EFFECTIVENESS OF RHIZOBIA STRAINS ISOLATED FROM SOUTH KIVU SOILS ON GROWTH OF SOYBEANS (*Glycine max*).

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DECLARATION

This thesis is my original work and has not been presented for award of a degree/research in any other university.

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DEDICATION

To my parents Victor Ndusha and Jeanne Bahati

&

My husband Norbert Mubalama and my daughter Glodie Nshokano, my sisters and brothers, cousins and friends, nieces and nephew

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TABLE OF CONTENTS

LIST OF APPENDICES

LIST OF TABLES

LIST OF FIGURES

ACRONYMS

ATP:	Adenosin triphosphate
BNF:	Biological Nitrogen Fixation
CIAT:	International Centre for Tropical Agriculture
DAPD:	Direct Amplified Polymorphic DNA
DNA:	Desoxyribo-nucleic Acid
DRC:	Democratic Republic of Congo
ELISA:	Enzyme-linked Immunosorbent Assay
FAO:	Food and Agriculture Organization
FAO:	Food and Agriculture Organization of the United Nations
FAOSTAT:	Food and Agriculture Organization of the United Nations Statistical Database
IAR:	Intrinsic Antibiotic Resistance
IITA:	International Institute of Tropical Agriculture
ppm:	part per million
RAPD:	Randomly Amplified Polymorphic DNA
RFLP:	Restriction Fragment Length Polymorphism
RNA:	Ribo-nucleic Acid
T/ha:	tons per hectare
TSBF:	Tropical Soil Biology and Fertility
U.C.B:	Universite Catholique de Bukavu (Catholic University of Bukavu).
YEMA:	Yeast extract Mannitol

ABSTRACT

Identification of effective indigenous *Bradyrhizobium* strains which nodulate soybean varieties could trigger development of an industry for inoculant production with use of strains adapted to local conditions. This study was conducted in South Kivu to identify and select effective rhizobial strains nodulating soybean present in South Kivu soils. One hundred and seven isolates from root nodules of legumes plants sampled in five villages of South Kivu were tested in sterile sand in the greenhouse in the modified Leonardôs jars. 10% of these isolates produced higher nodules and plant shoot dry weight (P' 0.001) compared to the commercial strain USDA110 .The effectiveness index of the isolates NAC10, NAC22, NAC37, NAC40, NAC42, NAC45, NAC46, NAC50, NAC67 and NAC75 was higher compared to the commercial isolates and were selected for further evaluation using soils in the greenhouse. From the potted soils experiment in the greenhouse the isolates NAC10, NAC22, NAC40 and NAC75 were classified as competitive and highly effective. Increments in nodule number and shoot dry weight were observed when the plant was inoculated with an effective rhizobial isolate compared to the controls. the best strains were tested also for carbon source utilization ability. Three sources of carbon were used namely mannitol, glucose and glycerol. From this experiment there was significant differences between the three carbon sources in sustaining viable counts of the tested isolates from South Kivu soils ($P<0.005$) with higher carbon substitution index with the glucose.

CHAPTER 1

1.0 General introduction

Soybean (*Glycine max*) grown by both commercial and subsistence farmers provides a valuable source of protein and thereby sustaining nutritional balances of low income populations (Appunu, 2009). The crop has high protein content (40%) and high gross output of vegetable oil (20%) among the cultivated crops in the world (Li-juan *et al*., 2010). The importance of soybean as a source of oil and protein and its ability to fix nitrogen from the atmosphere through root nodules formed symbiotic association with *Rhizobium* bacteria on low-N soils point to its continued status as a valuable grain legume in the world (Harold *et al*., 1992).

In the Democratic Republic of Congo (DRC), soybean cultivation has expanded as a result of its nutritive and economic importance and diverse domestic usage. Soybean is used for the production of oil, soymilk and soy meat. It is also used for production of infant food, spices and protein-fortified flour, for that it has been an important raw material for many small and medium enterprises. It is also consumed roasted and constitutes an important local business opportunity. Soybean is also used for production of concentrated milk for treatment of malnourished children. In South Kivu, the farmers produce and export soybean to neighboring countries, Rwanda and Uganda and therefore increase their income.

Soybean also improves soil fertility by capturing nitrogen from the atmosphere. An amount of 300 kg of nitrogen per hectare has been reported (Mulongoy, 1992; Hungrēa *et al.,* 2006) and range of 31-110kg per hectare reported by a study, Osunde and Bala (2005).This is a major benefit in South Kivu, where soils have become exhausted by the need to produce more food for

the increasing population, and where fertilizers are seldom available and too expensive for farmers (IITA, 2009).

Nitrogen is often the most limiting factor in plant growth and development (Albareda et *al.,* 2009). The use of nitrogen fertilizers is associated with severe pollution problems so the symbiosis between legumes and rhizobia for biological nitrogen fixation (BNF) is the best option because it uses photosynthetic energy and is environmentally cleaner (Alexandra *et al.,* 2006). The biological nitrogen fixation (BNF) is the process by which soil bacteria collectively called rhizobia enter into a symbiotic association with legumes which leads to the formation of nitrogen fixing root nodules. An important characteristic of this symbiotic interaction is host specificity, where defined species of rhizobia forms nodules on specific legumes (Ampomah *et al.,* 2008).

For successfully BNF and improve production of legumes, the inoculation of soils by the effective and compatible bacteria is required where the population of indigenous bacteria is low (Abaidoo *et al.,* 2007). The Soybean breeding program of IITA sought to eliminate the need for soybean inoculation in Africa by selecting soybean varieties for promiscuity with indigenous *Bradyrhizobium Spp.* (Abaidoo *et al*., 1999). The task of selecting rhizobial strains that match host legumes includes the following steps: 1) collection, isolation and maintenance of the *Rhizobium* germplasm 2) authentication and screening of the Rhizobial isolates for genetic compatibility and nitrogen fixation and 3) assessment of the *Rhizobium* germplasm for edaphic adaptation and *in situ* performance (Howieson *et al*., 2000).

1.1 Problem statement

Soybean is expanding rapidly in South Kivu and has been chosen by many humanitarian organizations to fight the malnutrition (World food program, 2011). The efficacy of soybean

protein is favorable in comparison to other traditional sources of protein. One kilogram of soybean contained as much protein as 2 kg of boneless meat or 5 dozen of eggs or 45 cups of cowôs milk and is relatively inexpensive (Dashiell, 1998). However Soybean production is affected by low soil fertility, poor agricultural practices, and lack of improved seed and extension services. Hence, in DRC there is low production of soybean which is estimated to be only 483 kg ha^{-1} (FAOSTAT, 2010). Compared to the other countries with the same agro ecological conditions, this production is very low: for instance in neighboring countries Rwanda and Uganda, the yields are 790kg ha^{-1} and 1,113kg ha^{-1} respectively.

Soybean grown in many African soils is characterized by low levels of biological nitrogen fixation (BNF) which often cannot support high soybean yields without addition of inorganic N fertilizers or inoculation with of soybean rhizobia (Abaidoo *et al.,* 2007). The inorganic fertilizers are mostly unaffordable by poor farmers and thus the success of soybean cultivation depends exclusively on effective nodulation by indigenous *Bradyrhizobium* (Abaidoo et al., 2002).

The indigenous Bradyrhizobium nodulating soybean remains mostly uncharacterized and the use of inoculants in South Kivu and DRC in general is not practiced however experimental inoculation indicates that soybean responds very well to commercial imported products. The need to inoculate soybean in Africa was, however, considered unfeasible because many African countries were not sufficiently equipped to deal with problems associated with rhizobia inoculum use in the tropics (Bala *et al.,* 2011).

The N2 Africa project has introduced the bio-fertilizer named Biofix Legume Inoculant, produced by MEA Fertilizer Company based in Kenya. BIOFIX Legume Inoculant contains rhizobia strains either USDA 110 or SEMIA 5019 singly. On-farm trials conducted in South

Kivu of DRC has yielded mixed results where the use of Biofix Legume Inoculant increased soybean yield from the currently 0.4 to above 1.5 t/ha in some soils and to no response to rhizobia inoculation at all in other (N2 Africa, 2010). A common approach to improve symbiotic nitrogen fixation and legume productivity has been the reliance on superior of very effective exotic rhizobia strains as inoculants. This approach has failed to achieve the desired responses in a lot of environment (Brockwell *et al.,* 2005).

1.2 Justification

Soybean is an important crop with clear attributes that positively contribute to soil health, human and livestock nutrition, household income, poverty reduction and overall improvements in livelihoods and ecosystem services that are critically needed in sub-Saharan Africa. BNF has the potential to increase world food production through biofertilizer, which is especially important in the developing countries where food shortage are common and prices of industrial nitrogen fertilizers are usually prohibitive (Kahindi and Karanja, 2009). The benefits accrued from use of *Rhizobium* inoculants show that a quite good deal of money can be saved by using quality tested inoculants on the farm.

The determination of rhizobia population is important because it highlights the need to inoculate (Thies *et al.,* 1991). It also allows isolation of indigenous rhizobial strains adapted to environment conditions. The use of strains adapted to environmental conditions can contribute to enhance BNF effect upon yield of soybean and the nitrogen status of the soil. The use of highly efficient and affordable fertilizers will contribute to increase the yield and so to reduce food insecurity in this part of DRC. In addition, producing inoculants locally in DRC is of high benefit for farmers since it facilitates the accessibility and use.

1.3 Objectives

The broad objective is to improve biological nitrogen fixation of soybean in South Kivu through provision of high quality rhizobia inoculants produced from locally isolated strains.

The specific objectives are:

1. To identify effective rhizobia strains in South Kivu soils suitable for use on locally accepted varieties of soybean,
2. To establish the competitive abilities of the most effective soybean rhizobia strains identified from South Kivu soils.
3. To test these strains for their ability to use cheaper carbon sources.

1.4 Hypotheses

1. South Kivu soils has effective and competitive rhizobia strains that could be commercialized for inoculants production

2. Glucose and/or glycerol could substitute mannitol in preparation of rhizobia culture media.

CHAPTER TWO

LITERATURE REVIEW

2.1. Rhizobia taxonomy

Rhizobia are gram negative bacteria, rod shaped that live in soil among other millions of bacteria. Their particularity is that they fix nitrogen in symbiosis with legumes through root nodules. Under the microscope they appear as short rods of 0.5-0.9 micrometers wide and 1.2 to 3 micrometer long. They require oxygen to live and move using special thread-like structures called flagella. They do not form spores but increase through cell division. Their life cycle consists in three phases: saprophytic, infective and symbiotic (Somasegaran and Hoben, 1994; Burton, 1984).

As saprophytes rhizobia live in the soil without their legume host. These are referred to as native rhizobia. The rhizobia that are introduced into the soil as inoculants are said to be introduced (Abaidoo *et al*., 1999). The population of native rhizobia can be very diverse with many distinct strains. However, in many cases and depending upon the legume host, the natural rhizobial population in the soil is too low or the strain of the rhizobial species is not effective (Sanginga and Woomer, 2010). In such circumstance, special mixtures of appropriate rhizobial inoculants may be applied (Brady and Weil, 2002).The number of a particular rhizobial species is usually greater in soils where the host is present or has been recently grown, probably due to the release of bacteria from the senescent nodules; in the absence of the host, numbers are usually low (Kuykendal *et al.,* 1982; Woomer *et al.,* 1988). Several Methods for rhizobia enumeration and measures of diversity were developed. The most probable number (MPN) technique is an important technique in estimating microbial populations in soils, water, and agricultural products

by the fact of its ability to estimate a microbial population size based on a process-related attribute. This method is based on four assumptions. First, inoculation of viable rhizobia on its specific host results in development of nodules. Second, nodulation on that inoculated plant becomes a proof of the presence of infective rhizobia. Third, absence of nodule is a proof of the absence of infective rhizobia. Finally, uninoculated plants are used as control, with absence of nodule (Woomer, 1994; Woomer *et al.,* 2011).

Most rhizobia only weakly absorb Congo red dye, which is included in culture media when isolating rhizobia; however, if the culture medium is not buffered, acid producing rhizobia causes the dye to turn purple. The optimal growth of most strains occurs at a temperature range of 25-30^{o}C and pH of 6.0-7.0 (Somasegaran and Hoben, 1994).

The Rhizobia strains are different from one environment to another. A study has shown that the *Bradyrhizobium spp*. isolates nodulating a Soybean cultivars in Africa are diverse and distinct from the Brabyrhizobia that nodulate North American soybeans (Abaidoo *et al*., 2000). There is an evident genetic diversity among the strains. Wasike *et al*. (2009) reported that indigenous strains are diverse and exhibit this diversity in competitiveness and effectivity with and between hosts.

Rhizobia currently consist of 61 species belonging to 13 different genera, namely *Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Methylobacterium, Burkholdera, Cupriavidus, Devosia, Herbaspirillum, Ochrobactrum* and *Phyllobacterium*. The taxonomy of rhizobia is in constant flux (Ahmad *et al*., 2008).

The genus *Rhizobium* (Family Rhizobiaceae) is genetically diverse and physiologically heterogeneous. These microorganisms are classified together by virtue of their ability to nodulate

groups of plants of the family Leguminoseae. Five genera (*Azorhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium* and *Rhizobium*) and 22 species are currently recognized (De Lajudie *et al*, 1994; Young, 1996). Willems (2006) has mentioned the rising number of species in the genera of rhizobia (53 species) and she recognized seven genera (*Agrobacterium, Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium* and *Allorhizobium*). More than one rhizobia type strain have been found from a single nodule, dual (or multiple) nodule occupancy (Odee, 1993). Rhizobia associated with legumes was described according the growth rate on YMA as very fast, fast, intermediate and slow (Odee *et al*., 1997). According to the classification of Rhizobiaceae in Bergeyôs Manual (Jordan, 1984), the main genera *Rhizobium* is characterized by fast growth on YMA. The *Bradyrhizobium genus* is characterized by slow growth.

Within species, millions of strains can be distinguished based on their effects on nodulation and growth of legumes. A study conducted in two soils of Ghana, whereby three strains of *Bradyrhizobium spp* were evaluated for their effects on nodulation and growth of two varieties of Soybean, has shown the significant difference induced on nodule number, nodule dry weight, N yield and shoot dry weight of plant (Kumaga *et al.,* 2000).

The study of rhizobial diversity has been achieved using several phenotypic and molecular approaches. Identification methods that often lead to phylogeny inference include DNAï DNA hybridization, 16S rDNA sequencing and 16S rDNA RFLP analysis. Some of the most commonly used differentiation methods, which have allowed the establishment of genetic relationships, are random amplified polymorphic DNA (RAPD), total protein profiles intrinsic antibiotic resistance (IAR) patterns , assimilation and biochemical test, and, more recently, direct amplified polymorphic DNA (DAPD) (Alexandra *et al.,* 2006). Mpepereki and Wollun (1991)

used the technique of enzyme-linked immunosorbent assay (ELISA) to characterize indigenous *Bradyrhizobium japonicum* in North Carolina soils.

