inoculated control. The reference strain CIAT 899 produced the second lowest RDM means of 0.391mg per plant.

The application of the treatments on the host plant significantly ($p<0.001$) affected the shoot dry matter. SDM means for all the treatments ranged from 1.099 to 6.139 mg per plant and non-supplemented control recorded the lowest and M7b inoculated plants recorded the highest SDM means. All the rhizobia isolate treatments scored higher SDM means than the CIAT 899 and the non-supplemented control. Three isolates M7b, B2ii and M2bii recorded higher SDM means compared to the nitrogen control.

Shoot nitrogen content was affected by inoculation of the rhizobia isolates on the host plant and the interaction was highly significant ($p<0.001$). B6ii inoculation influenced the fixation of the highest nitrogen content means of 1.985% and V9i gave the lowest SNC mean of 0.919%. Five isolates B6ii, V11i, B2i, V5ii and M2bii gave higher SNC means compared to the CIAT 899 and the nitrogen control. Four isolates V4, M7b, B2ii and V9i performed lower than the reference strain in nitrogen fixation and V4, M7b and V9i performed lower than the nitrogen control.

The interaction between the rhizobia isolate and the cowpea plant was highly significant ($p<0.001$) in the symbiotic efficiency of the plants. V9i isolate recorded the lowest SE score at (83.17%) and the highest SE score was observed in B6ii isolate inoculated plants at 179.64%. Five isolates B6ii, V11i, V5ii, M2bii and B2i were symbiotically more efficient than the reference strain while V4, M7b, B2ii and V9i were less efficient in reference to CIAT 899.

The SDM used as an indicator of relative effectiveness (expressed as percentage of shoot dry weight of each treatment compared with shoot dry weight of nitrogen supplemented control) indicated that B6ii was the most effective with SE of 179.6% (Table 4.3). The isolate V9i was found to be the least effective with 83.1% being lower than both reference strain and the negative control. The SE of the negative control was 84.1% of the dry weight of nitrogen control. The SE values for five isolates B6ii, V11i, V5ii, M2bii and B2i were more than that of CIAT 899 and their values were 179.6, 143.4, 127.3, 123.2 and 114.8% respectively and the reference strain recorded 109.3%. However, the SE values for V4, M7b, B2ii and V9i were lower than the chemical nitrogen control plants.

Table 4.3: Symbiotic Effectiveness of Selected Cowpea Rhizobia Isolates Under Greenhouse Conditions

Inoculation with Rhizobia	NN (plant^{-1})	NDM (mg plant^{-1})	RDM (mg plant^{-1})	SDM (mg plant $^{-1}$)	SNC (%)	SE (%)
K80 B6ii	94a	0.233ab	0.663f	3.569e	1.985a	179.6 a
K80 V4	63.67b	0.28a	1.299b	4.412c	1.028de	93.03h
K80 V11i	45c	0.263a	0.659f	3.255ef	1.584b	143.35b
K80 B2i	22.33d	0.198bc	0.810e	3.498e	1.268c	114.75e
K80 M7b	16de	0.096de	1.301b	6.139a	1.088d	98.46g
K80 V5ii	8.67ef	0.143cd	0.433g	3.092f	1.407c	127.33c
K80 B2ii	5.67fg	0.073ef	1.304b	5.168b	1.032de	93.39h
K80 V9i	2.33fg	0.0193fg	1.176c	3.949d	0.919e	83.17i
K80 M2bii	1.67fg	0.0153fg	1.076d	4.745c	1.361c	123.17d
CIAT 899	36cd	0.083de	0.391gh	3.022f	1.208cd	109.32f
K80 Ctrl	0g	0g	0.215h	1.099g	0.929e	84.07i
K80 Ntgn	0g	0g	1.93a	4.644c	1.105d	100g

Nodule number (NN), Nodule dry matter (NDM), Root dry matter (RDM), Shoot dry matter (SDM), total nitrogen content in shoots (SNC), control (Ctrl), nitrogen (Ntgn), and symbiotic efficiency (SE).

Means followed by same letters within column are not significantly different, by Turkey's test, at 5% probability

4.6 Molecular Identification of the Isolates

From symbiotic efficiency study, the *nifH* gene from the 6 cowpea rhizobia isolates B6ii, V11i and V5ii with the highest symbiotic efficiencies of 179.6%, 143.35% and 127.3% respectively and three isolates M7b, B2ii and V9i exhibiting lower SE of 98.46%, 93.3% and 83.17% respectively lower than the positive controls were amplified then sequenced with molecular identification determined using BLASTn tool of NCBI based on the sequence match with the reference strains.

