IDENTIFYING ELITE RHIZOBIA FOR COMMERCIAL SOYBEAN (*GLYCINE MAX*) INOCULANTS

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DECLARATION

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

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DEDICATION

To: my father, Chrispus W. Wanjala; my mother, Norah N. Wanjala; and my brothers and sisters. My parents' vision for a better tomorrow is unrivalled by the best educators in my life. Above all I extend my sincere gratitude to the Almighty God for making this journey possible.

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ABBREVIATIONS AND ACRONYMS

BNF	Biological nitrogen fixation
CRD	Complete randomized design
CAN	Calcium ammonium nitrate
C	Carbon
CIAT	International Center for Tropical Agriculture
DAP	Di-ammonium phosphate
DNA	Deoxyribonucleic acid
EI	Effectiveness index
FAME	Fatty acids methyl esters
GOK	Government of Kenya
K	Potassium
LARMAT	Land Resource Management and Agricultural Technology
MOA	Ministry of Agriculture
MPN	Most Probable Number

Mm	Millimolar
MPNES	Most Probable Number Enumeration System
Mt	Metric ton
N	Nitrogen
NaCI	Sodium Chloride
NO_3	Nitrate
NO	Nitric oxide
N_2O	Nitrous oxide
P	Phosphorus
PLWHA	People Living With HIV/AIDS.
PPM	Parts per million
PCR	Polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
YEMA	Yeast extract Mannitol agar
YEMB	Yeast extract Mannitol broth
µm	Micrometer

ABSTRACT

Existence of highly effective rhizobia in African soils is under exploited since commercial inoculants still contain exotic cultures from United States of America. Bio-prospecting was conducted in Kenya to identify elite strains of rhizobia capable of effectively nodulating and fixing large amounts of nitrogen with commonly grown soybean varieties. One hundred isolates were recovered from nodules of wild and cultivated legume hosts growing in different agro-ecological zones, namely coastal sand dunes and mangrove swamps, the uplands and Rift Valley highlands, to the Afro-montane zone of Mount Elgon and the Lake Victoria Basin, covering about 1045 km transect. These isolates were authenticated and tested for effectiveness on soybean (*Glycine max*) var. SB 19 in sterile vermiculite, and the twenty-four most promising isolates screened in potted soil to assess their competitive abilities using two contrasting varieties of soybean ("promiscuously nodulating" SB 19 and specific Safari). The six best performing isolates were evaluated under field conditions, comparing them to *Bradyrhizobium japonicum* strain USDA110, an industry standard. Test isolates were classified into five categories, non-infective (20%), ineffective (26%), partly effective (26%), effective (17%) and highly effective (11%) based on their performance relative to controls and commercial inoculants. The indigenous rhizobia that outperformed USDA110 were considered highly effective. In potted soil, all 24 native rhizobia isolates nodulated promiscuous soybean (SB19) but only 46% of them nodulated specific soybean (Safari). In the field experiment; at

Nyabeda in west Kenya, NAK 128 performed best on both promiscuous and specific soybean varieties, significantly ($P<0.05$) outperforming USDA110 by 29% and 24%, respectively. At another site in Butula, NAK 84 emerged as the most promising isolate on both promiscuous and specific soybean varieties and outperforming USDA 110 by 9% and 6%, respectively. The two best isolates from this investigation, NAK 84 and 128 outperformed the treatment receiving 78 kg N ha^{-1}, require further characterization and field testing but clearly have commercial potential.

CHAPTER ONE

INTRODUCTION

1.1 Background

Rhizobia are soil-inhabiting bacteria that form the root nodules where symbiotic biological nitrogen fixation occurs (Howieson and Brockwell, 2005; Weir, 2006). This process, where atmospheric N is captured for assimilation by plants is under-utilized by small-scale African farmers, in part because they do not understand its mechanism and management. For example, 95% of farmers in East and Southern Africa were familiar with legume root nodules but only 26% considered them beneficial (Woomer *et al.,* 1997). In another study, the percentage of farmers who use inoculants in Kenya was only 1% (Karanja *et al*., 2000).

Competitiveness of native rhizobia also pose a barrier to the benefits of inoculation (Shamseldin and Werner, 2004). Tropical soils are often rich in less-effective, native rhizobia and a key to overcoming their competitive advantage is through the composition and delivery of legume inoculants (Theis *et al*., 1991), especially for soybeans, a more specifically nodulating host legume (Sanginga *et al*., 2000). One pathway to improvement is to identify native rhizobia with superior symbiotic and competitive abilities and to use them in large doses within inoculants, building upon the biodiversity of indigenous rhizobial populations. The adaptability of indigenous rhizobia to their environment results in high levels of saprophytic competence, therefore continual identification of new, elite isolates offers the opportunity of improving BNF with fine-tuned geographical targets

(Zengeni *et al*., 2006; Appunu and Dhar, 2008). In this way, a wide diversity of rhizobial isolates ensures a sustainable source of strains for commercial application into the future (Musiyiwa *et al*., 2005).

One empirical approach to rhizobia strain selection focuses upon the stepwise collection, isolation and authentication of native rhizobia, screening of the isolates against reference strains for symbiotic effectiveness, assessment of their competitive abilities and evaluation of their performance under a range of field conditions Howieson *et al*., (2000), with each step eliminating the worst performing isolates from further consideration. In this way, the identified elite rhizobial strains are likely to colonize the soil, tolerate environmental stresses, and compete with background populations (Slattery and Pearce, 2002).

Ideally, this process identifies the elite strains of rhizobia across a range of agro-ecologies, mass produces them as inoculant and makes them available to legume farmers so that they become beneficiaries of native microbial biodiversity. Kenya is an excellent location to test this approach. It has a wide range of ecosystems, legume communities (White, 1983) and soils (Sombroek *et al*., 1982), and a large population of farmers cultivating legumes, including soybean as an increasingly important cash crop. Moreover, these farmers are in the process of advancing from subsistence to market-based agriculture and seeking to improve their field practices and yields (Woomer *et al*., 1998). This study evaluated the effectiveness of Kenya's native rhizobia on farmer accepted promiscuous and specific soybean

varieties and is for performance of soybean inoculants through identification of elite indigenous rhizobia.

1.2 Problem statement and justification

Naturally-nodulating, also known as "Promiscuous" soybean varieties were developed by the International Institute of Tropical Agriculture (IITA) between the mid-1970s and early 1990s and are being widely adopted in some African countries. Their promiscuous nature allows these varieties to nodulate freely with indigenous *Bradyrhizobium* spp. (Mpepereki *et al*., 2000; Sanginga *et al*., 2003). Although promiscuous soybean lines were bred on the basis that they would nodulate freely without artificial inoculation, studies by Okogun and Sanginga (2003) demonstrated that the indigenous rhizobia are not able to meet their full nitrogen (N) requirement therefore inoculation with rhizobia might be necessary.

Currently, USDA 110 is recommended for soybean cultivation (Somasegaran and Hoben 1984) and is likely to be effective upon recently introduced promiscuous soybean varieties in Kenya. However, it is also vital to assess the effectiveness and competitiveness of indigenous strains against this commercial strain (USDA 110) on promiscuous and specific soybean. The N_2-fixing capability of rhizobia varies greatly depending on the host plant species. Therefore selection of best strains must take rhizobia host compatibility into consideration for the production of suitable inoculants (Howieson *et al*., 2000). In addition, the ability of strains of rhizobia to survive, nodulate and fix nitrogen in soil environments varies widely; hence the selection of rhizobia with specific symbiotic and competitive attributes

suited to a range of soil environments must assume a high priority. Biodiversity and economic potential of African rhizobia is largely unexplored yet potential exists for native rhizobia to outperform exotic commercial strains. Given the poor fertility of many Kenyan soils and high demand for soybean, there is a need to select appropriate soybean-*Rhizobium* symbioses for Kenyan environments.

Demands for soybean are expected to rise to about 150,000 Mt per year over the next ten years (Jagwe and Nyapendi, 2004, MOA, 2006). Kenya is a very small soybean producer, even within the African context yet the conditions are suitable for soybean cultivation. The main factors include congenial agro-ecology, crop compatibility with existing farming systems, soybean's potential contribution in natural resource management, low cost of soybean protein, soybean's contribution to food security, its potential to contribute to bio-fuel energy, and its ability as an economic crop to create employment and generate income (Chianu *et al*., 2008). Soybean is one such crop that has the potential to make significant contributions to healthcare (Ohiokpehai and Osborne, 2003).

1.3 Objectives

1.3.1 Broad Objective

To improve biological nitrogen fixation of soybean through use of highly effective indigenous rhizobia.

1.3.2 Specific Objectives

1) Identify elite indigenous rhizobia for soybean through comparison with standard commercial strains.

2) Assess the effect of indigenous rhizobia on performance of specific and promiscuous soybeans.

1.3.3 Hypothesis

Highly effective rhizobia for inoculating soybeans exist in Kenyan soils and will outperform existing commercial inoculants strains.

CHAPTER TWO

LITERATURE REVIEW

2.1 Characteristics of rhizobia

A typical rhizobial cell is a small to medium sized (0.5 to 0.9 × 1.2 to 3.0 µm) gram negative, motile rod, these cells exhibit characteristic presence of copious β-hydroxybutyrate granules forming 40%-50% of the cell dry weight easily observed by using stains for metachromatic granules. Most strains produce sticky gum-like substances of varying composition. Rhizobia show a typical translucent, viscid, slimy growth on Yeast Mannitol Agar media with individual colonies having domed shape, elevated feature with entire margins. (Gupta *et al.*, 2007).

There is variation in specificity of interaction between rhizobia and legume species. Some rhizobia legume associations are very specific, where a host legume will only form nodules when infected with a specific rhizobium while other will form nodules with a range of rhizobia (Vance, 2000). Specificity involves the recognition of the bacterium by the host and of the host by the bacterium through the exchange of signal compounds, which induce differential gene expression in both partners (Broughton *et al.*, 2000). Rhizobia have been utilized in agriculture to increase the yield of leguminous plants (Wadhwa *et al.*, 2010) through their use as inoculants to seed or, less often, soil.

2.2 Rhizobial Taxonomy

Rhizobia are soil-inhabiting bacteria with the potential for forming specific root structures called nodules. In effective nodules the bacteria fix nitrogen gas (N_2) from the atmosphere into ammonia, which is assimilated by the plant and supports growth particularly where nitrogen availability in soils is limiting plant growth. In return the rhizobia are supplied with nutrients (predominantly dicarboxylic acids) by the host, and are protected inside the nodule structure (Weir, 2006).

Rhizobia currently consist of 61 species belonging to 13 different genera, namely *Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Methylobacterium, Burkholdera, Cupriavidus, Devosia, Herbaspirillum, Ochrobactrum* and *Phyllobacterium*. The taxonomy of rhizobia is in constant flux (Ahmad *et al*., 2008). *Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium* and *Allorhizobium* belong to the alpha-Proteobacterial subdivision of the purple bacteria, an extremely diverse group that includes pathogens, symbionts, photosynthetic organisms, bacteria that degrade environmental pollutants and the abundant marine organism *Pelagibacter ubique* (Pierre and Simon, 2010). *Rhizobium* contains 33 species, 24 of which were isolated from legume nodules, *Sinorhizobium* includes nine species isolated from legume nodules, *Bradyrhizobium* has seven species from legume nodules and *Azorhizobium* has two species nodulating legumes. The complete list of valid species of rhizobia is constantly updated (Khan *et al*., 2010). The technological advancements in morphological, biochemical, physiological, serological and

sequence analysis used for taxonomic classification could still make classification unstable (Manvika and Bhavdish, 2006). Several rhizobia are able to nodulate soybean (*Glycine max*) including *Bradyrhizobium liaoningense, Bradyrhizobium japonicum, Ensfer fredii, Ensifer xinjiangense, Mesorhizobium albiziae, Mesorhizobium temperatum,* and *Rhizobium oryzae* (Chen *et al*., 1988; Jordan, 1984; Gao *et al*., 2004; Wang *et al*., 2007; Peng *et al*., 2008).

2.3 Rhizobial Ecology and Diversity

Studies have targeted to uncover the nature of rhizobial symbionts in their native environments as it has been discovered that one of the major problems in the application of biological nitrogen fixation (BNF) technology is the establishment of introduced inoculant strains. Nodulation and nitrogen fixation in this symbiosis require that host and microorganism are compatible, but also that the soil environment be appropriate for the exchange of signals that precedes infection (Hirsch *et al*., 2003; Zhang *et al*., 2002). Earlier reviews have chronicled the influence of biotic and abiotic soil factors on rhizobium ecology (Amarger, 2001; Sessitsch *et al*., 2002). A problem in many of the reviews was in adequately describing change at the population level. Tools, such as intrinsic antibiotic resistance (Beynon and Josey, 1980), serology (Bohlool and Schmidt, 1973; Purchase and Vincent, 1977; Purchase *et al*., 1951), and multilocus enzyme electrophoresis (Pinero *et al*., 1988; Eardly *et al*., 1990), have all helped toward a more detailed examination of rhizobial population structure in soil, and how this is influenced by host and environment. However, only with the development of

molecular (Hirsch *et al*., 2003; Thies *et al*., 2001) and computational tools has the consideration of large populations of rhizobia on a routine basis been possible.

