NODULATION AND NITROGEN FIXATION POTENTIAL OF *SESBANIA* SPP. RHIZOBIA ON *SESBANIA SESBAN* (L.) MERR. AND ROSE COCO (*PHASEOLUS VULGARIS* L.)

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DECLARATION

Candidate's declaration

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

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This work is dedicated to my family Rose Nasimiyu (Mama Goddy), Linda D. Indakwa, Godfrey Musa, Alex Imbahala, Linda I. Makatiani, Diana Ihavi and Larisa Makatiani.

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ACRONYMS AND ABBREVIATIONS

ACIAR	Australian Centre for International Agricultural Research
AFORNET	African Forest Research Network
ALFP	Amplified Fragment Length Polymorphisms
ANOVA	Analysis of Variance
ARISA	Automated Ribosomal Intergenic Spacer Analysis
atm	atmospheres
ATP	Adenosine Triphosphate
BA	Beatrice Anyango
BMC	BioMed Central
BNF	Biological Nitrogen Fixation
bp	base pair
BSA	Bovine Serum Albumin
BTB	Bromothymol Blue
CA	California
CFN	Centro de Investigacion sobre Fijacion de Nitrogeno
CIAT	Centro Internacional d'Agricultura Tropical
CIRA	Centre International de Recherches sur l'Anarchisme
CIRAD	Center for International Cooperation in Agronomic Research for Development
CR	Congo Red
CTAB	Cetyl trimethyl ammonium bromide
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acids
DWO	David Warambo Odee
EDTA	Ethylenediaminetetra–acetic acid
EPS	Exopolysaccharide
FAO	Food and Agriculture Organization
FEMS	Federation of European Microbiological Societies
g	gram
GenAlEx	Genetic Analysis in Excel
GENSTAT	General Statistics
HSD	Honest Significant Difference

IAR	Intrinsic Antibiotic Resistance
IGS	Intergenic Spacer
IVS	Intervening Sequences
KARI	Kenya Agricultural Research Institute
kb	kilobase
KEFRI	Kenya Forestry Research Institute
KFR	Kenya Forestry Research
kJ	Kilo Juoles
LNB	Legume Nodulating Bacteria
masl	metres above sea level
MASS	Makatiani *Sesbania sesban*
MEGA	Molecular Evolutionary Genetics Analysis
MIRCEN	Microbiological Resources Centers
mL	Millilitre
µL	Microlitre
MLSA	Multilocus Sequence Analysis
MN	Makatiani Namibia
MoALF	Ministry of Agriculture, Livestock and Fisheries (Kenya)
MPa	Mega Pascals
NDWt.	Nodule Dry Weight
NGS	Next-Generation Sequencing
NiFTAL	Nitrogen Fixation by Tropical Agricultural Legumes
NJ	Neighbour Joining
NNo.	Nodule Number
PABRA	Pan-African Bean Research Alliance
PAST	PAleontological STatistics
PCR	Polymerase Chain Reaction
PGP	Plant Growth Promoting
pH	potential Hydrogen
PLOS	Public Library of Science
Psi	Pascal per square inch
PVP	Polyvinylpyrrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism

RISA	Ribosomal Intergenic Spacer Analysis
rRNA	ribosomal RNA
SDWt.	Shoot Dry Weight
SE	Symbiotic effectiveness
SNWt.	Specific Nodule Weight
SUA	Sokoine University of Agriculture
TE	Tris–EDTA
TGGE	Temperature Gradient Gel Electrophoresis
TSBF	Tropical Soil Biology and Fertility
VE	Very Efficient
UN	United Nations
UPGMA	Unweighted Pair Group Method with Average
USDA	United States Department of Agriculture
UVP	Ultraviolet Products
YEMB	Yeast Extract Mannitol broth
YEMA	Yeast Extract Mannitol Agar

ABSTRACT

Global crop and energy production are fast dwindling inversely to population growth. Common bean (*Phaseolus vulgaris* L.) yield has reduced worldwide due to soil infertility which can be reversed through application of chemical fertilizers. The chemical fertilizers used to ameliorate nitrogen, phoshorus and potassium are expensive and cause both deleterious physico–chemical modification of soil and water mass eutrophication. The common bean has ability to fix atmospheric nitrogen symbiotically with rhizobia but only a few effective strains have been recovered from African soils, most of them ineffective under field conditions. Prospecting from local pool of strains trapped by wild native species like sesbania can increase the number of elite inoculant production strains for both species in agroforestry systems practiced under diverse soil and eco-climatic conditions. The specific objectives of this study were: to assess the phenotypic and genotypic characteristics of rhizobia from root nodules of East Africa and Namibia *Sesbania* spp. (here after referred to as sesbania) using morpho-cultural characteristics and PCR–RFLP methods; to assess the nitrogen fixation potential of sesbania isolates on *S. sesban* using growth parameters; and to determine the infectiveness and symbiotic effectiveness of sesbania rhizobia on common beans. Experiments were carried out at the Kenya Forestry Research Institute, Muguga–Nairobi, Kenya. Morpho-cultural techniques were used to characterize and cluster 128 presumptive sesbania rhizobia collected from Kenya, Uganda, Tanzania and Namibia. The diverse growth characteristics of rhizobia on YEMA media, intrinsic antibiotic resistance and salt tolerance were used to select for 79 sesbania isolates that were later subjected to fingerprinting assays using PCR–RFLP of the 16S rDNA in comparison with 17 reference strains. The presumptive sesbania rhizobial isolates were used to inoculate *S. sesban* and common bean under glasshouse controlled conditions to test for their infectiveness and symbiotic effectiveness. Reference inoculants strains KFR647 and BA37 for *S. sesban* and bean respectively, were included in the test. Uninoculated positive control (70 ppm N as KNO_3) and a negative control (0 ppm N) were included for strain effectiveness comparison and to check for contamination. The sesbania rhizobia were grouped into nine morphotypes and various ribotypes per site. The rhizobia varied in their infectiveness and symbiotic effectiveness on *S. sesban* and the common bean resulting in three categories viz: (1) highly effective (2) effective and (3) ineffective. The mean shoot dry weight, nodule number and nodule dry weight were all significantly different ($p < 0.001$). The shoot N content range was 0.16–5.66 mg plant^{-1} and 0.34–3.08 mg plant^{-1} for *S. sesban* and common beans at 8 and 4 weeks of growth respectively. Based on shoot dry weight due to inoculation, rhizobial isolate KFR402 was preferred as a common inoculant production strain for both common beans and *S. sesban*. However, data in the present study shows that the highest shoot dry weight was obtained with strain MASS133 (*S. sesban*) inoculated on Rose coco bean variety (0.87 g plant^{-1}) and MASS172 (*S. sesban*) on *S. sesban* (1.06 g plant^{-1}). Rhizobia recovered from *sesbania* grown in East Africa and Namibia are phenotypically and genetically diverse. The isolates exhibit great variations in effectiveness and nitrogen fixation efficiency on *S. sesban* and common beans (variety: Rose coco). Prospecting for elite rhizobia inoculant strains should be prioritized and tested for effectiveness on both *S. sesban* and common bean grown in diverse edaphic and agro-ecological conditions under agroforestry systems.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Food production in heavily populated regions has remained steady, but at levels too low to mitigate widespread nutritional deficiencies. This has led to famines worldwide, which has resulted in the clearing of swathes of forest lands to pave way for crop production thereby reducing forest acreage to less than the recommended 10 % cover (FAO, 2012). Although at the onset, converted forest land is rich in soil nutrients, leaching and fixation of nutrients together with over–cultivation and crop harvesting methods like 'cut and carry' eventually diminishes the soil fertility (Yeshaneh, 2015). Apart from inadequate supply of water to crops, tropical soils have nitrogen as the number one deficient plant nutrient followed by phosphorus. The only environmental friendly and feasible way to alleviate nitrogen deficiency in soils is the inclusion of nodule forming herbaceous crops and tree legumes on farms, which constitutes agro–forestry.

Common beans, peas, groundnuts are among legume crops grown in East and Southern Africa. The common bean appears on many agricultural farmlands worldwide (Morosan *et al*., 2017). The pulse is an annual herb that matures between one and three months (Koskey *et al*., 2017), hence used to alleviate hunger during food scarcity. Although common bean also provides high protein content that replaces meat which has become very expensive for the poor (Romero-Arenas *et al*., 2013), its current production is far less than the expected yields. For instance, there was a common bean deficit of 26 kilo-tonnes in 2014 in the Kenyan market (MoALF, 2015). This is more often than not due to low soil fertility experienced in

East and Southern Africa. Therefore, a common bean crop must be supplemented with nitrogen–containing chemical fertilizers especially when growing in intensively farmed lands or naturally infertile soils. However, common bean is a legume that is expected to fix atmospheric nitrogen for their protein precursors if they successfully enter into symbiosis with compatible micro–symbionts (Ribeiro *et al*., 2013). But being a naturalised legume in African soils, it often fails to fix atmospheric nitrogen due to the presence of ineffective rhizobia or occurrence of inadequate effective strains or total absence of compatible rhizobia (Catroux *et al*., 2001). Such scenarios call for a mandatory inoculation of seeds before they are sown and hence the need to select for rhizobia of high nitrogen fixation effectiveness.

One of the methods used for selection of superior rhizobia is the determination of cross–inoculation groups that may consist of crops and wild legumes (Gaur and Sen, 1979) whose members have ability to share the symbionts. Few trials have been made to cross–inoculate the common beans with rhizobia baited using wild legumes including multipurpose trees grown on–farm (Bala and Giller, 2001). A search for elite strains begins with collection of nodules from roots of legumes which act as baits for rhizobia from a consortium of microbes found in the soil followed by cross–inoculation assays (Somasegaran and Hoben, 1994). Inoculation of hosts using a prospective inoculant strain is the only option to test for the symbiosis because identification and taxonomy does not globally reflect the symbiotic features of rhizobia, particularly their legume host range (Laguerre *et al*., 2001).

Leguminous trees are also grown on agricultural lands in East and Southern Africa but their choice is dependent on the benefits, suitability and interaction with crops.

Some of the common tree legumes include *Sesbania* spp., *Acacia* spp., *Leucaena* spp., *Calliandra* spp. and *Gliricidia* spp. Like the common beans, sesbania are members of sub–family Papilionaceae, family Leguminosae (Fabaceae) (Heuzé *et al*., 2015). *Sesbania sesban* is common in crop lands in East and Southern Africa intercropped with common beans and maize. *Sesbania sesban*, as well as other species of the genus have multiple uses that include fuelwood, soil nutrient enrichment fodder for both domestic animals and wild browsers and as medicinal plants (Gomase *et al*., 2012).

The common bean has been associated with a broad range of *Rhizobium* spp., all of which have been described in different areas where common beans have been introduced globally (Anyango *et al*., 1995). Although the common bean has its origin in the Mesoamerican it has been reported to nodulate with rhizobia native to Africa, some of them ineffective in N_2-fixation (Michiels *et al*., 1998). Such ineffectiveness in N_2-fixation has partly been attributed to the ability of *P. vulgaris* to perceive nodulation signals from diverse rhizobial strains some of which are ineffective (Dall'Agnol *et al*., 2014). However, rhizobia similar to *Rhizobium etli*, *R. phaseoli* and *R. tropici* have been described in African soils (Anyango *et al*., 1995; Aserse *et al*., 2012) and shown to nodulate common beans. It is routine to use common beans as trap hosts during rhizobial population studies and bioprospecting for elite strains in new bean growing fields. The native rhizobial populations may have superior traits of resistance to adverse abiotic conditions found in most soils of the tropics (Zahran, 1999). Hence, the key role of this research was to determine the diversity of sesbania rhizobia and identify rhizobial strain(s) to be used as common inoculant for both *S. sesban* and the common beans in agroforestry systems.

1.2 Problem statement

There is a great need to increase crop production and energy sources worldwide due to population increase (UN, 2013), which comes along with reduced purchasing power, especially for food. Global common bean production is way far below the expected yield mainly due to the ever declining soil fertility which can be reversed through application of chemical fertilizers (Fischer *et al*., 2014). The demand for common bean is on the increase as the world population and poverty increases (Porch *et al*., 2013). However, the use of chemical fertilizers is not only expensive, but has for long been implicated for both deleterious physico–chemical modification of soil and water mass eutrophication (Savci, 2012).

Common beans can establish symbiotic associations with several rhizobia species (Anyango *et al*., 1995); but the effectiveness of most strains under field conditions has been reported as very low (Ribeiro *et al.*, 2013). So far, the pulse has been symbiotically associated with members of the genera *Rhizobium* and *Sinorhizobium* (Laguerre *et al.*, 2001). The primary nitrogen fixation microsymbiont of the common beans is *Rhizobium tropici* which is found in native bean growing Mesoamerican and the Andean centre. *Rhizobium tropici* has been discovered associating with other legumes growing where the common bean is not native (Grange *et al*., 2007).

1.3 Justification

Unlike the common bean, sesbania is native to Africa and associate effectively with a number of rhizobia genera including *Rhizobium* spp., *Mesorhizobium* spp. and *Ensifer* (Odee *et al.*, 2002; Helene *et al*., 2015) among others. Exploration of new habitats and investigating nodulation of sesbania and other wild legumes cannot only

help to discover unidentified rhizobia, but also supports efforts of selecting effective combinations of legume–*Rhizobium* genotype to exploit the huge potential of increased nitrogen fixation. Both common bean and *S. sesban* rhizobia are affiliated to members of the genus *Rhizobium* and therefore have a likelihood of associating with similar strains in the absence of their specific types.

There are reports of frequent occurrence of common bean rhizobial isolates in East and Southern African soils that are more effective than CIAT899 (Anyango *et al*., 1995). However, little work has been carried out to determine cross-inoculating group containing *P. vulgaris* and other native legumes. Currently there is paucity of information on cross–inoculation regarding the common bean and sesbania. Discovery and identification of elite rhizobial strains through cross–inoculation experiments serve to make superior and multiple host inoculants hence reducing the cost of its production and purchase. Inoculation of legumes is not only environmental friendly way to enhance crop production but also a cheap way of reducing environmental pollution.

1.4 Research hypotheses

i. H_0: There are phenotypic and genotypic differences among the rhizobia isolated from various sesbania grown in East Africa and Namibia.
ii. H_0: Rhizobia from root nodules of sesbania are effective and efficiently fix nitrogen with *S. sesban*.
iii. H_0: Sesbania rhizobial isolates are effective and efficiently fix nitrogen with Rose coco bean variety.

1.5 Objectives

1.5.1 General objective

To screen for common and highly effective rhizobial isolates for *S. sesban* and common beans from sesbania grown in diverse conditions of East Africa and Namibia for inoculant production.

1.5.2 Specific objectives

i. To assess the phenotypic and genotypic characteristics of rhizobia isolates from root nodules of sesbania growing in East Africa and Namibia.
ii. To assess effectiveness and nitrogen fixation efficiency of sesbania rhizobial isolates on *S. sesban*.
iii. To determine the infectivity and nitrogen fixation potential of sesbania rhizobial isolates on Rose coco bean variety.

1.6 Significance of the study

Rhizobia differ in the ability to compete, infect and fix nitrogen due to host compatibility and a myriad of abiotic conditions (Zahran, 2001). Identified rhizobia isolates from nodules of sesbania growing in East Africa and Namibia from this study will increase the knowledge of the diversity of rhizobia capable of forming nodules on *S. sesban*. Common beans–*S. sesban* cross–inoculating elite strains identified in the present study will be used for production of a common *S. sesban*-beans inoculant instead of separate inoculants, thus reduce inoculant production cost and their purchase price.

A profusely nodulated perennial *S. sesban* crop following successful inoculation using a Rose coco–*S. sesban* elite rhizobial strain will perpetuate soil population of the strain when beans are off season hence, less need for inoculation of subsequent bean crop. Successful inoculation of these legumes using the selected elite strain(s) will lead to less use of nitrogenous fertilizers hence; mitigate eutrophication and hypoxia of water masses. The discovered rhizobial elite strain(s) for common beans from the present study will enhance production and hence alleviate the existing malnutrition.

CHAPTER TWO

LITERATURE REVIEW

2.1 Nitrogen reserves in the atmosphere and soil

Nitrogen (N) has globally received enormous attention probably much more than all other essential elements. It remains a requisite element for all living organisms for the reason that it determines the synthesis of nucleic acids which are essential for cellular functioning. Nitrogen represents about 2 % of the total plant dry matter that enters the food chain (Santi *et al*., 2013) while Bothe *et al*. (2007) estimated that all living organisms have nitrogen content of about 6.25 % dry weight. Naturally, approximately 10^{15} tonnes of dinitrogen gas (approximately 78 % N) is present in the atmosphere (Jordan, 1984) but unavailable to all living organisms. The available nitrogen reserves in the soil has remained small due to the comparatively large amounts withdrawn by plants, loss due to erosion, runoffs and leaching of nitrates (O'Leary *et al*., 1989).

Nitrogen exists in soils as soluble inorganic ammonium and nitrate compounds; organic nitrogen (associated with soil humus) and ammonium nitrogen fixed by clay minerals (Schulten and Schnitzer, 1998). Soluble inorganic ammonium and nitrate compounds are the only forms readily available to higher plants, but rarely exceed 1–2 % of the total N present in the soil. Most of the N in the soil is always metabolically unavailable to the higher plants leading to nitrogen deficiency-caused symptoms that may include yellowing and dropping of leaves, stunted growth, delayed flowering and fruiting (Dashora, 2011). The use of chemical nitrogen fertilizers in nitrogen deficient soils leads to a significant increase in crop yields. World fertilizer consumption trend has been on a steady increase since the 1960s.

For instance, between 2001 and 2009, the use of chemical nitrogenous fertilizers grew by 13 % (Stewart and Roberts, 2012). Food and Agricultural Organization (2012) estimated an annual increase of global chemical demand at 2 % between 2012 and 2016 with N fertilizer accounting for up to 60 % of the total costs of nitrogen, phosphorus and potassium (N+P+K) containing fertilizers. However, because of the inherent economical and environmental negative implications due to excessive use of nitrogen–containing fertilizers (Kanimozhi and Panneerselvam, 2010), efforts have consistently been made to maximize on biological nitrogen fixation (BNF) (Baddeley *et al*., 2013). Soil nitrogen is mainly lost through volatilization and drainage of solutions into water masses. According to Saikia and Jain (2007) and Erisman *et al.* (2008), some of the adverse environmental effects of overreliance on nitrogenous fertilizers include: (i) depletion of large amounts of fossil fuels, a non–renewable energy resources, (ii) eutrophication due to N in surface water, (ii) plant toxicity due to high levels of NO_2 and ammonia (NH_4) in soils and (iii) excessive plant growth due to excess nitrogen availability, depletion of stratospheric ozone due to nitric oxide (NO) and nitrous oxide (N_2O). Consequently, the overreliance on chemical nitrogen fertilizers is a serious problem for sustainability and can be avoided by augmented use of BNF.

2.2 Mechanisms of nitrogen fixation

The atmospheric diatomic nitrogen is inert due to its strong triple bond (N≡N) of energy 930 kJ mol^{-1} (Wagner, 2012) and consequently entirely unavailable to all living organisms. However, the high energy barrier can be broken through three nitrogen fixation processes. First, the atmospheric fixation which is a result of lightning that breaks the triple bond and allows oxidation of nitrogen to form

nitrogen oxides which in turn dissolve in rain and falls back to earth. Second, industrial fixation by the Haber–Bosch, a process in which nitrogen and hydrogen gases are heated to temperatures of 400–450 °C and 20 MPa (200 atm) pressure (Hopkins and Dungait, 2010) to form liquid ammonia used in the manufacture of commercial chemical fertilizer. Finally, BNF that reduce N_2 to NH_3 at atmospheric pressure and environmental temperature through an ATP–dependent process mediated by a multimeric enzyme complex, the nitrogenase found only in prokaryotes (Dighe *et al*., 2010). The Haber-Bosch process combines nitrogen from the air with hydrogen derived mainly from natural gas (methane) into ammonia. The reaction is exothermic, reversible and can be represented by the following equation:

$$N_2(g) + 3H_2(g) \rightleftharpoons 2NH_3(g) \quad \Delta H^{\ominus} = -92 \text{ kJ mol}^{-1}$$

2.2.1 Mechanisms of biological nitrogen fixation

Mobilization of nutrients has been studied for decades, focusing mainly on biological nitrogen fixation, in particular, on the symbiosis between microbes and higher plants. Whilst symbiotic nitrogen fixation is currently well known, it is a still mystery to how long ago the first legumes (family Fabaceae) engaged members of protobacteria. A biochemical interplay between legumes and rhizobia leads to infection and organogenesis of nodules on roots or stems of legumes as well as some non–legumes (Santi *et al*., 2013). The nodule forming microbes symbiotically fix atmospheric dinitrogen to the benefit of the plants and in return the plant supplies organic acids in particularly malate and succinate, which are produced from sucrose via sucrose synthase and glycolytic enzymes to rhizobia (Noisangiam *et al.*, 2012). Rhizobia form nodules on legumes while actinomycetes known as *Frankia* (genus) cause root nodules and fix nitrogen on actinorhizal plants (non–legume) such as *Alnus* spp.,

Comptonia spp., *Cassuarina* spp. and *Elaeagnus* spp. (Kumar and Rao, 2012). Infection by rhizobia and *Frankia* normally lead to the formation of root nodules however, a few species for instance, *Sesbania rostrata* can form stem nodules especially when grown in submerged environments (Goormachtig *et al*., 2004).

2.2.1.1 Nodule formation on legumes

Prokaryotes (bacteria or archaea) can fix nitrogen with or without direct association with eukaryotes (Figueiredo *et al*., 2013). The nitrogen fixation process is spontaneously switched on in presence of adequate carbon but limited nitrogen, resulting to estimated 1.44 x 10^8 tonnes N $year^{-1}$ globally (Kumar *et al*., 2007).

Divergent ideas and theories have attempted to explain the process of nodule formation and the subsequent nitrogen fixation in legumes. However, all agree to presence of a number of genes in bacteria that control different aspects of nodulation process and subsequent BNF. The complex process is orchestrated by a multitude of bacterial and plant signals (Ferguson *et al*., 2014) that starts with plant roots secreting specific cocktail of phenolic molecules, predominantly flavonoids and isoflavonoids into its rhizosphere. These molecules bind the transcriptional regulator NodD of compatible rhizobia and concomitantly stimulate a set of bacterial nodulation genes that lead to the synthesis of a highly specific signal molecule called *Nod* factors (lipochitooligosaccharide) which are perceived by legumes via LysM domain receptor kinases [lysin–motif (LysM)] present on the root (Mandal *et al*., 2010; Black *et al*., 2012). For instance, host determinants of symbiosis specificity in soybean are GmNFR1 and GmNFR5 as main Nod factor receptors (Indrasumunar *et al*., 2011). Although only a small percent of legumes has been surveyed for

nodulation to date, Odee and Sprent (1992) described *Acacia brevispica* as a *nod*-factor transducer mutant found among a prominently nodulating legume genus. Nod factor perception triggers a subsequent signaling cascade that is necessary for appropriate nodule establishment and maintenance. In addition to the nod factors, many other bacterial compounds including exopolysaccharides (EPS), lipopolysaccharides, K–antigen polysaccharides, cyclic β–glucan, high–molecular–weight neutral polysaccharides (glucomannan) and gel–forming polysaccharides affect different stages of the interaction (Lira *et al*., 2015).

The attachment on the root hair of nodule forming bacteria possessing the capacity to synthesize nod factors, initiates root tip curling which culminates in bacteria entrapment and formation of an infection thread followed by initiation of cortical cell division (Shtark *et al*., 2011). Legumes root nodules are either indeterminate or determinate depending on whether or not the meristem remains active for the entire life of the nodule respectively (Subramanian, 2013). The indeterminate nodules, as those formed on roots of pea plants have their cell division first observed in the inner cortex. Determinate nodules whose cell divisions are first observed in sub–epidermal cell layer are found on roots of common beans and sesbania among others legumes (Ferguson *et al*., 2010). In contrast to roots, both types of legume nodules have a peripheral vasculature.

2.2.1.2 The biochemical process of nitrogen fixation

The biochemical process of nitrogen fixation is catalyzed by the complex enzyme called nitrogenase which is composed of two component proteins (dinitrogenase and

dinitrogenase reductase) encoded by the family *nif* genes and whose presence is characterized by pink coloration of the nodule tissues. The universal reaction $N_2+8H^+ +8e^- +16ATP \longrightarrow 2NH_3+H_2+16ADP+16Pi$), requires hydrogen as well as energy in form of ATP to progress. Leghemoglobin maintains a high oxygen flux, but at a concentration of approximately 10^4 to 10^5 times lower than in aerobic cultures to avoid inactivation of oxygen–labile nitrogenase (Hunt and Layzell, 1993; Boyd and Peters, 2013). Excesses of oxygen hinder the transcription of the gene *nif*HDK or activity of the functional protein in aerobes but never a problem of anaerobic bacteria.

2.3 Legume–rhizobia symbiotic promiscuity

Legumes and rhizobia have the ability to select their symbionts alike. This is achieved through the complex chemical signalling pathways that determine symbiosis specificity between rhizobia and the host plants (Angus and Hirsch, 2010). The ability to form nodules on roots or stems of more than one host rely on the promiscuity either of the legumes, or their rhizobia, or both (Peix *et al*., 2015). Some rhizobial strains have ability to nodulate individual or an extremely limited number of host species while promiscuous types can form nodules with a wide range of hosts across subfamilies. In the same way, some legume species are extremely restrictive and tolerate only a very narrow range of rhizobia whereas others are promiscuous and are nodulated by a wide range of rhizobia. Perret *et al*. (2000) and Lira *et al*. (2015) suggested that the rhizobia of herbaceous host species can nodulate more host plant species than those of woody legumes and tropical legumes are typically more promiscuous than temperate legumes respectively.

2.4 The degree of specificity and nitrogen fixation effectiveness between legumes and rhizobia

Rhizobia represent great diversity between the genera with some closely related to non–nodulating bacteria such as *Agrobaterium* or *Brucella* compared to each other (Sprent, 2001; Maróti and Kondorosi, 2014). The common feature is their ability to form nodules on legumes but not on all. The disparity in the ability to form nodules and fix nitrogen among rhizobia is due to the difference in their genome (Okazaki *et al*., 2010) and it is termed as host specificity (Györgypal *et al*., 1988). According to Wang *et al*. (2012), host specificity can occur during bacterial infection and nodule development as well as at the nitrogen fixation stage. Rhizobia whose nod genes are not transcribed (*nod*-) initiate no nodules on their legume hosts. Similarly, legume mutants with no *nod* factor transducing mechanisms fail to form nodules even in presence of rich rhizobial diversity.

High degree of specificity between legumes and rhizobia has been described. For example, although the Nod factors produced by *Rhizobium etli* and *Rhizobium loti* are indistinguishable, the two species have distinct host ranges. *Phaseolus* spp. is restricted to *Rhizobium etli* while *Lotus* spp. forms symbiosis exclusively with *Rhizobium loti* (Cárdenas *et al*., 1995). Additionally, two *Rhizobium* strains that form nodules on one plant species may secrete different Nod factors. *Rhizobium tropici* and *R. etli* produce sulfated and acetylfucosylated Nod factors, respectively, yet both effectively form nodules on *Proteus vulgaris* (Mus *et al.*, 2016). On the other hand, *Bradyrhizobium japonicum*, *B. elkanii, Sinorhizobium fredii* (strain USDA257) and *Rhizobium* sp. (strain NGR234) have a number of common hosts, but their Nod factors vary considerably (Perret *et al*., 2000).

Nitrogen fixation efficiency always varies with different host–rhizobial combinations (Schumpp and Deakin, 2010). Rhizobia that offer insignificant benefits to their hosts are common in nature (El–Maksoud and Keyser, 2010). In such scenarios the microsymbionts have been referred to as parasites (Denison and Kiers, 2004) or cheats (Kiers *et al*., 2003). Several symbiotic phenotypes exist and can be manifested by mutants as: the nodule deficient mutants impaired in the first steps of infection (*nod*-); the bacterial mutants which induce nodules that present an early nodule senescence phenotype with blocked process of bacteroid differentiation (*nod*+ *fix*-); fully differentiated but unable to reduce nitrogen to ammonium (*nod*+*fix*-); differentiated into bacteroids less efficient in nitrogen fixation compared to the wild–type strain (*nod*+ *fix*+/-) (Maunoury *et al*., 2010; Liu *et al*., 2011; Saeki, 2011; Melino *et al*., 2012). Whilst, certain rhizobial strains can infect a host genotype and remain ineffective within the symbiosomes (*nod*+*fix*-), the same strains can establish an effective interaction (*nod*+*fix*+) with other alternative host genotypes (Simsek *et al*., 2007). A perfect match between host and the microsymbiont result in effective nodules that have a red pigmentation on cross–sections, signifying presence of leghemoglobin. Conversely, many isolates are known to produce ineffective nodules that border more on parasitism than symbiosis. Such ineffective rhizobia produce nodules that are greyish green or whitish on cross–sections (Pommeresche and Hansen, 2017).

Although symbiosis between rhizobia and legumes is a precise match, in some cases a certain level of mismatching is tolerated. A majority of rhizobia that form nodules on roots of wild legumes growing in most soils are diverse, most of which are promiscuous and a few with selective and specificity characteristics (Zahran, 2001).

Matching systems for many important legumes have been studied and categorized into cross–inoculation groups, each of which consists of all the species that develop nodules when inoculated with rhizobia obtained from any one of them (Gaur and Sen, 1979). Although the cross–inoculation matching system is used mostly as a guide for farmers, it can also be used for selecting the suitable rhizobial inoculant strain for the desired legume host(s). For instance, Syed *et al*. (2010) attempted to examine the host range of rhizobia isolated from nodules of *Tephrosia purpurea, Crotolaria medicaginea, Leucaena leucocephala* and *Sesbania sesban* in other legumes grown in Ajmer and Bikaner regions of Rajasthan. In this work, it was concluded that *S. sesban* was more specific with its rhizobia being compatible with few species outside the genus *Sesbania.* The only known way of testing for nitrogen fixation ability of a rhizobial isolate is when the host plant partners successfully forms nodules on the roots (infective) due to the inoculants (biofertilizers) with an ultimate alleviation of nitrogen deficiency symptoms (effective) of the host (Somasegaran and Hoben, 1994).

2.5 *Sesbania sesban* and legume nodulating bacteria

Sesbania sesban is a fast growing tree species (Evans and Macklin, 1990) that has the ability to associate and fix atmospheric nitrogen in presence of its compatible rhizobia and nodulates most effectively with homologous strains (Turk and Keyser, 1992; Bala *et al*., 2001). However, many soils are devoid of rhizobia that are compatible with *S. sesban*. For example, *S. sesban* growing in some soil of South African failed to nodulate due to the absence of effective native rhizobia (Bala *et al*., 2002). Makatiani and Odee (2007) emphasized the need to determine the symbiotic status of sesbania whenever they are cultivated for the first time in order to make

appropriate decisions on whether to inoculate or not, which requires proper selection of actively fixing strains for inoculants production. Whereas common bean forms nodules with only members of *Rhizobium* spp., the genus *Sesbania* can form root or stem nodules with *Azorhizobium* (Goncalves and Moreira, 2004; Lee *et al*., 2008), *Ensifer* spp. syn. *Sinorhizobium* (Sharma *et al*., 2005), *Mesorhizobium* (McInroy *et al*., 1999; Odee *et al*., 2002) and *Rhizobium* spp. (Bala *et al*., 2002; Vinuesa *et al*., 2005) but their nitrogen fixation effectiveness remain largely unknown. Other reports from tropical Africa, Asia, Puerto Rico, Central and South America indicate that sesbania can be nodulated effectively by *Bradyrhizobium* sp. (Doignon–Bourcier *et al*., 2000), *Rhizobium huautlense* (Wang *et al*., 1998), *R. gallicum* and *R. tropici* (Zurdo-Piñeiro *et al*., 2004), *Sinorhizobium saheli* and *S. terangae* (Lorquin *et al*., 1997), *Azorhizobium caulinodans* (Dreyfus *et al*., 1988), *Mesorhizobium* sp. and *M. plurifarium* (Wang and Martinez–Romero, 2000; Wang *et al*., 2003). Bala *et al*. (2002) and Odee *et al*. (2002) reported frequent recovery of strains closely related to the genus *Agrobacterium* from sesbania nodules.

2.6 Common beans

2.6.1 Origin and distribution of common beans

The common beans (*Phaseolus vulgaris*) has the Mesoamerican and the Andean as the two main centres of origin but are now widely distributed on many continents such as Asia, Europe and Africa (Becerra *et al*., 2011). It is the best known species of the genus *Phaseolus* in the family Fabaceae of about fifty plant species, all native to America (Romero-Arenas *et al*., 2013). Although the pulse was introduced to Africa approximately five centuries ago (Evans, 1976), common beans development to

increase on its yield through selection of genotypes suitable for diverse environmental conditions and their microsymbionts, is still under way.

2.6.2 Uses of common beans

The common bean is a source of high concentration protein complex (cheap substitute for meat globally), carbohydrates, fiber, prebiotic, vitamin B, and chemically diverse micronutrient composition recommended for human consumption (Câmara *et al*., 2013). Besides providing income for rural smallholder families in Kenya, common bean is also a popular food to both the urban and rural population. In Africa, bean products are consumed at different stages of plant development hence, offering a prolonged and spread out food supply in the form of leaves, green pods, fresh grain, as well as dry grains. The common bean containing food is distributed in many forms including unprocessed seeds, canned products and as part of mixes. The pulse matures rapidly (Koskey *et al*., 2017) and serves as a key component in intensifying production through intercropping systems. Its ability to form symbiosis and fix nitrogen with rhizobia provides a long term solution for soil fertility improvement.

2.6.3 Common beans production and its limitations

The world largest producer of common bean is Brazil with an estimated 3.3 million tons produced in 2016 (FAO, 2017). Although the common bean production in Kenya remained stable in 2014, 2015 and 2016 at 622.8, 714.4 and 616.0 kilo-tonnes respectively (FAO, 2017), its production is still way far below the expected yields due to the ever increasing population. The major limitation to common bean production in many smallholder farms is the declining soil fertility as a result of

continuous cropping with minimal inputs or rotation to replenish soil nutrients (Mungai and Karubiu, 2011). Other factors that influence bean production include seed density, chemical toxicities, pests and diseases, weeds, extreme environmental conditions among others (Beebe *et al*., 2013; Buruchara *et al*., 2011; Porch *et al*., 2013). For instance, in 2011 there was a net bean deficit in Kenya of 51.7 kilo-tonnes (FAO, 2013). In Kenya bean crop is mainly cultivated in rotation with maize and grows in diverse soil condition including low nitrogen and phosphorus, low pH, high salinity and low moisture (Maingi *et al*., 2001; Massawe *et al*., 2016).

2.6.4 Biology of common beans

Common beans grow twining or sub–erect and have numerous varieties which can be described in many shapes, sizes and colours. For example, kidney beans, lima beans, pinto beans, navy beans, green beans, wax beans and butter beans are just but a few of the many types found in the Americas (Gepts, 2014). Likewise, there also exists a number of *Phaseolus* spp. in Kenya each with a number of cultivars. *Phaseolus vulgaris* is the most dominant of all with over 100 cultivars grown in the country and its most common varieties include the *Nyayo*, *Amini*, Rose coco, *Nyayo* short, *Kakunzu*, *Mwezi moja*, *Mwitemania*, *Wairimu*, *Kitui*, *Kitui* small, *Kayellow*, *Ikoso* and *Kamwithiokya* which grow in the different eco–climatic zones of Kenya (Ramaekers *et al*., 2013). Apart from seed colour and size, the varieties are differentiated by the growth habit (determinate and indeterminate), seed shape and days to maturity.

2.6.5 Symbiosis of common beans with rhizobia

The common bean is known to form nodules with several *Rhizobium* species but the effectiveness of these strains under most field conditions is low (Dall'Agnol *et al*., 2013; Ribeiro *et al*., 2013). According to Hardarson *et al*. (1993), different varieties of common beans vary in their capacity to fix atmospheric nitrogen. Although common bean is generally considered more responsive than other legumes to nitrogen fertilization, it has been reported as a less nitrogen fixer than other crop legumes (Graham, 1981). Common bean is considered a promiscuous host that form nodules with *Bradyrhizobium* sp. (Han *et al*., 2005), *Sinorhizobium meliloti* (Zurdo-Piñeiro *et al*., 2009), *S. americanum* (Mnasri *et al*., 2012), *Rhizobium mesoamericanum* (Lopez–Lopez *et al*., 2012), *R. freirei* (Dall'Agnol *et al*., 2013), *R. etli*, *R. tropici*, *R. leguminosarum* bv. phaseoli, *R. gallicum*, *R. giardinii*, *R. lusitanum*, *R. phaseoli*, *R. azibense* (Mnasri *et al*., 2014) all in alpha–proteobacteria and *Burkholderia phymatum* (Gyaneshwar *et al*., 2011) in class beta–proteobacteria found in diverse soil ecosystems of the world. Although, *R. tropici* was originally isolated from host in native bean growing areas, it has been reported to associate with other legumes growing where the common bean is not native (Grange *et al*., 2007).

