POTENTIAL OF RHIZOBIA AND ARBUSCULAR MYCORRHIZAL FUNGI TO ENHANCE NITROGEN FIXATION AND GROWTH OF COWPEA GENOTYPES IN EASTERN KENYA

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DECLARATION

This thesis is my original work and has not been submitted for the award of a degree in any other university or any other award

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DEDICATION

This work is dedicated to my late father Benard Nyaga who was always my source of inspiration and strength for the better part of my academic journey and to my mother Jacinta Nyaga and siblings for continuously believing, supporting and encouraging me in every step.

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ABBREVIATIONS AND ACRONYMS

µl	Microliter
AFLP	Amplified Fragment Length Polymorphism
AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
ASL	Above Sea Level
BLAST	Basic Logical Alignment Search Tool
BNF	Biological Nitrogen Fixation
BTB	Bromothymol Blue
CRD	Completely Randomized Design
DNA	Deoxyribonucleic Acid
DNTP	Deoxynucleoside Triphosphate
EPS	Extracellular Polysaccharide
gDNA	Genomic DNA
HCl	Hydrochloric Acid
THSD	Tukey's Honest Significant Difference
KNO_3	Potassium Nitrate
KOH	Potassium Hydroxide

MPN	Most Probable Number
MPNES	Most Probable Number Enumeration System
N	Nitrogen
NCBI	National Center for Biotechnology Information
P	Phosphorus
PCR	Polymerase Chain Reaction
PPM	Parts per Million
RDA	Redundancy Analysis
rRNA	Ribosomal ribonucleic acid
RPM	Revolutions per Minute
RFLP	Restriction Fragment Length Polymorphism
SAS	Statistical Analysis Software
SC	Sandy Clay
SCL	Sandy Clay Loam
SD	Standard Deviation
SE	Symbiotic Efficiency
SL	Sandy Loam
TBE	Tris-Borate Ethylenediaminetetraacetic Acid

YEM	Yeast Extract Mannitol
YEMA	Yeast Extract Mannitol Agar

ABSTRACT

Semi-arid areas cover about 35% of the global landmass and support over 20% of the global population. Cowpea is an important crop in these areas due to its drought tolerance. However, its productivity is generally low owing to soil infertility, mainly due to nitrogen (N) and phosphorus (P) deficiency. Thus, cowpea associates with arbuscular mycorrhizal fungi (AMF) and rhizobia to supplement its P and N needs. However, low population of effective native rhizobia exists in these areas, which necessitates inoculation while the amount of native infective AMF propagules in the soil is highly variable. At the same time, the effective native rhizobia that can be used as cowpea inoculants in these areas remain unexplored, and the influence of rhizobia inoculation on native AMF association with cowpea remains unclear. The aim of this study was to determine the amount of AMF infective propagules and the influence of native rhizobia nodulating local cowpea genotypes on cowpea growth, production and AMF colonization on the smallholder fields. Soil samples were collected from fifteen smallholder farms across three semi-arid areas of Kenya (Embu, Kitui, and Tharaka Nithi counties) and assessed for physicochemical characteristics. The AMF infective propagules in the soil were estimated using the most probable number technique with Bermuda grass (*Cynodon dactylon*) as the trap host. Native rhizobia were trapped in the selected farms using three cowpea varieties (KVU 27-1, M-66 and K-80). Molecular identification of the isolates was done via the 16S rRNA gene sequencing using the universal primers 1492R and 27F. The symbiotic efficiency of the isolates was assessed relative to a commercial inoculant (Biofix), nitrogen supplemented control and uninoculated control. In the field, four rhizobial treatments, including native rhizobia, Biofix, native rhizobia + Biofix, and uninoculated control were tested. Field and greenhouse data were subjected to analysis of variance at a 5% level of significance. Rhizobial sequences were characterized based on bioinformatics tool, BLASTn and analysis of molecular variance and genetic differentiation computed using the Arlequin software version 3.5.2.2. The MPN values were related to the soil physicochemical characteristics using Redundancy Analysis. Results showed that the amount of AMF infective propagules varied in numbers (MPN values) across the fifteen farms. Based on the redundancy analysis, sand, clay and P were the most important soil parameters affecting the AMF-MPN values. Isolates were placed in twenty groups based on morphological characteristics. Thirteen groups initiated nodulation during authentication and belonged to the genus *Rhizobium*. Further assessment of their molecular diversity revealed a significant variation in the same population (County) but not among populations. In the greenhouse, 53.8% of the native isolates had a symbiotic efficiency of >80%, categorizing them as the most efficient isolates in nitrogen fixation. In the field, rhizobia inoculation significantly ($P < 0.05$) increased nodulation and shoot dry weight compared to the uninoculated controls. Native isolates led to the highest yield increase of 22.7% and 28.6% in season one and two, respectively. The performance of the cowpea genotypes was the same across all the rhizobia inoculants, although, it varied across different seasons and regions. Additionally, rhizobia inoculation significantly influenced AMF colonization only in the second season with no significant effect on individual cowpea genotypes in both seasons. The obtained native rhizobial isolates provide potential cowpea inoculants in the semi-arid areas compatible with the native AMF that can be used for sustainable cowpea production. Therefore, farmers in these areas should adopt rhizobia inoculation to boost the native rhizobia population and enhance cowpea production.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Cowpea [*Vigna unguiculata* (L) Walp], an indigenous leafy vegetable and grain legume, is widely grown in semi-arid areas of sub-Saharan Africa (SSA) (Da Silva *et al*., 2018). The crop is vital in these areas owing to its drought tolerance and ability to grow under water stress conditions, which are frequently experienced due to global climate change (Agbicodo *et al*., 2009; Carvalho *et al*., 2017). Cowpea production in Africa accounts for over 95% of the global production, thus serving as a major food source to millions of people globally (Samireddypalle *et al*., 2017). The crop provides nutritional and nutraceutical benefits, including quality and cheap dietary protein when consumed both as a vegetable and grain legume (Baptista *et al*., 2017). Considering its nutritional benefits and resilience under changing climate, cowpea is considered to have potential for alleviating food insecurity and malnutrition in SSA (Owade *et al*., 2020). Dual-purpose varieties also serve as a source of fodder for the livestock, making cowpea production an attractive venture to the farmers (Samireddypalle *et al*., 2017). When integrated into crop rotation systems, cowpea promotes the buildup of soil organic matter and carbon and nitrogen fixation. This, in turn, promotes soil fertility and improves soil physical characteristics such as water infiltration and retention capacity (Sánchez-Navarro *et al*., 2019a;2019b).

Growth and production of cowpea are greatly limited by adverse climatic conditions like drought and heat stress often occurring due to climate change (Farooq *et al*., 2017) as well as low soil nutrients (Oruru *et al*., 2018). Unlike drought, soil fertility in the semi-arid areas can be improved to enhance cowpea production. Nitrogen (N) and

phosphorus (P) are among the limiting nutrients in these regions, which adversely affect plant growth (Oruru *et al*., 2018; Kwena *et al*., 2019). Because the performance of cowpea largely depends on the rhizospheric characteristics, it can benefit from soil microbes existing naturally in the rhizosphere (Abdel-Fattah *et al*., 2016). Among these microbes are Arbuscular Mycorrhizal Fungi (AMF) and rhizobia which establish a tripartite symbiotic interaction with cowpea. This association with cowpea is not only crucial to meeting part of its nutrients need, but also key in enhancing plant tolerance to abiotic stresses (Ngakou *et al*., 2007).

Rhizobia are N fixing soil bacteria that inhabit the legume root nodules. They convert the atmospheric N to adequate amounts of ammonia, which are readily assimilated hence availing N to the plants (Leite *et al*., 2009). This reduces the reliance on inorganic fertilizers and the cost of production and provides a more sustainable technique for replenishing the soil N content. Leguminous crops also establish a symbiotic association with AMF, a symbiont in the phylum Glomeromycota (Schüßler *et al*., 2001; Muleta, 2017), which plays a key role in P absorption to meet the P needs of plants. This happens through AMF colonization on the host plant roots and the rhizospheric soils, leading to the formation of a hyphal network. The extraradical hyphae increase the capacity of plants to thrive under water stress due to the increased surface area for water absorption. This association with AMF enhances crop growth and survival under adverse and optimal growing conditions (Posta and Duc, 2019). Besides, AMF also enhances the N nutrition from the soil by contributing to the uptake of mobile nitrates.

Nitrogen and Phosphorus are essential nutrients whose deficiency can adversely affect crop productivity. This, coupled with the physical and biological characteristics of the

soil, significantly impact the agricultural output (Mabrouk *et al*., 2018). Therefore, for sustainable agricultural production, it is necessary to have efficient management of N and P, especially by using microorganisms that help fix and uptake these nutrients. More than 80% of the N utilized by plants in the agricultural setting comes from biological fixation through the legume-rhizobia symbiosis (Mabrouk *et al*., 2018). However, the effectiveness of rhizobia in fixing nitrogen in the soil is influenced by the genetic diversity of the rhizobia strains in the soil and the cowpea genotype (Leite *et al*., 2009). This is mainly because the rhizobia population greatly varies in composition and symbiotic efficiency in the different soils (Martins *et al*., 2003). Besides, efficient rhizobia inoculants can compete with the native rhizobia and the less efficient strains for attachment sites on the roots. This leads to nodulation induced by different strains occurring on the same plant, affecting the nitrogen supply to the plant.

Rhizobia inoculation has been reported to enhance soil nutrition leading to improved legume crops growth and yields (Ngakou *et al*., 2007; Takács *et al*., 2018). Nevertheless, in many smallholder agroecosystems, the indigenous rhizobia isolates are low in population or are not efficient in N fixation (Ngakou *et al*., 2007), necessitating inoculation with commercial isolates. That said, the compatibility between rhizobia inoculants and the host crop is vital since incompatibility has been linked to negative and neutral effects (Xavier and Germida, 2003; Wang *et al*., 2011). Host plant genotypes significantly influence this compatibility since the association between rhizobia and host plants have narrow specificity (Fauvart and Michiels, 2008). Therefore, the selection of rhizobia compatible with their host is key in optimizing the host nutrition for enhanced plant performance. This can be achieved by identifying the rhizobia strains forming the best association with cowpea with emphasis to the specific genotypes that have been bred to suit specific areas such as the adverse agro-ecological

and climatic conditions. Thus, the objectives of this study were to determine the diversity of native rhizobia and identify the effective isolates in enhancing nodulation, growth and production of cowpeas in the lower Eastern areas of Kenya where cowpea is mainly grown as well as the AMF infective propagules in these soils and how rhizobia inoculation influences the AMF colonization. The findings in this study will form a basis for modifying the soil physicochemical characteristics and management practices to enhance the AMF population and rhizobia diversity in the soil, respectively. Besides, it will inform the selection of rhizobia inoculant compatible with the existing AMF population in the arid and semi-arid areas for better cowpea performance

1.2 Statement of the problem

The arid and semi-arid zones cover about 35% of the global landmass and support over 20% of the worldwide population (Tchakerian, 2015). Most of these areas are highly degraded and eroded, hence underutilized due to low soil fertility, including N and P deficiency and drought. In Kenya, these areas experience prolonged drought periods and low rainfall amounts (Njoka *et al*., 2016). These create adverse growing conditions for the crops, affecting their growth and production, contributing to food insecurity (Huho and Mugalavai, 2010). Cowpea is highly suited for these areas. It is a major crop in the family farming systems in Kitui, Embu, and Tharaka Nithi counties, where it provides food and livelihood due to its drought-tolerance ability. However, it productivity in these areas continue to decline due to the poor nutritive value of the soil caused by soil degradation and the high cost of inorganic fertilizers, which limits the replenishment of the deficient nutrients.

Cowpea establishes a symbiotic association with native AMF and rhizobia, positively impacting its nutrition, growth and production (Ngakou *et al*., 2007; Silva *et al*., 2018). However, the existing native rhizobia strains in the rhizosphere do not effectively

promote plant nutrition, given the low number of efficient strains (Ngakou *et al*., 2007). This necessitates rhizobia inoculation to boost the activity of the existing native rhizobia population. Moreover, the specific native rhizobia strains interacting with locally grown cowpea genotypes in these areas remain uncharacterized, which could be exploited as rhizobial inoculants. In addition, the effect of using rhizobia inoculants on the performance of the locally grown cowpea genotypes that have been bred for these areas remains unclear.

Unlike rhizobia which associate only with legumes, AMF can associate with about 80% of plant families (Vlček and Pohanka, 2020). AMF also live in very unstable soils with low P, organic matter and water (Yang *et al*., 2008). Therefore, regions with soils having physicochemical parameters similar to these may be rich in AMF symbionts, hence only necessitating enrichment of rhizobia to improve the soil N content. However, the native AMF infection potential in these areas has not been explored. In addition, the influence of rhizobia inoculation on the indigenous AMF colonization on cowpea roots has not been established.

1.3 Justification

Cowpea, is a drought-tolerant crop suited for semi-arid regions. Improving soil fertility is imperative to enhancing its production to meet increasing food demands of the growing global population. Cowpea relies on rhizobia to fix N and AMF to absorb moisture and deficient nutrients (Ngakou *et al*., 2007). Numerous studies have reported the role of AMF in moisture and nutrient absorption (Mohammadi *et al*., 2011; Xu *et al*., 2018), suggesting that it may be an essential microorganism in the farming systems in Kitui, Embu and Tharaka Nithi counties, which experience frequent drought periods. Therefore, defining the native AMF infective propagules is imperative to unravelling the AMF potential to colonize the plant roots, which is vital in the absorption of the

soil, limiting nutrients and moisture. This is key in recommending AMF inoculation based on its level of existing AMF infectivity. Besides, it will help unravel how the changes in the physicochemical characteristic influence the AMF propagules in the soil. This will form a basis for modifying the soil physicochemical characteristics to enhance the AMF population in the soil.

With N deficiency also significantly limiting cowpea growth and yields, rhizobia inoculation is a sustainable technique in improving cowpea performance. However, considering the benefit of AMF in cowpea growth, it is important to determine the influence of rhizobia inoculation on native AMF association with cowpea. This is key in the selection of compatible rhizobia inoculant with the existing AMF population for better cowpea performance. This is because microorganisms in the soil function synergistically or antagonistically against each other, and the role of AMF in the host plants cannot be overlooked.

Besides, new drought-tolerant cowpea varieties such as K-80, M-66 and KVU 27-1 have been bred; hence, necessary to establish the effect of rhizobia inoculation on their growth and productivity. This will help identify the varieties that form the most effective association with the native rhizobia that can be recommended for the semi-arid areas. This is because cowpea is the most preferred legume in the semi-arid areas due to its ability to grow in relatively low soil moisture and drought tolerance (Njonjo *et al*., 2019). The use of 16S rRNA sequencing is key in identifying the specific rhizobia species associating with these cowpea varieties. Besides, understanding the genetic diversity of rhizobia in these areas is important for the management of such diversity.

1.4 Hypotheses

i. Soil physicochemical characteristics influence the AMF infective propagules in soils from Embu, Kitui and Tharaka Nithi counties

ii. Native rhizobia nodulating cowpea genotypes in Kitui, Embu and Tharaka Nithi counties are genetically diverse

iii. Native rhizobia isolated from smallholder farms of lower Eastern Kenya have different symbiotic efficiencies

iv. Rhizobia inoculation affects cowpea growth, production and native AMF colonization

1.5 General objective

To determine the diversity of native rhizobia and the effect of rhizobia inoculation on the nodulation, AMF colonization, growth and production of cowpeas in Embu, Kitui and Tharaka Nithi counties in Kenya

1.5.1 Specific objectives

i. To assess the effect of soil physicochemical characteristics on AMF infective propagules in Kitui, Embu and Tharaka Nithi counties

ii. To determine genetic diversity of native cowpea nodulating rhizobia in Kitui, Embu and Tharaka Nithi counties

iii. To evaluate the symbiotic efficiency of cowpea nodulating native rhizobia isolated from smallholder farms of lower Eastern Kenya

iv. To determine the effects of rhizobia inoculation on AMF colonization, cowpea nodulation, development and yield in smallholder farms of lower Eastern Kenya

1.6 Significance of the study

The most effective rhizobia species identified in this study can be used to develop rhizobia inoculants for cowpea in the semi-arid areas. These are expected to be well adapted to the adverse agro-ecological and climatic conditions experienced in the semi-arid regions. The inoculants can be introduced to the farmers as bio-fertilizers to improve soil nutrition for eco-friendly and sustainable cowpea production. In addition, the most compatible rhizobia inoculants with native AMF in relation to cowpea growth and yield can now be recommended to farmers to ensure maximum benefit on cowpea from its interaction with rhizobia and AMF.

CHAPTER TWO

LITERATURE REVIEW

2.1 Characteristics of cowpea

Cowpea [*Vigna unguiculata* (L) Walp] is a drought-tolerant leguminous crop. It is popular in the dry regions characterized by adverse conditions and declined legume crop yields, where it still achieves satisfactory performance (Singh *et al*., 2003; Ddamulira *et al*., 2015). The crop grows in low nutrient soils with low P content, low organic matter (below 0.2%) and sand content above 85% (Sanginga *et al*., 2000). Compared to other cultivated legume crops, cowpea is responsive to both favourable growth conditions and unfavourable ones, including heat and drought (Timko *et al*., 2007). Besides, it is shade tolerant hence can be intercropped with other cultivated crops maximizing production where land is scarce.

Cowpea germinates within 3 to 5 days. It has a growing season of fewer than 60 days for early maturing varieties, while the late-maturing types can take over 150 days depending on the photoperiod. The rapid growth of cowpea and its deep roots enhance the cowpea adaptability to hostile growing environments (Muli and Saha, 2008). The plants have a principal root with numerous lateral and adventitious roots spreading in the rhizosphere. Most of its accessions have an indeterminate stem that is smooth or slightly hairy and can grow up to 4 m. Depending on the genotype, cowpea has different growth forms dependent on the growth conditions and photoperiod. These growth forms include bushy, erect, trailing and climbing (Timko *et al*., 2007). The leaves are dark green and are usually alternate or trifoliate, with the first set of leaves being simple and alternate. It forms bisexual flowers borne on racemes that are self-pollinated or cross-pollinated. A distinguishing feature in cowpea is the presence of long peduncles

accommodating two or three pods or even more pods if the growth conditions are favourable, which facilitates hand harvesting of cowpea (Timko *et al*., 2007).

2.2 Importance and uses of cowpea

Cowpea has numerous economic, nutritional, agronomic, social and environmental benefits (Da Silva *et al*., 2018). Consumed across the globe, it is a source of food to millions of people providing both nutritional (dietary proteins and carbohydrates), and nutraceutical benefits (fibre, antioxidants, polyphenols and polyunsaturated fatty acids) (Phillips *et al*., 2003; Trinidad *et al*., 2010; Vilakati *et al*., 2016; Baptista *et al*., 2017). Cowpea leaves and grains are rich in proteins, with the protein content ranging between 17 and 43% in leaves and 21 and 33% in the grains (Santos *et al*., 2012; Ddamulira *et al*., 2015). Thus, cowpea acts as a quality and cheap protein to the urban and rural dwellers in many parts of Africa. In developing countries, cowpea provides a cheap source of nutrient-rich food contributing to food security. In developed countries, cowpea offers healthy substitutes to soya bean and meat due to its high fibre and low-fat content (Timko and Singh, 2008). With all the benefits associated with cowpea, it has been recommended as one of the crops with the potential to alleviate food insecurity and malnutrition in SSA (Owade *et al*., 2020).