The formation of nodules on the legume host continues to be regarded as the most important phenotypic trait because of the practical agricultural importance of rhizobia. Symbiotic phenotype, other phenotypic features such as FAME (fatty acids methyl esters), SDS-PAGE (whole-cell protein analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and MLEE (multilocus enzyme electrophoresis) followed by numerical taxonomy have successfully been used for grouping and characterization of unknown strains and for the description of novel species of rhizobia (Vandamme *et al.,* 1996).

Traditionally, variation has been determined using characteristics such as growth rate and colony morphology (size, shape, color, texture and general appearance) and antibiotic resistance methods (Graham *et al.,* 1991). However, these methods are not sufficiently discriminative to account for all the variation exhibited in the target species. They cannot delineate sources of observed phenotypic variation into its components that may be due to environmental factors or underlying genetic factors. Molecular tools have become available to analyze diversity and population structure of bacteria. The 16S rRNA gene sequences are an indispensable parameter in rhizobial taxonomy and methods based on differences in ribosomal RNA genes have been frequently applied to species identification (Laguerre *et al.,* 1994). Nevertheless, the conservative nature of 16S rRNA genes

limits its use for discrimination at the strain level. The intergenic spacer between 16S and 23S rRNA genes was described to be more variable (Massol-Deya *et al.*, 1995) and RFLP of the PCR-amplified IGS was used for the characterization of rhizobia (Nour *et al.*, 1994; Sessitsch *et al.*, 1997b). The development of the polymerase chain reaction (PCR) led to new fingerprinting methods. Arbitrary oligonucleotide PCR primers of random sequence (RAPD) have been used to generate strain-specific fingerprints of rhizobia (Selenska-Pobell *et al.*, 1995; Paffetti *et al.*, 1996). In addition, PCR primers based on short intergenic repeated sequences have been designed to fingerprint bacteria (de Bruijn *et al.*, 1992; Versalovic *et al.*, 1991) and this approach became a frequently technique for analysing bacterial communities (Laguerre *et al.*, 1996; Sessitsch *et al*., 1997).

Studies have shown that tropical rhizobia are diverse with sub-groups of varied symbiotic specificity and effectiveness (Thies *et al.* 1991; Mpepereki *et al.* 1996). Studies by Bala and Giller, (2007) showed rhizobia of the same phylogenetic grouping nodulating *Caliandra calothyrsus*, *Glyicidia. sepium* and *Leucaena leucocephala* in some soils, but failing to nodulate at least one of the hosts in other soil, thus suggesting that rhizobial phylogeny and host range (infectiveness) are only weakly linked. Rhizobia are heterotrophic, competent bacteria that can survive as large populations for decades in the absence of host legumes (Giller, 2001), but the presence of a compatible host legume confers protection to the microsymbionts against environmental stresses (Andrade *et al*., 2002). On the other hand, a greater diversity of rhizobia in soil populations broadens the range

of legume hosts that can be nodulated in such soils. Therefore a mutual benefit between aboveground (legume) and belowground (rhizobia) biodiversities exists.

2.4 Determinants of Host Specificity in Rhizobia

In rhizobia, host specificity plays a key role in the establishment of effective symbiosis. Although many host plants and rhizobia have the ability to enter into symbiosis with more than one partner, only a certain set of symbionts lead to the formation of nitrogen-fixing nodules. Exceptionally, tropical leguminous trees, such as Acacia, Prosopis or Calliandra can form nodulation symbioses with various rhizobia from different genera. However, the specificity between symbiotic partners minimizes the formation of ineffective, non-fixing nodules by the host plant (Perret *et al*., 2000).

The construction of root nodules requires extra energy and nutrient sources from the host. Rhizobia differ in their response to different signal molecules produced by legumes. Some rhizobia have a narrow host range and form nodules with a limited number of legumes. For example *Azorhizobium caulinodans*, *Sinorhizobium saheli* and the sesbaniae biovar of *Sinorhizobium terangae* nodulate only *Sesbania rostrata* (Boivin *et al.,* 1997) and *Rhizobium galegae* is the only symbiont of *Galega officinalis* and *Galega orientalis* (Lindström, 1989). In contrast some rhizobia have a broad host range and are capable of nodulating a wide spectrum of legumes with various degrees of promiscuity. For example, *Sinorhizobium* sp. NGR234 and the closely related *Sinorhizobium fredii*

USDA257 nodulates at least 112 and 77 legumes from two different tribes, respectively (Pueppke and Broughton, 1999). Conversely, legumes may also be host to only one kind of symbiont (*Galega* spp.) or establish symbioses with a wide range of rhizobia (*Leucaena leucocephala, Calliandra calothyrsus*, *Phaseolus vulgaris*). Distantly related rhizobia can nodulate the same host, e.g. *Sinorhizobium fredii*, *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* all nodulate *Glycine max*. Members of Rhizobium, Sinorhizobium and Bradyrhizobium are less related to each other than to other non-rhizobial genera. Stem and root-nodulating *Azorhizobium caulidonans* and root-nodulating *Sinorhizobium fredii* and *Sinorhizobium terangae* bv. sesbaniae, both symbionts of *Sesbania rostrata*, also represent two taxonomically distant genera.

2.5 Mechanisms of Biological Nitrogen Fixation

Biological nitrogen fixation, a process utilized only by certain prokaryotes, is catalyzed by a two-component nitrogenase complex (Yan *et al*., 2010). Nitrogenase catalyzes the simultaneous reduction of one N_2 and 2 H^+ to ammonia and a molecule of hydrogen gas.

$$N_2 + 8H_2 + 16\ ATP \longrightarrow 2NH_3 + 2NH_3 + 2H_2 + 16ADP + 16Pi$$

The immediate electron donor is the potent reducing agent ferredoxin and the reaction is driven by the hydrolysis of 2 ATP for each electron transferred (Wheelis, 2008).The best known Biological Nitrogen Fixation (BNF) system occurs between legumes and rhizobium bacteria (Carvalho *et al*., 2011). The symbiotic association between the roots of legumes and certain soil bacteria,

generally known as rhizobia, accounts for the development of a specific organ, the symbiotic root-nodule, whose primary function is nitrogen fixation. Root nodules make a crucial contribution to the nitrogen content of the soil playing a key role in agricultural practices (Alla *et al*., 2010).

Perception of legume root exudates triggers the production of rhizobial Nod factor signals which are recognized by compatible plant receptors leading to the formation of root nodules, in which differentiated bacteria (bacteroids) fix atmospheric nitrogen (Oldroyd and Downie, 2008). In the nodule, maintenance of nitrogenase activity is subject to a delicate equilibrium. Firstly, a high rate of oxygen respiration is necessary to supply the energy demands of the Nitrogen reduction process (Sanchez *et al*., 2011), but oxygen also irreversibly inactivates the nitrogenase complex. These conflicting demands are reconciled by control of oxygen flux through a diffusion barrier in the nodule cortex and by the plant oxygen carrier, leghemoglobin, which is present exclusively in the nodule (Minchin *et al*., 2008).

In addition to fixing nitrogen, some rhizobia species are able to grow under low oxygen conditions using nitrate as electron acceptor to support respiration in a process known as denitrification by which bacteria reduce sequentially nitrate (NO_3) or nitrite (NO_2) to Nitrogen (N_2). Nitrate is reduced to nitrite by either a membrane-bound or a periplasmic nitrate reductase, and nitrite reductases catalyse the reduction of nitrite to nitric oxide (NO). Nitric oxide is further reduced to nitrous oxide (N_2O) by nitric oxide reductases and, finally, N_2O is

converted to N_2 by the nitrous oxide reductase enzyme (Van Spanning *et al*., 2005, 2007). The significance of denitrification in rhizobia-legume symbiosis can be appreciated when oxygen concentration in soils decreases during environmental stress such as flooding of the roots, which causes hypoxia. Under these conditions, denitrifying activity could work as a mechanism to generate ATP for survival of rhizobia in the soil and also to maintain nodule functioning (Sanchez *et al*., 2011).

2.6 Significance of Biological Nitrogen Fixation

The atmosphere is a nearly homogeneous mixture of gases, the most plentifully is nitrogen 78.1% (Garrison, 2006). About 96% of the N taken up by the crop has been measured as nitrogen derived from atmosphere (Lo`pez-Bellido *et al*., 2006). Biological nitrogen fixation involves conversion of atmospheric nitrogen (N) to ammonium, a form of N that can be utilized by plants (Vessey *et al*., 2005). The nature of biological nitrogen fixation is that the dinitrogenase catalyzes the reaction, splitting triple-bond inert atmospheric nitrogen (N_2) into organic ammonia molecule (Cheng, 2008).

Biological nitrogen fixation is regarded as a renewable resource for sustainable agriculture as it helps to reduce fertilizer N requirements and thus increases economic returns to producers (Walley *et al*., 2007). Furthermore, it plays a key role in assessment of rhizobial diversity, contributes to worldwide knowledge of biodiversity of soil microorganisms, to the usefulness of rhizobial collections and to the establishment of long-term strategies aimed at increasing contributions of

legume-fixed N to agriculture. The fixation of N_2 by legumes has the potential to contribute greatly to more economically viable and environmentally friendly agriculture (Odair *et al*., 2006). It has been estimated that the 80–90% of the nitrogen available to plants in natural ecosystems originates from biological nitrogen fixation (Rascio and Rocca, 2008). Biological nitrogen fixation contributes to the replenishment of soil N, and reduces the need for industrial N fertilizers (Larnier *et al.,* 2005). It offers an economically attractive and ecologically sound means of reducing external N input (Yadvinder-Singh *et al.,* 2004). In recent years, agricultural systems have changed to improve environmental quality and avoid environmental degradation. One of the most promising techniques to avoid environmental degradation is the use of inoculants composed of diazotrophic bacteria as an alternative use of nitrogen fertilizers (Roesch *et al*., 2007).

2.7 Rhizobial Bio-prospecting Studies

The Agricultural Research Service (ARS) of the U.S. Department of Agriculture (USDA) has maintained a collection of nitrogen-fixing legume symbionts for most of the 20th century (Van Berkum, 2002). Although many rhizobial isolation studies appear in scientific literature, there has been little attempt to evaluate global trends across diverse strain collections (Bottomley, 1992). The most comprehensive studies focus on a particular rhizobial species recovered from several host legumes at multiple locations or on populations or communities of rhizobia recovered from a particular host legume over a wide geographic range

(Han *et al.,* 2008). The absence of a global synthesis can be attributed to the difficulty in comparing studies that use diverse methods for rhizobial sampling and strain typing. Use of diverse sampling strategies means that collections of isolates are rarely equivalent, except in related studies arising from individual research groups. Comparison of published studies is also difficult because strain typing methods vary in their discriminatory power (Schwinghamer and Dudman, 1980; Barnet, 1991; Bottomley, 1992) and therefore influence the number of strain types identified.

Microbial Resources Centre Network (MIRCEN) has centres across five continents, of which 3 are located in Africa: in Dakar (Senegal), Cairo (Egypt) and Nairobi (Kenya). The MIRCEN project reported to have isolated local strains of *Rhizobium leguminosarium* biovar *phaseoli* which tolerate temperatures as high as 42 degrees centigrade. In Kenya for almost two decades, the University of Nairobi has conducted research on biofertilizers. Rhizobium inoculants production in Kenya was initiated as part of Microbial Resources Centre Network (MIRCEN), supported by the United Nations Educational, Scientific and Cultural Organization (Odame, 1997). The year 2009 to 2013 bio-prospecting of rhizobia in Kenya was initiated as part of N2AFRICA program which was done by CIAT-TSBF team in collaboration with University of Nairobi-MIRCEN laboratory.

2.8 Factors affecting Nitrogen fixation

Environmental factors influence all aspects of nodulation and symbiotic N_2 fixation, in some cases reducing rhizobial survival and diversity in soil; in others affecting nodulation or nitrogen fixation and even growth of the host. Factors that are important include: Phosphorous (P) fertilization is the major mineral nutrient yield determinant among legume crops (Chaudhary *et al*., 2008). Phosphorus deficiency is a major constraint of effective nitrogen fixation because phosphorus is an important nutrient in the process of nodulation and nitrogen fixation (World Bank, 2006). The high requirement for P in legumes is consistent with the involvement of P in the high rates of energy transfer that must take place in the nodule. Under P shortage conditions, legumes may lose the distinct advantage of an unlimited source of symbiotic N (Sulieman *et al*., 2008). The more the supply of phosphorus, the more abundant are the nodules (Gowariker *et al*., 2009).