2.7 Characterization and identification of rhizobia

Preliminary identification of cultivable microbes often involves morphological and physiological characteristics (Somasegaran and Hoben, 1994), which is attainable through microbial isolation, culturing and later visualized by observation using microscopes. Typing of microbes into their respective biotypes, serotypes, bacteriocin and phage groups remain also crucial during identification of microbes residing in animals, plants and soil. Data is derived from well–established and

observable growth parameters, physiological and biochemical profiles. The physiological tests used for identification of microbes include fermentation of various carbohydrates, growth on carbon and nitrogen sources, determination of vitamin requirements, growth at various temperatures, growth on media containing various levels of sugar and sodium chloride, ability to hydrolyze urea and resistance to antibiotics (Fakruddin and Mannan, 2013).

Accuracy and speed of microbial characterization and identification have increased with the advent of molecular biology techniques. The genotypic techniques involved in identification of microbes include restriction digestion and PCR amplification or hybridization, all of which employ DNA bands visualization (Auch *et al*., 2010), multilocus sequence analysis (MLSA) of different protein–coding housekeeping genes and whole–genome sequence analysis (Berrada and Fikri–Benbrahim, 2014). These molecular tools are now used for investigating the diversity and structure of microbial communities including the uncultivable (Hill *et al*., 2000).

2.7.1 Methods for rhizobial identification

2.7.1.1 Phenotypic identification

Phenotypic identification methods comprise the study of the morphology and biochemical reactions by bacteria whose properties can be observed after incubation of the cultures grown on solid media for a definite time. Morphological characteristics include colony characteristics such as size, shape, pigmentation, elevation, opacity, margin, surface texture and consistency. The biochemical tests use specific growth media, nutrients, chemicals or growth conditions to elicit visible characteristics. Some simple tests like the Gram's reaction, acid-fast reaction,

motility, arrangement of flagella, presence of spores, capsules and inclusion bodies aid in identification of the organism. However, phenotypic characteristics like isoenzyme profile, antibiotic susceptibility profile and chromatographic analysis of cellular fatty acids are sensitive enough for strain characterization. Although vital in the preliminary classification of microbes, Petti *et al*. (2005) noted that phenotypic traits are always not static hence, can change with stress or evolution.

2.7.1.2 Molecular techniques used in rhizobial identification

Development of molecular techniques which complement the analysis of morpho-cultural traits has enhanced the ability to rapidly detect, identify and classify microbes. Moreover, these techniques have also been used to establish the taxonomic relationship among closely related genera and species (Mishra *et al*., 2016). Identification using molecular methods relies on the comparison of the nucleic acid (RNA and DNA) sequences or protein profiles of a microorganism with documented data on known organisms. The molecular methods are considered sensitive enough to allow detection of low concentrations of viable or non–viable microbes in both pure cultures and complex samples as in soils and water. These include methods such as DNA re-association, DNA–DNA and mRNA-DNA hybridization, DNA cloning and sequencing and other PCR-based methods such as 16S rRNA, comparisons of restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP) or G+C % content in the genomic DNA with corresponding data on known species or strains (Vinay *et al*., 2013). Other molecular currently used to delineate *in situ* microbes include denaturing gradient gel electrophoresis (DGGE) temperature gradient gel electrophoresis (TGGE) (Smalla *et al*., 2007), ribosomal

intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA) (Fakruddin and Mannan 2013; Namkeleja *et al*., 2016).

After elucidation of double helical structure of DNA by Watson and Crick, the quest to detect its sequences from many organisms including human and microbial genomes pioneered the discovery of molecular methods such as Maxam and Gilbert's technique (Maxam and Gilbert, 1977) and Sanger sequencing (Sanger and Coulson, 1975). As with many technologies, genomics has evolved at a remarkable pace leading to creation of dozens of next-generation sequencing companies, technologies and the corresponding field of bioinformatics. For instance, in the mid-to-late 1990s, microarrays were developed as highly parallel assays to measure RNA and DNA offering the first genome-scale parallel analysis of the nucleic acids (Heather and Chain, 2016). Second- and third-generation sequencing techniques emerged and have enabled unbiased means to scrutinize trillions of templates of DNA and RNA in a single instrument run. Coupled to current, high-performance computing and a host of bioinformatics tools that have been developed to analyze the data, whole genome sequences from an individual organism are generated (Jimenez-Lopez *et al*., 2013). Collectively these technologies are referred to as next-generation sequencing (NGS) and share several characteristics including massive parallelization of chemical sequencing reactions, micro- to nano-scale reaction volumes. Additionally, there is immense amount of computational power to capture the raw data and process to formats interpretable by analysis software running on external computers (Levy and Myers, 2016). The NGS has opened new frontiers of genomics research which include clinical applications (Rizzo and Buck, 2012) and identification of soil and plant microbial communities (Lakshmanan *et al*., 2014).

2.7.2 Morphological and molecular characterization of rhizobia

Distribution and diversity of rhizobia within tropical soils vary as their hosts and also with the ever changing diverse eco–climatic conditions of their natural habitats (Odee *et al*., 1997; Hungria and Vargas, 2000). The diversity among nodulating bacteria may be due to the dynamic structure of bacterial genomes. For example, the organization of symbiotic genes within easily transmissible symbiosis islands or plasmids can permit the conversion of non–symbiotic bacteria into nitrogen–fixing plant endosymbionts in a single step (Marchetti *et al.*, 2010). The host compatibility spectrum, cultural methods (media, morphology, antibiotic and other biochemical tests), serological methods, bacteriophage typing, molecular biology methods (nucleic acid hybridization and PCR-based techniques) and genomic sequencing have been used as criteria to consider during the study of rhizobia (Jia *et al*., 2015). The morphological properties include: size, shape, cell wall characteristics (Gram staining), surface characteristics and pigmentation, sporulation characteristics, mechanisms of motility, and other cellular inclusions and ultra–structural characteristics (Somasegaran and Hoben, 1994).

The recent advancement in molecular techniques have have been used to reorganized some genera and describe many other new genera and species of bacteria associated with tropical legumes (Odee *et al*., 2002; Menna *et al*., 2006; Toolarood *et al.*, 2012; Hassen *et al*., 2014). These molecular techniques are highly versatile and have better resolution over the traditional cultural methods which they complement (Pierre and Didier, 2002; Capote *et al*., 2012; Marinkovic *et al*., 2013). They have led to phylogeny inferences that include 16S rRNA PCR–RFLP analysis (Oger *et al*., 1998; Romdhane *et al*., 2005), 16S rRNA sequencing, RAPD (El–Fiki, 2006), DNA–DNA

hybridization (Murray *et al*., 2001; Auch *et al*., 2010), multilocus sequence analysis of different protein–coding housekeeping genes and whole–genome sequence analysis (Berrada and Fikri–Benbrahim, 2014).

2.7.2.1 The bacterial 16S rRNA gene

The rRNA genes play a great role in the protein synthesis and are therefore essential for the survival of all organisms. Among the rRNA genes is the 16S (small subunit) rRNA gene which is 1500 bp long with ten well conserved and ten divergent regions (Clarridge, 2004). However, cases of abnormally large 16S rRNA gene (larger than 1500 bp) have been documented and attributed to foreign DNA sequences, usually called intervening sequences (IVS) with about 140 bp long (Linton *et al*., 1994). For instance, Laguerre *et al*. (1994) found two strains, CFN299 and C–05–35 (both *Rhizobium tropici* type II) that produced a single band of 1600 bp when 16S rRNA was amplified which was attributed to insertion of 72 bp nucleotides. Constant mutation of the divergent regions of the 16S rRNA gene occurs at a rate of about 1 % every 50 years. Insertion or deletion of bases result in polymorphism of rRNA gene sequences and has been used in phylogenetic detection for more than two decades now (Moreira *et al*., 1998).

The taxonomy of Rhizobiaceae like many other bacteria has undergone significant changes with more importance put on genotypic rather than phenotypic methods for the identification of strains to their species level (Babalola, 2003). This has resulted in a change in the number of recognized species of rhizobia and the reorganization of the family. These genotypic methods rely on the conserved nature of rRNA such that isolates from the same species maintain the same sequence, whereas the more

phylogenetically diverse the species is, the greater the divergence in the sequences of their rRNA. More over, although the 16S rRNA is sufficiently conserved and contains conserved regions, it also contains variable and hypervariable sequences (Spratt, 2004).

2.7.2.2 PCR–RLFP of bacterial 16S rRNA

Bacterial strains identification has widely been carried out using PCR–RLFP of 16S rRNA, a technique which is currently regarded as quick and versatile (Wolde–Meskel *et al*., 2005). The PCR–RLFP procedures involve isolation of DNA, amplification of desired DNA sequence using primers that specifically target and amplify a region of desired genes of bacteria, digestion of the DNA with restriction endonucleases and size fractionation of the resultant DNA fragments by electrophoresis (Vinay *et al*., 2013). This method has extensively been used to assign bacterial collection to their likelihood phylogenetic groups. For instance, Manceau and Horvais (1997) used RFLP of rRNA operons to assess phylogenetic diversity among strains of *Pseudomonas syringae* pv. *Tomato* successfully established the close relationships existing between *P. syringae* and *P. viridiflava*. The PCR–RFLP of 16S rRNA gene has been used broadly in assessment of phylogenetic diversity among strains of rhizobia (Mwenda *et al*., 2011; Mnasri *et al*., 2012).

2.8 Characteristics and current classification of rhizobia

Rhizobia are non–spore forming, aerobic, heterotrophic and motile bacteria (Sheu *et al*., 2016). They are Gram negative microscopic rods and defined as nitrogen fixing bacteria (diazotroph) capable of forming root or stem nodules on legumes (Mia and Shamsuddin, 2010). The family Rhizobiaceae of order Rhizobiales in classes alpha–

and beta– protobacteria have generally been called rhizobia (from *rhiza–bios*; which live in a root). These Legume Nodulating Bacteria (LNB), to avoid confusion between the general term of *Rhizobium* and the genus name, grow as soil free–living organisms, but can also induce and colonize root nodules in legume plants resulting in symbiotic relationships that benefits both organisms (Zakhia *et al*., 2004). Ever since Beijerinck isolated the first *Rhizobium* culture which he named *Bacillus radicicola*, later changed to *Rhizobium leguminosarum* by Frank in 1889 and assigned to the genus *Rhizobium* (Young *et al*., 2001; Willems, 2006), more than 113 species belonging to 11 genera within the Proteobacteria classes had been described by 2013 using phenotypic features and molecular tools. The genera and species under α–Proteobacteria include: *Rhizobium* (43), *Bradyrhizobium* (15) *Azorhizobium* (3), *Mesorhizobium* (29) *Ensifer/Sinorhizobium* (13) *Neorhizobium*, *Devosia* (1) *Methylobacterium* (1) *Ochrobacterium* (2), *Phyllobacterium* (1) *Shinella* (1). Nine other species have been classified under β–Proteobacteria and they include *Burkholderia* (6), *Herbaspirillum* (1) and *Cupriavidus* (2) (http://edzna.ccg.unam.mx/rhizobialtaxonomy/node/4, http://www.bacterio.cict.fr/ and http://www.rhizobia.co.nz/taxonomy/rhizobia). Recently, the γ–Proteobacteria was also suggested and has been assigned the genera *Xanthomonas* and *Pseudomonas* (Berrada and Fikri–Benbrahim, 2014). Unlike other members of Rhizobiaceae, the genus *Agrobacterium* is mainly soil–inhabiting. However, some *Agrobacterium* strains possessing symbiotic plasmid as a result of horizontal or vertical transfer have ability to cause nodules on roots of legume plants (Cummings *et al*., 2009; Zhao *et al*., 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

Controlled experiments were carried out in the laboratory and glasshouses located at the KEFRI, Muguga, Nairobi, Kenya which is globally positioned at 1°15'34.29" S 36°37'36.05" E and 1267 metres above sea level.

3.2 Source of nodules

Nodules of varying sizes were randomely collected from roots of sesbania that were grown in diverse conditions of Namibia, Kenya, Uganda and Tanzania (Table 3.1). All the sites of nodule collection had no known previous sesbania inoculation using rhizobia. Nodules of *Sesbania sesban*, *S. pachycarpa*, *S. sphaerosperma*, *S. microphylla*, *S. rostrata*, *S. macowaniana* and *S. bispinosa* were collected by Dr. Herta Kolberg in Namibia, desiccated over unhydrous silica gel contained in air–tight capped plastic bottles and transported to KEFRI. Nodules of *S. sesban* grown in East Africa were collected by the author of this thesis. The nodules were desiccated over silica gel contained in air–tight capped plastic bottles before they were transported to KEFRI.

Table 3.1: Origin of sesbania nodules used in this study

Country	Origin	Latitude / Longitude	Sesbania	Isolates
Kenya	Bumala (Busia)	00° 18' 9.1" N 34° 12' 23.1" E	*S. sesban*	MASS120–128; 156–178.
	Kuinet (Eldoret)	00° 36' 7.8" N 35° 18' 28.0" E	"	MASS169–175.
	Kavutiri (Embu)	00° 25' 0.04" S 37° 30' 06.2" E	"	MASS129–155.
	Gituamba (Murang'a)	00° 52' 45.7" S 36° 54' 21.5" E	"	MASS110–117.
Tanzania	SUA (Morogoro)	06° 56' 8.3" S 37° 06' 3.6" E	"	MASS41–46.
	Lushoto	04° 07' 09" S 38° 26' 56.6" E	"	MASS29–40.
Uganda	Tororo	00° 63' 6.8" S 37° 19' 9.36" E	"	MASS47–53.
	Mbale	01° 06' 6.6" S 34° 17' 7.9" E	"	MASS54–61.
	Kabale	01° 25' 12" S 29° 53' 6.2" E	"	MASS62–69.
Namibia	Okahandja	21° 39' 35.5" S 16° 52' 22" E	*S. macowaniana*	MN1, MN15.
	Khorixas–Outijo	20° 30' 43" S 14° 57' 24.7"E	"	MN5, MN24, MN25, MN26, MN27, MN39.
	Swakop	22° 38' 23" S 14° 44' 35" E	*S. pachycarpa*	MN9, MN44, MN49, MN51.
	Rio Tinto Gorge	22° 27' 12.4" S 15° 07' 24.8" E	"	MN16, MN36, MN43, MN45, MN46, MN47, MN48, MN50, MN4, MN53, MN54, MN55, MN56, MN58, MN59, MN60, MN61, MN62.
	Epupa falls	16° 59' 39" S 13° 17' 28" E	*S. sesban*	MN7, MN8, MN13, MN18, MN19, MN21, MN22, MN23, MN30, MN35, MN38, MN57.
	Otjinungua	17° 49' 54" S 12° 31' 20" E	"	MN67, MN68.
	Suclabo	18° 07' 32.4" S 21° 35' 51" E	*S. cinerascens*	MN20, MN28, MN31, MN32, MN37, MN40.
	Omuramba	18° 05' 42" S 20° 23' 36" E	*S. bispinosa*	MN11, MN17, MN33, MN34.
	Bunya	17° 51' 29" S 19° 21' 49" E	*S. microphylla*	MN69, MN70.
	Rooidrom	17° 49' 53" S 12° 31' 22" E	*S. sphaerosperma*	MN2, MN10.
	Sesfontein	19° 02' 37" S 13° 45' 09" E	"	MN12, MN41, MN71.
	Korokoko	17° 59' 14" S 20° 57' 06" E	*S. rostrata*	MN50.

3.3 Media for culturing of rhizobia

Yeast Extract Mannitol broth (YEMB) or Yeast Extract Mannitol agar (YEMA) was used as an artificial media for culturing of the rhizobia recovered from root nodules. The recipe for YEMB included 10.0 g mannitol; 0.50 g di–Potassium orthophosphate (K_2HPO_4); 0.20 g magnesium sulphate ($MgSO_4.7H_2O$); 0.10 g sodium chloride (NaCl); 0.50 g yeast extract and distilled water (1.0 litre) while YEMA contained YEMB and agar (16.0 g) as described in Somasegaran and Hoben (1994). The pH of YEMB was adjusted to 6.8 and sterilized or before addition of agar when YEMA was desired. The media was sterilized at a temperature of 121 °C and a pressure of approximately 15 pounds per square inch for 15 minutes using an autoclave. YEMA containing 0.00125 mg kg^{-1} Congo red (Diphenyldiazo–α–naphthylaminesulfonate) dye (YEMA–CR) was used as differential media while acid reaction of isolates was determined on YEMA containing 0.00125 mg kg^{-1} bromothymol blue (BTB) as indicator.

3.4 Isolation, purification and preservation of root nodule bacterial isolates

The dry nodules preserved over silica gel were immersed in tap water contained in a petri dish for two hours to imbibe. Thereafter, isolation procedure was carried out according to Somasegaran and Hoben (1994) with minor modifications at the nodule sterilization step where acid was omitted and nodules were immersed for 60 seconds in 3.5 % sodium hypochlorite instead. Each of the nodules in which the rhizobia was isolated was then immersed in 95 % (v/v) ethanol for 10 seconds to break the surface tension and was followed by an immersion in 2 % (v/v) sodium hypochlorite (NaOCl) for two minutes to decontaminate the surface. The nodules were successively rinsed in five changes of sterile distilled water before being transferred

into a drop (approximately 100 µL) of sterile distilled water contained in a petri dish using a pair of sterile forceps. The nodules were squashed using a sterile blunt glass rod to release bacteroids and or bacteria. A sterile tungsten inoculating loop was used to lift a loopful of the nodule suspension and was streaked on YEMA media. The streaking was performed in a dilution manner to yield isolated colonies on the final streak–line. Inoculated plates were incubated in an inverted position in the dark at a temperature of 28±1 °C and monitored daily to observe colony emergence. Whenever more than one type of colony appeared growing on media from a single nodule, they were re–streaked on fresh YEMA media and considered as separate rhizobia isolates. A culture from each nodule was assigned MASS or MN and a numeral for East African or Namibian nodules respectively. However, in the case of dual or multiple nodule occupants these labels were followed by a consecutive lower case letters respectively (for example, MASS133a and MASS133b). Cultures from single nodules but stored as mixed forms were asigned a similar labels followed by respective lower case letters (for example, MASS133ab). Pure isolates were preserved in 16 % (v/v) glycerol–YEMB contained in well labeled autoclavable plastic vials at a temperature of -70 °C.

3.5 Phenotypic characteristics of sesbania rhizobia

During culturing, rhizobia isolates were characterized by their morphological traits as cell (Gram stain), colony (absorption of Congo red, form, elevation, margin, appearance, optical property, pigmentation, texture), mucous (extracellular polysaccharides) production and pH reaction on YEMA as described by Mpepereki *et al*. (1997) and Odee *et al*. (1997). All these traits were recorded and used to group rhizobia into their respective morphotypes.

3.5.1 Determination of rhizobial growth rate and colony characteristics

Yeast extract Mannitol Agar containing 25 mg L^{-1} (w/v) Congo red dye (YEMA–CR) was used as differential media for identification of typical rhizobia from other soil inhabiting bacteria (Somasegaran and Hoben, 1994). Morphological characteristics of the colonies were recorded as diameter, inability to absorb Congo red dye, form, elevation, margin, appearance, optical properties, pigmentation, texture and mucous (extracellular polysaccharides) production on YEMA.

3.5.2 YEMA–BTB medium colour change by sesbania rhizobial isolates

Rhizobial isolates were inoculated on YEMA media supplemented with 0.00125 mg kg^{-1} bromothymol blue (BTB) as a pH reaction indicator. Three- to seven-day old cultures were observed for their ability to change colour of YEMA–BTB medium at pH 6.8 (green) to yellow, blue or retain the green colour (Somasegaran and Hoben, 1994). The observed colour of YEMA–BTB media after three -seven days was recorded.

3.5.3 Gram staining

Gram staining was performed according to the method by Somasegaran and Hoben (1994). A loopful of actively growing rhizobia in liquid culture was transferred to the surface of a clean glass slide and spread over a small area. The culture film was air dried then fixed by passing the slide five times over a bunsen burner flame without exposing the dried film directly to the flame. The slide was flooded with crystal violet solution for one minute then washed off for 5 seconds using gently running tap water. The water was drained by by holding the slide in a vertical position before flooding the slide with Gram's iodine solution and allowed to act (as a mordant) for

one minute. The Gram's iodine solution was washed off using tap water. Excess water was drained from the slide by by holding the slide in a vertical position and blotted dry using a blotting paper. The slide was flooded with 95 % (v/v) alcohol for 10 seconds and washed off using tap water. The water was drained by holding the slide in a vertical position before the slide was flooded with safranin solution and allowed to counter stain for 30 seconds. The slide was washed in running tap water, drained and allowed to air–dry before bacterial examination using a total magnification of x600 on Olympus microscope (model: 1X2–ILL100 T5SN). The documentation system interphased to the microscope was then used to photograph the images.

3.6 Determination of genotype composition of sesbania rhizobia using PCR–RFLP

In this study, the number of sesbania isolates was reduced from 129 to 79 through selection of representative isolate(s). The selection was aided by dendrogram clusters obtained using UPGMA method based on combined similarity matrix of data from the diverse growth characteristics of rhizobia on YEMA media, intrinsic antibiotic resistance (IAR) and salt tolerance (NaCl). The 79 sesbania isolates were later subjected to fingerprinting assays using PCR–RFLP of the 16S rDNA in comparison with 17 reference strains.

3.6.1 Intrinsic antibiotic resistance assay

Resistance to antibiotics was evaluated by inoculating each rhizobial isolate on YEMA media supplemented with antibiotics. The isolates were tested for their

ability to resist two concentration levels [50 mg L^{-1} and 100 mg L^{-1} (w/v)] of each of the 12 different antibiotics.

3.6.1.1 Preparation of YEMA–antibiotics media

Yeast extract mannitol agar media supplemented with 12 antibiotics were prepared as described by Odee *et al*. (1997). Stock solutions of the following antibiotics were prepared separately: spectinomycin dihydrochloride ($C_{14}H_{24}N_2O_7$.2HCl), tetracycline ($C_{22}H_{24}N_2O_8$), penicillin–G sodium salt ($C_{16}H_{17}N_2O_4SNa$), novobiocin sodium salt ($C_{31}H_{35}N_2O_{11}Na$), kasugamycin hydrochloride ($C_{14}H_{25}N_3O_9$.HCl), neomycin sulphate ($C_{23}H_{46}N_6O_{13}.3H_2SO_4$), streptomycin sulphate $(C_{21}H_{39}N_7O_{12})_2.3H_2SO_4$), erythromycin ($C_{37}H_{67}NO_{13}$), rifampicin ($C_{43}H_{58}N_4O_{12}$), kanamycin mono sulphate ($C_{18}H_{36}N_4O_{11}.H_2SO_4$), ampicillin ($C_{16}H_{19}N_3O_4S$) and carbanicillin. All antibiotic stock solutions were prepared by dissolving 125 g of each antibiotic in 25 mL distilled water except erythromycin which was dissolved in 25 mL of 99 % ethanol due to its low solubility in water as compared to ethanol according to the method of Manna *et al.* (2004). Each of the stock solutions was then filter–sterilized using 0.45 µM millipore (Whatman cellulose nitrate membrane filter). Meanwhile, YEMA was prepared in aliquots of 500 mL, sterilized and allowed to cool to approximately 50 °C before addition of the sterile antibiotics to make either 50 mg L^{-1} or 100 mg L^{-1} of each of the antibiotics. The media was swirled clockwise and anticlockwise to uniformly disperse the antibiotics then poured in sterile plastic petri dishes under aseptic conditions and allowed to set.

3.6.1.2 Rhizobial culturing for intrinsic antibiotics resistance assay

A loopful of rhizobial culture growing on YEMA media was inoculated in a 10 mL YEMB and vigorously shaken to disperse the cells introduced in the media. The inoculated broth was placed on a horizontal orbital incubator shaker (Model: Tour–120–2) and aerated at 100 rpm, at a temperature of 28±1 °C for three and seven days for fast and slow growing types respectively. Zero point two mililitres (0.2 mL) of the broth culture was transferred to 10 mL of YEMB and allowed to grow for three and seven days for fast and slow growing rhizobia respectively.

3.6.1.3 Inoculation of rhizobial cultures on YEMA–antibiotics media

Rhizobia grown to turbid were aseptically transferred onto sterile wells. The well containing 0.5 mL of broth and a plate with YEMA containing antibiotics were put in the respective positions on a Denly multipoint inoculator. Multipoint inoculator pins were then lowered to pick the cultures and transfer to YEMA media supplemented with twelve antibiotics at two concentration levels of 50 mg L^{-1} and 100 mg L^{-1}. Three replicates per level of each antibiotics were allowed for each tested isolate. Inoculated media was incubated in the dark at a temperature of 28 ±1 °C. After 4 days of incubation growth of rhizobia was recorded in an excel spreadsheet as growth present (1) or no growth (0). During scoring, growth of rhizobial cultures on YEMA–antibiotics media was compared to those grown on control media (YEMA with no antibiotics).

3.6.2 Screening of rhizobial isolates for salt tolerance levels

Rhizobial isolates were grown in YEMB media to turbid and inoculated as described in sections 3.6.1.2 and 3.6.1.3 on YEMA media containing 0.1 %, 1 %, 3 %, 5 % 6 %, 7 %, 8 % and 10 % NaCl (w/v) using a multi–point inoculator then incubated at 28 ±1 °C. Four days–old rhizobia inoculated plates were recorded as growth present (1) or no growth (0) in an excel spreadsheet. During scoring, growth of rhizobial cultures on YEMA + salt levels was compared to those grown on control media (YEMA + 0.1 % NaCl).

3.6.3 Selection of sesbania rhizobia for PCR–RFLP assays

Respective intrinsic antibiotic resistance and salt tolerance data for isolates from each site in Excel format were merged and exported to statistical software PAST (Hammer *et al*., 2005) for clustering of the isolates into their similarity matrix using the Unweighted Pair Group Method with Average (UPGMA). Dendrograms were constructed for isolates per site and used to select for rhizobia samples used in PCR–RFLP fingerprinting practical. The clusters were then used to select characteristically unique rhizobial isolates per site. A maximum of three isolates were picked from each cluster of closely related isolates per site. Isolates from Namibia were merged to generate a single dendrogram. In cases of nodule co–occupancy, all the isolates were picked for PCR–RFLP assays even if they exhibited 100 % similarity in IAR and salt tolerance. Reference strains (Table 3.2) obtained from various culture collection centres were also included in the study.

Table 3.2: Reference strains used in PCR–RFLP assays, their host plants and sources

Strain	Host plant	Source
KFR8 (*Bradyrhizobium* sp.)	*Acacia nubica*	KEFRI
KFR84 (*Mesorhizobium* sp. type II)	*A. tortilis*	KEFRI
KFR459 (*Agrobacterium* sp. type I)	*A. polyacantha*	KEFRI
USDA9030 (*Rhizobium leguminosarum*)	*Phaseolus vulgaris*	USDA
BA37 (*R. leguminosarum*)	*P. vulgaris*	MIRCEN
DWO253 (*R. leguminosarum*)	*P. vulgaris*	KEFRI
DWO461 (*R. tropici* type IIB)	*Sesbania sesban*	KEFRI
KFR647 (*M. huakuii*)	*S. sesban*	KEFRI
KFR402 (*Mesorhizobium* sp. type I)	*S. sesban*	KEFRI
Azorhizobium sp.	*S. rostrata*	Senegal
USDA1002 (*Ensifer meliloti*)	*Medicago sativa*	USDA
ORS177 (*Bradyrhizobium* sp.)	*Faidherbia albida*	Senegal
KFR552 (*B. elkanii*)	*F. albida*	KEFRI
USDA76 (*B. elkanii*)	*Glycine max*	USDA
USDA110 (*B. japonicum*)	*G. max*	USDA
DWO100 (*Rhizobium* sp.)	*Prosopis juliflora*	KEFRI
USDA2370 (*R. leguminosarum* bv. Viciae)	*Pisum sativum*	USDA

KFR, Kenya Forestry Research – Rhizobial Culture Collection; MIRCEN, Microbiological Resources Centres; USDA, United states Department of Agriculture, National Rhizobium Culture Collection, Beltsville Agricultural Research Center, USDA, Beltsville, USA.

3.6.4 Rhizobial DNA extraction

Bacterial DNA was extracted using the method by Terefework *et al*. (2001) with minor alteration. The fast and slow growing rhizobial isolates were grown in 10 mL YEMB contained in McCartney bottles for three and seven days respectively. One hundred microlitres (100 µL) rhizobial cultures grown in YEMB to 10^9 cells mL^{-1} were aseptically seeded in fresh 10 mL YEMB. Two millilitres of 48 hours–old broth culture was aseptically transferred into an eppendorf tube and centrifuged at 10 000 x g (25 °C) for 10 minutes to pellet the rhizobial cells. The supernatant containing YEMB was carefully discarded by decanting leaving behind a pellet of bacteria at the

bottom of eppendorf tube. This process was repeated two more times for the slow growing rhizobia. The pellet was washed two times using sterile distilled water by centrifuging at 10 000 x g (25 °C) for 5 minutes. Cell pellets were re–suspended in 100 µL of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and vortexed for 5 seconds to mix. The suspension was subjected to boiling at 96 °C in a water bath for 10 minutes to lyse the bacteria and cooled for 30 minutes at temperature of 25 °C. Four hundred millilitres (400 mL) of 65 °C–preheated CTAB (Hexadecyltrimethylammonium bromide) extraction buffer (Table 3.3) was added to the lysed bacteria, mixed gently and incubated in a water bath at 65 °C for 20 minutes before cooling for 30 minutes at 25 °C. Four hundred microlitres (400 µL) of chloroform: isoamyl alcohol (24:1, v/v) was added and mixed gently then incubated at a temperature of -20 °C for 20 minutes. The samples were then centrifuged at 14 000 x g at a temperature of 4 °C for 10 minutes and the supernatant was transferred into a fresh eppendorf tube.

Table 3.3: Recipe for Hexadecyltrimethylammonium bromide extraction buffer

Reagent	**mL**
CTAB	1.28
PVP	2.56
5 M NaCl	3.60
0.5 M EDTA	0.51
1 M Tris–HCl, pH 7.6	1.28
Mercaptoethanol	0.064
H_2O (distilled sterile)	3.6
Total	12.894

CTAB, Hexadecyltrimethylammonium bromide; PVP, polyvinylpyrrolidone.

Four hundred microlitres of ice–cold 99.5 % ethanol was added and mixed well by inverting the tube 10 times. The DNA was precipitated by centrifugation at 14 000 x g at a temperature of 10 °C for 30 minutes. The supernatant was carefully discarded by aspirating the ethanol. The DNA pellet was washed in 400 µL of 70 % ice–cold ethanol by inverting the tube gently and then centrifuged at 14 000 x g (10 °C) for 10 minutes. The supernatant was carefully discarded by aspirating and the eppendorf tube was inverted over a styrofoam and placed on the bench at room temperature to dry the DNA pellet for 14 hours. The DNA pellet contained in the eppendorf tube was re–suspended in 100 µL of DNase/RNase free water and mixed by flicking the tube with a finger until DNA dissolved. The quality and quantity of the extracted DNA was measured using a spectrophotometer (Model: Biospec–nano for life science, Shimadzu Biotech–Japan) before each was diluted to 10 ng μL^{-1} using DNase and RNase free water. The bacterial DNA was stored under -20 °C before being used for 16S rRNA gene amplification.

3.6.5 PCR mastermix preparation

Bacterial DNA extracted using the method of Terefework *et al*. (2001) was used as a template to amplify the nearly full–length 16S rRNA gene using fD1 (5'–AGAGTTTGATCCTGGCTCAG–3') and rD1 5'–AAGGAGGTGATCCAGCC–3') primers (Weisburg *et al*., 1991). A 24 µL reaction PCR mastermix enough for 100 samples was prepared each to include 12.88 µL DNase and RNase free water; 2.5 µL of 10x PCR buffer; 2.92 µL of 2.5 mM $MgCl_2$; 1.0 µL of 10 mM dNTPs; 1.2 µL of 10 µM each of the universal oligonucleotide primers fD1 and rD1 which corresponds to *Escherichia coli* 16S rRNA gene position 8–27 and 1524–1540 respectively which amplified near

full–length rhizobia 16S rDNA; 2.0 µL Q–solution; 0.3 µL of 5 units μL^{-1} Taq polymerase and mixed using a vortex for 5 seconds. Twenty four microlitres (24 µL) of mastermix aliquots were transferred to DNase– and RNase–free thin–walled PCR 96 plate before 1 µL of 10 ng μL^{-1} rhizobial DNA was added using a Veriti® 96–well thermal cycler (Applied biosystems).

3.6.6 16S rRNA–PCR amplification conditions

PCR amplification of the 16S rRNA gene region of rhizobia from 79 sesbania, *P. vulgaris* and 16 reference strains was carried out as per the protocol described by Tan *et al*. (1997) using the following reaction conditions: initial denaturation at 94 °C for 5 minutes, 30 cycles (denaturation at 94 °C for 30 seconds, primer annealing at 53 °C for 40 seconds, extension at 72 °C for 90 seconds) and a final extension at 72 °C for 7 minutes.

3.6.7 Gel electrophoresis of rhizobial 16S rRNA PCR products

The size of the PCR amplicons were determined by staining the PCR products using SYBR® green dye then separated on 0.8 % agarose gel for 45 minutes at 90 volts using 0.5 x TBE buffer (0.484 g L^{-1} Tris, 0.037 g L^{-1} EDTA, pH 8). The PCR products were then viewed and documented using a gel documentation bioimaging system (Model: Gel Doc–it 300, UVP Bioimaging Systems, Upland, CA). The size of 16S rRNA band of each sample was recorded.

3.6.8 Restriction of 16S rRNA gene PCR amplicons

Restriction of the16S rRNA gene PCR amplicons was carried out in a mastermix that comprised of 2.4 µL DNase–free and RNase–free PCR water, 1.0 µL Cutsmart

buffer, 0.1 µL (100 ng 50 μL^{-1}) Bovine serum albumin (BSA) and 0.5 µL of each of the endonucleases *Hae*III (5′–GG/CC–3′), *Hinf*I (5′–G/ANTC–3 and *Msp*I (5′–C/CGG–3′) separately, as per the manufacturer′s instructions (Invitrogen™ Life Technologies). Six microlitres of the 16S rRNA gene PCR products were added to each of the 4 µL mastermix aliqouts in DNase– and RNase–free thin–walled un–skirted low profile 96 plates and incubated at 37 °C for 2 hours.

3.6.9 Gel electrophoresis of restriction fragments of rhizobial 16S rRNA

Separation of restriction fragments by electrophoresis was performed as described by Laguerre *et al*. (1994). Restriction fragments were stained using SYBR® green dye and separated by horizontal electrophoresis in a 2.5 % agarose gel for 2.5 hours at 90 V using 0.5 x TBE buffer. Restriction fragment profiles were then viewed and documented using a gel documentation bioimaging system (Model: Gel Doc–it 300, UVP Bioimaging Systems, Upland, CA). The restriction fragment patterns were used to generate binary data (1) or (0) and recorded in an Excel spreadsheet which was later separated per the four countries of origin and used to draw likelyhood dendrograms.

3.7 Isolates effectiveness test with *Sesbania sesban* and common bean plants using Leonard jar assembly

3.7.1 Plant materials

Quality *S. sesban* seeds were obtained from a raised seed stand in Malava forest, Kakamega, Kenya. A healthy seeded *S. sesban* tree was selected for seed harvesting. Seeds from a single mother tree were preferred to minimize genetic variations as opposed to the general collections. *Sesbania sesban* was chosen for this work since it

was not possible to acquire seeds for other local and exotic *Sesbania* species. Common bean seeds (variety Rose coco LOT Number 14–13–7561; REF Number 13/14/03/2116) were obtained from Simlaw Seeds Company Limited (Kenya).

3.7.2 Experimental design

The experiment was made in a randomized complete block design with four replicates each containing two plants. There were 115 treatments that consisted of one hundred and seven sesbania rhizobial isolates, six reference strain (DWO253 *Phaseolus vulgaris*, KFR269 Siratro, KFR209 *Faidherbia albida*, BA37 *P. vulgaris*, KFR647 *S. sesban* and KFR402 *S. sesban*), uninoculated plants supplemented with 0 ppm N and 70 ppm N (controls).