Apart from providing food for human consumption, haulms of dual-purpose varieties serve as a source of fodder for livestock. (Samireddypalle *et al*., 2017). This has become an attractive venture to the farmers, especially during the dry seasons when fodder scarcity is severe. This is considering the ability of cowpea to withstand drought, hence providing a constant fodder supply regardless of the climatological influence. Cowpea also contributes to soil fertility enhancement where no or minimal fertilizers are used by fixing the free atmospheric N_2 to a form that plants can efficiently utilise through biological nitrogen fixation (BNF) (Munjonji *et al*., 2018). Similarly, cowpea in the

crop rotation systems with cereals enhances the soil N hence meeting their own and subsequent cereal crop N requirements (Namatsheve *et al*., 2020). This cowpea ability to fix N ensures a sustainable supply of N in the soil, which could potentially replace N fertilization. Besides, it promotes the buildup of soil organic matter and carbon fixation. This, in turn, promotes soil fertility and improves soil properties such as the water infiltration and retention capacity of the soil (Da Silva *et al*., 2018). The fast growth and rapid ground cover of cowpea help protect the soil from wind and water erosion. Simultaneously, the decomposition of its residues in situ releases N rich residues in the soil refining the soil structure and fertility (Zougmore *et al*., 2000; Singh *et al*., 2003). In addition, considering the cowpea fast-growth, it provides a constant food supply and a source of livelihood to many cowpea growers and its produce and products traders (Langyintuo *et al*., 2003).

2.3 Cowpea production in Kenya

Cowpea originated from Central and West Africa, then spread to parts of Asia and America (Owade *et al*., 2020). Today, cowpea is produced in four continents, including Africa, which comes first in production, followed by Asia, then America, and Europe, where it is regarded as a minor crop (Carvalho *et al*., 2017). In East Africa, Kenya is the leading country in cowpea production, with 227,809 hectares under cowpea production (FAOSTAT, 2019). It has recorded a steady increase in cowpea production over the years. However, this has been attributed to the increased land under cultivation and not increased output per land area (FAOSTAT, 2019; Owade *et al*., 2020). This trend has been brought about by the frequent drought period, which has seen the farmers embrace cowpea farming more due to cowpeas ability to tolerate drought.

In Kenya, cowpea has been classified as the second most important legume crop after common beans (Muli and Saha, 2008). It is widely grown in the semi-arid regions of

Kenya consisting of 85% cowpea production area due to its ability to grow and produce in dry regions (Njonjo *et al*., 2019). Most of the cowpea production in Kenya is in the Eastern region, which accounts for more than 85% of the total cowpea produced in the country. The rest are produced in Western, Nyanza, Coast and Central parts of Kenya (Muli and Saha, 2008). The Cultivation of cowpea in these regions is mainly under small scale where it is intercropped with other cultivated crops, including sorghum, maize and millet (Kimiti, 2011).

Improved varieties and landraces have been widely utilized in cowpea production in Kenya. The common cowpea varieties that are commonly grown in Kenya include *Kaima-koko, Nyekundu, Macho, Khaki, Mwandato, Nyeupe,* KVU 27-1, KVU 419, Katumani-80 (K80) and Machakos 66 (M66) (Kimani *et al*., 2014; Ndiso *et al*., 2016; Oyoo *et al*., 2017; Nderi and Kamau, 2018). Katumani-80, KVU 27-1 and M66, which are drought-tolerant dual purpose varieties, are widely grown in semi-arid areas of Eastern Kenya. These varieties have a semi spreading growth habit and are suitable for both leaf and grain production. M66 forms purple flowers, while K80 and KVU 27-1 flower are purple-blue. While young pods in all varieties are green, they turn bright red and brown purple during grain filling and when dry, respectively, in M66. Mature pods turn white-brown and white in K80 and KVU 27-1, respectively. Under suitable growth conditions, all three varieties have a yielding potential of 800-1800 kilograms (kg) per hectare (ha) (Kimani *et al*., 2014; Oyoo *et al*., 2017).

However, cowpea production in Kenya faces several challenges, including adverse climatic conditions that lower the output. In addition, declining soil fertility due to erosion and over cultivation of land and the inability of the farmers to replenish the lost nutrient due to the high cost of fertilizer has continued to affect the cowpea yields

adversely. The use of low-quality seeds or varieties not well adapted for these regions has also contributed to declining cowpea production. Low-quality seeds have been attributed to farmers using certified seeds in one season and recycling the harvested grain over time, negatively affecting the seed quality and, subsequently, yields (Njonjo *et al*., 2019). In Kenya, the legume is produced under a small scale set up, limiting the total production at any given time (Ndungu *et al*., 2018).

2.4 Legume – rhizobia interaction

2.4.1 Rhizobia taxonomy and classification

Rhizobia consist of gram-negative bacteria in the rhizosphere that can form symbiotic interactions with leguminous crops. Many legume crops associate with different rhizobia strains in diverse taxonomic groups (Wolde-Meskel *et al*., 2004; Boakye *et al*., 2016). The assessment of these diverse rhizobia groups has helped link their species and genera and understand their symbiotic efficiency. However, the classification of rhizobia remains complex. It is occasionally revised to accommodate new findings that propose new genera or species. Rhizobia belongs to the order *Rhizobiales* and families *Rhizobiaceae* and *Bradyrhizobiaceae* (https://lpsn.dsmz.de/order/rhizobiales). To date, over 98 species of rhizobia distributed over 14 genera have been identified (Berrada and Fikri-Benbrahim, 2014). Rhizobia comprises the genera *Azorhizobium, Pseudomonas, Methylobacterium, Bradyrhizobium, Ochrobactrum, Rhizobium, Phyllobacterium, Cupriavidus, Devosia, Microvirga, Mesorhizobium, Burkholderia, Shinella* and *Sinorhizobium,* also referred to as *Ensifer* (Weir, 2011; Berrada and Fikri-Benbrahim, 2014; Mousavi, 2016). Out of the 14 genera, *Cupriavidus* and *Burkholderia* are in class beta proteobacteria, while *Pseudomonas* is in the class γ-rhizobia. The rest of the genera are in class alphaproteobacteria (Weir, 2011). Due to the genus *Burkholderia* harbouring phytopathogenic, clinically relevant bacterial species and

environmental species, including rhizobia, recommendations have been made to subdivide the genus into two. This has led to the birth of a new genus *Paraburkholderia* accommodating rhizobial species previous classified in the genus *Burkholderia* (Sawana *et al*., 2014). Thus, rhizobia species are classified in the genus *Paraburkholderia* while *Burkholderia* genus will encompass phytopathogenic and clinically relevant bacterial species.

2.4.2 Diversity of rhizobia nodulating cowpea

2.4.2.1 Morphological diversity of rhizobia

Various methods have effectively identified symbiotically effective rhizobia strains with a high N fixing ability, including morphological characteristics and biochemical assays. Although rhizobia have a narrow specificity for cowpea, cowpea genotypes have a discriminating effect on the rhizobia communities in the rhizosphere. Some isolates have specific characteristics associated with certain cultivars and not others (Leite *et al*., 2009). Therefore, in identifying effective rhizobia isolates nodulating cowpea with potential for use as bio inoculants, morphological and biochemical characterization of the isolates is a prerequisite. Morphological characteristics used to characterize rhizobia include the colour of the colonies on the media, extracellular polysaccharide (EPS) production, Congo red dye absorption, size, shape, transparency and margins of individual colonies (Ondieki *et al*., 2017). According to Ondieki *et al*. (2017), rhizobia colonies are either white or yellow. However, it is rare to find yellow rhizobia colonies (Soares *et al*., 2014). In addition, rhizobia do not absorb Congo red dye, which is a distinctive characteristic of rhizobia. However, the age of the rhizobia isolates or exposure to light may lead to low absorbance of the dye giving the rhizobia colonies a pale pink appearance (Somasegaran and Hoben, 1985; Somasegaran and Hoben, 1994).

The rhizobia colonies can be round or irregular in shape with EPS or no EPS production. The EPS production is an adaptive feature for rhizobia against fluctuating temperatures and edaphic conditions such as saline and acidic soil. It protects the rhizobial cells from desiccation during seasons of extreme temperatures (Batista *et al*., 2007). This ensures the rhizobia remains viable and infects legume plant roots, leading to increased nodulation and subsequent N fixation.

Gram staining of the isolates and the determination of pH change in the media are also widely used in rhizobia characterization. Rhizobia isolated from cowpea nodules are gram-negative, and the cells are rod-shaped. However, both acid and alkali producing rhizobia associate with cowpea (Ondieki *et al*., 2017). When grown in yeast extract mannitol agar (YEMA) media enriched with bromothymol blue (BTB) dye, acid-producing rhizobia change the media from green to yellow. In contrast, the alkali production turns the media from green to blue (Leite *et al*., 2009). The acid-producing are fast-growing while the alkali producing are slow-growing. The fast-growing feature is associated with rhizobia in arid and semi-arid areas as a survival strategy enabling them to tolerate abiotic stresses and multiply rapidly over a short time (Martins *et al*., 2003; Borges *et al*., 2010). However, morphological and biochemical techniques of identification are prone to errors due to morphological plasticity. Thus, other precise methods are used to identify and determine rhizobia diversity efficiently.

2.4.2.2 Molecular diversity of rhizobia

The use of molecular techniques enables identification of rhizobia strains associated with cowpea and settles the differences between species with the same phenotypic characteristics. Rhizobia nodulating cowpea belong to the genera *Bradyrhizobium*, *Rhizobium, Burkholderia* and *Sinorhizobium* (Pule-Meulenberg *et al*., 2010; Guimarães *et al*., 2012; Chidebe *et al*., 2018; Degefu *et al*., 2018; Mohammed *et al*., 2018).

Molecular tools such as restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR) – RFLP (Boakye *et al*., 2016), amplified fragment length polymorphism (AFLP) (Wolde-Meskel *et al*., 2004), PCR fingerprinting (Ondieki *et al*., 2017) and ribosomal DNA (rRNA) sequencing efficiently differentiate among the rhizobia genera, species and strains (Giongo *et al*., 2008).

The use of 16S rRNA excludes any ambiguity during the morphological characterization providing an easy and effective approach to elucidate genetic relationships between rhizobia species (Woo *et al*., 2003). This also helps to correctly characterize the rhizobia isolates with a high nitrogen-fixing capacity and establish their diversity in different environments.

2.4.3 Importance of rhizobia in legume production

Rhizobia have attracted much attention in the past decades because of their potential in contributing to sustainable legume production by improving soil fertility (Santos *et al*., 2019). Legume crops benefit from rhizobia through a symbiotic interaction between the crop and one or more rhizobia strains. Upon infection, rhizobia benefit by obtaining nutrients from the root exudates of the host legume crop. In exchange, the rhizobia play a critical role in biological nitrogen fixation (BNF), contributing to more than 80% of the N used in agricultural production (Mabrouk *et al*., 2018), where they fix the free atmospheric N to ammonia (Figure 2.1). In cowpea, a survey done on the farmers field established that up to 66 and 99% of N is derived from this interaction in Botswana and Ghana, respectively (Pule-Meulenberg and Dakora, 2009; Naab *et al*., 2009). Unlike inorganic N that is prone to leaching, volatilization and denitrification, BNF has N directly utilized by the plant (Sainju, 2017). Nevertheless, the benefits associated with rhizobia are, to a great extent, influenced by rhizobia strain and environmental

conditions. Under stress conditions, the selection and use of rhizobia strains that are stress-tolerant boost legume nodulation and production (Li *et al*., 2012).

Rhizobia also protects host plants by inducing defence and antagonistic mechanisms against pathogens and parasites. For example, chickpea inoculated with *Rhizobium* isolates enhances its defence against *Fusarium oxysporum* through increased activity of polyphenol oxidases and peroxidases (Arfaoui *et al*., 2007), while peas inoculated with rhizobia resist the parasitic plant (crenate broomrape) invasion through root lignification (Mabrouk et al., 2007). Rhizobia also promote plant growth through enhanced production of phytohormones such as auxins, cytokinins, abscisic acid and ethylene (Boiero *et al*., 2007; Senthilkumar *et al*., 2009) with *R. leguminosarum* able to increase auxin production in the vetch nodules by a 60-fold (Camerini *et al*., 2008). Some rhizobia such as *Bradyrhizobium japonicum, R. leguminosarum, R. tropici, Cupriavidus taiwanensis*, *Mesorhizobium* sp. and *R. etli* solubilize P hence compensate for the amount of P required for the growth of host plants (Marra *et al*., 2011; Imen *et al*., 2015).

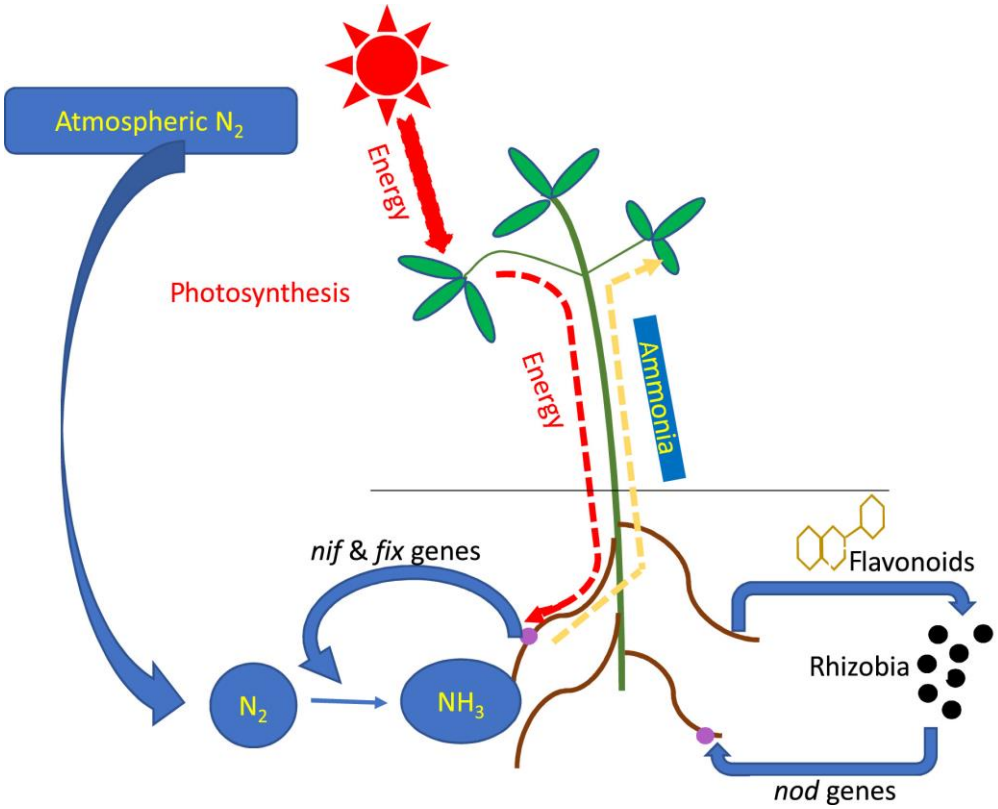

Figure 2.1: Biological nitrogen fixation by rhizobia (Lindstrom and Mousavi, 2020)

2.4.4 Native rhizobia as a source of inoculum in legume crops

Rhizobia inoculation is a viable technique in enhancing legume production in smallholder farming systems. It is a sustainable and cost-efficient technique for meeting the plants N needs and improving legume growth, nutrition, production, and abiotic stress tolerance (Ondieki *et al*., 2017; Takács *et al*., 2018; Aserse *et al*., 2020). However, the use of commercial inoculants and other proven rhizobia inoculants has at times failed to yield positive results when inoculation is done in regions with different environmental conditions as the original habitat. This has been associated with the potential competition with other rhizobia in the new habitat and poor persistence of the inoculant, reducing their potential in successful nodulation and N fixation (Martínez-Romero, 2003; Law *et al*., 2007; Stajković *et al*., 2011). In cowpea, failed response is associated with more effective native rhizobia than the inoculated strains (Mathu *et al*.,

2012). Under these circumstances, native rhizobia have been suggested as a better replacement for the development of legume crop inoculants. Besides, native rhizobia inoculated on cowpea, common bean, green grams, soy bean, and lentils showed enhanced legume productivity compared to the commercial inoculants (Mathu *et al*., 2012; Ouma *et al*., 2016; Tena *et al*., 2016; Koskey *et al*., 2017). This is because of the growth-enhancing traits and adaptation of native rhizobias to the soil and environmental stresses, making it form effective interactions with the host crops (Mwangi *et al*., 2011).

The resilience of native rhizobia strains under adverse agro-ecological conditions and their ability to establish positive interactions with other soil microorganisms, often explicates their superior characteristics over the introduced ones. Therefore, it is necessary to identify the native rhizobia strains that are more infective and more efficient in instating nodule formation and N fixation with the host crop (Tena *et al*., 2016; Ouma *et al*., 2016). Their use has been associated with the increased nodule numbers and dry nodule weights following inoculation. This consequently increases the rate and overall N fixed, enhancing the soil fertility and improving the legume production in the farmers farming systems (Koskey *et al*., 2017).

2.4.5 Persistence and re-inoculation of rhizobia inoculants in the soil

Rhizobia inoculants enhance N fixation and studies have demonstrated their predominance in nodules for 5–15 years after the initial inoculation (Lindström *et al*., 1990), confirming that they are effective colonizers persisting in soil for many years in the absence of their host (Sanginga *et al*., 1994). In Zimbabwe, rhizobia populations of up to 102 cells g^{-1} soil were found in soils inoculated 2-3 years before. Increased rhizobia persistence in the soils was attributed to higher clay content (>20%) and organic C (>1%), while sandy, and relatively less fertile soils have a lower rhizobia persistence (Zengeni *et al*., 2006). In addition, a low soil pH affects the rhizobia

survival and persistence after inoculation. Rhizobia are unable to survive in low pH soils leading to formation of infective nodules and low rhizobia populations (Stefan *et al*., 2018). Therefore, farmers with favourable soils can grow legumes for at least three years without the need for re-inoculation. Soil management practices, which build up organic matter such as addition of manure and liming to lower the soil pH are likely to create conditions that encourage survival and persistence of rhizobia in the soil. Still, although the rhizobia persistence after inoculation declines over time, the inoculation of legume seeds with effective and persistent rhizobial strains constitutes an evident advantage over inorganic nitrogen fertilizers, which have to be applied frequently for consistent high yields. For rhizobia re-inoculation is not necessary up to three years after the initial inoculation, and this period can be prolonged with efficient soil management practices.

2.4.6 Competition between rhizobia inoculants with indigenous rhizobia

The degree to which the rhizobia strains adapt to the local soil conditions strongly influence the competition between strains (Mendoza-Suárez *et al*., 2021) Rhizobial competitiveness has important practical implications for inoculants effectiveness, as differences in N fixation efficiency between strains can be large (Irisarri *et al*., 2019). Elite rhizobia inoculants must be highly effective in providing the plant with fixed N and, at the same time be highly competitive for nodule occupancy in a background of indigenous rhizobia that may show high competitiveness combined with a low N fixation efficiency (Onishchuk *et al*., 2017). In practice, competition between inoculants and native strains for nodule occupancy is a widely recognized challenge negatively impacting the efficiency of the inoculants. This results in, failures in establishment, and low productivity attributed to poor performance of the inoculant due to the absence of the inoculated strain in the nodules. Therefore, in the selection and

development of rhizobia inoculants, it is important to select highly competitive rhizobia strains for nodule occupancy with the native strains to ensure their effectiveness in nodulating and fixing N with the target host.

2.5 Importance of AMF in legumes growth and production

Arbuscular mycorrhizal fungi are important in the functioning of terrestrial ecosystems, where it associates with about 80% of plant families (Vlček and Pohanka, 2020). Leguminous crops are highly dependent on AMF, improving their overall performance in the different agro-ecological niches (Muleta, 2017). They are important microbes whose stable population is necessary for a healthy ecosystem through the restoration of the damaged ecosystems and enhanced productivity (Yang *et al*., 2008). AMF association with host plants lead to the modification of the host physiology and metabolism, which causes enhanced host growth and tolerance to the abiotic stresses (Smith *et al*., 2010). AMF colonization on the host plants occurs through hyphal growth on the host plant root hairs forming extensions that increase the root surface area for nutrients and water uptake (Mohammadi *et al*., 2011; Xu *et al*., 2018). This enhances moisture and limiting nutrient uptake by the host plant. Subsequently, this enhances the host plant adaptability to adverse conditions, better growth and increased yields.

