EFFECT OF RHIZOBIA INOCULANT ON SOYBEAN NODULATION AND ARBUSCULAR MYCORRHIZAL FUNGI COLONIZATION UNDER GREENHOUSE AND FIELD CONDITIONS

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DECLARATION

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

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Liliane Shukuru Bahati, Date
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DECLARATION BY THE SUPERVISORS

We confirm that the work reported in this thesis was carried out by the candidate under our supervision and has been submitted with our approval as university supervisors.

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DEDICATION

To Almighty Jehovah be the glory forever and ever

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ABBREVIATIONS AND ACRONYMS

AMF	Arbuscular Mycorrhizal Fungi
APT	America Peat Technology
BNF	Biological Nitrogen Fixation
CEC	Cation Exchange Capacity
Cfu	Colony forming unit
D.G	Director General
DRC	Democratic Republic of Congo
ECEC	Effective Cation Exchange Capacity
IITA	International Institute of Tropical Agriculture
ISFM	Integrated Soil Fertility Management
TSP	Triple Super Phosphate
WAP	Weeks After Planting
WHC	Water Holding Capacity

ABSTRACT

In the highland of South-Kivu, DR.Congo, soybean farming is increasingly practiced by smallholder farmers but it productivity remains low. Both productivity and N2-fixing abilities of legumes can be enhanced not only by *Rhizobium spp* but also by colonization of their roots by Arbuscular Mycorrhizal Fungi (AMF). The objectives of this study were:- to evaluate symbiotic effectiveness of bradyrhizobia isolates for soybean inoculant production, their effect on indigenous AMF root colonization, and the suitability of Walungu peat/DR. Congo for inoculants production. The study was carried out in the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Nine strains of *B. Japonicum* from IITA collection and two reference strains (USDA110 and USDA532c) used in commercial inoculant, two controls (Urea and un-inoculated, non fertilized plots), Walungu peat from D.R Congo and the commercial standards America Peat Technology (APT) were investigated. Strains effectiveness for soybean nodulation was evaluated in both screenhouse and field conditions. In the field experiment the study also evaluated the effect of each individual strain on indigenous AMF colonization with soybean roots. The experimental design used was a randomized complete block design (RCBD) replicated 4 times giving a total of 52 plots in the experiment. Data were recorded on leave chlorophyll content, nodulation parameters (number and dry weight of nodules) above ground biomass parameters (dry weight, nitrogen and phosphorus content), and AMF colonization (presence or absence of hyphae, arbuscular and vesicular).In the laboratory experiment, the suitability of Walungu peat as carrier material for inoculant production was assessed under sterile and unsterile conditions and at three different temperatures (4°C, 15°C and laboratory temperature), as compared to the standards APT, using plate count method. The results showed that RANI22, RACA6 and IRJ2180A were effective in improving most of the parameters under study (chlorophyll, above ground dry weight, above ground nitrogen and phosphorus content, nodule number and dry weight) in both screenhouse and field experiment. At 8WAP leaf chlorophyll content increased by 27, 25, and 23% for RANI22, RACA6, and IRJ2180A respectively in the screenhouse experiment and 40-47% in the field experiment as compared to the control (uninoculated- no fertilized plots). On another hand, nitrogen fertilizer also showed increase in some of the parameters especially chlorophyll and nitrogen content but not significantly different from one or another of the three strains. In screenhouse conditions for instance, nitrogen fertilizer, RANI22 and RACA6 increased the above ground nitrogen content by 43-51% ($p<.001$) as compared to the control, and in the field experiment the same parameter was increased by 69-66% ($p<.001$) by RANI22, RACA6, IRJ2180A, and nitrogen fertilizer relative to the control. None of *B. japonicum* strains used in the current study or nitrogen addition affected significantly the infection rate of indigenous AMF as indicated by 100% ($p>0.05$) hyphae in all the treatments. Walungu peat limed and APT were equally effective in increasing the growth and survival of the initial *B. japonicum* (USDA110) population ($5x\ 10^8$) over a period of 2 months. Higher population density ($3.9\ x10^9$ and $3.5x\ 10^9$) ($p<0.05$) was recorded in sterile condition at 4°C and 15°C respectively. RANI22, RACA6, IRJ2180A and Walungu peat amended are alternative inputs for high quality inoculant production and soybean performance

CHAPTER 1: INTRODUCTION

1.1. Background

Sub-Saharan Africa (SSA) accounts for about 9% of global population threatened by food and nutritional insecurity. This threat is partly due to poor soil fertility and low crop diversification (Sanchez and Swaminathan, 2005). The nitrogen reserves of arable soils must be replenished periodically in order to maintain an adequate level for crop production. This replenishment of soil nitrogen is accomplished through application of mineral fertilizer, or management of biological nitrogen fixation (BNF) (Sanginga and Woomer, 2009).

Fertilizer nitrogen (N) is one of the major agricultural inputs worldwide to meet the nitrogen requirement of several plants (Bohlool *et al*., 1992). However, in addition to being expensive for most small-scale farmers, heavy use of N-fertilizer is both harmful to the environment and results in depletion of fuels needed for its production (Bohlool *et al*., 1992). An alternative and more sustainable process is BNF by a group of symbiotic bacteria called rhizobia which fix atmospheric nitrogen (N_2) through symbiotic association with leguminous plants and make it available to their host (Zahran, 1999; Hassen *et al*., 2012)

Rhizobia influence crop growth, yield and nutrient uptake. They fix nitrogen, increase supply of other nutrients, such as phosphorus and iron, produce plant hormones, enhance other beneficial bacteria or fungi, control bacterial and fungal diseases and help in controlling insect pest (Trabelsi and Mhamdi, 2013). However, rhizobia are not

universally present in the soil and often those present are ineffective micro-symbionts. In order to take advantage of the wonderful bacteria-legume symbiosis, it is often necessary to provide reliable legume inoculants to ensure effective nodulation (Burton, 1984). In many countries, legumes such as soybean (*Glycine max* (Merr.) L.), are not fertilized with nitrogen, but only inoculated.

Integrated Soil Fertility Management (ISFM) well adapted incorporates biological soil amendments with improved agronomic and cropping systems and high yield crop varieties (Vanlauwe *et al*., 2010). Promotion of BNF whose products result in acceptable legume yields and offer residual fertility related benefits to crops in the subsequent seasons (Sanginga and Woomer, 2009) is key to ISFM. The N_2-fixing abilities of legumes can be enhanced not only by *Rhizobium* spp. but also by colonization of their roots by arbuscular mycorrhizal fungi (Asimi *et al*., 1980; Bayne and Bethlenfalvay, 1987).Goss *et al*. (2006) observed improved soybean nodule development with increased infection by indigenous arbuscular mycorrhizal fungi (AMF) indicating the importance of tripartite symbiosis legume-rhizobia-AMF. In most reported studies AMF have had a positive influence on biological nitrogen (N_2) fixation in grain and forage legumes (Azcon *et al*., 1979; Barea *et al*., 1987, 1989, 1992, 2002).

Soybean (*Glycine max* (L) Merrill) is a legume of tropical to subtropical origin and is one of the most important sources of food, feed and one of nature`s most versatile plants (Keyser and Li, 1992). Soybean contains 40–42% protein and 18–22% oil comprising of 85% unsaturated fatty acid and is free from cholesterol (Meghvansi *et al*., 2010). Soybean

protein provides all eight amino acids in the amount needed for human health; hence it is called meal of the field (Rathore, 2000). It is therefore, highly desirable in human and animal diet (Aslam *et al*., 1995; Haq *et al*., 2002). Inoculation of soybeans with rhizobia throughout the world is estimated to be in the range of 12-20 x 10^{6}year^{-1}ha^{-1}which results in the establishment of a large rhizobial population in the rhizosphere thereby enhancing nodulation and nitrogen fixation (Senevirante *et al*., 2000).