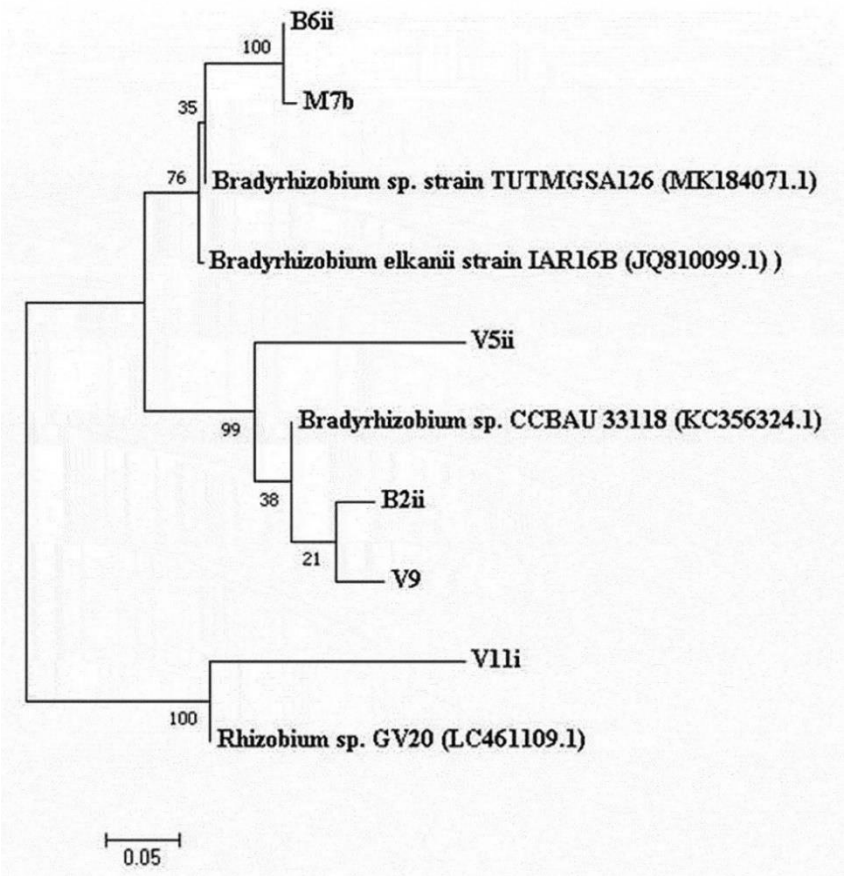

Figure 4.7: Phylogenetic Tree Generated on the Maximum-Likelihood Based on Cowpea Rhizobia Isolate nifH Gene Sequences

The importance of each branch is shown by the bootstrap value calculated for 1000 replicates. Scale bar indicates 5% substitution per site. GenBank accession numbers for the nucleotide sequences are given aside, B6ii - (MT948182), M7b - (MT948183) V5ii - (MT948184), V11i- (MT948185), B2ii- (MW218434) and V9i - (MW218435).

CHAPTER FIVE

DISCUSSION

5.1 Discussion

In this study, the pH of soils varied across the farms and ranged from 4.9-6.7 possibly due to leaching and fertilization rates. Lower pH increases solubility of Al, Mn, and Fe in soil bringing about toxicity to plants thus slowing or stopping root growth. Kawaka *et al*., (2014) reported that legumes require neutral or slightly acidic soil especially when they depend on symbiotic nitrogen fixation for growth. The soils nitrogen and organic carbon levels were lower than the recommended values by Okalebo *et al*., (2002), thus considered infertile. Soils from Vihiga have slightly low pH of 4.9 and possibly the reason for the low number of isolates since low pH has a detrimental effect on the growth of most rhizobia strains. Soil pH below 5.5 and high nitrogen values inhibit nodulation and nitrogen fixation (Koskey *et al*., 2017).

Growth of the isolates on YEMA produced white, mucoid, glistening and transparent colonies with entire margin confirming morphological characteristic of *Rhizobium spp*. (Plate 4.1). Congo red dye absorption (white to pale pink), elevated, gummy and shiny with entire margin colonies and Gram negative reaction during incubation are typical characteristics of cowpea nodulating rhizobia (Kawaka *et al*., 2014; Kaur et *al*., 2012).