The hyphal extensions also spread outside the rhizosphere connecting the plant roots to the nearby microhabitat, thus enlarging the area accessed by the root for moisture and nutrient uptake, especially P (He *et al*., 2010). This extensive hyphae development help the plant survive under adverse growing conditions (Kong *et al*., 2014). In addition, the physiological changes occurring in plants associating with AMF enhance these plants stress tolerance (Rapparini and Peñuelas, 2014). For example, AMF symbiosis has beneficial effects on physiological parameters, such as photosynthetic rate, stomatal conductance, and leaf water relations under saline conditions.

Arbuscular mycorrhizal fungi also increase nodule weights on legumes growing in soils with varying nutrient levels (Larimer *et al*., 2014). This leads to an increase in the plant shoot N following the increased N fixation. This high N content in the plant shoots allows the plant to maintain growth, develop and increase plant biomass production, and invest in defence against pathogens (Vannette and Hunter, 2011; Larimer *et al*., 2014; Yang *et al*., 2016; Muleta, 2017). Arbuscular mycorrhizal fungi also indirectly influence the acquisition of micronutrients and plant photosynthesis (Yang *et al*., 2016). However, this effect on nutrition and the growth of plants depends on the AMF species in the soil, with the *Glomus* sp. able to significantly enhance P absorption in the soil (Munkvold *et al*., 2004). Besides, the plants benefit from other microbial communities in the mycorrhizosphere (Artursson *et al*., 2006). Arbuscular mycorrhizal fungi are also an efficient tool in bioremediation. The hyphal extensions accumulate heavy metals such as lead and inorganic pollutants that damage the plants allowing continued growth (Leyval *et al*., 2002; Vodnik *et al*., 2008; Yang *et al*., 2016).

2.6 Rhizobia – AMF interaction in the soil

Legumes interact with N-fixing symbionts and AMF, which influences moisture and P absorption from the soil alleviating P deficiency. The influence of rhizobia and AMF on legume growth is independent of the soil N and P. Some leguminous plants largely depend on these micro-symbionts regardless of the N and P availability in the soil because of the strong synergistic effect of rhizobia and AMF co-infection on legume biomass production (Larimer *et al*., 2014). The synergistic interaction leads to better plant growth than when each of the symbionts works independently (Figure 2.2). However, potential competition between different AMF strains and rhizobia also exist, with some having antagonistic interactions. In some cases, AMF infection increases legume nodulation and nodule weight. At the same time, rhizobia inoculation decreases

the root colonization by AMF (Larimer *et al*., 2014). Plant growth traits may also influence the impacts of rhizobia and AMF interaction. Short-lived plants such as annual crops may exhibit reduced responsiveness and investment in their microbial partners compared to the long-lived plants. Thus, the microbial association and effects observed with short-lived plants may be more dependent on environmental factors than the mutualistic action of the symbionts (Larimer *et al*., 2010).

Nevertheless, these micro-organisms provide the host plants with the limited resources leading to a mutualistic interaction between the host and the symbiont. Arbuscular mycorrhizal fungi boost water absorption and uptake of limiting soil nutrients, such as P, while rhizobia fix the free atmospheric N_2 to ammonia for the plants (Schüßler *et al*., 2001; Smith and Read, 2010; Oruru and Njeru, 2016; Poole *et al*., 2018; Posta and Duc, 2019). In addition, AMF association with legume crops lead to enhanced host nodulation and functioning subsequently enhancing the N supply to the host (Takács *et al*., 2018).

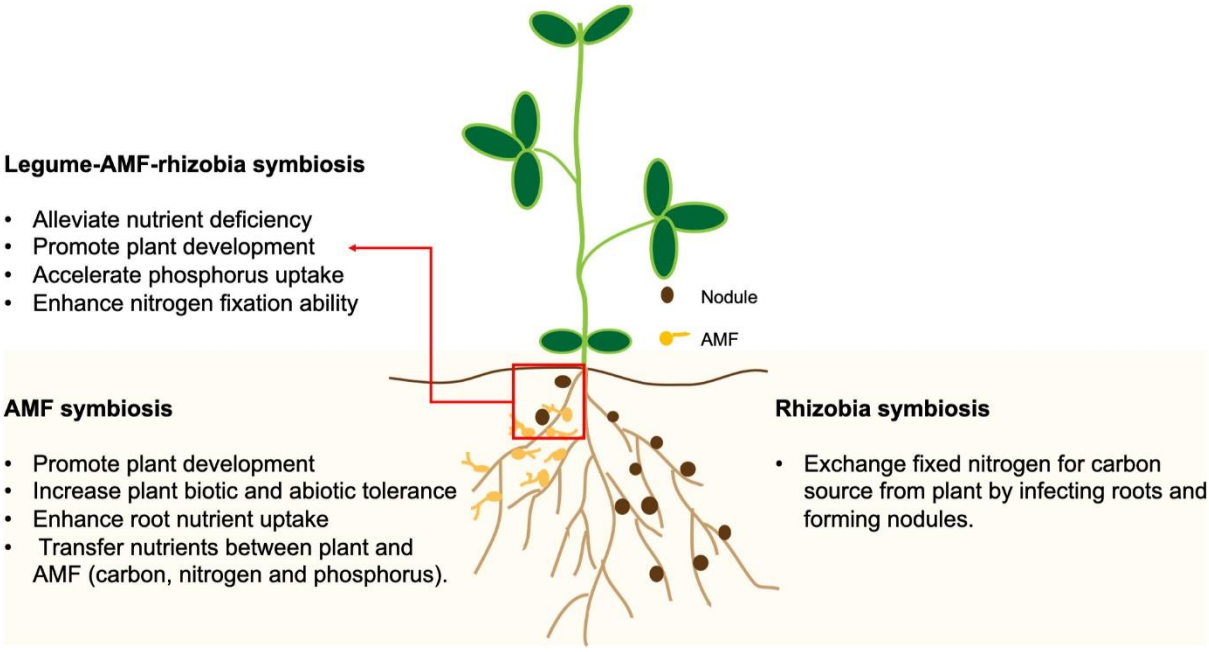

Figure 2.2: Effects of rhizobia – AMF association on legumes (Liu *et al*., 2020)

2.7 Arbuscular mycorrhizal fungi infective propagules in the soil

The propagules of AMF include the extraradical mycelia, spores and colonized plant roots (Guadarrama *et al*., 2008). The effectiveness of AMF to benefit plants depends on its ability to infect and colonize the plant roots. Colonization is determined by the presence of infective propagules in the roots. These infective propagules include the vesicles and the arbuscules. Arbuscules are characterized by intraradical hyphae and are sites for resource exchange; thus, many arbuscules in the roots indicate effective nutrient exchange between AMF and the host plant. Unlike arbuscules, the role of vesicles is to store resources, and a high vesicle number indicates fungal resource hoarding (Denison and Kiers, 2011).

The infectivity of AMF has been determined by quantifying the total colonization percentage of the infective propagules (Guadarrama *et al*., 2008), while the abundance of the infective propagules in the soils are quantified using the most probable number (MPN) technique (Porter, 1979). Soil properties and plant responsiveness influence the abundance of AMF in any ecosystem. In a study by Casazza *et al*. (2017), the abundance of AMF in *B. subacaulis* was greatly affected by both the slope and the physicochemical characteristics of the soil. On the other hand, plant responsiveness in a given ecosystem is dependent on the specificity among AMF species to the plant host, which is characterized by the species numbers in the soil, the AMF traits and plants ability to discriminate among AMF in space and time (Smith and Read, 2010; Powell and Rillig, 2018).

2.8 Gaps in knowledge addressed in the study

Although rhizobia inoculants have been proven to enhance legume productivity using commercially available inoculants (Ondieki *et al*., 2017; Takács *et al*., 2018; Aserse *et al*., 2020), there is limited knowledge on the potential of native rhizobia as inoculants.

At the same time, the efficiency of commercial inoculants under unfavourable soil conditions, including sandy and low fertile soils has been reported as considerably poor (Zengeni *et al*., 2006). In Kenya, cowpea is cultivated in low-fertile areas characterized by sandy soils, drought and low pH. Given cowpea is able to grow under such condition with no use of inorganic fertilizers, there is a possibility of the existence of superior and highly efficient rhizobia strains, well adapted to these conditions, which establish effective symbiotic association with cowpea. Therefore, there is need to isolate these strains for use as rhizobia inoculants in regions with similar conditions to improve cowpea production.

Microbes co-exist in the soil, with some like rhizobia and AMF forming a tripartite symbiosis with a host plant. However, not all rhizobia-AMF species/strains are compatible, and introduction of new strains can destabilize this association leading to inefficient associations that do not benefit the host plant (Trabelsi and Mhamdi, 2013). Therefore, with rhizobia inoculation it is crucial to establish the tripartite rhizobia symbiosis and the impact of introducing new rhizobia strains on the AMF population. At present, no studies have evaluated the effect of rhizobia inoculation on the AMF population especially in the dry areas where AMF plays a crucial role in nutrient and water uptake.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Description of the study area

The study was carried out in the semi-arid areas of Eastern Kenya, which is part of SSA. Field experiments were set up in 15 smallholder farms situated in the semi-arid zones of three counties, namely, Tharaka Nithi, Kitui, and Embu (Figure 3.1). The major crops cultivated in the three areas include maize, sorghum, millet, common beans, green grams, cowpea, and mangos. However, cowpea is the most preferred legume due to its ability to grow in relatively low soil moisture and drought tolerance. The farms were selected based on their management history (no prior use of rhizobia inoculants, and inorganic chemicals, including herbicides and pesticides) and prior cowpea cultivation. Experiments were conducted during the short and long rain seasons, running from October 2019 to February 2020 and March 2020 to July 2020, respectively. The five farms in Tharaka Nithi were situated in the Tunyai area (0◦10′33″ S, 37◦50′12″E), which lies at an elevation of 600–1,500 m above sea level (asl). The site is characterized by shallow, stony, and low fertile soil, which requires frequent replenishment of nutrients through the application of organic or inorganic fertilizer (Smucker, 2002; Jaetzold *et al*., 2006).

In Kitui, the five farms were situated in Matinyani (1◦18′30.6″ S 37◦59′30.2″E), which is located at 400–1,800 m asl, with soils characterized by a loose structure and a high water infiltration rate. In Embu County, the farms were located in the Kasafari area (0◦29′12″ S, 37◦41′50″ E), which lies at an elevation of 1,174 m asl. The three sites are in the lower midland agro-ecological zone, receive bimodal rainfall, and are relatively hot and dry. The mean monthly precipitation and the maximum and minimum

temperature during the experimental period are given in Appendix 1. Greenhouse and laboratory experiments were conducted at Kenyatta University (geographical position: 1.18°S 36.93°E).

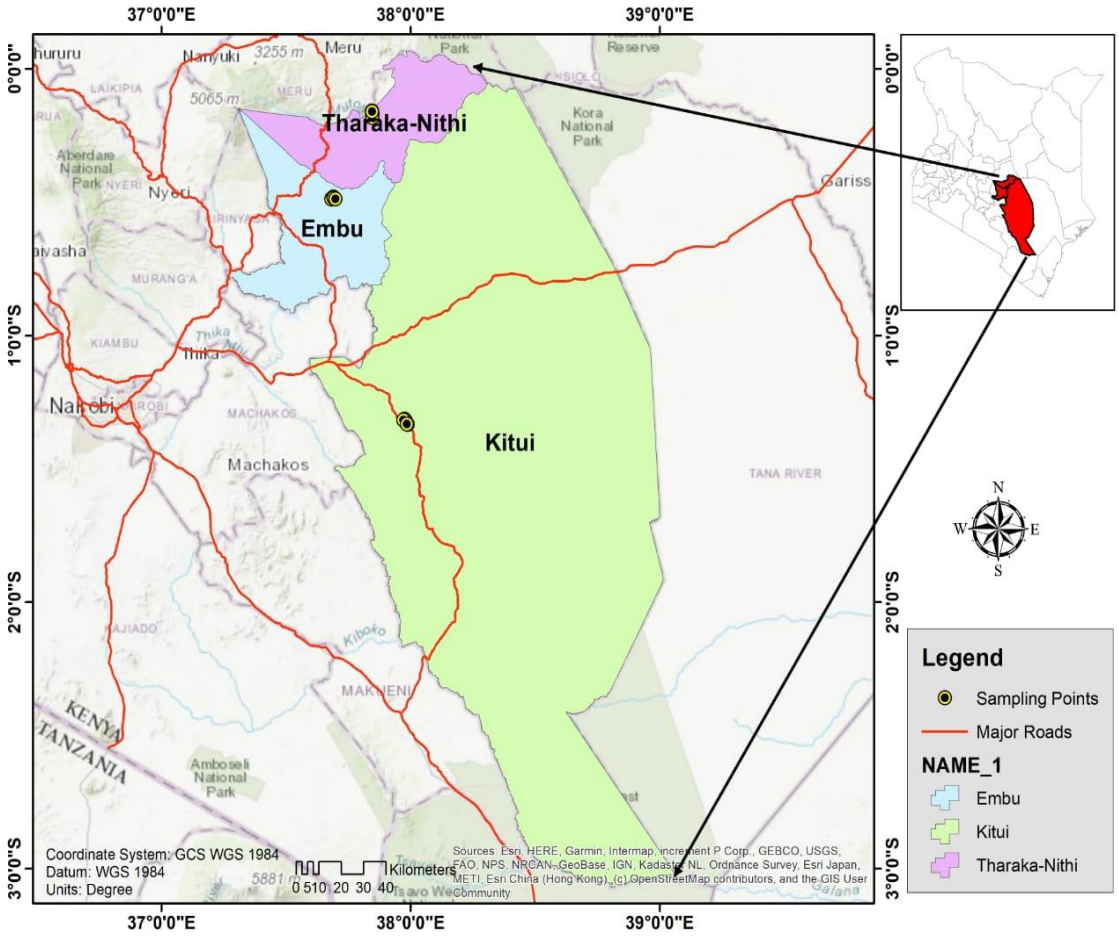

Figure 3.1: A map showing the study sites in the three counties. Map generated by ArcGIS (version 10.7.1) using the GCS WGS 1984 coordinate system.

3.2 Soil sampling

Soil sampling across the selected five farms in each County was done in June 2019 before onset of the rains. This was done on 20 points distributed in each farm using a shovel at a depth of 0-20 cm following the zigzag method of soil sampling. Before sampling at each point, the plant debris and surface materials were cleared, and the shovel sterilized using 70% ethanol and 3% sodium hypochlorite, then rinsed with sterile distilled water. The sampled soil from 20 points was then thoroughly mixed into

a single sample. One kilogram of the homogenous sample was placed in a khaki bag for transportation to Kenyatta University.

3.3 Physicochemical analysis

The sampled soils were air-dried at 110 ºC to a constant weight in the laboratory. Once dry, the physicochemical parameters of the soil were examined. These parameters included the soil texture, soil pH, total organic carbon (TOC), available soil P, total soil N and the exchangeable cations including magnesium (Mg^{2+}), sodium (Na^{+}), calcium (Ca^{2+}), potassium (K^{+}) and soil micronutrients {copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn)}. The total organic carbon was determined through an oxidation process using concentrated sulphuric acid and aqueous potassium dichromate ($K_2Cr_2O_7$) following the description given by Okalebo *et al*. (2002). After oxidation, the residual $K_2Cr_2O_7$ was titrated against ferrous ammonium sulphate to establish the content of the used $K_2Cr_2O_7$ during oxidation, which gives the TOC present in the soil. The total soil N was examined using the Kjeldahl method (Sáez-Plaza *et al*., 2013). The exchangeable cations present in each soil sample were assessed by flame photometry for Na^{+} and K^{+} and atomic absorption spectrophotometry for Ca^{2+} and Mg^{2+} following descriptions by Okalebo *et al*. (2002). Available P was estimated using a colourimeter following soil digestion with hydrogen peroxide, sulphuric acid, selenium and salicylic acid as described by Okalebo *et al*. (2002). The soil pH was determined in 2.5:1 soil and water suspension using a pH meter. The micronutrients present in the soil were extracted in 1% ethylenediaminetetraacetic acid (EDTA) and their levels in the soil determined by atomic absorption using a spectrophotometer as described by Okalebo *et al*. (2002).

3.4 Determination of AMF infection potential

3.4.1 Trapping of AMF

To determine the AMF propagules richness in the sampled soils, a greenhouse experiment was set to establish the AMF infection potential on a model plant. The infection potential of the AMF was estimated using the MPN technique described by Lehmann *et al*. (2012). Precisely, from the sampled soil in each of the farms, 20 g of soil was used as the AMF inoculum. The 20 g was serially diluted in 180 g sterile 1:1 soil and sand mix sterilized by oven drying for 48 hours to obtain 200 g in a ten-fold dilution series. Six levels of serial dilution (10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) replicated four times for each dilution were performed to create a six-by-four MPN matrix for each sampled soil.

Bermuda grass (*Cynodon dactylon*), used as the host plant, was grown in 150 cm^3 plastic pots in the greenhouse for 60 days. The pots were arranged in a completely randomized design (CRD), with re-randomizations after every two weeks to avoid positional effects (light and temperature distribution). Watering was done twice per week. After 60 days, the Bermuda grassroots were harvested and washed under running water to remove adhered soils, then packaged in khaki bags and stored in a freezer at -28°C in the laboratory.

3.4.2 Staining and enumeration of the AMF infective propagules

All the harvested roots were stained with trypan blue in the laboratory to establish the AMF colonization following the standard procedure given by Phillips and Hayman (1970). The grassroots were cut into 2 cm long pieces, washed in distilled water and placed in falcon tubes. Potassium hydroxide (KOH) was added to the falcon tubes to cover the roots and heated in a water bath at 80°C. After 45 min, the KOH was poured out, and the roots rinsed with distilled water. The roots were then covered with 2%

hydrochloric acid (HCl) for 10 min and heated in a water bath at 80°C, after which the HCl was poured out and 0.05% Trypan Blue stain added on the roots until the roots were completely covered. The roots were heated in the water bath at 80°C for five min. The Trypan Blue was emptied, and 10% lactic acid added for destaining. The stained roots in each dilution were spread on the petri dish surface and scored for presence or absence of arbuscules and vesicles in the roots by observing under a dissecting microscope (Olympus Optical Co. Ltd., Tokyo, Japan) at a 45X magnification.

3.5 Rhizobia trapping in the field

Field trap cultures were set in selected smallholder farms in the three counties using three cowpea varieties: K80, M66, and KVU 27-1 between June and July 2019. The plots (measuring 2x2 m) were ploughed and demarcated with a 1 m gap between them. The Cowpea varieties were planted at a spacing of 40x15 cm, and each replicated three times. Six weeks after planting, three cowpea plants per plot were randomly uprooted. The root nodules were detached and wrapped with an absorbent paper towel and stored in labelled khaki bags. They were transported to the Kenyatta University Microbiology Laboratory for rhizobia isolation.

3.6 Rhizobia isolation

The detached nodules were washed with sterile distilled water to remove adhering soil particles and soaked for two hours to imbibe and soften for eased crushing. The nodules were surface sterilized using 70% ethanol for 20 s, followed by rinsing in with sterile distilled H_2O and final sterilization in 3% sodium hypochlorite (NaOCl) for 2 min. The nodules were then serially rinsed in six changes of sterile distilled water, crushed and plated on the yeast extract mannitol agar (YEMA) containing Congo red dye (0.025 g in 1 l) media. The cultures were incubated at 28±1°C for 48 h in the dark. Single

colonies identified on the plates were restreaked 3-4 times on plates containing freshly prepared YEMA until pure isolate cultures were obtained.