Many inoculants are made with a solid carrier. Peat is the most frequently used carrier for the rhizobial inoculants industrial because of its high water holding capacity and high surface area that support rhizobial growth and survival in large numbers (Smith, 1992). Good inoculants must be prepared with a strain of rhizobium selected for high N fixation efficiency and competitive ability for nodulation (Rebah *et al*., 2007). The strain must survive, maintain its properties during storage, and be tolerant to stress factors such as acidity, desiccation, high temperature and chemical pesticides (Rebah *et al*., 2007).The most important factor for inoculants quality is a high number of live rhizobia (greater than 1x10^{9} rhizobia g^{-1}), and no or minimal contamination by microorganisms detrimental to rhizobia or pathogen to plants and humans (Lupwayi *et al*., 2000). According to quality control criteria for inoculants product, any inoculant which does not comply with the standards are rejected for marketing (Strejidom and Van Rensburg, 1981). The inoculant is rejected if the number of rhizobia is less than5x10^{8} cfu g^{-1} for peat, less than 6.5x 10^{8} cfu g^{-1} for perlite, and less than 2x10^{9} cfu ml^{-1} for liquid inoculants. If the contaminants present in the 10^{-5} dilution plates at 6.5 or less than 7.5 pH carrier, rhizobial strain is doubtful (Strejidom and Van Rensburg, 1981). Hence, there is

need to evaluate symbiotic effectiveness of bradyrhizobia isolates for soybean inoculants production, their effect on indigenous AMF root colonization, and the suitability of local carrier materials for inoculants production.

1.2. Problem statement

Fertilizer nitrogen (N) is one of the major agricultural inputs worldwide used to meet the nitrogen requirement of crops (Bohlool *et al.*, 1992). In contrast to expensive chemical nitrogen fertilizer, the use of nodulated legumes in smallholder farming systems is often a more attractive and practical alternative (Sanginga and Woomer, 2009). Their ability to fix atmospheric N allows them to grow in N impoverished soils (Giller, 2001).BNF serves as either a direct source of nitrogen to symbiotic crops, or as an indirect source through decomposition of legume residues (Sanginga and Woomer, 2009). For less developed countries in Asia, Africa, Central and South America, including D.R Congo and Nigeria, inoculant technology has had no significant impact on productivity of the smallholder farmers, because inoculants are not used or are of poor quality (Bashan, 1998). The basic problem in acquiring quality inoculants is the low number of effective rhizobia in terms of nodulation and nitrogen fixation of the host plant.

Also, high- quality peat-based inoculant is the most dependable and commonly used especially for small-scale farmers than any other legume inoculants. Unfortunately, good quality peat is not available in many countries (Miličić *et al*., 2006), DR Congo included and Nigeria. Thus, it is advisable to use an effective rhizobium strain and high quality peat when producing legume inoculants. Therefore, there is need to identify and select an

effective rhizobium strain and the best locally available peat material for large scale rhizobia inoculants production in DR Congo and Nigeria, as an easy and inexpensive way of enhancing soil fertility and productivity of soybean.

1.3. Justification of the study

Soybean is relatively a new crop to many African countries, and over the past 10 –15 years, its cultivation and utilization in many sub-Saharan Africa (SSA) countries is gaining popularity. This is probably as a result of increasing need for protein in food and animal fodder (Sanginga, 2003; Ado *et al.*, 2006). In the highlands of South-Kivu/DR. Congo, soybean farming is increasing among smallholder farmers, but its productivity remains low (Pypers *et al*., 2011). In order to produce higher yields, larger amounts of nitrogen fertilizer are required. However, the cost of such fertilizer is prohibitive. Thus, very few use it on their farms (Haque and Jutzi, 1984; Haque *et al*., 1988; Savory, 1972; Thomas, 1973; Thomas and Addy, 1977). Biological Nitrogen Fixation is a less expensive alternative of overcoming this challenge, and results in soil nitrogen replenishment and crop productivity improvement.

1.4. Objectives

1.4.1. General objective

The general objective of this study was to determine the effect of Rhizobia inoculant on soybean nodulation and Arbuscular Mycorrhizal Fungi colonization under greenhouse and field conditions.

1.4.2. Specific objective

i) To evaluate eleven elite bradyrhizobia for their symbiotic effectiveness with soybean under greenhouse conditions

ii) To evaluate eleven elite bradyrhizobia for their symbiotic effectiveness with soybean under field conditions

iii) To determine the effect of eleven elite bradyrhizobium on indigenous AMF colonization with soybean roots under field conditions

iv) To determine the effect of Walungu peat on *B. Japonicum* under different storage conditions.

1.3.2. Research hypothesis

The research was guided by the following hypotheses:

i) *Bradyrhizobium japonicum* strains vary in their symbiotic effectiveness with soybean under greenhouse conditions

ii) *Bradyrhizobium japonicum* strains vary in their symbiotic effectiveness with soybean under field conditions

iii) *Bradyrhizobium* strains vary in their ability to enhance soybean colonization with indigenous AMF.

iv) Walungu peat used as a carrier in legume inoculants performs as well as a widely commercialized peat source from North America (America Peat Technology, APT).

1.4. Conceptual framework

Low agricultural productivity leading to low farmers livelihood as a result of low soil fertility especially nitrogen deficiency due to low external inputs, low biological nitrogen fixation and low quality inoculants (poor carrier material and ineffective rhizobia) may be improved by an effective symbiosis of soybean with both rhizobia and AMF through good quality inoculants (good carrier material and high density of an effective rhizobia) production and application in both controlled and uncontrolled conditions (Figure1.1).