The *Rhizobium* strains were further tested on BTB-YEMA plates and colonies produced were both fast and slow growers which were raised or convex, white, circular, with entire margins, 1-5 mm in diameter and this is in agreement with the findings of the study done by (Koskey et al., 2018) which says that the colony color was milky white, with entire margin but the colony elevation varied consistently with convex and raised colonies being observed on YEMA media**.** All slow growing *Rhizobium* species are alkali producers which turn bromothymol blue dye blue from green while fast growing *Rhizobium* species are acidic in nature and turn the dye in the media yellow (Kaur *et al*., 2012). In this study 28 isolates turned the media blue showing an alkaline reaction, 22 isolates produced acid reaction and 4 isolates gave a

neutral reaction. This observation is in agreement with the findings of Costa *et al*., (2014) and Kawaka *et al*., (2014) which indicate that different rhizobia species will give either acidic, alkaline or neutral reactions on BTB-CR media

Temperature, pH and salinity are among the abiotic stresses affecting morphological characteristics of rhizobia. The rhizobia isolates that can adapt widely to the environmental stresses (such as extreme pH, temperature and salinity) and maintain higher ability to fix nitrogen can be considered for inoculum development (Koskey *et al*., 2017)**.** In this study the optimum pH for rhizobia growth was observed to be 6-8. Twenty three isolates showed significant growth at pH 4 and most of the isolates thriving in this pH were from Vihiga County which had a soil pH of 4.9. This finding is similar to that observed by Ali *et al*. (2009) who reported the existence of acid tolerant fast growing strains. Further studies on strains nodulating cowpea (*Vigna unguiculata)* tolerant to pH as low as 4 (Kabede *et al.,* 2021) support the existence of acid tolerant fast growing strains. The tolerance of isolates from Vihiga County to acidic conditions in the media is probably due to their ability to grow in acidic soil and this trait makes them suitable for inoculum development for acid soils. (Emmanuel *et al*., 2017).

In temperature tolerance evaluation all the isolates grew optimally at 30°C and 35°C which disagrees with the findings of Harpreet *et al*., (2012) and Rodrigues *et al*., (2006) which reported optimum growth temperature for root nodulating bacteria as 25°C to 30 °C. The effect of increased temperature was clearly seen on the growth response of rhizobia as only 10 isolates showed significant growth at 40°C and above. This study clearly demonstrated that very high temperatures have a negative effect on the rhizobia populations which corroborates with the findings of Ali *et al*., (2009) and Kaur *et al*., (2012) where survival of the majority of Bradyhizobium strains reduced drastically above 40 ^{0}C**.** Sub cluster I of cluster A of the dendogram on temperature similarity of the isolates has two isolates (V5ii, M7a) which thrived in all the temperature ranges and this may be due to very high exopolysaccharide producing ability. The production of exopolysaccharide by the isolates could be an adaptive feature in providing protection to the bacteria against factors like salinity, temperature and pH fluctuations in soil. This supports the findings of Shraddha *et al*

(2013) and Kawaka *et al*., (2014) which reported that the production of exopolysaccharides (EPS) by the isolates could be an adaptive feature in providing protection to bacteria against factors like temperature, salinity, and pH fluctuations in the soil.

Tolerance to Nacl stress is complex and involves the bacteria's ability to tolerate and adapt to the changing environmental conditions. In this study decreasing growth was observed with increasing NaCl concentrations. This observation is similar to the report by Ali *et al*., (2009), Nagales *et al*., (2002) and Thrall *et al*., (2008) who reported that NaCl concentration may have negative effect on rhizobia as a result of direct toxicity and osmotic stress. There was optimum growth at 0 and 0.01% NaCl apart from V9 and V11ii which did not grow at 0% NaCl. Significant growth was noted at 1 and 3% NaCl concentration for majority of the isolates from Mumias and Vihiga County. The NaCl similarity dendogram classified the isolates into 2 clusters A and B. Cluster B had all the 20 isolates showing growth at 0 and 0.01% NaCl concentration but generally unable to grow in 1-5% NaCl. Thirty five bacterial isolates presented the ability to grow in different NaCl concentrations at different rates. This result is in agreement with the findings of other studies evaluating tolerance of rhizobia from pigeon pea (Costa, 2014), in which isolates grew optimally at 0 and 0.01% sodium chloride concentration and growth decreased with increasing concentrations of sodium chloride. Seventeen isolates grew at 5% salt concentration with V9, M1bi and M3aii showing the highest growth and these isolates were grouped in cluster A sub cluster I.