2.2 Importance of Biological nitrogen fixation in agriculture.

Plant-associated nitrogen fixation fixation currently contributes 50ï 70 million tonnes annually to the global agricultural N budget (Unkovich *et al.,* 2008), this account for from 40 to 70% of total global nitrogen input (Kahindi and Karanja, 2009). Augmenting nitrogen supply through BNF is a viable option for resource-poor farmers of developing countries (Rattan, 1995); it is a ñfree" source of nitrogen for agriculture (Giller and Cadisch, 1995). Nitrogen-fixing systems offer an economically and ecologically attractive means of reducing external inputs and improving internal resources (Bohlool *et al*., 1992; Albareda *et al.,* 2009).

All organisms require nitrogen for their growth because it is an essential constituent of proteins and nucleic acid, which is a basis for their life. It has been reported that healthy plant contains 3 to 4% of nitrogen in their above ground tissues. This is a higher concentration than other nutrients, except carbon, oxygen and hydrogen. Because it is a constituent of amino acids which are required to synthesize proteins, nitrogen plays a role in almost all plant metabolic processes (Mckee, 1962). Nitrogen is essential for the function of biochemical agents like chlorophyll (which make photosynthesis possible), enzymes (which helps organisms carry out biochemical processes and assimilate nutrients) and nucleic acids such as DNA and RNA (which are involved in reproduction) (Kramer, 2000). Plant lacking nitrogen shows slow stunted growth and their foliage is pale green

Nitrogen gas makes up 78% of the air. In spite of its abundance in the atmosphere, it is not available to plants in a form that is suitable for metabolism until it is converted to ammonia or other reduced forms. Nitrogen in the atmosphere occurs mostly in the form of dinitrogen,

essentially inert, with a very strong triple bond (N≡N). In order for nitrogen to be used for growth it must be "fixed" (combined) in the form of ammonium (NH_4) or nitrate (NO_3) ions. Atmospheric N_2 can be fixed industrially, through Haber Bosch process, or through biological nitrogen fixation. BNF is the process whereby atmospheric nitrogen is reduced to ammonia in the presence of nitrogenase (Mulongoy, 1992). It is also an important process within the nitrogen cycle, one of the important biogeochemical cycles for living things.

BNF is important because it presents the potential to reduce the manufactured fertilizers N in certain cropping systems. It is an inexpensive valuable resource for farmers because it cost is low than the N-fertilizers. Crops legume can fix between 30 to 150 kg of nitrogen per crop per hectare (Unkovich *et al.,* 2008*).* BNF has economic and environment advantages compared to N-fertilizers. Economically it reduces costs of production. Field trials have shown that the N captured by crops due to the use of rhizobia inoculants costing $3/ha is equal to fertilizer N costing $87 (Silva and Uchida, 2000). Environmentally, the use of inoculants as alternatives to N fertilizer avoids problems of contamination of water resources from leaching and runoff of excess fertilizer. It has been demonstrated that fertilization with ammonia leads to increase soil acidity (Werner *et al.,* 2005). Utilizing BNF is part of responsible natural resource management. The production of nitrogen fertilizers by industrial fixation generates large quantities of carbon dioxide, contributing to earth warming. The natural process of BNF offers an economic means of reducing environmental problems and improving internal resources (Kahindi and Karanja, 2009).

The importance of BNF is explained also by the increase of yields. A study has been conducted in China where the use of inoculants based on *B.japonicum* on Soybean has increased the yield up to 13% compared to the control (Werner *et al.,* 2005). A study has shown also that the use of biological fixation in flooded paddies can yield up to 50 kg ha-1 crop -1 (Stoltzfus, *et al.,* 1997).

In addition, BNF may be a solution for the nutrients losses from soils. It has been reported that an average of 660 kg N ha-1, 75 kg P ha-1, and 450 kg K ha-1 has been lost during the last 30 yr from about 200 million ha of cultivated land in 37 African countries (Sanchez et al., 1997). The soybean-*Bradyrhizobium* symbiosis can fix about 300 kg N ha^{-1} under good conditions (Harold and Fudi 1992). This also may improve soil organic matter important for the soil health.

2.3. Mechanism of BNF

Biological nitrogen fixation is mediated by the nitrogenase enzyme system that catalyses the ATP dependent reduction of atmospheric dinitrogen to ammonia. Nitrogenase consists of two component metalloproteins, the MoFe-protein with the FeMo-cofactor that provides the active site for substrate reduction, and the Fe-protein that couples ATP hydrolysis to electron transfer (Rees *et al*., 2005). It is a process that changes inert N_2 to biologically useful NH_3. This process is mediated in nature only by bacteria. Other plants benefit from nitrogen-fixing bacteria when the bacteria die and release nitrogen to the environment or when the bacteria live in close association with the plant (Lindemann and Gloves, 2003). Legume BNF involves a symbiosis between legume plants and the rhizobia that live in nodules on their roots.

Atmospheric Nitrogen (N) is a molecule composed by two atoms of nitrogen linked by a very strong triple bond. Large amount of energy are required to break this bond (1) before they become reactive (2).

$$N_2 + 6H^+ + ATP \longrightarrow 2NH_3 + ADP + Pi.$$

$$N{\equiv}N + 8H+ + 8e^- + 16\ ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi$$

The triple bond must be broken and three atoms of hydrogen must be added to each of the nitrogen atoms. Nitrogen fixing bacteria use energy derived from oxidation (burning) of carbohydrate to reduce molecular nitrogen (N_2) to ammonia (NH_3). The industrial reaction of biological fixation involves burning of fossil fuel to obtain the electrons, hydrogen atoms and energy needed to reduce molecular nitrogen (Hubbel and Kiddel, 2000).

The factors that control the amount of nitrogen fixed include available soil N, genetic determinant of compatibility between symbiotic partners, and absence of other yield-limiting factors. Response to inoculation is controlled by the level of native rhizobia, the N demand and yield potential of the host, and the N availability in the soil (Keyser and Li, 1992).

Formation of symbiotically effective root nodules involves signaling between host and microsymbiont. Flavonoids and/or isoflavonoids released from the root of the legume host induce transcription of nodulation genes in compatible rhizobia, leading to the formation of lipo-chitooligosaccharide molecules that, in turn, signal the host plant to begin nodule formation (Long, 1996). Lipo-chitooligosaccharide nodulation factors (nod factors) produced by rhizobia determine the host range. These factors play a pivotal role in the molecular signal exchange, infection and induction of symbiotic developmental responses in legumes leading to the formation of a nodule in which rhizobia carry out N_2 fixation (Reddy *et al.,* 1998). Nitrogenase is the enzyme responsible of Nitrogen fixation. This enzyme consists of two proteins namely, an iron protein and a molybdenum-iron protein.

Once the recognition signal is induced Rhizobia present in the rhizosphere begin to multiply on the surface of young root of an emerging legume plant. They enter the roots through root hairs. But other entry pathways are also known, for example wound entry for groundnut, stem nodulation for sesbania. After about two weeks, small bumps appear on the roots. These bumps

eventually become larger and mature into fully functional nodules. The nodules produce nitrogen for the legume throughout the growing season with peak activity usually at about the time of flowering (Singleton *et al*., 1990).

2.4 Factors limiting BNF

Nodulation and symbiotic fixation of nitrogen is strongly related to the nutritional and physiological state of the host plant. Nutrient deficiency, mineral toxicity, salinity, unfavorable pH, plant diseases, weed competition, extreme temperature and moisture are factors that affect the legume host vigor, receptive physiology, nodulation and/or symbiotic nitrogen fixation in the soil (Giller, 2001).

Excess salinity is a threat to the initiation of symbiotic nitrogen fixation (Rao *et al.* 2002). It is a serious threat to agriculture in general and to the production of grain legumes in particular as a result of their low tolerance to salinity. Salinity does not affect colonization of root by rhizobia (Singleton and Bohlool, 1984) but does retard the initiation or growth of new nodules, reduce the efficiency of fully formed nodules which had developed earlier under non saline conditions (Rao *et al*. 2002) and decrease the proportion of those nodules that are initiated in saline conditions that are able to differentiate fully in to active nitrogen fixing nodules (Yousef and Sprent, 1983).

Soil acidity limits symbiotic nitrogen fixation by reducing rhizobium survival and persistence in soils, as well as reducing nodulation (Taylor *et al*., 1991). In most of the cases, a pH sensitive stage in nodulation occurs early in the infection process and that *Rhizobium* attachment to root hairs is one of the stages affected by acidic conditions in soils. At or below pH 4.8, aluminum will reduce root growth while manganese disrupts photosynthesis and other functions of growth resulting in the reduction of nitrogen fixation by rhizobia (Duncan, 2002).

Phosphorous (P) fertilization is the major mineral nutrient yield determinant among legume crops (Chaudhary, 2008). Its deficiency is usual in tropical Africa and reduces plant growth, nodulation and nitrogen fixation (Mulongoy, 1992). Plants dependent on symbiotic nitrogen by roots fixation have special ATP requirements for nodule development and function and need additional P for signal transduction and membrane biosynthesis (Ribet and Drevon, 1996). The increase of whole plant growth and plant nitrogen concentration in response to increased soil P supply has been noted for several leguminous species. Decreased specific-nitrogenase activity in nodules of leguminous plants was associated with decreased energy status of host plant cells of nodules.

In legume symbiosis, the host legume rather than the microsymbionts mediates regulation of N_2 fixation in response to available fixed nitrogen (Giller, 2001). Mineral nitrogen inhibits the *Rhizobium* infection process and also inhibits N_2-fixation in active nodules. The former condition probably results from impairment of the recognition mechanisms by nitrates, while the latter is probably due to diversion of photosynthates toward assimilation of nitrates (Mulongoy, 1992). The phenomenon of inhibition of symbiotic N_2-fixation by nitrate appears to occur in three stages. In the first stage, nitrate is restricted to the cytoplasm of nodule cells and the immediate decreases in nitrogenase activity due to an increase in resistance of the O_2 diffusion barrier. It is followed by a reduction in carbon metabolism (Arrese-Igor *et al.,* 1997). Irreversible nodule senescence follows, perhaps encouraged by toxic effects of nitrite on nitrogenase and leghemoglobin (Becana and Sprent, 1987). Application of large quantities of fertilizer N inhibits N_2 fixation, but low doses (<30 kg N ha^{-1}) of fertilizer N can stimulate early growth of legumes and increase their overall nodulation and nitrogen fixation. The amount of this starter N_2 must be defined in relation to available soil (Mulongoy, 1992). Tubb (1976) reported a depressed

synthesis of nitrogenase in *Rhizobium* in the presence of NH_4^+. So nitrate is a preferred form of starter nitrogen.

Water logging prevents the development of root hair and sites of nodulation, and interferes with a normal diffusion of O_2 in the root system of plants. The lack of O_2 is a major problem for root respiration and results in loss of nitrogenase activity (Sprent and Gallacher, 1976). Depression of growth in soybean plants caused by water logging is greater in plants dependant on N_2 fixation than on plants supplied with nitrate, suggesting that N_2 fixation is more sensitive to the effect of water logging than plant growth (Bacanamwo and Purcell 1999). Waterlogging also interferes with diffusion of nitrogen to root nodules.

The number of rhizobia in the soil declines drastically as soil dries (Giller, 2001). Apart from its effect on population number, drought also affects the process of infection and fixation. A significant decrease in number of infection threads and complete inhibition of nodulation under low moisture potential has been reported, although the number of rhizobia in the rhizosphere is unaffected (Worrall and Roughley, 1976). Prolonged drought will promote nodule decay. Deep-rooted legumes exploiting moisture in lower soil layers can continue fixing N_2 when the soil is drying. The rate of N_2 fixation is more sensitive to reduction in soil water content than other process such as photosynthesis, transpiration, leaf growth rates or nitrate assimilation (Giller, 2001).

The two important climatic determinants affecting BNF are the soil temperature and light. The effect of high temperature could be explained in terms of reducing the rhizobial population in the soil (Marshal, 1964). The maximum rate of nitrogen fixation has been obtained at high soil temperature of 33^oC. This is easy to understand because N_2 fixation is an enzymatic process.

However, there are differences between symbiotic systems in their ability to tolerate high (>35°C) and low (<25°C) temperatures (Mengel and Kirkby, 1987). Optimum temperature for growth and nitrogen fixation vary widely among legume species and reflect environmental adaptation (Giller, 2001). For instance, critical temperatures for N_2 fixation are 30°C for pea which is a high land pulse and range between 35 and 40°C for soybean, groundnut, and cowpea (low land pulse) (Micheils *et al*., 1994).

The availability of light regulates photosynthesis, upon which biological nitrogen fixation depends. This is demonstrated by diurnal variations in nitrogenase activity. Very few plants can grow and fix N_2 under shade. In alley farming, if hedgerows are not weeded, or if trees are planted with food crops like cassava, their nitrogen fixation and growth will be reduced due to shading. Early growth of legume trees is slow and they cannot compete successfully for light (Mulongoy, 1992). In most cases, the microsymbiont is the more affected partner (Hungria and Vargas, 2000).

The response to inoculation can be controlled by the number of effective native rhizobia present in the soils. When native rhizobia are sufficient in number and effectiveness, the cropôs N requirement may be met without inoculants addition

2.5 Biological Nitrogen Fixation (BNF) in Soybean

Improving BNF component has been identified as part of the overall strategy for increasing productivity of soybean. BNF in soybean will be improved by mastering the functioning system of symbiosis rhizobia-soybean. The symbiosis involving Soybean and *Bradyrhizobium* is a well organized system, beginning at root surface and resulting in nitrogen fixing nodules. The host plant provides carbon substrates as a source of energy to the bacteria, and the bacteria reduces

atmospheric N_2 to NH_3 which is exported to plant tissues for eventual protein synthesis (Keyser and Li, 1992).

A study conducted by Osunde and Bala (2005) has shown that the proportion of N derived from fixation of N by Soybean is in the range of 31-110 kg N ha^{-1}. The requirement of soybean to fix nitrogen is a good soil with an optimum level of nutrients such as phosphorous, potassium and micronutrients such as zinc, molybdenum and cobalt. Nitrogen must be in low quantity called starter N (Mulongoy, 1992).