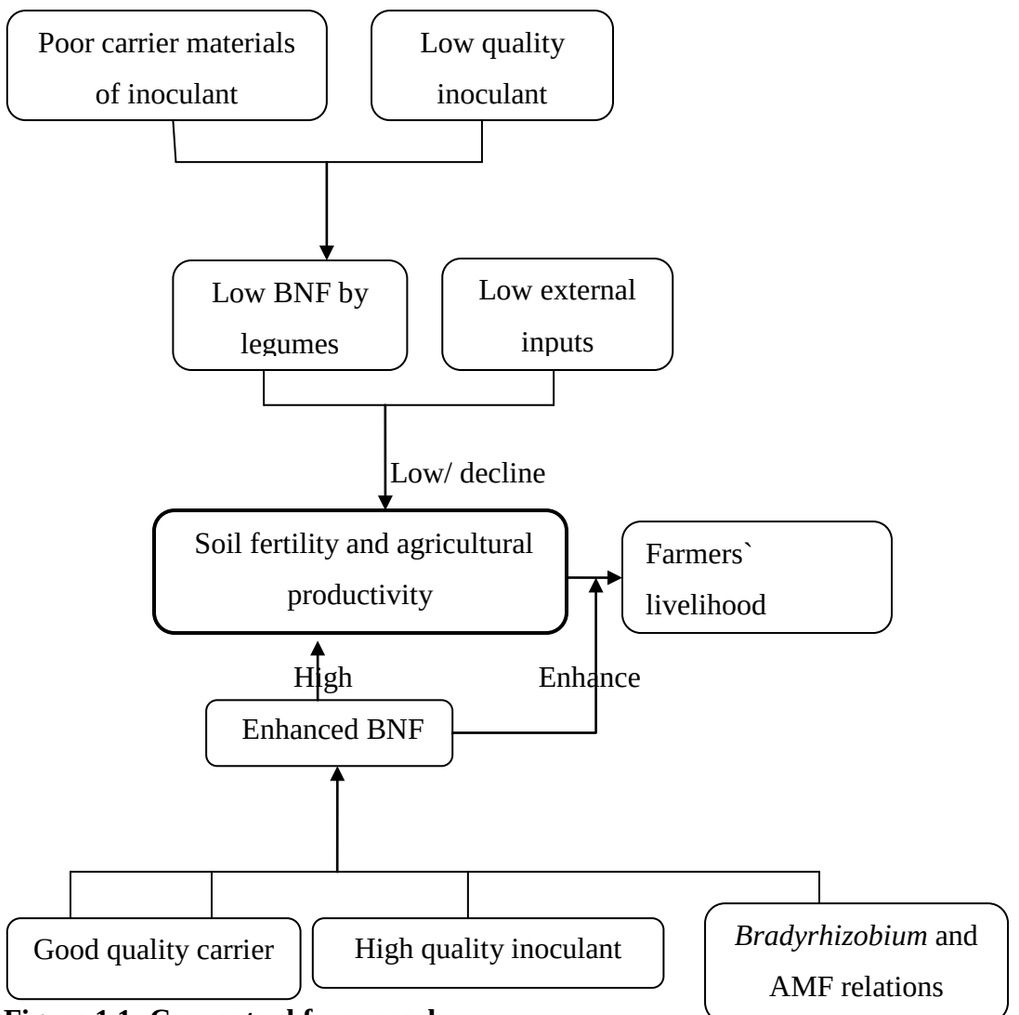

Figure 1.1: Conceptual framework

CHAPTER 2: LITERATURE REVIEW

2.1. Overview

Rhizobia bacteria are among the most useful soil microorganisms for crop production. They form symbiotic relation with legume plants in root nodules and fix atmospheric nitrogen which becomes the source of nitrogen for the plant. In order to get maximum benefit from the relation, inoculation with live effective rhizobial cell is needed and the environmental conditions such as soil temperature and acidity must not be detrimental to their survival. When biological nitrogen fixation is successful, leaf chlorophyll content of the legume plant increases due to the presence of high available nitrogen derived from the process. Phosphorus is among the macronutrient mostly needed for a successful biological nitrogen fixation because of high energy required for the process and the doubtless role of phosphorus compound in storing and transferring energy in the plant from photosynthesis. It is evident that AMF plays a great role in supplying plant phosphorus. Therefore, agricultural systems that involve AMF are the most sustainable and most reliable because of the beneficial effect of AMF on nutrient use efficiency. Many researchers while assessing the effect of dual inoculation have reported no effect of rhizobial performance on AMF, with AMF having a great effect on nodulation and nitrogen fixation. However, the effect of rhizobial inoculation on indigenous AMF is very limited. Rhizobia inoculant is a formulation of both carrier and rhizobia. Many carriers have been tested and used for good quality rhizobia inoculants but peat remained the widely used though good quality peat is not universally available.

2.2. Elite bradyrhizobium`s nitrogen fixing efficiency

2.2.1. Under field conditions

Rhizobia are unique in that they are the only nitrogen fixing bacteria living in a symbiotic relationship with legume (Jadhav, 2013). Biological nitrogen fixation by rhizobia is one of the most effective methods to improve the growth and productivity of grain legumes (Deshwal *et al*., 2013). The erratic performances of bio inoculants under field conditions have raised concerns about the practical potential offered by microbial releases into soil (Arora *et al*., 2010).

Soils usually lack *Bradyrhizobium japonicum* strains unless soybean is grown on them for at least five or more years (Tamiru *et al*., 2012). Inoculation responses are associated primarily with the first planting of a legume in soil having no prior history of the crop (Herridge *et al*., 1987; Brockwell *et al*., 1988; Juliana *et al*., 2013). In any case, to get the maximum benefit out of inoculation there is a need to follow correct and careful inoculation procedures, and the inoculant should carry live and effective bacterial cells (Solomon *et al*., 2012).

2.2.2. Under different soil types

Successful nodulation of leguminous crops by rhizobium largely depends on the presence of a specific and compatible strain in soil for a particular legume (Tamiru *et al*., 2012.). Several soil and environmental stresses affect the performance of the legume-rhizobium symbiosis and may limit nodulation and nitrogen fixation (Rebah, 2007). Rhizobia in the soil are exposed to a series of variable stresses in their natural environments, including nutrient limitation and/or exposure to physical stresses, such as elevated temperature,

acidity, high osmolarity, or oxidative shock (Zahran, 1999). It is known that soil acidity affects rhizobial persistence in the soil and rhizosphere of plants, as well as the efficiency of nodulation, especially in tropical areas (Graham *et al*., 1994). As a result, rhizobia have evolved adaptive strategies designed to minimize acid induced damage because inducible acid tolerance mechanisms are vital to plant symbionts growing in acidic soils (Foster, 2000). In the majority of cases, the bacterial response leads to transcriptional activation of genes, the products of which cope with a given physicochemical stress (Foster, 2000; Vinuesa, 2003). Rhizobial populations, the host plant species, agronomic practices and climatic conditions are among the important factors (Rebah, 2007). When a new crop is introduced, rhizobia may be absent in soils or indigenous populations may be low due to unfavorable soil conditions, especially low pH (Catroux *et al*., 2001). Since rhizobia should be in sufficient numbers to ensure optimal nodulation and efficient nitrogen fixation, inoculation of legumes with high populations of specific and efficient rhizobial strains has proven to be a valuable agronomic strategy to improve crop productivity (Rebah, 2007).

2.3. Effect of nitrogen, phosphorus and B. japonicum on soybean leaf chlorophyll content

Nitrogen is a major constituent of chlorophyll, the most essential pigment needed for photosynthesis and amino acids. It is also found in other bio-molecules such as ATP and nucleic acids (Weisany *et al*., 2013). Nitrogen is a factor in many biological compounds that plays a major role in photosynthetic activity. It is part of the enzymes associated with chlorophyll synthesis which reflect relative crop N status present in plants (Hokmalipour and Darbandi, 2011). Nitrogen is also a major part of the chlorophyll molecules and plays

a necessary role in photosynthesis (Reza *et al*., 2013). Nitrogen supply has significant effect on leaf growth because it increases the leaf area of plants and consequently influences photosynthesis function (Bojović and Marković, 2009). Furthermore, in legumes and other leafy vegetables, N improves the quality and quantity of dry matter and protein (Uchida, 2000). However, Nitrogen deficiency vanish the leaf green color and this may cause the decrease in leaf area and intensity of photosynthesis. This deficiency is also associated with symptoms of yellowing, dropping of leaves, poor growth, delayed flowering and fruiting (Wu *et al*., 2005). Inoculation with appropriate strain(s) of *B. japonicum* may be an effective way of increasing growth and leaf chlorophyll content in legumes (Eutropia *et al*. 2013).