3.7.3 Seed pretreatment and pre–germination

Healthy *S. sesban* and common bean seeds with uniform seed size were selected before they were pre–treated. Common bean–seeds were cleaned in several changes of tap water to remove seed preservatives. The seeds were surface sterilized using 3 % sodium hypochlorite for ten minutes, rinsed in five changes of sterile distilled water, soaked in sterile distilled water for 12 hours to imbibe and break the dormancy before they were aseptically pre–germinated on 0.5 % (w/v) water agar contained in petri dishes for three days at a temperature of 28 °C.

3.7.4 Preparation of Leonard jar assembly

The assembling of the high density (autoclavable) Leonard jars was accomplished as described by Somasegaran and Hoben (1994). Leonard jar assembly was made up of a reservoir (bottom) containing plant nutrient solution and a substrate holder (top).

Medium size vermiculite, an inert substrate, was soaked for 48 hours and cleaned in several changes of tap water before its pH was adjusted to 6.8 using either 1 N sodium hydroxide or 1 N hydrochloric acid. The top part of the assembly was parked with vermiculite then placed over the reservoir which contained 500 mL nitrogen–free plant nutrient solution. The entire assembly was inserted in a *khaki* paper bag No. 4 and covered using an aluminum foil. Assembled jars were sterilized using an autoclave at a temperature of 121 °C and pressure of 1.2 bars for 60 minutes. Sterile jars were allowed to cool overnight.

3.7.5 Preparation of nitrogen–free plant nutrient solution

Five millilitres of N–free plant nutrient stock solutions (Broughton and Wilworth, 1971) containing 294.1 g L^{-1} $CaCl_2.2H_2O$; 136.1 g L^{-1} KH_2PO_4; 6.7 g L^{-1} Fe–citrate; 123.3 g L^{-1} $MgSO_4.7H2O$; 87.0 g L^{-1} K_2SO_4; 0.338 g L^{-1} $MnSO_4.H_2O$; 0.247 g L^{-1} H_3BO_3; 0.288 g L^{-1} $ZnSO_4.7H_2O$; 0.100 g L^{-1} $CuSO_4.5H_2O$; 0.056 g L^{-1} $CoSO_4.7H_2O$ and 0.048 g L^{-1} $Na_2MoO_2.2H_2O$ was each added to 5 litres of distilled water, stirred using a glass rod to mix then diluted to ten litres. The pH of the nutrient solution was adjusted to 6.8 using 1 N NaOH or 1 N HCl before it was added to Leonard jar reservoir.

3.7.6 Transfer of pre–germinated seeds to vermiculite in Leonard jars

The aluminum foil on the sterile Leonard jar was removed before three holes deep enough for inserting 2 cm–long radicles were made into the substrate of each Leonard jar using a sterile pair of forceps. Three pre–germinated seeds were then inserted gently into holes made in each jar and covered using vermiculite of the same

Leonard jar. The aluminum foils were placed back to cover the jars until emergence of the seedlings.

3.7.7 Inoculant preparation and inoculation of the host plant

One hundred and seven representative sesbania rhizobial isolates of various morphological groups and origin of sesbania isolates and reference strains (DWO253, *Phaseolus vulgaris*, KFR269 Siratro, KFR209 *Faidherbia albida*, BA37 *P. vulgaris*, KFR647 *S. sesban* and KFR402 *S. sesban*, used for inoculants preparation for their respectively hosts at KEFRI) were grown in YEMB for 3–7 days on a rotary shaker with a rotation of 100 rpm depending on their rates of growth. Slow growing rhizobia were inoculated in broth three days before the fast growers. Turbid cultures at late exponential phase, with approximately 10^9 rhizobial cells mL^{-1} broth were used to inoculate the pre–germinated *S. sesban* and common bean seedlings. One millilitre of the rhizobial culture was introduced at the root collar of each one of the three–day old seedling growing in Leonard jar using a pipetman with a sterile pipette tip. Four replicates per treatment of sesbania rhizobial cultures and a similar set of uninoculated Minus N treatments (controls) replenished with N–free plant nutrient and uninoculated Plus N treatments (controls) replenished with 70 ppm N applied as KNO_3 solution were allowed. Vermiculite in all jars was covered with a layer of sterile ballast as a barrier against external contamination. Ten–day old seedlings were thinned to two per jar using a sterile scalpel blade. The four replicates of each treatment were arranged into a completely randomized design on a clean bench in a glasshouse with controlled conditions (70 % shaded, 80 % humidity and at mean temperatures of 30/18 °C day/night). Plants were replenished

with 500 mL of respective sterilized nutrient solution every two weeks after pouring off the previous contents of the Leonard jar reservour until harvest.

3.7.8 Harvesting of *Sesbania sesban* and common beans

Sesbania sesban and common beans were destructively harvested after 8 and 4 weeks of growth respectively. The substrate holding plant roots were pulled out of the Leonard jar then immersed in tap water contained in a spacious trough to wash off the vermiculite. Plants with washed roots were transferred to the laboratory where nodules were detached from the roots and roots separated from the shoots. Nodules were counted and recorded. Shoots, roots and nodules were then dried separately at a temperature of 70 °C for 72 hours. Shoot dry weight, nodule dry weight per plant and specific nodule dry weight were determined and recorded separately.

3.7.9 Determination of shoot total nitrogen

Shoots of all nodulated common beans and selected *S. sesban* treatments were ground to fine powder using a hammer grinder (Model; Retsch Gmbh MM400, Germany) and their nitrogen content determined using Kjeldhal method as described by Anderson and Ingram (1993) and Okalebo *et al*. (2002). A 0.3 g plant sample was added to a digestion tube containing 4.4 mL of dissolved 0.42 g of selenium (Se) powder and 14 g of lithium sulphate in 350 mL of 30 % hydrogen peroxide and 420 mL. of concentrated sulphuric acid and digested in a block digester (Skalar Block Digester System, Model SA 5640). The digestion tubes were heated at a temperature of 360 ºC for 2 hours. Twenty five millitre (25 mL) of water was added to the cooled sample and topped to the 50 mL mark using distilled water. The sample was allowed to settle and a 10 mL clear solution was transferred to the distillation tubes, 10 mL of

40 % NaOH added to the sample then distilled. The extract was steamed immediately into 5 mL of 1 % boric acid and 4 drops of mixed indicator. Distillation was continued for 2 more minutes after the indicator turned green then followed by titration using 0.1 M HCl until the colour changed from green through grey to a definite pink. Nitrogen content in shoot sample was calculated using the following formula by Okalebo *et al.* (2002):

N (%) = Titre x HCl Molarity x Extract Volume x 0.014 / aliquot volume x plant weight (where 0.014 is milliequivalent weight of nitrogen).

3.7.10 Determination of symbiotic efficiencies (%)

Symbiotic efficiencies percent (SE %) of the sesbania rhizobial isolates on *S. sesban* and common beans was determined as described by Yaman and Cinsoy (1996). Nitrogen content was calculated as: (shoot dry weight x N concentration) per plant and the N fixed (plant N content in inoculated treatments - plant N content in un–inoculated treatments). Symbiotic effectiveness was estimated by comparing each inoculated plant with the plus 70 ppm N treatments (i.e. plant N content in inoculated plant/plant N content in 70 ppm N plant) x 100 (Beck *et al*., 1993). The sesbania isolates forming symbiosis with *S. sesban* were then arbitrarily rated as: very efficient (VE) when the shoot dry weight (SDWt.) of the nodulated host in the Leonard jar was higher than the total mean of all inoculation treatments added to the standard deviation (0.867 + 0.179); efficient (E) when its yield was between that of the mean + standard deviation (between 0.867 + 0.179) and mean - standard deviation (0.867 - 0.179) and inefficient (I) when its yield was smaller than the mean - standard deviation (0.867 - 0.179), as described by Lalande *et al*. (1990). This

rating was repeated using the total mean of shoot dry weight of nodulated common beans and the standard deviation (0.789 + 0.109 and 0.789 - 0.109).

3.8 Data analysis

The combined binary data from antibiotic resistance and salt tolerance recorded in an Excel spreadsheet was exported to statistical computer software (Hammer *et al*., 2005) for dendrogram construction using the UPGMA. The binary data obtained from PCR–RFLP fragment profiles and recorded in an Excel spreadsheet was exported to the statistical programs GenAlex 6.2 (Peakall and Smouse, 2006; 2012) and statistical program MEGA version 4 (Tamura *et al*., 2007) for phylogenetic diversity analysis and dendrogram construction using the UPGMA. Isolates that had identical RFLP patterns were designated as one ribotype. Data for shoot dry weight, root dry weight, nodule number and nodule dry weight per plant were assessed for normality of distribution before they were subjected to a one-way ANOVA using GenStat computer software 16^{th} Edition (VSN International, 2012). Pearson correlation coefficient was used to determine the relationship between plant growth parameters. Tukey's HSD pairwise comparison at $p \leq 0.05$ was used to separate the means.

CHAPTER FOUR

RESULTS

4.1 Morphological characterization

One hundred and twenty eight nodule forming bacteria (rhizobia) were isolated from root nodules of sesbania that were grown in diverse conditions in Kenya, Uganda, Tanzania and Namibia (Appendices I–IV). The isolates were either flat, raised or dome shaped with entire margins. They were grouped into nine unique morphotypes based on their growth characteristics on YEMA–CR (Table 4.1). Morphotypes I–V were fast growers (2–3 days), morphotypes VI–VII (4–6 days) were moderate growers while morphotypes VIII and IX exhibited slow growth (7–9 days) on growth media incubated at 28±1 °C. Morphotypes II, V and VI produced copious exopolysaccharides (EPS), morphotypes I, III, IV and VII secreted moderate EPS with gummy properties while morphotypes VIII and IX were non–EPS producers (Table 4.1).

Table 4.1: Characteristics of sesbania rhizobial isolates on YEMA–CR media

Characteristics	**Morphotype**
3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	I
5 mm diameter, milky, translucent, shiny, dome and copious friable exopolysaccharides	II
4 mm diameter, red, opaque, shiny, raised and moderate gummy exopolysaccharides	III
2 mm diameter, brown centre, clear margin, raised, shiny, moderate gummy purple exopolysaccharides	IV
5 mm diameter, pink suspensions, opaque, raised, dull and copious watery exoplysaccharides	V
4 mm diameter, transparent, shiny, dome and copious viscous exopolysaccharides	VI
2 mm diameter, milky opaque, raised, shinny and gummy moderate exopolysaccharides	VII
1 mm diameter, milky opaque, dome, shiny and no exopolysaccharides	VIII
< 1.0 mm diameter, pink, translucent, flat, dull, dry and no exopolysaccharides	IX

Colony size of the rhizobia ranged from < 1.0 mm to 5.0 mm in diameter. Morphotype VI colonies were transparent. Morphotypes I, II and IX were translucent, morphotypes III, V, VII and VIII were opaque while morphotype IV had a brown centre with clear margins. With the exception of rhizobia clustered in morphotype III, all others were nonchromogenic during growth on YEMA media. All the isolates were shiny except those in morphotypes V and IX which were dull (Plate 4.1).

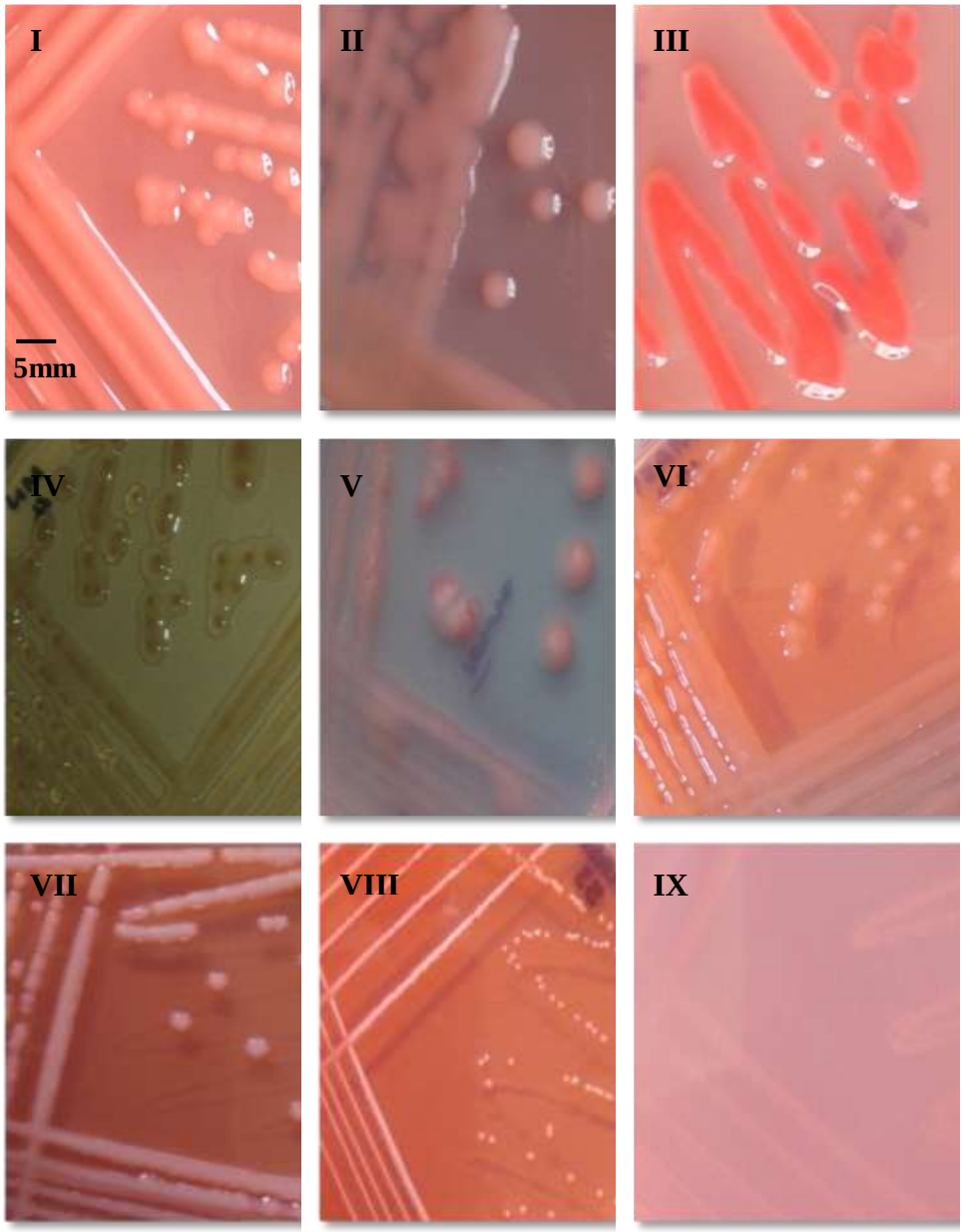

Plate 4.1: Growth of sesbania rhizobial on YEMA–CR media showing colony morphotypes I – IX.

During culturing of the sesbania rhizobia on YEMA media, some cultures developed hollow centres with uneven margins irrespective of the number of sub–culturing onto fresh media (Plate 4.2).

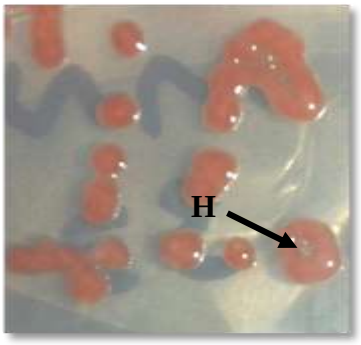

Plate 4.2: Rhizobial colonies on YEMA media showing H, hollow centre.

Sesbania rhizobia assigned to the nine morphotypes had the following percent composition: morphotype I (23.7 %), II (5.3 %), III (5.3 %), IV (2.6 %), V (18.4 %), VI (14.5 %) VII (14.5 %), VIII (6.6 %) and IX (9.2 %) (Table 4.2).

Table 4.2: Percent composition of sesbania rhizobial isolates in each of the nine morphotypes

Morpho–type	Phenotype groups of isolates	Percent isolates
I	MASS37a, MASS51a, MASS59, MASS137a, MASS140, MASS177a, MASS170, MN2, MN10, MN18, MN26, MN27, MN35, MN39, MN50, MN51, MN56, MN62.	23.7
II	MASS171, MASS57, MASS138, MN4.	5.3
III	MASS31a, MASS130, MASS147, MN22.	5.3
IV	MASS40a, MN44.	2.6
V	MASS31b, MASS40b, MASS62, MASS114, MASS117a, MASS126, MASS127, MASS159, MASS174, MASS175, MN19, MN31, MN34, MN38.	18.4
VI	MASS117b, MASS141, MASS172, MASS177b, MASS37b, MASS41, MASS49, MASS53, MN9, MN36, MN71.	14.5
VII	MASS42, MASS43, MASS51b, MASS60, MASS65, MASS112, MASS132, MASS133, MASS137b, MASS176, MN12.	14.5
VIII	MASS30, MASS33, MASS50, MASS117c, MN13.	6.6
IX	MASS29, MASS36, MASS136, MASS160, MN11, MN17, MN26.	9.2

Sesbania rhizobial isolates were grouped into nine distinct morphotypes (I–IX) as per the Neighbour joining (NJ) dendrogram constructed based on colony morphological traits using UPGM A (Figure 4. 1).

Figure 4.1: Neighbour joining dendrogram constructed based on colony morphological traits using UPGMA showing relatedness of sesbania rhizobial isolates.

Rhizobial isolates recovered from Kenyan sites were distributed in all morphotypes except IV (Table 4.3a). Morphotype VIII contained a single isolate (MASS117c). Morphotype VI was universal in the four Kenyan sites. No rhizobia isolated from Kavutiri were grouped into morphotypes IV, V and VIII. The three Kuinet rhizobial isolates were distributed in morphotypes I, II and VI. Two isolates from Bumala site were grouped into morphotype V (MASS177b) and morphotypes VI (MASS176) while three isolates were grouped into morphotype IX. No isolates from Bumala conformed to characteristics of morphotypes II, III, IV and VIII. Gituamba isolates were grouped into morphotypes V, VI, VII and VIII with two isolates in morphotype V and one each in morphotypes VI, VII and VIII. No rhizobial isolates from Gituamba were grouped into morphotypes I, II, III, IV and IX (Table 4.3a).

Table 4.3a: Distribution of *S. sesban* rhizobial isolates from four Kenyan sites in nine morphotypes

Morphotype group	Kenya			
	Kavutiri	**Kuinet**	**Bumala**	**Gituamba**
I	MASS137a MASS140	MASS170	MASS177a	–
II	MASS138	MASS171	–	–
III	MASS130 MASS147	–	–	–
IV	–	–	–	–
V	–	–	MASS126 MASS127 MASS159 MASS174 MASS175	MASS114 MASS117a
VI	MASS141	MASS172	MASS177b	MASS117b
VII	MASS132 MASS133 MASS137b	–	MASS176	MASS112
VIII	–	–	–	MASS117c
IX	MASS136	–	MASS168 MASS178 MASS160	–

The only two rhizobial isolates from Kabale were grouped each in morphotypes V and VII (Table 4.3b). Tororo isolates were distributed in groups I, VI, VII and VIII. Mbale nodules had isolates that conformed to characteristics similar to those of morphotypes I, II and VII only.

Table 4.3b: Distribution of *S. sesban* rhizobial isolates from three Ugandan sites in nine morphotypes

Morphotype group	**Uganda**		
	Kabale	**Tororo**	**Mbale**
I	–	MASS51a	MASS59
II	–	–	MASS57
III	–	–	–
IV	–	–	–
V	MASS62	–	–
VI	–	MASS49	–
		MASS53	
VII	MASS65	MASS47	MASS60
		MASS51b	
VIII	–	MASS50	–
IX	–	–	–

The three isolates from SUA in Tanzania were grouped into morphotypes VI (one isolate) and VIII (two isolates) (Table 4.3c). Lushoto isolates were spread in all morphotypes except in morphotypes II and VII.

Table 4.3c: Distribution of *S. sesban* rhizobial isolates from two Tanzanian sites in nine morphotypes

Morphotype group	**Tanzania**	
	SUA	**Lushoto**
I	–	MASS37a
II	–	–
III	–	MASS31a
IV	–	MASS40a
V	–	MASS31b
		MASS40b
VI	MASS41	MASS37b
VII	MASS42	–
	MASS43	
VIII	–	MASS30
		MASS33
IX	–	MASS29
		MASS36

All the rhizobial isolates recovered from Khorixas Outijo and Rooidrom were grouped into morphotype I (Table 4.3d). Isolates from Swakop were distributed equally in morphotypes I, IV and VI. Rio Tinto Gorge isolates were clustered into morphotypes I (3 isolates) and one isolate in each of morphotypes II and VI. Isolates from Epupa falls were grouped into morphotypes I, III, V and VIII. Suclabo and Omuramba isolates were grouped into V and IX while those from Sesfontein were grouped in morphotypes VI and VII.

Table 4.3d: Distribution of sesbania rhizobial isolates from eight Namibian sites in nine morphotypes

	Namibia							
Morphotype	**Khorixas Outijo**	**Swakop**	**Rio Tinto Gorge**	**Epupa falls**	**Suclabo**	**Omuramba**	**Rooidrom**	**Sesfontein**
I	MN26 MN27 MN39	MN51	MN50 MN56 MN62	MN18 MN35	–	–	MN2 MN10	–
II	–	–	MN4	–	–	–	–	–
III	–	–	–	MN22	–	–	–	–
IV	–	MN44	–	–	–	–	–	–
V	–	–	–	MN19 MN38	MN31	MN34	–	–
VI	–	MN9	MN36	–	–	–	–	MN71
VII	–	–	–	–	–	–	–	MN12
VIII	–	–	–	MN13	–	–	–	–
IX	–	–	–	–	MN28	MN11 MN17	–	–

4.2 Characteristics of sesbania rhizobia on YEMA–BTB media

Actively growing sesbania rhizobia characteristically changed the green colour of YEMA–BTB at pH 6.8 to blue or yellow colour. Some of the isolates did not change the green colour to either blue or yellow (Plate 4.3).

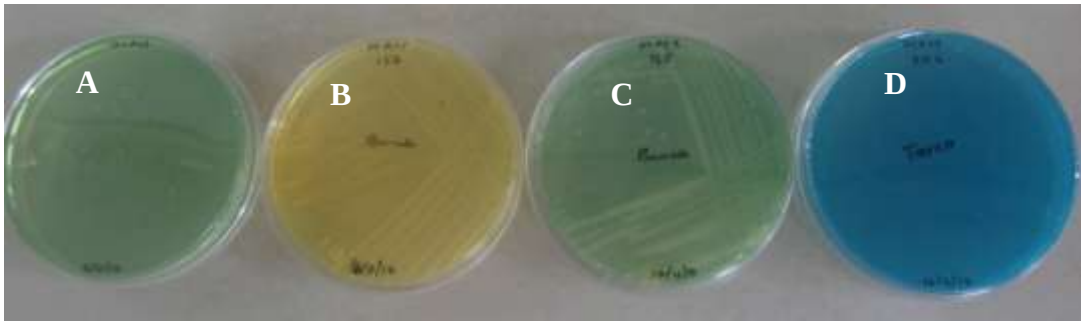

Plate 4.3: pH reaction characteristics of sesbania rhizobia on YEMA–BTB media. A, control plate (with no rhizobia); B, yellow colour; C, green colour; D, Blue colour.

Seventy eight sesbania rhizobial isolates were acid producers, 25 were neutral and 21 were alkaline producers (Table 4.4).

Table 4.4: pH reaction of sesbania rhizobial isolates on YEMA–BTB media

pH reaction	**Isolate**
Acid	MASS30, MASS31a, MASS32, MASS33, MASS34, MASS35, MASS37, MASS38, MASS39, MASS40, MASS41, MASS42, MASS43, MASS44, MASS45, MASS46, MASS47, MASS48, MASS49, MASS50, MASS51, MASS53, MASS54, MASS55, MASS57, MASS60, MASS62, MASS63, MASS65, MASS66, MASS67, MASS68, MASS69, MASS110, MASS111, MASS112, MASS113, MASS114, MASS115, MASS161, MASS162, MASS167, MASS168, MASS170, MASS172, MASS173, MASS176, MASS177, MN1, MN4, MN8, MN13, MN16, MN18, MN19, MN20, MN21, MN22, MN24, MN25, MN26, MN27, MN28, MN31, MN34, MN37, MN38, MN40, MN41, MN42, MN43, MN44, MN45, MN51, MN58, MN59, MN9, MN60.
Neutral	MASS116, MASS131, MASS132, MASS133, MASS134b, MASS135, MASS136, MASS137b, MASS156, MASS158, MASS159, MASS169, MASS171, MASS174, MASS181, MN2, MN10, MN12, MN15, MN39, MN49, MN56, MN57, MN62, MN68.
Alkaline	MASS29, MASS31b, MASS36, MASS61, MASS64, MASS117, MASS130, MASS157, MASS163, MASS164, MASS165, MASS166, MASS178, MASS180, MN11, MN17, MN35, MN50, MN69, MN70, MN71.

Out of the 124 isolates tested on YEMA-BTB media, the colour change from green to yellow accounted for 64.3 % followed by the green colour (19.4 %) and blue at 16.3 % (Figure 4.2).

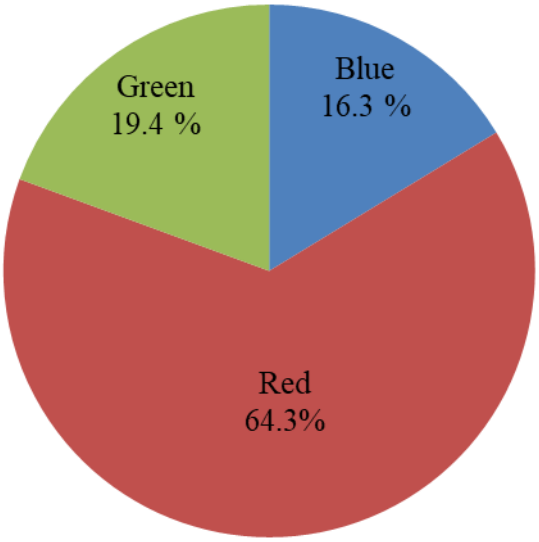

Figure 4.2: Colour change of YEMA-BTB by sesbania rhizobial isolates.

4.3 Gram staining

All the sesbania isolates retained the pink color on counter staining using safranin, typical of legume root nodule forming bacteria. The stained isolates were rod–shaped on observation under a microscope (Plate 4.4).

Plate 4.4: Gram negative rod–shaped rhizobia (Magnification x 600).

4.4 Selection of rhizobia for PCR–RFLP using intrinsic antibiotic resistance (IAR) and sodium chloride (NaCl) tolerance assays

4.4.1 Intrinsic antibiotic resistance of sesbania isolates

A total of 128 rhizobia from root nodules of sesbania that were tested for their intrinsic antibiotic resistance on YEMA supplemented with 12 different synthetic antibiotics each at two concentration levels of 50 mg L^{-1} and 100 mg L^{-1} were variously sensitive to the type and concentration of antibiotics used. For instance, isolate MASS140 was totally suppressed by 100 mg L^{-1} of kanamycin but produced copious EPS on 100 mg L^{-1} penicillin compared to control plate. Also, MASS50a produced copious EPS on plates with 100 mg L^{-1} penicillin compared to the controls (Plate 4.5).

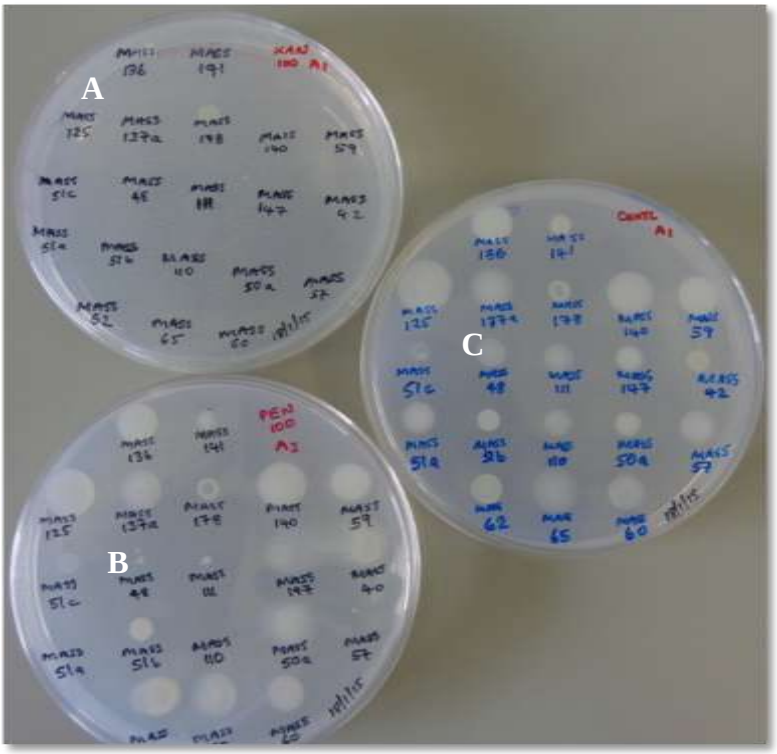

Plate 4.5: Sensitivity of sesbania isolates to antibiotics. A, kanamycin; 100 mg L^{-1}; B, penicillin 100 mg L^{-1}; C, control.

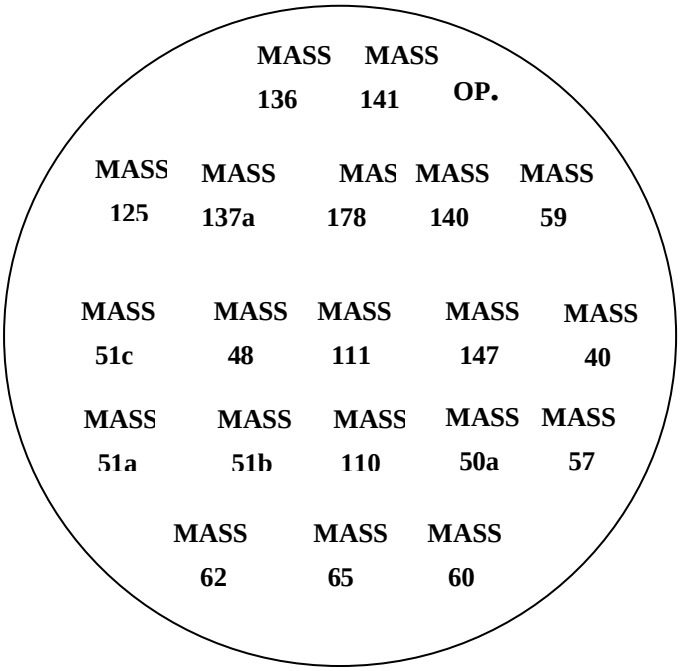

Figure 4.3: Schematic diagram showing orientation of isolates on Plate 4.5. OP = orientation point).

Sixty one point three percent (61.3 %) of the sesbania rhizobia tested were resistant to all the antibiotics except spectinomycin and streptomycin at a concentration of 50 mg L^{-1} (Figure 4.4). Spectinomycin and streptomycin at a concentration of 50 mg L^{-1} allowed 26.8 % and 49.3 % respectively of sesbania isolates to grow. The isolates were most sensitive to concentrations of 100 mg L^{-1} of rifampicin, spectinomycin, streptomycin, neomycin and kanamycin with resistance of 14.8 %, 12 %, 12 %, 10.6 % and 8.5 % respectively.

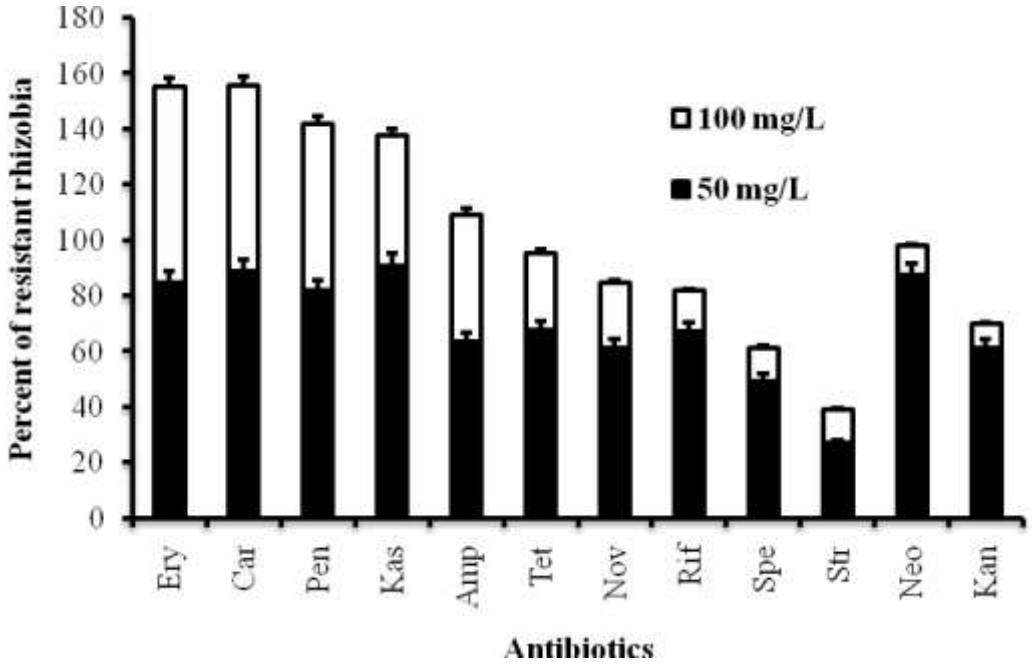

Figure 4.4: Resistance of sesbania rhizobial isolates to antibiotics. Kas, kasugamycin; Car, carbanicillin; Neo, neomycin; Ery, erythromycin; Pen, penicillin–G; Tet, tetracycline; Rif, rifampicin; Amp, ampicillin; Nov, novobiocin; Kan, kanamycin; Spe, spectinomycin; Str, streptomycin.

Rhizobia from Bumala, Gituamba, Kuinet and Kavutiri (Kenya) had varying resistance to the 12 antibiotics at the two concentration levels 50 mg L^{-1} and 100 mg L^{-1} (Table 4.5a). The mean percent resistance ranged from 64.6 % to 81.1 % and 26.7 % to 44.2 % for antibiotics concentration level of 50 mg L^{-1} and 100 mg L^{-1}

respectively. Bumala site isolates had a mean percent resistance of 79.5 % and 44.2 % to antibiotics at a concentration of 50 mg L^{-1} and 100 mg L^{-1} respectively. All isolates from Bumala were resistant to 50 mg L^{-1} rifampicicn, kasugamycin, neomycin and carbanicillin. Rhizobia from Bumala had the least resistance (7.7 %) to spectinomycin and neomycin. Gituamba site isolates had a mean percent resistance of 64.6 % and 29.2 % on antibiotics at a concentration of 50 mg L^{-1} and 100 mg L^{-1} respectively. There was a total growth inhibition of isolates from Gituamba site by streptomycin and spectinomycin at a concentration of 50 mg L^{-1} and kanamycin, streptomycin, spectinomycin, novobiocin and neomycin at a concentration of 100 mg L^{-1}. An equal number of isolates from Gituamba had similar resistance to ampicillin and penicillin (37.5 %) and erythromycin (87.5 %). Kuinet site isolates had a mean percent resistance of 66.7 % and 26.7 % to antibiotics at a concentration of 50 mg L^{-1} and 100 mg L^{-1} respectively. All the isolates from Kuinet grew on media supplemented with kanamycin, penicillin and novobiocin at a concentration of 50 mg L^{-1}. Kanamycin, streptomycin, spectinomycin and neomycin at a concentration of 100 mg L^{-1} totally inhibited growth of Kuinet site isolates. Kavutiri site isolates had a mean percent resistance of 81.1 % and 39.4 % to antibiotics at a concentration of 50 mg L^{-1} and 100 mg L^{-1} respectively. All the isolates from Kavutiri were resistant to penicillin, kasugamycin, erythromycin, neomycin and carbanicillin at a concentration of 50 mg L^{-1}. Kanamycin at a concentration of 100 mg L^{-1} had a total growth inhibition to Kavutiri isolates (Table 4.5a).