Soybean production in many African countries depends on indigenous *Bradyrhizobium* spp. populations as sources of fixed N (Abaidoo *et al.,* 1999). Nodulation of soybean (*Glycine max)* requires specific Bradyrhizobium species. Compatible populations of these bradyrhizobia are seldom available in soils where the soybean crop has not been grown previously (Abaidoo *et al.,* 2007). *Bradyrhizobium japonicum* populations required for effective nodulation of soybeans are not endemic to African soils (Hadley and Hymowitz, 1973), reason why inoculation with effective rhizobia is needed to achieve good production of soybean. Appunu *et al.,* (2008) stated that Soybean-nodulating rhizobia are genetically diverse and are classified into different genera and species. Rhizobia able to nodulate soybean include six species belonging to three different genera, *Bradyrhizobium, Mesorhizobium* and *Sinorhizobium* (Albareda *et al.,* 2009). The slow growers are distributed in three species of the *Bradyrhizobium* genus, namely, *Bradyrhizobium japonicum*, *Bradyrhizobium liaoningense* and *Bradyrhizobium elkanii*. Fast growers belong to *Sinorhizobium fredii* and *S. xinjiangense* and also include other unclassified rhizobia. Soybean rhizobia with a variable generation time were classified into *Mesorhizobium tianshanense*. Otherwise Abaidoo *et al.* (2000) stated that *Bradyrhizobium* spp. (TGx) isolates nodulating the

new soybean cultivars in Africa are diverse and distinct from bradyrhizobia that nodulate North American soybeans.

Some varieties of soybean may be promiscuous (nodulate a wide range of rhizobia) or specific to a rhizobia strains. Promiscuous soybeans called TGX were bred by the breeding program of IITA to nodulate freely with indigenous soil *Brabyrhizobium*, hereby eliminating the need for inoculation (Osunde and Bala, 2005). A study conducted by Yusuf *et al.,* (2009) has shown that soils of many potential soybean fields in Africa are characterized by low levels of biological nitrogen fixation (BNF) activities and often cannot support high soybean yields without addition of inorganic N fertilizers or external application of soybean rhizobia. A study conducted by Mpepereki *et al.,* (2000) has shown that promiscuously nodulating varieties of soybean nodulate abundantly and effectively in most soils in Southern Africa.

Host-strain compatibility must be respected; a study has shown that some strains may nodulate well with some specific germplasm of Soybean (Appunu *et al*., 2006). Keyser and Li (1992) have proposed the research strategy to increase BNF in soybean. The strategy should focus on selection and engineering of elite rhizobia, selection and breeding of soybean genotype, improvement inoculation techniques. The production strategy to increase BNF in soybean should focus on inoculants production and quality control and training, matching soybean genotype to the environment and management of other inputs.

Several studies have been conducted in relation to the symbiotic nitrogen fixation potential of soybean and the diversity of the associated *Bradyrhizobium* strains. Abaidoo *et al.,* (1990) measured the fixed N by promiscuous soybean varieties by the isotope dilution methods. According to this study the current selection procedure for enhanced N_2 fixation based on an assessment of nodule formation does not directly quantify the proportions of crop N derived

from the atmosphere. Appunu *et al.,* (2006) studied the symbiotic interactive effect of different *Bradyrhizobium japonicum* strains with six soybean cultivars under field conditions. On the basis of analysis of correlation coefficients, they stated that plant dry matter accumulation emerged as best criterion for selection of most effective legume-Rhizobium associations for given physical and biological conditions. Otherwise, Unkovich *et al.,(*2008), in his study, measuring plant-associated nitrogen fixation in agricultural systems, stated that there is no single ócorrectô way to measure N_2 fixation, and since all current methodologies have limitations, measuring the exact amount of N_2 fixed continues to be a challenge. Ideally, several different methods should be used simultaneously, particularly if they are complementary.

Legume inoculation is a process through which leguminous crops are provided with the effective bacterial strain of the genous of *Rhizobium* which results in an effective symbiotic relationship that brings about fixation of atmospheric nitrogen into organic nitrogenous compounds in the plant (Ayuke *et al*, 2012). About 10,000 tonnes of rhizobial inoculants for inoculating soybean are produced in Kenya, South Africa, Zambia and Zimbabwe (Karanja *et al.,* 1995). Many regional Microbiological Resources Centre (MIRCENS) were established in Africa by UNESCO to promote high value, low-cost technologies that improve rural agricultural practices, creating rural market economies and providing more technological avenues for employement, increased incomes and ultimate feeder industries to the urban sector (Bala *et al.,* 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the study area

South Kivu is located in Eastern of Democratic Republic of Congo, approximately between 1°36ô - 5° South and 26°49ô- 29°20ô East. It covers an area of 69,130km^2 with the population estimated at 3.5 millions peoples with population density is estimated at 51 people per km^2. The relief is essentially highland with numerous mountains; the climate is sub-humid moderate by the altitude with a long rain season of nine months (Kakura, 2005). Soils in South-Kivu are rather infertile Dystric Humic Nitisols or Humic Ferralsols (FAO/UNESCO, 1988), developed one ruptive formations from the Pliocene or Pleistocene and characterized by a heavy clay texture, low soil pH, low base saturations and high organic carbon contents(Hecq, 1961). In some parts soils are more fertile Humic Nitisols and Ferralsols resulting from recent rejuvenation by volcanic ashes or mud flow deposits; these soils have high organic matter content, favourable pH and larger nutrient reserves (Lunze, 2000).

The rainfall in Sud-Kivu is bimodal and allows crop cultivation during two subsequent seasons: the ñAò season starts mid September and ends mid-January, while the ñBò season lasts from mid-February to mid-June, followed by a short dry period, often referred to as the ñCò season, when farmers cultivate in valleys and drained marshlands. The area receives on average 1500ï1800 mm per year, and the growing period extends to over 325 days per year (Hijmans *et al.,* 2005).

Soils for potted field soils experiment were obtained in two N2 Africa action sites in South Kivu; the North axis and South axis. The North axis is characterized by volcanic soils named *civu* by

the farmers and the South axis with highly degraded soils rich in oxide ferrous named k*alongo* by the farmers.

The samples were taken in five villages namely: Walungu, Katana, Uvira, Burhinyi, kalehe, Birava, etc. Walungu and Katana are located in high altitude (1600 and 1400m respectively) and Uvira in low altitude (400m).

3.2. Collection, isolation, characterization and authentication of indigenous rhizobia strains.

Nodules were collected from cultivated and uncultivated legumes (appendix 1) which were randomly selected along a transect (between 400m and 1600m of elevation). In the field, the legumes were identified using botanical key and plants were uprooted using a knife, avoiding detaching secondary roots from plant as nodules may be found on lateral roots as well as the tap root. Carefully the nodules were excised using a blade with lateral roots and placed in the vials containing silica gel and covered with a layer of cotton wool. The nodules were transported to the laboratory in a cooler. The location of the sampling points or farms was determined using the global positioning system (GPS) (Woomer et al., 2011). The growth media used for rhizobia isolation is the Yeast Extract mannitol Media (YEMA) (Vincent, 1970). Composition of YMA media is as follows: 10.0 g Mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g Nacl, 1.0 g Yeast Extract, 15.0 g of agar and 1 litre of distilled water. Adjustment was done on pH of the media to 6.8 using 0.1N NaOH. Various dyes were incorporated in the Media: Congo Red (CR) and Bromothymol blue (BTB) that were added at a concentration of 25 ppm.

Surface sterilization of rhizobia from the nodules was performed immersed in 95% ethanol for 5-10 seconds, then transferred to a 2.5-3% (v/v) solution of sodium hypochlorite, and soaked for 3-4 minutes. The nodules were rinsed in five changes of sterile water using clean forceps. After

surface sterilization the nodule was crushed with a pair of blunt tipped forceps in a large drop of sterile water in a petri-dish. The drop of suspension was streaked onto the agar surface so that suspension is progressively diluted. The plates were then be incubated at 25-30°C in the dark (Antoun and Prevost, 2006). After growth, a single colony was re-streaked again on YMA CR for purification (Somasegaran and Hoben, 1985).

Typical rhizobia were recognized by their appearance, the growth rate and the production of alkalinity or acidity. The growth media contained a pH indicator; for example in YMA with initial pH 6.8, Bromothymol blue was incorporated, which turns to yellow on the production of acid, and blue on the production of alkali. As an alternative to this pH indicator, Congo red was incorporated into the media to help distinguish rhizobia with other bacteria. Usually *Rhizobium* colonies take up milky color whereas colonies of other bacteria take the color dark red (CIAT, 1988).

The cultures were examined microscopically by gram staining; rhizobia are gram negative, motile rods (CIAT, 1988). For gram staining, thin smears of various bacteria was made and heated for fixation; then the smears was stained with the Crystal Violet for one minute; washed lightly with water and flooded with iodine; immediately drained and flooded again with iodine for one minute; drained with iodine and decolorize with 95% alcohol for 30-60 seconds ; the smear was washed with water and blotted dry carefully; the stain was then countered with safranin for one minute, finally washed with water and air dried; the preparation was observed under oil immersion (Somasegaran and Hoben, 1985). Growth rate for rhizobia, colony characteristics on Congo red -YMA and reaction on Bromothymol Blue were recorded. For the nodulation test, two suitable growth units (modified Leonardôs jars) for each of the isolates plus at least two extra units of uninoculated controls was set up. Seeds were surface sterilized and

pre-germinated by immersing in 95% alcohol for 10 seconds to remove waxy material and trapped air. Seeds were then immersed in 3% sodium hypochlorite solution for 3-5 minutes in an Erlenmeyer flask, and then the seeds were washed in six change of distilled sterile water. The seeds were left in the final change of sterile water for four hours until they were fully imbibed. After wards seeds were washed in two more changes of sterile water and transferred aseptically with forceps to the surface of a 2% water agar petri-dish and incubated at 25°C until the radicals were 0.5 to 1.0 cm long. Surface sterilized and pre germinated seeds were inoculated with 1.0 ml of broth culture for each isolate in two growth units. Planting and inoculation was done in a clean area. The plants were removed from the rooting medium and the presence or absence of nodules was noted. The cultures of presumptive isolates were confirmed as rhizobia and were given NAC numbers (Woomer *et al.,* 2011). From this nodulation test one hundred and seven isolates were collected.

3.3. Screening of isolates for nitrogen-fixing effectiveness in aseptic conditions.

3.3.1 Modified Leonard's jars preparation.

A commercially available 1.5 capacity water bottle was cut into two halves; one portion used for holding the nutrient solution and the other part was inverted for the growth media (sterile sand). The assembly was covered by a grey paper bag to protect roots and nutrients solution from light. A centrally positioned lantern wick made from braided cotton which runs through the length of the bottle and extends out of the mouth of the bottle into the reservoir containing the nutrient was used to irrigate the growth medium. Well washed and autoclaved sands were used like growth medium (Burton, 1984). Nitrogen-free nutrient solution (Broughton and Dilworth, 1970) was used for growth of Soybean (appendix 12). For plus N control treatment, KNO_3 (0.05%) was added in the nutrient solution giving a nitrogen concentration of 70 ppm.

3.3.2 Cultures preparation and seeds pre germination.

Each of the strains to be evaluated was cultured for 5-7 days in advance of planting time. Rhizobia were grown in 100 ml Erlenmeyer flasks containing 20 ml of yeast-mannitol broth. Then the cultures were incubated at a room temperature (25-30°C) on a rotary shaker for 7 days (Somasegaran and Hoben, 1985).

For seed pre-germination, uniform seeds were surface sterilized by immersing in 95% alcohol for 10 seconds to remove waxy material and trapped air. Seeds were then immersed in 3% sodium hypochlorite solution for 3-5 minutes in sterile Erlenmeyer flask, and then seeds were washed in six changes of distilled sterile water. The seeds were left in the final change of sterile water for four hours until they were fully imbibed. After being imbibed, seeds were washed in two more changes of sterile water and transferred aseptically with forceps to the surface of a 2% water agar petri-dish and incubate at 25℃ until the development of a radical of 0.5 to 1cm long (Woomer *et al.,* 2011; Burton, 1984).

3.3.3 Planting and inoculating.

Three well-spaced holes were made in the rooting medium to a depth that will accommodate seeds one centimeter below the surface. Pre-germinated seeds were picked up with sterile forceps and one seed in each hole was placed with the radical entering first. After placement of the seed, the hole was covered with the rooting medium (sand). Seeds were then inoculated with 1ml of broth using a fresh pipette for each isolate (Somasegaran and Hoben, 1985). Five days after emergence, the plants were thinned to two uniform plants per jar. During the thinning process the disturbing of rooting medium was avoided by cutting the plant instead of uprooting (Burton, 1984)

3.3.4 Conducting the trial and harvesting.

Water and nutrients were supplied three times per week. The leaf color and plant growth was noted frequently. After seven weeks (early flowering), the tops were excised and their dry matter determined by drying for 48 hours at 70°C. The roots were removed from the jars and tubes and washed carefully with stream water. Where nodules were present, the shape, size, pigmentation and distribution was described. The nodules were detached, counted, their total fresh weight determined. After that, they were placed in an aluminum foil weighing boats for drying to constant weight at 70°C for 2 days. Nodule harvest from each jar was treated individually as well as shoots. The dry weight was determined for shoots and for nodules.

3.3.5 Experimental design and treatments.

The screening of isolates under aseptic conditions was set up as a split plot design with a Randomized Complete Block (RCBD) arrangement replicated four times. The treatments were assigned as follows: the 107 isolates from South Kivu soils, two commercial strains (USDA110 and SEMIA 5019) and two controls (a non-inoculated control with nitrogen and without nitrogen). The factors under study are: Rhizobia strains assigned on the sub-plot and soybean promiscuous varieties (two: SB19 and SB24) assigned on the main plot. For the plus-nitrogen control 70 ppm of nitrogen was applied as a 0.05 % KNO_3 (w/v) solution. The nitrogen was added to the nutrient solution in the reservoir of the Leonard jar assembly.

3.3.6 Data collection and analysis

Nodules were scored using 0 to 5 scoring system where 0 = no nodules, 1 = 1 to 5 nodules (rare), 2 =6 to 10 nodules (few), 3 = 10 to 20 nodules (moderate), 4 = 20 to 50 nodules (abundant) and 5 = >50 nodules (extra abundant). Nodule distribution was also scored as crown (many at upper

tap root) or diffuses (Woomer *et al.,* 2011). The greenness of plants was qualitatively scored by comparing the plant color with the color of the uninoculated plants and the inoculated plants which they were assigned scores 1 and 3, respectively, with a color in between the controls assigned as 2. The Effectiveness Index (EI) was calculated for each strain, where, Effectiveness index (E.I) = isolate shoot dry weight/USDA 110 shoot dry weight (Pinto *et al.,* 1974). The Analysis of Variance was done with GENSTAT and the means were compared using Least Significant Difference test at 5% (p=0.05)

3.4 Selecting effective strains of rhizobia in potted field soil in greenhouse

The experiment was conducted at IITA research centre Kalambo station in a greenhouse, temperature ranging between 19^0C and 38^0C. Ten best performing isolates were selected from the 107 isolates that were tested in sterile sand. The isolates were tested for their symbiotic effectiveness using soil as media on two promiscuous soybean varieties (SB19 and SB24). Soil was collected from a field with no history of use of rhizobia inoculations and cultivation with the soybean or any other legume, no water-logging or salinity problems.