Phosphorus is a fundamental component of the substances that are the building blocks of genes and chromosomes (Uchida, 2000). It is an essential part of the process of carrying the genetic code from one generation to the next, giving the blueprint for all characteristic of plant growth and development (Longstreth and Nobel, 1980). Plants need phosphorus for growth throughout their life cycle, especially during the early stages of growth and development (Eutropia *et al*., 2013). The primary role of phosphorus compounds in plants is to store and transfer energy produced by photosynthesis to be used for growth and reproduction (Leidi *et al*., 2000). Sufficient phosphorus is also required to enhance different plant organs growth, promote nodulation and early maturity in legumes (Kamara *et al*., 2010). Studies by Shahid *et al*. (2009) indicated that, increased phosphorus application enhanced plant height significantly. Apart from growth, Ganga-Suresh *et al*. (2010) noted that, phosphorus is a crucial element in legume crop production which plays

an important role for many characteristics such as sugar and starch utilization, photosynthesis, cell division and organization and nodule formation. Phosphorus is required in large quantities in young cells particularly shoots tips where metabolism is high and cell division is rapid. Insufficient levels of phosphorus may hinder plant growth; lower the chlorophyll accumulation which limits photosynthesis (Lambers *et al*., 2006). Furthermore, studies involving different types of crops have revealed that when phosphorus is limited, the most prominent effects are a reduction in leaf expansion, leaf surface area and the number of leaves (Bekere *et al*., 2012).

2.4. Effect of rhizobium inoculants on indigenous mycorrhizal colonization

Optimum growth of leguminous plants is usually enhanced by symbiotic relationships with mycorrhizal fungi and N_2-fixing bacteria (Xavier *et al*., 2003). AMF stimulate growth, improve pathogen, heavy metal and salinity resistance and influence the content of secondary metabolites in plants (Nikli *et al*., 2012). They also play an important role in the formation and stabilization of soil aggregates (Smith and Read, 2008; Gianinazzi *et al*., 2010). Mycorrhiza have strong mycelia, which expand the area of roots available for absorption of nutrients (especially phosphorus) (Jia *et al*., 2004; Shockley *et al*., 2004), and then stimulate rhizobium infection, improving nitrogen-fixation ability and legume plant growth (Siviero *et al*., 2008). Studies have shown that AM fungi can enhance the ability of soybean to absorb nutritional elements while improving both the N_2-fixing ability of rhizobium and the colonization structure in the rhizosphere niche, thus increasing yields and economic efficiency of soybean (Tian *et al*., 2013). However, Goss *et al*.(2006) observed no significant difference in soybean root colonization by indigenous AMF between inoculated and uninoculated plants with *B. Japonicum*,

indicating that the presence of *B. Japonicum* did not affect the establishment of fungal symbionts. Yanjun *et al*. (2010) also found out that infection rate of lucerne (*Medicago sativa*) with fungal mycelium was not significantly affected by inoculation with rhizobium. However, AMF are influenced by numerous factors such as soil type, plant species being cultivated, fertilization, pesticide use and/or ploughing (Nikli´ *et al*., 2012). The potential of AMF to colonize roots, microbial biomass and soil enzyme activities are often found to decrease in conventionally managed soils (Bending *et al*., 2004; Entz *et al*., 2004; Oehl *et al*., 2004; Fliebach *et a*l., 2007; Galván *et al*., 2009; Moeskops *et a*l., 2010). AMF colonization rates and the abundance of arbuscules are usually higher in plants grown on organically managed soils than in conventional cultivation (Bending *et al.*, 2004; Entz *et al.*, 2004; Galván *et al.*, 2009; Kahiluoto *et al.*, 2009). High soil phosphorus content usually results in low AMF colonization (Smith and Read, 2008). Moreover, long-term P fertilization, even at low levels, can reduce mycorrhiza formation (Mäder *et al.*, 2000; Bending *et al.*, 2004). For instance, Duan *et al*. (2010) found low colonization levels in maize, soybean, and wheat grown on fertilized soils. Similarly, Entz *et al*. (2004) observed lower colonization rates in flax from conventional treatments than from organic. Furthermore, the use of other readily soluble fertilizers, particularly nitrogen fertilizers, has similarly been found to have a negative impact on AMF colonization in some cases (Liu *et al.*, 2000; Burrows and Pfleger, 2002; Treseder and Allen, 2002), though not in others (Ryan and Ash, 1999; Jumpponen *et al.*, 2005).

2.5. Material suitability as an inoculants' carrier

Variety of materials used as carriers has been shown to improve the survival and biological effectiveness of inoculants by protecting bacteria from biotic and abiotic

stresses (van Veen *et al.*, 1997). Suitable carrier should be cheap, easily used, mixable, "packageable", and available. Also, the carrier must permit gas exchange, particularly oxygen, and has high organic matter content and high water holding capacity as well (Bashan, 1998; Rebah *et al*., 2002). According to Somasegaran and Hoben (1994), the good carrier material must be non-toxic either to the bacterial inoculants or to the plant itself. Furthermore, according to Stephens and Rask (2000) and Ferreira and Castro (2005) the carriers should have near neutral or readily adjustable pH, be abundant locally at a reasonable cost and can be easily sterilized. These properties only indicate the potential for a good carrier, while final selection of carrier must be based on microbial multiplication and survival during storage, the general method of planting, equipment used for planting, and acceptable cost (Ferreira and Castro, 2005).

2.5.1. Malt sprouts

The by-product of the malt industry (malt sprouts) has been used as a culture medium for rhizobia (Boiardi and Ertola, 1985). Malt sprouts extract is prepared by heating a mixture of malt sprouts and water at 100°C for 30 minutes. After centrifugation, a liquid is obtained that contains 3.75% w/v of solids, 0.18% w/v of nitrogen and 0.6% w/v of total reducing sugars. Many rhizobial species (*R. leguminosarum* bv. *phaseoli* and bv. *viciae*, *Sinorhizobium meliloti*, *B. japonicum*) have been tested in media containing different concentrations of malt sprouts extract. The malt sprouts extract at concentrations varying from 0 to 80% v/v of the growth medium were used to replace yeast extract in the medium containing: glycerol, yeast extract, peptone, K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, NaCl, $MnSO_4$ and biotin. In batch culture, all strains grew well in the malt sprouts based medium and reached a concentration higher than $5x10^9 cfuml^{-1}$. Differences

between slow and fast-growers were observed: cell yields of the fast-growing rhizobia increased with increasing concentration of the malt sprouts extract, while a concentration of 40% v/v inhibited the growth of the slow-growing rhizobia (*B. japonicum*) (Rebah *et al.,* 2007).

2.5.2. Cheese whey

The by-product of the cheese industry has been used for culture of the fast-growing rhizobium, *S. meliloti* (Bissonnette *et al.*, 1986). Whey contains the majority of elements included in the standard media at high enough concentrations to allow rhizobial growth (Vincent, 1974). According to Cerbulis *et al*. (1982), lactose in whey represents approximately 70–75% of the total solids while nitrogen represents 1.82–2.4%. The lactose uptake system of *S. meliloti* is inducible, and its catabolism involves cleavage to monosaccharide (Glenn and Dilworth, 1981; Bissonnette *et al.,* 1986). To avoid any latency on whey, rhizobium inoculum is produced under inducing conditions. Hence, whey allows rapid growth of *S. meliloti* and gives a cell population similar to that obtained in Yeast Mannitol Broth (YMB). In whey media, stationary growth is reached after 48 hours, and the maximum cell number of $6.3x10^{9}$cfuml^{-1} is obtained within 72 hours. Whey supplemented with yeast extract (1g/l) and phosphate (0.5g/l) can increase the cell numbers to $1x10^{10}$cfu cfuml^{-1} after 48 hours (Bissonnette *et al.,* 1986). According to Rebah *et al.* (2007) and Bio-resource Technology (2007) whey protects rhizobial cells from freezing and thawing damage. S. *meliloti* grown in whey survived freezing at 18°C better than cells grown on mannitol or sucrose (Bissonnette and Lalande, 1988). Moreover, rhizobia produced in whey and stored in peat survive better at various temperatures of storage as shown by 100% survival after 23 weeks at – 4°C. Therefore,

the by-product of the dairy industry offers an interesting potential as growth media and as protectant for industrial inoculants.