Table 4.5a: Percent intrinsic antibiotic resistance of *S. sesban* rhizobial isolates from Kenya

	Bumala		**Gituamba**		**Kuinet**		**Kavutiri**	
Antibiotics	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**
Amp	84.6	61.5	37.5	37.5	40.0	40.0	80.0	53.3
Kan	84.6	30.8	87.5	0	100.0	0	66.7	0
Rif	100.0	53.8	100.0	37.5	80.0	20.0	66.7	20.0
Str	23.1	15.4	0	0	20.0	0	40.0	26.7
Pen	92.3	69.2	37.5	37.5	100.0	40.0	100.0	66.7
Tet	84.6	61.5	37.5	25	40.0	20.0	80.0	26.7
Kas	100.0	46.2	100.0	62.5	80.0	20.0	100.0	40.0
Spe	15.4	7.7	0	0	20.0	0	53.3	33.3
Nov	76.9	23.1	100.0	0	100.0	40.0	86.7	13.3
Ery	92.4	76.9	87.5	87.5	80.0	80.0	100.0	93.3
Neo	100.0	7.7	87.5	0	60.0	0	100.0	6.7
Car	100.0	76.9	100.0	62.5	80.0	60.0	100.0	93.3
Mean %	79.5	44.2	64.6	29.2	66.7	26.7	81.1	39.4

Rhizobial isolates from Tororo, Mbale and Kabale (Uganda) exhibited variation in their resistance to the 12 antibiotics used in this study (Table 4.5b). The mean percent resistance ranged from 66.7 % to 91.7 % and 26.7 % % to 45.8 % for antibiotics concentration level of 50 mg L^{-1} and 100 mg L^{-1} respectively. Isolates from Tororo had a mean percent resistance of 66.7 % at 50 mg L^{-1} and 29.2 % at 100 mg L^{-1}. inhibition to Tororo isolates exhibited the lowest resistance of 20 % and 0 % to streptomycin at concentrations 50 mg L^{-1} and 100 mg L^{-1} had the highest growth respectively. All isolates from Tororo were resistant to carbanicillin at a concentration of 50 mg L^{-1} while 90 % of the isolates were resistant at 100 mg L^{-1}. Mbale isolates had a mean percent resistance of 75 % and 26.7 % at 50 mg L^{-1} and

100 mg L^{-1} respectively. All isolates from Mbale were susceptible to kanamycin, rifampicin, streptomycin, spectinomycin, novobiocin and neomycin at a concentration of 100 mg L^{-1}. Mbale isolates were all resistant to 50 mg L^{-1} and 100 mg L^{-1} carbanicillin. Rhizobial isolates from Kabale had a mean percent resistance of 91.7 % and 45.8 % at 50 mg L^{-1} and 100 mg L^{-1} respectively. All isolates from Kabale site were susceptible to kanamycin, rifampicin, streptomycin, spectinomycin and neomycin at a concentration of 100 mg L^{-1}. All Kabale isolates were resistant to ampicillin, penicillin, erythromycin and carbanicillin at concentrations 50 mg L^{-1} and 100 mg L^{-1}.

Table 4.5b: Percent intrinsic antibiotic resistance of *S. sesban* rhizobial isolates from Uganda

	Tororo		**Mbale**		**Kabale**	
Antibiotics	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**
Ampicillin	70.0	60.0	60.0	60.0	100.0	100.0
Kanamycin	60.0	0.0	40.0	0	50.0	0
Rifampicin	80.0	10.0	100.0	0	100.0	0
Streptomycin	20.0	0	40.0	0	50.0	0
Penicillin	80.0	70.0	100.0	60.0	100.0	100.0
Tetracycline	40.0	10.0	60.0	20.0	100.0	50.0
Kasugamycin	70.0	20.0	100.0	40.0	100.0	50.0
Spectinomycin	20.0	10.0	40.0	0	100.0	0
Novobiocin	80.0	10.0	60.0	0	100.0	50.0
Erythromycin	90.0	60.0	100.0	40.0	100.0	100.0
Neomycin	90.0	10.0	100.0	0	100.0	0
Carbanicillin	100.0	90.0	100.0	100.0	100.0	100.0
Mean %	66.7	29.2	75.0	26.7	91.7	45.8

Rhizobial isolates from Lushoto and SUA sites (Tanzania) varied in their intrinsic resistance to the 12 antibiotics (Table 4.5c). The mean percent resistance ranged from 73.6 % to 77.8 % and 30.6 % to 34.0 % for antibiotics concentration level of 50 mg L^{-1} and 100 mg L^{-1} respectively. Lushoto site isolates had a mean percent resistance of 73.6 % and 34 % to antibiotics at concentrations of 50 mg L^{-1} and 100 mg L^{-1} respectively. At a concentration of 100 mg L^{-1} rifampicin inhibited the growth of all the isolates from Lushoto. The highest IAR was recorded with Lushoto isolates at 100 mg L^{-1} erythromycin (91.7 %). A 58.3 % resistance was recorded for rhizobial isolates from Lushoto on YEMA media containing 50 mg L^{-1} and 100 mg L^{-1} concentrations of ampicillin. Similarly, an equal resistance of 91.7 % was recorded for rhizobial isolates recovered from Lushoto on YEMA media containing erythromycin at concentrations of 50 mg L^{-1} and 100 mg L^{-1}. Rhizobia from SUA site had a mean percent resistance of 77.8 % and 30.6 % at concentration of 50 mg L^{-1} and 100 mg L^{-1} respectively. Kanamycin and streptomycin conferred inhibition to all SUA isolates at both concentrations. SUA isolates recorded 100 % resistance to all antibiotics at 50 mg L^{-1} except ampicillin, kanamycin, streptomycin and kasugamycin. Kanamycin, rifampicin, streptomycin, tetracycline spectinomycin and neomycin at a concentration 100 mg L^{-1} had total inhibition of SUA isolates.

Table 4.5c: Percent intrinsic antibiotic resistance of *S. sesban* rhizobial isolates from Tanzania

Antibiotics	**Lushoto**		**SUA**	
	50 mg L^{-1}	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**
Ampicillin	58.3	58.3	66.7	66.7
Kanamycin	83.3	8.3	0	0
Rifampicin	75.0	0	100.0	0
Streptomycin	33.3	16.7	0	0
Penicillin	83.3	66.7	100.0	66.7
Tetracycline	58.3	33.3	100.0	0
Kasugamycin	100.0	25.0	66.7	33.3
Spectinomycin	50.0	16.7	100.0	0
Novobiocin	75.0	16.7	100.0	66.7
Erythromycin	91.7	91.7	100.0	66.7
Neomycin	75.0	16.7	100.0	0
Carbanicillin	100.0	58.3	100.0	66.7
Mean %	73.6	34.0	77.8	30.6

The mean percent resistance ranged from 25 % to 92 % and 8 % to 75 % for antibiotics concentration level of 50 mg L^{-1} and 100 mg L^{-1} respectively (Table 4.5d). Fifty milligrams per litre and one hundred milligrams per litre (50 mg L^{-1} and 100 mg L^{-1}) kanamycin and streptomycin totally inhibited the growth of sesbania isolates from Okahandja while 50 mg L^{-1} and 100 mg L^{-1} kanamycin inhibited growth of isolates from Khorixas–Outijo. Fifty milligrams per litre and one hundred milligrams per litre (50 mg L^{-1} and 100 mg L^{-1}) ampicillin, streptomycin, tetracycline, spectinomycin and novobiocin totally inhibited the growth of sesbania isolates from Rio Tinto Gorge. Likewise, 50 mg L^{-1} and 100 mg L^{-1} ampicillin, streptomycin, tetracycline and novobiocin inhibited the growth of isolates from Suclabo. Fifty milligrams per litre and one hundred milligrams per litre (50 mg L^{-1} and 100 mg L^{-1}) kanamycin and streptomycin inhibited the growth of isolates from

Bunya while 50 mg L^{-1} and 100 mg L^{-1} novobiocin totally inhibited the growth of isolates from Rooidrom. Also 50 mg L^{-1} and 100 mg L^{-1} neomycin totally inhibited the growth of isolates from Sesfontein. Apart from the complete resistance to tetracycline, kasugamycin and spectinomycin at a concentration of 50 mg L^{-1} and to kasugamycin at a concentration of 100 mg L^{-1}, all isolates from Korokoko were susceptible to the antibiotics used in the present study.

Penicillin, novobiocin, erythromycin and carbanicillin at concentrations of 50 mg L^{-1} and 100 mg L^{-1} did not affect sesbania isolates from Okahandja (Table 4.5d). Erythromycin and carbanicillin did not affect growth of sesbania isolates from Rio Tinto Gorge. Sesbania isolates from Epupa falls were all resistant to kasugamycin while ampicillin, streptomycin and carbanicillin at concentrations 50 mg L^{-1} and 100 mg L^{-1}did not affect growth isolates from Otjinungua. Growth of all the isolates from Rooidrom was not affected by concentrations 50 mg L^{-1} and 100 mg $L^{-1}$of ampicillin, streptomycin, penicillin, tetracycline, kasugamycin, spectinomycin, erythromycin, neomycin and carbanicillin. Growth of all the sesbania isolates from Sesfontein and Korokoko were not affected by concentrations 50 mg L^{-1} and 100 mg $L^{-1}$of kasugamycin. Isolates from Rooidrom had the highest mean percent survival at antibiotics concentration 100 mg L^{-1} (75.0 %) while those from Korokoko had the lowest mean percent survival (8 %).

Table 4.5d: Percent antibiotic resistance of *Sesbania* spp. rhizobial isolates from Namibia

	Okaha-ndja		**Khorixas Outijo**		**Swakop**		**Rio Tinto Gorge**		**Epupa falls**		**Otjinu-ngua**		**Suclabo**		**Omura-mba**		**Bunya**		**Rooi-drom**		**Sesfon-tein**		**Koro-koko**	
Antib-iotics	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**
Amp	50	0	25	25	57	6	0	0	50	7	100	100	0	0	50	20	100	50	100	100	7	3	0	0
Kan	0	0	0	0	43	0	100	0	17	0	50	0	33	0	30	0	0	0	100	0	67	33	0	0
Rif	100	50	25	0	57	14	100	0	50	0	50	0	33	0	40	0	100	0	100	0	67	0	0	0
Str	0	0	50	25	29	14	0	0	50	17	50	0	0	0	20	0	0	0	100	100	33	0	0	0
Pen	100	100	25	25	71	71	100	0	67	67	100	100	33	0	70	40	50	50	100	100	67	67	0	0
Tet	100	0	25	25	86	0	0	0	100	17	50	0	0	0	60	30	100	0	100	100	100	0	100	0
Kas	100	50	100	75	100	43	100	0	100	100	100	0	100	67	80	60	100	50	100	100	100	100	100	100
Spe	100	0	75	25	100	14	0	0	100	0	50	0	67	0	70	10	50	0	100	100	100	0	100	0
Nov	100	100	50	25	57	43	0	0	33	17	50	0	0	0	40	20	100	0	0	0	67	33	0	0
Ery	100	100	25	25	71	57	100	100	50	50	100	50	33	33	70	60	100	50	100	100	67	0	0	0
Neo	100	0	25	25	57	0	100	0	50	33	50	0	33	0	50	10	100	0	100	100	0	0	0	0
Car	100	100	25	25	71	71	100	100	50	17	100	100	33	33	80	50	100	50	100	100	67	0	0	0
Mean %	79	42	38	25	31	11	55	25	67	30	58	17	60	28	71	29	75	21	92	75	72	33	25	8

4.4.2 Rhizobia tolerance to sodium chloride

Sesbania rhizobial isolates growth on YEMA containing salt levels (NaCl w/v) of 1 %, 3 % and 5 % were similar to those on 0.1 % NaCl (control). Higher salt levels (6 % to 10 %) increasingly inhibited growth of the rhizobial isolates (Plate 4.6).

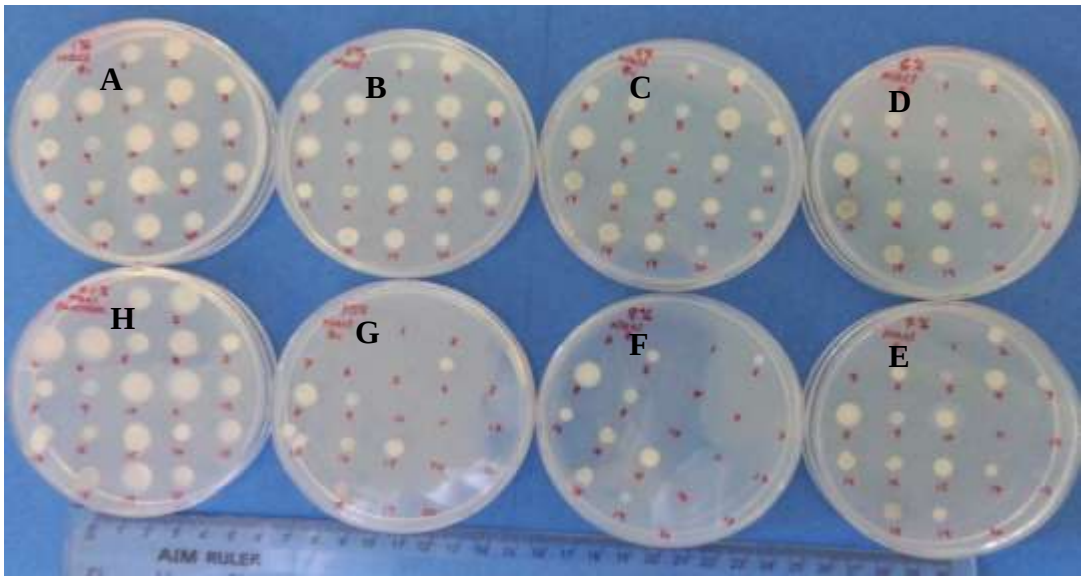

Plate 4.6: Sensitivity of sebania rhizobial isolates to salt levels (NaCl w/v) compared to controls. A,1 %; B, 3 %; C, 5 %; D, 6 %; E, 7 %; F, 8 %; G, 10 % (NaCl levels); H, 0.1 % (control).

Rhizobia obtained from all the study sites were tolerant to 0.1–3 % NaCl (Figure 4.4), there after an increase in salt concentration became inversely proportional to the percent of tolerant sesbania rhizobia (3 % NaCl = 99.2 %, 5 % NaCl = 98.4 %, 6 % NaCl = 93 %, 7 % NaCl = 80.1 %, 8 % NaCl = 64.3 % and 10 % NaCl = 54.3 % of the isolates (Figure 4.5).

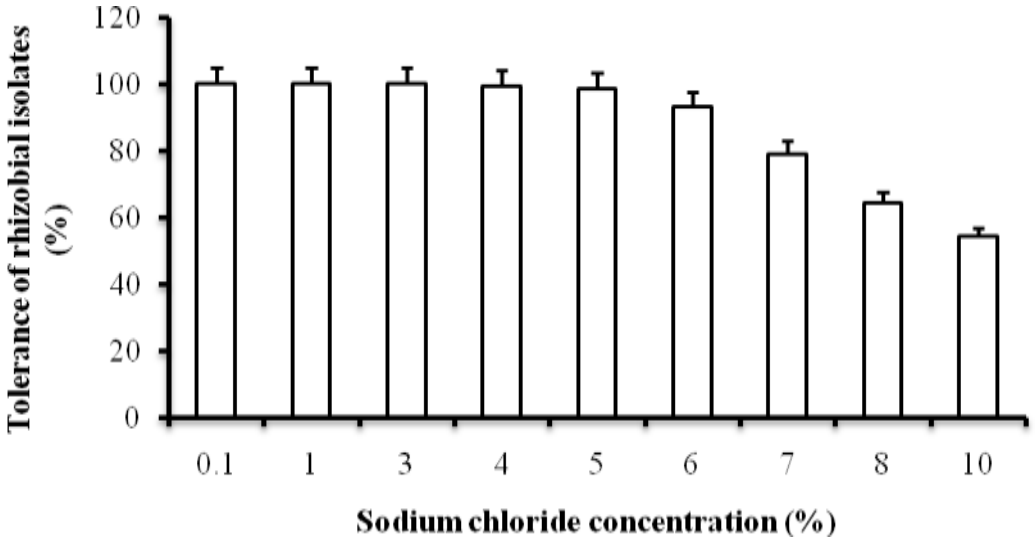

Figure 4.5: Tolerance of sesbania rhizobial isolates to different sodium chloride concentrations. Error bars with 5 %.

4.4.3 Selection of rhizobial isolates for PCR–RFLP

Combined binary patterns generated by growth (1) or no growth (0) of rhizobial isolates on YEMA media supplemented with various levels of antibiotics or NaCl and analysed using UPGMA method resulted in dendrograms per site with distinctive clusters (Figures 4.6a–j). Thirteen Bumala isolates were split into clusters I and II. Cluster I had one isolate MASS176. Cluster II comprised of split into two groups of nine isolates (MASS165, MASS157, MASS126, MASS127, MASS167, MASS177a, MASS178, MASS125 and MASS168) in one group and three MASS160, MASS123 and MASS177b in the other group at node with 26 % similarity index. Two isolates MASS165 and MASS157 were split within cluster Cluster II at nodes with 6 % and 9 % respectively. Isolates MASS126, MASS127, MASS167, MASS177a and MASS178 had a similarity index of 89 %. Similarly, isolates MASS125 and MASS168 had a similarity index of 89 % (Figure 4.6a).

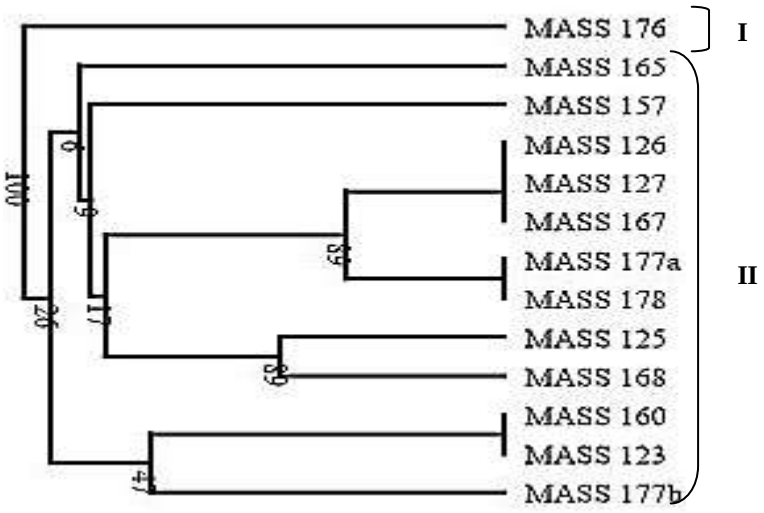

Figure 4.6a: Unrooted neighbour joining dendrogram constructed based on IAR and salt tolerance using UPGMA method showing relatedness clusters I and II of sesbania rhizobial isolates from Bumala. Numbers on branches are bootstrap % from 1000 replicates.

Rhizobial isolates from Kuinet formed two similarity clusters. Cluster I consisted of two perfectly similar isolates (MASS117a and MASS117c) at similarity index 100 %) but were 72 % similar to MASS117b. Cluster II contained MASS112, MASS114 and MASS115 (100 % similar) and MASS111 and MASS110 (100 % similar) but the two groups had a nodal similarity index of 97 % (Figure 4.6b).

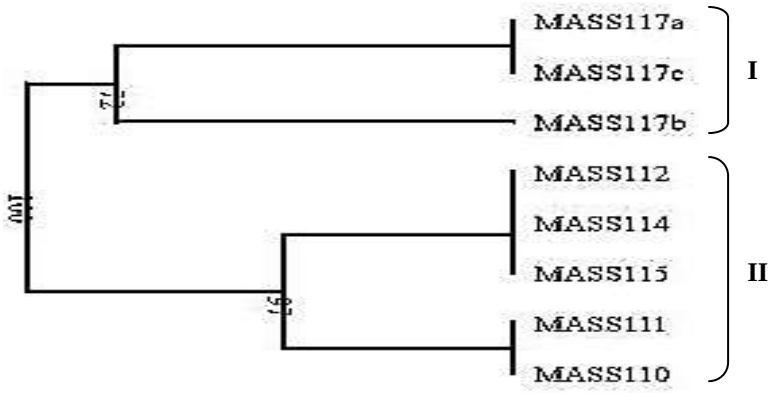

Figure 4.6b: Unrooted neighbour joining dendrogram constructed based on IAR and salt tolerance using UPGMA method showing relatedness clusters I and II of sesbania rhizobial isolates from Kuinet. Numbers on branches are bootstrap % from 1000 replicates.

Gituamba isolates MASS174 and MASS170 shared the same similarity node at 76 % in cluster I. Isolates MASS172 and MASS173 were similar (100 %) but also shared a cluster with MASS171 at a node with similarity index of 63 % in cluster II (Figure 4.6c).

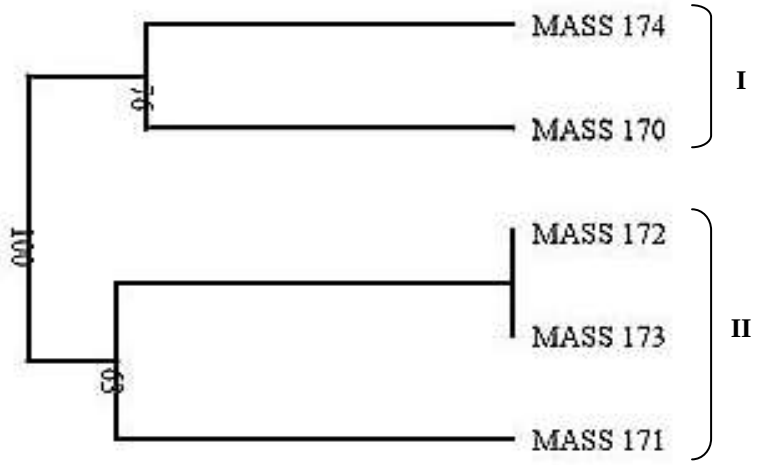

Figure 4.6c: Unrooted neighbour joining dendrogram constructed based on IAR and salt tolerance using UPGMA method showing relatedness clusters I and II of sesbania rhizobial isolates from Gituamba. Numbers on branches are bootstrap % from 1000 replicates.

Rhizobial isolates from Kavutiri were grouped into cluster I and II (Figure 4.6d). Cluster I contained isolates split into two groups at similarity index 34 %. Rhizobial isolates MASS134, MASS130 and MASS129 were similar (100 %) and had a similarity index of 82 % with MASS141. The similarity index of isolate MASS140 to isolates MASS138, MASS147, MASS149 and MASS131 was at 42 %, isolate MASS131 to isolates MASS138, MASS147 and MASS149 was at 44 % and isolate MASS138 to isolates MASS147and MASS149 was 58 %. Rhizobial isolates in cluster II had a node at 61 % and they included MASS132, MASS133 (100 % similarity index) and MASS137a and MASS 137b (100 % similarity index). Isolate MASS136 was similar to isolates MASS132 and MASS133 by 52 % while

MASS142 was similar to isolates MASS137a and MASS 137b by 42 % (Figure 4.6d).

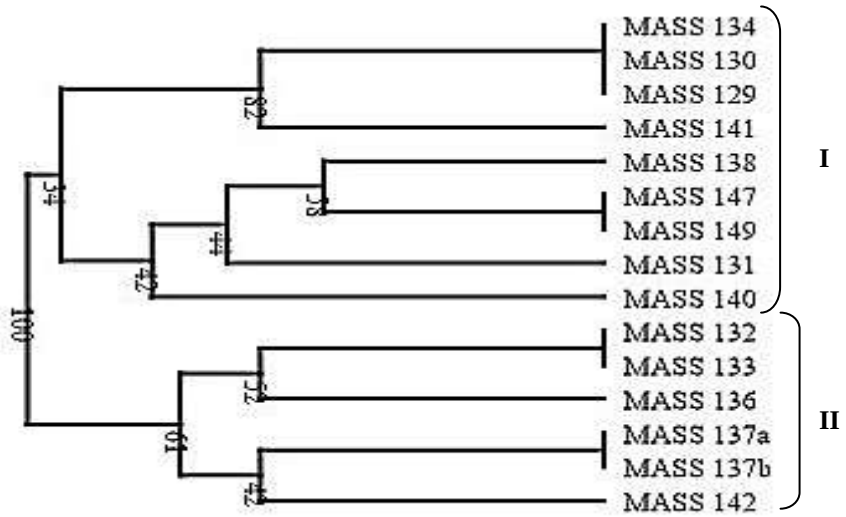

Figure 4.6d: Unrooted neighbour joining dendrogram constructed based on IAR and salt tolerance using UPGMA method showing relatedness clusters I and II of sesbania rhizobial isolates from Kavutiri. Numbers on branches are bootstrap % from 1000 replicates.

Rhizobial isolates of nodules collected from Tororo segregated into two clusters (I and II). Cluster I had a node at 38 % relatedness which split the isolates into two groups. Isolates MASS50b and MASS47 with relatedness of 21 % formed one group while a node with 7 % relatedness formed another group of six isolates. Isolates MASS48 and MASS49 were 100 % similar but differed with MASS51b and MASS53 (29 % similarity) at a node with 14 % similarity. Isolates MASS50c and MASS51a were similar by 44 %. Custer II contained isolates MASS50a and MASS52 which had a relatedness index of 57 % **(**Figure 4.6e).

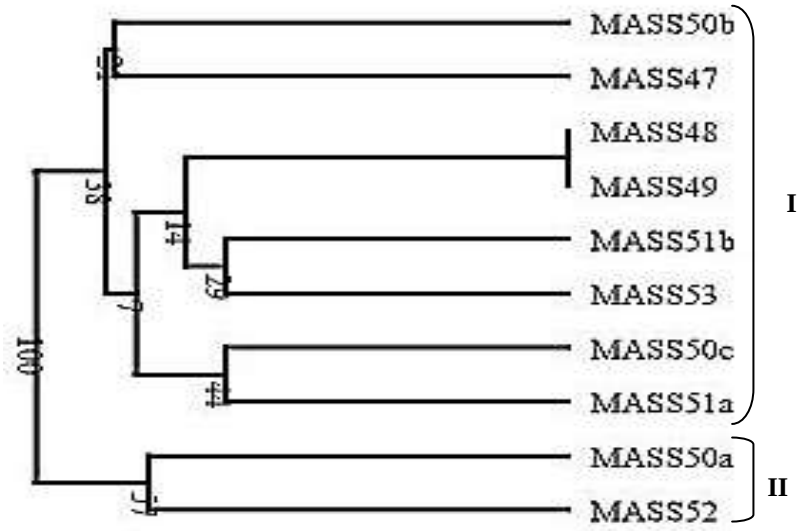

Figure 4.6e: Unrooted neighbour joining dendrogram constructed based on IAR and salt tolerance using UPGMA method showing relatedness clusters I and II of sesbania rhizobial isolates from Tororo. Numbers on branches are bootstrap % from 1000 replicates.

Five *S. sesban* rhizobial isolates from Mbale were segregated into two clusters (I and II). Cluster I contained isolates MASS60 and MASS54 which had a similarity of 100 %. Cluster II had a node with similarity of 58 % and split isolate MASS59 from MASS57 and MASS61. Rhizobial isolates MASS57 and MASS61 had a similarity of 100 % (Figure 4.6f).

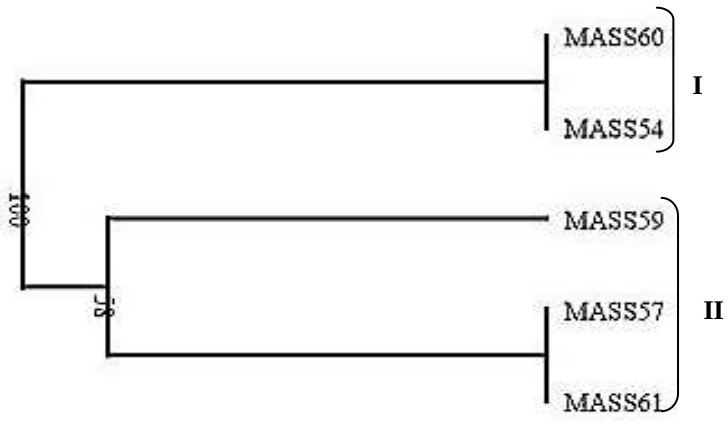

Figure 4.6f: Unrooted neighbour joining dendrogram constructed based on IAR and salt tolerance using UPGMA method showing relatedness clusters I and II of sesbania rhizobial isolates from Mbale. Numbers on branches are bootstrap % from 1000 replicates.

Four *S. sesban* rhizobial isolates from Kabale site were segregated into clusters I and II. Cluster I contained isolates MASS69 and MASS62 with 100 % similarity. Cluster II consited of MASS65 and MASS68 with 100 % similarity (Figure 4.6g).

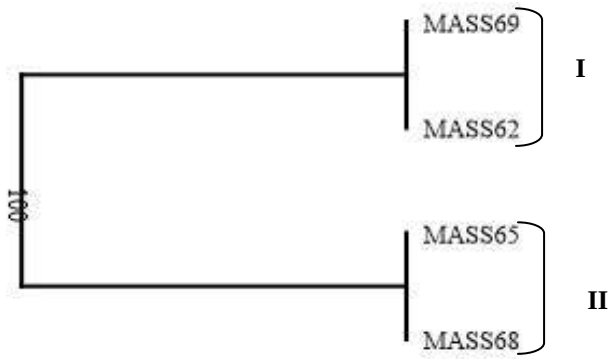

Figure 4.6g: Unrooted neighbour joining dendrogram constructed based on IAR and salt tolerance using UPGMA method showing relatedness clusters I and II of sesbania rhizobial isolates from Kabale. Numbers on branches are bootstrap % from 1000 replicates.

Isolates from Lushoto were separated into two clusters I and II. Cluster I contained five isolates which were split into two groups of isolate MASS37a and four isolates MASS38, MASS29, MASS30 and MASS31a respectively at node with 75 % similarity. The group with four isolates was again split into two isolates each (MASS38; MASS29) and (MASS30; MASS31a) at node 38 % similarity with similarity of 83 and 100 % repectively. Cluster II was again split into two subclusters with a single isolate (MASS36) on one side and six isolates on ther other side at node 35 %. The six were split into four and two isolates respectively at node with similarity of 22 % (MASS33, MASS40b, MASS40a and MASS37b) and (MASS31b and MASS39). The clusters containing isolates MASS33 and MASS40b and isolates MASS40a and MASS37b were split at node with similarity of 22 %. Isolates MASS33 and MASS40b; MASS40a and MASS37b; MASS31b and MASS39 had similarities of 46 %, 33 % and 46 % respectively (Figure 4.6h).

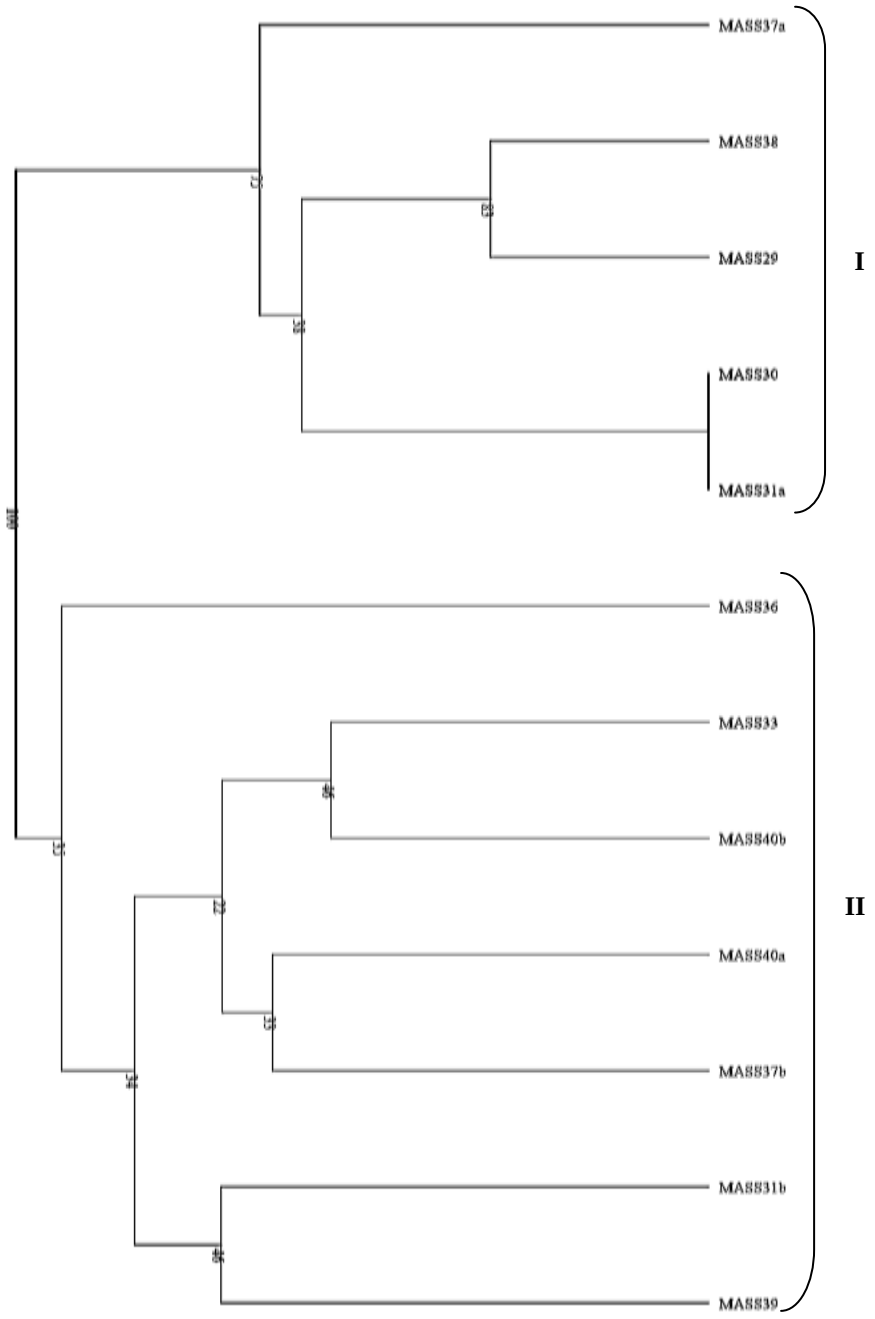

Figure 4.6h: Unrooted neighbour joining dendrogram constructed based on IAR and salt tolerance using UPGMA method showing relatedness clusters I and II of sesbania rhizobial isolates from Lushoto. Numbers on branches are bootstrap % from 1000 replicates.

The three *S. sesban* rhizobial isolates from SUA (Sokoine University of Agriculture) were grouped into clusters I and II (Figure 4.6i). Cluster I contained isolates MASS42 and MASS43 (100 % similar) while cluster II had one isolate (MASS41).

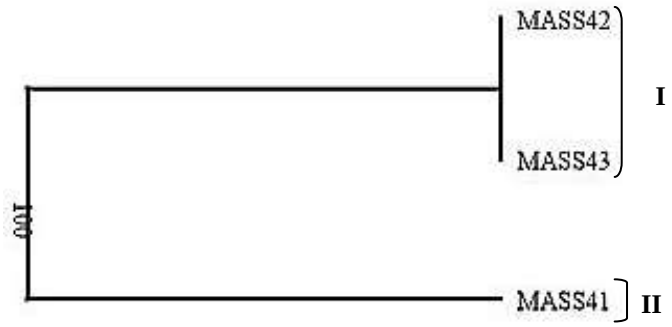

Figure 4.6i: Unrooted neighbour joining dendrogram constructed based on IAR and salt tolerance using UPGMA method showing relatedness clusters I and II of sesbania rhizobial isolates from SUA. Numbers on branches are bootstrap % from 1000 replicates.

Sesbania rhizobial isolates from Namibia clustered into I–V (Figure 4.6j). Cluster I included isolates MN2, MN10, MN31, MN12, MN18, MN39, MN45, MN11, MN62, MN57, MN13, MN15, MN19, MN40, MN70, MN71, MN1, MN4, MN8, MN28 and MN58. Isolates MN28 and MN58 with similarity index 45 % were separated from other isolates in cluster I by a node with 16 % similarity. Two other groups were separated by a node with 9 % similarity. A subcluster comprising isolates MN2, MN10, MN31, MN12, MN18, MN39, MN45, MN11, MN62 and MN57 had similarities varying between 4 and 100 %. The subcluster containing isolates MN13, MN15, MN19, MN40, MN70, MN71, MN1, MN4 and MN8 had similarities between 18 and 98 %. Cluster II contained isolates MN9, MN16, MN51, MN25, MN26, MN24, MN20, MN32, MN60, MN38, MN59, MN43, MN27, MN37, MN17, MN21 and MN36. The isolates in this cluster had similarities that varied from 2 to 100 % at different nodes. Isolates MN17 and MN21 were 100 % similar. Cluster III contained a single isolate MN69. Cluster IV comprised of three isolates separated by a node with 90 % similarity into MN44 and MN68 (62 % similarity) and MN47. Cluster V comprised of MN50 and MN56 with a node with 26 % similarity (Figure 4.6j).