Phosphorus is a key structural and functional element in the plant: its efficiency has effects on plant morphology but also on its metabolism. These effects are of special importance to legumes where phosphorus is needed not only for growth but also for nitrogen fixation. Thus, a decrease in phosphorus requirement mainly reflects on reducing the leaf area, shoot dry matter and phosphorus content in shoot and root. However, root growth has inverse relations with P because under deficiency, root growth is stimulated as a strategy to improve the phosphorus nutrition (Ahmed, 2007). The strength of total nitrogen, or the concentration and composition of certain phloem sap nitrogenous compounds (amino acid and

amide) have been suggested to play the messenger role that signal nitrogen status to the nodule and down-regulating nitrogenase activity (Ahmed, 2007). It was also suggested that plant nitrogen is in mobile status and there is a permanent circular flow from the leaves via the phloem to the nodules and back to the leaves. This circular flow is supposed to form the signal for regulating nitrogen fixation (Fischinger *et al*., 2006).

Drought, as defined in agronomy, is a temporary or durable change in the plant water status, affecting its functioning, and it is related to a decrease in soil water content (Katerji *et al*., 2011). Drought is by far the most important environmental factor contributing to crop yield loss, including soybean (*Glycine max* (L.) Merr.) where symbiotic fixation of atmospheric nitrogen (N_2) is sensitive to even modest soil water deficits **(**Sinclair *et al*., 2007). Drought-related inhibition of biological nitrogen fixation (BNF) seriously limits legume yield in many arid and semiarid regions of the world. Three major factors have been proposed to be involved in drought effects on BNF: oxygen limitation, carbon shortage, and regulation by nitrogen metabolism (Ladrera *et al*., 2007). Decline of N_2 fixation with soil drying causes yield reductions due to inadequate N for protein production, which is a critical seed product **(**Sinclair *et al*., 2007). Several studies have shown that drought stress reduced nitrogen fixation in leguminous species. In soybean, lines with high nitrogen fixation at pod filling stage were found to have higher yield under water stress than those having low nitrogen fixation (Pimratch *et al.*, 2008). This suggested that maintaining high N_2 fixation under drought stress could be a

means for a legume genotype to achieve high yield under water-limited conditions.

Among the most common effect of soil salinity include growth inhibition by Na^+ and Cl^-. Elevated Na^+ in soil solution inhibits the uptake of other nutrients (e.g., P, K, Fe, Cu, and Zn) directly by interfering with various transporters in the root plasma membrane (Giri *et al*., 2007). Mineral nitrogen deficiency is also an important limiting factor for plant growth in arid zones, and rhizobia–legume symbioses are the primary sources of fixed nitrogen in these habitats (Verdoy *et al*., 2006). Salinated soil has very little nitrogen and therefore not suitable for cultivation of most plants. An appropriate solution to this situation would be the cultivation of plants that are able to fix nitrogen through biological nitrogen fixation (Chen *et al*., 2000). Most leguminous plants, however, are sensitive to even low levels of salinity.

Increasing salt concentrations may have a detrimental effect on soil microbial populations as well, either due to direct toxicity or through osmotic stress (Yadav *et al*., 2010). For example, most rhizobia are sensitive to moderate and higher levels of salinity during both the free-living stage and the symbiotic process. Legumes used in the reclamation of degraded lands (e.g. salt-affected lands) include *Prosopis. juliflora*, *Acacia nilotica*, *Acacia auriculiformis*, *Dalbergia sisso*, and *Gliricidia maculata*. However, both legume growth and the process of nodule formation are more sensitive to salinity than are rhizobia (Singleton *et al.*,

1982; Zahran, 1999). For instance, *Sinorhizobium meliloti* tolerated up to 300 mM NaCl, while nodulation and nitrogen fixation of its host (*M. sativa*) was inhibited at about 100 mM salt (Graham and Vance, 2000).

Optimum temperature for nitrogen fixation and soybean growth, range from 25^0 C to 30^0 C. In this temperature range, the bacteria can begin actively fixing nitrogen within seven days of forming nodules. A continually cool root zone temperature can significantly delay the onset of nitrogen fixation compared to an optimum soil temperature (Abendroth *et al*., 2006). The optimal soil pH is between six and seven. A soil pH significantly outside this range (less than five or greater than 8) is detrimental because it disrupts the communication process leading to root hair infection, thereby limiting nodule development. Soil pH also affects the amount of nitrogen fixed. For example, in a very acidic soil (pH 4), nitrogen fixation can be reduced up to 30 percent (Abendroth *et al*., 2006). Apparent effect of soil pH and exchangeable acidity on the relative dominance of rhizobial types have been reported Bala and Giller, (2007).

2.9 Nitrogen Fixation in Soybean

Soybean (*Glycine max* (L) Merrill) is of subtropical origin and member of the family Fabaceae (Ahmed, 2007). For optimal symbiotic activity, the soil temperature should be between 25-30°C (Bohner, 2009). The protein content in soybean (*Glycine max* (L) Merrill) seed is approximately 40% and the oil content is approximately 20%. This crop has the highest protein content and the highest

gross output of vegetable oil among cultivated crops in the world. The origin of soybean cultivation is China (Singh, 2010) but has since spread to various continents of the world including Africa. Soybean is a facultative short-day plant (Wu *et al*., 2006) with many latitude-specific maturity groups including day-neutral types suitable for equatorial areas.

Soybean has been categorized into three types, determinate, indeterminate and semi-indeterminate based on their growth and flowering characteristics. In determinate types, the vegetative development of the shoot apical meristem ceases when the plant begins to produce terminal inflorescence. This type of inflorescence has a terminal raceme bearing a rather dense terminal cluster of pods at maturity. Indeterminate types can continue their vegetative development after flowering. The inflorescence of this type has a set of sparse and rather evenly distributed pods on all nodes with a diminishing frequency toward the tip of the stems at maturity. Semi-determinate types are intermediate between the determinate and indeterminate types. Since stem termination determines plant type and pod/seed distribution in soybean plant, it is considered as an important agronomic trait for the productivity of soybean (Jiang *et al*., 2011).

Soybean varieties with various types of stem termination have unique geographic distribution (Tian *et al*., 2010). Considering attention has been given to increase symbiotic nitrogen fixation activity of legumes. However, much of the research, especially with the advent of molecular genetics, has focused on improving rhizobia (Ahmed, 2007). Nevertheless, the performance of *rhizobium* inoculants

as bio-fertilizers is limited under field conditions, unless the rhizobia are stuck to the seeds in carrier formulated inoculants, where it can reduce the competition of indigenous rhizobia (Huang and Erickson, 2007).

Rhizobia require plant hosts, they cannot independently fix nitrogen. Soybean plants get nitrogen from bacteria while plant supplies carbohydrates, protein and sufficient oxygen so as not to interfere with fixation process. Nodules that fix nitrogen, are pink or red inside and they are healthy. The significance of rhizobia forming root nodules and growth enhancement in soybean was widely studied by many scientists in the recent past (Saeki *et al.*, 2006; Sharma, 2006). Until 1980, *Bradyrhizobium japonicum* was considered the sole symbiont of soybean (Jordan, 1982). However, the isolation of rhizobia from new areas around the world and the availability of molecular techniques allowed researchers to identify and/or classify, already known rhizobia, as new symbionts of soybean. There is a wide array of responses of soybean to rhizobium. While some cultivars are fully incompatible with rhizobia, others might exclude or restrict nodulation by bacteria belonging to certain serogroups of *Bradyrhizobium japonicum* (Van *et al*., 2007).

Alternatively, soybean cultivars might be highly promiscuous like TGX African soybean cultivars (Vanlauwe *et al*., 2003). However, Sanginga *et al*. (2000) noted that promiscuous soybean is incapable of nodulating effectively with indigenous rhizobia in all locations in the moist savanna zone of Nigeria. Similarly, Bala (2008) observed that it is also not clear whether promiscuous soybean cultivars

are effectively nodulated by indigenous rhizobial populations in all soils and under all conditions. However, it is important to note that even promiscuously nodulating soybeans (that often do not require inoculation), developed and cultivated in some parts of Africa, sometimes respond to inoculation (Lichtfouse *et al*., 2011). A study carried out by Osunde *et al*, (2003) at five sites in the moist savanna region of Nigeria showed that promiscuous soybean varieties responded to inoculation. However, 'Magoye' an exceptionally promiscuous line released in Zambia in 1981, nodulates readily in all soils of Southern Africa where it has been tested and rarely responds to inoculation in Zambia and Zimbabwe (Mpepereki *et al*., 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Site description

Three sets of experiments were performed to identify elite rhizobia from the MIRCEN–N2Africa culture collection; authentication and effectiveness screening of rhizobial isolates in potted sterile media (vermiculite) under greenhouse conditions, subsequent evaluation of the better isolates in a representative potted soil also in the greenhouse, and finally on-farm testing of the best isolates in areas where soybean enterprise is being rapidly adopted by small-scale farmers in west Kenya. Greenhouse studies were conducted at University of Nairobi field station, Kabete Campus, situated about 15 km to the West of Nairobi at $1^0$15'S and 36^0 44' E, in the Central Kenyan Highlands (Sombroek *et al*., 1982.). The field experiment was conducted at two sites in west Kenya (Butula and Nyabeda). Both are situated within smallholder farming communities. Nyabeda is located at 00^0 08'N and 034^0 24' E, 1331 m above sea level and Butula is located at 00^0 18'N and 34.11" 034^0 17' E. Vermiculite free of rhizobia obtained from a horticultural supply company in Athi river, Kenya was used as media for authentication and soil collected from a farm at Butula was used as media for the effective screening in the greenhouse experiment. Indigenous rhizobia populations in non-sterile media (soil) used in the greenhouse and soil at the field (Butula and Nyabeda) were determined using the plant infection technique (Woomer *et al.,* 1994) before installing the experiments.

3.2 Authentication and screening of indigenous rhizobia isolates in the greenhouse collected from soils under different land uses in Kenya.

3.2.1 Authentication of indigenous rhizobia isolates in the greenhouse collected from soils under different land uses in Kenya.

The rhizobia isolates were recovered from 13 different genera: *Glycine* spp., *Vigna* spp., *Stylosanthes fruiticosa, Phaseolus vulgaris, Crotalaria* sp., *Desmodium* sp., *Mimosa pudica, Eriosema* sp., *Arachis hypogaea, Indigofera* sp. *Tephrosia* sp. *and Macroptilium* sp. Most of the isolates (37%) were isolated from *Glycine* spp., 25% from *Vigna* spp., 12% from *Macroptilium* sp., 7% from *Crotalaria* sp. and 19% from the other mentioned hosts (Appendix 1). Two standard commercial rhizobia strains (USDA110 and SEMIA5019) obtained from MIRCEN Laboratory, University of Nairobi, served as positive controls in the authentication experiment.

3.2.1.1 Design used for the authentication experiment in greenhouse

A Completely Randomized Design (CRD) experiment with three replicates was established in the greenhouse comprising of 104 treatments which were: 100 indigenous rhizobial isolates, two standard commercial strains SEMIA5019 and USDA110 and, non-inoculated plants with and without mineral N. Promiscuous soybean SB19 was used as test crop. Total experimental units were 312. Plants were grown for eight weeks in a greenhouse with a minimum temperature of 17^0C a maximum temperature of 50^0C recorded.

3.2.1.2 Preparation of potting media

Horticultural grade vermiculite was obtained from Athi River in Kenya and was used as media. Before the experiment, the vermiculite was tested for rhizobia using promiscuous soybean (SB19) as a trap host in three litre plastic pots with negative results. Gravel was washed with water then 3.5 % sodium hypochlorite and finally rinsed with sterile water. A 2.5 cm layer of gravel was placed on the bottom of each pot, followed by 750 g of sterile vermiculite then covered with a clean plastic plate with two holes to accommodate the test crop and a watering tube. The details of the set up are in Figure 1.