3.7 Morphological and biochemical characterization

The pure rhizobia isolates were grouped into distinct groups according to their morphological and biochemical features as described previously based on their size, shape, colour, elevation, transparency, and margin (Ondieki *et al*., 2017). The isolates were also classified as fast or slow-growing based on the colour changes when plated on YEMA containing BTB (0.025 g/l) followed by a 3-day incubation at 28±1°C in the dark (Boakye *et al*., 2016). Gram staining of the isolates was also done as described by Beck *et al*. (1993). A thin layer of 24 h old cultures was spread on a glass slide, lightly heated, Crystal violet stain added, left to stand for a minute and washed under gently running tap water. Iodine (mordant) was then added and maintained for another minute, followed by washing under gently running tap water. Three drops of 95% ethyl alcohol were added to decolourize, maintained for 10 s, and Safranin added for counterstaining for 60 s. The slides were washed under running tap water, dried using a filter paper and observed under oil immersion using a light microscope at 40X. Isolates with similar morphological and biochemical characteristics were grouped together.

3.8 Authentication of the nodule isolates

One representative isolate across each group were authenticated as rhizobia based on their ability to induce nodule formation on cowpea.

3.8.1 Greenhouse conditions

Authentication assessment was done in an even span greenhouse with roll-up sides having natural lighting of 12 h. The greenhouse temperature and relative humidity ranged from 22-28°C and 61-80%, respectively.

3.8.2 Preparation of Leonard jars assembly

Authentication was done using the Leonard jars assembly. The assembly consisted of a small plastic cup with an 8 cm and 4 cm top and bottom diameter inserted in a larger plastic container. A sponge wick running through the middle of the small cup was used to connect the small cup to the larger container. Before the setup, the small cup and the large container were swapped with 70% ethanol while the sponge wick was dipped in 3% sodium hypochlorite for four min, then rinsed in five changes of sterile distilled water.

3.8.3 Preparation of the plant growth medium

Sterile N-free vermiculite was used as the growth medium during the authentication assessment. The vermiculite was soaked in water overnight to dissolve any nutrients that were present. It was then washed by mixing and changing the water until the water was clear followed by autoclaving. Next, the small plastic cups in the Leonard jars assembly were filled with sterile vermiculite and covered with aluminium foil swapped with 70% ethanol. In each larger container, 700 ml of sterile N-free nutrient solution (Broughton and Dilworth, 1971) (Table 3.1) was then added. The assemblies were placed in khaki bags for insulation.

Table 3.1: Composition of the N-free nutrient solution

Stock Solution	Nutrient	Form	Gram/Liter
1	P	KH_2PO_4	136.10
2	Ca	$CaCL_2.2H_2O$	294.10
3	Fe	Fe-Citrate	6.70
4	Mg	$MgSO_4.7H_2O$	123.30
	K	K_2SO_4	87.00
	Mn	$MnSO4.H_2O$	0.338
5	Zn	$ZnSO_4.7H_2O$	0.288
	Cu	$CuSO_4.5H_2O$	0.100
	Mo	$Na_2MoO_2.2H_2O$	0.048
	B	H_3BO_3	0.247
	Co	$CoSO_4.7H_2O$	0.056

(Broughton and Dilworth, 1971).

3.8.4 Planting and inoculation of cowpea seeds

Two seeds (K-80 variety) of the same size, shape and colour sterilized using 70% ethanol and 3% sodium hypochlorite for 20 s and 1 min respectively, then rinsed in 6 changes of sterile distilled water were planted in each of the Leonard jars. The nodule isolates for authentication were cultured in yeast extract mannitol (YEM) broth containing 2.5 g mannitol, 0.125 g K_2HPO_4, 0.05 g $MgSO_4$. $7H_2O$, 0.025 g NaCl, 0.125 g yeast extract and 250 ml distilled water and incubated at 28±1°C for three days before inoculation. One ml of an axenic broth culture of the isolates was then inoculated on 7-day-old cowpea seedlings. Uninoculated plants served as negative controls. Those inoculated with a commercial strain USDA 3456 (Biofix, MEA Limited, Kenya) served as a positive control. The set up was laid out in a CRD with four replicates in the greenhouse. The nutrient solution in the jars was replenished once every week. The plants were maintained in the greenhouse for 45 days after inoculation, after which they were uprooted and scored for nodulation. Isolates that initiated nodule formation were selected to assess their molecular identity and symbiotic efficiency.

3.9 Genetic diversity and characterization of the isolates

3.9.1 DNA extraction

Genomic DNA was extracted from rhizobia isolates using Zymo quick DNA™ min prep kit, Lot No: ZRC185271 (ZYMO Research Corporation, CA, USA) following the manufacturers' instructions. In summary, pure rhizobia isolates grown on YEMA media at 28±1°C in the dark for three days were suspended in 400 µl of normal saline in 1.5 ml sterile microcentrifuge tubes to remove excess EPS. The suspension was then vortexed for 20 s to get a homogenous solution followed by 10-min centrifugation at 13,000 rpm. The liquid phase was gently decanted, and the washing process repeated four times. The pellets were then re-suspended in 400 µl of lysis buffer followed by

vortexing for 10 min and incubation at room temperature for 30 min before transferring into zymospin column in collection tubes. The zymospin column was then transferred to new collection tubes, and 200 µl DNA pre-wash buffer added to the supernatant and centrifuged for a minute at 13,000 rpm to the columns. Another centrifugation at 10,000 rpm for 1 min was done after adding 500 µl of genomic DNA (gDNA) wash buffer. The supernatant was then carefully transferred into sterile microcentrifuge tubes, and 50 µl of DNA elution buffer added and incubated at room temperature for 10 min to dissolve the DNA. The solution was centrifuged at 10,000 rpm for 30 s to collect the dissolved DNA. The gDNA was stained using SYBR green (Invitrogen) and visualized in a 1% agarose gel run in 0.5X TBE (Tris-borate EDTA) buffer at 80 V for 30 min to determine the quality and quantity of DNA. The DNA bands were captured using a digital camera (Nikon) for documentation. The DNA was stored at -20ºC before any further analysis.

3.9.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed on the extracted DNA as previously described (Krasova-Wade *et al*., 2003), targeting the 16S rRNA gene and using the following primers; 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'. Amplification was done in a volume mixture consisting of 10 ng/µl of genomic DNA, 0.5 µl of 5 U/µl Taq DNA polymerase, Native (ThermoFisher Scientific), 2.5 µl of 10x Taq standard buffer, 1 µl of 10 µM dNTPs and 0.5 µl of 10µM of both the forward and reverse primers. The mixture was topped up to a volume of 25 µl with sterile nuclease-free water. Amplification was performed in a Techgene programmable Thermocycler, FTGENE5D model (Techne, United Kingdom), under the following conditions; initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s for denaturation, annealing at 51.8 °C for 45 s,

extension at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR products were then stored at -4 °C awaiting purification and sequencing.

3.9.3 Gel electrophoresis

Amplification was confirmed on a 1.5% agarose gel. Amplicons were visualized after running on a UV trans-illuminator lighting (Simmon *et al*., 2004) in a 1.5% agarose gel stained with SYBR green (Invitrogen) using 0.5X TBE buffer at 80 V for 30 min and bands photographed using a digital camera (Nikon Inc). The sizes of the amplicons band were estimated using a 1 kb DNA ladder.

3.9.4 Sequencing and bioinformatics analysis

The PCR products were purified, then sequenced on the Sanger platform, using 27F and 1492R primers, at Macrogen-Inc (Amsterdam, Netherlands). Raw reads were first edited, and consensus sequences generated using BioEdit software (version 7.2.5) (Hall, 1999). Obtained contigs were used as querries for nucleotide BLAST (blastn) at National Center for Biotechnology Information (NCBI), and homologous sequences retrieved. The obtained sequences were then aligned alongside those obtained from NCBI, using the CLUSTAL-W tool in MEGA X software (version 10.1.8) (Kumar *et al*., 2018), and a phylogenetic tree constructed using the neighbour-joining algorithm with 1000 bootstrap replication (Tamura *et al*., 2004). Sequences obtained herein were deposited to Genebank at NCBI, for accession numbers. DnaSP 6 software was used to calculate nucleotide diversity of the isolates across regions, then computed analysis of molecular variance and genetic differentiation using Arlequin version 3.5.2.2.

3.10 Symbiotic efficiency assessment of the obtained isolates

Symbiotic efficiency assessment was done in the greenhouse as described by Somasegaran and Hoben (1994). Cowpea seeds (K-80 variety) were sterilized, as described in section 3.8. The sterile seeds were planted in sterile N-free vermiculite in

the Leonard jar assemblies (two seeds per jar) and maintained in the greenhouse as described in section 3.8. Rhizobia isolates that induced nodulation in section 3.8 and a commercial strain (USDA 3456) were cultured in YEM broth and incubated for three days at 28±1°C. Seven days after planting, the cowpea seedlings were inoculated with 1 ml of the broth culture per plant. Uninoculated plants supplemented with potassium nitrate (KNO_3) (1 g/l) and those inoculated with the commercial strain served as positive controls. Uninoculated plants with the N-free nutrient solution served as the negative control. Each treatment had four replicates arranged in a CRD.

The plants were harvested 45 days after inoculation. The nodules were detached from the roots, counted, wrapped in an absorbent tissue paper and air-dried to a constant weight at room temperature. Further, the roots were separated from the shoots. Dry nodules were weighed to determine their weight. The roots and shoots were oven-dried at 65°C for 48 hours, after which their weights were determined. The symbiotic efficiency (SE) of the rhizobia isolates was determined following the procedure described by Karaca and Uyanöz (2012) given below.

$$\frac{\text{Total dry shoot weight of inoculated plants}}{\text{Total dry shoot weights of N}-\text{supplemented plants}} \times 100$$

3.11 Effect of rhizobia inoculation on cowpea productivity, and AMF colonization

The farms were prepared as mentioned in section 3.5 before the onset of rains in September 2019 and February 2020 during the short (mid-October- early January) and long-rain seasons (late March - early June), respectively. The cowpea (K80, M66 and KVU 27-1) seeds were inoculated with rhizobia under shade before planting. The rhizobial treatments included native isolates, Biofix, a mixture of the Biofix and native isolates (Consortium), and uninoculated control. The native isolates consisted of a consortium of three highly effective isolates identified from the greenhouse experiment,

which also associated with all the three cowpea genotypes in all the three study sites, which were JN44, JN4, and JN19.

Native isolates were inoculated on the seeds as broth using sugar as the sticker material for the rhizobia inoculum. A 250 ml of YEM broth was prepared for each of the regions and incubated for three days at 28±1°C. In the field, the broth containing the indigenous rhizobia was mixed with 0.5 kg of sugar as the coating agent of rhizobia cells on the cowpea seeds and then mixed with the seeds separately for each variety. Biofix was applied to the seeds following the manufacturers' guidelines (100 g of inoculum per 15 kg of seeds). The control plots with no rhizobia treatment were planted first to avoid cross-contamination, followed by other treatments. Two cowpea seeds were planted per planting hole, and the experiment was arranged in a randomized complete block design (RCBD) with three replications.

The plants were gapped immediately after germination in both seasons and kept free of weeds by occasional weeding. The plants were sprayed with Aceprid 20 WSP (Acetamiprid 200 g/kg) to prevent attack by the thrips, which infest the cowpea flowers, causing flower abortion and subsequently leading to declined yields (Oladejo *et al*., 2017). The plants were also sprayed with Evisect (Thiocyclam hydrogen oxalate) during the first season to control the whiteflies which had infested the cowpea plants. The experiment was rain-fed with the rainfall and temperature averages during the growing seasons given in fig. 3.2.

Data on the cowpea growth, nodulation and AMF colonization were taken six weeks after germination at the onset of flowering. Three plants per plot were randomly selected, uprooted and roots separated from the shoots. The nodules were prepared as described in section 3.5. The heights of the plants were measured using a tape measure,

and the number of leaves per plant counted. The shoots and roots were then placed in separate plastic courier bags and labelled. The obtained shoot, root and nodule samples were transported to Kenyatta university microbiology laboratory, where the roots were stored in a freezer. The shoots were oven-dried at 65°C for 48 hours while the nodules were air-dried to a constant weight at room temperature, before their dry matter were weighed.

To determine the effect of rhizobia inoculation on indigenous AMF colonization on the different cowpea varieties, the roots were stained as described in section 3.4.2. After destaining, 30 root fragments per sample (plot) were observed using the gridline intersect method under a dissecting microscope (Giovanneti and Mosse, 1980). For each root fragment, AMF colonization was determined by checking for arbuscules and vesicles. The number of root fragments that were positive for AMF colonization per sample was recorded. The AMF colonization intensity (arbuscules and vesicles) on the cowpea roots was calculated as below (Hashem *et al*., 2019).

$$\text{Colonization (\%)} = \frac{\text{Total number of AM positive segments}}{\text{Total number of segment studied (30)}} \times 100$$

Matured cowpea was harvested in February 2020 and July 2020 in the first and second season, respectively, when the plants had reached physiological maturity. Three cowpea plants plot^{-1} were randomly selected and harvested. For each plant, the seeds were detached from the pods. The seeds and the stover were then transported to the laboratory, where they were dried and weighed. In addition, the 100 seed weight per plot was determined.

3.12 Data analysis

The AMF infective propagules in the different farms were estimated using the MPN technique. The MPN values for each farm were calculated using the Most Probable Number Enumeration System (MPNES). Further, the obtained MPN values per farm were correlated to the soil physicochemical characteristics by Redundancy Analysis (RDA) using Canoco software version 5.0. The morphological and biochemical characteristics of the nodule isolates were scored numerically, then subjected to cluster analysis based on the Jaccard similarity index using the neighbour-joining method in the Darwin 6 software.

All the data on nodule number, dry nodule weight, shoot dry weight, root dry weight, AMF colonization on cowpea roots, yield and stover weight were analyzed using the statistical analysis software (SAS) version 9.1. Before analysis, the data were tested for homogeneity of variance using the Bartlett test and log-transformed. Comparisons among groups of greenhouse data were analyzed using a one-way analysis of variance (ANOVA) (CRD) whereas the field data were analyzed using a three-way ANOVA (RCBD). The means were separated at a 5% level of significance by Tukey's honest significance difference (THSD).

CHAPTER FOUR

RESULTS

4.1 Soil physicochemical parameters

The soil physicochemical parameters varied across the farms in Embu, Kitui and Tharaka Nithi counties. The highest pH was 7.12 recorded in farm T5 in Tharaka Nithi, while the lowest was recorded in farm E3 in Embu County (Table 4.1). Similarly, the highest percentage (%) N was in farm T4 in Tharaka Nithi. However, farms in Kitui had a very low N content, recording as low as 0.07 and 0.05% N in farms K1 and K5, respectively. Although slight differences were observed in % TOC among the farms in Embu and Tharaka Nithi, this was not the case in Kitui farms which recorded as low as 0.42% TOC (Table 4.1). Additionally, the available P in the study areas was highly variable. Farm E2 in Embu had the least available P, and farm K5 in Kitui County had the highest available P concentration of 245ppm (Table 4.1).

Exchangeable cations in the study farms were also highly variable across the farms. The calcium (Ca^{2+}), sodium ions (Na^{+}), potassium ions (K^{+}) and magnesium ions (Mg^{2+}) ranged between 1 to 16.6, 0 to 1.22, 0.3 to1.62 and 1.4 to 5.82% in that order. The micronutrients analyzed in the soil included manganese (Mn), copper (Cu), iron (Fe) and zinc (Zn) and their concentrations ranged between 0.3 to 1.09%, 1.01 to 6.42 ppm, 1.07 to 66.2 ppm and 0.26 to 1.04 ppm respectively in the farms (Table 4.1). The soil textural class of soils in Embu was sandy clay (SC) except for farm E3. In Tharaka Nithi, all farms had a sandy loam (SL) texture except for farm T5, which has a clay (C) soil texture. Additionally, in Kitui, the soil textural class was SCL except for farm K4, which had an SL texture (Table 4.1).

Table 4.1: Physico-chemical properties of soils in the study sites

Farm	pH	TN (%)	TOC (%)	P (ppm)	K (%)	Ca (%)	Mg (%)	Mn (%)	Cu (ppm)	Fe (ppm)	Zn (ppm)	Na (%)	Sandy (%)	Clay (%)	Silt (%)	STC
E1	5.81	0.16	1.75	50	1.62	2.6	3.1	0.67	1.01	29.2	10.4	0.5	46	42	12	SC
E2	5.84	0.17	1.77	3	1	2.6	2.41	0.5	0.39	10.8	6.96	0.68	48	46	6	SC
E3	5.5	0.15	1.73	30	1.12	2	2.05	0.65	1.4	14.8	6.75	0.54	60	26	14	SCL
E4	6.06	0.18	2.2	25	1.54	3.6	3.03	0.56	0.39	16.4	7.66	1.22	50	42	8	SC
E5	5.38	0.14	1.33	25	1.16	2	2.02	1.09	1.21	21.4	9.66	0.48	50	44	6	SC
T1	7.01	0.13	1.18	23	0.42	16.6	5.38	0.39	1.98	52.3	1.28	0.58	72	18	10	SL
T2	7.12	0.12	1	15	0.3	13.2	5.82	0.45	2.15	1.07	1.03	0.54	70	18	12	SL
T3	6.9	0.13	1.22	165	0.54	11.2	3.86	0.89	2.32	66.2	2.08	0.6	76	12	12	SL
T4	7.1	0.18	2.07	16	0.7	17	4.86	0.64	2.58	16	0.31	0.52	76	14	10	SL
T5	5.68	0.13	1.37	20	0.62	1	2.84	0.5	4.04	23.9	0.26	0.52	44	48	8	C
K1	6.66	0.07	0.67	100	0.95	4	2.6	0.5	4.9	23.5	4	0	68	28	4	SCL
K2	6.44	0.11	1.04	60	0.88	4.4	2.09	0.32	5.64	13.6	9.85	0.64	64	30	6	SCL
K3	5.74	0.11	0.97	20	0.88	1.4	2.05	0.34	6.42	14.2	4.82	0.62	62	34	4	SCL
K4	6.74	0.11	1	70	0.48	5	1.4	0.3	4.6	2.11	8.04	0.58	82	16	2	SL
K5	6.22	0.05	0.42	245	1.02	3	1.84	0.55	3	62.3	5.16	0.64	72	24	4	SCL

Note: %, percentage; C, clay; Ca, calcium; Cu, copper; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; Na, sodium; P, phosphorus; pH, potential of hydrogen; SC, sandy clay; SCL, sandy clay loam; SL, sandy loam; STC, soil textural class; TN, total nitrogen; TOC, total organic carbon; Zn, zinc.

4.2 Relationship between MPN values and soil physicochemical characteristics

The AMF infective propagules in the sampled soils from the study locations were enumerated using Bermuda grass as the host plant. The number of propagules in the soils varied in numbers (MPN value), with the highest MPN values found in soils from farm E3 in Embu County and the least in farm K3 (500.83) from Kitui County (Table 4.2). Redundancy analysis (RDA) was used to assess the relationship between the AMF-MPN values in the soil and the soil physicochemical characteristics (Figure 4.1). The majority of the soil physicochemical characteristics were present in the lower part of the RDA ordination figure, indicating that the AMF-MPN values of the soil were negatively affected by these characteristics. The soil characteristics negatively affecting AMF MPN values in the soil included P, Fe, Mn, K, Zn, Na, TOC, TN and clay %. In contrast, the silt, Mg, soil pH, Ca, sand %, and Cu positively correlated with the AMF-MPN values of the soils. Based on the RDA (Figure 4.1), sand %, clay % and P were the most critical soil characteristics affecting the AMF MPN values in the soil.