2.5.3. Industrial-grade yeast extract

With the aim to produce cell concentrates of *R. leguminosarum* bv. *viciae*, Meade *et al.* (1985) compared standard YMB with a medium using industrial-grade yeast extract as the carbon and nitrogen source under industrial-scale conditions. The growth yield in yeast extract (5g/l) medium was $2.4x10^9$ and $8.1x10^9 cfuml^{-1}$ for medium containing 0.6 and 0.9g/l of phosphate buffer respectively, and these values were similar to that of $3x10^9 cfuml^{-1}$ obtained in standard YMB medium. Yeast extract has an advantage for cell recovery with a continuous centrifugation system. The recovery is up to 63% and 70% in yeast extract medium in comparison to only 0.6% in standard YMB medium. The better recovery in yeast extract medium is attributed to the absence of polysaccharides which, in the YMB medium, trap cell within the viscous supernatant (Rebah *et al.*, 2007).

2.5.4. Pea husk, molasses and water hyacinth

Agriculture wastes were used to formulate a non-synthetic medium that contained pea husk, molasses, water hyacinth, mineral salts (NaCl, $CaCO_3$, K_2HPO_4, $MgSO_4$) and yeast extract (Gulati, 1979). A mixture of pea husk and water hyacinth (saccharized with mycelium of Trichoderma viride) boiled for 4 hours with 5% HCl used to partially replace the yeast extract normally present in standard YMB. Many strains of *R. leguminosarum* bv. *Trifolii* and *B. japonicum* tested in flask and in Fermentor for large-scale production showed equal growth in standard and in formulated non-synthetic media. The suitability of the formulated medium can be attributed to the presence of

required amino acids such as tryptophan, glutamic acid and carbon sources such as glucose, arabinose and cellobiose. The large-scale production (25 and 135 l Fermentor) was adequate for commercial use (Rebah *et al.*, 2007).

2.5.5. Alginate

Alginate is dry, synthetic, simple to use, uniform, biodegradable by soil microorganisms, and non-toxic in nature (Bashan, 1998). It contains a large uniform bacterial population and provides slow release of the bacteria for long periods (Bashan, 1998). It causes no ecological pollution and can be produced on large scale by the proper industry. The beads can be stored for long periods in a relatively small volume without any apparent effect on the size of the bacterial population (Bashan and Gonzalez, 1999).

2.5.6. Wastewater sludge

Waste water sludge, a worldwide recyclable waste, has shown good potential for inoculants production as a growth medium and as a carrier (dehydrated sludge) (Rebaha *et al*., 2007). Sludge usually contains nutrient elements at concentrations sufficient to sustain rhizobial growth and heavy metals are usually below the recommended level. In some cases, growth conditions can be optimized by sludge pre-treatment or by the addition of nutrients. Inoculants produced in wastewater sludge are efficient for nodulation and nitrogen fixation with legumes as compared to standard inoculants (Rebaha *et al*.,, 2007).

2.6. Peat as carrier material

Peat is the most widely used carrier, and it can sustain high numbers of rhizobia (greater than 10^8 cells g^{-1}) during storage at a temperature range from 3 to 28 °C (Burton, 1967; Van Schreven, 1970). Peat usually contains certain essential nutrients which not only help survival of the cultures but also allow further growth and multiplication during storage (Pugashetti *at al.*, 1971), but is not universally available (Tilak and Subba Rao, 1978). Peat (or humus) is used as a carrier in either a granular form, which is applied in-furrow, or in a powder form, which is applied to the seed at planting (Abendroth *et al*., 2006). The choice of inoculants carrier material depends largely on its suitability as a good carrier medium for rhizobia and the criteria for suitability have been summarized by Burton (1978) as non-toxicity to Rhizobium species, good absorption qualities, easy preparation and sterilization, good adhesion to seeds and ready availability at moderate costs.

2.7. Inoculants' shelf life as influenced by storage temperature

Legume inoculants are perishable and can lose their effectiveness when exposed to a temperature of 40°C or higher for a few hours (Herridge *et al*, 2002). High-quality inoculants should retain their effectiveness for six months or longer when stored at a temperature around 20°C. This period can be extended if inoculants are refrigerated near 4°C. Warning should be given regarding the harmful effects of high temperatures on inoculants. Warning should also be given about the harmful effects of deep freezing. Deep freezing of inoculants may damage the *rhizobium* cell membranes. Inoculants should be stored in a cool place until ready to use. Storage in a refrigerator is good. Cold cannot harm the bacteria but they cannot tolerate high temperatures. Leftover inoculant

stored in a refrigerator at 4°C or lower will remain effective for several months (Herridge *et al*, 2002). Survival is also affected by the initial condition of the cells in the inoculant, particularly the moisture status, age, purity, the initial number, the strain and the type of inoculant (Deaker *et al*., 2004). Changes in the physiological and morphological characteristics of cells during the maturation of the peat inoculant have been shown to affect survival (Dart *et al.*, 1969; Feng *et al.*, 2002). In addition, contaminants in inoculant carriers are known to suppress growth of rhizobia during inoculant production (Roughley *et al.*, 1967).

2.8. Summary of literature and isolation of research gaps

Successful nodulation of leguminous crops by rhizobia largely depends on the presence of a specific and compatible strain in soil for a particular legume (Tamiru *et al*., 2012.).Soils usually lack *B. japonicum* strains unless soybean is grown on them for at least five or more years (Tamiru *et al*., 2012). However, *B. japonicum* strains vary in their effectiveness for biological nitrogen fixation (Kadiata *et al.*, 2012). Therefore, there is a need to select effective strains in order to get maximum advantage from biological nitrogen fixation process. Optimum growth of leguminous plants is usually dependent on symbiotic relationships with mycorrhizal fungi and N_2-fixing bacteria (Xavier *et al.*, 2003). Research on the effect of rhizobial inoculants on indigenous AMF is very limited. Since AMF are reported to improve biological nitrogen fixation of legume with rhizobia inoculation, there is a need to assess the effect of elite bradyrhizobium on AMF.

A wide range of carrier has been used as a base for rhizobium inoculants production, but peat has remained the preferred material because of the protection it offers to cultures

against high storage temperatures and its ability to maintain hydration of the cultures(Burton, 1982),but is not universally available (Tilak and Subba Rao, 1978). Since, Walungu peat is locally available at cheaper cost in DR Congo, assessing its suitability as carrier material for inoculants production may help to overcome the challenge of APT`s exportation currently been faced by IITA.

CHAPTER 3: MATERIALS AND METHODS

3.1. Study area

The study was carried out in soil microbiology laboratory, screenhouse and experimental field (plot A2West) of IITA headquarters, Nigeria. IITA headquarters is in Ibadan, the capital city of Oyo State, located in the Southwestern part of Nigeria on longitude $3^{o}54^{'}$ of Greenwich Meridian East and latitude 7° $54^{'}$ North. The study area is about 234 meters above sea level and situated on gently rolling hills running in a northwest/ southeast direction (Agbola and Olurin, 2000). Ibadan enjoys the characteristic West African monsoon climate which has two major seasons (rain- March and October and dry- November and February season). According to Oluwasamni (1967), patterns of land use in Ibadan bear the imprint of both Western and Traditional Yoruba practices. Traditionally, very little farming went on within the limits of a Yoruba town while the belt surrounding a town was used for cultivation of yield.