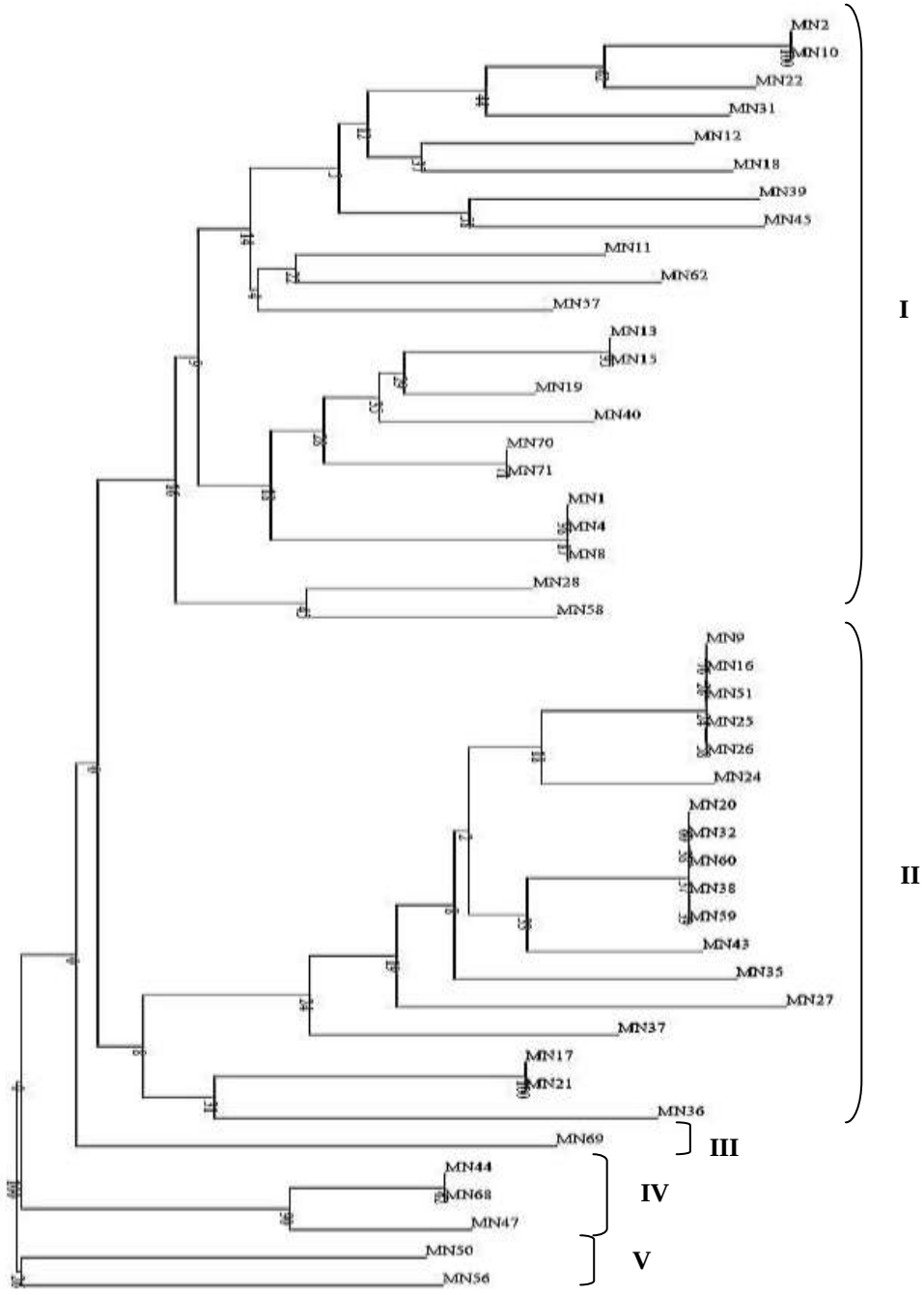

Figure 4.6j: Unrooted neighbour joining dendrogram constructed based on combined IAR and salt tolerance using UPGMA method showing relatedness clusters I–V of sesbania rhizobial isolates from Namibia. Numbers on branches are bootstrap % from 1000 replicates.

A total of 79 test rhizobia from East Africa and Namibia, were selected for PCR–RFLP assays. In addition, 17 reference strains were included in the assays, two of them a *S. sesban* and common bean inoculant strain each (Table 4.6).

Table 4.6: List of sesbania rhizobial isolates and reference strains used for PCR–RFLP assay

No.	Isolate	No.	Isolate	No.	Isolate	No.	Isolate
1.	MASS29	25.	MASS117b	49.	MASS172	73.	MN39
2.	MASS30	26.	MASS117c	50.	MASS174	74.	MN44
3.	MASS31a	27.	MASS126	51.	MASS114	75.	MN50
4.	MASS31b	28.	MASS127	52.	BA37	76.	MN51
5.	MASS36	29.	MASS159	53.	MN2	77.	MN56
6.	MASS37a	30.	MASS160	54.	MN4	78.	MN62
7.	MASS37b	31.	MASS168	55.	MASS53	79.	MN71
8.	MASS40a	32.	MASS175	56.	MN9	80.	MASS33
9.	MASS40b	33.	MASS176	57.	MN10	81.	KFR459
10.	MASS41	34.	MASS177a	58.	MN12	82.	USDA9030
11.	MASS42	35.	MASS177b	59.	MN13	83.	KFR84
12.	MASS43	36.	MASS178	60.	MN11	84.	DWO253
13.	MASS47	37.	MASS130	61.	MN17	85.	DWO461
14.	MASS49	38.	MASS132	62.	MN18	86.	USDA1002
15.	MASS51a	39.	MASS133	63.	MN19	87.	ORS177
16.	MASS51b	40.	MASS136	64.	MN34	88.	KFR647
17.	MASS50	41.	MASS137a	65.	MN22	89.	KFR402
18.	MASS57	42.	MASS137b	66.	MN26	90.	KFR8
19.	MASS59	43.	MASS138	67.	MN27	91.	*Azorh.* sp.
20.	MASS60	44.	MASS140	68.	MN28	92.	KFR552
21.	MASS62	45.	MASS141	69.	MN31	93.	DWO100
22.	MASS65	46.	MASS147	70.	MN35	94.	USDA337
23.	MASS112	47.	MASS170	71.	MN36	95.	USDA76
24.	MASS117a	48.	MASS171	72.	MN38	96.	USDA110

Nos. 1–51 and 53–80, sesbania rhizobial isolates; No. 52, common bean inoculant strain; Nos. 81–96, reference strains; *Azorh.* sp., *Azorhizobium* sp.

4.5 Genotype composition of sesbania rhizobia

4.5.1 Estimation of rhizobial 16S rRNA amplicons using gel electrophoresis

The DNA samples extracts from the 79 rhizobial isolates and 17 reference strains had OD260/280 of 1.78–2.04. The PCR amplicons of the 16S rRNA gene amplified by universal primers fD1 and rD1 yielded similar bands of approximately 1500 bp when quantified on 0.8 % agarose gel (Plate 4.7).

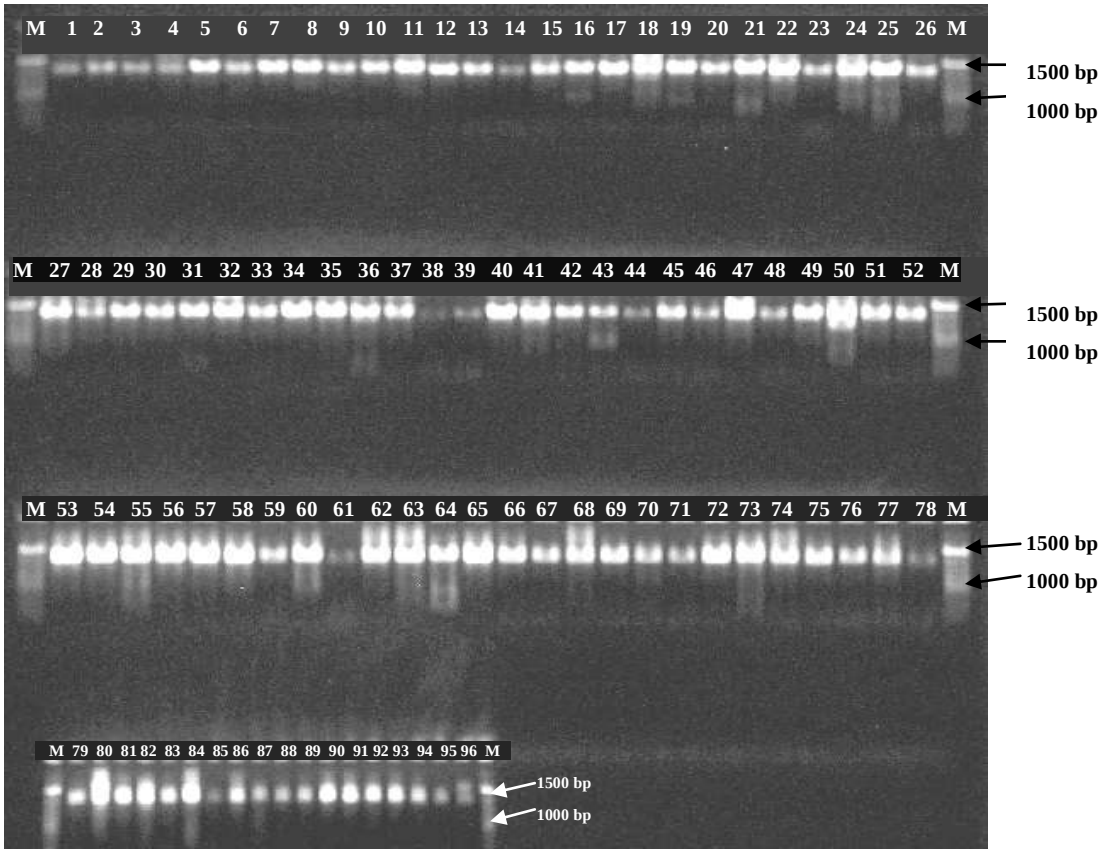

Plate 4.7: Agarose gel electrophoresis showing amplicon bands of 16S rRNA region.

Lanes 1–51 sesbania rhizobial isolates, Lane 1, MASS29; 2, MASS30; 3, MASS31a; 4, MASS31b; 5, MASS36; 6, MASS37a; 7, MASS37b; 8, MASS40a; 9, MASS40b; 10, MASS41; 11, MASS42; 12, MASS43; 13, MASS47; 14, MASS49; 15, MASS51a; 16, MASS51b; 17, MASS50; 18, MASS57; 19, MASS59; 20, MASS60; 21, MASS62; 22, MASS65; 23, MASS112; 24, MASS117a; 25, MASS117b; 26, MASS117c; 27, MASS126; 28, MASS127; 29, MASS159; 30, MASS160; 31, MASS168; 32, MASS175; 33, MASS176; 34, MASS177a; 35, MASS177b; 36, MASS178; 37, MASS130; 38, MASS132; 39, MASS133; 40, MASS136; 41, MASS137a; 42, MASS137b; 43, MASS138; 44, MASS140; 45, MASS141; 46, MASS147; 47, MASS170; 48, MASS171; 49, MASS172; 50, MASS174; 51, MASS114; Lane 52, common bean inoculant strain, BA37; Lanes 53–80 sesbania rhizobial isolates; 53, MN2; 54, MN4; 55, MASS53; 56, MN9; 57, M10; 58, MN12; 59, MN13; 60, MN11; 61, MN17; 62, MN18; 63, MN19; 64, MN34; 65, MN22; 66, MN26; 67, MN27; 68, MN28; 69, MN31; 70, MN35; 71, MN36; 72, MN38; 73, MN39; 74, MN44; 75, MN50; 76, MN51; 77, MN56; 78, MN62 79, MN71; 80, MASS33; Lanes 81–96, reference strains 81, KFR459; 82, USDA9030; 83, KFR84; 84, DWO253; 85, DWO461; 86, USDA1002; 87, ORS177; 88, KFR647; 89, KFR402; 90, KFR8; 91, *Azorhizobium* sp.; 92, KFR552; 93, DWO100; 94,USDA337; 95, USDA76; 96, USDA110; M, 100 bp (Invitrogen).

4.5.2 Separation of 16S rRNA restriction bands using gel electrophoresis

Digestion of the 16S rRNA amplicons using endonucleases *Msp*I, *Hinf*I and *Hae*III, resulted in specific and unique band patterns for DNA extracts from the 79 test isolates and 17 reference strains. Restriction using each of the three endonucleases yielded one to five polymorphic bands (Plates 4.8a, 4.8b and 4.8c). The 50 bp bands were not included in analysis since most were not clear.

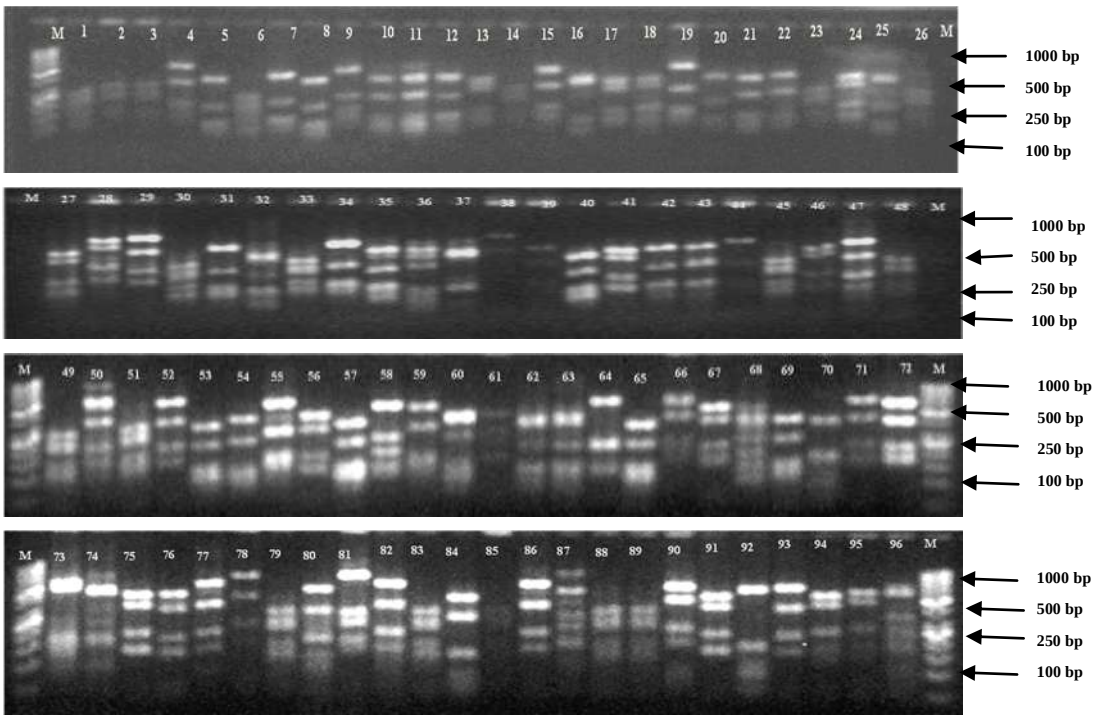

Plate 4.8a: Agarose electrophoresis patterns of 16S rRNA gene region of sesbania rhizobial isolates generated as a result of restriction digestion using endonuclease *Msp*I. Samples in lanes 1–96 are as in Plate 4.7. M, 50 bp marker (Invitrogen).

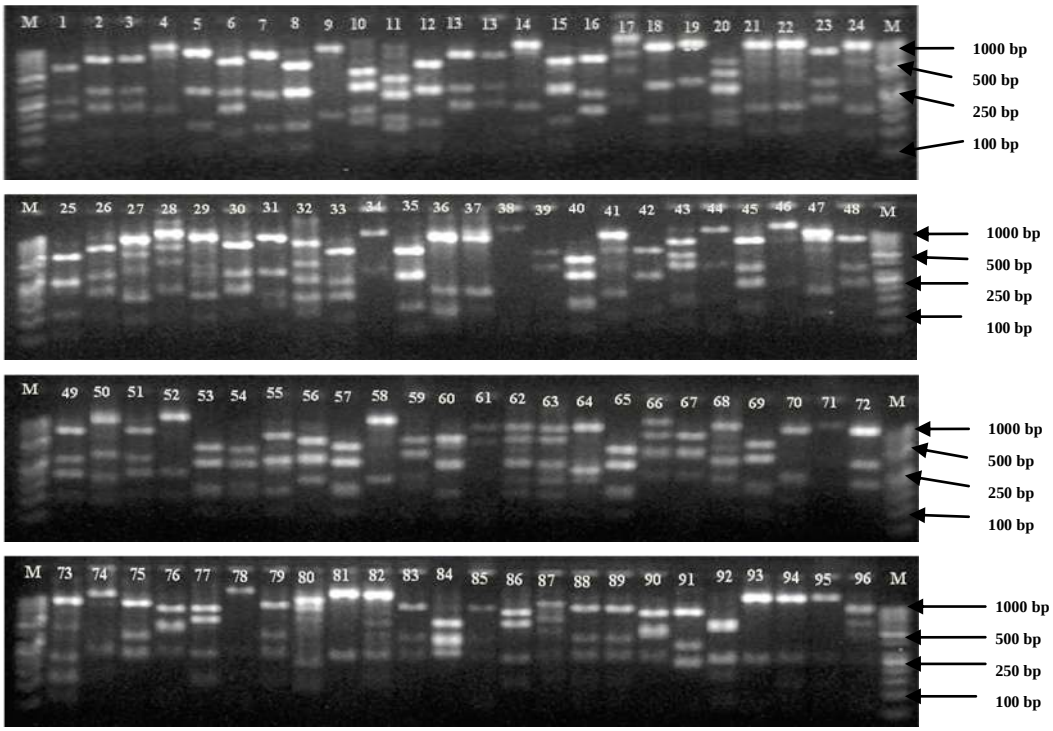

Plate 4.8b: Agarose electrophoresis patterns of 16S rRNA gene region of sesbania rhizobial isolates generated as a result of restriction digestion using endonuclease *Hinf*I. Samples in lanes 1–96 are as in Plate 4.7. M, 50 bp marker (Invitrogen).

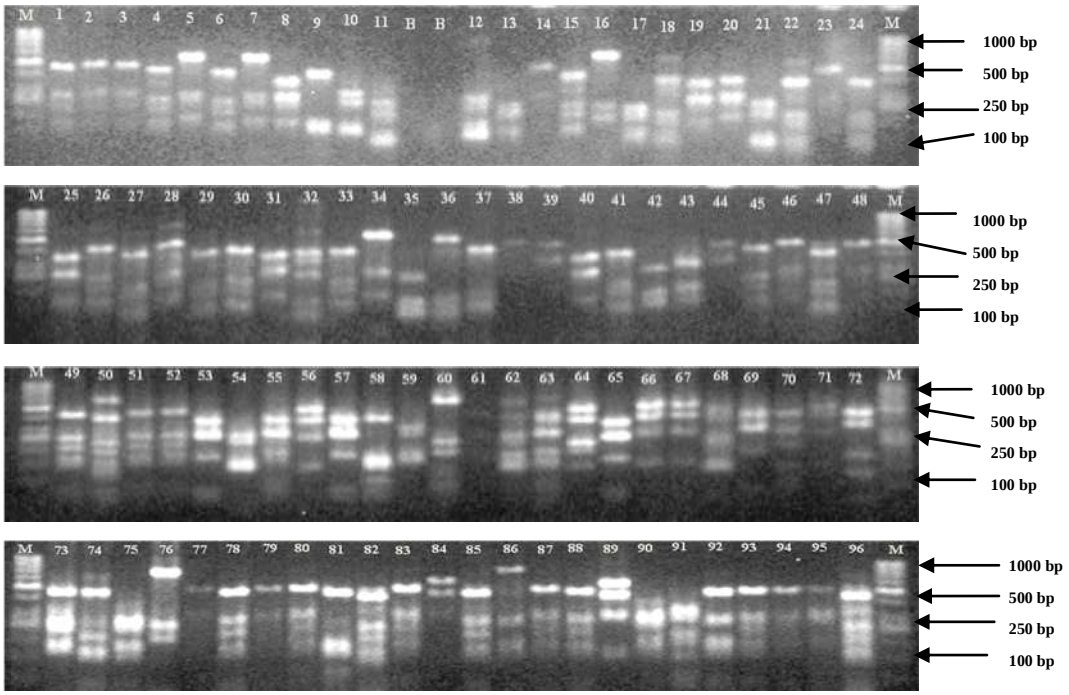

Plate 4.8c: Agarose electrophoresis patterns of 16S rRNA gene region of sesbania rhizobial isolates generated as a result of restriction digestion using endonuclease *Hae*III. Samples in Lanes 1–96 are as in Plate 4.7. M, 50 bp marker (Invitrogen).

4.5.2 Phylogenetic clusters of sesbania rhizobia

The combined data for presence (1) or absence (0) of fragments obtained from restriction of 96 PCR products of the 16S rRNA using endonucleases (*Msp*I, *Hinf*I and *Hae*III) separated into country of origin and subjected to cluster analysis using UPGMA by GenAlex statistical program and MEGA version 4 statistical programs resulted in phylogenetic diversity dendrograms. The mean cophenetic correlation coefficient was 62.1 % after 1000 bootstrap replications were applied to the analysis to determine branch support in the consensus trees.

Rhizobia isolated from nodules of *S. sesban* grown in Kenya were grouped into nine clusters which comprised of ribotypes I–IX (Figure 4.7a). Ribotype I contained five isolates closely affiliated to *Rhizobium* spp. Type A. Isolate MASS132 had a similarity of 40 % with *R. leguminosarum* DWO253 but shared a branch support at 3 % similarity with other *Rhizobium* reference strains. A cluster of four isolates sharing the same node at 16 % split into MASS126 and MASS159 with similarity of 84 % and MASS117a and MASS174 with similarity of 34 %. No *S. sesban* rhizobial isolates from Kenya clustered with *Bradyrhizobium* spp. that formed ribotype II. Ribotype III was split into two sub-clusters. The first sub-cluster contained isolates that were unmatched to any of the reference strains and contained two pairs of isolates with similarity indeces of 44 % (MASS141 and MASS172) and 53 % (MASS177a and MASS178) repectively but separated by a common branch support of 23 % similarity. The second sub-cluster of ribotype III was similar to the first sub-cluster ribotype by a branch support with only 1 % similarity. Two isolates were clustered in the second sub-cluster and were similar to *Sinorhizobium meliloti* USDA1002 by 24 % (MASS170) and 6 % (MASS177b) (Figure 4.7a).

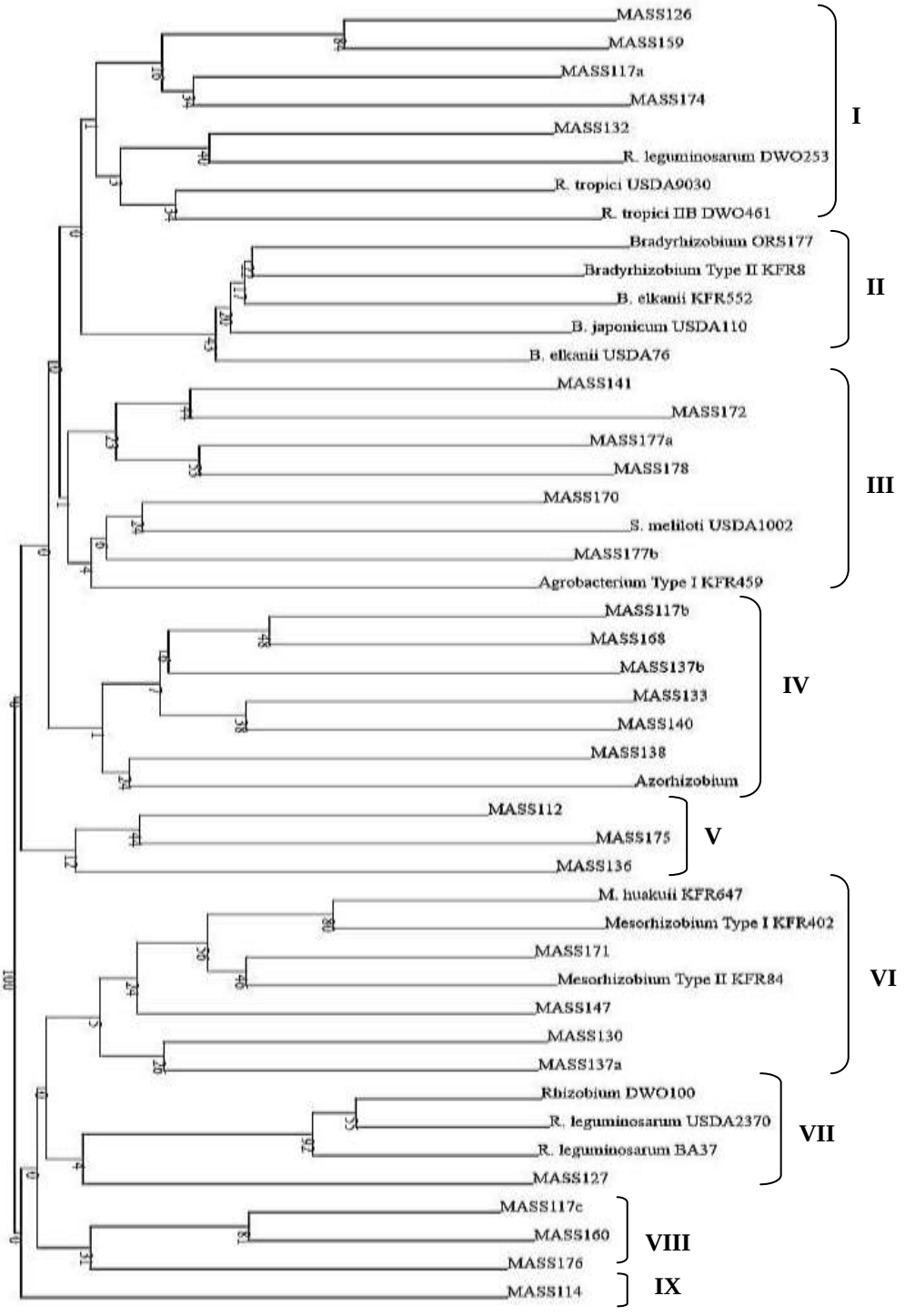

Figure 4.7a: Unrooted UPGMA dendrogram showing clusters generated by combined restriction patterns of amplified 16S rRNA (*HinfI*+ *Hinf*I + *Hae*III) of *S. sesban* rhizobia from Kenya and reference strains. Ribotypes are indicated by Roman numbers I–IX. Numbers on branches are bootstrap % from 1000 replicates.

Agrobacterium sp. shared a branch support at 6 % similarity index with the three isolates in the second sub-cluster. Two sub-clusters of isolates with a branch support of 1 % formed ribotype IV. The first sub-cluster contained isolates MASS117b, MASS168, MASS137b, MASS133 and MASS140 with varying similarities of between 1 % and 48 %. The second sub-cluster comprised of MASS138 which was similar to *Azorhizobium* sp. by 24 %. The unmatched ribotype V contained isolate MASS112 by a bootstrap of 44 % to MASS175 and similar to MASS136 by 12 %. Isolates MASS171, MASS147, MASS130 and MASS137a were all grouped in ribotype VI with varying similarities between 5 % and 56 % and were clustered together with *Mesorhizobium* spp. Ribotype VII contained MASS127 which was similar to *Rhizobium* spp. Type B with a similarity of 4 %. Ribotype VIII consisted of three isolates that were not matched to any of the reference strains. Isolate MASS117c was similar to MASS160 by a bootstrap of 81 % while MASS176 had a bootstrap of 31 %. Ribotype IX had a single isolate (MASS114) that was unmatched to any of the reference strains used in the present study (Figure 4.7a).

The highest percentage of isolates (20.70 %) was in ribotype III and IV (Table 4.7a). No rhizobial isolate from the Kenyan sites clustered with *Bradyrhizobium* spp. Bumala isolates were clustered with all ribotypes except VI. Isolates MASS117a, MASS117b and MASS117c from a single nodule collected from Gituamba site were grouped into ribotypes I, IV and VIII respectively. Isolate MASS137a and MASS137b (Kavutiri) clustered into ribotypes VI and IV respectively. Dual isolates MASS177a and MASS177b were both grouped with III (Table 4.7a).

Table 4.7a: *Sesbania sesban* rhizobial isolates from Kenya clustered using UPGMA based on combined patterns of 16S rRNA PCR–RFLP (*Msp*I, *Hinf*I and *Hae*III) compared to reference strains

Ribo-type	Reference	*Sesbania sesban* rhizobial isolate clusters	% ribo-type
I	*Rhizobium* spp. Type A	MASS126 (BK), MASS159 (BK), MASS117a (GK), MASS174 (BK), MASS132 (KvK).	17.20
II	*Bradyrhizobium* spp.	-	0.00
III	*Agrobacterium* spp./ *Sinorhizobium* spp.	MASS141 (KvK), MASS172 (KuK), MASS177a (BK), MASS178 (BK), MASS170 (KuK), MASS177b (BK).	20.70
IV	*Azorhizobium* spp.	MASS117b (GK), MASS168 (BK), MASS137b (KvK), MASS133 (KvK), MASS140 (KvK).	20.70
V	Unmatched	MASS112 (GK), MASS175 (BK), MASS136 (KvK).	10.30
VI	*Mesorhizobium* spp.	MASS130 (KvK), MASS137a (KvK), MASS147 (KvK), MASS171 (KuK).	13.80
VII	*Rhizobium* spp. Type B	MASS127 (BK).	3.50
VIII	Unmatched	MASS117c (GK), MASS160 (BK), MASS176 (BK).	10.30
IX	Unmatched	MASS114 (GK).	3.50

Sites of nodule collection, KuK, Kuinet; KvK, Kavutiri; GK, Gituamba; BK, Bumala; GK, Gituamba. (All in Kenya).

Rhizobial isolates from Uganda formed two clusters (I and II) (Figure 4.7b). Cluster I comprised of *Bradyrhizobium* spp., *Rhizobium* spp., *Sinorhizobium* spp., *Azorhizobium* sp. and isolates MASS60, MASS51a and MASS53. Isolate MASS60 was similar to *R. leguminosarum* DWO253 by 33 % similarity index and with *S. meliloti* USDA1002 by 6 %. Isolate MASS51a and MASS53 had a similarity of 39 % and shared the same branch support with *R. tropici* USDA9030 and *R. tropici* IIB DWO461 at a similarity of 6 %.

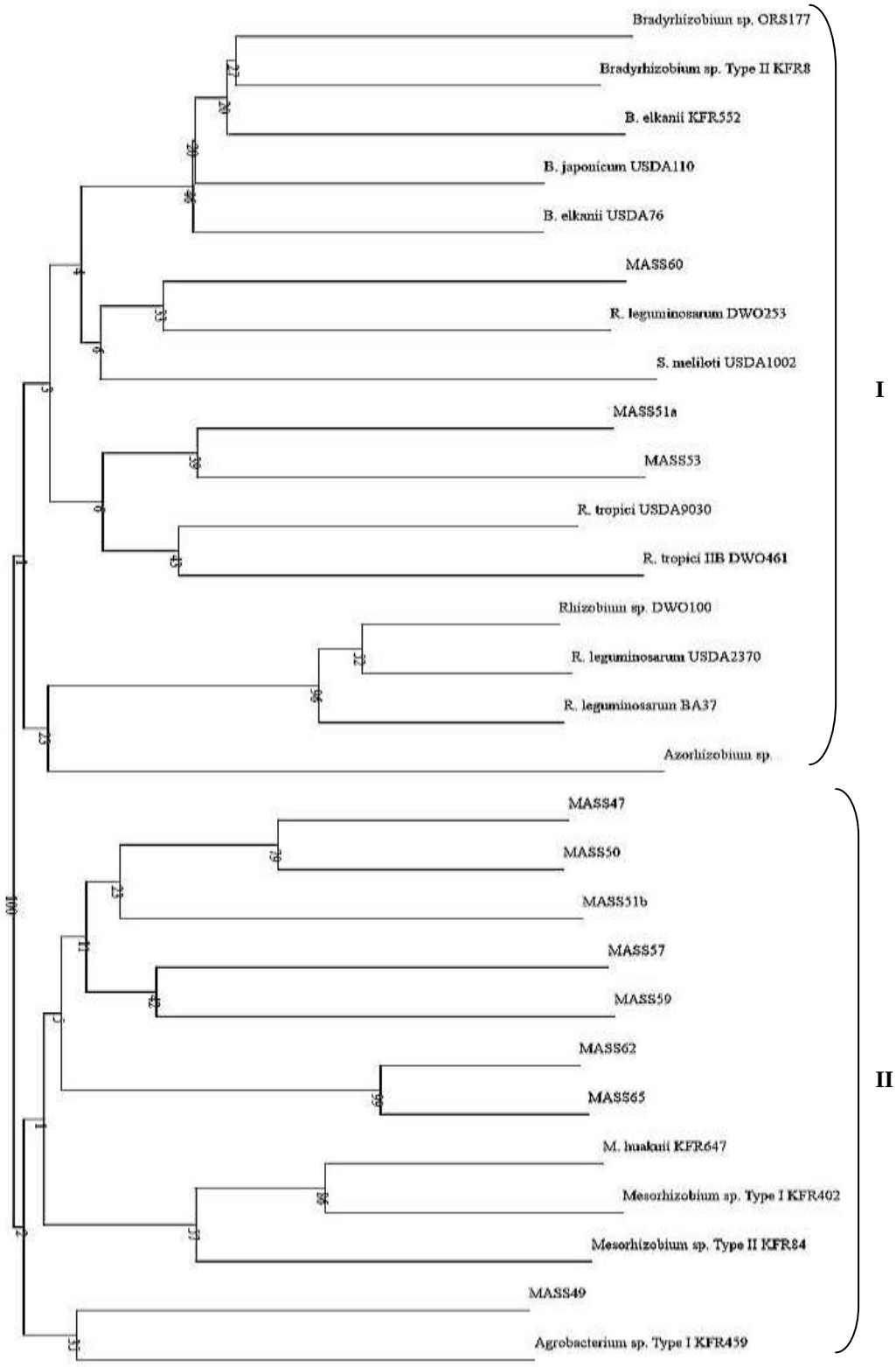

Figure 4.7b: Unrooted UPGMA dendrogram showing clusters generated by combined restriction patterns of amplified 16S rRNA (*HinfI*+ *Hinf*I + *Hae*III) of *S. sesban* rhizobial isolates from Uganda and reference strains. Ribotypes are indicated by Roman numbers I–II. Numbers on branches are bootstrap % from 1000 replicates.

The isolates MASS47, MASS51b, MASS57, MASS59, MASS62 and MASS65 with similarity between 5 % and 99 % shared a branch support at 1 % with *Mesorhizobium* spp. Isolate MASS49 was similar by 35 % to *Agrobacterium* sp. Type I KFR459 which also shared a branch support with *Mesorhizobium* spp. at 2 % similarity index (Figure 4.7b).

Ribotype I contained isolates MASS51a (Tororo), MASS53 (Tororo) and MASS60 (Mbale) which were closer to *Rhizobium* spp. Type A than *Bradyrhizobium* spp. (Table 4.7b). Isolates MASS47, MASS50, MASS51b and MASS49 from Tororo (Uganda), MASS57 and MASS59 from Mbale (Uganda), MASS62 and MASS65 from Kabale (Uganda) were in one cluster with *Mesorhizobium* spp. and *Agrobacterium* sp. and accounted for 72.70 % of all isolates.

Table 4.7b: *Sesbania sesban* rhizobial isolates from Uganda clustered using UPGMA based on combined patterns of 16S rRNA PCR–RFLP (*Msp*I, *Hinf*I and *Hae*III) compared to reference strains

Ribo-type	**Reference rhizobial strains**	***Sesbania sesban* rhizobial isolate clusters**	**% ribo–type**
I	*Bradyrhizobium* spp./ *Rhizobium* spp. Type A	MASS51a (TU), MASS53 (TU), MASS60 (MU).	27.30
II	*Mesorhizobium* spp./ *Agrobacterium* spp.	MASS47 (TU), MASS50 (TU), MASS51b (TU), MASS57 (MU), MASS59 (MU), MASS62 (KU), MASS65 (KU), MASS49 (TU).	72.70

Sites of nodule collection in parenthesis, TU, Tororo; KU, Kabale; MU, Mbale. (All in Uganda).

Rhizobial isolates from nodules of *S. sesban* grown in Tanzania were grouped into four clusters of ribotypes I–IV (Figure 4.7c). Cluster I comprised of *Bradyrhizobium*

spp. and was supported by a branch with similarity of 5 % with MASS42, *Sinorhizobium meliloti* USDA1002 and *Agrobacterium* Type I KFR459.