Field soils were obtained from a depth of 0-15cm with a steel spade and hoes in the two sites. Soil were collected and transported in strong plastic bags to the laboratory. In the laboratory, large pieces of clean cardboard were spread on the floor and covered with thick plastic sheets. The bags of soil were emptied onto the prepared substratum to pool all the collected soil. The soil was mixed thoroughly debris (stones, roots, leaves, etc.) removed and lumps broken. The soil was sifted using a 0.5 cm mesh screen. Lime was added to the South axis soils to bring the pH to 6.5. Soil and lime were mixed thoroughly and allowed to equilibrate for seven days. During the equilibrating period, the soil was covered with a plastic sheet.

Polyvinylchloride (PVC) pots with of 15-18 cm diameter and 18 cm height with a capacity of 3 liters and with at least three holes on the bottom were used. Pots held approximately 2.5 kg of soil. The soil in all pots occupied the same volume to achieve similar bulk density. 250 grams of soil must was set aside in the refrigerator for MPN count of the native rhizobial population and 1kg was taken to the Universite Catholique de Bukavu (U.C.B) laboratory for physic-chemical analysis (N,P and K) (Okalebo *et al.,* 2002).

3.4.1Methods for soil characterization

3.4.1.1 Soil pH

Soil pH was measured on a 1:2.5 soil suspension to water using a pH meter as described by Okalebo *et al., (*2002). Twenty grams of air dried soil were passed through 2 mm sieve, weighed and put into plastic bottles. Fifty ml of distilled water was added and the bottles tightly corked. The mixture was shaken for 30 minutes with the mechanical shaker and allowed to stand for 30 minutes. The pH of the soil suspension was then measured using a pH meter.

3.4.1.2 Total Nitrogen

Soil total N was determined by the Kjeldahl method. Air dried samples was passed through a 0.5 mm sieve and 5g of soil obtained. These were put into boiling tubes and a 0.5g selenium mixture catalyst added followed by 3.5 ml of concentrated sulphuric acid to start the digestion process. The set-up was left in digestion chamber for 2 hours. After 2 hours, the digests were retrieved from digestion chamber and allowed to cool for 30 minutes after which they were transferred into distillation flask and 40 ml of 10N NaOH were added then followed by distillation process. NH_3 released was collected into 20 ml of 1% Boric acid and titration done against 0.01 M H_2SO_4 (Okalebo *et al.,* 2002).

3.4.1.3 Phosphorus

Available soil P was determined by Olsen method. Five grams of air dried soil was passed through 2 mm sieve and weighed into a 100 ml extracting tube and 50 ml double acid reagent added. The tubes were corked tightly, placed horizontally in a rack on a mechanical shaker and shaken for 30 minutes. The soil was filtered through Whatman filter paper No. 42 and filtrate collected in specimen bottles. A suitable aliquot of the soil extract was measured and put into a 50 ml volumetric flask. Twenty-five ml of distilled water was added to each tube followed by 8 ml of reagent B and immediately distilled water was added to the mark and mixed thoroughly. The solution was allowed to stand 25 minutes before readings (Okalebo *et al.,* 2002).

3.4.1.4 Potassium

Five gram of air dried soil was passed through 2 mm sieve; the samples were put into plastic containers and leached with 100 ml of 1M NH_4OAc at pH 7.0. The leachate was diluted ten times; 5 ml of the leachate was pipetted into a 50 ml volumetric flask. One hundred ml 1N KCl was added in each container and the contents diluted with 1M NH_4OAc. Potassium (K) was measured using flame photometry (Okalebo *et al.,* 2002).

3.4.1.5 Indigenous rhizobial populations in soil

The serial dilution was done by adding 900 ml of sterile water into 100 g of soil. This was mixed thoroughly on a rotary shaker for 20 minutes to disperse the soils. Serial dilutions were up to 10^{-6}. Soybean seeds were surface sterilized and pre-germinated in sterile vermiculite. Two pre-germinated seeds were planted in each growth pouch with 4 replications per dilution. Seedlings in each growth pouch received 30ml of Nitrogen-free nutrient solution (Broughton and Dilworth, 1970). Each growth pouch was inoculated with 1 ml of the appropriate soil dilution set starting

from the highest dilution and proceeding down the series. The uninoculated control was included. Harvesting was done at 28 days after inoculation. The pot where there is nodule was recorded as positive and where there is no nodule negative (Woomer, 1994).

3.4.1.6 Rhizobia population in the culture used in pot soil experiment

The bacterial culture population was determined in the ten isolates. A serial dilution was done for each isolate by adding 100µl of the culture into 900µl of diluent. Eight tubes each containing 9 ml of sterile diluent (YM broth, pH 6.8) were settle out. One ml of the broth culture was diluted in steps, tenfold each time (10^{-1} through 10^{-8}). Dilution 10^{-6}, 10^{-7}, 10^{-8} were plated using pour plate method and the volume used was 1µl (Woomer et al., 2011).

3.4.2 Planting and inoculating the seeds

At sowing the soil moisture was maintained at field capacity for better plant performance. The fertility of the soil was adjusted to optimal level to obtain good growth of the plant by adding the following fertilizers: phosphorous (100 kg ha^{-1}) as triple superphosphate ; Potassium (200 kg ha^{-1}) as KCl; magnesium (5 kg ha^{-1}) as $MgSO_4.7H_2O$; Nitrogen (100 kg ha^{-1}) applied as urea in the plus nitrogen control. The fertilizers (except the TSP) were prepared as solution and pipette on the soil surface and allowed to dry the day of planting. The soil was mixed thoroughly to ensure uniform distribution of the nutrients.TSP was added to the soil three days before planting but the other fertilizers were added at planting (Burton, 1984).

The seeds were planted at the planting rate of eight seeds per pot thinned to two plants after emergence. The seeds were sterilized as described in section 3.2.0. After the process of sterilization, the seeds were planted at a depth of 2cm. Each seed was inoculated with 1 ml of the culture. The treatments were labeled and the number of blocks assigned.

3.4.2 Watering of the pots, periodic observations and harvesting

Watering was done frequently with sterile water. The plants were harvested at 35 days (early flowering). The tops of the plants were cut excised and put in the paper bag. Soils were removed carefully and the nodules number, distribution, pigmentation determined. Nodules were detached and their fresh weight determined. Shoots and nodules were drying in the oven at 70°C for 48hours and the dry weight was determined for each treatment.

3.4.3 Experimental design and treatments

The potted-field soil experiment was laid out on a randomized complete block design with split-split-plot arrangement replicated four times. The factors under study were soils as the main plot, the soybean varieties were the sub plot and rhizobia strains were the sub-sub-plot. The treatments include ten isolates; eight rhizobia isolates from South Kivu and two standards strains (USDA110 and SEMIA5019) from Biofix Legume Inoculant and the two controls (a non-inoculated control plus-nitrogen and non-inoculated control without nitrogen).

3.4.4 Data analysis.

The data to be collected include nodule number, size and distribution scored according to 0-6 scale (Corbin *et al*., (1977), the fresh weight of nodules and the dry weight of shoots and nodules. The greenness of plants was qualitatively scored by comparing the plant color of treatments with the color of the uninoculated pots plus nitrogen and the uninoculated pots without nitrogen plants which they were assigned scores 1 and 3, respectively, with a color in between the controls assigned as 2.The Plants were carefully uprooted so that no nodules were left in the soil. The table 1 below shows how nodule score for nodule distribution on roots was done on a 0-6 scale.

Table 1: Nodule score (Modified Corbin, *et al.,* 1977).

Nodule score	Distribution of nodules on roots	
	Crown (top 5cm of root system)	Elsewhere
0	0	0
0.5	0	1-4
1	0	5-9
1.5	0	Ó10
2	< 5	0
2.5	< 5	< 10
3	< 5	> 10
4	> 5	0
5	> 5	< 10
6	> 5	> 10

The competitiveness index was calculated for each strain, where competitiveness index (C.I) = Shoot dry weight strain/ shoot dry weight of native rhizobia (control without nitrogen). All data from the experiment were entered into excel spreadsheet and were subjected to analysis of variance (ANOVA) using GenStat statistical package. Regression and correlation analyses were established between plant dry weight and nodule number. The statistical significance was determined at P Ò0.05. The MPNES program (Woomer *et al*., 1990) was used to calculate the indigenous rhizobial populations in soil.

3.4 Screening of isolates for cheaper carbon source utilization.

The determination carbon source utilization ability of our selected best strains is important because for inoculant production it is necessary to identify a less expensive carbon source that is locally available, cheaper and able to support a large viable count during the shelf life without losing the effectiveness of the strains (Okereke and Okeh, 2007). The eight rhizobia strains screened for competitiveness was screened for carbon source utilization compared to the references strains (USDA 110 and SEMIA 5019). The different carbon sources included mannitol, glycerol and glucose. The isolates: NAC 17, NAC 22, NAC 37, NAC40, NAC 42, NAC 46, NAC 67, NAC 75, USDA 110 and SEMIA 5019 maintained on yeast extract agar slants at 4°C, prepared as described by Somasegaran (1992), were transferred to the Yeast manitol congo red and incubated at 25°C for seven days.

The different broth media prepared containing 10.0g mannitol, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.1g NaCl, 1.0g Yeast Extract, 15.0g of agar and 1 liter of distilled water . Adjustment was done on pH of the media to 6.8 using NaOH 1M for the mannitol as carbon source. For glucose as carbon source, the media was containing 10.0g glucose, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.1g NaCl, 1.0g Yeast Extract, 15.0g of agar and 1 liter of distilled water. For glycerol as carbon source, glucose was replaced by 10ml of glycerol 92.8% in the above composition. Single colony were picked aseptically from Congo red and transferred to 20 ml of different broth media in triplicate. The broth cultures were placed on rotary shaker and allow growing for seven days at 27°C (Okereke and Okeh, 2007).

To enumerate the survival of the cultivated strains, the serial dilution of different broth was prepared by aseptically transferring 10ml of each broth culture into 90ml of saline solution 0.85%. After thorough mixing, the solution was serially diluted in 9ml of saline solution in the

McCartney bottles ten times. Ten milliliters of dilution 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} was placed in petri-dish containing yeast mannitol agar Congo red with sterile micropipettes with sterilized tips using the drop plate method described by Miles *et al.,* (1938). The drops were allowed to dry by absorption into the agar and the petri-dishes were inverted and incubated at 27°C for ten days for colony development. The colonies were then counted and the original broth concentration determined by the above formula (Somasegaran and Hoben, 1994).The original broth culture concentration = (number of colonies) X (dilution factor) X (vol. of inoculum).

Results from the carbon source utilization ability experiment were analyzed as completely randomized design with three replications. The data collected was culture concentration or population per milliliter of broth. Data was submitted to the analysis of variance and LSD was used to make comparisons among the means at p (0.05) level of significance using GENSTAT software. The carbon substitution index was calculated by dividing the population obtained with glucose and glycerol by the population obtained with mannitol.

CHAPTER FOUR

4.0 RESULTS

4.1 Potted soil chemical properties.

Some chemical properties of potted soil samples are presented in Table 2. According to Desta Beyene (1986), who classified soils based on their N content as total N < 0.05% - very low, 0.05-0.10% - low, 0.10-0.15% medium, 0.15-0.20%-high and > 0.20% very high, the soil at Kalehe and Walungu have very high nitrogen content, respectively 0.29% and 0.17%. . According to Pypers *et al.,* (2010), the range of olsen P in the site soils is between 6 and 31. Thus, the P content of the experimental soils was well above the critical level. But the amount of P (14.32 ppm) at Kalehe was almost by half more than at Walungu soils (6.29 ppm). Generally it can be concluded that the available P contents at Kalehe have optimum available P concentration that would help to enhance nodulation and nitrogen fixation in the grain legumes. The pH values indicate that the soils have neutral (6.8) soil reaction at Kalehe but acidic at Walungu. The rhizobia soils concentration was respectively 10^3 and 10^2 at Kalehe and Walungu.

Table 2: Chemical properties of potted soils

Properties	Unit	Kalehe	Walungu
pH(H_2O)		6.57	5.1
Total N	%	0.29	0.17
Olsen P	(ppm)	14.32	6.29
Rhizobia cells concent.	cells/g of soil	10^3	10^2

4.2 Effectiveness of rhizobia isolated from south Kivu soils in nodulation of soybean in sterile medium in the greenhouse.

4.2.1 Leaf color score and nodule score.

A total of one hundred and seven rhizobia strains were isolated from root nodules of wild and cultivated legumes. The trap hosts, location, number and code of isolated strains are presented in (appendix 1).Significant differences in leaf color and nodule score were observed (P’ 0.001) among plants inoculated with different strains and non-inoculated controls (ī N and +N treatments). The scores for leaf color showed that plants inoculation with the isolates NAC45, NAC22, NAC75, NAC38, NAC20, USDA110, NAC40, NAC14, NAC37 AND NAC23 improved the green color than the un-inoculated without nitrogen control but lower than plus N control. There was no difference between the two varieties but the interaction between variety and rhizobial isolate are highly observed in plant greenness (P<0.001). The nodule scores were higher with these same isolates. The highest nodules scores were recorded with the isolates NAC40, NAC14, NAC19, NAC46, NAC45, NAC37, NAC17, NAC22, NAC51, USDA110 and NAC66. The leaf color positively correlated with the nodules scores (r=0.65) and P<0.001). The correlation between score of plant greenness is presented in figure 1.