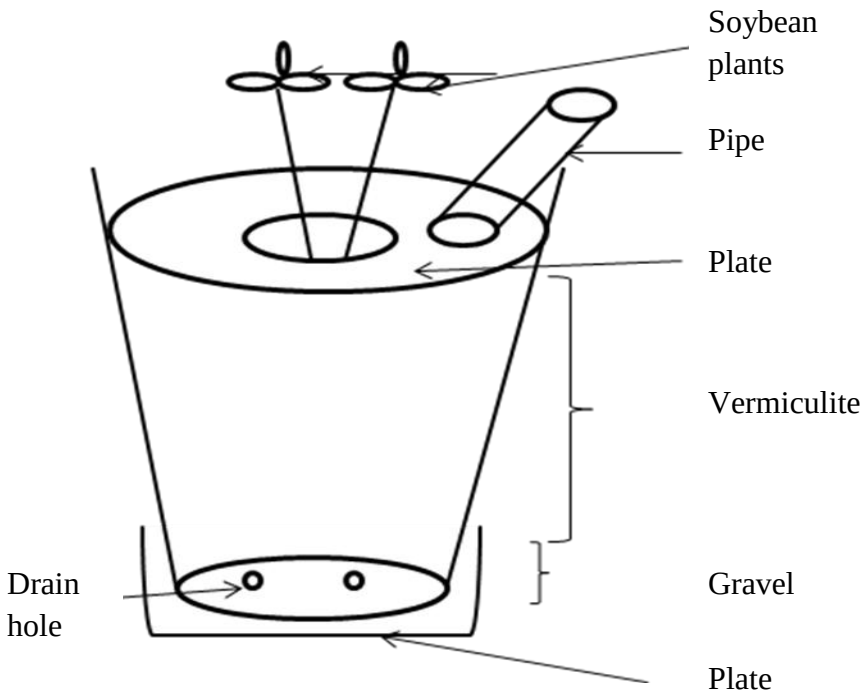

Figure 1: Arrangement of gravel and sterile media (vermiculite) in pots used for authentication of indigenous rhizobia isolates in the greenhouse.

3.2.1.3 Pre-germination of seeds

Soybean seeds were placed in a 250 ml Erlenmeyer flask and covered with sterilized aluminum foil. Alcohol of 95% concentration was added to the seeds to remove waxy materials and trapped air and drained away after 10 seconds. Sodium hypochlorite was added in sufficient volume to immerse the seeds completely. Contents were swirled gently to bring the seeds and sterilant into contact. After 5 minutes, the sterilant was drained off. Rinsing was done with six changes in sterile water. Aseptic procedures were observed throughout the rinsing (Somasegaran & Hoben, 1994).

Vermiculite was placed in a shallow autoclavable polypropylene tray then moistened to field capacity, covered with aluminum foil and sterilized by autoclaving for 15 minutes. The vermiculite was allowed to cool overnight and furrows made in the vermiculite with a sterile spatula where the sterilized soybean seeds were planted. The aluminum foil cover was replaced on the tray and incubation was done at 28^0C for three days, until uniform early seed germination was observed.

3.2.1.4 Planting and inoculation of bacterial isolates

Pots for authentication of indigenous rhizobial isolates were watered and allowed to drain to field capacity for one day. Three seeds were planted per pot and after seven days seedlings were thinned to two plants per pot. From glycerol preserved rhizobia culture, a loop of cells was streaked on yeast mannitol agar in petri-dishes (Vincent, 1970). The petri-dishes were incubated at 28^0C and checked for

the presence of any contamination. A loop of rhizobia cells was then transferred from the YEMA plate after seven days in the incubator and placed in nine milliliter of sterile yeast mannitol (YEMB) broth (Vincent, 1970). The mixture of yeast mannitol broth and bacterial suspension were incubated at 28 ^{0}C and rotated at 150 rpm on a rotary shaker. One ml of bacterial culture was inoculated in each pot seven days after planting. Constituents of YEM broth (Vincent, 1970) were Mannitol 10.0 g, K_2HPO_4 0.5g, $MgSO_4.7H_20$ 0.2 g, Yeast Extract 0.5 g, distilled water 1.0 liter. Constituents of yeast Mannitol Agar were One liter of Yeast mannitol broth and 15g of agar (Vincent, 1970).

3.2.1.5 Nutrient solution

Nutrient solution application rates were calculated based on media in pot. Full strength solution was used throughout the experiment until final harvest. For plus N controls treatment, KNO_3 (0.05%) was added giving an N concentration of 70ppm. Stock solutions were prepared separately and mixed at the required application rate when required for fertilization following procedure described by Broughton and Dillworth (1970) (Appendix 2) from which the nutrient solution for feeding the seedling was prepared.

3.2.1.6 Harvesting authentication experiment

After eight weeks, plants were carefully uprooted so that no nodules were left in the vermiculite. The nodules were separated, collected and enumerated. Nodule color was determined by scoring as follows; 0- no nodules, 1- white, 2- green, 3- pink, and 4- red. Shoot and nodule materials were oven dried at 70°C for 48

hours. Oven-dried weight of shoot and nodule were recorded. An Effectiveness Index (EI) of rhizobia isolates was calculated by shoot biomass of test strain divided by shoot biomass from the standard USDA110 strain. Non-infective isolates did not nodulate the soybeans hence were dropped for further consideration. Based on Effectiveness Index rhizobia isolates were placed under four different categories: highly effective, effective, partly effective and ineffective.

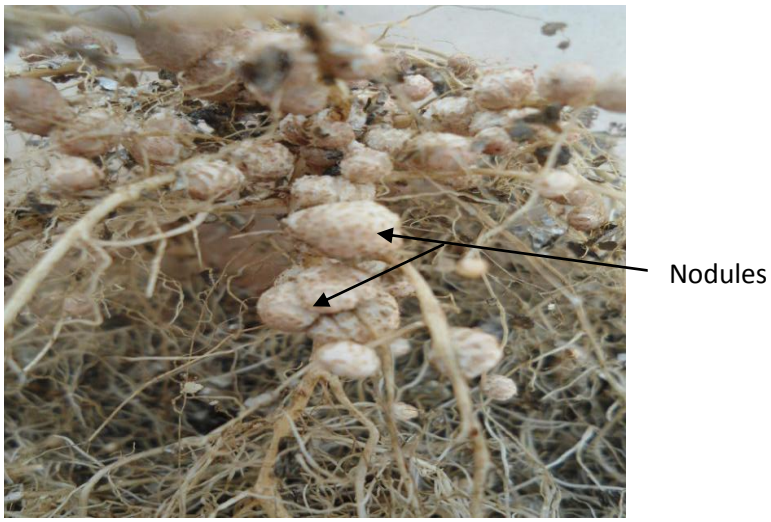

Plate 1: Nodules from soybean grown in vermiculite after 56 days from planting.

3.3. Screening of indigenous rhizobia isolates in the greenhouse on performance of specific (Safari) and promiscuous (SB19) soybean grown in non sterile soil from Western Kenya

An experiment was established in the greenhouse at the University of Nairobi Field Station, Kabete Campus using soil collected from a farm at Butula-Western Kenya. The soil had the following characteristics: pH 5.5, 1.86% total carbon, 0.08 % total nitrogen, 73.30 ppm potassium, 4.91 ppm phosphorus. Based on

MPN the soil had an indigenous rhizobia population of 2.7×10^3 per gram of soil. Twenty-four indigenous isolates which constituted 24% of the best performers from the earlier authentication test were evaluated alongside two standard commercial isolates; USDA110 and SEMIA5019 on both promiscuous (SB19) and specific Safari soybean varieties.

3.3.1 Experimental design of screening test in greenhouse using non sterile soil

Split Plot arrangement in a Completely Randomized Block Design (RCBD) was used with promiscuous and specific soybeans as the main plot. The total number of experimental units was 224; 24 native isolates, two commercial (USDA110 and SEMIA5019) and two controls (controls were uninoculated plus nitrogen and uninoculated minus nitrogen) with four replicates.

3.3.2 Rhizobia used for potted non-sterile soil experiment in greenhouse

The 24 indigenous rhizobia isolates which constituted 24% of the total indigenous isolates were selected from the earlier authentication activity which was conducted in the greenhouse, selection of isolates was based on effectiveness index and nodulation characteristics. Six indigenous rhizobia isolates which constituted 6% of the total indigenous isolates were later selected from the potted soil experiment again in addition to USDA110 to simulate farmer conditions and tested their effectiveness in the field.

Table 1: Indigenous rhizobia isolates screened in the greenhouse where non sterile soil was used as media, host the isolates were isolated and geographical area they were collected from.

Rhizobia isolate	County	Host	Growth characteristic	Rhizobia isolate	County	Host	Growth characteristic
NAK 10	Embu	*Macroptilium* sp.	Slow	NAK 161	Kwale	*Vigna* sp.	Slow
NAK 115	Teso	*Glycine max*	Slow	NAK 176	Msambweni	*Vigna* sp.	Slow
NAK 117	Teso	*Glycine max*	Slow	NAK 179	Ramisi	*Eriosema* sp.	Slow
NAK 122	Busia	*Glycine max*	Slow	NAK 182	Ramisi	*Vigna* sp.	Slow
NAK 127	Bungoma	*Glycine max*	Slow	NAK 30	Embu	*Macroptilium* sp.	Slow
NAK 128	Bungoma	*Glycine max*	Slow	NAK 84	Bungoma	*Glycine max*	Slow
NAK 135	Bungoma	*Glycine max*	Slow	NAK 9	Embu	*Macroptilium sp.*	Slow
NAK 139	Diani	*Macroptilium* sp.	Fast	NAK 96	Butula	*Glycine max*	Slow
NAK 144	Diani	*Vigna* sp.	Slow	NAK12	Embu	*Macroptilium* sp.	Slow
NAK 146	Diani	*Vigna* sp.	Slow	NAK 83	Bungoma	*Glycine max*	Slow
NAK 149	Diani	*Vigna unguiculata*	Slow	NAK89	Mumias	*Glycine max*	Slow
NAK152	Kwale	*Macroptilium* sp.	Slow	NAK160	Kwale	*Vigna* sp.	Slow
USDA110*				SEMIA5019*			

* Standard isolates

Source: N2 AFRICA rhizobia isolate database, 2012

3.3.3 Performance of 24 indigenous rhizobia isolates inoculated on soybean grown in Butula soil in the greenhouse

Twenty four indigenous rhizobia isolates selected from authentication experiment were screened using promiscuous soybean SB19 and specific soybean Safari in soil collected from Butula. Fertilizer containing K, P, Ca, Mg and S was added to the soil as a basal fertilizer at a rate of 500 kg ha^{-1} or 1 g pot^{-1}. Calcium ammonium nitrate (CAN) was added to soil which was used for the uninoculated plus nitrogen treatment. CAN was applied at a rate of 100 kg N ha^{-1} or 0.77 g pot^{-1} in three times application (at planting, after 26 and 33 days from planting). Three litre pots were filled with 2 kg of soil and cleaned gravels at the bottom of the pot. Pots were watered to field capacity. Promiscuous and specific soybean seeds were pre-germinated as described in the potted vermiculite (authentication) experiment (Chapter 3 section 3.2.1.3). Three seeds were planted per pot and thinned to two plants after six days. The bacterial isolates were prepared as described by Vincent, (1970). One milliliter of rhizobia culture was inoculated in each pot at the base of the plants using micro-pipette seven days after planting. Tap water was used for watering the plants and harvested eight weeks after planting.

Plants were carefully uprooted so that no nodules remained in the soil. The nodules on roots were manually collected and placed on a plate and counted. The shoot, root and nodule samples were oven dried at 70°C for 48 hours. Oven-dried weights were recorded.

3.4 Field trials to assess performance of promising indigenous rhizobia using promiscuous (SB19) and specific (SB97) soybean

From each site, soil samples were taken randomly then mixed thoroughly and two composite samples taken, labeled and transported to laboratory for analyses. The parameter measured from the samples included; total nitrogen, phosphorous, organic carbon, potassium, pH and population of indigenous rhizobia. Field experiments were established in western Kenya at Butula and Nyabeda during the 2012-2013 short rains (September to January). Six indigenous (NAK 84, 89, 115,117,128 and 135) and USDA110 rhizobia were compared on promiscuous SB19 and specific SB97 soybeans varieties at Butula and Nyabeda. Before installing the experiment, maize had been planted during the previous long rain season 2012.

3.4.1 Experimental design used in the field assessment of the indigenous rhizobia isolates

Split Plot arrangement in a Randomized Complete Block Design (RCBD) was installed with plot size being 5.4 m^2, each plot consisted 6 rows 45 cm apart, 5-8 cm intra-row spacing. Each plot was separated by a non-inoculated row to reduce cross contamination. The treatments included: inoculation of six native rhizobia and USDA110, two non-inoculated controls with (plus N) and without mineral nitrogen (minus N).

3.4.2 Planting and Inoculation

The inoculants were prepared using sterilized sugarcane mill filter mud as a carrier, cured for 14 days and applied at 10 g per kg of seeds with 16% gum arabic as an adhesive using the two-step method (Woomer, 2010). In the first step, the pre-weighed seeds in a container were uniformly coated with 16% (w/v) gum arabic (sticker), then the container was closed and swirled until all the seeds were uniformly wet. In the second step, the inoculant was added to the sticky seeds and the container was closed and swirled gently until seeds were uniformly black. Seeds were air dried on clean paper to enhance adhesion before planting.