Table 4.2: AMF infective propagules in the individual farms used during the study

Farm	Study location	MPN Value	The range at P <0.05	Confidence Factor
E1	Embu	2129.69	560.17-8096.87	3.80
E2	Embu	3301.45	868.37-12551.77	3.80
E3	Embu	120000.00	31563.22-456227.30	3.80
E4	Embu	5469.20	1438.55-20793.33	3.80
E5	Embu	5000.50	1315.27-19011.37	3.80
T1	Tharaka Nithi	16250.19	4274.24-61781.48	3.80
T2	Tharaka Nithi	3399.10	894.05-12923.01	3.80
T3	Tharaka Nithi	13125.35	3452.32-49901.17	3.80
T4	Tharaka Nithi	21250.22	5589.38-80791.07	3.80
T5	Tharaka Nithi	748.61	196.90-2846.12	3.80
K1	Kitui	3301.451	868.37-12551.77	3.80
K2	Kitui	13125.35	3452.32-49901.10	3.80
K3	Kitui	500.83	131.73-1904.10	3.80
K4	Kitui	2676.51	703.99-10175.82	3.80
K5	Kitui	5469.20	1438.55-20793.33	3.80

Note: MPN – Most probable number.

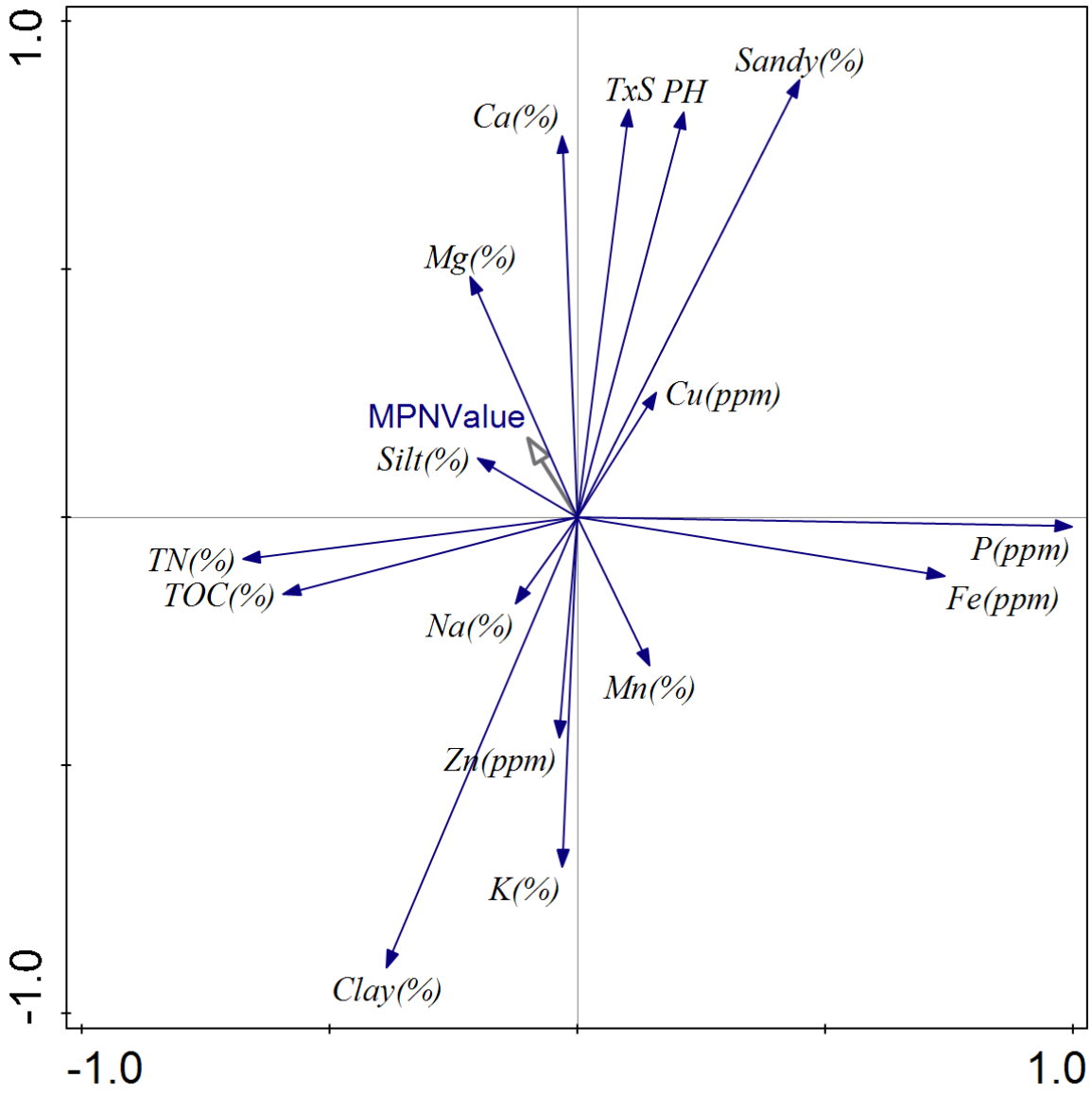

Figure 4.1: Redundancy analysis of AMF-MPN values and soil characteristics.

4.3 Morphological and biochemical characterization of the nodule isolates

One hundred and three isolates were obtained from the three cowpea varieties (K80, M66 and KVU 27-1) in the three study regions. Out of these, 38 isolates were from Tharaka Nithi County, 36 from Embu County and 29 from Kitui County. Based on these characteristic, the isolates were grouped into 20 distinct groups. Group JN19 had the highest number of isolates (16.5%), while group JN2, JN18 and JN32 had the least

with (0.9%) (Table 4.3). All the isolates were gram-negative. They did not absorb Congo red dye when cultured in YEMA containing the dye. Still, they turned YEMA-BTB media from green to yellow (Plate 4.1). Sixteen isolates changed the media from green to yellow within three days. The other four had media change between seven and 10 days. The isolates exhibited diverse characteristics, as indicated in Table 4.3.

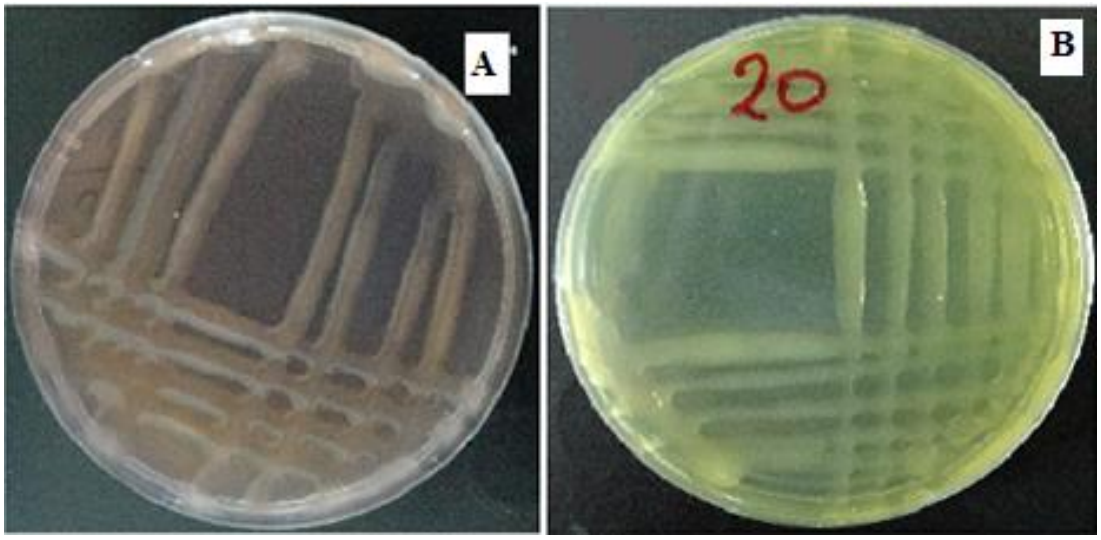

Plate 4.1: Colonies of JN20 rhizobia isolate. A, In YEMA; B, In YEMA containing BTB.

Table 4.3: Morphological and biochemical characteristics of the isolates

ISOLATE CHARACTERISTICS												
Isolate Group	Transparency	Texture	Shape	Size (mm)	Colour	Margin	Gram stain	Growth	BTB reaction	Congo red absorption	Elevation	% Isolates
JN30	TO	SG	R	4	MW	SC	-ve	F	Y	NA	C	5.5
JN4	T	SM	C	3	MW	S	-ve	F	Y	NA	C	3.7
JN20	TO	SG	C	6	CW	SC	-ve	F	Y	NA	RA	4.6
JN1	TO	FlG	R	2	CW	SC	-ve	SL	Y	NA	C	2.8
JN5	T	SG	R	4	CW	S	-ve	SL	Y	NA	C	1.8
JN39	O	SM	C	2	W	S	-ve	F	Y	NA	C	3.7
JN44	TO	SG	R	3	CW	SC	-ve	F	Y	NA	RA	3.7
JN8	T	FlG	C	6	MW	S	-ve	F	Y	NA	FL	5.5
JN6	T	SG	I	6	CW	S	-ve	F	Y	NA	RA	1.8
JN9	T	SG	C	4	W	S	-ve	F	Y	NA	C	8.3
JN11	O	SG	C	1	CW	S	-ve	F	Y	NA	RA	1.8
JN2	TO	SG	C	4	CW	S	-ve	F	Y	NA	C	0.9
JN18	TO	SG	C	5	CW	SC	-ve	F	Y	NA	RA	0.9
JN29	O	SG	R	4	CW	S	-ve	F	Y	NA	C	6.4
JN28	T	SG	R	4	MW	S	-ve	F	Y	NA	C	4.6
JN36	O	SG	R	2	W	SC	-ve	F	Y	NA	RA	5.5
JN17	TO	SG	C	4	W	SC	-ve	SL	Y	NA	RA	5.5
JN19	O	FG	R	3	CW	S	-ve	F	Y	NA	RA	16.5
JN3	TO	SG	R	4	CW	S	-ve	F	Y	NA	C	15.6
JN32	T	SG	C	2	W	S	-ve	SL	Y	NA	C	0.9

Note: C, circular; C, convex; CW, cream white; F, fast growing; FG, firm gummy; FlG, flowing gummy; FL, flat; I, irregular; MW, milky white; NA, Congo red non-absorbance; O, opaque; R, rod shaped; RA, raised; S, smooth; SC, smooth clear; SG, soft gummy; SL, slow growing; SM, soft mucoid; T, translucent; TO, translucent with opaque centre; W, white; Y, yellow.

4.4 Authentication of the isolates from cowpea nodules

Authentication was based on the presence of at least one nodule in the roots of the cowpea plants. The nodulated plants were green, and the uninoculated nodulation yellow (Plate 4.2). Out of the 20 representative group isolates, nodulation was positive in 13 groups. These groups were JN30, JN4, JN20, JN39, JN44, JN9, JN2, JN18, JN29, JN28, JN36, JN19 and JN3. Groups characterized by the absence of nodule formation included JN17, JN1, JN32, JN5, JN8, JN6 and JN11. Nodule isolates from the K-80 cowpea variety were represented in each of the 13 groups, which nodulated with cowpea (Appendix 2). Some groups, such as JN28, JN4, JN19, JN39 and JN44, were isolates from all three cowpea varieties (Appendix 2). However, some groups comprised isolates from only one cowpea variety, such as JN2, JN29, JN3, JN18 and JN30 (Appendix 2). With regards to regions, isolates from Tharaka Nithi had the most representation in the groups. Out of the 13 groups, isolates from Tharaka Nithi were present in nine groups. In contrast, Embu and Kitui isolates were each present in eight groups (Appendix 2). Additionally, isolates represented by JN4, JN19 and JN44 were from all three regions (Appendix 2). Interestingly, these isolates were also isolated from nodules of all three cowpea varieties (Appendix 2).

Plate 4.2: Inoculated cowpea plant (K-80 variety) and inoculated control (negative control) rhizobia authentication

4.5 Morphological resemblance among the nodule forming isolates

The nodule forming isolates were classified into three main clusters, A, B and C, based on the similarities of their morphological and biochemical characteristics using cluster analysis. (Figure 4.2). Group A comprised seven isolates which were further subdivided into two major sub-clusters, A1 and A2, with five and two isolates, respectively. Group B was composed of five isolates subdivided into two sub-clusters, with four isolates in B1 and one in B2. Only one isolate, JN36, clustered in cluster C, which was isolated from Kitui.

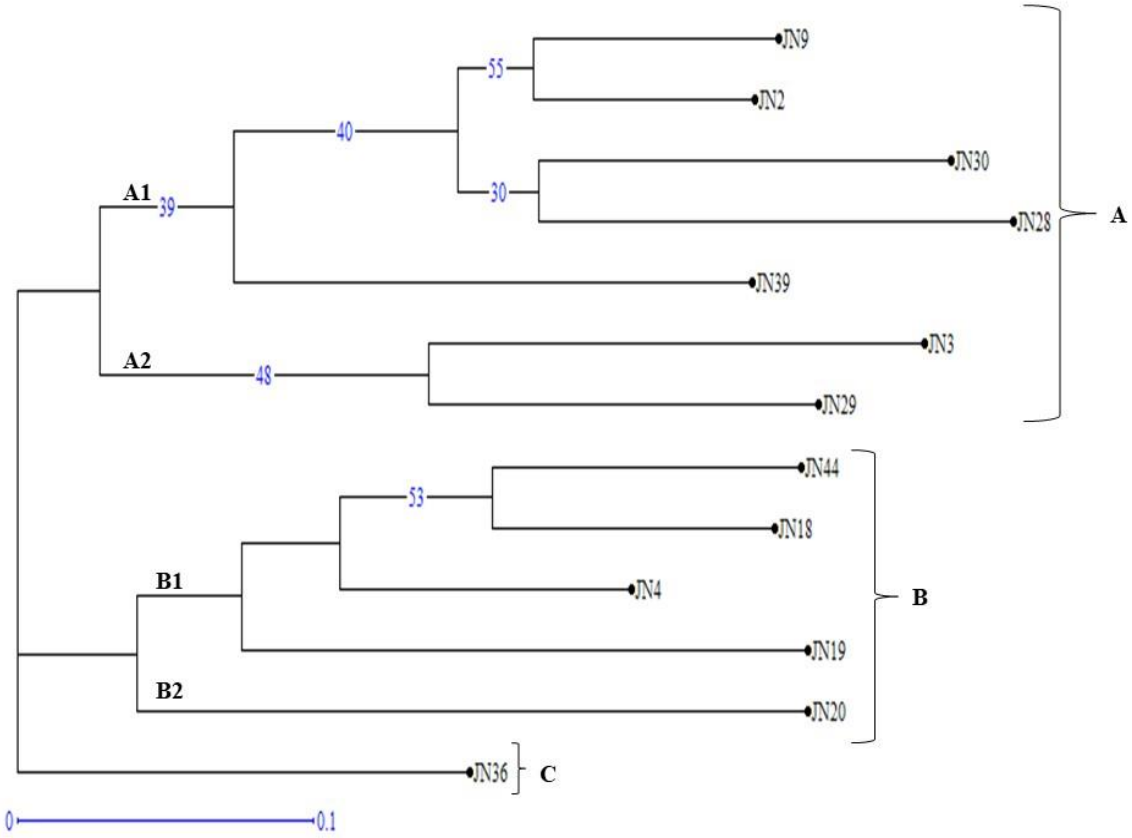

Figure 4.2: Cluster analysis showing the three clusters (A, B and C) of the native rhizobia isolates morphological and biochemical characteristics relatedness based on the neighbor joining method

4.6 Genetic diversity of the rhizobia isolates

4.6.1 Molecular identification of isolates

The DNA fragments of the nodule forming isolates were amplified using the 27F/1492R primer pair targeting the 16S rRNA gene (Plate 4.3). At the species level, these isolates belonged to five rhizobia species (Table 4.4). The majority of the isolates were affiliated to *Rhizobium pusense*, with nine isolates consisting of the various strains in this rhizobia species. These isolates include JN20, JN3, JN28, JN9, JN2, JN18, JN19, JN39 and JN30. Other species identified were *Rhizobium mesosinicum*, *Rhizobium tropici*, *Rhizobium oryzicola*, and *Rhizobium* sp.

According to the neighbour-joining method tree, the sequenced isolates fell in two major clades (Figure 4.3). Clade A had the majority of the isolates with 11 isolates, further subdivided into two sub-clades, A1 and A2. Clade B consisted of the remaining two isolates, consisting of isolates JN36 and JN29 identified as *Rhizobium* sp. and *Rhizobium oryzicola*, respectively.

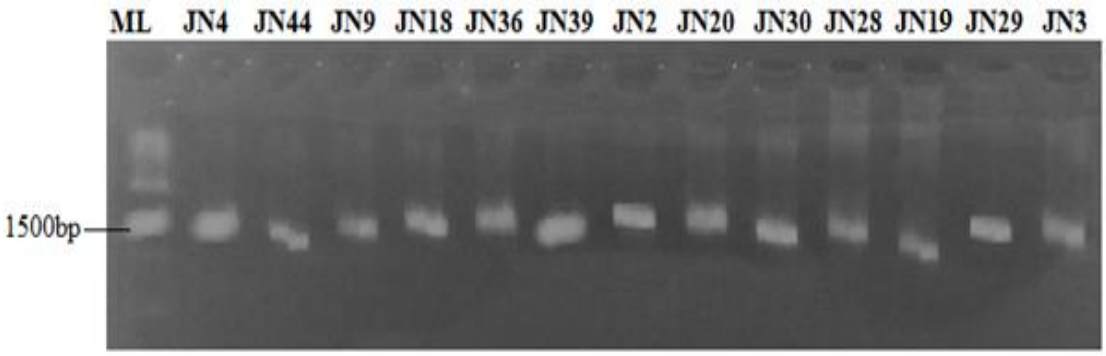

Plate 4.3: PCR products of the amplified 16S rRNA gene of rhizobia. ML, DNA ladder (Biolabs).