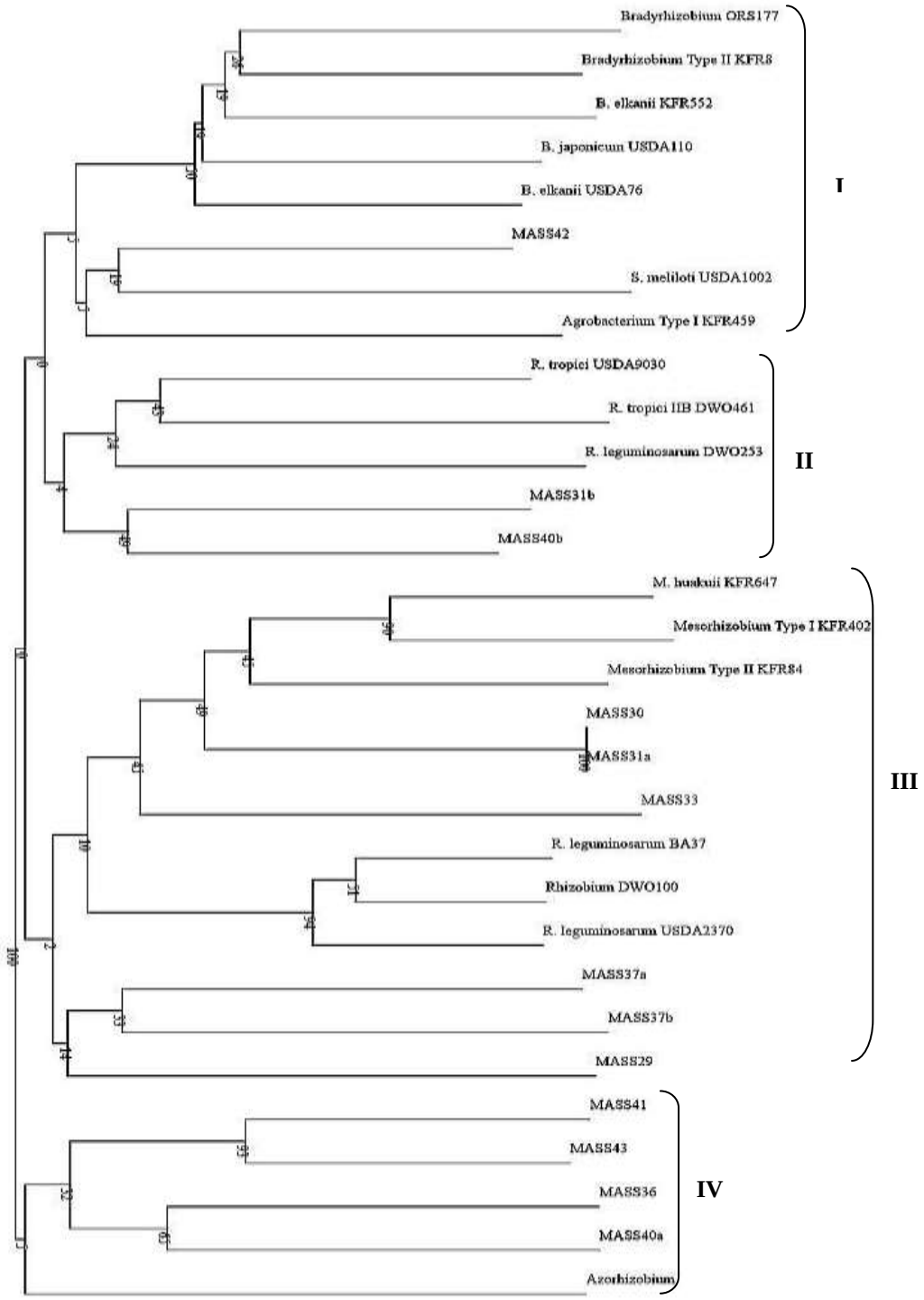

Figure 4.7c: Unrooted UPGMA dendrogram showing clusters generated by combined restriction patterns of amplified 16S rRNA (*HinfI*+ *Hinf*I + *Hae*III) of *S. sesban* rhizobial isolates from Tanzania and reference strains. Ribotypes are indicated by Roman numbers I–IV. Numbers on branches are bootstrap % from 1000 replicates.

Isolate MASS42 was closely clustered with *S. meliloti* USDA1002 with a similarity of 19 % than with *Agrobacterium* Type I KFR459 with 5 % similarity. Cluster II was composed of MASS31b and MASS40b with a similarity of 49 % and shared a branch support of 4 % with *Rhizobium* spp. Isolates MASS30 and MASS31a (100 % similar) and were grouped in cluster III together with *Mesorhizobium* spp. Isolate MASS33 shared one node with *Mesorhizobium* spp. with a similarity of 45 %. *Rhizobium* spp. shared a branch support with *Mesorhizobium* spp. at a similarity of 10 % while isolates MASS37a and MASS37b had a similarity of 33 % and shared the same branch support at similarity of 14 % with MASS29 in cluster III. *Mesorhizobium* spp. and *Rhizobium* spp. shared a branch support with isolates MASS37a, MASS37b and MASS29 at 2 % similarity. Cluster IV comprised of isolates MASS36, MASS40a, MASS41 and MASS43 which shared a branch support at 5 % with *Azorhizobium* sp. Isolates MASS41 and MASS43 had a similarity of 93 % while MASS36 and MASS40a had a similarity of 65 %. The two set of isolates of cluster IV shared a similarity branch support at 32 % (Figures 4.6c).

A *S. sesban* rhizobial isolate MASS42 collected from SUA (Tanzania) clustered in ribotype I while isolates MASS41 and MASS43 from the same site clustered in ribotype IV (Table 4.7c). Rhizobial isolates from Lushoto clustered in ribotype II and IV. Two pairs of nodule co–occupants recovered from Lushoto segregated separately into different ribotypes i.e. MASS31b (II) and MASS31a (III), MASS40b (II) and MASS40a (IV). Nodule co–occupants MASS37a and MASS37b were both clustered in ribotype III. Ribotype III had the highest percentage (46.10 %) of rhizobial isolates and comprised of isolates from Lushoto only. No isolate clustered with ribotype I.

Table 4.7c: *Sesbania sesban* rhizobial isolates from Tanzania clustered using UPGMA based on combined patterns of 16S rRNA PCR–RFLP (*Msp*I, *Hinf*I and *Hae*III) compared to reference strains

Ribo-type	Reference rhizobial strains	*Sesbania sesban* rhizobial isolate clusters	% ribo–type
	Bradyrhizobium spp.	-	0.00
I	*Sinorhizobium* spp./ *Agrobacterium* spp.	MASS42 (SUAT).	7.70
II	*Rhizobium* spp. Type A	MASS31b (LT), MASS40b (LT).	15.40
III	*Mesorhizobium* spp./ *Rhizobium* spp. Type B	MASS29 (LT), MASS30 (LT), MASS31a (LT), MASS33 (LT), MASS37a (LT), MASS37b (LT).	46.10
IV	*Azorhizobium* spp.	MASS36 (LT), MASS40a (LT), MASS41 (SUAT), MASS43 (SUAT).	30.80

Sites of nodule collection in parenthesis, LT, Lushoto; SUAT, Sokoine University of Agriculture. (All in Tanzania).

Rhizobia from Namibia were clustered into ten groups (I-X) (Figure 4.7d). Cluster I comprised of MN17, MN9, MN28, MN12 and *Bradyrhizobium* spp. Isolate MN17 had 83 % similarity with *Bradyrhizobium* sp. ORS177 and shared a branch support of 26 % with MN9 and MN28 which had a similarity of 41 %. Isolate MN12 shared a 7 % branch support with the *Bradyrhizobium* spp. Isolate MN34 was similar to the *Rhizobium* spp. by a similarity of 38 % in cluster II. Cluster III with three isolates MN51, MN50 and MN71 were similar to *Mesorhizobium* sp. Type II KFR84 by 13 %, 27 % and 37 % respectively. Cluster IV comprised of isolate MN56 which was similar by 46 % to *S. meliloti* USDA1002. Cluster V contained isolates MN18, MN19, MN35, MN36 and MN39 which did not match with the reference strains and had similarity indices of between 1 % and 31 %. Cluster VI had one isolate MN38 which had a similarity of 71 % with *R. tropici* IIB DWO461.

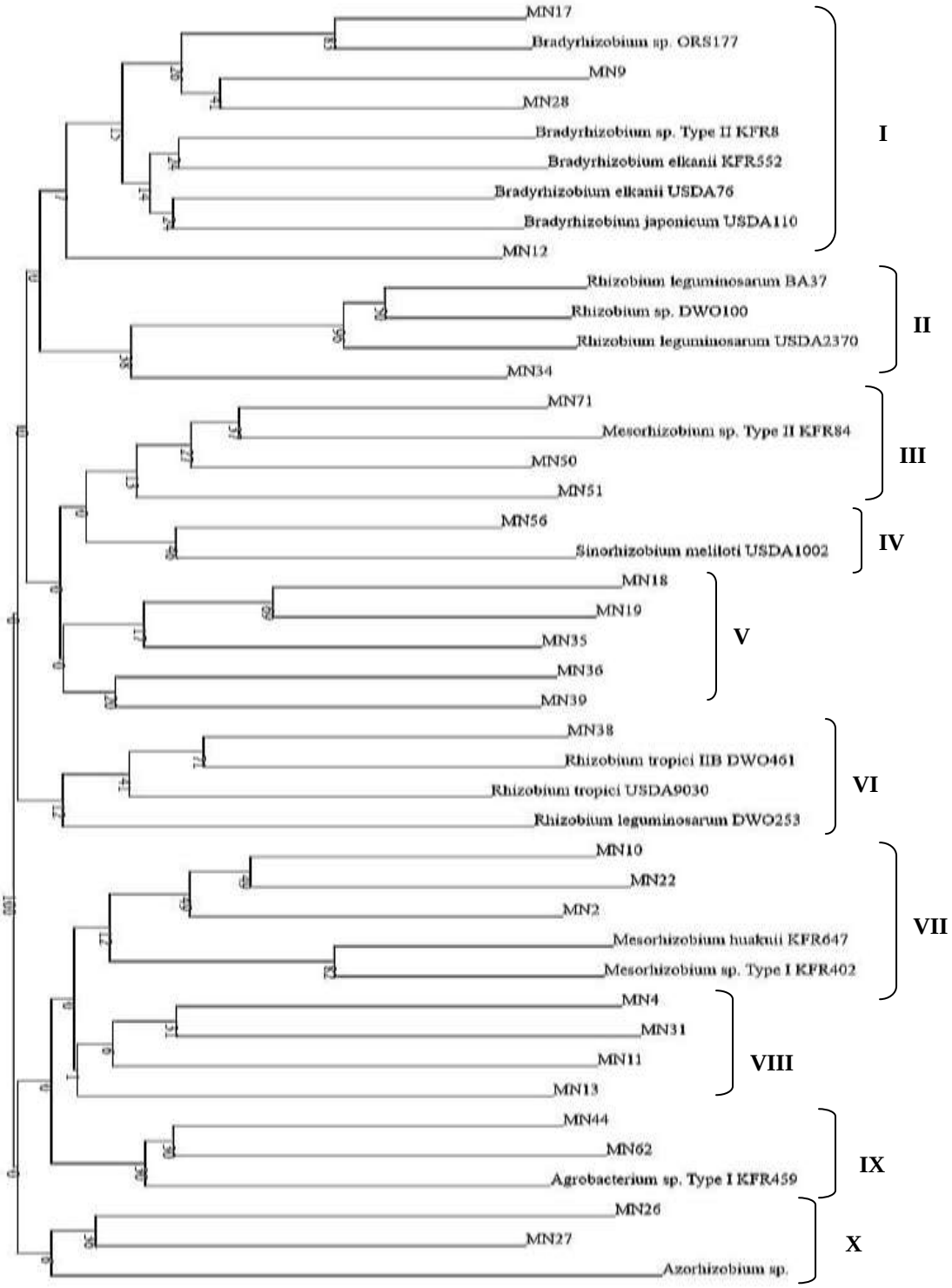

Figure 4.7d: Unrooted UPGMA dendrogram showing clusters generated by combined restriction patterns of amplified 16S rRNA (*HinfI*+ *Hinf*I + *Hae*III) of sesbania rhizobial isolates from Namibia and reference strains. Ribotypes are indicated by Roman numbers I–X. Numbers on branches are bootstrap % from 1000 replicates.

Isolates MN10, MN22 and MN2 in cluster VII had a similarity of 49 % and shared a branch support with *Mesorhizobium* spp. at a similarity of 12 % (Figure 4.6d). The isolates in cluster VIII comprised MN4, MN31, MN11 and MN13 which did not match with the reference strains and had similarity of between 1 % and 31 %. The two isolates in cluster IX were both similar to each other by 30 % and to *Agrobacterium* sp. Type I KFR459 by 30 %. Cluster X comprised of isolates MN26, MN27 and *Azorhizobium* sp. MN26 was similar to MN27 by 36 % and were both similar to *Azorhizobium* sp. by 6 %.

Sesbania rhizobial isolates recovered from Namibia were not ribotype specific apart from isolates from Khorixas Outijo (MN26, MN27) which entirely formed ribotype X and MN39 in ribotype V (Table 4.7d). Isolates MN9, MN51 and MN44 from Swakop were distributed in ribotypes I, III and IX respectively. Five isolates MN50, MN56, MN36, MN4 and MN62 from Rio Tinto Gorge were distributed in ribotypes III, IV, V, VIII and IX respectively. Isolates from Epupa falls were distributed in ribotypes V (MN18, MN19 and MN35), VI (MN38), VII (MN22) and VIII (MN13). Isolates MN28 and MN31 from Suclabo were clustered in ribotypes I and VIII respectively. Isolates MN17 in ribotype I, MN34 in ribotype II and MN11 in ribotype VIII were all isolated from sesbania root nodules collected from Omuramba. Both isolates MN2 and MN10 from Rooidrom were grouped into ribotype VII. Isolates MN12 and MN71 from Sesfontein were distributed in ribotypes I and III respectively. Ribotype V had the highest percent (19.30 %) of isolates while ribotypes II, IV and VI had the lowest percent (3.80 %) of isolates (Table 4.7d).

Table 4.7d: *Sesbania* spp. rhizobial isolates from Namibia clustered using UPGMA based on combined patterns of 16S rRNA PCR–RFLP (*Msp*I, *Hinf*I and *Hae*III) compared to reference strains

Ribo-type	Reference strains	Sesbania isolate clusters	% ribo–type
I	*Bradyrhizobium* spp.	MN9 (Nb), MN12 (Nh), MN17 (Nf), MN28 (Ne).	15.40
II	*Rhizobium* spp. Type B	MN34 (Nf).	3.80
III	*Mesorhizobium* spp. Type A	MN50 (Nc), MN51 (Nb), MN71 (Nh).	11.60
IV	*Sinorhizobium* spp.	MN56 (Nc).	3.80
V	Unmatched	MN18 (Nd), MN19 (Nd), MN35 (Nd), MN36 (Nc), MN39 (Na).	19.30
VI	*Rhizobium* spp. Type A	MN38 (Nd).	3.80
VII	*Mesorhizobium* spp. Type B	MN2 (Ng), MN10 (Ng), MN22 (Nd).	11.60
VIII	Unmatched	MN4 (Nc), MN11 (Nf), MN13 (Nd), MN31 (Ne).	15.40
IX	*Agrobacterium* spp.	MN44 (Nb), MN62 (Nc).	7.70
X	*Azorhizobium* spp.	MN26 (Na), MN27 (Na).	7.70

Sites of nodule collection in parenthesis, Na, Khorixas Outijo; Nb, Swakop; Nc, Rio Tinto Gorge; Nd, Epupa falls; Ne, Suclabo; Nf, Omuramba; Ng, Rooidrom; Nh, Sesfontein. (All in Namibia).

4.6 Symbiotic efficiency test of sesbania rhizobia on *Sesbania sesban* plants using Leonard jars in the greenhouse

Eighty three percent (83 %) of the 79 sesbania rhizobial isolates formed nodules on roots of *S. sesban*. Leaf colour of the eight-week old *S. sesban* plants varied from dark green to yellow. The most effective isolates caused dark green colour akin to the

Plus N treatment plants while the non-effective isolates caused yellow plants as those of Minus N treatment (Plate 4.9).

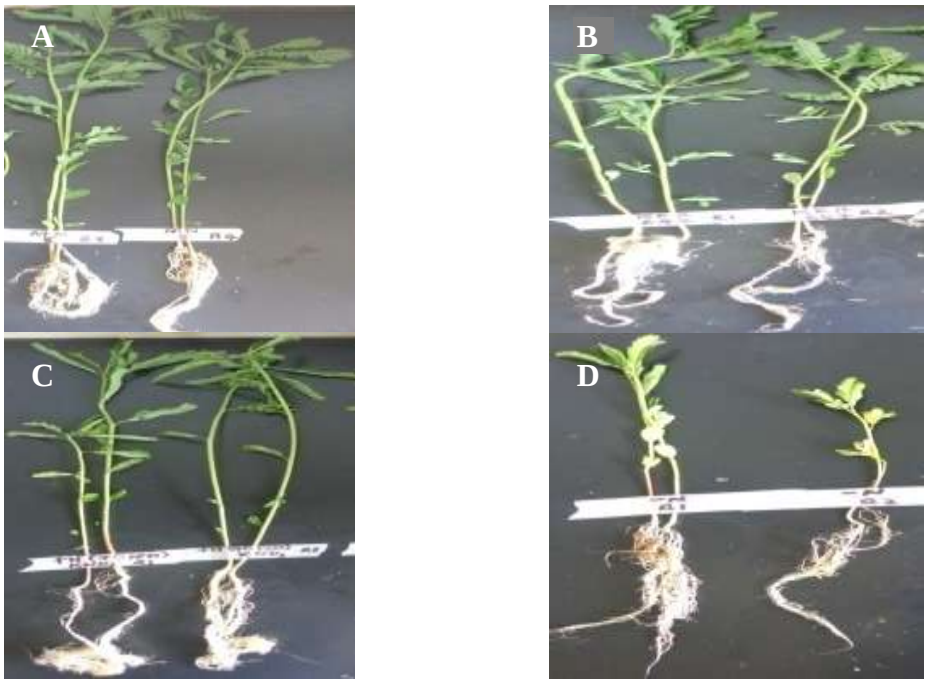

Plate 4.9: Eight week-old *S. sesban* plants inoculated with sesbania rhizobial isolates (A, MN8; B, KFR647); C, Plus N treatment (70 ppm N) (control) and D, Minus N treatment (control).

The nodules formed by all the isolates were morphologically similar but differed in symbiotic effectiveness. Cross-sections of nodules formed by effective isolates on roots of *S. sesban* were pink (Leghemoglobin present). Nodules formed by ineffective isolates were pale to white on their cross-sections (Leghemoglobin absent) (Plate 4.10). Nodules on roots of *S. sesban* were either oval or spherical with smooth surfaces.

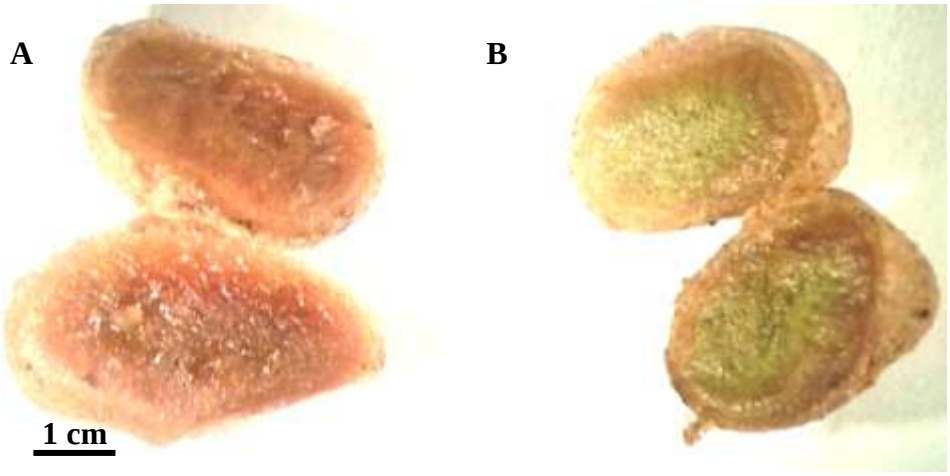

Plate 4.10: Transverse section of nodules formed by sesbania isolates on *S. sesban*. A, Leghemoglobin present; B, Leghemoglobin absent.

Shoot dry weight of *S. sesban* caused by inoculation using sesbania rhizobia ranged between 0.06 g (isolate MN17) and 1.06 g (isolate MASS172) (Table 4.8). Apart from MN19 (*S. macowaniana*) which caused a mean shoot dry weight of 0.79 g, fifteen *S. sesban* isolates recorded higher shoot dry weight compared to 0.36 g Minus N treatments (Control) of between 0.66 g (MASS177b) and 1.06 g (MASS172). Nodule dry weight ranged between 1.0 mg (MASS177a) and 94.0 mg (MASS125) while nodule number per plant ranged between 1 (MASS50) and 36 (MASS31a) (Table 4.8). Rhizobia included in this experiment from legumes other than sesbania [strains BA37 and DWO253 (*Phaseolus vulgaris*), KFR269 (Siratro) and KFR209 (*Faidherbia albida*)] also elicited nodules on roots of *S. sesban*. Plus N (uninoculated) treatments had a mean dry weight of 0.98 g while non-inoculated Minus N (control) treatments had 0.36 g. Both control treatments did not nodulate.

Table 4.8: Effect of sesbania rhizobial isolates from Kenya, Uganda, Tanzania and Namibia on shoot dry weight, nodule dry weight and nodule number of *S. sesban*

Isolate–Origin	SDWt. (g)	NDWt. (mg)	NNo.
Kuinet (Kenya)			
MASS170 *Sesbania sesban*	0.36±0.13	34.00±15.00	11.00±4.00
MASS171 *S. sesban*	0.75±0.06	64.00±14.00	24.00±4.00
MASS172 *S. sesban*	1.06±0.10	91.00±12.00	23.00±4.00
MASS173 *S. sesban*	0.44±0.05	53.00±10.00	21.00±4.00
MASS174 *S. sesban*	0.20±0.04	24.00±11.00	3.00±2.00
Kavutiri (Kenya)			
MASS129 *S. sesban*	0.89±0.17	84.00±20.00	23.00±4.00
MASS130 *S. sesban*	0.72±0.13	58.00±26.00	14.00±3.00
MASS133 *S. sesban*	0.21±0.06	31.00±19.00	3.00±1.00
MASS134 *S. sesban*	0.79±0.18	72.00±14.00	20.00±3.00
MASS136 *S. sesban*	0.09±0.03	16.00±7.00	20.00±7.00
MASS137b *S. sesban* .	0.12±0.04	14.00±4.00	6.00±2.00
MASS138 *S. sesban*	0.20±0.03	21.00±3.00	18.00±3.00
MASS141 *S. sesban*	0.61±0.10	62.00±15.00	15.00±2.00
MASS147*S. sesban*	0.82±0.22	87.00±11.00	29.00±5.00
MASS149 *S. sesban*	0.25±0.03	7.00±3.00	2.00±1.00
Gituamba (Kenya)			
MASS110 *S. sesban*	0.57±0.18	78.00±12.00	21.00±5.00
MASS111 *S. sesban*	0.72±0.08	73.00±13.00	29.00±4.00
MASS114 *S. sesban*	0.37±0.07	33.00±8.00	11.00±2.00
MASS115 *S. sesban*	0.38±0.06	39.00±8.00	14.00±2.00
MASS117a *S. sesban*	0.16±0.08	19.00±1.00	5.00±4.00
MASS117b *S. sesban*	0.16±0.05	3.00±1.00	1.00±0.00
MASS117c *S. sesban*	0.40±0.08	51.00±13.00	18.00±3.00
MASS117abc *S. sesban*	0.34±0.11	26.00±15.50	11.00±6.00
Bumala (Kenya)			
MASS123 *S. sesban*	0.33±0.06	44.00±9.00	21.00±3.00
MASS125 *S. sesban*	0.60±0.14	94.00±16.0	19.00±5.00
MASS126 *S. sesban*	0.14±0.02	9.00±3.00	2.00±1.00
MASS160 *S. sesban*	0.31±0.04	35.00±8.00	11.00±3.00
MASS168 *S. sesban*	0.15±0.06	23.00±16.00	10.00±5.00
MASS177a *S. sesban*	0.10±0.02	1.00±0.20	5.00±2.00

MASS177b *S. sesban*	0.66±0.12	67.00±22.00	21.00±5.00
MASS178 *S. sesban*	0.24±0.03	20.00±16.00	6.00±3.00
Lushoto (Tanzania)			
MASS29 *S. sesban*	0.11±0.02	14.00±3.00	10.00±2.00
MASS30 *S. sesban*	0.32±0.05	27.00±6.00	11.00±3.00
MASS31a *S. sesban*	0.47±0.08	59.00±13.00	36.00±5.00
MASS31b *S. sesban*	0.09±0.02	1.00±0.10	1.00±1.00
MASS33 *S. sesban*	0.09±0.03	2.00±0.70	1.00±1.00
MASS36 *S. sesban*	0.10±0.02	12.00±3.00	6.00±1.00
MASS37a *S. sesban*	0.07±0.02	8.00±2.00	8.00±2.00
MASS37b *S. sesban*	0.30±0.08	30.00±11.00	15.00±3.00
MASS38 *S. sesban*	0.22±0.05	17.00±5.00	12.00±6.00
MASS39 *S. sesban*	0.68±0.03	64.00±8.00	24.00±5.00
MASS40b *S. sesban*	0.35±0.10	50.00±15.00	13.00±3.00
MASS42 *S. sesban*	0.13±0.02	6.00±3.00	2.00±1.00
SUA (Tanzania)			
MASS41 *S. sesban*	0.38±0.05	36.00±5.00	13.00±1.00
MASS43 *S. sesban*	0.28±0.05	29.00±5.00	15.00±2.00
Tororo (Uganda)			
MASS47 *S. sesban*	0.33±0.05	39.00±10.00	17.00±4.00
MASS48 *S. sesban*	0.16±0.03	4.00±2.00	3.00±1.00
MASS49 *S. sesban*	0.22±0.04	19.00±9.00	4.00±2.00
MASS50ab *S. sesban*	0.08±0.03	1.00±0.30	1.00±1.00
MASS50b *S. sesban*	0.45±0.07	47.00±11.00	13.00±4.00
MASS51a *S. sesban*	0.15±0.03	10.00±5.00	4.00±2.00
MASS51abc *S. sesban*	0.14±0.02	4.00±1.00	4.00±1.00
MASS51c *S. sesban*	0.18±0.02	9.00±6.00	2.00±1.00
MASS53 *S. sesban*	0.33±0.06	42.00±8.00	19.00±4.00
Kabale (Uganda)			
MASS62 *S. sesban*	0.42±0.07	63.00±12.00	20.00±3.00
MASS65 *S. sesban*	0.17±0.03	12.00±6.00	3.00±2.00
MASS69 *S. sesban*	0.46±0.18	50.00±7.00	19.00±3.00
Mbale (Uganda)			
MASS54 *S. sesban*	0.35±0.05	33.00±5.00	15.00±4.00
MASS57 *S. sesban*	0.24±0.06	43.00±21.00	6.00±4.00
MASS59 *S. sesban*	0.83±0.18	71.00±17.00	27.00±5.00

MASS60 *S. sesban*	0.16±0.04	15.00±5.00	3.00±2.00
MASS61 *S. sesban*	0.74±0.12	61.00±8.00	19.00±3.00
Namibia			
MN1*Sesbania macowaniana*	0.14±0.04	20.00±8.00	8.00±2.00
MN2 *S. sphaerosperma*	0.13±0.04	12.00±6.00	4.00±2.00
MN8 *S. sesban*	0.78±0.12	73.00±9.00	30.00±4.00
MN10 *S. sphaerosperma*	0.22±0.05	48.00±35.00	5.00±3.00
MN11 *S. sphaerosperma*	0.21±0.06	25.00±10.0	26.00±3.00
MN12 *S. sphaerosperma*	0.27±0.04	18.00±7.00	10.00±4.00
MN13 *S. sesban*	0.06±0.02	16.00±0.20	1.00±1.00
MN17 *S. bispinosa*	0.06±0.01	6.00±1.00	10.00±1.00
MN18 *S. sesban*	0.84±0.19	75.00±18.00	25.00±5.00
MN19 *S. macowaniana*	0.79±0.12	65.00±7.00	21.00±2.00
MN21 *S. sesban*	0.16±0.04	18.00±9.00	4.00±3.00
MN22 *S. macowaniana*	0.18±0.03	12.00±9.00	6.00±3.00
MN28 *S. cinerascens*	0.06±0.01	8.00±1.00	14.00±2.00
MN31 *S. cinerascens*	0.17±0.02	24.00±5.00	23.00±6.00
MN39 *S. macowaniana*	0.14±0.03	2.00±1.00	2.00±1.00
MN40 *S. cinerascens*	0.37±0.10	34.00±10.00	9.00±3.00
MN44 *S. pachycarpa*	0.32±0.06	43.00±19.00	7.00±3.00
MN45 *S. pachycarpa*	0.15±0.02	14.00±4.00	7.00±2.00
MN49 *S. pachycarpa*	0.28±0.06	29.00±5.00	11.00±3.00
MN50 *S. pachycarpa*	0.18±0.02	18.00±3.00	20.00±4.00
MN51 *S. pachycarpa*	0.23±0.01	28.00±2.00	24.00±3.00
MN57 *S. sesban*	0.59±0.13	43.00±11.00	14.00±3.00
MN58 *S. pachycarpa*	0.32±0.41	20.00±15.00	4.00±1.00
MN62 *S. pachycarpa*	0.13±0.02	9.00±8.00	2.00±2.00
MN68 *S. sesban*	0.93±0.14	54.00±10.00	32.00±6.00
MN69 *S. microphylla*	0.19±0.03	22.00±5.00	19.00±3.00
MN70 *S. microphylla*	0.13±0.01	19.00±1.00	31.00±3.00
MN71 *S. microphylla*	0.15±0.04	10.00±6.00	3.00±1.00
Reference strains			
Kibwezi (Kenya)			
DWO253 *Phaseolus vulgaris*	0.21±0.02	18.00±4.00	11.00±5.00
KFR269 Siratro	0.59±0.03	72.00±17.00	25.00±5.00
Loruk (Kenya)			

KFR209 *Faidherbia albida*	0.60±0.17	52.00±12.00	16.00±3.00
BA37 *P. vulgaris*	0.16±0.07	15.00±3.00	28.00±3.00
Nyamonye (Kenya)			
KFR647 *S. sesban*	0.60±0.19	60.00±11.00	22.00±3.00
KFR402 *S. sesban*	0.70±0.04	85.00±12.00	20.00±3.00
Controls			
0 ppm N	0.36±0.17	-	0
70 ppm N	0.98±0.21	-	0

SDWt., shoot dry weight; NDWt., nodule dry weight; NNo., nodule number; Sokoine University of Agriculture Tanzania. Values represent the mean ± SD of eight replicates.

Ten of the 13 isolates from Kavutiri elicited nodules on roots of *S. sesban* but only five had effective nodules (*nod*+*nif*+ phenotypes) (Table 4.9a). All isolates from Kuinet nodulated *S. sesban*, but only two exhibited *nod*+*nif*+ phenotypes. Eight of the 12 isolates from Bumala had *nod*+ but only two had *nod*+*nif*+ phenotypes. All isolates from Gituamba nodulated *S. sesban*, two of the isolates (MASS110 and MASS111) were effective while one of the isolates (MASS117c) was partially effective. A pair of nodule co–occupants were isolated each from *S. sesban* growing in Kavutiri and Bumala while triple isolates co–occupied a nodule collected from Gituamba. The co–occupant isolates included MASS137a and MASS137b (Kavutiri), MASS177a and MASS177b (Bumala) and MASS117a, MASS117b and MASS117c (Gituamba). Co–occupant isolates MASS137a and MASS137b of nodules collected from Kavutiri site were non–infective (*nod*-) and infective/ineffective (*nod*+*nif*-) phenotypes respectively. The Bumala isolate MASS177a was *nod*+*nif*- while its nodule co–occupant MASS177b was both infective and effective (*nod*+*nif*+) phenotype. The three Gituamba nodule co–occupant isolates were of phenotypes *nod*+*nif*- (MASS117a and MASS117b) and partially effective (*nod*+*nif*+/-) phenotype (MASS117c).

Table 4.9a: Nodulation and nitrogen fixation phenotypes of *Sesbania sesban* rhizobial isolates from Kenya on *S. sesban*

Site	***nod-***	***nod+ nif-***	***nod+nif+/-***	***nod+ nif+***
Kavutiri	MASS131 MASS137a MASS137ab MASS140	MASS133 MASS136 MASS137b MASS138 MASS149	–	MASS129 MASS130 MASS134 MASS141 MASS147
Kuinet	–	MASS170 MASS173 MASS174	–	MASS171 MASS172
Bumala	MASS127 MASS157 MASS167 MASS176 MASS177ab	MASS123 MASS126 MASS160 MASS168 MASS177a MASS178	–	MASS125 MASS177b
Gituamba	–	MASS114 MASS115 MASS117a MASS117b MASS117abc	MASS117c	MASS110 MASS111

nod-, non–infective; *nod+*, infective; *nif-*, ineffective; *nif+/-*, partially effective; *nif+*, effective.

Two isolates from Kabale (Uganda) were partially effective on *S. sesban*; one was ineffective while one failed to nodulate *S. sesban* (Table 4.9b). Similarly, isolates MASS50b and MASS53 from Tororo were partially effective; five isolates (MASS47, MASS48, MASS49, MASS51a and MASS51c) were ineffective while three isolates (MASS50a, MASS51b and MASS52) were non–infective on *S. sesban*. Mixed isolates from single nodules (MASS50ab and MASS51abc) were non–effective on their host of origin. Two isolates (MASS59 and MASS61) recovered from nodules from Mbale were effective on *S. sesban* while MASS54 isolate was partially effective.

Table 4.9b: Nodulation and nitrogen fixation phenotypes of *Sesbania sesban* rhizobial isolates from Uganda on *S. sesban*

Site	***nod-***	***nod+nif-***	***nod+nif+/-***	***nod+nif+***
Kabale	MASS68	MASS65	MASS62	–
			MASS69	
Tororo	MASS50a	MASS47	MASS50b	–
	MASS51b	MASS48	MASS53	
	MASS52	MASS49		
		MASS50ab		
		MASS51a		
		MASS51c		
		MASS51abc		
Mbale	–	MASS57	MASS54	MASS59
		MASS60		MASS61

nod-, non–infective; *nod+*, infective; *nif-*, ineffective; *nif+/-*, partially effective; *nif+*, effective.

Three isolates from SUA formed nodules on the host of origin but only MASS41 was partially effective (Table 4.9c). Eleven of the twelve isolates from Lushoto formed nodules on *S. sesban*. Isolate MASS39 was effective while MASS31a and MASS40b were partially effective. Mixed isolates from single nodules (MASS37ab and MASS40ab) were ineffective on *S. sesban*.

Table 4.9c: Nodulation and nitrogen fixation phenotypes of *Sesbania sesban* rhizobial isolates from Tanzania on *S. sesban*

Site	***nod-***	***nod+nif-***	***nod+nif+/-***	***nod+nif+***
SUA	–	MASS42	MASS41	–
		MASS43		
Lushoto	MASS37ab	MASS29	MASS31a	MASS39
	MASS40a	MASS30	MASS40b	
	MASS40ab	MASS31b		
		MASS33		
		MASS36		
		MASS37a		
		MASS37b		
		MASS38		

nod-, non–infective; *nod+*, infective; *nif-*, ineffective; *nif+/-*, partially effective; *nif+*, effective.

Five isolates from nodule collection sites in Namibia did not form nodules on *S. sesban* (Table 4.9d). Twenty one isolates formed ineffective nodules. Two isolates (MN44 and MN49) from Swakop were partially effective on *S. sesban*. Four isolates (MN8, MN18, MN19 and MN57) from Epupa falls and a single isolate (MN68) from Otjinungua were effective on *S. sesban.*

Table 4.9d: Nodulation and nitrogen fixation phenotypes of sesbania rhizobial isolates from Namibia on *S. sesban*

Site	***nod-***	***nod+nif-***	***nod+nif+/-***	***nod+nif+***
Khorixas Outijo	–	MN39	–	–
Swakop	MN9	MN51	MN44	–
			MN49	
Rio Tinto Gorge	MN54	MN45	–	–
	MN56	MN58		
		MN62		
Epupa falls	–	MN13	–	MN8
		MN21		MN18
		MN22		MN19
				MN57
Suclabo	MN37	MN28	–	–
		MN31		
		MN40		
Omuramba	–	MN11	–	–
		MN17		
Rooidrom	–	MN2	–	–
		MN10		
Sesfontein	–	MN12	–	–
		MN71		
Okahandja	MN15	MN1	–	–
Otjinungua	–	–	–	MN68
Bunya	–	MN69	–	–
		MN70		
Korokoko	–	MN50	–	–

nod-, non–infective; *nod+*, infective; *nif-*, ineffective; *nif+/-*, partially effective; *nif+*, effective.