3.2. Initial soil characteristics

The soils at the experimental field was moderately acidic in reaction (pH = 5.6) with a sandy texture (Table 1). The effective cation exchange capacity of the soil was 2.12 Cmol (+)/kg which is very low according to Tisdale *et al.* (2002). This low CEC may be attributed to the higher percentage (80.8%) of sand in the soil. The total nitrogen and organic carbon contents of the soil before sowing were found to be 0.11% and 1.39%, respectively, whereas available phosphorous content was 2.38 ppm (Table 4.1).

Table 3.1: Selected Physicochemical Properties of Soil.

% Particle size			Exchangeable cations				Cmol+/Kg ECEC	pH	%OC	%N	ppm P
SAN	SIL	CLA	Ca	Mg	K	Na					
80.8	6	13.2	1.39	0.45	0.2	0.09	2.12	5.6	1.39	0.11	2.38

3.3. Rainfall information

Figure (4.1) shows daily rainfall during the experimental period (6th May-6th August). Daily rainfall varied with the maximum (85mm) recorded at the end of the experiment (6th August 2014).

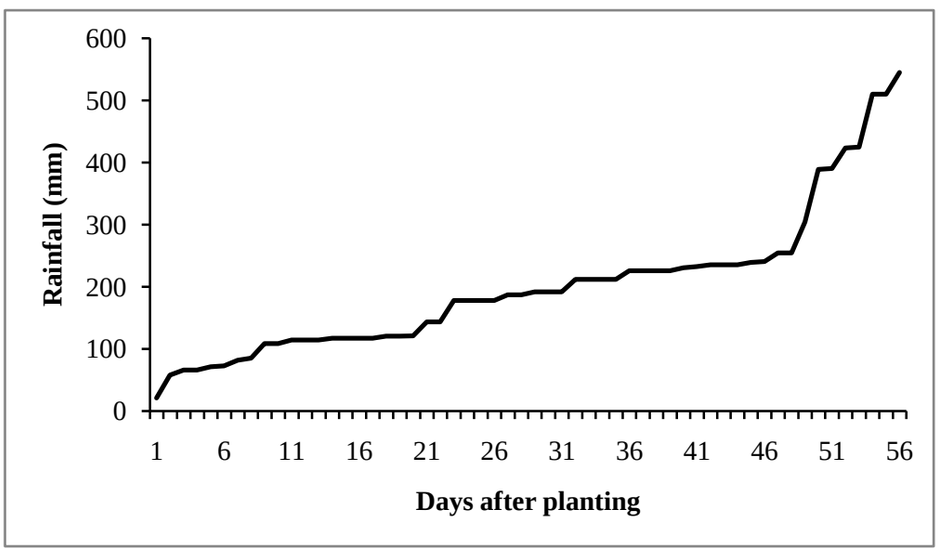

Figure 3.1: Rainfall information during the experimental period

3. 4. Research design

3. 4. 1. Screenhouse experiment

A screenhouse experiment was set to evaluate eleven *Bradyrhizobium japonicum* strains, nine strains from IITA collection and two reference strains (USDA110 and USDA532c) used in commercial inoculant products: were screened for their symbiotic effectiveness with soybean. The treatments were compared against a control (no inoculation) and inorganic nitrogen fertilizer application at the rate of 100 kg N ha^{-1}(in the form of urea). This led to a total of 13 treatments (9 isolates of IITA, USDA 532c, USDA 110, and two controls +N and -N).

Table 1: Experimental treatments

Number of treatments	**Treatments**
1	USDA110
2	USDA532c
3	RANI22
4	RACA6
5	FA3
6	CC511
7	IRJ2180A
8	USDA9032
9	MAR1495
10	USDA138
11	USDA136
12	Urea
13	Control

The experimental design was a completely randomized design (CRD) replicated 4 times giving a total of 52 pots in the experiment. Variables observed in the course of the experiment were; leave chlorophyll content (17-25), nodulation (number of nodules and nodule dry weight) and above ground biomass parameters (dry weight, nitrogen and phosphorus contents).

3. 4. 2. Field experiment

A field experiment was set to study the symbiotic effects of selected *Bradyrhizobium* strains inoculation on soybean under natural environment conditions in the experimental field of IITA headquarter/ Ibadan (plot A2West). The selected *Bradyrhizobium* strains were eleven and two controls (9 isolates of IITA , USDA 532c, USDA 110, and two controls +N and -N) leading to 13 treatments in total. The experimental design used was a randomized complete block design (RCBD) replicated 4 times giving a total of 52 plots in the experiment. Data were recorded on leave chlorophyll content, nodulation parameters (number and dry weight of nodules) and above ground biomass parameters (dry weight, nitrogen and phosphorus contents), AMF colonization (Hyphae and vesicles, Arbuscular).

3.4. 3. Peat experiment

The peat experiment was carried out in the laboratory. Three factors were considered ;(1) peat type (Walungu peat not limed, Walungu peat limed and America peat), (2) sterilization (as sterilization and no sterilization) and (3) storage temperatures (4 ^{o}C, 15^{o}C and 22-30^{o}C). This resulted to eighteen treatments replicated three times in the experiment. Plate- count for rhizobia was performed.

3. 5. Experimental management

3. 5. 1. Strains purification, isolation and broth preparation

This investigation was carried out to determine the purity of inoculum material collected from N2Africa rhizobiology laboratory in DR Congo (NAC41, NAC49, NAC35, NAC15, NAC37, NAC22, NAC51 and NAC43), Kenya (NAK128 and NAK 115), IITA headquarter collection (CC511, USDA 9032, RACA6, RANI22, FA3, USDA138, MAR1495, IRJ2180Aand USDA136), and the standard commercial inoculant strains (USDA110 and USDA 532c), and produce broth inoculants used in the screenhouse and field experiments. The rhizobium cultures were streaked on both Congo red-YEMA and Nutrient Agar plates and incubated for three to five days at 28^{o}C. Young colonies with little or no absorption of Congo red and no growth on Nutrients Agar were confirmed as *Rhizobium* species. All the cultures from Congo and Kenya were highly contaminated with fungi. Since, bradyrhizobium takes at least 3 days to grow; fungi were already spread on the plate media preventing bradyrhizobium growth. We assumed that bradyrhizobium cells might be present in the initial culture but not able to grow because of the ability of fungi to overspread on the plate media. Based on this confirmation/conclusion, and given the fact that soybean can only nodulate with *B. japonicum*, soybean was grown in growth chamber and inoculated with the initial culture collected from rhizobiology team in both Congo and Kenya and hereafter, isolate from the nodule formed to obtain pure culture of bradyrhizobium for future research. Broth inoculants used in the current study were prepared for each of the eleven good strains according to Vincent (1970).Six weeks after planting, out of 10 strains collected from DR Congo and Kenya, only four strains from DR Congo (NAC22, NAC35, NAC37 and

NAC49) formed nodules. Nodules were then collected and bradyrhizobium strains isolated according to the rhizobia strain isolation and characterization protocol of N2Africa (Bala *et al*., 2011), for further research in this study.