Genotypic characterization of rhizobia isolates revealed wide diversity among the isolates. The dendograms generated from morphological traits, physiological characteristics and ERIC- PCR fingerprinting were almost similar, grouping the isolates into 2 distinctive clusters despite the difference in the origin of the isolates. The clustering was random and not geograghical based and the difference was in parameter tested. Isolates from the same region were grouped in different clusters and isolates from different study sites were clustered together. This was distinctively exemplified by two isolates V9 and M3aii which were clustered together in all the dendograms despite being from different geographical locations except in salinity

tolerance dendogram. This finding is in corroborates with results obtained by (Sanja & Sulejman, 2003) stating that the dendrograms derived from PCR- -RFLP, rep-PCR and RAPD analysis were similar and this consistency provided confidence that strain grouping reflected true relationships among rhizobial strains tested

The dendogram constructed from the analysis of the DNA fingerprinting from the amplification of the repetitive DNA sequences by the ERIC primer generated two major clusters at 53% similarity indicative of the genetic diversity of the strains. The high degree of diversity in the rhizobia strains analyzed is possibly attributed to the promiscuous nature of the cowpea plant since a wide range of strains from the various *Rhizobium* genera have the ability to nodulate this plant species. This is supported by the findings of the study by Ligiane, *et al*. (2010) and Amanda *et al*., (2012) who observed the promiscuity of the cowpea plant species through the demonstration of high symbiotic and genetic diversity among the bacterial strains studied. The reference strain CIAT 899 diverged into cluster A which had 42 isolates.

The highest genetic similarity of 100% was observed in three pairs of isolates B2ii and V5ii, M3aii and V9, V2 and V11ii even though members of the first two pairs were isolated from soils of different Counties. Despite the high genetic similarity between these isolates, B2ii and V5ii, displayed a lot of morphological variations and only matched by the fact that both are Congo red dye absorbers. They displayed similar physiological traits which include ability to grow in neutral to near neutral pH (6-8) only, growth in all temperature ranges and growth in absence and in low NaCl concentrations. The major morphological differences between these isolates could be attributed to the environmental influence since they were isolated from different environmental conditions. This result strongly confirmed the other important feature of indigenous strains, their close association with environmental factors as reported by Sanja and Sulejman, 2003. M3aii and V9 displayed similarity in a number of physiological characteristics such as ability to grow in high NaCl concentrations, growth in high temperatures up to 40 °C and the acidifying ability on YEMA-BTB media. These isolates' ability to thrive in these conditions could be attributed to their high genetic similarity (Sanja & Sulejman, 2003). The isolates in the last pair V2 and

V11ii were isolated from the Vihiga County and were similar in their ability to grow in high alkaline conditions (up to pH 10) possibly due to their high genetic similarity. They also displayed very similar phenotypic (morphological and physiological) characteristics which may be attributed to environmental influence since both were isolated from the same County. ERIC-PCR profiles demonstrated high level of genetic diversity among the rhizobia isolates (Koskey *et al*., 2017, Sanja & Sulejman, 2003).

Inoculant strains characterized by high nitrogen fixation ability are usually applied in cowpea production. However in field conditions, inoculated strains of rhizobia are at a survival disadvantage by virtue of their low competitive ability for nodulation sites in comparison to indigenous soil strains. Moreover a major advantage of the indigenous strains is their adaptability to diverse edapho-climatic conditions (Ligiane *et al*., 2010). Analysis of the NN, NDM, SDM and SNC showed a lot of variability among the rhizobia strains inoculated plants and controls. The difference in the shoot dry weights and nodulation among the rhizobia strains could largely be due to the genetic composition of the isolates and the ineffectiveness of some strains under the enclosed and controlled green house conditions. This concurs with findings of the study done by Apunnu *et al*., (2008) and Musiyiwa *et al*., (2005) which reported significant differences between rhizobial strains for parameters such as nodule dry weight and N2 -ase, and total plant dry weight in soybean under growth room, green house and phytotron conditions.

The number of nodules had a highly significant influence on the shoot nitrogen content. Out of the 9 rhizobia isolates tested, 5 strains B6ii, V11i, V5ii, M2bii and B2i were highly effective in nitrogen fixation compared to CIAT 899 (reference strain) and nitrogen supplemented control. At harvest time inoculated cowpea had higher SDM compared to the controls an indication that the inoculated indigenous strains enhanced plant growth hence efficient in nitrogen fixation. This observation is in agreement with results of Kawaka *et al*., (2018) The symbiotic efficiency (SE) of native isolates varied and exhibited comparable or superior BNF compared to the local commercial inoculants (CIAT 899 and Strain 446). B6ii was observed to record the highest number of nodules (94) which fixed the highest level of nitrogen