All the reference strains (KFR209, DWO253, KFR269, KFR647 and KFR402) used in *S. sesban* nodulation test were effective except the common bean inoculant strain, BA37 which was infective but ineffective (Table 4.9e).

Table 4.9e: Nodulation and nitrogen fixation phenotypes of reference strains on *S. sesban*

Site	***nod-***	***nod+nif-***	***nod+nif+/-***	***nod+nif+***
Gituamba	–	BA37 (*P. vulgaris*)	–	–
Loruk	–	–	–	KFR209 (*F. albida*)
Kibwezi	–	–	–	DWO253 (*P. vulgaris*), KFR269 (Siratro)
Yala swamp	–	–	–	KFR647 (*S. sesban*)
Nyamonye	–	–	–	KFR402 (*S. sesban*)

nod-, non–infective; *nod+*, infective; *nif-*, ineffective; *nif+/-*, partially effective; *nif+*, effective.

There were significant differences ($p < 0.001$) in mean percent nitrogen and nitrogen content per shoot of *S. sesban* inoculated using selected rhizobia from various legume hosts (Table 4.10). The lowest percent nitrogen was 1.35 (isolate MASS134) while the highest was 7.9 (isolate MN19). Common bean inoculant strain (BA37) caused the lowest nitrogen content per shoot (0.16 mg shoot^{-1}) while isolate MASS129 caused the highest (5.66 mg shoot^{-1}). Nitrogen fixation ratio varied from -0.28 (BA37) to 5.23 (isolate MASS129). The symbiotic effectiveness of selected rhizobia on *S. sesban* also varied with the rhizobial isolate used. Three isolates were ineffective, twenty effective and three highly effective.

Table 4.10: Effect of some sesbania rhizobial isolates on percent nitrogen, N content, N fixation ratio and the symbiotic effectiveness of *S. sesban*

Isolate/Origin	% N shoot $^{-1}$	N content (mg) shoot^{-1}	N fixation ratio	SE
Kuinet (Kenya)				
MASS171 *S. sesban*	4.08±0.18fg	4.15±0.49de	3.71	E
MASS172 *S. sesban*	1.87±0.23ab	1.99±0.31b-e	1.56	HE
Kavutiri (Kenya)				
MASS129 *S. sesban*	5.65±0.67h	5.66±0.64e	5.23	HE
MASS130 *S. sesban*	5.56±0.11h	2.78±0.29a-e	4.54	E
MASS134 *S. sesban*	1.35±0.10a	1.03±0.56abc	0.14	E
MASS141 *S. sesban*	4.09±0.13fg	2.43±0.32abc	1.99	E
MASS147*S. sesban*	4.00±0.42fg	3.10±0.49a-e	2.66	E
Gituamba (Kenya)				
MASS110 *S. sesban*	2.12±0.20abc	1.11±0.24a-d	0.68	E
MASS111 *S. sesban*	2.12±0.15abc	1.52±0.17ab	0.64	E
Bumala (Kenya)				
MASS125 *S. sesban*	5.41±0.23h	2.00±0.41b-e	1.57	E
MASS177b *S. sesban*	3.22±0.12c–f	2.03±0.07a-e	1.59	E
Lushoto (Tanzania)				
MASS39 *S. sesban*	1.98±0.18ab	1.35±0.10ab	0.46	E
Kabale (Uganda)				
MASS62 *S. sesban*	3.39±0.03ef	1.13±0.26ab	0.70	I
MASS69 *S. sesban*	2.36±0.09a–e	1.52±0.19abc	1.08	I
Mbale (Uganda)				
MASS59 *S. sesban*	2.25±0.13a–d	1.86±0.36abc	1.62	E
MASS61 *S. sesban*	1.37±0.08a	1.01±0.01ab	0.13	E
Namibia				
MN8 *S. sesban*	5.46±0.17h	4.01±0.40cde	3.58	E
MN18 *S. sesban*	2.96±0.18b–f	2.47±0.32a-e	1.59	E
MN19 *S. macowaniana*	7.90±1.02i	4.14±0.02a-d	3.71	E
MN57 *S. sesban*	3.88±0.20fg	3.80±0.41b-e	3.36	E
MN68 *S. sesban*	3.35±0.28def	2.71±0.12a-e	2.28	HE
Reference strains				
Kibwezi (Kenya)				
KFR269 Siratro	1.66±0.14a	0.97±0.14ab	0.09	E

Loruk (Kenya)				
KFR209 *Faidherbia albida*	4.54±1.15gh	1.74±0.26a-e	1.31	E
BA37 *P. vulgaris*	2.04±0.19ab	0.16±0.03a	-0.28	I
Nyamonye (Kenya)				
KFR647 *S. sesban*	3.23±0.20c–f	2.19±0.16a-d	1.76	E
KFR402 *S. sesban*	2.20±0.20abc	1.20±0.21abc	0.77	E
Controls				
0 ppm N	2.45±0.10a–e	0.43±0.05a	0.00	–
70 ppm N	3.93±0.15fg	3.70±0.39b-e	0.00	–

Means within a column followed by the same letter(s) are not significant different according to Tukey HSD at 5 %. Values are means of four replicates ± standard errors. SE (%), symbiotic effectiveness; I, ineffective; E, effective; HE, highly effective.

Rhizobial isolates MN57, MN8, MN19, MASS171, MASS130, MASS129 and MASS172, recovered from sesbania had a higher SE on *S. sesban* compared to plus N control (Figure 4.8). Isolate MASS172 had the highest SE compared to all other isolates used in this study.

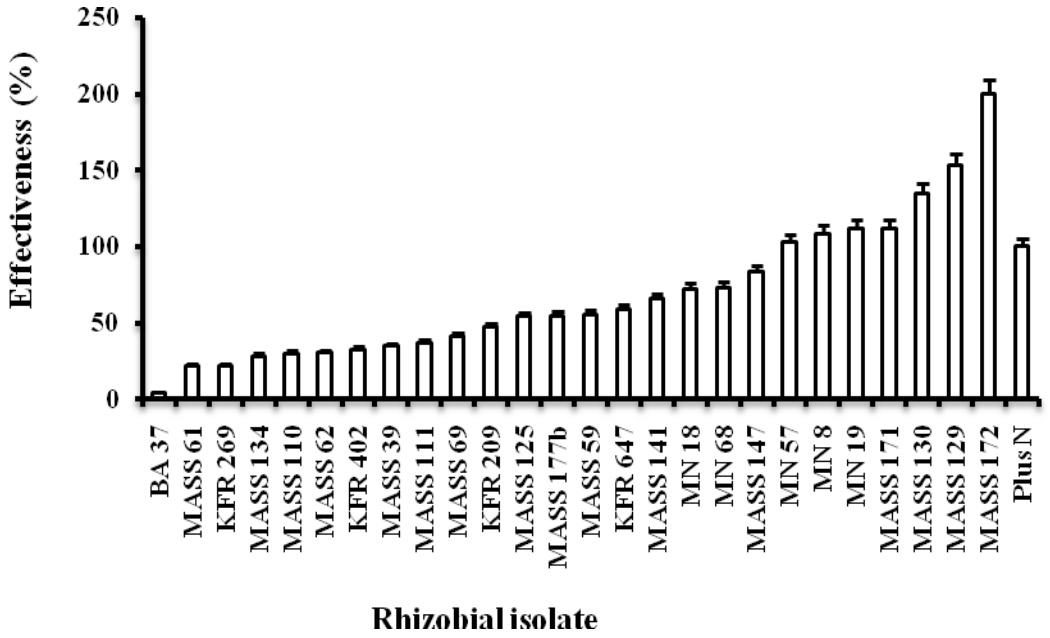

Figure 4.8: Effectiveness of some selected sesbania rhizobial isolates on *S. sesban* plants. Error bars with 5 %.

4.6.1 Correlation among shoot dry weight, nodule number, nodule dry weight, nitrogen concentration and SE of *S. sesban* plants

There was no correlation ($r^2 = 0.191$, $p = 0.097$) between the number of nodules per *S. sesban* plant and the respective shoot dry weight (Table 4.11). There was a very strong positive correlation ($r^2 = 0.836$, $p < 0.001$) between shoot dry weight of *S. sesban* inoculated using sesbania rhizobia and the respective nodule dry weight. There was also a strong positive correlation ($r^2 = 0.818$, $p < 0.001$) between nodule number per plant and dry weight of nodules following inoculation using sesbania rhizobia. There was no correlation ($r^2 = 0.171$, $p = 0.395$) between percent N per shoot of *S. sesban* and shoot dry weight. No correlation (correlation ($r^2 = -0.142$, $p = 0.473$) was recorded between percent N per shoot of *S. sesban* and number of

nodules per plant (Table 4.11). Similarly, there was no correlation (r^2 = 0.257, p = 0.089) between percent N per per shoot and nodule dry weight. Higher symbiotic effectiveness significantly correlated positively (r^2 = 0.499, $p < 0.05$) with shoot dry weight. There was no significant correlation (r^2 = 0.083, p =0.224) between SE and the number of nodules per *S. sesban* plant. A significant correlation (r^2 = 0.479, $p < 0.05$) was recorded between SE and nodule dry weight per plant. Likewise, there was a significant correlation (r^2 = 0.933, $p < 0.001$) between % N per plant and SE in *S. sesban* (Table 4.11).

Table 4.11: Correlation between shoot dry weights, nodule number, nodule dry weight, nitrogen concentration and symbiotic effectiveness of rhizobia in *S. sesban*

		SDWt.	**NNo.**	**NDWt.**	**% N shoot^{-1}**	**SE**
SDWt.		1				
NNo.	Pearson Correlation	0.191	1			
	Sig. (2–tailed)	0.097				
NDWt.	Pearson Correlation	0.836**	0.818**	1		
	Sig. (2–tailed)	<0.001	< 0.001			
% N shoot^{-1}	Pearson Correlation	0.171	–0.142	0.257	1	
	Sig. (2–tailed)	0.395	0.473	0.089		
SE	Pearson Correlation	0.499*	0.083	0.479*	0.933**	1
	Sig. (2–tailed)	<0.05	0.224	< 0.05	< 0.001	

*,** correlation is significant at 0.05 and 0.001 level (2–tailed) respectively. SDWt., shoot dry weight; NDWt., nodule dry weight; NNo., nodule number; SE (%), symbiotic effectiveness.

4.7 Symbiotic effectiveness test of sesbania rhizobial isolates on Rose coco bean plants using Leonard jars

The deliberate introduction of rhizobial isolates from sesbania on roots of common bean seedlings allowed determination of microsymbionts' infectivity and effectiveness. The leaf colour of common beans inoculated using sesbania rhizobial isolates were yellowish green while those of Minus N treatment (controls) were yellow at harvesting time (Plate 4.11).

Plate 4.11: Four week–old common bean plants inoculated with sesbania rhizobial isolates (A, MASS133); B, BA37 (common bean inoculant strain); C, Plus N treatment (70 ppm N) (control) and D, Minus N treatment (control).

Nodules recovered from the roots of common bean were globose, determinate and measured between 1–5 mm in diameter. Some of the nodules had a smooth surface as those found on roots of sesbania while others had a rough surface typical of bean nodules. Nodules were observed on entire fibrous roots of the rhizobia infected common beans (Plate 4.12).

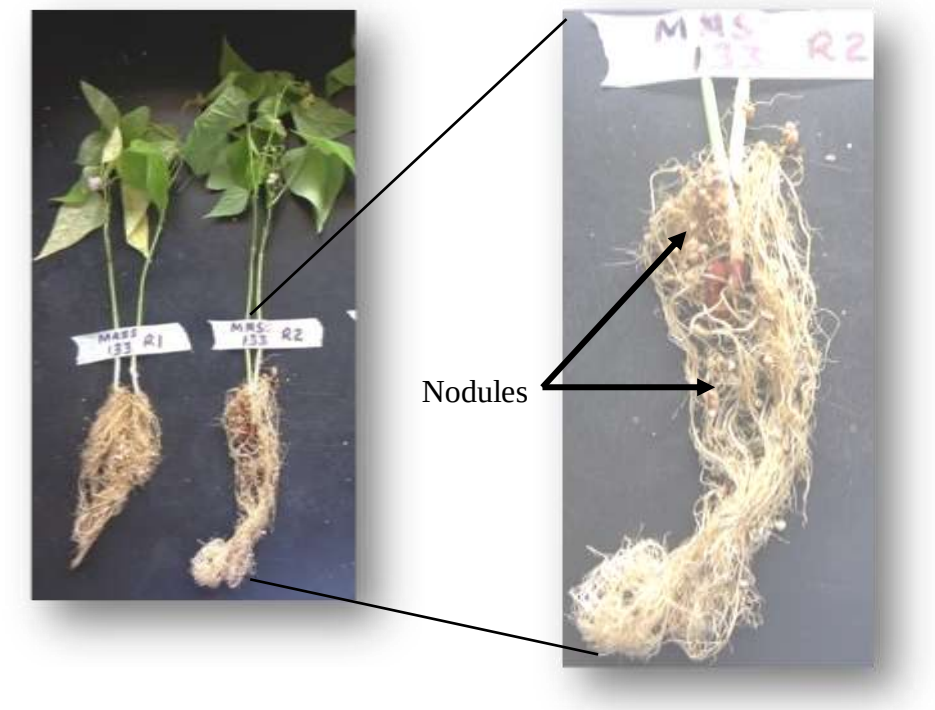

Plate 4.12. Nodules on roots of four–week old common bean inoculated with *S. sesban* rhizobial strain MASS133.

Cross section of common bean nodules formed by effective isolates were large and pink (Leghemoglobin present) while those formed by ineffective isolates were small and green (Leghemoglobin absent) (Plate 4.13). Nodules borne on roots of common beans were spherical with either rough or smooth surfaces.

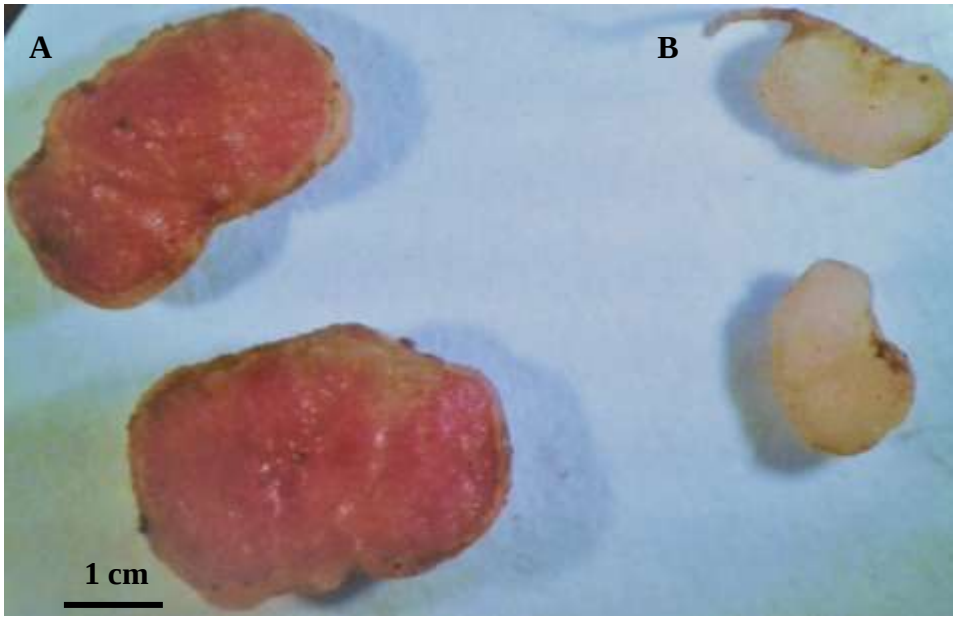

Plate 4.13: Transverse section of nodules formed by sesbania isolates on common bean. A, Leghemoglobin present; B, Leghemoglobin absent.

Fifteen out of the 79 rhizobia recovered from root nodules of various sesbania elicited nodules on the roots of common bean. There was no nodulation on roots of Plus N and Minus N treatments (control). The number of nodules on roots inoculated bean treatments ranged between 3 and 63 and was significantly different ($p < 0.001$). The nodule dry weight varied significantly ($p < 0.001$) and ranged between 0.5 mg (MASS31a) and 153 mg (MASS149). Seventy three percent of the infective isolates originated from *S. sesban* root nodules. The mean shoot dry weight accumulated by nodulated common bean was not significantly different ($p = 0.244$) and ranged between 0.45 g (MASS31a) and 0.87 g (MASS133) per plant (Table 4.12).

Table 4.12: Effect of sesbania rhizobial isolates on shoot dry weight, nodule dry weight and nodule number of common beans

Isolate–Origin	SDWt. (g)	NDWt. (mg)	NNo.
Kavutiri (Kenya)			
MASS133 *Sesbania sesban*	0.87±0.03abc	145.6±44.20bc	63.00±15.00b
MASS137a *S. sesban*	0.69±0.09ab	61.8±20.10a–c	36.00±9.00ab
MASS149 *S. sesban*	0.55±0.05a	153.0 ±62.40c	33.00 ±16.00ab
Lushoto (Tanzania)			
MASS30 *S. sesban*	0.51±0.11a	5.0±1.90a	5.00±2.00a
MASS31a *S. sesban*	0.45±0.06a	0.5±0.30a	3.00±2.00a
MASS38 *S. sesban*	0.81±0.13abc	7.0± 4.90a	3.00±2.00a
MASS40ab *S. sesban*	0.79±0.08abc	5.8±2.80a	5.00±2.00a
MASS42 *S. sesban*	0.55±0.11a	5.4±3.30a	13.00±9.00a
Tororo (Uganda)			
MASS51c *S. sesban*	0.67±0.07ab	20.4±11.70a	8.00±3.00a
Mbale (Uganda)			
MASS57 *S. sesban*	0.76±0.06bc	16.7±4.80a	26.00±7.00a
Namibia			
MN2 *S. sphaerosperma*	0.69±0.10abc	12.9±9.30a	5.00±3.00a
MN28 *S. cinerascens*	0.74±0.04ab	10.6±4.40a	6.00±3.00a
MN39 *S. macowaniana*	0.74±0.07ab	16.8±9.40a	10.00±4.00a
MN69 *S. microphylla*	0.75±0.07ab	47.1±26.70ab	25.00±10.00a
Reference strains			
Kibwezi (Kenya)			
DWO253 *Phaseolus vulgaris*	0.70±0.10a	30.9± 15.30a	18.00±9.00a
KFR269 Siratro	0.68±0.10ab	16.2±4.80a	29.00±9.00ab
Loruk (Kenya)			
KFR209 *Faidherbia albida*	0.60±0.17	52.00±12.00	16.00±3.00
Nyamonye (Kenya)			
KFR402 *S. sesban*	0.71±0.10a	34.1±16.20a	13.00±6.00a
Gituamba (Kenya)			
BA37 *P. vulgaris*	0.60±0.10ab	36.0±10.50a	20.00±6.00a
Controls			
0 ppm N	0.36±0.17	-	-
70 ppm N	0.98±0.21	-	-

SDWt., shoot dry weight; NDWt., nodule dry weight; NNo., nodule number; Means within a column followed by the same letter(s) are not significant different according to Tukey HSD at 5 %. Values represent the mean ± SD of eight replicates.

There was a significant difference ($p < 0.001$) of shoot % N due to inoculation of common bean plants using rhizobial isolates from sesbania and some reference strains from various hosts (Table 4.13). Isolate MASS133 caused the highest shoot N content (3.14 %). Nitrogen content per plant ranged between 0.34 mg (MASS31a) and 3.08 mg (MASS57) and had a significant difference ($p < 0.001$). Similarly, the nitrogen fixation ratio of nodulated common beans plants ranged between -0.4–2.16. Fifteen isolates were symbiotically effective, four of them described as highly effective. Four isolates MASS149, MASS30, MASS31a and MASS42 were ineffective on the common bean plants. The computed symbiotic effectiveness (SE) of four elite strains (MASS38, MASS57, MASS133 and MASS40ab) was rated as very effective (Table 4.13).

Table 4.13: Effect of sesbania rhizobial isolates on percent nitrogen, N content, N fixation ratio and the symbiotic effectiveness of common beans

Isolate	% N shoot $^{-1}$	N content (mg) shoot^{-1}	N fixation ratio	SE
Kavutiri (Kenya)				
MASS133	3.14±0.28g	2.75±0.22e	2.01	HE
MASS137a	1.92±0.16c–f	1.32±0.27a-e	0.58	E
MASS149	2.03±0.13c–f	1.11±0.07a-e	0.37	I
Lushoto (Tanzania)				
MASS30	2.05±0.31c–f	1.04±0.19abc	0.30	I
MASS31a	0.75±0.04a	0.34±0.08a	-0.40	I
MASS38	2.05±0.36c–f	1.65±0.17a-e	0.91	HE
MASS40ab	1.90±0.33b–f	1.50±0.37a-e	0.76	HE
MASS42	1.36±0.06a–d	0.75±0.19abc	0.01	I
Tororo (Uganda)				
MASS51c	1.24±0.26abc	0.83±0.28abc	0.09	E
Mbale (Uganda)				
MASS57	2.69±0.09f–g	3.08±0.23abc	2.16	HE
Namibia				
MN2	1.89±0.21b–f	1.86±0.32c-e	1.12	E
MN28	2.51±0.08e–g	3.03±0.19b-e	1.13	E
MN39	2.85±0.12f–g	2.12±0.20d-e	1.38	E
MN69	2.65±0.10e–g	1.98±0.22b-e	1.24	E
Reference strains				
Kibwezi (Kenya)				
DWO253 *Phaseolus vulgaris*	1.65±0.20a–e	1.16±0.30a-d	0.42	E
KFR269 Siratro	1.11±0.11abc	0.76±0.12abc	0.02	E
Loruk (Kenya)				
KFR209 *Faidherbia albida*	2.41±0.25d–g	1.59±0.34b-e	0.85	E
Nyamonye (Kenya)				
KFR402 *S. sesban*	2.31±0.19d–g	1.64±0.40a-e	0.90	E
Gituamba (Kenya)				
BA37 *P. vulgaris*	2.10±0.14c–f	1.25 ±0.27a-e	0.51	E
0 ppm N	1.10±0.04abc	0.90±0.10ab	0.00	–
70 ppm N	1.48±0.12a–d	2.33±0.10 a-e	0.00	–

Mean % N: $f_{(20, 83)} = 12.37$, p–value < 0.001, l.s.d = 0.4402. Mean N content (mg) plant $^{-1}$: $f_{(20, 83)} = 5.21$, p–value < 0.001, l.s.d = 0.8348.

Means within a column followed by the same letter(s) are not significant different according to Tukey HSD at 5 %. Values are means of four replicates ± standard errors. SE (%), symbiotic effectiveness; I, ineffective; E, effective; HE, highly effective.

Symbiotic effectiveness of rhizobia that nodulated common bean varied significantly ($p < 0.05$) and ranged between 28.4 % for MASS31a and 229.6 % for MASS133 when compared to uninoculated Plus N treatments (control) rated as 100 %. The common bean inoculant (BA37) caused a symbiotic effectiveness of 104.4 % (Figure 4.9).

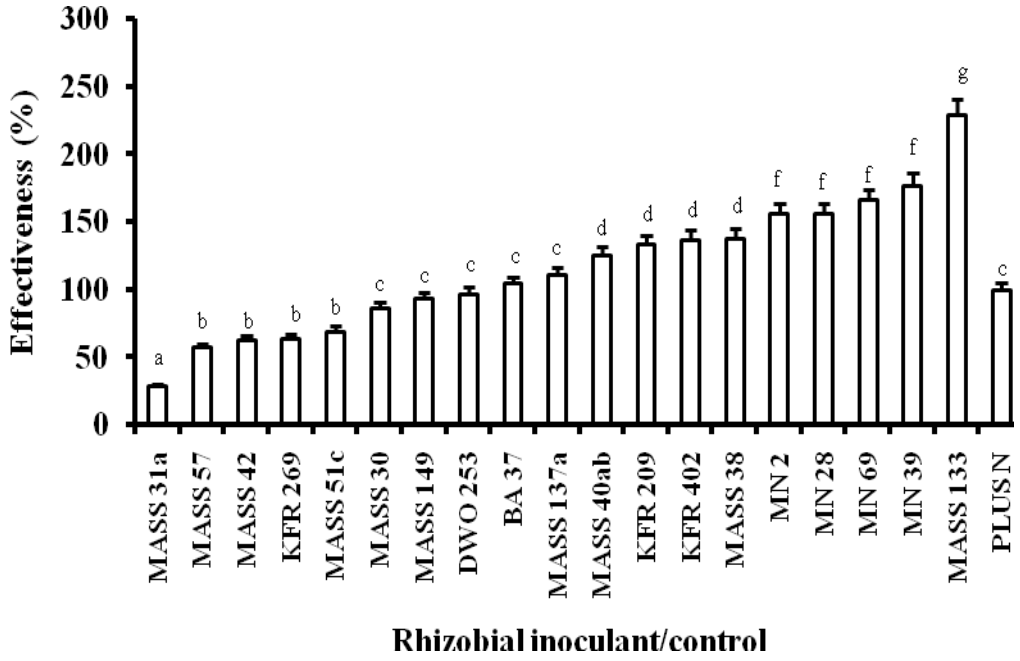

Figure 4.9: Effectiveness of sesbania rhizobial isolates on common beans. Error bars with 5 % value.

Apart from MASS133 and MASS137a sesbania rhizobial isolates that were effective nitrogen fixers (*nod+nif+* phenotypes) in common beans, all other isolates recovered from the Kenyan sites were non-infective (*nod-*) on the common bean (Table 4.14a).

Table 4.14a: Nodulation and nitrogen fixation phenotypes of *S. sesban* rhizobial isolates from Kenya on common beans

Site	*nod-*	*nod+nif-*	*nod+ nif+/-*	*nod+ nif+*
Kavutiri	MASS129, MASS130, MASS131, MASS134, MASS136, MASS137b, MASS137ab, MASS138, MASS140, MASS141, MASS147.	MASS149	-	MASS133, MASS137a,
Kuinet	MASS160, MASS168, MASS170, MASS171, MASS172, MASS173, MASS174.	-	-	-
Bumala	MASS123, MASS125, MASS126, MASS127, MASS157, MASS167, MASS176, MASS177a, MASS177b, MASS177ab, MASS178.	-	-	-
Gituamba	MASS110, MASS111, MASS114, MASS115, MASS117a, MASS117b, MASS117c, MASS117abc.	-	-	-

All isolates affiliated to *S. sesban* from Ugandan soils did not form nodules on common beans (*nod-* phenotypes) except MASS51c and MASS57 which were infective and effective (*nod+nif+* phenotypes) on the common beans. None of the *S. sesban* rhizobial isolates obtained from Kabale, Tororo and Mbale sites in Uganda caused ineffective or partially effective nodules (*nod+nif-* or *nod+nif+/-* phenotypes) on common beans (Table 4.14b).

Table 4.14b: Nodulation and nitrogen fixation phenotypes of *S. sesban* rhizobial isolates from Uganda on common beans

Site	**nod-**	***nod+nif-***	***nod+nif* +/-**	***nod+nif+***
Kabale	MASS62 MASS65	–	–	–
	MASS68 MASS69			
Tororo	MASS47 MASS48	–	–	MASS51c
	MASS49 MASS50a MASS50b MASS50ab MASS51a MASS51b			
	MASS51abc MASS52 MASS53			
Mbale	MASS54 MASS59	–	–	MASS57
	MASS60 MASS61			

Sesbania sesban isolates MASS38 and MASS40a + MASS40b (as a mix inoculant of a nodule co–occupants) from Lushoto formed effective nodules (*nod+nif+* phenotypes) on common beans while MASS42 (SUA), MASS30 and MASS31a (Lushoto) formed ineffective nodules on common beans (*nod+nif-* phenotypes) (Table 4.14c). There were no partially effective nodules formed on the common beans by *S. sesban* rhizobial isolates from the two Tanzanian sites. All other isolates from the two sites did not form nodules (*nod-* phenotypes) on common bean plants.

Table 4.14c: Nodulation and nitrogen fixation phenotypes of *S. sesban* rhizobial isolates from Tanzania on common beans

Site	***nod*–**	***nod*+*nif*–**	***nod*+*nif*+/–**	***nod*+*nif*+**
SUA	MASS41 MASS43	MASS42	–	–
Lushoto	MASS29 MASS31b MASS33 MASS36 MASS37a MASS37b MASS37ab MASS39 MASS40a MASS40b	MASS30 MASS31a	–	MASS38 MASS40ab

Four isolates (MN2, MN28, MN39 and MN69) from various Namibian sites were effective (*nod*+*nif*+ phenotype) on common beans (Table 4.14d). All other sesbania rhizobia from Namibia were non–infective (*nod*– phenotypes) on common bean plants. None of the sesbania rhizobial isolates from Namibia caused ineffective or partially effective nodules.

Table 4.14d: Nodulation and nitrogen fixation phenotypes of sesbania rhizobial isolates from Namibia on common beans

Site	***nod-***	***nod+nif-***	***nod+nif* +/-**	***nod+nif+***
Khorixas Outijo	–	–	–	MN39
Swakop	MN9, MN44, MN49, MN51.	–	–	–
Rio Tinto Gorge	MN45, MN50, MN54, MN56, MN58, MN62.	–	–	–
Epupa falls	MN8, MN13, MN18, MN19, MN21, MN22, MN57.	–	–	–
Suclabo	MN31, MN37, MN40.	–	–	MN28
Omuramba	MN11, MN17	–	–	–
Rooidrom	MN10.	–	–	MN2
Sesfontein	MN12, MN71.	–	–	–
Okahandja	MN1, MN15.	–	–	–
Otjinungua	MN68	–	–	–
Bunya	MN70	–	–	MN69
Korokoko	MN50	–	–	–

Five reference isolates viz: BA37 (*P. vulgaris*), KFR209 (*F. albida*), DWO253 (*P. vulgaris*), KFR269 (Siratro) and KFR402 (*S. sesban*) exhibited *nod+nif+* phenotype on common bean plants (Table 4.14e). A *S. sesban* inoculant production strain (KFR647) failed to induce nodules on the common bean plants. None of the reference rhizobial strains from Kenya caused ineffective or partially effective nodules on common beans.

Table 4.14e: Nodulation and nitrogen fixation phenotypes of reference rhizobial strains from Kenya on common beans

Site	*nod-*	*nod+nif-*	*nod+nif+/-*	*nod+nif+*
Gituamba	–	–	–	BA37 (*P. vulgaris*)
Loruk	–	–	–	KFR209 (*F. albida*)
Kibwezi	–	–	–	DWO253 (*P. vulgaris*), KFR269 (Siratro)
Yala swamp	KFR647 (*S. sesban*)	–	–	–
Nyamonye	–	–	–	KFR402 (*S. sesban*)

4.7.1 Correlation among shoot dry weight, nodule number, nodule dry weight, nitrogen concentration and SE of common bean plants

There was a significant positive correlation (r^2 =0.323, $p < 0.05$) between number of nodules per plant and the shoot dry weight of the plants (Table 4.15). There was no significant correlation ($r^2 = 0.025$, $p = 0.0529$) between nodule dry weight and shoot dry weight following rhizobial inoculation of common bean using sesbania rhizobia. There was also a significant positive correlation ($r^2 = 0.461$, $p < 0.05$) between % N per shoot and shoot dry weight. A strong positive significant correlation ($r^2 = 0.6943$, $p < 0.001$) between symbiotic effectiveness (SE) and shoot dry weight. There was a strong positive correlation ($r^2 = 0.676$, $p < 0.001$) between number of nodules and nodule dry weight. No significant correlation ($r^2 = 0.204$, $p = 0.089$) was recorded between % N per shoot and number of nodules per plant. There was a weak correlation ($r^2 = 0.302$, $p < 0.05$) between SE and number of nodules per plant. There was a significant positive correlation (r^2 =0.386, $p < 0.05$); (r^2 =0.388, $p < 0.05$) between % N per shoot and nodule dry weight; SE and nodule dry weight

respectively (Table 4.15). A strong positive correlation ($r^2 = 0.946$, $p < 0.001$) was recorded between nitrogen concentration (% N) per shoot and symbiotic effectiveness on common bean treatments due to inoculation with sesbania isolates (Table 4.15).

Table 4.15: Correlation between shoot dry weight, nodule number, nodule dry weight, nitrogen concentration and SE on common beans

		SDWt. (g)	**NNo.**	**NDWt. (mg)**	**% N shoot^{-1}**	**SE**
SDWt. (g)	Pearson Correlation	1				
	Sig. (2–tailed)					
NNo.	Pearson Correlation	0.323*	1			
	Sig. (2–tailed)	< 0.05				
NDWt. (mg)	Pearson Correlation	0.025	0.676**	1		
	Sig. (2–tailed)	0.529	< 0.001			
% N shoot^{-1}	Pearson Correlation	0.461*	0.204	0.386*	1	
	Sig. (2–tailed)	< 0.05	0.089	< 0.05		
SE	Pearson Correlation	0.694**	0.302*	0.388*	0.946**	1
	Sig. (2–tailed)	< 0.001	< 0.05	< 0.05	< 0.001	

*, ** correlation is significant at 0.05 and 0.001 level (2–tailed) respectively. SDWt., shoot dry weight; NDWt., nodule dry weight; NNo., nodule number; SE (%), symbiotic effectiveness.

Fourteen (14) sesbania rhizobial isolates from *S. sesban*, *S. sphaerosperma*, *S. macowaniana*, *S. cinerascens* and *S. microphylla* were infective on common bean while 97 isolates were non–infective (Table 4.16). Isolate KFR402 (*S. sesban*) caused nodules on roots of common beans. Rhizobial isolates from *S. pachycarpa*, *S. bispinosa* and *S. rostrata* did not form association with common beans. Other infective isolates with origin from non–sesbania included BA37, DWO253 (common bean), KFR209 (*Faidherbia albida)* and KFR269 (Siratro). Isolates from *Acacia tortilis* and *A. xanthophloea* were non–infective on common beans.

Table 4.16: Sesbania rhizobia with infective traits on common beans

Host of origin	Infective rhizobia	Non–infective rhizobia
Sesbania sesban	MASS30 MASS31a MASS38 MASS40ab MASS42 MASS51c MASS57 MASS133 MASS137a MASS149 KFR402	MASS29, MASS31b, MASS33, MASS36, MASS37a, MASS37b, MASS37ab, MASS39, MASS40a, MASS40b, MASS41, MASS43, MASS47, MASS48, MASS49, MASS50a, MASS50b, MASS50ab, MASS51a, MASS51b, MASS51abc, MASS52, MASS53, MASS54, MASS59, MASS60, MASS61, MASS62, MASS65, MASS68, MASS69, MASS110, MASS111, MASS114, MASS115, MASS117a, MASS117b, MASS117c, MASS117abc, MASS123, MASS125, MASS126, MASS127, MASS129, MASS130, MASS131, MASS134, MASS136, MASS137b, MASS137ab, MASS138, MASS140, MASS141, MASS147, MASS157, MASS160, MASS167, MASS168, MASS170, MASS171, MASS172, MASS173, MASS174, MASS176, MASS177a, MASS177b, MASS177ab, MASS178, KFR647, MN8, MN13, MN18, MN21, MN57, MN68
S. pachycarpa		MN9, MN44, MN45, MN49, MN51, MN56, MN58, MN62
S. sphaerosperma	MN2	MN10, MN12
S. macowaniana	MN39	MN1, MN22, MN15, MN19, MN71
S. cinerascens	MN28	MN31, MN37, MN40
S. microphylla	MN69	MN70
S. bispinosa		MN11, MN17
S. rostrata		MN50
P. vulgaris	BA37 DWO253	
Acacia tortilis		KFR84
Faidherbia albida	KFR209	
Siratro	KFR269	
A. xanthophloea		DWO75

4.8 Multiple rhizobial occupancy in root nodules of *Sesban sesban*

Several rhizobial isolates from *S. sesban* grown in East African sites were found to co–occupy the nodules. Test for ability of the co–occupants to infect *S. sesban* resulted in non–infective or sub–obtimal nitrogen fixation. Nodulated *S. sesban* inoculated using either of the nodule rhizobial companions or in their mixed forms resulted in significantly ($p < 0.001$) lower shoot dry weight compared to Plus N (control) and the *S. sesban* inoculant production strain (KFR647) treatments. Isolate MASS177b had the highest nodule dry weight (0.67 mg) and specific nodule weight of 3.14 mg. A mixed inoculant MASS177a + MASS177b (MASS177ab) was non–infective on *S. sesban* while in their separate forms isolates MASS177a and MASS177b initiated 4.6 and 21.4 nodules respectively. Similarly, a mixture of isolate MASS31a + MASS31b (MASS31ab) was non–infective on *S. sesban*. Isolate MASS31a caused 36 nodules per plant with shoot dry weight of 0.47 g while MASS31b elicited only one nodule per plant with shoot dry weight of 0.09 g. A combined rhizobial inoculation using companions MASS117a + MASS117b + MASS117c (MASS117abc) of a single nodule recorded insignificant difference of shoot dry weight 0.34 g compared to a single isolate MASS117c (0.40 g). The shoot dry weight 0.16 g caused by isolates MASS117a and MASS117b was not significantly different ($p > 0.05$) from shoot dry weight of Minus N treatment (Table 4.17).