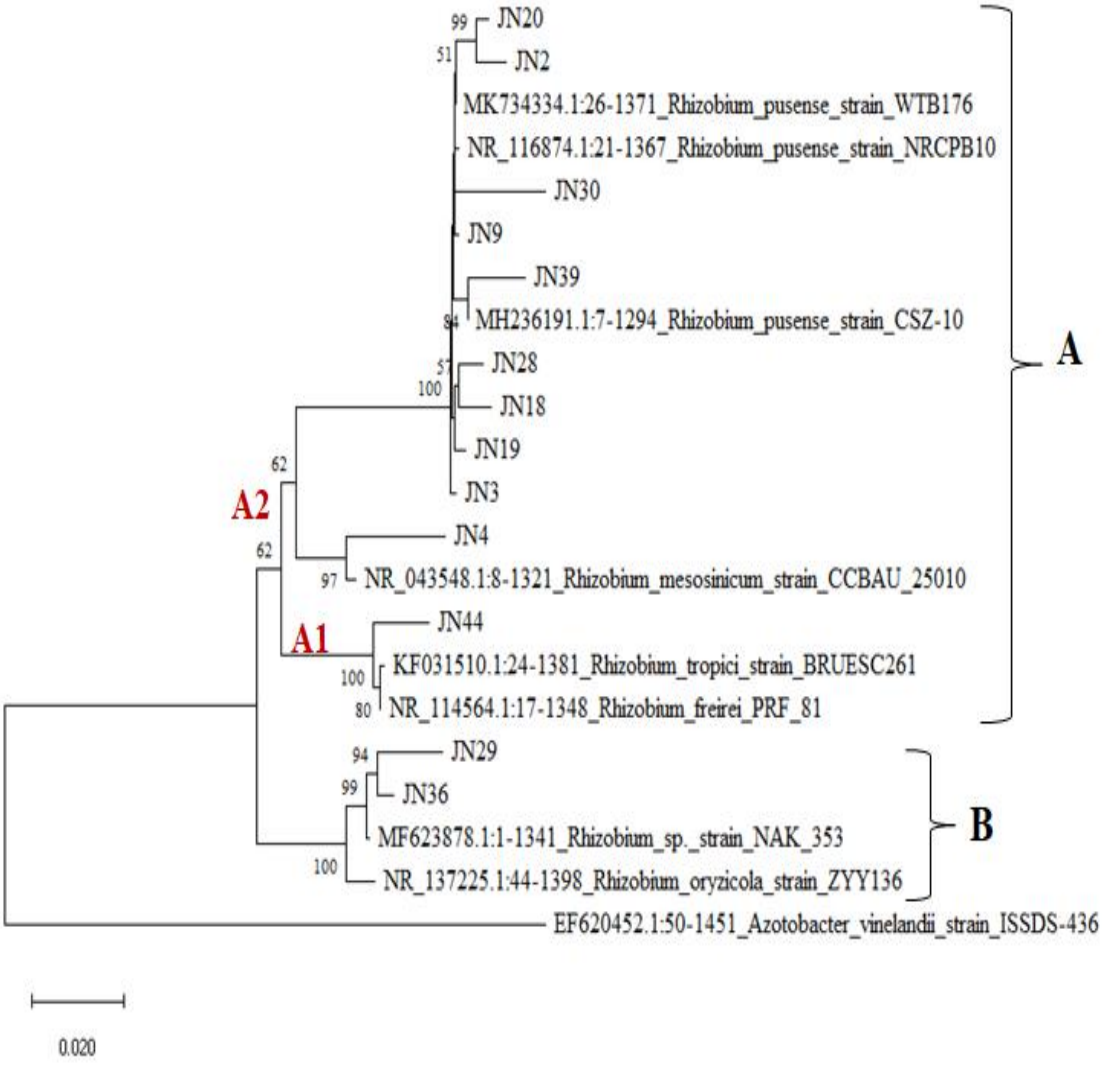

Figure 4.3: Phylogenetic tree based on 16S rRNA gene of the 13 isolates derived from the neighbour joining method on the Jaccard parameter model in MEGA X software.

Table 4.4: Phylogenetic resemblance and similarity of isolates and neighbour on NCBI

Isolate	Molecular identity	Accession No.	Closest type identity in NCBI	Accession No.	Similarity %	Sequence Length
JN4	*Rhizobium mesosinicum*	MW497597	*Rhizobium mesosinicum* strain R1-99	JQ659535.1	97.86	
JN44	*Rhizobium tropici*	MW497602	*Rhizobium tropici* clone H53	EF054892.1	98.46	
JN29	*Rhizobium* sp.	MW497594	*Rhizobium* sp. Strain NAK 353	MF623878.1	99.11	
JN36	*Rhizobium oryzicola*	MW497603	*Rhizobium oryzicola* strain ZYY136	NR137225.1	97.94	
JN20	*Rhizobium pusense*	MW497591	*Rhizobium pusense* strain NRCPB10	NR_116874.1	99.04	
JN3	*Rhizobium pusense*	MW497596	*Rhizobium pusense* strain CSZ-10	MH236191.1	99.77	
JN28	*Rhizobium pusense*	MW497592	*Rhizobium pusense* strain CSZ-10	MH236191.1	99.77	
JN9	*Rhizobium pusense*	MW497598	*Rhizobium pusense* strain NRCPB10	NR_116874.1	99.78	
JN2	*Rhizobium pusense*	MW497593	*Rhizobium pusense* strain WTB7176	MK734334.1	98.81	
JN19	*Rhizobium pusense*	MW497600	*Rhizobium pusense* strain CSZ-10	MH236191.1	99.48	
JN18	*Rhizobium pusense*	MW497599	*Rhizobium pusense* strain WTB7176	MK734334.1	98.89	
JN39	*Rhizobium pusense*	MW497601	*Rhizobium pusense* strain CSZ-10	MH236191.1	98.30	
JN30	*Rhizobium pusense*	MW497595	*Rhizobium pusense* strain WTB7176	MK734334.1	94.13	

4.6.2 Genetic differentiation and diversity

Analysis of molecular variance of the rhizobia isolates from the three populations (Embu, Kitui and Tharaka Nithi) revealed the highest variation within the populations of 107.58% (Table 4.5). The variation among the populations did not have a significant genetic variation at ($P<0.05$) (-7.58%) (Table 4.5).

Table 4.5: Analysis of molecular variance of rhizobia isolates from the three regions

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among populations	2	449.505	23.17759 Va	-7.58
Within populations	11	3619.567	329.05152 Vb	107.58
Total	13	4069.071	305.87393	100

Fixation index F_{ST} : -0.07577

P = 0.87390±0.00995

Significance tests (1023 permutations)

Va and F_{ST} : P (rand. value > obs. value) = 0.87390

P(rand. = obs.) = 0.00000

Further estimation of the population pairwise genetic differentiation revealed no significant differentiation between the populations (Table 4.6). All the pairwise analysis yielded negative F_{ST} values at ($P<0.05$) (Table 4.6).

Table 4.6: Population pairwise (F_{st}) difference between populations

Population 1	Population 2	Population pairwise (Fst)
Kitui	Embu	-0.07281
Kitui	Tharaka Nithi	-0.13473
Embu	Tharaka Nithi	-0.05757

Kitui County recorded the highest nucleotide diversity (Pi and PiJC), while Embu County had the least (Table 4.7). Rhizobia isolates from Tharaka Nithi County had the

highest number of segregating sites. Embu County had the least with 1065 sites (Table 4.7). No difference in haplotype diversity among the three populations was observed.

Table 4.7: Diversity of rhizobia nodulating cowpea in semi-arid areas of Kenya

Population	Number of segregating sites, (S)	Number of haplotypes, (h)	Haplotype diversity, (Hd)	The average number of differences, (K)	Nucleotide diversity, (Pi)	Nucleotide diversity with (JC, PiJC)
Tharaka Nithi	1264	6	1	693	0.519	1.433
Embu	1065	5	1	551.2	0.413	0.878
Kitui	1068	3	1	784.667	0.588	1.687

4.7 Symbiotic efficiency of isolates under greenhouse conditions

The symbiotic efficiency of the isolates was determined by their effect on dry shoot weights upon inoculation following their effectiveness on nodulation and influence on the growth of cowpea. Significant differences at ($P<0.05$) in nodule number (Nod NO), dry nodule weight (Nod DW), shoot dry weight (DW), root DW and symbiotic efficiency (SE) were observed (Table 4.8). Isolate JN18 recorded the highest Nod NO (50.00±8.15) (Plate 4.4). However, this was not significantly different from the other isolates. Biofix recorded the highest Nod DW, similar to all the tested isolates except JN39 and JN28. As expected, uninoculated control and KNO_3 supplemented plants did not form any nodules. Inoculation with the different native rhizobia isolates increased the shoot DW. Isolates JN30, JN4, JN20, JN29, JN28 and JN19 recorded shoot DW statistically similar to that of Biofix and KNO_3 supplemented plants. The rest of the isolates recorded shoot DW significantly lower than that of Biofix and KNO_3 supplemented plants, with the uninoculated control having the least shoot DW (0.20±0.03 g plant^{-1}) (Table 4.8). Isolate JN18 recorded the highest root DW, which was statistically similar to JN30, JN4, JN44, JN29, JN28, JN19 and JN20. The uninoculated control recorded the least root DW. Out of the thirteen isolates tested,

seven (53.85 %) had SE higher than 80 % hence classified as very effective (Table 4.8). Four other isolates with SE ranging between 51-80 % were rated as effective. Only two isolates had SE below 50% (JN39 and JN2) and were rated as lowly effective in fixing N.

Table 4.8: Symbiotic effectiveness of native rhizobia isolates

Treat Ment	Nod No	Nod DW (g)	Shoot DW (g)	Root DW (g)	SE (%)
JN30	28.25±4.13^{abc}	0.03±0.00^{ab}	0.84±0.07^{abcde}	0.41±0.09^{abc}	81.52±8.33^{abcde}
JN4	20.75±11.72^{abc}	0.06±0.02^{ab}	1.05±0.05^{ab}	0.42±0.06^{abc}	102.06±5.17^{ab}
JN20	39.50±4.25^{ab}	0.11±0.01^{ab}	0.85±0.04^{abcde}	0.33±0.07^{bcd}	82.33±3.39^{abcde}
JN39	9.25±5.25^{abc}	0.00±0.00^{b}	0.41±0.06^{gh}	0.19±0.04^{cd}	39.62±5.95^{hg}
JN44	25.00±10.12^{abc}	0.07±0.02^{ab}	1.20±0.14^{a}	0.53±0.13^{ab}	116.81±13.57^{a}
JN9	18.75±7.97^{abc}	0.02± 0.00^{b}	0.53±0.08^{efgh}	0.32±0.02^{bcd}	51.45±7.98^{efgh}
JN2	29.25±5.04^{abc}	0.08±0.00^{ab}	0.45±0.04^{fgh}	0.19±0.03^{cd}	43.93±3.90^{fgh}
JN18	50.00±8.15^{a}	0.13±0.01^{ab}	0.57±0.07^{defgh}	0.63±0.05^{a}	54.99± 6.82^{defg}
JN29	17.00±11.36^{abc}	0.05±0.03^{ab}	0.95±0.10^{abc}	0.36±0.03^{abcd}	92.67±9.37^{abc}
JN28	22.75±4.94^{abc}	0.01±0.00^{b}	0.89±0.07^{abcde}	0.44±0.08^{abc}	86.50±6.68^{abcde}
JN36	31.±3.54^{abc}	0.04±0.00^{ab}	0.79±0.03^{bcdef}	0.26±0.02^{bcd}	76.25±2.45^{bcdef}
JN19	26.25±2.78^{abc}	0.08±0.00^{ab}	0.97±0.04^{abc}	0.40±0.04^{abc}	94.53±4.31^{abc}
JN3	28.00±3.24^{abc}	0.06±0.00^{ab}	0.07±0.00^{bcdefg}	0.43±0.02^{abc}	70.49±7.77^{bcdefg}
Biofix	34.00±3.54^{ab}	0.17±0.10^{a}	0.91±0.05^{abcd}	0.32±0.08^{bcd}	88.22±5.1^{abcd}
+KNO$_3$	0	0	1.03±0.09^{abc}	0.33±0.02^{abcd}	100.00±8.67^{abc}
Control	0	0	0.20±0.03^{h}	0.09±0.03^{d}	19.02±3.35^{h}
P-Values	<.0001	0.0006	<.0001	<.0001	<.0001

Key: Nod NO, Nodule number; Nod DW, Nodule dry weight; Shoot DW, dry shoot weight; Root DW, dry root weight; g, grams; +KNO3, plants supplemented with potassium nitrate; USDA 3456, commercial inoculant. Mean±SD values followed by the same letters within a column are not statistically different (Tukey`s Honest Significant Difference (THSD)) at P <0.05.

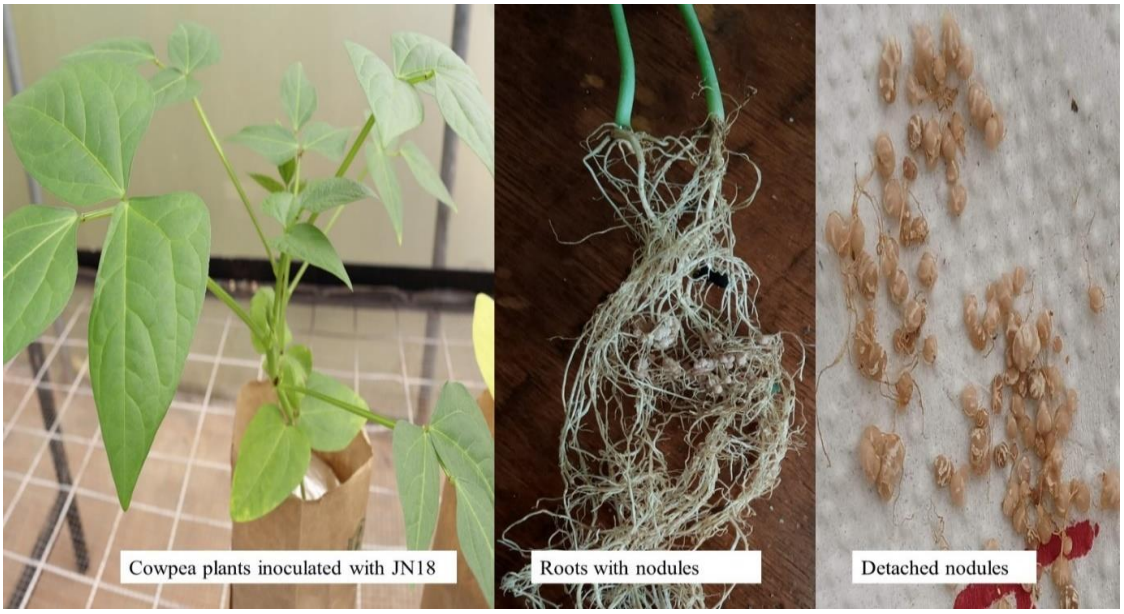

Plate 4.4: Nodulation on cowpea plant inoculated with a native rhizobia isolate

4.8 Effect of rhizobia inoculation on cowpea growth and production in the field

4.8.1 Effect on nodule number and dry weight

The Nod NO and Nod DW were significantly enhanced by rhizobia inoculation ($P<0.0001$) (Plate 4.5) during season one relative to controls (Table 4.9). However, in season two inoculation had no significant effect on the Nod NO recorded. Nevertheless, native isolates and consortium recorded the highest Nod DW while the control had the least Nod DW in season two. Overall, better nodulation was observed in season one when compared to season two. For example, the consortium had the highest number of nodules in both seasons, recording 41.58±3.60 and 14.02±0.82 nodules plant^{-1}in season one and two, respectively. Of the three varieties, K-80 and KVU 27-1 recorded statistically high Nod NO compared to the M-66 cowpea variety in both seasons (Table 4.9). While no significant difference in Nod DW among the three varieties was present in season one, KVU 27-1 recorded the highest (0.05±0.01gplant^{-1}) Nod DW while M-66 had the least (0.036±0.00 gplant^{-1}) in season two. The Nod DW in the K-80 variety

was not significantly different from the other two varieties. Although rhizobia inoculation increased the Nod NO and Nod DW in the individual cowpea varieties when compared to their uninoculated counterparts in the three sites, variety × rhizobia inoculant interaction was not statistically significant ($P<0.05$) across the locations (Table 4.9). The nodule number and dry weight also differed significantly among the three sites in the two seasons. In season one, Tharaka Nithi recorded the highest Nod NO and Nod DW. In season two, Embu registered the highest Nod NO and Nod DW (Table 4.9). Kitui County recorded the least Nod NO in both seasons.

Additionally, the site × rhizobia inoculant interaction was significant ($P<0.05$) for Nod NO in season one (Table 4.9). During this season, plants inoculated with consortium recorded a high nodule number in all the sites than when inoculated with native isolates and Biofix. Moreover, the site × inoculant interaction was also significant for Nod DW in both seasons, whereby plants inoculated with native isolates showed increased Nod DW in both seasons.

Table 4.9: Effect of rhizobia inoculation on nodulation at the flowering stage of cowpea

	Season 1		Season 2	
	Nodule number	**Nodule dry weight (g)**	**Nodule number**	**Nodule dry weight(g)**
Site				
Tharaka Nithi	60.59±3.16[a]	0.42±0.02[a]	12.76±0.69[b]	0.04±0.00[b]
Embu	23.86±0.70[b]	0.10±0.00[b]	18.36±0.76[a]	0.07±0.01[a]
Kitui	17.34±0.92[c]	0.12±0.01[b]	9.87±0.35[c]	0.03±0.00[b]
Variety				
K-80	36.13±2.52[a]	0.23±0.02[a]	14.37±0.61[a]	0.046±0.00[ab]
M-66	30.16±2.10[b]	0.19±0.02[a]	11.71±0.55[b]	0.036±0.00[b]
KVU 27-1	30.16±2.10[ab]	0.22±0.02[a]	14.91±0.78[a]	0.05±0.01[a]
Inoculant				
Native	34.88±2.65[a]	0.22±0.02[a]	14.44±0.81[a]	0.05±0.00[a]
Consortium	41.58±3.60[a]	0.27±0.03[a]	14.02±0.82[a]	0.05±0.01[a]
Biofix	38.30±2.87[a]	0.25±0.02[a]	13.48±0.74[a]	0.04±0.00[ab]
Control	20.98±0.97[b]	0.12±0.01[b]	12.71±0.71[a]	0.03±0.00[b]
P values of the main factors and their interactions				
Variety	0.0215	0.1163	0.0004	0.0055
Inoculant	<.0001	<.0001	0.3359	0.0006
Site	<.0001	<.0001	<.0001	<.0001
Variety*Inoculant	0.5348	0.7414	0.9419	0.7226
Site*Variety	0.1261	0.1554	0.1437	0.4722
Site*Inoculant	<.0001	<.0001	0.2143	0.0004
Site*Variety*Inoculant	0.0988	0.2387	0.9362	0.8976

Key: g, grams. Mean±SD values followed by the same letters within the columns are not statistically different (Tukey`s HSD at P<0.05).

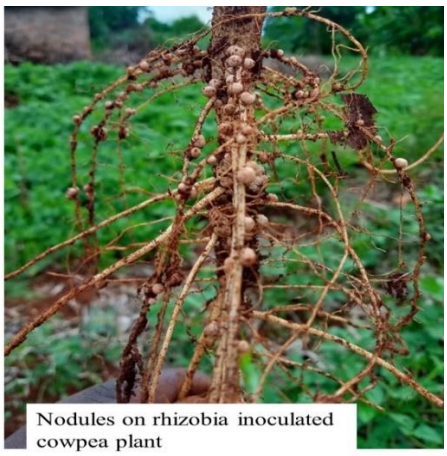

Plate 4.5: Cowpea plants (M-66 variety) and nodulation in Tharaka Nithi County during sampling

4.8.2 Effect rhizobia inoculation on above-ground biomass of cowpea

Rhizobia inoculation significantly enhanced the shoot DW at flowering compared to the controls but had no significant effect on the number of leaves recorded plant^{-1} and plants' height in both seasons (Table 4.10). Additionally, the number of leaves, plant height, and shoot DW were statistically similar among the three cowpea varieties in season one. However, while the shoot DW and plant height did not differ among the three varieties in season two, K-80 and KVU 27-1 were more productive in terms of the number of leaves with the least number found in M-66 (Table 4.10). Cowpea inoculated with rhizobia in Tharaka Nithi County produced the highest shoot DW with the least found in Kitui in season 1. Embu county recorded the highest number of leaves and plants heights, followed by Tharaka Nithi and Kitui (Table 4.10) during the first season. In season two, the number of leaves and shoot DW recorded in Embu, and Tharaka Nithi were not statistically different. However, cowpea plants in Kitui County recorded the least number of leaves, plants height and shoot DW (Table 4.10).

Additionally, the site × inoculant interaction on the shoot DW was significant at $P < 0.05$ in both seasons (Table 4.10). In season one, plants inoculated with the consortium and Native isolates recorded significantly high shoot DW in all three sites. In comparison, in season two, cowpea plants inoculated with native isolates recorded the highest shoot DW in all three areas.