Furrows were prepared and Sympal fertilizer containing P, K, Ca, Mg and S was applied at the rate of 200 kg ha^{-1} or 108 g $plot^{-1}$. Bagasse was added at the rate of two tonnes ha^{-1} in the furrows to immobilize inorganic N in the soil. Calcium ammonium nitrate (CAN) fertilizer was applied at the rate of 78 kg N ha^{-} to plus N treatment. Soybean seeds were planted first in plots with Plus N and minus N and plots where seeds were inoculated were planted after control treatments to avoid cross contamination.

The first sampling was carried out at two months after planting which coincided with 50% flowering of the plants. The second sampling was done at crop maturity one hundred and sixteen days from sowing. The first rows on either side of the plots were omitted to eliminate border effects. During the first sampling; plants were carefully uprooted, samples were taken from an area of 0.225 m^2. Roots were separated from the shoots and then soil was washed off in gently running

water to ensure that roots and nodules remained intact and nodules were recovered and counted. Shoots and nodules were oven dried for 48 hours at 70^0C and dry weight noted. At harvest one hundred and sixteen days after planting, soybean grains and were harvested from an area of 5.4 m^2. Plant biomass and grain yield were recorded.

3.5 Procedures for chemical characterization of soil and vermiculite

Soil and vermiculite used in the potted experiments were characterized. Soil sampling for data analysis was done before planting started. Vermiculite analysis was also done before planting. Soil parameters measured were total N, organic carbon, Phosphorus, Potassium and pH. Total N was determined using steam distillation method (Bremner and Keeney, 1965) and organic carbon (C) measured by wet oxidation using modified Walkley-Black method as described by Nelson and Sommers (1982). Phosphorus (P) and potassium (K) were extracted by Mehlich-3 procedure (Mehlich, 1984) and then measured by automated colorimetry using an Inductively Coupled Plasma Atomic Emission Spectrophotometer (Kalra and Maynard, 1991).

3.5.1 Total Nitrogen

The procedure involved digestion and distillation. The soils were digested in concentrated H_2SO_4 with a catalyst mixture that raised the boiling temperature and promoted the conversion from organic-N to ammonium-N. Ammonium-N from the digest was obtained by steam distillation, using excess NaOH to raise the pH.

The distillate was collected in saturated H_3BO_3; and then titrated with dilute H_2SO_4 to pH 5.0 (Bremner and Mulvaney, 1982).

3.5.2 Soil pH

Soil pH was measured in a 1:2.5 soil: water ratio using a glass electrode pH meter. Twenty (20) g of soil were weighed into 120 ml plastic shaking bottle and 50 ml of deionised water was added to the soil. The soil-water solution was shaken thoroughly for 30 minutes after which the suspension was allowed to stand for 20 minutes. After calibrating the pH meter with buffers of pH 4.00 and 7.00, the pH was read by immersing the electrode into the upper part of the soil suspension and the pH values recorded.

3.5.3 Organic Carbon

Organic carbon was determined by the modified Walkley-Black method as described by Nelson and Sommers (1982). This involved wet combustion of the organic matter with a mixture of potassium dichromate and sulphuric acid. After the reaction, the excess dichromate was titrated against ferrous sulphate ($FeSO_4$). One (1.0) g of finely ground air-dried soil was weighed into a clean and dry 250 ml Erlenmeyer flask then 2 ml of water was added to the flask. A reference sample and a blank were included. Ten (10) ml 0.1667M potassium dichromate ($K_2Cr_2O_7$) solution was accurately dispensed into the flask. The flask was swirled gently to form a uniform mixture. Twenty (20) ml of concentrated sulphuric acid (H_2SO_4) was dispensed into the soil suspension. The mixture was digested at 150°C for 30 minutes after which the digest was allowed to cool. One hundred (100) ml of distilled water was added and mixed well. Ten (10) ml of ortho-

phosphoric acid and 1 ml of diphenylamine indicator was added and titration completed by adding ferrous sulphate drop-wise from a burette until the solution turned dark green at end-point from an initial purple color. The volume of $FeSO_4$ solution used was recorded and organic carbon calculated as follows:

$$\%C \text{ in soil} = \frac{(V_{Blank} - V_{Sample}) \times \text{Molarity of } FeSO_4 \text{ solution} \times 0.39}{\text{Weight of oven-dried soil sample digested (g)}}$$

Where:

V_{Sample} = volume (ml) of the standard H_2SO_4 used in titrating the sample

V_{Blank} = volume (ml) of the standard H_2SO_4 used in titrating the blank

3.5.4 Potassium and Extractable P

Potassium and available P were extracted using Mehlich-3 (M-3) procedures. Three (3.0) g of air-dried soil samples, ground and passed through 2 mm sieve were weighed into 125 ml Erlenmeyer flasks and 30 ml of M-3 extraction solution (a mixture composed of 0.2M CH_3CHOOH, 0.25M NH_4NO_3, 0.015M NH_4F, 0.013M HNO_3 and 0.001M EDTA) at a ratio of 1:10 (soil: solution) was added into the samples. The flasks were covered and shaken on a reciprocating shaker at 120 oscillation min^{-1}. The suspension was filtered into plastic vials using an M-3 extractant-rinsed Whatman filter paper number 42. Analysis was done by colorimetry using an Inductively Coupled Plasma Atomic Emission Spectrophotometer (Kalra and Maynard, 1991).

3.6 Statistical analysis

Data from the experiment were entered into excel spreadsheet. Calculation of Effectiveness Index was done based on plant biomass and means for internal nodule color and nodule number for authentication experiment in the greenhouse. Screening in pot experiment; means for plant biomass, nodule number were calculated and data for nodule number was analyzed by one way analysis of variance (ANOVA). Field data (nodule number and biomass, plant biomass and grain yield) were analyzed per site using two-way analysis of variance (ANOVA) and interaction determined using combined analysis of variance (ANOVA). The statistical package used was GenStat (GenStat Release 6.1 Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK). Means were separated using Duncan multiple range test and coefficients of variation were also calculated. The MPNES program (Woomer *et al*., 1990) was used to calculate the indigenous rhizobial populations in soil.

CHAPTER FOUR

RESULTS

4.1 Characteristics of media used in the greenhouse and field level

The population estimate of indigenous rhizobia in soil was 2.7×10^3, 1.9×10^3 and 2.8×10^3 per gram of soil in the greenhouse, Nyabeda and Butula respectively. The chemical characteristics of media used in the greenhouse and field are indicated in Table 2.

Table 2: Characteristics of media used in greenhouse and at field level (Butula and Nyabeda).

Media	pH	C	N	K	P	IRP
	(H_2O)	------- % ----------		-------- (ppm) ------		
Vermiculite (sterile media)	8.8	0.44	0.08	117	115.0	0
Greenhouse (soil from Butula)	5.5	1.86	0.08	73	4.9	2.7×10^3
Butula farm (soil)	5.2	1.50	0.24	101	3.9	2.8×10^3
Nyabeda farm (soil)	5.7	2.32	0.21	397	5.0	1.9×10^3

IRP refers to Indigenous rhizobia population

4.2 Authentication and screening indigenous rhizobia isolates collected from soils under different land uses in Kenya.

4.2.1 Authentication of indigenous rhizobia isolates in the greenhouse using sterile media

Twenty isolates out of the hundred test isolates did not form nodules and were eliminated from further consideration. The remaining isolates were classified as ineffective (26%), partly effective (26%), effective (17%) and highly effective (11%) based upon their performance compared to the non-inoculated control and USDA 110 (Figure 2). The Effectiveness Index of the isolates ranged from 0.10 to 1.30 and nodule number from zero to 151 per pot, suggesting that the growth system allowed for large differences between treatments (Figure 2). The internal nodule color from the category of highly effective, effective and partly effective were pink and some red while some of the nodules from ineffective category had white nodules (Figure 2 and Table 3).

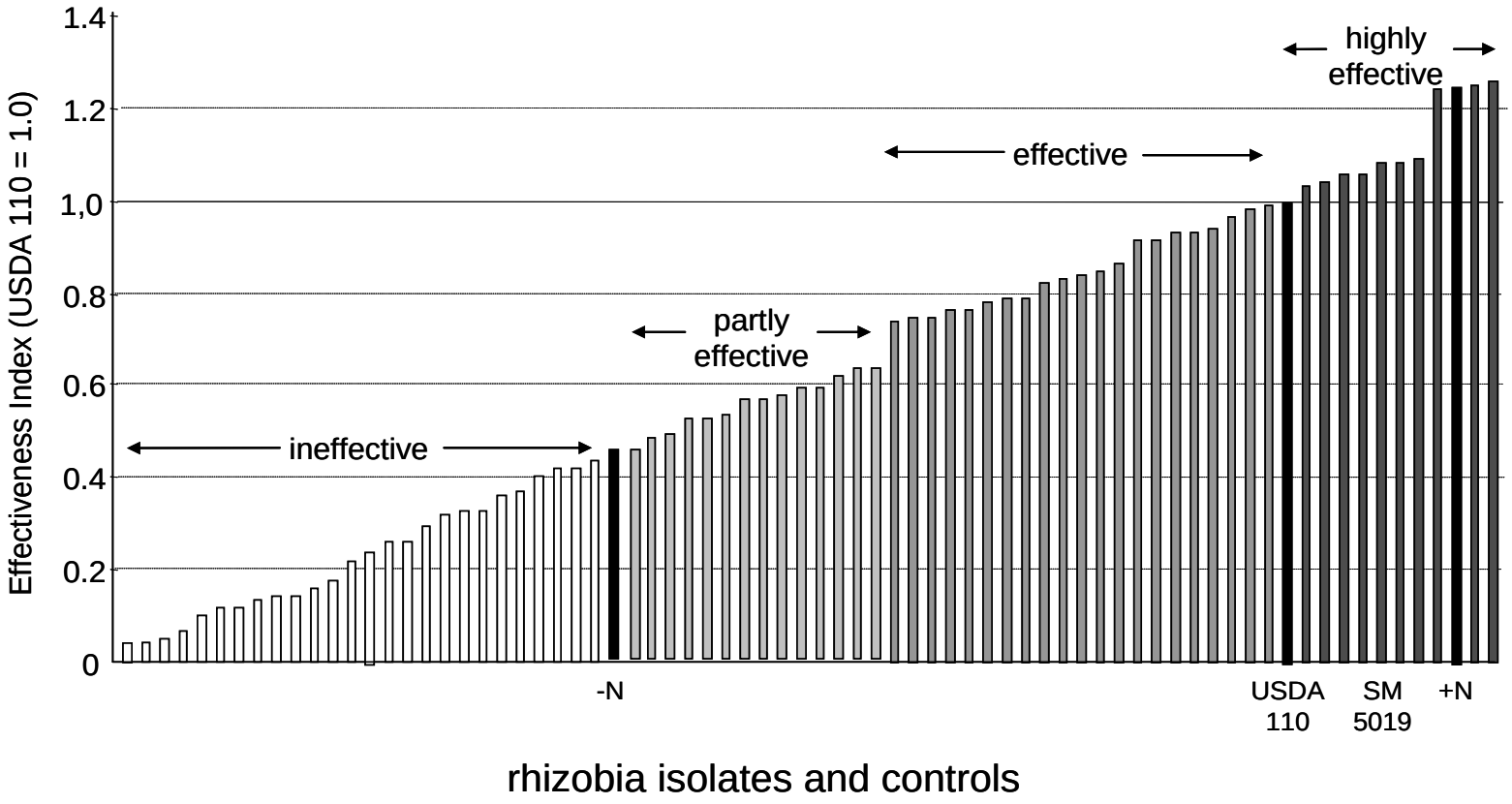

Figure 2: Effectiveness Index of indigenous rhizobia isolates on soybean variety SB 19 grown in rhizobia-free vermiculite.

4.2.1.1 Nodule number and internal nodule color

Out of one hundred isolates authenticated, twenty did not nodulate the soybean variety SB19 and they included NAK86, 102, 119, 155, 156, 159, 163, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 177,180 and 181. The twenty isolates that did not nodulate soybean were left out from further consideration. Generally, nodules were not observed in uninoculated treatment in both positive and negative control treatment (Table 3). Number of nodule was affected significantly by inoculated isolate ($p<0.001$). Seven native isolates (84, 89, 128, 135, 137, 140, 179) produced significantly higher number of nodules than USDA110 and the uninoculated control. Of the seven rhizobia isolates three were highly effective, two effective and two partly effective. Indigenous rhizobia isolates categorized as ineffective, produced significantly lower nodule number than USDA110 (Table 3).

Internal nodule color was affected significantly by treatment ($p<0.001$) (Table 3). The category highly effective, effective and partly effective showed that nitrogen fixation took place because the internal nodule color was pink and some red. Out of twenty six ineffective rhizobia isolates twelve of them did not fix nitrogen they produced nodules which were white and some green internally (Table 3).

Table 3: Parameters of eighty indigenous rhizobia isolates and controls authenticated using sterile media (vermiculite) and SB19 as host plant.