(1.985%) far much higher than the controls. B6ii is significantly different from the rest of the other 4 effective strains in nitrogen fixation (V11i, B2i, V5ii and M2bii) and this is likely to be attributed to its high exopolysaccharide producing ability, ability to grow in acidic conditions and its alkalizing ability while the other three strains are acidifiers. This result is in agreement with the observation made by Kawaka *et al*., (2014) who reported the production of exopolysaccharides (EPS) by an isolate as an adaptive feature in providing protection to bacteria against factors like temperature, salinity, and pH fluctuations in the soil. Even though V4, M7b, B2ii and V9i induced nodulation, their symbiotic efficiency was lower than the reference strain and the nitrogen supplemented control. This result supports the finding that most of the soils are rich in less effective or ineffective rhizobia native strains and a solution to overcoming their uncompetitive nature is to compose and deliver legume inoculants (Simon *et al*., 2014; Onyango *et al*., 2015). V4 induced the second highest number of nodules (64) but performed lower than the reference strain CIAT 899 in SNC possibly due to the smaller number of nodules, and this concurs with the findings of Barauna *et al*., (2014), who attributed the lower performance of the isolate to the presence of many small nodules, apparently still in formation. Cultivation times longer than the tested 60 days may result in more fully formed. The size of the nodules in V4 isolate may be possibly increased by growth in acidic soils and in high temperature conditions since the isolate was extracted from acidic soils. 5 isolates were highly effective in nitrogen fixation and 4 were as good as the controls.

The superior ability of the isolated indigenous strains in this study compared to *CIAT 899* recommended as inoculant strain could have been due to presence of more effective and efficient isolated native strains. The occurrence of more effective *Rhizobium* strains in nitrogen fixation than *CIAT 899* has been reported in Eastern and Southern African soils indicating potential benefits of isolates from natural environments (Kawaka *et al*., 2014). Based on the differences in SDM and SNC of the inoculated and nitrogen supplemented plants as a measure of SE, 56% of the isolates were effective nitrogen fixers and 44% were as good as the positive controls.

Results on molecular identification of the isolates revealed the greatest sequence similarity with genus *Bradyrhizobium* and *Rhizobium*. Three groups were

established; Group A comprised of B6ii and M7b rhizobia isolates which showed similarity with reference strain *Bradyrhizobium sp.* Strain TUTMGSA126 (MK184071.1) and *Bradyrhizobium elkani* strain IAR16B (JQ810099.1) placed in a neighbouring branch. Three rhizobia isolates (V9i, B2ii and V5ii) of group B were observed in the same clade with reference strain of *Bradyrhizobium sp*. CCBAU33118 (KC356324.1). Group C comprised of one isolate (V11i) which was placed with reference strain *Rhizobium sp*. GV20 (LC461109.1) and therefore identified as a *Rhizobium sp*.

5.2 Conclusions

i. The cowpea rhizobia isolates from Western Kenya soils are morphologically different.
ii. The cowpea rhizobia isolates from Western Kenya are tolerant to abiotic stress.
iii. The cowpea rhizobia isolates from Western Kenya are genetically diverse because different isolates had different number of ERIC amplified fragments of varying sizes from 75bp - 5000 bp.
iv. The selected abiotic stress tolerant cowpea rhizobia isolates from Western Kenya soils are symbiotically efficient.
v. The symbiotically efficient cowpea rhizobia strains are Bradyrhizobia and Rhizobia species and therefore I reject the null hypothesis.

5.3 Recommendations

i. The identified symbiotically efficient rhizobia isolates should be further evaluated for tolerance to abiotic stress under field conditions.
ii. There is need to genetically characterize the identified selected symbiotically efficient rhizobia isolates using different markers, for example the enterobacterial repetitive sequences (REP).
iii. There is need to isolate and characterize rhizobia from other regions of the Western Kenya
iv. The selected symbiotically efficient abiotic stress tolerant isolates should be formulated into inoculants for cowpea production in Kenya

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APPENDICES

Appendix I: Preparation of Media

Preparation of YEMA media

Into 600 ml of distilled water in a 1 litre conical flask, 0.1g of NaCl, 0.2g of $MgSO_4.7HO_2$, 0.5 g OF K_2HPO_4, 10 g of mannitol and 1g of yeast extract was added then mixed. The pH was adjusted to 6.8 then 15 g of agar was added. The volume was adjusted to 1 litre and the media was sterilized at 121 °C for 15 minutes.

YEMA-CR media

1g of Congo red dye was dissolved in 400 ml of distilled water. 10 ml of the stock solution was added to make 1 litre of YEMA medium and the media was sterilized at 121 °C for 15 minutes.

YEMA-BTB media

0.5 g of bromothymol blue was weighed and dissolved in 100 ml of ethanol. 5 ml of the stock solution was added to make 1 litre of YEMA then sterilized at 121°C for 15 minutes.

Appendix II: Preparation of Reagents

2% CTAB extraction buffer

Into 300 ml of distilled water in a 500 ml flask, 50ml of 1M tris base (pH 8) was added. 10g of CTAB was added to the above solution slowly while stirring at 60 °C. 100 ml of 1M EDTA, 25 ml of 5M NaCl and 2.5ml of proteinase K was added. The volume was adjusted to 500 ml then labeled with name, strength and date.