Table 4.10: Effect of rhizobia inoculation on above-ground cowpea growth at flowering

	Season 1			Season 2		
	Height	**Leaf No**	**Shoot DW(g)**	**Height**	**Leaf No**	**Shoot DW (g)**
Site						
Tharaka Nithi	65.21±1.80[b]	25.08±0.73[b]	32.51±1.40[a]	52.46±1.12[a]	24.08±0.77[a]	9.74±0.40[a]
Embu	74.75±1.82[a]	30.49±1.31[a]	15.75±0.65[b]	51.57±0.82[a]	18.58±0.53[b]	9.81±0.36[a]
Kitui	45.09±0.71[c]	21.15±0.59[c]	8.18±0.29[c]	38.86±0.67[b]	6.01±0.29[c]	2.01±0.14[b]
Variety						
K-80	60.46±1.68[a]	27.05±0.83[a]	18.71±1.21[a]	46.45±0.89[a]	17.56±0.82[a]	7.01±0.41[a]
M-66	62.61±1.84[a]	25.11±1.28[a]	19.26±1.22[a]	47.35±1.02[a]	14.38±0.71[b]	6.91±0.40[a]
KVU 27-1	62.61±1.84[a]	25.11±1.28[a]	18.48±1.11[a]	49.09±1.09[a]	16.74±0.85[a]	7.63±0.46[a]
Inoculant						
Native	61.38±2.05[a]	25.71±0.90[a]	19.82±1.48[a]	48.34±1.30[a]	15.55±0.89[a]	8.29±0.53[a]
Consortium	60.73±1.86[a]	25.16±0.77[a]	20.14±1.52[a]	46.97±1.05[a]	16.23±0.90[a]	7.66±0.48[a]
Biofix	64.87±2.15[a]	27.46±1.69[a]	19.45±1.35[a b]	47.59±1.25[a]	16.87±1.85[a]	7.20±0.48[a]
Control	59.76±2.19[a]	23.96±0.87[a]	15.84±1.03[b]	47.63±1.03[a]	16.25±0.85[a]	5.60±0.43[b]
P values of the main factors and their interactions						
Variety	0.5931	0.1465	0.8148	0.1080	0.0002	0.2021
Inoculant	0.1888	0.1452	0.0099	0.8318	0.5559	<.0001
Site	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Variety*Inoculant	0.1198	0.6994	0.5564	0.8529	0.9981	0.9978
Site*Variety	0.6374	0.7966	0.9057	0.9472	0.3803	0.3624
Site*Inoculant	0.2361	0.7097	0.0011	0.4092	0.3913	<.0001
Site*Variety*Inoculant	0.3103	0.8064	0.1208	0.2711	0.5332	0.9839

Key: Nod NO, Nodule number; Nod DW, Nodule dry weight; Leaf NO, Leaf number; Shoot DW, shoot dry weight; g, grams. Mean±SD values followed by the same letters within the columns are not statistically different (Tukey`s Honest Significant Difference (THSD)) at P<0.05

4.8.3 Effect of rhizobia inoculation on AMF colonization on cowpea roots

Rhizobia inoculation had no significant impact on the AMF colonization % during the first growing season. However, it significantly influenced colonization (P = 0.0370) during the second growing season (Table 4.11). Inoculation with Biofix showed the highest AMF colonization with 48.74±1.25 during the second season, while cowpea

plants inoculated with native isolates had the least AMF colonization with 45.46±1.29. The AMF colonization percentage in the plants inoculated with the consortium and the uninoculated plants did not differ significantly with those inoculated with Biofix and native isolates (Table 4.11). Overall, AMF colonization was high in season one when compared to the second season.

All three cowpea varieties did not show a preference for AMF colonization following rhizobia inoculation in both seasons (Table 4.11). However, the AMF colonization in season one in all three varieties was higher than in the second season. There existed a significant ($P = 0.0262$) interaction between the cowpea varieties and the study sites during the second growing season and a significant ($P = 0.0396$) three-way interaction of the cowpea variety × rhizobia inoculant × study site.

Among the three study locations, a significant ($P = 0.0048$) AMF colonization percentage was observed during the first growing season (Table 4.11). During this season, Kitui and Tharaka Nithi counties recorded the highest AMF colonization percentage with 50.88±1.02 and 50.86±1.01, respectively. Conversely, Embu recorded a significantly low AMF colonization percentage compared to the other two locations with 47.73±1.02. Unlike in season one, there was no significant difference in AMF colonization in the three areas in season two (Table 4.11). In addition, a comparison between the two seasons indicates a significantly higher AMF colonization in season one than in season two.

Table 4.41: Percentage AMF colonization on cowpea roots inoculated with rhizobia

	% AMF root colonization	
	Season 1	**Season 2**
Site		
Kitui	50.88±1.02[a]	48.37±1.22[a]
Tharaka Nithi	50.86±1.01[a]	48.30±1.26[a]
Embu	47.73±1.02[b]	46.20±1.28[a]
Variety		
K-80	48.77±1.02[a]	47.94±1.25[a]
M-66	50.41±1.02[a]	47.87±1.26[a]
KVU 27-1	50.25±1.01[a]	47.04±1.25[a]
Inoculant		
Native	49.83±1.02[a]	45.46±1.29[b]
Biofix	49.48±1.02[a]	48.74±1.25[a]
Consortium	50.99±1.02[a]	48.60±1.25[ab]
Control	48.93±0.02[a]	47.72±1.23[ab]
P values of the main factors and their interactions		
Variety	0.4085	0.6658
Inoculant	0.3403	0.0370
Site	0.0048	0.0848
Variety*Inoculant	0.3723	0.3346
Site*Variety	0.6037	0.0262
Site*Inoculant	0.1012	0.1113
Site*Variety*Inoculant	0.2409	0.0396

Key: %, percentage. Mean±SD values followed by the same letters within the columns are not statistically different (Tukey`s Honest Significant Difference (THSD)) at $P<0.05$

4.8.4 Effect on yield and stover weight

Rhizobia inoculation significantly ($P<0.05$) increased cowpea yields. In season one, native isolates recorded the highest yields with 940.90±71.88 kg ha^{-1} while the control had the least (766.60±61.86 ha^{-1}), which is a 22.74% increase compared to the uninoculated control. Consortium and Biofix recorded an 18 and 7.8% increase, although this was no significantly different from the uninoculated control (Table 4.12). Similarly, native isolates recorded the highest yields in season two with 800±79.75 kg ha^{-1}, which is a 28.6% increased yields over the uninoculated control. This was not significantly different from yields of plants inoculated with the consortium (716.83±68.77 kg ha^{-1}). Still, it differed considerably from those inoculated with Biofix

and the uninoculated controls. (Table 4.12). However, cowpea plants inoculated with Biofix had seeds with the highest 100 seed weight in season one. Their weights did not differ significantly with those inoculated with native isolates and consortium at $P < 0.05$. Uninoculated plants recorded the least 100 seed weight in season one. In contrast, no significant influence on 100 seed weight was observed in season two (Table 4.12). Similarly, the impact of rhizobia inoculation on the stover weight was only present in season two (Table 4.12).

The yield performance of the three cowpea genotypes was not influenced by rhizobia inoculation (Table 4.12). However, the seeds' quality in terms of the 100 seed weight depended on the cowpea genotype. KVU 27-1 and K-80 recorded the highest and lowest 100 seed weight across the two seasons. A significant variety × inoculant interaction was present on the 100 seed weight in both seasons. In this case, plants inoculated with native isolates and Biofix yielded the highest 100 seed weight in season one and two, respectively. While no significant difference in stover weight was observed in season one among the three genotypes, K-80 recorded the highest stover weight at harvest in the second season.

Additionally, the yields, 100 seed weight and the stover weight were significantly influenced by the study site in both seasons ($P<0.0001$). In the first season, Tharaka Nithi and Kitui regions recorded the highest yields while Embu recorded significantly low yields 55.62 ± 7.23 kg ha^{-1}. Tharaka Nithi recorded the highest yields during the second season, which was significantly higher than that of Embu, while Kitui recorded the lowest yields (Table 4.12). In addition, there was a significant site × inoculant interaction in both seasons (Table 4.12). In both cases, inoculation with native isolates led to better yields when compared to the other inoculants. Correspondingly, Tharaka

Nithi and Kitui regions had the highest 100 seed weight while Embu had the least during the first season. In the second season, Kitui recorded a significantly low 100 seed weight compared to Tharaka Nithi and Embu. The highest stover weight was recorded in the Embu region during the two seasons, which was significantly higher than in the other two areas. However, no significant difference in stover weight between Kitui and Tharaka Nithi was observed in season one, although they differed significantly in season two. A significant site and variety interaction on stover were present in the second season. In this case, K-80 plants in Embu and Tharaka Nithi recorded the highest stover weight, while in Kitui, M-66 plants recorded significantly high stover weight.

Table 4.52: Effect of rhizobia inoculation on cowpea yield, and dry stover weight

	Season 1			Season 2		
	Yield (Kg ha^{-1})	**Seed weight (per 100 seeds)**	**Stover weight (g)**	**Yield (Kg ha^{-1})**	**Seed weight (per 100 seeds)**	**Stover weight (g)**
Site						
Tharaka Nithi	1294.35±40.19^{a}	11.91±0.14^{a}	12.53±0.39^{b}	1338.34±53.71^{a}	12.27±0.11^{a}	18.89±0.73^{b}
Embu	55.62±7.23^{b}	4.94±0.30^{b}	22.21±0.65^{a}	781.28±62.65^{b}	11.69±0.27^{a}	39.39±1.37^{a}
Kitui	1228.94±50.56^{a}	11.91±0.13^{a}	14.08±0.51^{b}	17.27±2.49^{c}	2.63±0.28^{b}	0.33±0.04^{c}
Variety						
K-80	834.43±57.40^{a}	8.81±0.35c	16.51±0.63^{a}	771.46±61.28^{a}	8.16±0.38^{b}	21.21±1.55a
M-66	854.43±53.41^{a}	9.63±0.28b	16.19±0.60^{a}	663.79±54.84^{a}	8.91±0.40^{ab}	17.33±1.33b
KVU 27-1	890.06±59.17^{a}	10.32±0.32a	16.13±0.61^{a}	761.63±70.29^{a}	9.52±0.43^{a}	20.08±1.56ab
Inoculant						
Native	940.90±71.88^{a}	9.77±0.38^{ab}	15.86±0.69^{a}	800.62±79.75^{a}	8.88±0.46^{a}	21.56±1.90^{a}
Consortium	904.85±64.79^{ab}	9.58±0.38^{ab}	16.50±0.69^{a}	716.83±68.77^{ab}	8.85±0.46^{a}	20.69±1.87^{a}
Biofix	826.20±62.17^{ab}	9.97±0.36^{a}	16.94±0.77^{a}	709.22±76.94^{b}	9.02±0.46^{a}	19.10±1.69^{ab}
Control	766.60±61.86^{b}	9.04±0.36^{b}	15.81±0.69^{a}	622.51±61.36^{b}	8.71±0.48^{a}	16.82±1.34^{b}
P values of the main factors and their interactions						
Variety	0.5531	<.0001	0.8604	0.3491	0.0002	0.0046
Inoculant	0.0168	0.0286	0.5089	0.1557	0.8734	0.0044
Site	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Variety*Inoculant	0.9440	0.7429	0.8096	0.8362	0.7941	0.4990
Site*Variety	0.4348	0.0176	0.8060	0.5254	0.0314	0.0461
Site*Inoculant	<.0001	0.0042	0.5948	0.0212	0.9684	<.0001
Site*Variety* Inoculant	0.4965	0.8654	0.6848	0.9527	0.9444	0.6005

Key: Kg ha^{-1}, cowpea yields in kilograms achieved in one hectare; g, grams. Mean±SD values followed by the same letters within the column are

not statistically different (Tukey`s HSD at P <0.05).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMEDATIONS

5.1 Discussion

5.1.1 Influence of soil characteristics on AMF infective propagules in the soil

The AMF colonization enumerated as infective propagules (arbuscules and vesicles) on Bermuda grassroots showed different AMF infections on the soil. The choice to use Bermuda grass was based on the grass fast growth and its characteristic fibrous root, which provides a better association with AMF (Abd Rahim *et al*., 2016). The MPN values of the AMF infective propagules varied substantially among the 15 study farms. The variation could be attributed to the broad range of geographical variables such as soil type, pH and available P (Wang *et al*., 2008).

The MPN values in the sampled soil were positively correlated with silt %, Mg, Ca, sand %, soil pH and Cu and negatively correlated with P, Fe, Mn, K, Zn, Na, TOC, TN and clay %, which suggests that they could have impacted on the AMF infectivity on Bermuda grass. The low AMF infective propagules in clay soil are due to its high water retention capacity, making it remain wetter for more extended periods and has been demonstrated in clay-rich soils in India (Rathore and Singh, 1995; Mathimaran *et al*., 2005). Nevertheless, AMF are abundant in drier regions (De Souza *et al*., 2013), with sandy soils, low in nutrients and organic matter (Alarcón and Cuenca, 2005), confirming the current finding.

The variance in MPN values of the AMF infective propagules and the soil chemical properties in the different study areas suggest that the chemical properties of the soil influence the AMF densities in the soil and the infective propagules present in the plant roots. This could be because the soil chemical properties are the key determinants of

soil fertility. The negative correlation between the MPN values and the soil available P could be due to high P content in the soil. This reduces the densities of the AMF limiting its infectivity, which consequently influence the infective propagules estimates (Collins and Foster, 2009). Phosphorus content in soil is also crucial in structuring the fungal communities in the soil, including the AMF richness (Siciliano *et al*., 2014). Thus, at low P, AMF plays a critical role in accessing P hence higher AMF infectivity in soil with low P than those rich in P.

Soil pH positively correlated with MPN values. The soils were lowly acidic, with a few farms having a pH above 7.0. Soil acidity is unfavourable for soil P availability, with a pH of 6.0 – 6.5 being optimal for plant P absorption (Smith and Read, 2008). In acidic soils, exchangeable cations such as Fe^{2+}, Mn^{2+} and Al^{3+} block the soils' available P, making it unavailable for plant absorption (Oteino *et al*., 2015). So, acidic soils are characterized by low available P (Kazadi *et al*., 2020). In such soils, AMF plays an important role in extracting the unavailable P from the soil and availing it to the crops (Kluber *et al*., 2012; Jan *et al*., 2014). Therefore, it is expected that soils with low available P have a high AMF colonization, as shown in the current study, where the available P was negatively correlated to the AMF-MPN values. Similarly, the exchangeable cations were negatively correlated to the AMF-MPN values, possibly because of their hindrance on the availability of P in the soil, which resulted in the increased AMF colonization on the Bermuda grass.

5.1.2 Morphological and biochemical characteristics of the root nodule isolates

All the rhizobia isolates identified were Gram-negative and did not absorb Congo red dye when incubated in the dark which are characteristics of rhizobia (Somasegaran and Hoben, 1985). Rhizobia lacks the ability to absorb the Congo red dye producing white to cream colonies on media supplemented with the dye, unlike other soil bacteria. The

isolates were white, cream white, or milky white and turned YEMA-BTB media from green to yellow, indicating that they are fast-growing and acid-producing (Boakye *et al*., 2016). Although cowpea forms an association with both slow and fast-growing rhizobia, most of the rhizobia nodulating cowpeas in the semiarid areas of Kenya are fast-growing (Ondieki *et al*., 2017). This fast-growth characteristic is a survival strategy of rhizobia in semiarid areas to thrive under unfavourable agro-climatic conditions. This enables them to multiply rapidly over a short period, ensuring they remain extant in the soil even under adverse conditions (Borges *et al*., 2010).

The size of the colonies also significantly varied across the different isolates. While most isolates had a gummy texture, a few produced EPS. Production of EPS is an adaptive and protective feature of rhizobia under a hostile environment that helps prevent cells' desiccation primarily due to high temperatures (Adriana Giongo et al., 2010). Also, it suppresses the host plant defense reactions such as reactive oxygen species production upon plant infection (Fraysse *et al*., 2003; Scheidle *et al*., 2004). It is an essential feature in which isolates that produce mucus show a competitive advantage during infection and nodulation (Batista *et al*., 2007). The significant difference in the isolates' morphological characteristics is an indicator of diverse indigenous rhizobia-nodulating cowpea in the study locations. Isolates well adapted to the local agro-ecological and climatic conditions with a high BNF could be considered to develop commercial rhizobia inocula (Berrada *et al*., 2012).

5.1.3 Authentication of cowpea root nodule isolates

Although all the isolates were initially placed in 20 groups based on their morphological characteristics, only 13 isolates representing the distinct groups could re-infect the host during their authentication. This indicates that 35% of the isolates from root nodules were not nodule forming rhizobia and could potentially be bacterial endophytes co-

occupying the cowpea nodules with rhizobia. The existence of non-rhizobia endophytes in the root nodules has been previously reported (Abdelnaby *et al*., 2015). These non-rhizobia bacterial endophytes promote plant growth by solubilizing P and Zn and produce siderophore, IAA, and ammonia. They also have ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, act as biological control agents against plant pathogens (Egamberdieva *et a*l., 2017; Raja and Uthandi, 2019; Bakhtiyarifar *et al*., 2020) and enable the plant to tolerate salt stress in semiarid areas (Leite *et al*., 2017).

5.1.4 Diversity of rhizobia isolates based on sequencing of 16S rRNA gene

The 16S rRNA gene sequencing revealed that different rhizobia species and strains nodulate cowpea. Some were specific to one cowpea variety, while others were isolated from all three cowpea varieties. This could indicate the existence of specificity in the rhizobia-cowpea interaction. Generally, rhizobia have a narrow specificity in the host-rhizobia symbiosis. Hence, the host identity plays a crucial role in determining the rhizobial diversity, effectively fixing N with a given host (Laguerre *et al*., 2003; Miranda-Sánchez *et al*., 2016). Moreover, different plant varieties of the same host plant species can select and discriminate against some rhizobia species in their symbiotic interactions (Gubry-Rangin *et a*l., 2010; Sachs *et al*., 2010). Therefore, identifying the diversity of rhizobia nodulating a given host crop is the first step for selecting and mass-producing rhizobia inoculants.

In the semi-arid regions of Kenya, only one genus of rhizobia was found to nodulate the three cowpea genotypes. All the rhizobia nodulated cowpea were affiliated to different species of the genus *Rhizobium*. No genetic differentiation in rhizobia nodulating cowpea was present when these areas were compared; instead, the genetic differentiation was present in the rhizobia species within a population. This indicates that the rhizobia diversity varied over a small geographical location (within a study site)

rather than a large geographical setting. This point out the possibility of the rhizobial populations in these areas having a common ancestral origin (Mwenda *et al*., 2011).

Additionally, the absence genetic differentiation (Fst) in all three populations shows that genetic diversity is not necessarily dependent on the site of origin (Rejili *et al*., 2009). This could have been attributed to the regions having similar climatological conditions such as temperature and rainfall, which are important to the occurrence and diversity of rhizobial species. The areas independently differ in soil characteristics, including acidity levels which contributes to the genetic variation of the rhizobia population present in each region. For example, rhizobia populations have varying levels of tolerance to acidity/alkalinity of the soil; hence the rhizobia diversity will vary depending on the soil pH (Stefan *et al*., 2018).

The genus *Rhizobium* nodulates with cowpeas (Zhang *et al*., 2007; Guimarães *et al*., 2012; Castro *et al*., 2017) and other legume crops in semi-arid areas (Li *et al*., 2012). In this study, *R. oryzicola*, *R. pusense*, *R. mesosinicum* and *R. tropici* nodulated cowpea. *Rhizobium mesosinicum* and *R. tropici* were the best isolates with symbiotic efficiency above 100%. To my knowledge, this is the first case of *R. tropici* and *R. mesosinicum* nodulating cowpea. *Rhizobium tropici* is competitive and efficiently fixes N in common bean (Mostasso *et al*., 2002; Pinto *et al*., 2007), and a commercial inoculant *R. tropici* CIAT 899 is available for the legume (Martinez-Romero *et al*., 1991). This also shows the significance of this strain as a potential inoculant for the cowpea. The isolation of *R. tropici* from cowpea nodules could be attributed to other legume crops grown in the areas such as the common bean and sharing of the rhizobia symbionts.