Treatment	Category	NC	NN	Treatment	Category	NC	NN	Treatment	Category	NC	NN
+N	C	0	0.00	NAK 129	E	3	0.67	NAK 93	PE	3	16.00
-N	C	0	0.00	NAK 98	E	3	63.00	NAK 95	PE	3	12.00
SEMIA 5019	C	3	72.00	NAK 4	E	3	2.33	NAK 164	I	3	46.67
USDA110	C	3	56.33	NAK111	E	3	39.50	NAK116	I	2	9.00
NAK127	HE	3	99.00	NAK 125	PE	3	79.00	NAK 109	I	1	0.67
NAK89	HE	3	129.00	NAK 82	PE	3	89.67	NAK 147	I	3	7.33
NAK 176	HE	3	79.33	NAK 90	PE	3	77.67	NAK85	I	3	18.67
NAK 179	HE	4	133.50	NAK 122	PE	3	81.50	NAK94	I	2	1.00
NAK 96	HE	3	33.33	NAK 130	PE	3	0.67	NAK114	I	3	9.33
NAK 149	HE	4	81.00	NAK 182	PE	3	61.50	NAK136	I	3	7.33
NAK 115	HE	3	11.33	NAK 139	PE	4	86.00	NAK99	I	3	3.00
NAK 128	HE	3	112.67	NAK 161	PE	3	25.00	NAK388	I	1	3.00
NAK 9	HE	4	38.03	NAK 146	PE	3	49.67	NAK 100	I	2	0.50
NAK 12	HE	3	2.00	NAK 112	PE	3	29.33	NAK134	I	3	8.33
NAK 83	HE	3	43.00	NAK 137	PE	3	120.00	NAK118	I	2	7.67
NAK 30	E	3	1.67	NAK 145	PE	3	19.00	NAK120	I	1	3.00
NAK 84	E	3	123.33	NAK 153	PE	3	61.00	NAK 141	I	1	0.33
NAK 10	E	3	1.67	NAK 107	PE	3	11.00	NAK 124	I	3	76.00
NAK 135	E	3	123.67	NAK 150	PE	3	7.00	NAK87	I	3	3.67
NAK 117	E	3	20.00	NAK 140	PE	3	151.33	NAK110	I	3	22.50
NAK 151	E	3	33.67	NAK 106	PE	3	33.00	NAK 131	I	3	71.33
NAK 132	E	3	3.67	NAK 138	PE	4	81.33	NAK 142	I	1	5.00
NAK 88	E	3	1.33	NAK 148	PE	3	2.67	NAK 157	I	3	21.33
NAK 105	E	3	26.33	NAK 143	PE	3	10.00	NAK 101	I	1	6.00
NAK 133	E	3	12.67	NAK 154	PE	3	76.67	NAK 123	I	2	6.67
NAK 152	E	3	39.33	NAK 113	PE	3	30.33	NAK 178	I	3	15.67
NAK 144	E	4	59.00	NAK 160	PE	3	5.00	NAK 158	I	3	8.00
NAK 121	E	3	2.33	NAK 38	PE	3	1.67	NAK 183	I	2	8.33
								p-value		<0.001	<0.001
								$LSD_{0.05}$		1	37.15

NC- internal nodule color, NN-nodule number, C-control, HE- highly effective, E-effective, PE- partly effective, I-ineffective

4.2.2 Screening of indigenous rhizobia isolates in the greenhouse on performance of soybean varieties grown in soil from Butula-Western Kenya

4.2.2.1 Nodulation of promiscuous (SB19) and specific (Safari) soybean

Nodule numbers were significantly ($P<0.001$) influenced by soybean varieties. Soybean variety (SB19) produced significantly higher nodule number (40.49 g pot^{-1} than Safari which recorded 15.31pot^{-1} (Figure 3). Inoculation of rhizobial isolates also showed significant ($P<0.001$) difference with respect to nodule number. The interaction between soybean varieties and rhizobia isolates was significant ($P<0.001$) in terms of nodule number. The native population and many of the test isolates (NAK9, 30, 96, 122, 127, 139, 144, 146, 152, 160, 176, 179 and 182) failed to nodulate Safari, while all (NAK9, 10, 12, 30, 83, 84,89, 96,115,117, 122, 127,128,135, 139, 144, 146,149, 152, 160, 161, 176, 179 and 182,) nodulated SB 19, reaffirming the latter's "promiscuous" pedigree (Figure 3). Many isolates formed more nodules than the uninoculated control, 67% and 33% on SB19 and Safari, respectively (Figure 3.) NAK83, 84, 89, 115,117,128 and 135 formed nodules comparable to USDA110 which ranged between 38.25 to 164.25 for promiscuous soybean (SB19) and 12.75 to 103.25 for specific soybean (Safari).

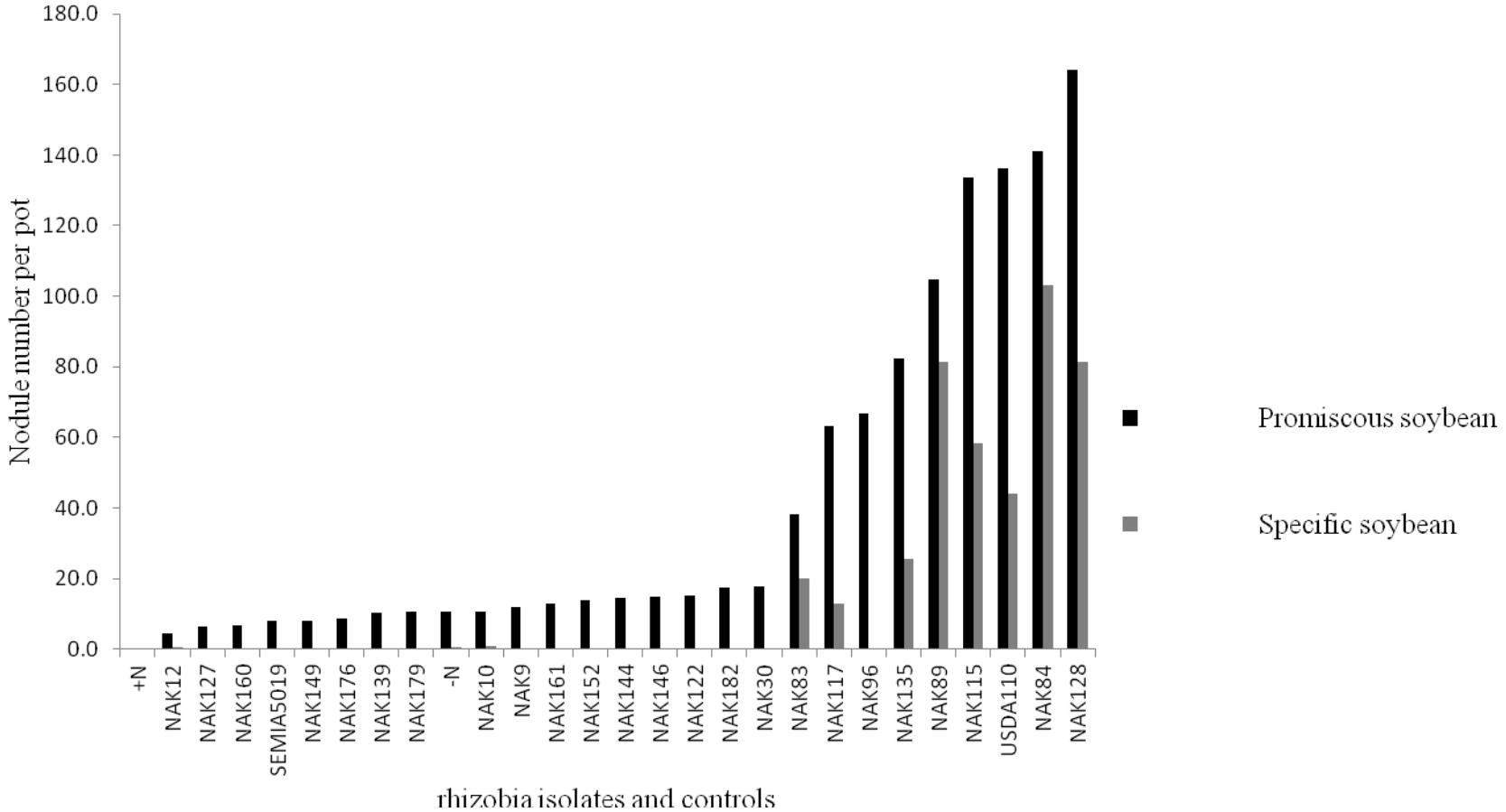

Figure 3: Nodule number on roots of promiscuous (SB19) and specific (safari) soybean varieties grown in Butula soil inoculated with 24 indigenous rhizobia isolates from Kenya.

4.2.2.2 Plant biomass for SB19 and Safari inoculated with promising rhizobia isolates grown in soil collected from Butula at greenhouse level

Plant biomass was significantly ($P<0.001$) influenced by the main effect of soybean varieties. Soybean variety SB19 produced significantly higher amount of plant biomass. Inoculation of rhizobial isolates also showed significant ($P<0.001$) difference with respect to plant biomass (Figure 4). The interaction between soybean varieties and rhizobia isolates was significantly high ($P<0.001$) in terms of plant biomass. Soybeans variety SB19 inoculated with rhizobia isolates had plant biomass ranging from 9.16 g - 14.89 g while for Safari 6.96 g - 13.75 g (Figure 4).

The native population control produced plant biomasses of 13.12 g and 12.43 g for SB19 and Safari soybeans respectively (Figure 4). Only 25% and 16% of the isolates outperformed the native rhizobia population in terms of plant biomass on SB19 and Safari respectively which were referred to as more competitive (Figure 4). Three of the indigenous isolates outperformed commercial isolate (USDA110) and five outperformed SEMIA5019 with the soybean variety SB19. Twenty one of the indigenous isolates outperformed USDA110 and fifteen outperformed SEMIA5019 with specific soybean Safari. The rhizobia isolates classified as more competitive for soybean variety SB19 were less competitive for soybean variety Safari and the rhizobia isolates classified as more competitive for soybean Safari were less competitive for soybean SB19. The indigenous rhizobia isolates

NAK128 and NAK135 outperformed USDA110 for SB19 and Safari) soybeans in terms of plant biomass (Figure 4).

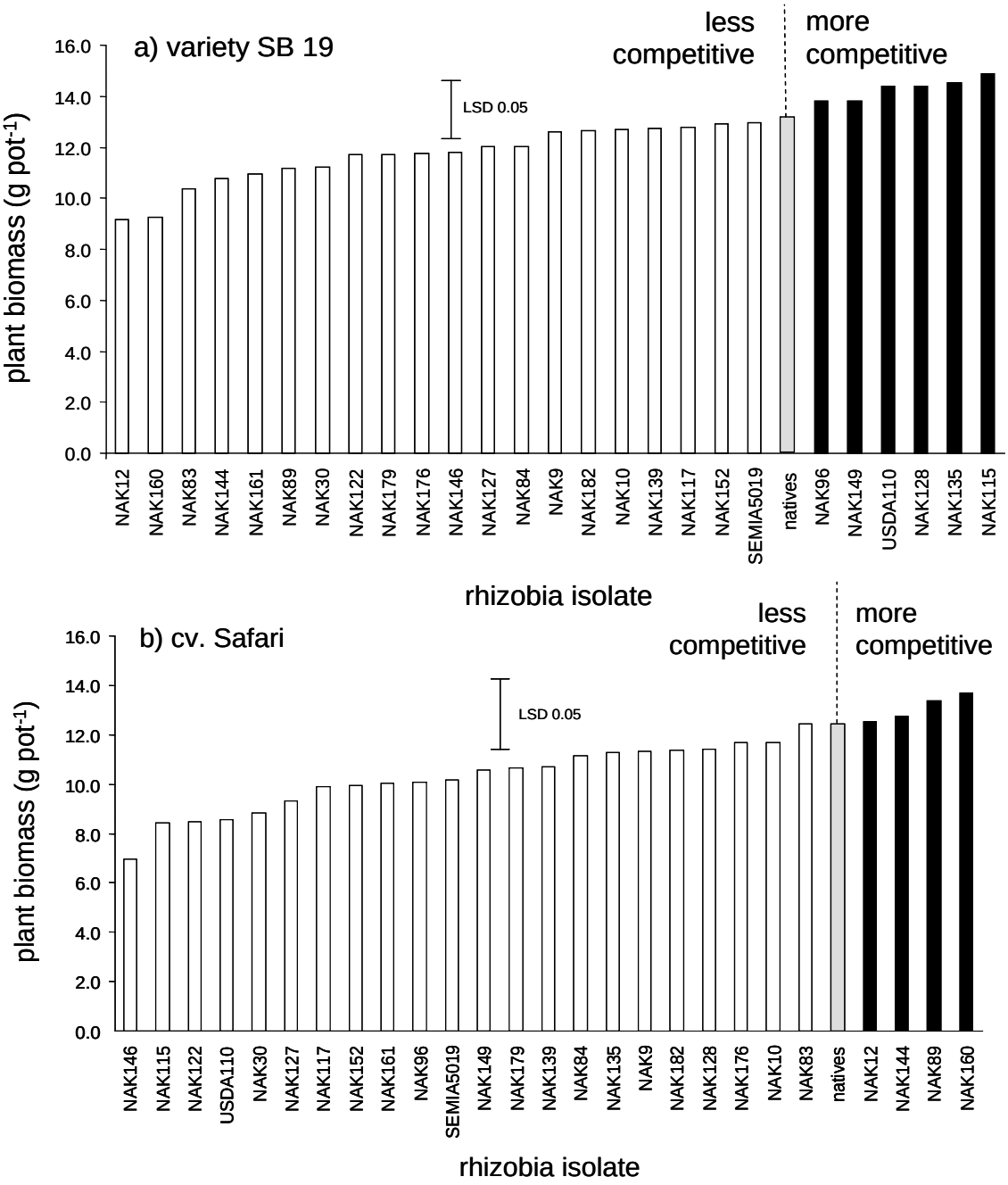

Figure 4: Perfomance of the 24 isolates on the soybean varieties grown in potted Butula soil.