3. 5. 2. Screenhouse experiment

The screen house trial adopted the pot experiment approach. The growth media was prepared by mixing sterile one part washed sand, 12 parts fine washed granite, and one part standard American peat. The planting media were put in one kilogram pots to form the treatment units. Before planting, soybean seeds were surface sterilized with 95% ethanol for 10 seconds and 3% sodium hypochlorite for 2 minutes, then washed thoroughly with sterile distilled water and sown. At planting 0.15 g of Sympal (14 parts Triple Super Phosphate (TSP), 5 parts KCl and 1 part $MgSO_4$) was applied per pot in a hole between seeds. Three seeds were planted in each pot and thinned to two after emergence and inoculated with 1 ml broth inoculants containing $5x10^9$ rhizobia cells ml^{-1} per seedling using 1 ml pipette. Sterile distilled water was added daily depending on the need. Six weeks after planting, 50ml N- free nutrient solution was daily added to each pot for two week.

Data collection

Four weeks after planting, leaf chlorophyll content was determined for a period of three weeks using the chlorophyll meter Spad-502(79923113, KONICA MINOLTA SENSING, ING. Japan). The device was first of all switched on, calibrated by pressing the measuring head closed without inserting a leaf, and then the leaf chlorophyll content was measured by inserting the leaf and pressing the measuring head closed.

Measurements were taken weekly on two medium leave (6 leaflets) per plant. Eight weeks after planting, each plant through its leaves color was classified either as vigorous or stunted. Plants with dark green leaves classified as vigorous indicated effective nodulation and positive N_2-fixation whereas, those with chlorotic/yellow leaves indicated ineffective nodulation. All the plants were then harvested and separated into shoots, roots and nodules. All the nodules were washed and carefully rinsed with water and their number determined. Then shoot and nodules were allowed to dry at 60^oC for 48 hours in the oven and their dry weight recorded. The treatments combinations and layout is shown in appendix1

3. 5. 3. Field experiment

An experimental area where soybean had not been grown before was used. Plots measuring 3 m by 0.75 m was prepared with furrows measuring 0.20 m wide and 0.15 m deep separated by a 0.20 m wide unplanted ridge on either side to prevent plot to plot cross contamination. The control +N plot received 100 Kg ha^{-1}, split applied at planting and flowering. Potassium in the form of muriate of potash (MOP) and phosphorus in the form of TSP was blanket applied at planting at the rate of 50Kg ha^{-1} and 30 Kg ha^{-1}, respectively. Broth inoculum of each of the eleven strains containing rhizobia population of $5x10^9$ Cells ml^{-1} broths was used for inoculation of the seed at planting. After adding 1 ml of inoculum with 1 ml pipette, each seed was immediately covered with soil.

Data collection

Leaf chlorophyll content was measured as previously described. Eight weeks after planting, each plant through its leaves color was classified either as vigorous or stunted.

Plants with dark green leaves classified as vigorous indicated effective nodulation and positive N_2 fixation whereas, those with chlorotic/yellow leaves indicated ineffective nodulation. At pod filling, all the plants in the net plot (1.5 m^2) were harvested and separated into shoots, roots and nodules. All the nodules were washed and carefully rinsed with water and their number determined. Then shoot and nodules were allowed to dry at 60^oC for 48h in the oven and their dry weight recorded. Fresh roots were used for mycorrhizal colonization examination using light microsco`pe (Kormanik and Mc Graw, 1982; Brundrett *et al*., 1994). The treatments combinations and layout is shown in Appendix 2

3.5.4. Peat experiment

Natural peat from Walungu territory /DR Congo obtained from the Catholic University of Bukavu, was used in this study as carrier material for *Bradyrhizobium Japonicum* (USDA110) inoculum in comparison with standard peat from United State of America received from IITA Headquarters. Walungu peat was prepared by drying, grinding in a hammer mill and passing through a 100 µ mesh sieve before use. Selected physical-chemical properties (Water holding capacity, pH and Organic carbon), was determined for each peat type. For some treatments, Walungu peat was amended with 4.1% lime to raise the pH to 6.14 (pH of America peat). With each of the three peat type (America peat, Walungu peat and Walungu peat amended), 18 bags of 50g polyethylene bags were packed. The survival of *B. Japonicum* was evaluated under sterile and unsterile conditions. Therefore, out of 18 bags of each peat type, nine bags were sterilized by autoclaving at 121^o C for 3 hours (24 hours interval after autoclaving for 1 hour). *B. Japonicum,* USDA110 strain obtained from the soil microbiology laboratory of IITA

headquarter/ Ibadan was used for inoculation. The strain was grown on yeast extract mannitol broth (Vincent, 1970) in a Fermentor at 28^{o}C for 7 days. In 50 g package of sterilized and unsterilized peat, 50% of the broth culture (25 ml) was injected aseptically under laminar flow bench with 25 ml syringe. Three replicates per each carrier were used and stored at three different temperatures (4^{o}C, 15^{o}C and laboratory temperature), giving a total of 54 bags in the experiment.

Data collection

The mean number of viable *B. Japonicum* cells in the fresh culture used for injection was 5 x 10^{8} cellsg^{-1} peat as determined by the plate count method (Vincent, 1970). The inoculants were cured for 14 days in the incubator at 28^{o}C and then stored at three different temperatures (4^{o}C, 15^{o}C and laboratory temperature).

Number of viable *B. Japonicum* cells on yeast extract mannitol agar with 25 ppm Congo red were determined by plate count (Vincent, 1970), every 15 days interval for 60 days. For each sample eight fold dilutions was performed. Three plates were used for each sample (two Congo red plate and one Nutrient Agar plate; for assessing the contamination level).The treatments combinations and layout are shown in appendix3.

3. 5. 5. Soil sampling and Plants Chemical analyses

Soil samples were collected at planting using a W design. The soil samples were collected and mixed thoroughly to get one composite soil sample per block for initial soil characterization analysis. Soils and plant tissue were analyzed according to Okalebo *et al.* (2002). Soil pH was measured in both KCl and water at a ratio of 1:2.5 soil to solution

ratio using pre-calibrated pH meter. Organic carbon was determined using modified Walkley and Black method. Available P was determined following Olsen method. Total nitrogen was determined using Kjehdal distillation method. Available N (NH_4^+ and NO_3) was determined according to Bremner (1956), by filtration, steam distillation and titration method.

3.6. Statistical analysis

Microsoft Excel was used for data management. All the quantitative data collected from the various experiments were subjected to analysis of variance (ANOVA) to examine the effect the treatments on the measured parameters. The ANOVA was conducted using the Mixed procedure in SAS 9.2 (SAS Institute, 2004). Where significant treatment differences were observed, least significant difference (LSD) test ($p \leq 0.05$) was used to evaluate how the treatments differed from each other

CHAPTER 2 : RESULTS AND DISCUSSION

4.1. Overview

In this chapter, the results and discussions are presented logically following the study objectives. It starts with the evaluation of elite *B. japonicum* strains under study for their effectiveness in improving soybean chlorophyll content; above ground biomass dry weight, tissue nitrogen and phosphorus content, and nodulation (number and dry weight); under greenhouse conditions. This is followed by field evaluation, of the effect of *B. japonicum* strains on indigenous AMF root colonization. Finally, in this chapter, the of Walungu peat, a local peat on the survival of *B. japonicum* are presented and discussed.