0.5M EDTA (pH 8)

Into 700 ml of distilled water 186.1g of disodium ethylene tetra acetic acid was added. pH was adjusted to 8.0 with 10M NaOH and the volume was adjusted to 1 litre.

TE buffer (pH 8)

Into 400 ml of distilled water 5ml of 1M tris Cl (pH 8) and 1 ml of 0.5 ml EDTA was added. The volume was topped up to 500 ml then sterilized at 121 °C for 15 minutes.

10% SDS

10g of sodium dodecyl sulphate (SDS) was dissolved in 90 ml of distilled water. The volume was adjusted to 100 ml and the solution was autoclaved at 121 °C for 15 minutes.

Proteinase K

Into 5 ml of 10 mM tris Cl (pH 7.5) 100g of proteinase K was dissolved. 20 mM calcium chloride was added.

6X loading dye/buffer (10ml)

Into 7 ml of double distilled water 3ml of 100% glycerol was added. 0.0125g of bromophenol blue and 0.0125g of xylene / cyanol were added then mixed. The volume was adjusted to 10 ml.

10X Tris borate EDTA (TBE) buffer - (1000 ml)

The solution was prepared by dissolving 121.1g of tris base in 600 ml of distilled water. 55.6 g of boric acid was added then 40 ml of 0.5M EDTA. The pH of EDTA was adjusted to 8 for dissolution. The volume was adjusted to 1 litre.

Mixed indicator

Into 100 ml of ethanol 0.099 g of bromocresol green, 0.066 g methyl red and 0.011 g of thymol blue were dissolved.

Sulphuric acid and selenium powder

Into 1 litre of sulphuric acid 3.5 g of selenium powder was dissolved. By heating at 330 °C while covering the beaker with a watch glass. The originally blackish colour of selenium suspension turns via green blue to clear light yellow.

Digestive mixture

3.2 g of salicylic acid was dissolved in 100 ml of sulphuric acid selenium mixture. This mixture should not be stored for more than 48 hours.

Ammonium acetate (1M NH4OAc)

77.08g of NH_4OAc was dissolved in distilled water and then volume made to 1 litre. The pH was adjusted to 7 with acetic acid.

26.8% Lanthanum chloride solution

134g of lanthanum chloride ($LaCl_3.7H_2o$) was dissolved in distilled water and volume was made to 500ml in distilled water. 1ml contains 0.1 g La.

Potassium standard stock solution, 250 ppm K.

0.4678 g of KCl dried at 105 °C was weighed and dissolved in distilled water and volume made to the mark in a 1 litre volumetric flask. The solution contains 250 mg K/100 ml (250 ppm K).

Standard solution, 100 ppm K: 200 ml of the stock solution (250 mmpK) was diluted into a 500 ml volumetric flask and the volume was made up to mark with distilled water.

Potassium standard solutions: Into each 100 ml volumetric flasks the following amounts of standard solutions were added 0, 1.25, 2.5, 5.0, 7.5 and 10.0. To each flask 1 ml of 26.8% lanthanum chloride solution and 10 ml of the 1M NH_4OAc extraction solution was added.

Soil extracting solution or Bray P2 solution

300 ml of 1N ammonium fluoride solution and 2000 ml of 0.5 N HCl were added to 7700 ml of distilled water placed in a 10 litre container. This gives a solution of 0.03N ammonium flouride and 0.1N HCl which keeps for more than one year.

Ammonium molybdate/ Antimony potassium tartrate solution (mixed reagent)

12 g of ammonium molybdate (AR) was dissolved in 250 ml of warm (50) distilled water. 0.291 g of antimony potassium tartrate was dissolved in 100 ml of 5 N H_2SO_4. The two solutions were mixed thoroughly and then diluted to 2 litres with distilled water. The solution was transferred to a brown reagent bottle and stored in a dark, cool place. The mixture keeps for 2 months.

Ascorbic acid reducing agent

The solution was prepared by dissolving 1.054 g of ascorbic acid in 200 ml of ammonium molybdate. Antimony potassium tartrate solution (above) then mixed well. This must be prepared as required on the day of analyses. The solution keeps for about 24 hours.

250 ppm P standard stock solution

The solution was prepared by dissolving 1.0982 g of oven dry KH_2PO_4 (AR) into 1000 ml volumetric flask and making the volume to 100 ml mark with distilled water. The phosphorous content in this solution is 250 mg P per litre or 0.0250 mg ml^{-1} (250 ppm P)

Standards

The standards were prepared by pipetting 0, 1, 2, 5, 10, 15 and 20 ml of phosphate standard stock solutions (above) into 500 ml volumetric flasks. 100 ml of Bray 2 extracting solution was added then filled to 500 ml mark with distilled water. The standard series solutions contain 0, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 mg P per litre (ppm p).