Table 4.17: Growth response of *S. sesban* on inoculation using multiple nodules co-occupant rhizobial isolates from roots of *S. sesban*

Isolate	SDWt.(g)	NNo.	NDWt.(mg)	SNWt. (mg)
Bumala (Kenya)				
MASS177a	0.10±0.02ad	4.60±2.40ab	0.01±0.01a	0.25±0.12a
MASS177b	0.48±0.12def	21.40±4.70cde	0.67±0.02d	3.14±0.85c
MASS177ab	0.11±0.01ad	-	-	-
Gituamba (Kenya)				
MASS117a	0.16±0.08ad	4.60±3.90ab	0.09±0.01ab	0.57±0.28ab
MASS117b	0.16±0.05ad	0.60±0.40a	0.01±0.01a	0.85±0.63ab
MASS117c	0.40±0.08ae	17.60±3.00bcd	0.51±0.01bcd	2.69±0.30bc
MASS117abc	0.34±0.11ae	11.00±6.00ad	0.26±0.02ad	1.42±0.40abc
Lushoto (Tanzania)				
MASS31a	0.47±0.08 cf	36.00±4.90e	0.59±0.11cd	1.74±0.26abc
MASS31b	0.09±0.02abc	0.90±0.30a	0.01±0.01a	0.49±0.16ab
MASS31ab	0.14±0.03ad	-	-	-
MASS37a	0.07±0.02a	6.80±2.00abc	0.06±0.02ab	1.24±0.70abc
MASS37b	0.30±0.08ae	15.00±3.00ad	0.30±0.01ad	1.99±0.40abc
MASS37ab	0.12±0.03ad	-	-	-
Tororo (Uganda)				
MASS50a	0.15±0.03ad	-	-	-
MASS50b	0.45±0.07bf	13.00±4.00abcd	0.04±0.01ad	1.96±0.44abc
MASS50ab	0.08±0.03ab	1.00±0.50a	0.01±0.01a	0.25±0.14a
MASS51a	0.15±0.03ad	3.90±2.30ab	0.05±0.03ab	0.73±0.32ab
MASS51b	0.10±0.03ad	-	-	-
MASS51c	0.18±0.02ad	2.30±1.00a	0.06±0.04ab	1.13±0.50abc
MASS51abc	0.14±0.02ad	4.00±1.00ab	0.04±0.01ab	1.23±0.49abc
Reference strains				
KFR647	0.58±0.09ef	22.00±3.30de	0.60±0.01d	2.69±0.24bc
Controls				
0 ppm N	0.15±0.03ad	-	-	-
70 ppm N	0.98±0.14f	-	-	-

SDWt. (g) $f_{(22, 159)} = 7.01$, p–value < 0.001, l.s.d = 0.2141, NNo. $f_{(22, 159)} = 10.47$, p–value < 0.001, l.s.d = 8.185, NDWt. (mg), $f_{(22,159)} = 6.39$, p–value < 0.001, l.s.d = 0.026 and SNWt. (mg), $f_{(22,159)} = 4.72$, p–value < 0.001, l.s.d = 1.213.

Means within a column followed by the same letter(s) are not significant different according to Tukey HSD at 5 %. Values are means of four replicates ± standard errors. SDWt., shoot dry weight; NNo., nodule number; NDWt., nodule dry weight; SNWt., specific nodule weight.

4.9 *Sesbania sesban* and common bean cross–inoculating isolates

All the isolates with cross–inoculating ability on *S. sesban* and the common beans caused low shoot dry weight on both species compared to the respective Plus N treatments (control). There was no significant difference ($p \leq 0.05$) in shoot dry weight of *S. sesban* inoculated using strains KFR269, KFR209 and KFR647 (*S. sesban* inoculant strain). Similarly, there was no significant difference ($p \leq 0.05$) in relative effectiveness of isolate MASS133, DWO253 and BA37 (inoculant strain for common beans) on *S. sesban* (Figure 4.9a). Isolate KFR402 showed the highest relative effectiveness (0.35) on *S. sesban*.

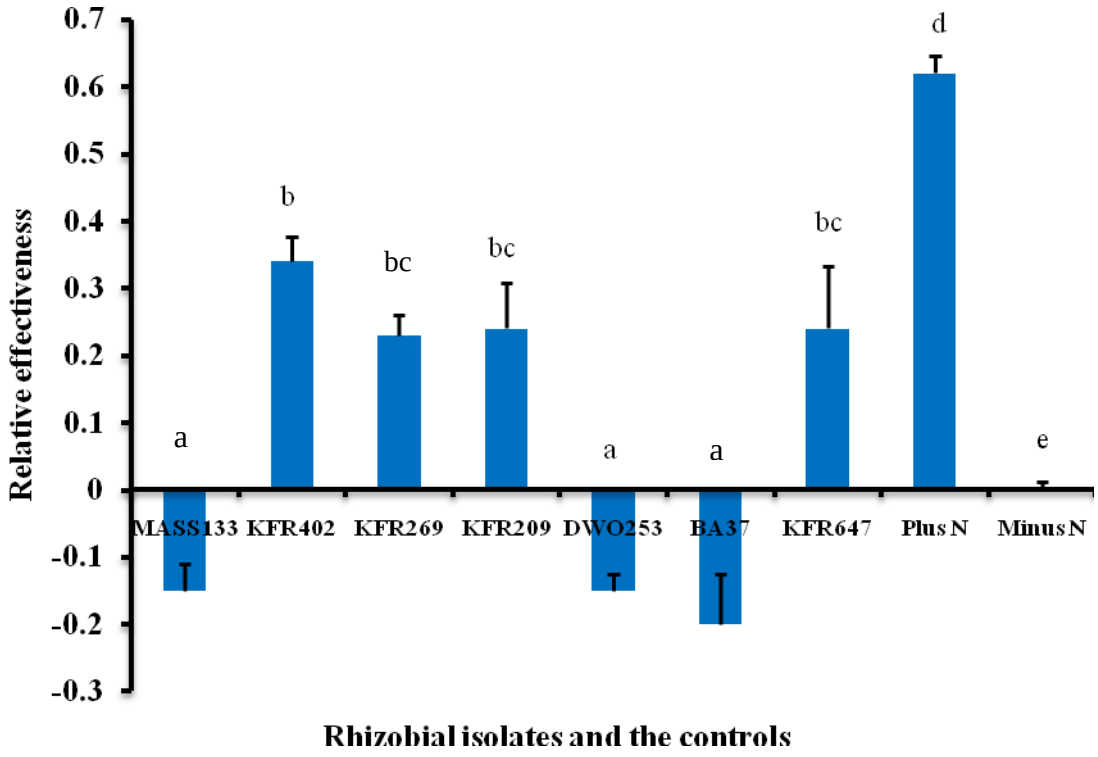

Figure 4.10a: Response of inoculation on shoot dry weight (g) of *S. sesban*. Standard error (SE) bars with the same letters are not significantly different ($p > 0.05$) according to Tukey's HSD test.

The isolate MASS133 caused a significant difference ($p \leq 0.05$) of a higher relative effectiveness of 0.2 on common bean compared to all other isolates. The common

bean inoculant production strain BA37 caused the least relative effectiveness (-0.07) compred to Plus N treatment (control). Common bean shoot dry weight caused by isolates KFR402, KFR269, DWO253 and BA37 were not significantly different ($p > 0.05$) from Minus N treatment (control) (Figure 4.9b).

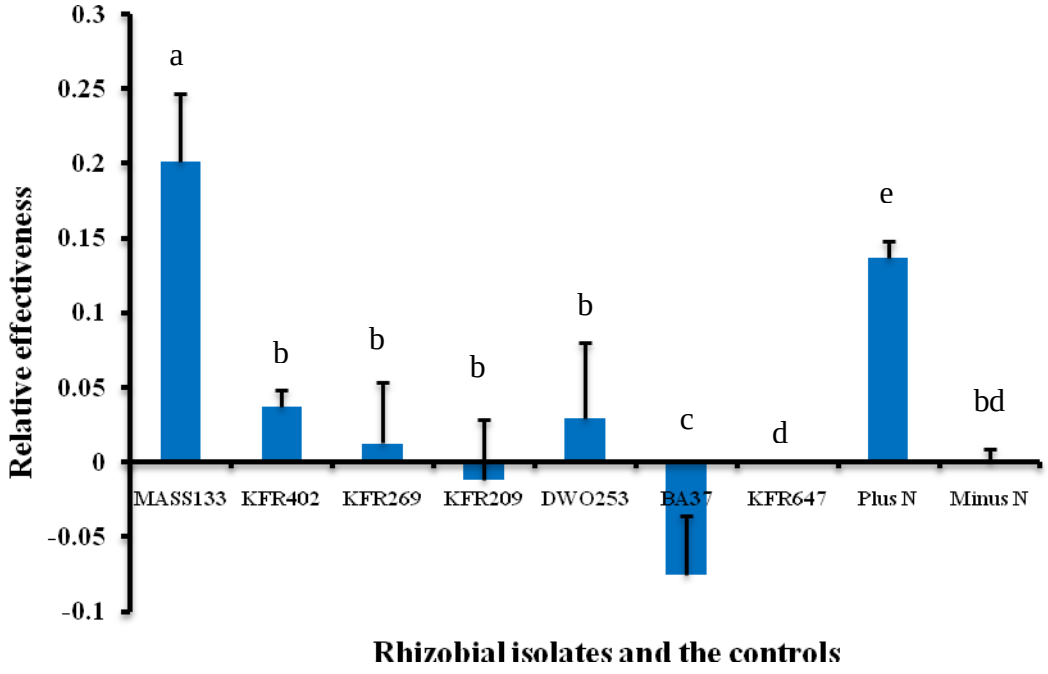

Figure 4.10b: Response of inoculation on shoot dry weight (g) of common bean. Bars with the same letters are not significantly different ($p > 0.05$) according to Tukey's HSD test.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Morpho–cultural characterization of sesbania rhizobia

The morpho-cultural characteristics exhibited by sesbania isolates including colonies with either flat, raised or dome shaped (all with entire margins), fast, moderate or slow growth, production of copious exopolysaccharides (EPS), moderate EPS with gummy properties or non–EPS producers, acid, alkaline or neutral on YEMA–BTB media were similar to those of root nodules isolated from legumes grown worldwide (Somasegaran and Hoben, 1994; Legesse and Assefa, 2014; Bhargava *et al*., 2016; Nahar *et al*., 2017). Majority of fast growing rhizobia were acid producers while the slow growers were alkaline producers on YEMA medium. However, the pH reaction for fast and slow growers was not universal, findings that agree with those by Okaron *et al*. (2017). The time taken for sesbania rhizobial colonies to attain maximum growth (i.e. 2–3 days, 4–6 days and 7–9 days for fast, moderately fast and slow growers respectively) with a colony size range from < 1.0 mm to 5.0 mm in diameter on YEMA medium incubated in the dark at 28±1 °C were in close agreement with Dereje *et al*. (2015).

Bacteria recovered from the nodules of sesbania were Gram negative, a characteristic shared by all members of α- and β-protobacteria (Berrada and Fikri-Benbrahim, 2014). This characteristic aided in the presumptive identification of rhizobia from the study sites. However, the recovery of fast and slow growing rhizobia from root nodules of sesbania contrasted in the slow growth with the observations by Atangana *et al*. (2014) that sesbania form nodules with fast-growing strains of *Rhizobium*.

Most of the sesbania rhizobial isolates recovered were clustered in morphotypes I, II, VII, VIII and IX (Figure 4.1), which are probably the most compatible morphotypes possessing high affinity for sesbania as opposed to morphotypes III, IV, V and VI. The high diversity of morphotypes observed with sesbania isolates could be as a result of horizontal gene transfer which confers survival mechanisms over time to bypass adverse conditions found in some tropical soils. For instance, the presence of rhizobial isolates grouped in morphotypes II, IV and V produced copious exopolysaccharides (EPS) a property also characterized by Hewedy *et al*. (2014) and which is essential for infection process and nodule formation; protect rhizobia in the soil against deleterious biotic and abiotic stress factors and which restrict oxygen diffusion through the nodular cells to protect the oxygen sensitive nitrogenase in the nodules (Bhargava *et al*., 2016). Rhizobia falling in morphotype III presented a unique characteristic of chromogenic colonies which is contrary to growth of most rhizobia. The results also point out to non-specificity of the rhizobial morphotypes to sesbania used in the study and that the infection process is not stringently controlled by a single variant of flavanoids or isoflavanoids even within the same host (Liu and Murray, 2016). The recovery of rhizobia with heterogeneous colony characteristics in the present study indicates that sesbania symbiotically associate with diverse rhizobial strains. Hence the acceptance of hypothesis that there are phenotypic differences among the rhizobia isolated from various sesbania grown in East Africa and Namibia.

Lack of site specificity for morphotypes implies that rhizobial strains compatible to sesbania are readily available in many soils of East Africa and Namibia. However, this finding contradicts that of Bala *et al*. (2002) in which they found no rhizobia

compatible with *S. sesban* in 39 out of 53 soils sampling sites of Southern Africa. The similarities of colony traits of *Mesorhizobium* and *Sinorhizobium* (morphotype II) and *Bradyrhizobium* and *Azorhizobium* (morphotype IX) together with the variation of traits for the genus *Rhizobium* (morphotypes V and VII) suggest presence of colony dimorphism within rhizobia nodulating sesbania which may lead to identification inconsistencies when only morphological traits are relied upon.

An intriguing observation in the present study is the evidence of hollow centres (Plate 4.2) in some rhizobial colonies isolated from Namibian sesbania nodules. The hollow centres could be a symptom of rhizobial infection by rhizobiophages. Rhizobiophages are important vectors for rhizobial transformation, a potential means of genetic diversity among rhizobia through conferring of traits like resistance to antibiotics, tolerance to salts and infectiveness (Santamaría *et al*., 2013). However, rhizobiophages could as well have a negative effect on elite strains in the soil through elimination of susceptible rhizobial populations or transform infective and effective strains into non-infective forms (Msimbira *et al*., 2016).

5.1.2 Intrinsic antibiotic resistance and salt tolerance

The intrinsic antibiotic resistance (IAR) and salt tolerance are important techniques used for discrimination and identification of rhizobial strains. Sesbania rhizobial isolates showed different degrees of susceptibility of between 26.8 % and 90.8 % (Figure 4.3) to the twelve antibiotics which can be attributed to genetic variation in target genes and resistance genes acquired through horizontal gene transfer (Bhargava *et al*., 2016). Notable is the less resistance to kanamycin and which support findings by The isolates also showed a generally low IAR for kanamycin, which is a very common characteristic

for tropical rhizobia. This phenotypic trait is highly desired for inoculant strains persistence amidst antibiotics producing microbes present in many agricultural soils (Shetta *et al*., 2011; Adegboye and Babalola, 2013). Sesbania grow in diverse conditions that include water-logged and dry soils with varying salinity levels in different parts of the world. Salinity is one of the adverse conditions that affect rhizobia–legume interactions leading to poor nitrogen fixing through inhibition of the initial steps of the symbiosis. It was intriguing to note that some of the sesbania isolates were highly halotolerant at a concentration of 10 % NaCl (w/v) which is higher than the 5 % recorded by Messaoud *et al*. (2014) but agrees with the findings of Bouzeraa-Bessila *et al*. (2015). In their findings, the most salt-tolerant strains MSC2 and MSC9 isolated from Chatt soil and MSF21, MSF20 and MSF19 isolated from Fetzara soil were tolerant at 10 % of NaCl. This might be as a result of the soil chemical characteristics of the site where sesbania root nodules were recovered. Results of the present study suggest that IAR and salt tolerance dendrogram patterns (Figures 4.5a–4.5j) were sufficiently used in grouping of rhizobial isolates from sesbania grown in diverse habitats of East Africa and Namibia, which supports the use of some phenotypic traits for strain identification (Chanway and Holl, 1986).

5.1.3 Molecular characterization of sesbania rhizobia

According to the 16S rRNA PCR–RFLP unrooted tree topologies (Figure 4.6a–4.6d), rhizobial isolates from root nodules of sesbania were tentatively grouped into the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium* and *Agrobacterium* with an overall mean cophenetic correlation coefficient of 62.1 % (bootstrap value) compared to reference strains. Except for Bradyrhizobium, these results support those of Young and Haukka (1996) that *S. sesban* rhizobia are

genetically diverse, with isolates represented in the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Azorhizobium*. The presence of unmatched isolates (dissimilar to all the reference strains used in this study) from Kenya and Namibia was an indication that there exists rhizobial genera other than *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Agrobacterium* that also form symbiosis with sesbania. These data demonstrated a high diversity of rhizobia associated with sesbania and therefore the hypothesis that there are genotypic differences among the rhizobia isolated from various sesbania grown in East Africa and Namibia is accepted. These results agree with those of Nahar *et al*. (2017) where rhizobia affiliated with *S. bispinosa* showed genotypic diversity using 16S rRNA sequences. Results from the present study have also shown that the PCR-RFLP markers were not able to resolutely split *Agrobacterium* spp. and *Sinorhizobium* spp. isolated from sites in Kenya and Tanzania; *Bradyrhizobium* spp. and *Rhizobium* spp. Type A, *Mesorhizobium* spp. and *Agrobacterium* spp. isolated from sites in Uganda; *Sinorhizobium* spp. and *Agrobacterium* spp., *Mesorhizobium* spp. and *Rhizobium* spp. Type B from sites in Tanzania. This may possibly indicate that some of the strains in the sites of nodule collection are actively exchanging their genome or acquiring genes from the surroundings to suit both host and abiotic conditions for survival.

The phylogenetic disimilarities range of 2 % – 100 % is an indication of a rich diversity of rhizobia affiliated symbiotically to sesbania growing in East Africa and Namibia. Findings of the present study show that the bulk of sesbania isolates from various sites were of the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Mesorhizobium.* With the exception of *Bradyrhizobium* spp., these results are in line

with those of Bala and Giller (2001) who reported that *S. sesban* are effectively infected by rhizobia that belong to the genera *Rhizobium*, *Mesorhizobium* and *Sinorhizobium.* However, these contrast with those of Singh *et al*. (2013) where only fast growers (*Rhizobium* spp.) were recovered from *S. sesban* which were grown in Mumbai and its suburban areas. In the present study isolates with *Agrobacterium*-like 16S rRNA PCR-RFLP patterns were recovered from sesbania of Namibia which is an indication that some *Agrobacterium* spp. acquired the ability to nodulate legumes through transformation, a finding that supports that of Kondorosi *et al*. (1982) that *Agrobacterium* mutants supplied with symbiotic plasmids of rhizobia are conferred the ability to form nodules and fix nitrogen. However, the 16S rRNA PCR-RFLP clustering together of *Agrobacterium*-like rhizobia from Kenya and Tanzania with *Sinorhizobium* spp. and those from Uganda clustered with *Mesorhizobium* spp. might be a pointer to the source of symbiotic genes that transforms *Agrobacterium* spp. to legume nodulating bacteria.

5.1.4 Nodulation and nitrogen fixation potential of sesbania rhizobia on *S. sesban*

A high number of sesbania rhizobia (83 %) caused nodulation on *S. sesban* an indication that members of this genus *Sesbania* easily cross-inoculate with similar rhizobia. Sesbania rhizobial infection and the associated nodule formation on roots of *S. sesban* were not host or site specific which can plausibly be attributed to wide distribution of sesbania and their compatible rhizobia in the tropics following widespread seed dispersal to new sites mainly accompanied by compatible microsymbionts as suggested by Grange *et al*. (2007).

In the present study *S. sesban* responded to inoculation using sesbania rhizobia with the four possible nodulation and nitrogen fixation phenotypes *nod-*, *nod+fix–*, *nod+fix+/-* and *nod+fix+* as was earlier described by Maunoury *et al*. (2010), Liu *et al*. (2011), Saeki, (2011) and Melino *et al*. (2012). Symbiotic effectiveness test of sesbania rhizobia on *S. sesban* revealed a huge disparity in the ability to fix nitrogen as indicated by shoot dry weight range of 0.06 g – 1.06 g per plant. This variation in effectiveness supports the hypothesis that not all rhizobia from root nodules of sesbania are effective in fixing nitrogen with *S. sesban*.

The unexpected failure of isolates recovered from *S. sesban* to nodulate the host of isolation resulting in negative Koch's postulates may be attributed to premature abortion of bacteroid differentiation process, failed bacterial endocytosis from infection threads into cortical cells or a reduced pool of functional bacteroids after they underwent premature senescence (Melino *et al*., 2012). The highly positive significant correlation (r^2 = 0.836, $p < 0.001$) between shoot dry weight of *S. sesban* and the respective nodule dry weight imply that high nodule biomass is responsible for fast growth of *S. sesban* in the absence of external nitrogen sources. The high nodule biomass observed in treatments with efficient nitrogen fixation could be as a consequence of rhizobial affinity for root cortex cells and the subsequent entry into the cells resulting in higher hyperplasic and greater hypertrophic effects on the symbiosomes. Results in the present study also show no significant correlation (r^2 = 0.191, $p = 0.097$) between nodule number and shoot dry weight of *S. sesban* which is inconsistent with that of Chemining'wa *et al*. (2011) where they reported a positive and significant correlation between the two parameters. In the present study, the high weight of nodules can have compensated for high number of nodules and the incidences of low plant biomass of profusely nodulated legume plants

could imply failure of host to sanction ineffective *nod+fix-* or *nod+fix+/-* phenotypes rhizobial strain(s) (Sachs *et al*., 2010). Six isolates recovered from *S. sesban* [MASS59 (Mbale–Uganda), MN18 and MN68 (Nambia), MASS147 and MASS129 (Kavutiri–Kenya) and MASS172 (Kuinet–Kenya)] caused high shoot dry weight compared to the uninoculated plus N (control) treatments. Most of the isolates had significant ($p < 0.001$) low shoot dry weights compared to the uninoculated minus N (control) treatments (0.36 g per plant) denoting parasitism nature of some highly infective microsymbionts which concurs with the findings by Sachs *et al.* (2010) that legumes have no ability to detect effectiveness of rhizobia at the point of infection.

The significant differences ($p < 0.001$) in mean percent nitrogen and nitrogen content per shoot of *S. sesban* inoculated using selected rhizobia from various legume hosts indicate polymorphism in genes involved in nitrogen fixation present in different rhizobial isolates. Nodulation of *S. sesban* by rhizobia from legumes other than sesbania [i.e. strains BA37 and DWO253 (*P. vulgaris*), KFR269 (Siratro) and KFR209 (*F. albida*), all included in the present study] indicates that *S. sesban* and the common beans fall in a common cross-inoculation group with a wide host range inluding other herbaceous legumes and tree species. These results match and support those of Wolde-Meskel *et al*. (2016) where most of the rhizobia strains identified as effective symbionts of sesbania were originally isolated from trees and leguminous food crops other than sesbania suggesting an availability of broad range isolates in soils where other legumes are grown.

5.1.5 Nodulation and nitrogen fixation potential of sesbania rhizobia on Rose coco bean variety

Sesbania isolates formed root nodules on Rose coco bean plants (Table 4.16) which plausibly indicate that common beans together with sesbania fall within the same cross-inoculation group. These findings support the hypothesis that sesbania rhizobial isolates infect common beans. The cross nodulation ability of the two legumes was further confirmed by the reciprocal nodulation of *S. sesban* by strains of common bean origin (Table 4.9e). These results support the idea that tropical legumes nodulate with rhizobial strains found outside their centre of diversity as a result of promiscuity in host range (Giller, 2001). A limited number of 14 out of the total 128 test sesbania rhizobial isolates formed nodules on roots of Rose coco bean cultivar. The most probable explanation for these results is the lack of preference by sesbania rhizobia for the Rose coco common bean cultivar used in the present study (Gicharu *et al*., 2013).

The symptomatic nitrogen deficiency frequently manifests in plants growing in nitrogen poor soils, non-nodulated legumes or legumes associated with ineffective rhizobial strains. Unexpectedly Rose coco bean variety nodulated by sesbania rhizobial *nod+nif+* phenotypes with pink nodules (Plate 4.13) and which rated as highly effective (Table 4.13) had yellowish green leaves compared to the dark green colour of the non–inoculated Plus N treatments (control) (Plate 4.11). However, the yellow leaf colour of the bean plants with effective nodules not only points to the high nitrogen demand by bean plants especially during the onset of flowering and podding but also to the low nitrogen fixation ability of the pulse compared to other crop legumes (Graham, 1981; Hardarson *et al*., 1993). Inspite of the leaf yellow

colour of nodulated common bean, these findings support the null hypothesis that sesbania rhizobial isolates infect and effectively fix nitrogen with Rose coco beans variety. These results contrast with those of Degefu *et al*. (2011) where none of the rhizobial strains recovered from the tree species *S. sesban*, *Acacia abyssinica*, *A. tortilis* and *A. senegal* were able to induce nodules in crop legume species.

Rose coco bean variety was also nodulated by non-sesbania isolates KFR209 (*F. albida*) and KFR269 (siratro), findings which support observations by Michiels *et al*. (1998) and Perret *et al*. (2000) that common bean has the ability to recognize signals that trigger the nodulation process from many rhizobia although the resultant interactions are often not effective. The negative relative effectiveness of MASS133, DWO253 and BA37 on *S. sesban* and KFR209 and BA37 on Rose coco beans can be attributed to failure of host-entry restriction mechanisms and symbiotic selection centres against 'parasitic' rhizobial strains (Perret *et al*., 2000) hence the rejection of the hypothesis that sesbania rhizobial isolates efficiently fix nitrogen with Rose coco bean variety.

5.1.6 Multiple nodule occupancy

Two or three rhizobial isolates were found co–occupying nodules collected from roots of East African *S. sesban,* results that support earlier findings by Nguyeni *et al*. (2010) where 10–50 % of nodules were simultaneously occupied by two or three genotypes. When and how the diverse genotypes of nodule forming rhizobia enter and co–occupy a single nodule has remained unclear. The significant difference ($p < 0.001$) between shoot dry weight caused by two nodule companions and the absence of additive effect in mixed inoculants could be attributed to detection and subsequent

elimination of the 'cheater' strains as earlier described by Jones *et al*. (2015) and Checcucci *et al*. (2016). The frequent low shoot dry weight of *S. sesban* inoculated using mixed co-occupants compared to one of the co-occupants support (Friesen and Mathias, 2010) that legume hosts discriminate between strains within a nodule resulting in emergence of polymorphism that cause precipitous decline in host benefit.

5.2 Conclusions

i. Rhizobia nodulating sesbania grown in East Africa and Namibia are phenotypically and genetically diverse but not site specific.

ii. Most sesbania isolates are infective on *S. sesban*. However, they exhibit great variations in nodulation and effectiveness.

iii. Rose coco bean variety fall in the same cross-inoculation group as sesbania but only 14 out of the total 128 sesbania rhizobial isolates caused nodulation.

5.3 Recommendations

i. Sesbania are nodulated by diverse rhizobia which calls for keen selection before they are used as inoculant production strains for legumes within its cross-inoculating group.

ii. Prospecting for sesbania-common bean cross-inoculating rhizobia in tropical soils where sesbania are endemic should be prioritized in order to recover even more superior strains.

iii. Superior genetic tools for example DNA and gene sequencing should be employed to evaluate the genetic diversity of rhizobia nodulating *S. sesban* and common bean to species level.

iv. Isolates MASS172 and MASS133 from root nodules of *S. sesban* grown in Kuinet and Kavutiri (Kenya) respectively and KFR402 (reference strain from *S. sesban*) have the potential as inoculant production strains for both species. However, the isolates should be evaluated for effectiveness in soils from different agro-ecological zones where common beans and *S. sesban* are grown.

v. The isolates MASS172, MASS133 and KFR402 should be evaluated for compatibility and nitrogen fixing effectiveness on many other common bean varieties given that there are frequent reports of variations in nodulation and biomass accumulation among bean varieties under controlled greenhouse conditions.

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APPENDICES

Appendix I: Growth characteristics of *Sesbania sesban* rhizobial isolates from Kenya on YEMA media

Isolate/Host	Characteristics per study site	BTB
Gituamba		
110 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
111 *S. sesban*	5 mm diameter, milky, gummy, dome shaped shinny	A
112 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
113 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
114 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
115 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
116 *S. sesban*	2 mm, milky suspensions, shiny, raised and watery	N
117 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	B
Kavutiri		
129 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	B
130 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	B
131 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	N
132 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	N
133 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	N
134a *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
134b *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	N
135 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	N
136 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	N
137a *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	N

137b *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	N
Bumala		
156 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
157 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	B
158 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
159 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
161 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
162 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
163 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	B
164 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	B
165 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	B
166 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	B
167 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
168 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	A
176 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
177 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
178 *S. sesban*	<1 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	B
179 *S. sesban*	<1 mm, transparent, dome shaped, tiny	B
180 *S. sesban*	<1 mm diameter, transparent, dome shaped, tiny	B
181 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	N
Kuinet		
169 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
170 *S. sesban*	3 mm diameter, pink, translucent, milky centre,	A

	dome, shinny and moderate gummy exoplysaccharides	
171 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
172 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
173 *S. sesban*	3 mm diameter, translucent, raised shinny, watery	A
174 *S. sesban*	3 mm diameter, translucent, raised shinny, watery	N

BTB, Bromothymol blue; A, acid; B, alkaline; N, neutral.

Appendix II: Growth characteristics of *Sesbania sesban* rhizobial isolates Uganda on YEMA media

Isolate/Host	**Characteristics per study site**	**BTB**
Tororo		
MASS47 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
MASS48 *S. sesban*	4 mm, milky suspensions, shiny, raised and watery	A
MASS49 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
MASS50 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
MASS51 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
MASS52 *S. sesban*	1.0 mm diameter, milky opaque, dome, shiny, and no exopolysaccharides	A
MASS53 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
Mbale		
MASS54 *S. sesban*	2.5 mm diameter, milky suspensions, shiny, raised and watery	A
MASS55 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A

MASS56 *S. sesban*	1.0 mm diameter., milky opaque, dome, shiny, and no exopolysaccharides	A
MASS57 *S. sesban*	2.5 mm diameter, milky suspensions, shiny, raised and watery	A
MASS59 *S. sesban*	1.0 mm diameter, milky opaque, dome, shiny, and no exopolysaccharides	A
MASS60 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
MASS61 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	B
Kabale		
62 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
63 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
64 *S. sesban*	<1.0 mm diameter, transparent, dome shaped, tiny	B
65 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
66 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
67 *S. sesban*	<1.0 mm diameter, transparent, dome shaped, tiny	A
68 *S. sesban*	<1.0 mm diameter, transparent, dome shaped, tiny	A
69 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A

BTB, Bromothymol blue; A, acid; B, alkaline.

Appendix III: Growth characteristics of *Sesbania sesban* rhizobial isolates from Tanzania on YEMA media

Isolate/Host	Characteristics per study site	BTB
SUA		
MASS41 *S. sesban*	<1.0 mm diameter, transparent, dome shaped, tiny	A
MASS42 *S. sesban*	<1.0 mm diameter, transparent, dome shaped, tiny	A
MASS43 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
MASS44 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
MASS45 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
MASS46 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
Lushoto		
MASS29 *S. sesban*	<1.0 mm diameter, pink, translucent, flat, dull and dry, no exopolysaccharides	B
MASS30 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
MASS31a *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
MASS31b *S. sesban*	<1.0 mm diameter, pink, translucent, flat, dull and dry, no exopolysaccharides	B
MASS32 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
MASS33 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
MASS34 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
MASS35 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
MASS36 *S. sesban*	1.0 mm diameter, milky opaque, dome, shiny, and no exopolysaccharides	B

MASS37 *S. sesban*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
MASS38 *S. sesban*	4 mm diameter, red, opaque, shiny, raised and moderate gummy exopolysaccharides	A
MASS39 *S. sesban*	2 mm diameter, milky suspensions, shiny, raised and watery	A
MASS40 *S. sesban*	4 mm diameter, red, opaque, shiny, raised and moderate gummy exopolysaccharides	A

BTB, Bromothymol blue; A, acid

Appendix IV: Growth characteristics of sesbania rhizobial isolates from Namibia YEMA media

Isolate/Host	**Characteristics per study site**	**BTB**
Namibia		
1 *S. macowaniana*	2 mm diameter, pink translucent, flat, dull	A
2 *S. sphaerosperma*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
4 *S. pachycarpa*	4 mm diameter, transparent, margin brown centre, raised, gummy, mucoid, shinny	A
8 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
9 *S. pachycarpa*	0.5 mm diameter, red, raised dry, shinny	A
10 *S. sphaerosperma*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
11 *S. bispinosa*	<1 mm diameter, pink, translucent, flat, dull and dry, no exopolysaccharides	B
12 *S. sphaerosperma*	1 mm diameter., milky opaque, dome, shiny, and no exopolysaccharides	N
13 *S. sesban*	4 mm diameter, transparent, raised, watery mucous, banana smell	A
15 *S. macowaniana*	<1 mm diameter, pink, translucent, flat, dull and dry, no exopolysaccharides	N
16 *S. pachycarpa*	<1 mm diameter, clear, raised, dry, shinny	A
17 *S. bispinosa*	0.5 mm, pink, translucent, raised, dull, dry	B
18 *S. sesban*	4 mm diameter, transparent, shiny, dome and copius viscous EPS	A
19 *S. macowaniana*	3 mm diameter, pink translucent, milky, raised, mucoid, shinny	A

20 *S. cinerascens*	1 mm diameter., milky opaque, dome, shiny, and no exopolysaccharides	A
21 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
22 *S. macowaniana*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
24 *S. macowaniana*	<1 mm diameter, pink, translucent, flat, dull and dry, no exopolysaccharides	A
25 *S. macowaniana*	0.5 mm diameter, red, flat, dry, shinny	A
26 *S. macowaniana*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
27 *S. macowaniana*	<1.0 mm diameter, red, flat, dry, shinny	A
28 *S. cinerascens*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
31 *S. cinerascens*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
34 *S. sphaerosperma*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
35 *S. sesban*	<1.0 mm diameter, pink, translucent, flat, dull and dry, no exopolysaccharides	B
36 *S. pachycarpa*	4 mm diameter, red, opaque, shiny, raised and moderate gummy exopolysaccharides	B
37 *S. cinerascens*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
38 *S. sesban*	2 mm diameter, pink translucent, flat, dull, dry	A
39 *S. macowaniana*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	N
40 *S. cinerascens*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	A
41 *S. sphaerosperma*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
42 *S. macowaniana*	3 mm diameter, milky, translucent, flat, dry	A
43 *S. macowaniana*	2 mm diameter, milky, translucent, flat, dry	A
44 *S. pachycarpa*	2 mm diameter, pink opaque, raised, dry, dull	A
45 *S. pachycarpa*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
49 *S. pachycarpa*	4 mm diameter, red, opaque, shiny, raised and moderate gummy exopolysaccharides	N

50 *S. rostrata*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	B
51 *S. pachycarpa*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
56 *S. pachycarpa*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
57 *S. sesban*	<1.0 mm diameter, pink, translucent, flat, dull and dry, no exopolysaccharides	N
58 *S. pachycarpa*	4 mm diameter, red, opaque, shiny, raised and moderate gummy exopolysaccharides	A
59 *S. pachycarpa*	4 mm diameter, red, opaque, shiny, raised and moderate gummy exopolysaccharides	A
60 *S. pachycarpa*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	A
62 *S. pachycarpa*	3 mm diameter, pink, translucent, milky centre, dome, shinny and moderate gummy exoplysaccharides	N
68 *S. sesban*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	N
69 *S. microphylla*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	B
70 *S. microphylla*	4 mm diameter, transparent, shiny, dome and copious watery exopolysaccharides	B
71 *S. macowaniana*	5 mm diameter, milky, translucent, shiny, dome and copious friable exopolysaccharides	B

BTB, Bromothymol blue; A, acid; B, alkaline; N, neutral.