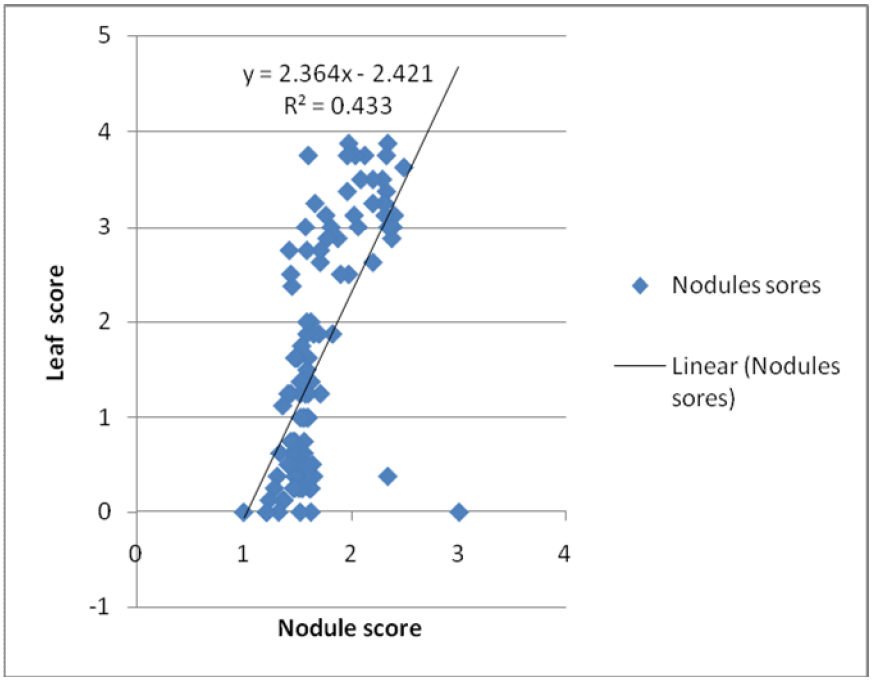

Figure1: The relationship between leaf scores and nodules scores of the treatments

4.2.2 Nodulation of soybean as affected by inoculation with indigenous rhizobia in sterile sand.

Indigenous rhizobia isolated from South Kivu soils were able to nodulate the soybean varieties SB24 and SB19. Only the treatment +N and ï N produced any nodule (figure 2). There were more nodules on the variety SB24 than SB19 ($P<0.05$). The nodules induced by different isolates were significantly different ranging from 1 to almost 30 (P' 0.001). The isolates NAC42, NAC40, NAC46, NAC19 and NAC66 produced the highest nodules number. Figure 4 shows the nodules number produced by the best performing rhizobial isolates on the two test varieties (SB24 and SB19).

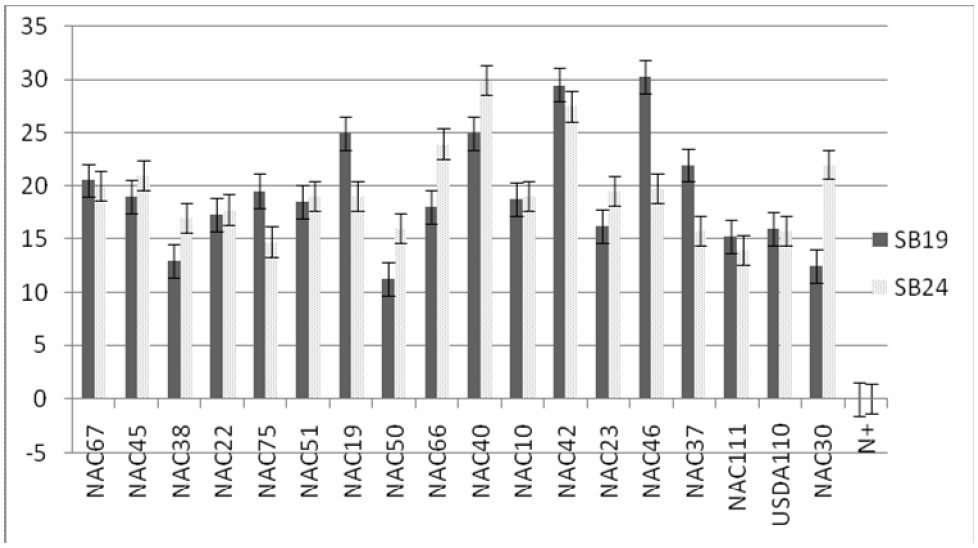

Figure 2: Nodules number produced on varieties SB24 and SB19

4.2.3. Shoot dry weight and effectiveness index of Soybean as affected by nodulation with indigenous and commercial rhizobia in sterile sand.

The shoot weight also varied significantly between strains ($P<0.001$). The interaction of the two factors was significant ($P<0.05$) on the shoot weight. The effectiveness index of isolates NAC67, NAC45, NAC38, NAC22, NAC75, NAC51, NAC19, NAC50, NAC66, NAC40, NAC10, NAC42, NAC23, NAC46 and NAC 37 outperformed the reference strain USDA110. The shoot weight means and effectiveness index of best performing isolates are presented in table 3; the complete table is presented in appendix 2.

Table 3: Shoot weight (in grams) and effectiveness index (E.I) induced by best performing strains on the two promiscuous soybean varieties

Rhizobia isolates	Plant shoot dry weight		Effectiveness index
	SB19	SB24	
NAC67	9.83	11.28	2.92
NAC45	8.99	9.29	2.53
NAC38	8.96	9.24	2.51
NAC22	8.68	9.35	2.49
NAC75	7.95	9.90	2.46
NAC51	8.07	9.77	2.46
NAC19	8.70	9.04	2.45
NAC50	8.37	8.96	2.39
NAC66	7.40	8.57	2.21
NAC40	7.31	8.38	2.17
NAC10	7.52	8.06	2.15
NAC42	7.70	7.40	2.09
NAC23	7.23	7.55	2.04
NAC46	6.24	7.39	1.88
NAC37	5.96	6.42	1.71
NAC111	4.25	4.19	1.17
USDA110	4.11	3.13	1
NAC30	3.31	3.35	0.92
N+	3.12	3.39	0.90
SEMIA5019	3.23	3.09	0.87
N-	0.47	0.79	0.18
CV:	12.20%		
Significance: variety:	0.006		
Strain:	<0.001		
Variety*strain :	0.019		

Analysis of variance was done to test the hypothesis that indigenous rhizobia strains isolated from South Kivu outperform commercial strains. Results show this hypothesis to be true for Nodule number and shoot dry weight primarily because of the significant differences between the strains NAC67, NAC45, NAC38, NAC22, NAC75, NAC51, NAC19, NAC50, NAC66, NAC40, NAC10, NAC42 and the commercial strain USDA 110 for these parameters.

Nodules number and plant shoot weight positively correlated; the correlation results are presented in the figure 5.

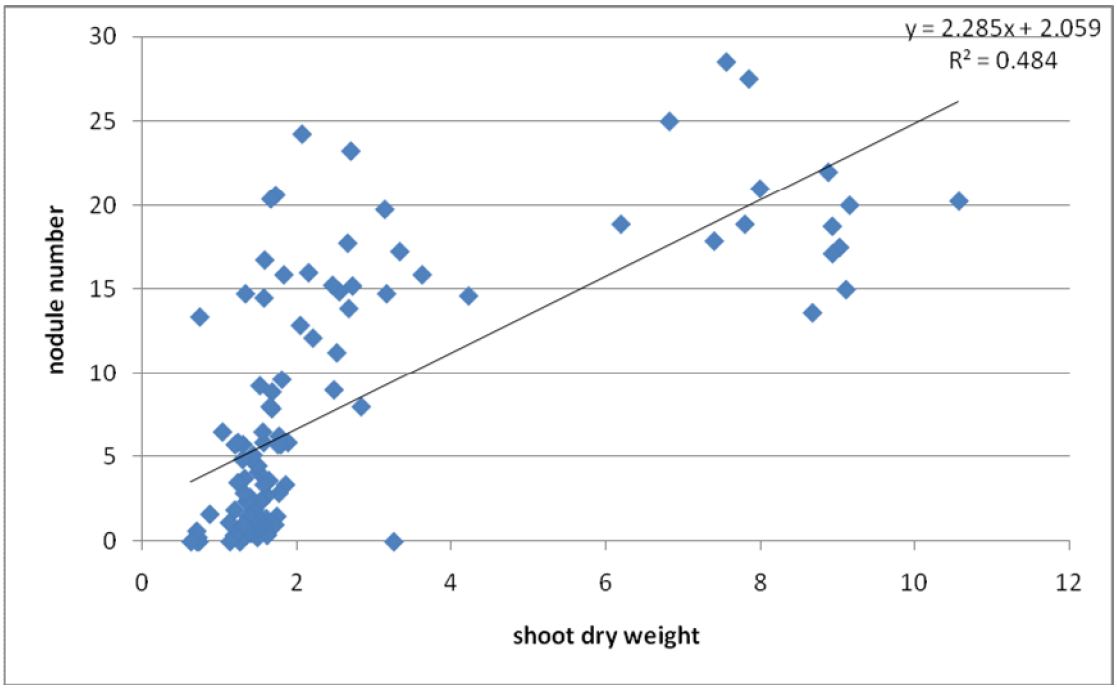

Figure 5: Relationship between nodules number and shoot dry weight

There was a positive linear relationship between nodule number and shoot dry weight (Figure 5, correlation coefficient r=0.54 and P' 0.001). These results suggest that nodule number is important in estimating the amount of the shoot weight.

4.3 The competitiveness of best ten isolates in potted greenhouse experiment

4.3.1 Nodulation and shoot dry weight of soybean as affected by inoculation with indigenous rhizobia

Nodulation of promiscuous varieties with indigenous rhizobia isolates across treatments were observed in both sites soils and for all treatments, even the non inoculated control nodulated because of the presence of native rhizobia in the potted soils. There were more nodules per plant and shoot dry weights at Kalehe than Walungu soils (P' 0.001) (Table 4). When averaged over treatments, nodule numbers ranged from 7.0 to 170 nodules, shoot dry weight ranged from 3 to 17 grams per plant. There was a significant interaction ($P<0.05$) between the soil and rhizobial strain.

Table 4: Nodules number and plant shoot dry weight (in grams) produced by promiscuous varieties inoculated with indigenous rhizobia in the two soils.

Soils	Rhizobia strains		SB24		SB19	
			nodules number	shoot dry weight	nodules number	shoot dry weight
Kalehe	N+		5.25	9.97	7.24	14.23
	N-		75.00	8.83	76.5	7.29
	NAC10		46.25	14.81	56.75	14.72
	NAC22		54.00	17.10	70.00	17.11
	NAC37		46.00	16.93	81.50	17.11
	NAC40		37.50	16.92	89.00	16.50
	NAC42		46.75	11.49	89.00	10.95
	NAC45		49.50	9.53	56.25	9.19
	NAC46		53.50	6.74	76.50	5.05
	NAC50		35.50	9.79	39.50	9.91
	NAC67		62.25	13.42	66.25	13.89
	NAC75		34.25	17.07	51.75	15.91
	USDA110		54.25	13.98	71.75	13.29
	SEMIA5019		62.00	9.36	88.50	10.38
Walungu						
	N+		20.75	10.18	7.75	8.39
	N-		25.50	3.97	12.25	3.79
	NAC10		57.50	9.77	31.00	9.38
	NAC22		26.50	14.09	11.50	14.45
	NAC37		20.25	9.84	54.50	9.833
	NAC40		62.00	13.61	42.50	12.63
	NAC42		20.50	8.77	5.75	9.38
	NAC45		34.25	8.77	9.50	9.38
	NAC46		20.50	5.92	22.50	6.08
	NAC50		40.50	3.19	28.75	3.47
	NAC67		91.25	11.68	28.75	11.07
	NAC75		55.75	12.53	170.50	13.34
	USDA110		61.50	5.95	7.50	7.79
	SEMIA5019		33.00	9.65	14.50	8.99
	CV(nod num):		13.90%			
	CV(SDW)		8.50%			
	Signif. (nod.num)	site:	<0.001			
		variety:	<0.001			
		strain:	<0.001			
		site*strain:	<0.001			
	Signif. (SDW)	site:	<0.001			
		variety:	0.86			
		site*strain:	<0.001			

CV = coefficients of variation, signif.= significance, nod.no= nodules number, SDW=shoot dry weight.

The table shows that at Kalehe, the highest nodule number were recorded with the strains SEMIA5019 and NAC67 compared to the commercial strain USDA110. The non inoculated control produced highest number of nodules in these but these nodules were of small size and produced lower shoot dry weight. The highest shoot dry weight, in Kalehe, was recorded with the strains NAC22, NAC75 and NAC75. At Walungu, the highest nodule number was recorded with the strain NAC67 compared to the commercial strain. The highest shoot dry weight was recorded by the strains NAC22, NAC40 and NAC67.

4. 3.2. Competitiveness ability of indigenous rhizobia strains using non inoculated pot and commercial strain (USDA110) as references.

The indigenous rhizobia strains were classed into four classes: Competitive and highly effective, Less competitive and highly effective, Less competitive and less effective, Competitive and less effective. The figure 4 shows the competitiveness of selected indigenous rhizobia in potted field soils and their effectiveness in sterile sand.

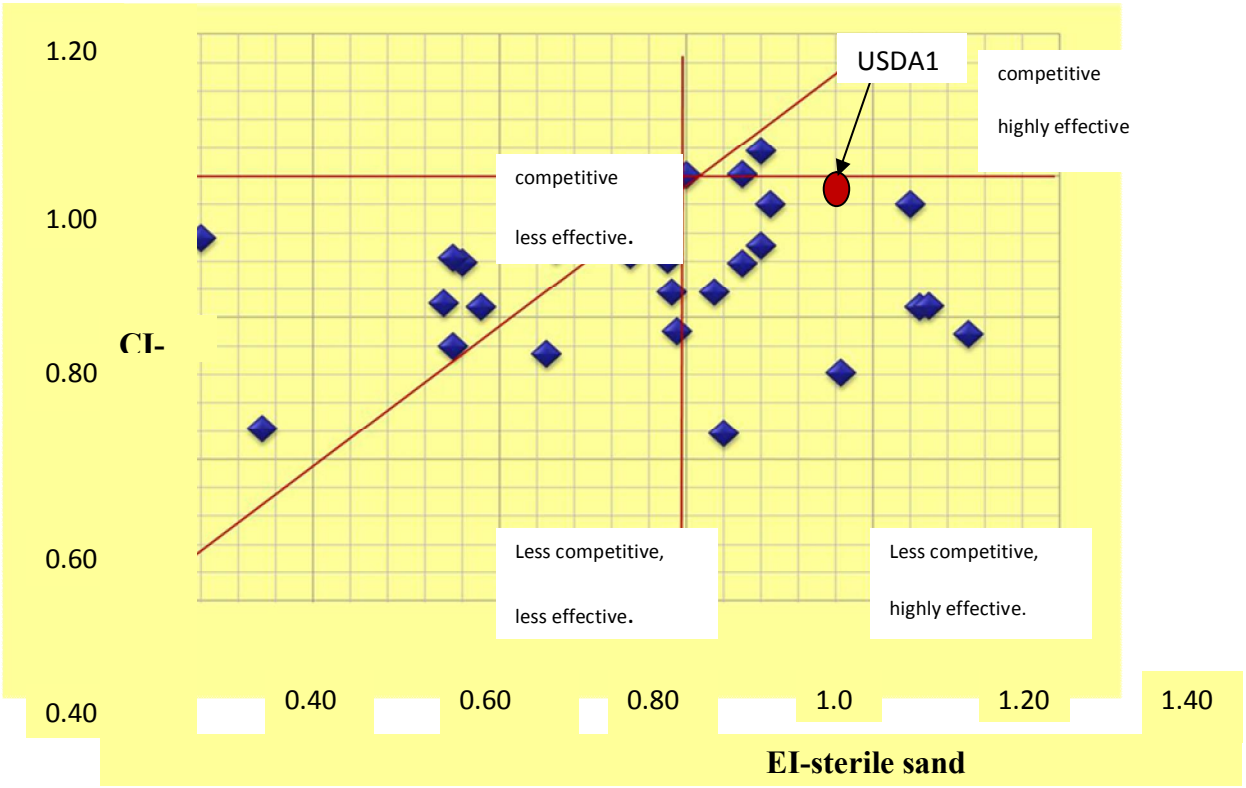

Figure 4: Competitiveness ability of indigenous rhizobia strains

The isolates NAC10, NAC22, NAC40 and NAC75 were classified as competitive and highly effectives, where NAC10 had the competiveness index of 2.04 and the effectiveness index of 2.15; NAC22 had the C.I= 2.68 and E.I= 2.48; NAC40 had the C.I= 2.49 and the E.I= 2.16, NAC75 had the C.I= 2.45 and the E.I= 2.60.