Appendix III: Growth of Rhizobia Isolates in YEMA Media of Different pH Levels

Table 1: Growth of Rhizobia Isolates in YEMA Media of Different pH Levels

Isolate	PH 4	PH 6	PH 7	PH 8	PH 10
M1ai	+	++++	++++	++++	_
M1aii	+	++++	++++	++	_
M1bi	+	++++	+++	++++	+
M1bii	++	+++	++++	++++	+
M2a	+	++	++++	++	+
M2bi	++	++	++++	++++	_
M2bii	+	++	+	++++	_
M3ai	+	++++	++++	++	++
M3aii	+	+++	+++	+++	_
M3bi	++	++	++++	+++	++
M3bii	++	++	+++	++	+
M4ai	+	++++	+++	++	+
M4aii	_	+++	++++	++	+
M4bi	+	++++	++	++++	+
M4bii	+	+++	++++	+++	+
M6a	+	++	++++	+++	++
M6b	+	++++	+++	+++	+
M7a	+++	++	+++	_	_
M7b	+++	+++	++	+	+
M9	++	++++	+++	+++	+++
M10	++	+++	++++	+++	+++
M11	++	++++	+++	++	++
M12	+	++++	++	++++	+
M13i	+	++++	+++	++++	+
M13ii	+	+++	+++	+	_
M14i	+	++++	+++	+++	+
M14ii	+	++++	+++	++++	+++
B1i	++	+++	+++	++++	+
B1ii	+++	+++	+++	+++	+++
B2i	+++	++++	++++	++++	++++
B2ii	_	+++	+++	++	_
B2iii	_	+++	++	++	+++
B3	_	+	++	+++	+
B4i	++	++++	++++	++++	_
B4ii	++	++	+++	++++	++
B5	_	_	++	++++	++

Isolate	PH 4	PH 6	PH 7	PH 8	PH 10
B6i	_	++++	++	++++	_
B6ii	++	+++	+++	+++	_
B7	+	_	++	++++	_
B8i	+++	+++	+++	+++	+
B8ii	+++	+++	+++	++	++
B9ii	+	++	+++	+	_
B10	_	++	++	+++	_
B11	_	+	++	++	+
V2	_	+	++	++++	++++
V4	+++	++++	++++	++++	+++
V5i	_	+++	+++	+++	++++
V5ii	++	+++	+++	++++	++
V7	++++	++++	+++	++	+
V9	_	_	++++	+++	_
V10	++++	++++	+++	++++	++++
V11i	++++	++++	++++	++++	+++
V11ii	++	+++	+++	++++	++++

(-) = No growth, (+) = Slight growth, (++) = Moderate growth, (+++) = High growth, (++++) = Very high growth

Table 2: Growth of Rhizobia Isolates in Various Temperature Levels

Isolate	20 ° C	25 ° C	30 ° C	35 ° C	40 ° C	45 ° C
M1ai	+	+++	+++	+	_	++
M1aii	_	++	+++	++	_	+
M1bi	+	+++	++++	+++	_	+++
M1bii	+++	++	++++	++	_	_
M2a	+++	+++	++++	+++	_	_
M2bi	++	++	++++	++	_	_
M2bii	++	+++	++++	+++	_	_
M2biii	+	++	++++	++++	_	_
M3ai	+++	+++	+++	+++	_	_
M3aii	++++	++++	+++	++++	++	_
M3bi	+++	+++	+++	+++	_	_
M3bii	+	+++	+++	+++	_	_
M4ai	++++	++	+++	+++	_	_
M4aii	+	++++	+++	+++	_	_
M4bi	+	+++	+++	+++	+	_
M4bii	+	++	+++	+++	_	_
M6a	+	+++	+++	+++	++	_
M6b	+	++	++	+++	+	_
M7a	++++	+++	++++	+++	+++	+++
M7b	+++	++++	++++	+++	_	_
M9	+++	++	++++	++	_	_
M10	++	+++	++	++	+	_
M11	++++	++++	+++	+++	_	_
M12	+	++++	++	+++	+	_
M13i	+	+++	+++	++	+	_
M13ii	+++	++	+++	+++	_	_
M14i	+	++	++	+++	+	++
M14ii	+	+	+++	+++	+	_
B1i	_	_	++++	++++	_	_
B1ii	+++	+++	++++	+++	_	_
B2i	+++	+++	++++	++++	+++	+
B2ii	_	+	++++	+++	++	_
B2iii	+	+	++++	+++	+	+
B3	+	_	++++	+++	_	_
B4i	_	+++	++++	+++	_	_
B4ii	_	+	++++	++++	_	_
B5	++	++	+++	+++	_	_
B6i	_	+	+++	+++	_	_
B6ii	_	_	++++	+++	_	_