4.3 Field trials to assess on performance of promising indigenous rhizobia using promiscuous (SB19) and specific (SB97) soybean at Butula and Nyabeda

4.3.1 Effect of different rhizobia isolates on plant biomass

There was no interaction observed between rhizobia isolates and soybean variety at both Butula and Nyabeda farms (Table 4). The control treatments produced more plant biomass at Nyabeda as compared to Butula with both SB19 and SB97 soybean varieties (Table 4). At Nyabeda plant biomass production was 2040 kg ha^{-1} and 2271 kg ha^{-1} for SB19 and SB97 soybeans respectively. At Butula plant biomass production for SB19 and SB97 soybeans were 1775 kg ha^{-1} and 1897 kg ha^{-1} respectively. NAK84 and 135 gave higher biomass than USDA110 at Butula while NAK128 and 135 gave higher biomass than USDA110 at Nyabeda (Table 4).

Table 4: Plant biomass for SB19 and SB97 inoculated with best performing rhizobia isolate at Butula and Nyabeda.

Treatment	Plant biomass kg ha^{-1}			
	Promiscuous soybean (SB19)		Specific soybean (SB97)	
	Butula	Nyabeda	Butula	Nyabeda
+N	1986 a	2320 a	2501b	2719 a
-N	1970 a	2206 a	1912 ab	2626 a
NAK115	1667 a	2030 a	1987 ab	2552 a
NAK117	1759 a	2576 a	1518 a	2001 a
NAK128	1606 a	2792 a	1349 a	3020 a
NAK135	1977 a	2286 a	2094 ab	2874 a
NAK84	1684 a	2204 a	2122 ab	2458 a
NAK89	1494 a	2118 a	1734 ab	2584 a
USDA110	1830 a	2213 a	1853 ab	2973 a

	Butula	Nyabeda
p-value for Rhizobia isolates	0.121	0.697
p-value for Soybean variety	0.313	0.042
p-value for Rhizobia isolates* Soybean variety	0.769	0.699
$LSD_{0.05}$	721	667

Means in a column followed by same type of letter (s) are not significantly different (P=0.05) by Duncan's multiple range test.

4.3.2 Effect of different rhizobia isolates on yield of two soybean varieties grown at Butula and Nyabeda

Differences in yield were significant at Butula and Nyabeda. Generally the yield production at Nyabeda was significantly higher as compared to Butula. At Nyabeda, yield from soybeans inoculated were significantly different (P=0.01) while at Butula no significant difference was observed in yield from soybeans inoculated (Table 5). The grain yield at Butula was 1121 kg ha^{-1} and 1147 kg ha^{-1} while at Nyabeda was 1242 kg ha^{-1} and 1225 kg ha^{-1} for SB19 and SB97 respectively. The rhizobia isolate NAK84 performed better than USDA110 with both varieties at Butula (Table 5). There were five isolates at Nyabeda that outperformed USDA110 (Table 5).

Table 5: Grain yield by two soybean varieties inoculated with different rhizobia isolates at Butula and Nyabeda.

Treatment	Grain yield kg ha^{-1}			
	Promiscuous soybean (SB19)		Specific soybean (SB97)	
	Butula	Nyabeda	Butula	Nyabeda
+N	1214 a	1299 ab	1294 b	1304 bc
-N	1193 a	1057 a	1142 ab	1301 bc
NAK115	987 a	1153 ab	1148 ab	1140 ab
NAK117	1270 a	1210 ab	1017 ab	1086 a
NAK128	874 a	1462 b	921 a	1416 c
NAK135	1028 a	1212 ab	1194 ab	1230 ab
NAK84	1232 a	1339 ab	1319 b	1182 ab
NAK89	1176 a	1317 ab	1089 ab	1230 ab
USDA110	1212 a	1129 ab	1196 ab	1139 ab

	Butula	Nyabeda
p-value for Rhizobia isolate	0.083	0.010
p-value for Soybean variety	0.620	0.667
p-value for Rhizobia isolate* Soybean variety	0.571	0.443
$LSD_{0.05}$	313	232

Means in a column followed by same type of letter (s) are not significantly different (P=0.05) by Duncan's multiple range test.

CHAPTER FIVE

DISCUSSION

5.1 Characteristics of soil at Butula and Nyabeda

In general, the fertility status of Nyabeda soil was higher than that of Butula soil. In addition, Butula soil was more acidic than Nyabeda soil. The MPN results show that both sites (Nyabeda and Butula) had high population of native rhizobia. Meade *et al*. (1985) reported that successful competition for nodule sites by native rhizobia is one reason for the failure to achieve a response to inoculation with elite rhizobial strains. However, Giller (2001) found out that the presence of large population density of compatible native rhizobia does not preclude the possibility that responses to inoculation can be obtained if competitive and highly effective strains are introduced.

5.2 The indigenous rhizobia isolates from different Agro-ecological zones in Kenyan soils

Nodulation of leguminous crops by *Rhizobium* largely depends on the presence of a specific and compatible strain in soil for a particular legume. There was considerable variation in nodulation and Effectiveness Index of SB19 grown in vermiculite indicative of varying symbiotic ability of different rhizobia isolates. Efficiency in nitrogen fixing symbioses can vary from those that fix little or no nitrogen to those that fix at levels equivalent to even greater than plants provided

with mineral N as was also demonstrated by Terpolilli *et al*., (2008) and Karaca and Uyanöz (2012).

Twenty isolates recovered from *Crotalaria* sp., *A. hypogaea*, *Mimosa pudica*, *P.vulgaris*, *V. unguiculata*, *Macropitillium* sp, *Vigna* sp., *Tephrosia* sp., *V. membranacea*, *G. wightii*, *V. radiate*, and *Eriosema* sp. did not nodulate the test soybean variety and this might be due to the isolates lacking genes responsible for nodulation in soybean variety used. Sullivan *et al*., (1996) reported that rhizobia lacking genes for infecting legumes are common in rhizosphere of some suitable host legumes.

Eighty isolates were authenticated to be rhizobia as they nodulated the soybean roots grown in sterile vermiculite. Brockwell (1980) suggested that an isolate cannot properly be regarded as a species of *Rhizobium* until its identity has been confirmed through plant infection test on an appropriate host. Number of nodules alone did not reflect the effectiveness of the rhizobia isolate because some ineffective isolates produced high number of nodules meaning nitrogen fixation was very little. Isolates in ineffective category nodulated the plant implying that the full set of standard nod genes and accessory symbiosis components were present however some isolates did not fix nitrogen at all in the ineffective category where white and green nodules were produced which concurred with Quigley *et al.* (1997) observation. Ford and Toby (2004) suggested that rhizobia that fix little or no nitrogen could exhibit parasitic behaviour.

5.3 Performance of indigenous isolates in Butula soil

The nature of soil rhizobial populations may affect the N_2 fixation potential of legumes. First, the number of available invasive rhizobia may be insufficient to nodulate the host adequately. Second, the average effectiveness of the population may be inadequate to support the host's fixed-N_2 requirements. When one or both conditions are present, we might reasonably expect that successful inoculation with an effective *Rhizobium* isolate would enhance N_2 fixation (Bergersen, 1970). Both SB19 and Safari varieties nodulated in the uninoculated treatment, which suggest that native rhizobia capable of nodulating the host were present in soil used for the experiment and they competed with inoculated isolates in the inoculated treatments (Corbin *et al*., 1977).

Rhizobia isolates which produced higher number of nodules were not necessarily more effective than those that produced less nodule number to the inoculated soybeans. Similar findings were found by (Abd El-Maksoud and Keyser, 2010), who reported that a great number of nodules can be formed by a strain fixing little or no nitrogen, even in the presence of effective strains. Rhizobial isolates differed in their nodulating competitiveness when SB19 and Safari were inoculated in soil containing native rhizobial populations and this was supported by (Laguerre *et al*., 2003). Amerger and Lobreau (1982) reported that it is noteworthy that, the choice made by the plant is not dependent on the nitrogen ability of the strain.The inoculum strain fails to occupy a significant proportion of

nodules as the introduced inoculant strains have to compete with the indigenous rhizobial populations (Al-Falih, 2002).

Specific interactions between soybean varieties and rhizobia isolates in nodule formation were noted. The same findings were found by Pazdernik *et al*. (1997) who indicated that soybean genotype may be used to exclude specific isolates. Caldwel and Vest, (1970) reported that at nodule formation, the legume may favor one of the number of strains of Rhizobium to form the nodules. *Rhizobium* spp. may therefore differ in their capacity to be selected by the plant host in nodulating competitiveness. Alternatively, SB19 and Safari might be having different levels of preference which exclude certain rhizobia (Kvien *et al.,* 1981). Different soybean genotype performed differently with rhizobia for example NAK179 performed well in terms of nodule number on SB19 but did not nodulate SB97 because of specificity between soybean line and rhizobia isolate which is supported by (Tukamuhabwa *et al*., 2012). Gandanegara *et al*. (1992) reported similar findings.

The background native rhizobia had competitive ineffective rhizobia as this was noted on Safari when USDA110 produced higher nodule number and was poorer in terms of plant biomass (Eaglesham, 1985). Hahn (1986) reported that legumes cannot consistently recognize and exclude non-fixing rhizobia, especially those that are closely related to their usual symbiotic partners. Therefore USDA110 is not the best *Rhizobium* for Safari.

5.4 Performance of selected rhizobia isolates with SB19 and SB97 soybeans grown in Butula and Nyabeda farms

The first criterion for a *Rhizobium* used as a biofertilizer or nitrogen inoculum is that it must be superior in their symbiotic effectiveness and have a highly effective nitrogen-fixing ability, forming a symbiotic association with the host legume (O'Hara *et al.* 2002). Elite indigenous rhizobia were found for two sites (Butula and Nyabeda) and this is supported by Appunu and Dhar (2006) and Appunu *et al.* (2008), they reported that indigenous rhizobia can be symbiotically more effective than reference strains of foreign origin.

Yield varied at two sites (Butula and Nyabeda) and the yield difference at two sites could be attributed to fertility level of the sites which is supported by (Burdon *et al*. 1999). O'Hara *et al*. (1988) reported that mineral nutrient deficiencies are the major constraints limiting legume N_2 fixation and yield. Alternatively, the yield difference at the two sites could be attributed to different acidity level of the sites (Brady *et al*. 1990). Hecht-Buchholz *et al*. (1990) reported that, in acid soils aluminium toxicity is the limiting factor for BNF. Due to excess of aluminium, plants grow very poorly, the root systems are poorly developed, they form little fine branching roots, which may result in a low number of infection sites, and therefore limit the infection process of the *Bradyrhizobium.*

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

With step-wise approach of selecting effective rhizobia isolates for promiscuous and specific soybeans from one hundred isolates, two of them (NAK84 and NAK128) are elite rhizobia compared to USDA110 for Butula and Nyabeda respectively. The current rhizobia selection process, suggests that best isolates warrant effectiveness testing across a wider range of soybean germplasm and location. For example, NAK 179, performed very well with SB19 in potted soil and very poorly with Safari, and was dropped from further testing and NAK 128 performed well at Nyabeda while NAK 84 performed well at Butula.

The six indigenous rhizobia isolates (NAK84, 89, 115, 117, 128 and 135) have potential for fixing nitrogen at field level, though they might be site-specific. The good symbiotic performance of imported rhizobial strains encourages the identification of new competitive and efficient *Rhizobium* isolates for soybean crops in Kenya. Nodulation were improved with rhizobia inoculation; nodulation depended on both the macro- and micro-symbiont.