4.2.3 Correlation between nodules number –shoot dry weight

Nodule number was positively and significantly (P’ 0.001) correlated with shool dry weight in both promiscuous and specific soybeans (P’ 0.001 and r=0.46).

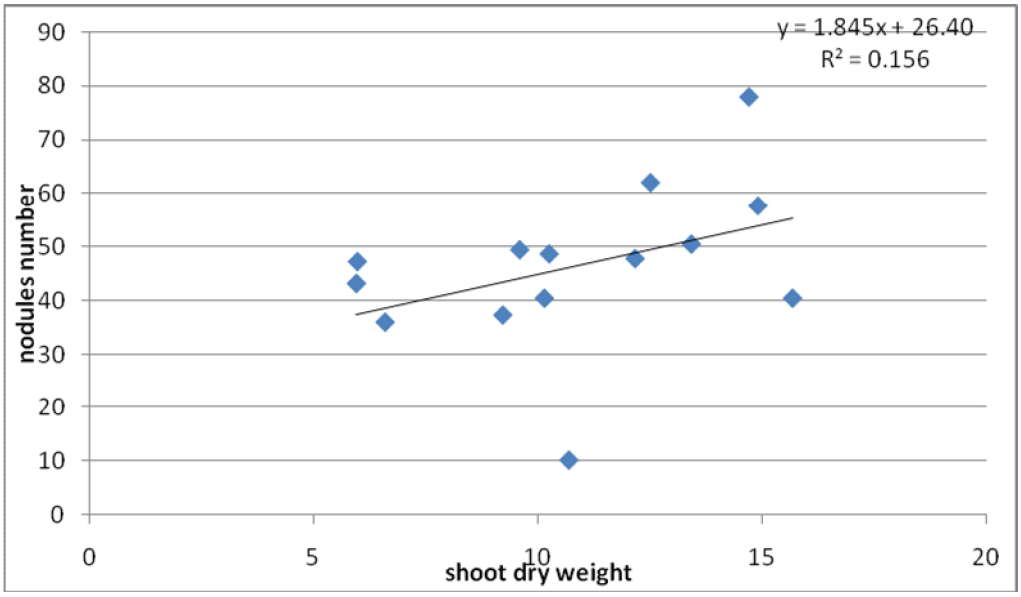

Figure 5: Relationship between the nodules number and shoot dry weight

The above figure shows a positive linear relation between nodule number and shoot dry weight but this relationship is less apparent suggesting that other parameters such as nodule weight may be more important than nodule number in estimating the nitrogen fixation.

4.3 The performance of indigenous rhizobia strains to use alternative carbon sources

There was difference in the ability of the three carbon sources to sustain growth of the South Kivu isolates ($P<0.05$). The interaction between the factors strain and carbon source produced significant difference (P' 0.001). Mannitol as a carbon source in YMA produced highest viable count followed by glucose while glycerol supported the lowest count. The results showed also that the strain SEMIA 5019 and NAC 67 produced the highest rhizobia population when glucose was used as carbon source in the YMA. The means of rhizobia population obtained are presented in table 5.

Table 5: Growth of rhizobia strains in media with the three carbon sources in log_{10} cells per milliliter

Rhizobia strain	Carbon source			Means
	Glucose	Glycerol	Mannitol	
NAC22	9.81	9.74	9.80	9.78
NAC37	9.73	9.79	9.79	9.77
NAC40	9.52	9.80	9.82	9.71
NAC42	9.79	9.80	9.80	9.80
NAC45	9.73	9.70	9.80	9.74
NAC46	9.77	9.75	9.80	9.77
NAC67	9.84	9.68	9.81	9.78
NAC75	9.81	9.81	9.80	9.81
SEMIA5019	9.85	9.73	9.76	9.78
USDA110	9.78	9.75	9.80	9.78
Means	9.77	9.75	9.80	9.77
CV: 0.5%				
Signif.:carb sour.	0.003			
Strain	0.006			
carb. Sour*strain	<0.001			

In the present investigation, the highest number of rhizobia population was observed when mannitol was used as carbon source. However the population was high also when glucose was

used as carbon source with some rhizobia strains such as SEMIA5019, NAC67, NAC75 and NAC22. The lowest population of rhizobia was observed when glycerol was used as carbon source.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Screening of rhizobia isolates from South Kivu soils in sterile media

The results showed that there was a significant difference in green color of plants inoculated with different rhizobial isolates compared to the control without nitrogen. Nitrogen is the major constituent of chlorophyll that confers green color to the plant. Nitrogenous compounds resulting from nitrogen fixation process are exported from root nodules in the form of ureides (allantoin and allantoic acids) and translocated to the leaves where they are catabolized and used for the biosynthesis of chlorophyll and proteins (Winkler *et al.,* 1988). The same results were found by Sardokie-Addo *et al.* (2006). They found that there is a high significant difference in nitrogen measured in seeds, in crop residue but also in total plant nitrogen on different plants nodulated by different isolates. Abaidoo *et al.* (2007) classified the isolates tested into four symbiotic phenotypic groups based on their symbiotic effectiveness as follows: ineffective, less effective, moderately effective and effective. The group less effective was composed by isolates that were likely to have caused rhizobiotoxine-induced chlorosis on the soybean genotypes.

The difference in shoot weight produced by different varieties is due to the genetic differences. The same results were found by Appunu *et al.,* (2008) in their study, the variation in symbiotic performance of *Bradyrhizobium japonicum* strains and soybean cultivars under field conditions. By this study the biomass accumulated by different cultivars was highly different. The same differences have been experimented by Abaidoo *et al*., (1999) and Jemo *et al.,* (2006).

The difference in nodulation as well as in shoot dry weight observed with different indigenous isolates is due to the difference in the genetic but also in effectiveness of each strain since the work was conducted in the greenhouse, where some climatic variations were controlled.

Musiyiwa *et al.,* (2005) found the same results. From the 129 indigenous isolates tested, only three isolates had significantly higher nitrogen fixing potential in comparison to the commercial strains. This was also observed in this study where 15 isolates only out of 107 were highly effective compared to the reference strain USDA110 used in the industry standard. Abaidoo *et al.* (1999) stated that the indigenous rhizobia that nodulates promiscuous soybeans are present in low numbers in many soils. Tropical soils are often rich in less-effective, native rhizobia and a key to overcoming their competitive advantage is through the composition and delivery of legume inoculants (Thies *et al.* 1991).

Some isolates induced higher shoot dry weight compared the nitrogen plus control and this confirmed observations by other authors in which the application of small amounts of N-fertilizer did not provide a major benefit. Even more, it was observed that the application of 200 kg N/ha did not improve seed yields in comparison with soybean rhizobial inoculation, as it was previously demonstrated (Hungrēa *et al.,* 2006; Albareda *et al.,* 2009). Rhizobium inoculation is a cheaper and usually a more effective agronomic practice for ensuring an adequate supply of nitrogen for legume-based crop than the application of fertilizer nitrogen (Marufu *et al.,* 1995).

The interaction between the variety and the rhizobial isolates was also observed on shoot dry weight. This may be explained partly by the host specificity of some rhizobial strains and soybean germoplasm. The difference in preference may be due to the quality and quantity of exudates produced by different varieties (Kahindi and Karanja, 2009).

5.2 The competitiveness of best performing rhizobia isolates on native rhizobial in potted-field soils

Plant growth and microorganismōs activity depend upon soil reaction and possible condition of the soil *i.e.* soil acidity, neutrality and alkalinity. Soil management practice, which build up

organic matter content and arrest pH declining are likely to create soil condition that encourage survival, persistence and higher population of *Rhizobium* in soil. Phosphorus is known to stimulate the *Rhizobial* growth (Subba Rao, 1999). A study conducted by Jemo *et al.,* (2006) showed that P application highly significantly increased shoot dry matter, P uptake, nitrogen fixation and grain yields of the grain legumes. Two of the soybean and two of the cowpea genotypes were more efficient at using P. Another study conducted by Wasike *et al.,* (2009) showed that P improved nodulation across tested varieties at both sites although the magnitude of this response was higher at Bungoma which had a low inherent soil P status and most of the nodules contained leghaemoglobin indicating active nitrogen fixation.

The significant interaction between site and treatments (rhizobial strains) in nodulation response at both sites suggests that some strains may be pH sensitive and may require relatively specific amount of phosphorus than others for optimal nodulation (Munns and Keyser 1981). Soil type affects the ability of introduced organisms to colonize the rhizosphere or root soil interface (Kluepfel, 1993). It was reported that the survival of *Rhizobium leguminosarum* in natural soil was greatly affected by certain protozoa, fungi and bacteriophages (Chonkar and Subba Rao, 1966; Subba Rao, 1999) and the number in these organisms varied according to soils.

The study showed also that there is a high significant difference between the selected rhizobial isolates from South Kivu soils. Their differential abilities in nodule number and plant shoots dry weight might be due to their genotypic differences since soil and climatic variations were minimal. Kasasa (1999) and Musiyiwa *et al*. (2005) reported the presence of indigenous rhizobia nodulating promiscuous soybean varieties in many soils in Zimbabwe. Some of the isolates were as good or superior in N_2 fixation effectiveness to commercial inoculants strains under greenhouse conditions.

Among the tested isolates, some were classified as effective in sterile sand but less effective in potted-soils because of the presence of indigenous rhizobia. Streeter (1994) stated that the presence of *B. japonicum* in the inoculated soil sites may compromise nodulation competitiveness of the introduced rhizobia (Streeter, 1994). The survival of *Rhizobium* depends on their ability to compete favorably with indigenous soil *Rhizobia* and subsequently from a large proportion of nodule (Elkins *et al,.* 1976). This may be also associated with ability of rhizobia to induce signals for nodulation with many types of soybean germoplasm (Martẽnez-Romero, 2003). Some authors stated that the survival of *Rhizobium* is lower in natural soil than in sterile soil or media. Soil is a complex matrix that is difficult to manipulate and control (Young and Burns, 1993). From a study conducted by Saleh (2013) the highest number of rhizobial population was observed in sterile soil than in non sterile, suggesting that the viability of *Rhizobium* was more in sterile soil.

The two varieties, SB24 and SB19 produced no significant differences in shoots weight. This suggested that as both of them are promiscuous, they nodulated freely with different isolates. This result is similar to that obtained by Appunu *et al.,* (2008). They tested six soybean cultivars for variation in symbiotic performance with five *B. japonicum*. Analysis of data revealed that among the six cultivars tested, only two cultivars recorded higher dry matter accumulation after inoculation with different rhizobia strains. Similar dry matter production trends were observed for other cultivars.

The significant interaction was observed between variety and site on nodules number. Soybean varieties differ significantly in their tolerance to acidity, salinity and different level of nutrients. Assa (2002) has studied the effect of salinity stress on growth and nutrient composition of three

soybeans cultivars. By this study, there was a high significant difference on shoots dry weights between the three cultivars under different conditions.

The nodules number and plant shoots weight positively correlated suggesting that there is a clear response of the soybean variety to inoculation. Although Kipenolt and Giller (1993) stated that evaluation of nodulation provides useful tool to the measurement of nitrogen fixation, but there is often no simple relationship between nodule number, nodule weight and total nitrogen fixed. Nodules number also positively correlated with the nodules dry weight highlighting the rhizobial isolates effectiveness observed in the sterile sand. This may be due to the fact that nodules of introduced effective strains are often numerous as well as of large size. Addo *et al*., (2006) found the contrary with the nodulation of some soybean lines by the indigenous rhizobial present in the soil. In their study, the nodules number and nodules dry weight negatively correlated (r=-45) suggesting that the lines that produced more nodules produced small-sized nodules while those that produced fewer nodules produced bigger well-filled nodules.

Different concentration of broth cultures used for inoculation was not considered in this study because in the study conducted by Albareda *et al.,* (2009), all determined parameters (nodule dry weight, seed yield and seed N content) of soybean inoculated with different isolates were not significantly different ($P < 0.05$) among the bacterial concentrations tested. In the case of USDA110, nodulation and seed yield of soybean were not statistically different when the inoculum rate exceeds 10^5 rhizobia/seed.

5.3. The ability of isolates to use glucose and glycerol as carbon sources

The results showed that there were differences in mannitol, glucose and glycerol in sustaining the viable counts of *Bradyrhizobia* in the broth cultures. Their differences may be due to their composition but also to the ability of different strains to metabolize these carbon compositions. The carbon is important for growth of bacteria in general and specially for rhizobia because these organisms produce ATP from organic molecules. Evans *et al.,* (1980) stated that the *Rhizobium* requires tremendous amount of energy for the reduction of atmospheric nitrogen. Mannitol and glucose products are composed by six carbon atoms while glycerol is composed by only three atoms. Mannitol produced highest viable count with most of the strains but glucose substitution index was higher with certain strains. This result is similar to the results found by Singh *et al.,* (2008) where strains were able to utilize glucose more efficiently than mannitol medium. Glycerol produced the lowest viable counts with all South Kivu soybean rhizobial strains. The same results were found by Arias *et al.,* (1976); according to them, slow-growing rhizobia have lower generation times when growing on glycerol than other carbon sources such as glucose and mannitol. Numerous investigations have examined the nutritional diversity of carbon utilization by rhizobial. From many of them, mannitol and glucose were established as useful carbon sources for rhizobia (Sowers, 1985).