Rhizobium mesosinicum has been isolated in legumes growing in the deserts as a potential inoculant under such unfavourable conditions (Abd-Alla *et al*., 2017). This

suggests that this strain effectively fixes N and is well adapted to the adverse climatic and agro-ecological conditions present in the semi-arid areas of Kenya. Nevertheless, most of the rhizobia nodulating cowpea consisted of different strains of the species *Rhizobium pusense*. This suggests that this strain has better adaptability to the prevailing semi-arid conditions, although it had low efficiency in N fixation.

These findings contradict previous findings that *Bradyrhizobium* is the primary symbionts of cowpea (Wade *et al*., 2014; Tampakaki *et al*., 2017; Ndungu *et al*., 2018). *Bradyrhizobium* is slow-slowing rhizobia that have extensively been reported to associate with cowpea in many parts of the world (Tampakaki *et al*., 2017). Low numbers of *Bradyrhizobium* strains have been reported to nodulate legumes in the semi-arid areas compared to other genera (Li *et al*., 2012). This could suggest that *Bradyrhizobium* species are not well adapted for the harsh agro-climatic conditions experienced in the semi-arid areas compared to other genera.

5.1.5 Symbiotic efficiency of native rhizobia isolates

Results from the greenhouse experiment showed that inoculation with the native rhizobia isolates significantly increased cowpea nodulation, shoot DW and root DW compared to the uninoculated controls. The potential of the symbiotic interaction in benefiting the legume crop is dependent on the effectiveness of the rhizobia to fix N into ammonia. The most effective rhizobia strains on the legumes are utilized as biofertilizers, a sustainable technique for promoting legume production (Stajković *et al*., 2011). Most native isolates exhibited superior performance in nodulation, shoot DW, root DW, and SE. This reflects the existence of superior rhizobia strains in the family farming systems, which can be exploited as low-cost inoculants to improve cowpea productivity. The isolates that yielded the highest nodule numbers also translated to a high nodule dry weight. This mirrors effective symbiosis between the

host and rhizobia, especially when it translates to higher biomass production (Gyogluu *et al*., 2018). However, for isolate JN18, the high nodule number did not translate to a high shoot DW. This indicates that the nodule number alone may not reflect the effectiveness of an isolate in fixing N. Some rhizobia strains can form a large number of nodules but have little or no N fixing abilities (Abd El-Maksoud and Keyser, 2010). These strains may have parasitic behaviours; hence, they nodulate with their host but are not efficient in N fixation (Denison and Kiers, 2004). This is because legumes cannot consistently discriminate against strains with low N fixation abilities.

Cowpea inoculation with native rhizobia increased the shoot DW and root DW. The increased shoot DW upon inoculation is due to legumes enhanced plant growth leading to increased biomass production (Kawaka *et al*., 2014; Jalloh *et al*., 2020; Matse *et al*., 2020). The high root DW and high shoot DW of native rhizobia treated plants could result from plant-growth-promoting hormones such as 3-indoleacetic acid (IAA) produced by the native isolates (Mabrouk *et al*., 2018). In KNO_3-treated plants and the negative controls, no nodules were formed, which confirmed the absence of any contamination. The use of sterile vermiculite ensured the absence of any rhizobia contaminants in the controls, hence the lack of N fixation attributing to the reduced biomass formation and, subsequently, the low shoot DW in the negative control (Muleta *et al*., 2017). The better growth of cowpea recorded in native isolates compared to the Biofix indicate that the native isolates are superior, hence have the potential for exploitation as biofertilizers to enhance cowpea growth and production.

5.1.6 Effectiveness of rhizobia under field conditions

Rhizobia inoculation on cowpea with the consortium, native isolates and Biofix enhanced cowpea nodulation and shoot DW at flowering than the control plants. The native isolates recorded higher Nod and shoot DW compared to the other inoculants.

The superiority of the native isolates could be linked with good adaptability to the local agro-ecological environment. Therefore, their reintroduction increases their numbers in the fields, leading to increased N fixation. Reintroduction of well-adapted rhizobia strains on legumes from which they were isolated has been reported to result in effective symbiotic associations leading to increased nodule formation and nodule dry weight (Matse *et al*., 2020).

Nodule formation on the roots of control plants is due to the free-living rhizobia present in the soil. Therefore, the increased nodule formation following inoculation could result from increased rhizobia occupancy in the cowpea root nodules due to increased rhizobia in the soil. The increased nodule occupancy leads to better N fixation necessary for shoot development in plants (Abou-Shanab *et al*., 2019). Among the three cowpea genotypes, K-80 and M-66 formed the highest and lowest number of nodules and dry nodule weight, respectively. The differences in legume varietal response to inoculation in terms of nodulation have been reported as an important trait in legume crop that plant breeders can exploit to have crops with a high N fixation ability (Hossain *et al*., 2016). This is especially in cowpeas, where nodulation has been reported to be dependent on the cowpea genotype (Njeru *et al*., 2020). Although the M-66 genotype had the least nodule number and nodule dry weight, the shoot DW did not differ significantly with the other two genotypes. This could be because all the three genotypes are well-adapted to both the study regions' environmental and ecological conditions (Recha *et al*, 2013). In addition, it seems all three genotypes did not discriminate against the inoculants; thus, no interaction of inoculant by genotype was present, which suggests that the cowpea genotypes' response to inoculation was the same across the three study sites. However, in the study locations, significant differences were present in the nodulation and shoot dry weight. This could be associated with the varying soil physicochemical

characteristics in the study sites. The performance of individual genotype did not differ across the location. However, a better response to inoculation was present in Tharaka Nithi than in the other two regions.

Similarly, the season of planting greatly influenced nodulation and the shoot dry matter. Tharaka Nithi and Kitui regions recorded a significantly low nodule number and dry weight in the second season. This could be associated with the unexpected low rainfall received in these regions during the second season. Moisture stress and drought have been reported to limit nodule formation, subsequently limiting N fixation (Sindhu *et al*., 2020). This has been linked to increased acid phosphatases and antioxidant activity in the root nodule during drought conditions (Mouradi *et al*., 2018).

Cowpea yield in the field increased with inoculation. Remarkably, native isolates *Rhizobium. tropici*, *Rhizobium. mesosinicum*, and *Rhizobium. pusense* recorded the highest yield per hectare. Simultaneously, the consortium and Biofix performance did not differ significantly from the control plants. This superiority of the native rhizobia isolates indicates that there exist effective rhizobia in these regions with the potential to enhance cowpea production. This could be associated with the better adaptability of native rhizobia to the ecological surrounding, hence their ability to infect and form a positive association with cowpea under the prevailing conditions (Koskey *et al*., 2017; Matse *et al*., 2020). Besides, the native rhizobia can form positive interactions with the naturally occurring soil microbiota, enhancing the host crop nutrition and health. This includes interactions with plant-growth-promoting bacteria and bio-enhancers such as arbuscular mycorrhizal fungi, which increase the supply and access to other nutrients (Karthikeyan and Arunprasad, 2019). According to Koskey *et al*. (2017), inoculation of common bean with native rhizobia outperformed other inoculants in respect to yield,

which is in agreement with the findings in this study. The average performance of Biofix is linked to moderate adaptability and unfavourable agro-ecological setting in the study area, which negatively impacted the Biofix–cowpea association (Koskey *et al*., 2017). This is also seen in the cowpea response to inoculation with the consortium. This is mainly because the effectiveness of rhizobia inoculants on the improvement of yields is not dependent on the diverse rhizobia population in the inoculant. Inoculation with a diverse rhizobia population increases chances of incompatibility and hostile interactions, which occurs at the expense of N fixation (Martínez-Romero, 2003). Additionally, cowpea is a promiscuous legume that enables it to nodulate with many rhizobia strains, including the ineffective strains that lead to poor responses to rhizobia inoculation (Kanonge-Mafaune *et al*., 2018). For these reasons, it is necessary to use effective rhizobia adapted to the agro-ecological condition for maximum cowpea production. Besides the influence on yield, all three inoculants significantly increased the stover weight at harvest during the second season when compared to the control. The stover is important. It supplements the soil with nutrients and organic matter upon decomposition needed for the subsequent crop and soil structure improvement.

All the inoculants did not discriminate against the cowpea genotypes with reference to yields, as no significant interaction of rhizobia inoculant and cowpea genotype was observed. This could be attributed to the fact that all three genotypes have been bred to suit the study sites' environmental and climatic conditions. There is a likelihood that these genotypes have been empirically selected to form efficient interaction with the indigenous rhizobia (De Freitas *et al*., 2012). This opposes a finding by Karasu and Dogan, (2011), who reported a significant genotype × rhizobia inoculant interaction. Further differences in the 100 seed weight could be attributed to the seeds' varying sizes in each of the cultivars.

Additionally, the associated difference in stover weight among the genotypes could be linked to the different growth forms of these genotypes in the field. Similarly, significant differences were recorded in all the parameters across the three study sites between the two seasons. These differences can be linked to the three locations' varying climatic conditions in the two growing seasons. The significant differences in yields between seasons could be attributed to the extreme rainfall during the flowering and podding stage during the first season. The associated rainfall impact on the flowers and small pods causes flower and pod abortion, reducing the number of pods that reach maturity. This consequently affects the yields achieved per plant and, subsequently, the yield achieved per hectare.

5.1.7 Effects of rhizobia inoculation on AMF colonization on cowpea roots

The interaction of the cowpea with rhizobia and AMF in the rhizosphere helps the plant to acquire P and N, which mitigate its needs (Njeru *et al*., 2020). In the arid and semi-arid areas, where cowpea is the main crop and the land is unstable and degraded, native AMF has demonstrated potential in restoring the ecological functionality of such areas (Ait-El-Mokhtar *et al*., 2020). Rhizobia and AMF co-exist in the rhizosphere and benefit the plants in a synergistic manner (Larimer *et al*., 2014). Rhizobia inoculation also plays a significant role in catering the plant N needs in the semi-arid areas leading to the overall improvement of plant growth and production. However, plants' benefits following rhizobia inoculation and interaction with native AMF are dependent on specific rhizobia-AMF species combination (Xavier and Germida, 2003). This study focused on evaluating whether inoculation with rhizobia significantly affected the native AMF colonization on cowpea roots.

During the first season, rhizobia inoculation had no significant impact on the AMF colonization on the cowpea plants. During the second season, plants inoculated with

Biofix had increased AMF colonization compared to the native isolates. However, the variation in % AMF colonization did not influence the overall cowpea growth and production, with plants inoculated with native isolates having higher yields than Biofix-inoculated ones. This shows that the tripartite symbiosis formed between Biofix and native AMF with cowpea plants was incompatible and not effective as that formed with the native rhizobia, which drastically reduces the growth performance of inoculated plants (Xavier and Germida, 2003). Thus, the overall growth and yield greatly depended on the rhizobia rather than AMF. The enhanced growth and yield could also be due to the adaptation of native isolates to the agro-ecological conditions, making them fix more N leading to low AMF colonization.

The colonization % among the three cowpea varieties did not significantly differ in both seasons. The absence of AMF varietal preference confirms that the ability of AMF to interact, infect and benefit these varieties has been maintained in modern breeding (Wang *et al*., 2020). However, their performance in the individual study location differed as depicted in the significant site × variety during the second season. Despite all the three varieties being bred to suit the semi-areas, one cowpea variety recorded a significantly high AMF colonization in each location compared to the other varieties. In Embu, K-80 recorded the highest AMF colonization, while in Kitui and Tharaka Nithi, KVU 27-1 and M-66 had the highest AMF colonization. This may suggest the suitability of each of the varieties in the three areas in terms of AMF infectivity and colonization.

In terms of study locations, Kitui and Tharaka Nithi counties recorded a significantly higher AMF colonization during the first season than Embu. This significant difference could be related to the climatological differences experienced during the first season.

Although AMF root colonization has been reported to be influenced by soil characteristics, climatic conditions, including temperature and rainfall, influence root colonization the most (Jerbi *et al*., 2020). Embu experienced extremely high rainfall while Kitui and Tharaka Nithi experienced some period of drought during this season. AMF enhances plant tolerance to abiotic stress such as drought, potentially increasing AMF colonization in these regions (Kavadia *et al*., 2020; Diagne *et al*., 2020). In the second cropping season, no significant difference in AMF colonization was observed in the three locations. Similarly, this could be linked to the climatological conditions, which didn't differ across the locations before sampling was done.

5.2 Conclusion

i. The soils in the semi-arid areas of Kenya have varying physicochemical characteristics that influence the amount of AMF infective propagules. Amongst them, sand, clay composition as well as available phosphorus were the most significant characteristics impacting the AMF infective propagules in the soil.

ii. Based on the biochemical characteristics, all rhizobia isolated from cowpea nodules were fast-growing rhizobia. Authentication experiment in the greenhouse confirmed that majority of the nodule isolates (65%) were rhizobia based on their ability to re-infect cowpea and initiate nodule formation and all were from the genus *Rhizobium*. These isolates were narrowly diverse with a higher genetic variability within the populations (same region) than among populations (different regions).

iii. Cowpea nodule isolates authenticated as rhizobia had significant different symbiotic efficiency ($p < 0.05$) with the best isolates having nitrogen fixation

capacity similar to the commercial strain (Biofix) and nitrogen treatment (KNO3) under greenhouse conditions.

iv. Rhizobia inoculation in the smallholder farms significantly ($p < 0.05$) enhanced cowpea growth and yields with native isolates (*Rhizobium. tropici*, *Rhizobium. mesosinicum*, and *Rhizobium. pusense*) outperforming Biofix and the uninoculated controls. However, the influence of rhizobia inoculation on AMF colonization was dependent on the AMF-rhizobia compatibility under the prevailing climatic conditions. The selected cowpea genotypes had no preference for a specific rhizobial inoculant. They did not differ in performance and AMF colonization following inoculation. Thus, the three genotypes are suited for the semi-arid conditions.

5.3 Recommendations

5.3.1 Recommendations from the study

i. Farmers should adopt organic farming practices to boost the soil physical and chemical characteristics which can help maintain or increase the AMF population in the soil.

ii. Rhizobia inoculation significantly increased the cowpea yields in the field. Farmers should be trained and encouraged to adopt the use of bio-fertilizers to enhance soil fertility and legume production.

iii. The most symbiotically effective rhizobia species (*Rhizobium tropici* and *Rhizobium mesosinicum*) should be packaged and availed to the farmers to enhance cowpea growth and production.

5.3.2 Areas for further studies

i. Compatible rhizobia-AMF species in the field with potential to enhance cowpea growth and production should be determined and utilized as inoculants in cowpea production.

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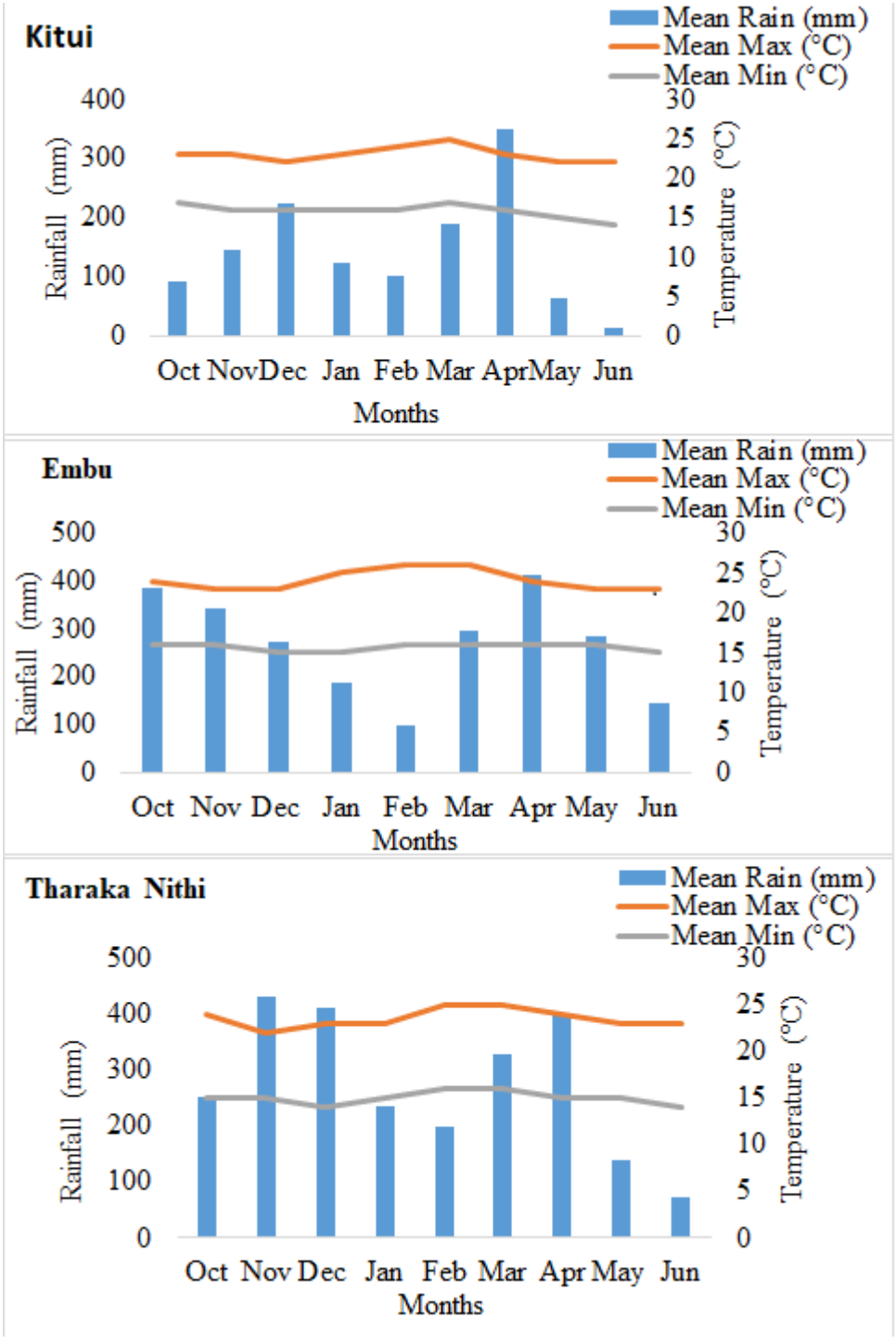
Appendix 1: Rainfall and temperature distribution in the study areas



Rainfall and temperature in Kitui, Embu and Tharaka Nithi counties during the months of the field experiment. (Source: https://www.worldweatheronline.com/weather-averages/eastern/ke.aspx)

Appendix 2: Identity of the isolates per County

Representative isolate	Cowpea variety	County
JN2	K-80	Tharaka Nithi
JN20	K-80, KVU 27-1	Kitui
JN28	K-80, KVU 27-1, M-66	Embu, Kitui
JN29	K-80	Kitui, Tharaka Nithi
JN3	K-80	Embu
JN4	K-80, M-66, KVU 27-1	Tharaka Nithi, Kitui, Embu
JN9	KVU 27-1, K-80	Embu, Tharaka Nithi
JN19	K-80, M-66, KVU 27-1	Embu, Kitui, Tharaka Nithi
JN18	K-80	Tharaka Nithi, Kitui
JN36	K-80, M-66	Kitui
JN39	KVU 27-1, K-80, M-66	Tharaka Nithi, Embu
JN44	K-80, M-66, KVU 27-1	Tharaka Nithi, Kitui, Embu
JN30	K-80	Embu, Tharaka Nithi