6.2 Recommendation

1. There is need for extensive agro ecological zone evaluation for six (NAK84, 89,115,117,128 and 135) rhizobia isolates because rhizobia isolates are site-specific.
2. Cultivar selection should be a major component of future work related to the soybean-rhizobia symbiosis in Kenya.
3. Continued research on the effectiveness of commercially available rhizobia inoculants in Kenya is crucial for the farmer to get the best for yield increase.
4. Further research; molecular characterization of NAK84, 89,115,117,128 and 135 should be done for appropriate recommendation to farmers.
5. Need to avoid blanket inoculants, there should be specific inoculants for specific soybean variety. Therefore, consideration of soybean variety when producing inoculants is crucial since differences in response to the commercial standard strain was evident.

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APPENDICES

Appendix 1: One hundred indigenous rhizobia isolates authenticated in the greenhouse using sterile media (vermiculite)

Isolate code	Longitude	Latitude	Cultivated/wild	Geographical area	Ecological zone	Host	Growth rate	Rank
NAK140	039° 32.934' E	04° 23.119' S	wild	Diani	Coastal plain	Macropitillium sp	Fast	1
NAK179	039° 23.064' E	04° 31.671' S	wild	Ramisi	Coastal plain	Eriosema sp.	Slow	2
NAK89			cultivated	Mumias	Midlands	G. max	Slow	3
NAK135			cultivated	Bungoma	Midlands	G. max	Slow	4
NAK84			cultivated	Bungoma	Midlands	G. max	Slow	5
NAK137			cultivated	Bungoma	Midlands	G. max	Slow	6
NAK128			cultivated	Bungoma	Midlands	G. max	Slow	7
NAK127			cultivated	Bungoma	Midlands	G. max	Slow	8
NAK82			cultivated	Bungoma	Midlands	G. max	Slow	9
NAK139	039° 33.892' E	04° 22.102' S	wild	Diani	Coastal plain	Macropitillium sp	Fast	10
NAK122			cultivated	Busia	Midlands	G. max	Slow	11
NAK138			cultivated	Bungoma	Midlands	A. hypogaea	Slow	12
NAK149	039° 31.438' E	04° 23.896' S	cultivated	Diani	Coastal plain	V. unguiculata	Slow	13
NAK176	039° 27.051' E	04° 29.431' S	wild	Msambweni	Coastal plain	V. unguiculata	Slow	14
NAK125			cultivated	Bungoma	Midlands	G. max	Slow	15
NAK90			cultivated	Mumias	Midlands	G. max	Slow	16
NAK154	039° 28.849' E	04° 18.511' S	wild	Kwale	upland plateau	M. pudica	Fast	17
NAK124			cultivated	Busia	Midlands	G. max	Slow	18
NAK131			cultivated	Bungoma	Midlands	G. max	Fast	19
NAK98			cultivated	Butula	Midlands	G. max	Slow	20
NAK182	039° 23.064' E	04° 31.671' S	cultivated	Ramisi	Coastal plain	V. unguiculata	Slow	21
NAK153	039° 28.736' E	04° 18.761' S	wild	Kwale	upland plateau	Macropitillium sp	Slow	22
NAK144	039° 31.608' E	04° 24.002' S	wild	Diani	Coastal plain	Vigna sp	Slow	23
NAK146	039° 31.608' E	04° 24.002' S	wild	Diani	Coastal plain	Vigna sp	Slow	24
NAK164	039° 33.509' E	04° 22.071' S	wild	Diani	sand dune	Macropitillium sp	Slow	25
NKA83			cultivated	Bungoma	Midlands	G. max	Slow	26
NAK111			wild	Bondo	Lake basin	V. unguiculata	Slow	27
NAK152	039° 28.888' E	04° 20.124' S	wild	Kwale	upland plateau	Macropitillium sp	Slow	28
NAK9	37° 19' - 37°	00° 08' - 00° 35' S		Embu	montaine	M. atropurpureum	Slow	29

	40' E							
NAK151	039° 31.489' E	04° 22.617' S	cultivated	Diani	Coastal plain	V. radiata	Slow	30
NAK96			cultivated	Butula	Midlands	G. max	Slow	31
NAK106			cultivated	Butula	Midlands	G. max	Slow	32
NAK113			wild	Bondo	Lake basin	V. unguiculata	Slow	33
NAK112			wild	Bondo	Lake basin	V. unguiculata	Slow	34
NAK105			cultivated	Butula	Midlands	G. max	Slow	35
NAK161	039° 28.850' E	04° 18.499' S	cultivated	Kwale	upland plateau	V. subterania	Slow	36
NAK110			wild	Bondo	Lake basin	V. unguiculata	Slow	37
NAK157	039° 28.849' E	04° 18.511' S	cultivated	Kwale	upland plateau	P. vulgaris	Fast	38
NAK117			cultivated	Teso	Upper midlands	G. max	Slow	39
NAK145	039° 31.608' E	04° 24.002' S	wild	Diani	Coastal plain	Vigna sp	Fast	40
NAK85			wild	Bungoma	Midlands	Desmodium sp.	Fast	41
NAK93			cultivated	Butula	Midlands	G. max	Slow	42
NAK178	039° 23.064' E	04° 31.671' S	wild	Ramisi	Coastal plain	Eriosema sp.	Slow	43
NAK133			cultivated	Bungoma	Midlands	G. max	Slow	44
NAK95			cultivated	Butula	Midlands	G. max	Slow	45
NAK115			cultivated	Teso	Upper midlands	G. max	Slow	46
NAK107			cultivated	Butula	Midlands	G. max	Slow	47
NAK143	039° 32.714' E	04° 24.508' S	wild	Diani	sand dune	Indigofera sp	Slow	48
NAK114			wild	Bungoma	Midlands	M. pudica	Fast	49
NAK116			cultivated	Teso	Upper midlands	G. max	Slow	50
NAK134			cultivated	Bungoma	Midlands	G. max	Slow	51
NAK183	039° 32.893' E	04° 23.677' S	wild	Diani	sand dune	Terminalia sp	Intermediate	52
NAK158	039° 28.850' E	04° 18.499' S	cultivated	Kwale	upland plateau	V. unguiculata	Fast	53
NAK118			cultivated	Teso	Upper midlands	A. hypogaea	Slow	54
NAK147	039° 31.608' E	04° 24.002' S	wild	Diani	Coastal plain	Vigna sp	Slow	55
NAK136			cultivated	Bungoma	Midlands	G. max	Slow	56
NAK150	039° 31.438' E	04° 23.896' S	cultivated	Diani	Coastal plain	V. unguiculata	Fast	57
NAK123			cultivated	Busia	Midlands	G. max	Slow	58
NAK101			cultivated	Butula	Midlands	Crotalaria sp.	Slow	59
NAK160	039° 28.850' E	04° 18.499' S	cultivated	Kwale	upland plateau	V. Subterania	Slow	60
NAK142	039° 32.934' E	04° 23.119' S	wild	Diani	Coastal plain	Crotalaria sp.	Slow	61
NAK132			cultivated	Bungoma	Midlands	G. max	Slow	62
NAK87			wild	Bungoma	Midlands	Crotalaria sp.	Slow	63
NAK99			cultivated	Butula	Midlands	G. max	Slow	64

NAK338	039° 35.778' E	04° 15.509' S	Wild	Congo river	mangroove flat	Tephrosia sp	Slow	65
NAK120			wild	Busia	Midlands	Albezia sp.	Fast	66
NAK148	039° 31.438' E	04° 23.896' S	cultivated	Diani	Coastal plain	V. unguiculata	Slow	67
NAK121			cultivated	Busia	Midlands	G. max	Slow	68
NAK4	37° 19' - 37° 40' E	00° 08' - 00° 35' S		Embu	montaine	M. atropurpureum	Slow	69
NAK12	37° 19' - 37° 40' E	00° 08' - 00° 35' S		Embu	montaine	M. atropurpureum	Slow	70
NAK30	37° 19' - 37° 40' E	00° 08' - 00° 35' S		Embu	montaine	M. atropurpureum	Slow	71
NAK10	37° 19' - 37° 40' E	00° 08' - 00° 35' S		Embu	montaine	M. atropurpureum	Slow	72
NAK38	37° 19' - 37° 40' E	00° 08' - 00° 35' S		Embu	montaine	M. atropurpureum	Slow	73
NAK88			cultivated	Mumias	Midlands	G. max	Slow	74
NAK94			cultivated	Butula	Midlands	G. max	Slow	75
NAK129			cultivated	Bungoma	Midlands	G. max	Slow	76
NAK130			cultivated	Bungoma	Midlands	G. max	Slow	77
NAK109			cultivated	Bondo	Lake basin	P. vulgaris	Fast	78
NAK100			cultivated	Butula	Midlands	Crotalaria sp.	Slow	79
NAK141	039° 32.934' E	04° 23.119' S	wild	Diani	Coastal plain	Crotalaria sp.	Slow	80
NAK86			wild	Bungoma	Midlands	Crotalaria sp.	Slow	NA
NAK102			cultivated	Butula	Midlands	Crotalaria sp.	Slow	NA
NAK119			cultivated	Busia	Midlands	A. hypogaea	Slow	NA
NAK155	039° 28.849' E	04° 18.511' S	wild	Kwale	upland plateau	M. pudica	Fast	NA
NAK156	039° 28.849' E	04° 18.511' S	cultivated	Kwale	upland plateau	P. vulgaris	Fast	NA
NAK159	039° 28.850' E	04° 18.499' S	cultivated	Kwale	upland plateau	V. unguiculata	Fast	NA
NAK163	039° 28.798' E	04° 18.608' S	wild	Kwale	upland plateau	S. fruticosa	Slow	NA
NAK165	039° 33.509' E	04° 22.071' S	wild	Diani	sand dune	Macropitillium sp	Slow	NA
NAK166	039° 35.778' E	04° 15.509' S	wild	Congo river	mangroove flat	Vigna sp	Fast	NA
NAK167	039° 35.778' E	04° 15.509' S	wild	Congo river	mangroove flat	Vigna sp	Fast	NA
NAK168	039° 35.778' E	04° 15.509' S	wild	Congo river	mangroove flat	Tephrosia sp	Slow	NA
NAK169	039° 35.778' E	04° 15.509' S	wild	Congo river	mangroove flat	V. membranacea	Fast	NA
NAK170	039° 26.910' E	04° 29.282' S	wild	Msambweni	Coastal plain	G. wightii	Slow	NA
NAK171	039° 26.910' E	04° 29.282' S	wild	Msambweni	Coastal plain	G. wightii	Fast	NA
NAK172	039° 26.910' E	04° 29.282' S	wild	Msambweni	Coastal plain	G. wightii	Fast	NA

NAK173	039° 27.051' E	04° 29.431' S	cultivated	Msambweni	Coastal plain	V. radiata	Fast	NA
NAK174	039° 27.051' E	04° 29.431' S	cultivated	Msambweni	Coastal plain	V. radiata	Slow	NA
NAK177	039° 23.064' E	04° 31.671' S	wild	Ramisi	Coastal plain	Eriosema sp.	Slow	NA
NAK180	039° 23.064' E	04° 31.671' S	cultivated	Ramisi	Coastal plain	V. unguiculata	Fast	NA
NAK181	039° 23.064' E	04° 31.671' S	cultivated	Ramisi	Coastal plain	V. unguiculata	Slow	NA

Source: N2 Africa rhizobia isolate database

Rank: 1-80 nodulated SB19 while NA did not

Appendix 2. Nitrogen-free Nutrient Solution (Broughton and Dillworth, 1970)

Stock Solutions	Element	M	Form	MW	g/l	M
1	Ca	1000	CaCl2•2H2O	147.03	294.1	2.0
2	P	500	KH2PO4	136.09	136.1	1.0
3	Fe	10	Fe-citrate	355.04	6.7	0.02
	Mg	250	$MgS0_4•7H_2O$	246.5	123.3	0.5
	K	250	K2SO4	174.06	87.0	0.5
	Mn	1	$MnSO_4•H_2O$	169.02	0.338	0.002
4	B	2	H_3BO_3	61.84	0.247	0.004
	Zn	0.5	$ZnSO_4•7H_2O$	287.56	0.288	0.001
	Cu	0.2	$CuSO_4•5H_2O$	249.69	0.100	0.0004
	Co	0.1	$CoSO_4•7H_2O$	281.12	0.056	0.0002
	Mo	0.1	$Na_2MoO_2•2H_2O$	241.98	0.048	0.0002

For each 10 liters of full strength culture solution, 5 milliliter each of solutions 1to 4 was taken, then added to 5.0 liters of water, and then dilute to 10 liters. One normal NaOH was used to adjust the pH to 6.6-6.8. For plus N control treatments, KNO3 (0.05%) was added giving an N concentration.