Isolate	20 ° C	25 ° C	30 ° C	35 ° C	40 ° C	45 ° C
B7	+	_	++++	+++	_	_
B8i	_	_	+++	++++	_	_
B8ii	_	+	+++	+++	_	_
B9ii	_	_	++++	+++	_	_
B10	_	+	+++	+++	_	_
B11	+	++	+++	+++	_	_
V2	+	+++	+++	+++	+	_
V4	+	+	+++	++++	+	_
V5i	+	++++	+++	+++	_	_
V5ii	++++	++++	++++	++++	++++	++
V7	++++	+	++++	++++	++	_
V9	+	+++	++++	++++	++++	_
V10	++++	++	+++	++++	++	_
V11i	++	++++	++++	++++	++	_
V11ii	++++	++++	+++	+++	_	_

(-) = No growth, (+) = Slight growth, (++) = Moderate growth, (+++) = High growth, (++++) = Very high growth

Table 3: Growth of Rhizobia Isolates in YEMA Media with Different NaCl Concentrations

Isolate	0%	0.01%	1%	3%	5%
M1ai	+++	++	_	_	_
M1aii	++++	+++	++	++	_
M1bi	+++	+++	++	+++	+++
M1bii	++	+++	++	+	++
M2a	++	++	++	++	_
M2bi	+	++	++	++	_
M2bii	++	+++	+++	+	_
M2biii	+++	++	++	++	_
M3ai	++++	+++	+++	+++	+
M3aii	+++	+++	++	++	++
M3bi	++++	++++	+++	++	_
M3bii	++++	+++	++	++	_
M4ai	++++	++++	+++	++	+
M4aii	++++	++	++	++	+
M4bi	+++	+++	++++	+	+
M4bii	++++	+++	_	_	_
M6a	++++	++++	++	++	+
M6b	++	+++	+++	+	+
M7a	+++	+++	+++	+++	_
M7b	+++	+++	+	+	+
M9	++	++	+++	++	+
M10	++	+	+++	++	+
M11	++	+	++	+	_
M12	+++	++++	+++	++	_
M13i	++	++	_	++	_
M13ii	+	+	+	+++	+
M14i	++	+++	++	+++	+
M14ii	++	++	++	+	_
B1i	+++	++	_	_	_
B1ii	+++	+++	_	_	_
B2i	++++	++++	+++	++	_
B2ii	+++	+++	_	_	_
B2iii	++++	+	_	_	_
B3	+++	+++	_	_	_
B4i	++	++	_	_	_
B4ii	+++	++	_	_	_
B5	++	++	+	+	_

Isolate	0%	0.01%	1%	3%	5%
B6i	+++	+++	_	_	_
B6ii	+++	+++	_	_	_
B7	++	++	_	_	_
B8i	+++	++	_	_	_
B8ii	+++	++	_	_	_
B9ii	+++	+++	_	_	_
B10	+++	++	_	_	_
B11i	++	++	_	_	_
V2	++	++	_	_	_
V4	++++	+++	+++	++	_
V5i	+	+++	+++	++	_
V5ii	+	+++	++	+	_
V7	+++	+++	+++	++	_
V9	_	++++	+++	+++	++
V10	++++	++++	+++	+	+
V11i	+++	++++	++	++	_
V11ii	_	+++	+++	++	_

(-) = No growth, (+) = Slight growth, (++) = Moderate growth, (+++) = High growth, (++++) = Very high growth

Appendix IV: Preparation of PCR Master Mix (50 samples)

Components	No. of sample	Total
4 µl of pre-mix	4 x 50	200 µl
1 µl of forward primer (ERIC 1)	1 x 50	50 µl
1 µl of reverse primer (ERIC 2)	1 x 50	50 µl
12 µl of nuclease free water	12 x 50	600 µl
18 µl		900 µl

Appendix V: Preparation of the Nutrient Solution

The nutrient solution consisted of 5 stock solutions containing in g/litre

0.1$CaCl_2$, 0.12 $MgSO_4.7H_2O$, 0.1 KH_2PO_4, 0.15 $Na_2HPO_4.2H_2O$, 0.005 ferric citrate and 1.0 ml

0.2 of trace elements stock solution.

The trace elements stock solution contained 2.86 H_3BO_3, 2.03 $MnSO_4.7H_2O$, 0.1 KH_2PO_4 and 0.15 $NaMoO_2.2H_2O$ in g/litre.

The pH of the solution was adjusted to 6.8 using NaOH (1.0M) or HCl (1.0 M)

All solutions were sterilized by autoclaving at 121 OC for 15 minutes (Fanuel K. et al., 2014)