In a study conducted by Estrella *et al.,* (2004), the concept of media optimization was to establish a medium which shows the optimum conditions for the growth of the organism at cheap cost as compared to normal media. Rhizobium strains were able to utilize glucose and sucrose more efficiently than normal YEM medium .The interaction between carbon source and rhizobia strains was highly significant. The differences between strains have been documented in the previous sections. Differences of rhizobial strains in carbon nutrition have been reported by

several authors. A study conducted by Tittabur *et al.,* (2005), cassava as a cheap source of carbon for rhizobial showed that some strains tested could grow in the prepared medium but others could not and preferred other kind of media. In a study ñmodification of yem broth for medium scale production of legume inoculantsò conducted by Ormeño and Zúñiga (1998), the growth of the *Bradyrhizobium* strain was different in all the tested carbon sources. Some tested rhizobia strains grew similarly well on mannitol and sucrose but poorly on glycerol.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

1. From the study, 10% of isolates were highly effective compared to the commercial strain USDA110 in the sterile sand but only 5% were effective and competitive in unsterile soil. This suggests that effective and competitive indigenous strains with potential for use as commercial rhizobia inoculants by farmers in South Kivu.

2. The two soybean varieties (SB24 and SB19) nodulated freely even without inoculation. The variety SB24 nodulated better with the rhizobial isolates from South Kivu soils than the variety SB19.

3. Soils from Kalehe responded well to inoculation while the Walungu soils failed to respond to inoculation with certain isolates. This suggests that for better benefit from inoculation, phosphorous fertilization and pH adjustment is needed for Walungu soils.

5. Glucose as carbon source can substitute for mannitol in media for growth of rhizobia that is used for production of inoculants.

6.2 Recommendation

The best performing indigenous strains from South Kivu need to be evaluated under diverse edaphic adaptation. Upon verification of their potential genetic diversity and stability should be conducted.

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8.0 **APPENDICES**

Appendix 1: Isolates codes, host plant, Geographic origin and Isolates characteristics

Isolates	**Host plant**	**Geographic origin**	**Isolates characteristics**		
			reaction on CR	**growth rate**	**reaction on BTB**
NAC1	*Tephrosia sp.*	Idjwi	NA	S	NR
NAC2	*Trifolium sp.*	Kalehe	P	S	B
NAC3	*Trifolium sp.*	Kalehe	NA	S	B
NAC4	*Trifolium sp.*	Uvira	NA	S	B
NAC5	*Vigna sp.*	Walungu	NA	S	NR
NAC6	*Crotalaria sp.*	Walungu	CA	S	B
NAC7	*Rhyncosia sp.*	Kalehe	NA	S	NR
NAC9	*Indigofera sp*	Kalehe	P	S	B
NAC10	*Glycine. Max*	Walungu	P	S	NR
NAC11	*Glycine. Max*	Walungu	NA	S	NR
NAC13	*Glycine. Max*	Kalehe	NA	S	NR
NAC14	*Glycine. Max*	Kalehe	NA	S	A
NAC15	*Tephrosia sp.*	Walungu	P	S	B
NAC16	*Phaseolus. Vulgaris*	Kalehe	CA	F	A
NAC17	*Phaseolus. Vulgaris*	Idjwi	CA	F	A
NAC18	*Crotalaria sp.*	Walungu	NA	S	NR
NAC19	*Tephrosia sp.*	Idwi	CA	F	A

NAC21	*Tephrosia sp.*	Walungu	NA	F	A
NAC22	*glycine max*	Kalehe	CA	S	NR
NAC23	*Crotalaria. Incana*	Kalehe	P	S	NR
NAC24	*Tephrosia sp.*	Kabare	NA	S	B
NAC25	*Tephrosia sp.*	Kabare	NA	S	B
NAC26	*Sesbania. Sesban*	Idjwi	P	F	NR
NAC27	*Sesbania. Sesban*	Kalehe	NA	F	NR
NAC28	*Calliandra sp.*	Idjwi	P	F	A
NAC29	*Calliandra sp.*	Kalehe	CA	F	A
NAC30	*Mimosa. Pudica*	Idjwi	CA	F	A
NAC31	*Glycine sp.*	Kalehe	NA	S	B
NAC32	*Vigna sp.*	Walungu	CA	S	B
NAC33	*Vigna sp.*	Uvira	NA	S	NR
NAC34	*P. vulgaris*	Kalehe	CA	F	A
NAC35	*P. vulgaris*	Kalehe	CA	F	A
NAC36	*Glycine. Max*	Kalehe	CA	S	NR
NAC37	*Glycine. Max*	Kalehe	P	S	NR
NAC38	*P. vulgaris*	Walungu	NA	F	A
NAC39	*P. vulgaris*	Katana	NA	F	A
NAC40	*Desmodium sp.*	Katana	NA	S	NR
NAC41	*G. max*	Uvira	NA	S	B
NAC42	*G. max*	Katana	P	S	NR
NAC43	*G.max*	Kalehe	NA	S	B

NAC44	*P. vulgaris*	wal16	P	F	A
NAC45	*G.max*	Walungu	P	S	B
NAC46	*G.max*	Walungu	NA	S	B
NAC47	*Uknown*	Katana	P	S	A
NAC48	*Glycine max*	Kalehe	NA	S	B
NAC49	*Glycine max*	Katana	P	S	A
NAC50	*Glycine max*	sange3	NA	S	B
NAC51	*Glycine max*	Walungu	P	S	B
NAC52	*Rhyncozia sp.*	Walungu	P	F	A
NAC53	*Phaseolus.vulgaris*	Kalehe	NA	F	A
NAC54	*Arachis.hypogeae*	Walungy	NA	S	B
NAC55	*Sesbania. Sesban*	Uvira	NA	F	A
NAC56	*Rhyncosia hirta*	Kalehe	P	F	A
NAC57	*Tephrosia.vogelii*	Uvira	NA	S	B
NAC58	*Indigofera repens*	Kalehe	NA	S	B
NAC59	*Glycine wightii*	Kalehe	NA	S	B
NAC60	*A.hypogeae*	Walungu	A	S	B
NAC61	*Cassia mimosoides*	Walungu	NA	S	A
NAC62	*Rhyncosia hirta*	Walungu	NA	F	A
NAC63	*Cassia.mimosoides*	Walungu	NA	S	B
NAC64	*Phaseolus.vulgaris*	Walungu	CA	F	A
NAC65	`	Walungu	CA	F	A
NAC66	*Peas*	Kabare	NA	S	A

NAC67	*Glycine max*	Kalehe	CA	S	A
NAC68	*Glycine max*	Kalehe	CA	S	B
NAC69	*Arachis monticola*	Uvira	CA	S	B
NAC70	*Glycine max*	Uvira	P	B	B
NAC71	*Sesbania sesban*	Idjwi	NA	F	B
NAC72	*Glycine max*	Walungu	NA	S	B
NAC73	*Phaseolus vulgaris*	Walungu	NA	S	B
NAC74	*Arachis hypogeae*	Uvira	NA	F	NR
NAC75	*Glycine max*	Katana	P	F	A
NAC76	*Arachis hypogeae*	Katana	NA	F	A
NAC77	*Phaseolus vulgaris*	Walungu	P	S	NR
NAC78	*Glycine max*	Kalehe	P	I	A
NAC79	*Desmodium barbatum*	Uvira	NA	I	B
NAC80	*Glycine max*	Kabare	NA	I	A
NAC81	*Phaseolus vulgaris*	Walungu	P	VS	A
NAC82	*Crotalaria incana*	Walungu	NA	F	A
NAC84	*Cassia mimosoides*	Kalehe	P	S	NR
NAC85	*Desmodium barbatum*	Katana	NA	S	A
NAC86	*Unknown*	Katana	NA	I	B
NAC87	*Indigofera repens*	Walungu	NA	VS	B
NAC88	*Phaseolus vulgaris*	Walungu	P	F	NR

NAC91	*Rhyncosia hirta*	Kalehe	NA	VS	NR
NAC92	*Sesbania sesban*	Walungu	NA	VS	NR
NAC93	*Desmodium adsendens*	Walungu	NA	VS	NR
NAC94	*Unknown*	Walungu	A	VS	B
NAC95	*Unknown*	Walungu	NA	S	NR
NAC96	*Unknown*	Walungu	NA	VS	B
NAC97	*Unknown*		NA	VS	NR
NAC98	*Vigna vexilatta*	Kalehe	P	F	A
NAC99	*Crotalaria incana*	Walungu	P	S	A
NAC100	*Glycine wightii*	Katana	NA	F	A
NAC101	*Vigna vexilatta*	Kalehe	P	S	NR
NAC102	*Indigofera repens*	Walungu	NA	VS	NR
NAC103	*Indigofera arrecta*	Walungu	P	F	A
NAC104	*Glycine wightii*	Walungu	CA	S	A
NAC105	*Acacia monticola*	Katana	NA	S	NR
NAC106	*Unknown*	Katana	CA	S	A
NAC107	*Indigofera repens*	Uvira	P	F	A
NAC105	*Glycine wightii*	Katana	NA	S	NR
NAC106	*Unknown*	Walungu	NA	S	B
NAC107	*Glycine max*	Katana	P	S	B
NAC108	*Crotalaria incana*	Walungu	P	S	B
NAC109	*Unknown*	Idjwi	NA	S	A

NAC110	*Vigna vexilata*	Kalehe	NA	VS	NR
NAC111	*Glycine max*	Idjwi	NA	S	B

Growth rate: VS = very slow (small), S = slow (Bradyrhizobia), I = intermediate, F = fast (Rhizobia), VF = very fast (Burkeholderia)

CR YMA: colony characteristics on Congo Red YMA, NA = non absorbant, P = partly absorbant, CA = center absorbant, A = fully absorbant

BTB YMA: Reaction on bromothioblue YMA, A = acid forming (yellow), NR = non-reactive (green), B = basic (blue)

Appendix 2: Nodules number and shoot weight produced by the indigenous isolates in sterile sand

Rhizobia isolates	Nodules numbe		Shoots dry weight		Effectiveness index
	SB19	SB24	SB19	SB24	
NAC67	20.5	20	9.832	11.281	2.915
NAC45	19	21	8.999	9.290	2.525
NAC38	13	17	8.959	9.238	2.513
NAC22	17.25	17.75	8.677	9.351	2.489
NAC75	19.5	14.75	7.946	9.898	2.464
NAC51	18.5	19	8.071	9.770	2.464
NAC19	25	19	8.700	9.036	2.449
NAC50	11.25	16	8.372	8.957	2.393
NAC66	18	24	7.400	8.572	2.205
NAC40	25	30	7.309	8.377	2.166
NAC10	18.75	19	7.524	8.059	2.152
NAC42	29.5	27.5	7.704	7.401	2.086
NAC23	16.25	19.5	7.235	7.554	2.042
NAC46	30.25	19.75	6.237	7.394	1.882
NAC37	22	15.75	5.961	6.420	1.710
NAC111	15.25	14	4.250	4.193	1.166
USDA110	16	15.75	4.112	3.130	1.000
NAC30	12.5	22	3.317	3.349	0.921

N+	0	0	3.123	3.389	0.899
SEMIA5019	12.5	17	3.231	3.093	0.873
NAC109	19.75	19.75	2.905	3.371	0.867
NAC34	6.5	9.5	2.979	2.688	0.783
NAC103	13.75	16.67	2.483	2.961	0.752
NAC14	22	24.5	2.431	2.970	0.746
NAC70	11.75	16	2.392	2.955	0.738
NAC102	20.75	14.75	3.173	2.146	0.734
NAC59	11.75	18	1.325	3.779	0.705
NAC86	9.75	12.75	2.677	2.362	0.696
NAC61	6.5	11.5	1.846	3.120	0.686
NAC73	8.75	21.75	2.282	2.645	0.680
NAC85	11	13.25	2.356	2.065	0.610
NAC101	14.25	17.75	1.813	2.495	0.595
NAC74	22.75	25.75	2.439	1.702	0.572
NAC87	15.75	10	2.124	1.972	0.565
NAC97	6	5.75	1.779	1.996	0.521
NAC36	1.75	5	1.561	2.156	0.513
NAC35	14.75	17	2.064	1.606	0.507
NAC88	7	12.25	1.468	2.151	0.500
NAC92	2.75	8.75	1.466	2.126	0.496
NAC49	2.75	9.75	1.530	2.015	0.490
NAC57	2	3.75	1.381	2.164	0.489

NAC29	4.5	7	1.635	1.882	0.486
NAC53	2.25	0.75	1.777	1.713	0.482
NAC17	22.5	18.75	1.234	2.222	0.477
NAC91	0	2	1.477	1.959	0.474
NAC104	7	10.75	1.614	1.752	0.465
NAC72	7.75	8	1.632	1.730	0.464
NAC100	20.25	20.5	1.657	1.675	0.460
NAC47	5.5	10.5	1.652	1.663	0.458
NAC65	1.25	6	1.557	1.728	0.454
NAC68	0	1	1.080	2.162	0.448
NAC6	0.25	0.5	1.804	1.435	0.447
NAC107	3.73	1.75	1.517	1.710	0.446
NAC108	0.75	2	1.594	1.620	0.444
NAC4	17.25	16.25	1.098	2.078	0.438
NAC76	14	15	1.599	1.559	0.436
NAC81	5.5	1.25	1.537	1.619	0.436
NAC77	4.25	7.5	1.638	1.511	0.435
NAC15	6	7	1.501	1.626	0.432
NAC13	1.25	1	1.652	1.437	0.427
NAC28	3.5	1.25	1.055	2.018	0.424
NAC99	3.75	1	1.530	1.524	0.422
NAC39	8.25	10.25	1.438	1.614	0.421
NAC33	1	0.25	1.518	1.491	0.415

NAC11	3	5.25	1.092	1.916	0.415
NAC79	5.5	3.5	1.244	1.756	0.414
NAC56	0	0.5	1.315	1.675	0.413
NAC96	0	2.75	1.293	1.660	0.408
NAC63	0	2.25	1.455	1.486	0.406
NAC1	0.25	2.5	1.276	1.654	0.405
NAC27	2.755	1	1.476	1.434	0.402
NAC55	0.25	0.75	1.207	1.691	0.400
NAC64	0.5	3	1.247	1.649	0.400
NAC18	0.25	1	1.643	1.253	0.400
NAC31	2	1	1.396	1.483	0.398
NAC26	5.25	5	1.373	1.503	0.397
NAC80	0.25	0.75	1.320	1.510	0.391
NAC82	4.75	5	0.974	1.855	0.391
NAC94	1.75	0	1.425	1.384	0.388
NAC24	0.5	0.5	1.389	1.409	0.386
NAC48	3.5	1.75	1.138	1.658	0.386
NAC98	0.25	0.75	1.440	1.312	0.380
NAC7	1.5	1.5	1.508	1.237	0.379
NAC9	3.5	1.25	1.561	1.141	0.373
NAC69	1.75	0	1.339	1.361	0.373
NAC60	0.25	0.5	0.945	1.751	0.372
NAC20	13	16.5	1.607	1.072	0.370

NAC2	1	0.5	1.306	1.363	0.369
NAC5	1.75	0	1.101	1.554	0.367
NAC62	5	2.5	1.007	1.648	0.367
NAC52	1	0.75	1.294	1.357	0.366
NAC95	1.75	4	1.124	1.522	0.365
NAC3	1	0.25	1.246	1.389	0.364
NAC32	4	7.5	1.099	1.518	0.361
NAC58	0.25	1.75	1.236	1.377	0.361
NAC105	7.5	2.25	1.045	1.558	0.360
NAC54	0	0	1.068	1.467	0.350
NAC84	7.5	4.25	1.215	1.269	0.343
NAC41	2.75	4.25	1.087	1.397	0.343
NAC25	5	6.5	1.230	1.181	0.333
NAC43	0.5	3.25	1.117	1.286	0.332
NAC106	0.75	0	1.234	1.157	0.330
NAC16	0	0	0.762	1.516	0.315
NAC78	0	2.25	1.019	1.243	0.312
NAC44	6	7	0.807	1.278	0.288
NAC93	0	3.25	0.791	0.967	0.243
NAC21	14	12.75	0.760	0.739	0.207
NAC71	0	0	0.685	0.794	0.204
NAC110	0	0.5	0.756	0.693	0.200
NAC8	0	0	0.743	0.679	0.196

NAC89	0	1.25	0.715	0.703	0.196
N-	0	0	0.474	0.798	0.176

$LSD_{0.05}$inter	6.707	0.899
CV	8.8	12.2

Appendix 3: Table of mean of nodules scores and leaf scoring of the isolates in sterile sand

Rhizobia type	Leaf scores		Nodules scores	
	SB19	SB24	SB19	SB24
N+	3	3	0	0
N-	1	1	0	0
NAC1	1.75	1.55	0.25	0.5
NAC2	1.5	1.6	0.25	0.25
NAC3	1.625	1.3	0.25	0.25
NAC4	1.825	1.7	3.25	3
NAC5	1.45	1.5	0.75	0
NAC6	1.825	1.425	0.25	0.25
NAC7	1.55	1.325	0.75	0.75
NAC8	1.2	1.225	0	0
NAC9	1.675	1.5	1.25	0.75
NAC10	2.175	2.425	3.25	3.25
NAC11	1.525	1.675	1	1.5
NAC13	1.375	1.25	0.5	0.25
NAC14	2.2	2.45	3.5	4
NAC15	1.625	1.675	1.75	2
NAC16	1.175	1.25	0	0
NAC17	2.25	2.15	3.75	3.25
NAC18	1.675	1.325	0.25	0.5
NAC19	2.05	2.2	3.75	3.75

NAC20	2.35	2.375	2.75	3.25
NAC21	1.625	1.55	2.75	2.75
NAC22	2.275	2.525	3	3.25
NAC23	2.075	2.55	3	3.25
NAC24	1.45	1.55	0.5	0.25
NAC25	1.55	1.425	1.5	1.75
NAC26	1.65	1.4	1.25	1.25
NAC27	1.55	1.275	0.75	0.25
NAC28	1.5	1.525	1	0.25
NAC29	1.65	1.55	1.5	1.75
NAC30	2.125	2.45	2	3.25
NAC31	1.475	1.2	0.75	0.5
NAC32	1.575	1.475	1	1.75
NAC33	1.45	1.55	0.75	0.25
NAC34	1.9	1.75	1.75	2
NAC35	1.675	1.65	3.25	3.25
NAC36	1.6	1.6	0.75	1.25
NAC37	2.25	2.4	3.75	3
NAC38	2.15	2.6	2.5	3.25
NAC39	1.6	1.3	2.25	2.5
NAC40	2.05	2.625	3.75	4
NAC41	1.5	1.325	1.25	1.25
NAC42	2.05	1.9	4	3.75

NAC43	1.5	1.675	0.25	0.75
NAC44	1.5	1.45	1.75	1.5
NAC45	2.175	2.8	3.5	3.75
NAC46	1.975	2.1	3.75	3.75
NAC47	1.675	1.725	1.5	2.25
NAC48	1.475	1.575	1.25	0.75
NAC49	1.65	1.525	1	2
NAC50	2	2.4	2.5	2.75
NAC51	2.2	2.425	3.25	3.25
NAC52	1.475	1.475	0.25	0.25
NAC53	1.625	1.6	0.5	0
NAC54	1.625	1.625	0	0
NAC55	1.625	1.6	0.25	0.25
NAC56	1.375	1.35	0	0.25
NAC57	1.325	1.6	0.5	1
NAC58	1.725	1.5	0.25	0.5
NAC59	1.95	2.175	2.75	3.25
NAC60	1.525	1.575	1.5	1.25
NAC61	1.55	1.7	1.5	2.5
NAC62	1.35	1.375	1.5	0.75
NAC63	1.525	1.5	0	0.5
NAC64	1.6	1.675	0.25	0.75
NAC65	1.575	1.525	0.5	1.5

NAC66	2.125	2.45	3.25	3.75
NAC67	2.175	2	3.75	3.25
NAC68	1.45	1.6	0	0
NAC69	1.45	1.625	0.5	0.5
NAC70	1.825	2.125	2.5	2.5
NAC71	1.25	1.4	0	0
NAC72	1.55	1.625	1.5	2.25
NAC73	1.825	1.6	2	3.25
NAC74	1.975	1.95	3.5	4
NAC75	2.225	2.55	3.25	2.75
NAC76	1.375	1.475	2.75	2.75
NAC77	1.675	1.575	1	1.75
NAC78	1.525	1.625	0	0.75
NAC79	1.725	1.7	1.5	1
NAC80	2.35	2.325	0.25	0.5
NAC81	1.675	1.45	1	0.25
NAC82	1.575	1.575	1.25	1.25
NAC84	1.575	1.5	0.75	0.5
NAC85	1.5	1.375	2.5	2.5
NAC86	1.925	1.875	2.5	2.5
NAC87	1.675	1.75	3	2.5
NAC88	1.525	1.65	1.5	2.5
NAC89	1.175	1.3	0	0.25

NAC91	1.675	1.45	1.25	0.75
NAC92	1.575	1.575	2.75	3.25
NAC93	1.575	1.5	0	0.5
NAC94	1.5	1.375	0.75	1.75
NAC95	1.4	1.275	0.5	0.75
NAC96	1.5	1.475	0	0.75
NAC97	1.3	1.7	1.5	1.75
NAC98	1.5	1.4	0.25	0.75
NAC99	1.5	1.4	1	0.25
NAC100	1.65	1.55	3.75	3.75
NAC101	1.975	1.775	2.75	3
NAC102	2.375	2.025	3.5	3
NAC103	1.75	1.8	2.75	3
NAC104	1.575	1.5	1.5	2
NAC105	1.4	1.75	1.75	0.75
NAC106	1.125	1.45	0.5	0
NAC107	1.45	1.675	1	0.5
NAC108	1.4	1.55	0.75	0.75
NAC109	2.15	1.775	3.25	3.5
NAC110	1.45	1.3	0	0.25
NAC111	1.75	1.875	3.25	2.75
USDA110	2.1	1.95	3.25	3
SEMIA5019	2.35	2.325	2.75	3.25

$LSD_{0.05}$	6.707	0.899
CV	60.7	33.9

Appendix 4: Table of Analysis of variance: shoot dry weight in sterile sand

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
repetition stratum	3	37.1582	12.3861	41.8	
repetition.variety stratum					
Variety	1	14.2349	14.2349	48.04	0.006
Residual	3	0.8889	0.2963	0.7	
repetition.variety.strains stratum					
Strains	112	687.7124	6.1403	14.6	<.001
variety.strains	112	62.7104	0.5599	1.33	0.019
Residual	672	282.5928	0.4205		
Total	903	1085.298			

Appendix 5: Table of analysis of variance for nodules number in sterile sand

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
repetition stratum	3	184.85	61.62	4.36	
repetition.variety stratum					
Variety	1	153.08	153.08	10.84	0.046
Residual	3	42.38	14.13	0.62	
repetition.variety.strains stratum					
Strains	112	54418.43	485.88	21.38	<.001
variety.strains	112	2378.17	21.23	0.93	0.667
Residual	672	15268.27	22.72		
Total	903	72445.18			

Appendix 6: Table of analysis of variance for carbon source utilization

Variate: carbon source in log10					
Source of variation	d.f	s.s	m.s	v.r	Fpr.
Repetition stratum	2	0.000835	0.000418	0.18	
Strain	9	0.058	0.0064	2.014	0.009
carbon source	2	0.0299	0.014	6.5	0.003
strain.carbon source	18	0.245	0.013	5.93	<.001
Residual	58	0.133	0.0023		
Total	89	0.468			

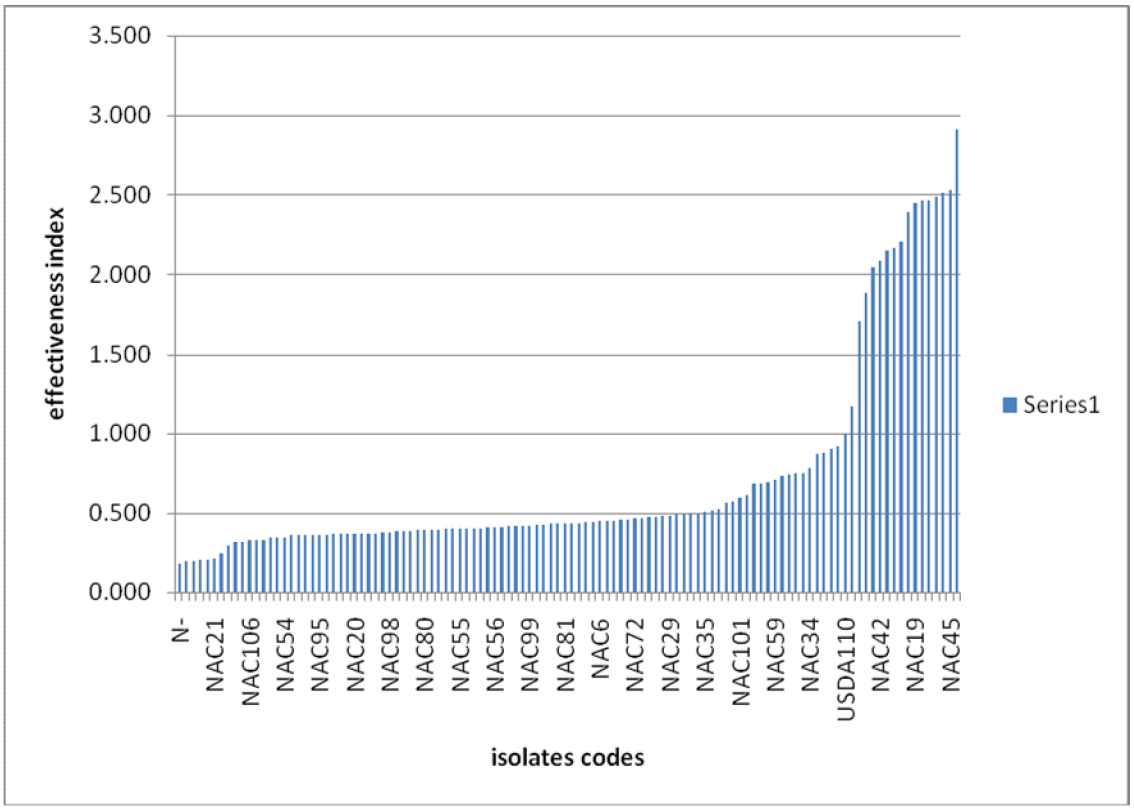
Appendix 7: Table of analysis of variance for shoot dry weights in potted soils

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
repetition stratum	3	0.804	0.268	0.5	
repetition.site stratum					
Site	1	726.6336	726.6336	1359.99	<.001
Residual	3	1.6029	0.5343	0.61	
repetition.site.varieties stratum					
Varieties	1	0.0263	0.0263	0.03	0.868
site.varieties	1	0.1471	0.1471	0.17	0.697
Residual	6	5.2884	0.8814	1.04	
repetition.site.varieties.strains stratum					
Strains	13	2446.883	188.2218	223.07	<.001
site.strains	13	90.855	6.9888	8.28	<.001
varieties.strains	13	26.2141	2.0165	2.39	0.006
site.varieties.strains	13	52.0146	4.0011	4.74	<.001
Residual	156	131.6296	0.8438		
Total	223	3482.098			

Appendix 8: Table of analysis of variance of nodules number for potted soils experiment

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
repetition stratum	3	99.89	33.3	0.6	
repetition.site stratum					
Site	1	29440.29	29440.29	529.66	<.001
Residual	3	166.75	55.58	1.42	
repetition.site.varieties stratum					
Varieties	1	1410.02	1410.02	36.04	<.001
site.varieties	1	10615.02	10615.02	271.31	<.001
Residual	6	234.75	39.12	0.89	
repetition.site.varieties.strains stratum					
Strains	13	30033.46	2310.27	52.6	<.001
site.strains	13	55503.46	4269.5	97.22	<.001
varieties.strains	13	28655.23	2204.25	50.19	<.001
site.varieties.strains	13	22837.73	1756.75	40	<.001
Residual	156	6851.11	43.92		
Total	223	185847.7			

Appendix 9: Effectiveness index of indigenous rhizobia strains inoculated from South Kivu soils in sterile sand



Appendix 10: Selected physico-chemical soil properties of the two sites (Pypers et al., 2010)

Properties	Units	North axis (katana axis)		South axis(walungu axis)	
		Mean	Range	Mean	Range
pH		5.59	5.14-6.01	5.31	4.62-5.86
Organic C	%	2.14	1.75-2.48	2.48	1.23-4.24
Total N	%	0.19	0.17-0.23	0.22	0.10-0.41
Olsen P	$mgPKg^{-1}$	18.7	11.7-34.5	8.20	3.59-21.7
Exchang.K	$CmolKg^{-1}$	0.83	0.50-1.77	0.91	0.24-2.47
Exchang. Mg	$CmolKg^{-1}$	2.19	1.57-3.06	1.22	0.15-1.90
Exchang. Ca	$CmolKg^{-1}$	5.85	4.34-7.32	3.80	0.97-6.50
Exchange.acidity	$CmolKg^{-1}$	0.26	0.00-0.60	0.68	0.00-2.46
ECEC	$CmolKg^{-1}$	9.18	7.51-10.6	0.60	3.06-9.85
Clay	%	38	21-57	32	17-58
Silt	%	19	17-22	17	14-24
Sand	%	43	25-59	51	28-64

Appendix 11: Nodules sampling point's location