4.2. Evaluation of elite Bradyrhizobia for their symbiotic effectiveness with soybean under greenhouse conditions.

4.2.1 Soybean chlorophyll content

Leaf chlorophyll content was significantly ($p<.001$) affected by nitrogen fertilizer. Nitrogen fertilizer had the highest leaf chlorophyll content which was 39%, 65%, and 79% for measurements taken 4WAP, 6WAP and 8WAP respectively compared to the control (Table 4.1). This was followed by RANI22 and RACA6 strains which were the highest as far as the strains are concern. They were both not significantly ($p< 0.001$) different from each other but stood out superior compared to control and the other seven strains throughout the three interval periods when the content was determined. At 8WAP, leaf chlorophyll content increased by 19.2% for RANI22 and 18.6% for RACA6 as compared to the control. The strains that had least effect on chlorophyll content throughout the period of measurements were MAR1495, USDA9032 and USDA136. However, leaf chlorophyll varied within strains and decreased at 6WAP for all strains

including control (uninoculated-no fertilized pot). The highest value was recorded in the pot inoculated with RANI22 and the lowest with MAR1495, USDA9032, and USDA136. At 8WAP, leaf chlorophyll increased by 27% for RANI22 and decreased 29-25% for MAR1495, USDA9032, USDA136 and the control.

Table 4.1: Chlorophyll content during the growth period of Soybean inoculated with different bradyrhizobium strains

Treatments	Chlorophyll Content		
	4WAP*	**6WAP**	**8WAP**
Urea	34.2[a]	41.18[a]	50.7[a]
RANI22	31.8[b]	21.8[b]	29.8[b]
RACA6	29.8[bc]	21.9[b]	29.2[b]
IRJ2180A	28.9[cd]	20.6[c]	27.0[c]
USDA110	27.8[cde]	19.3[d]	25.1[d]
USDA532c	27.0[ed]	18.6[de]	24.1[d]
FA3	26.2[ef]	18.1[ef]	22.5[e]
USDA138	24.4[fg]	17.3[fg]	20.7[f]
CC511	23.0[gh]	16.7[g]	17.3[g]
MAR1495	22.0[hi]	16.2[gh]	11.5[h]
USDA9032	21.3[hi]	15.3[h]	11.5[h]
USDA136	21.1[hi]	15.3[hi]	10.9[h]
Control	20.8[i]	14.2[i]	10.6[h]
LSD	2.14	1.1427	1.323
P	<.0001	<.0001	<.0001

*WAP : Weeks After Planting.

Qrbanly *et al.* (2006) found a positive correlation between nitrogen and the chlorophyll content of leaves, mainly due to presence of nitrogen in the structure of chlorophyll molecules. The leave chlorophyll decrease at 6WAP recorded for some of the strains (RANI22, RACA6, IRJ2180A, USDA110, USDA 532c, FA3, USDA138) was probably as a result of the decrease in biological nitrogen fixation as a response to some micro and macro nutrients stress generated by low nutrients availability in the experimental media, that showed their response by increasing the leave chlorophyll by 27, 25, 24, 23, 23, 20, 16 and 4% for RANI22, RACA6, IRJ2180A, USDA110, 532C, FA3, USDA138, and

CC511 respectively after adding N-free nutrients solution to each pot for two weeks. The decrease in chlorophyll content recorded for the USDA9032, USDA136 and MAR1495 strains which are statistically similar to the control may be a result of ineffectiveness of the strains to form effective symbiosis with soybean. Kadiata *et al*. (2012), reported that rhizobial strains may vary in their symbiotic effectiveness for legume performance. Tairo and Ndakidemi (2013) showed that rhizobial inoculation significantly increases the total leaf chlorophyll content in both glasshouse and field experiment. Anjum *et al.* (2006) also reported that beneficial rhizobia bacteria may influence the physiological growth conditions of leguminous plants by increasing chlorophyll contents in leaves.

4.2.2. Soybean above ground biomass.

Soybean shoot dry matter accumulated was significantly affected (P<.0001) by *B. Japonicum* strains and nitrogen fertilizer added as compared to the control, uninoculated-no fertilized plot. Maximum dry weight per plant (2.41g) was observed in nitrogen fertilizer treatment. However, inoculation of soybean with the strains; (RACA6, RANI22, IRJ2180A, USDA532c, FA3, USDA138, and CC511) also induced significant increase in above ground dry weight per plant by 42, 42, 39, 35, 31, 21, 17, and 12% for RACA6, RANI22, IRJ2180A, USDA 532c, FA3, USDA138, and CC511 respectively. Lowest dry weight was recorded in the control and MAR1495 strain which were statistically not different from USDA9032 and USDA136.

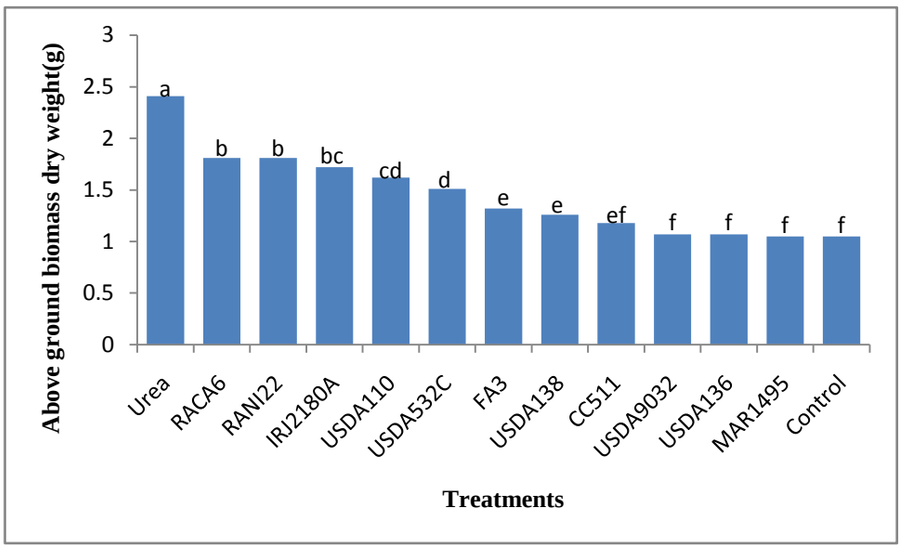

Figure 4.1: The above ground biomass of soybean as influenced by inoculation with different bradyrhizobium strains, $p<.0001$ value and $LSD = 0.1542$.

The difference between strains in accumulating above ground dry matter might be the result of different strain potential for biological nitrogen fixation with soybean. Kadiata, *et al.* (2012) reported that rhizobial strains may vary in their symbiotic effectiveness for legume performance. Tamiru *et al.* (2012) also reported significant difference between strains in terms of soybean dry matter production. The higher dry weight biomass recorded with nitrogen fertilizer treatment might be the results of biomass production due to sufficient available nitrogen in the rhizosphere. Sulochana and Prashanth (2008) observed significant effect of nitrogen fertilizer on above ground dry weight of green gram, Bengal gram and groundnut as compared to the control.

Those strains were divisible into three clusters with regard to their effect on above ground dry biomass (Table 4.2). RACA6 and RANI22 formed the higher cluster, IRJ2180A, USDA110 and 532C; the middle cluster and FA3, USDA138, CC511, MAR1495, USDA9032, and USDA136, the lower cluster. The high above ground biomass dry

weight induced by RACA6 and RANI22 might be the result of effective biological nitrogen fixation that provided enough nitrogen to the plant leading to high biomass formation and high dry matter accumulation. Lukiwati and Simanungkalit (2002) reported significant increase in dry matter of soybean plants by bradyrhizobia inoculation in sterilized soil. The difference between strains in their effect on soybean above ground dry matter might be the results of their potential difference for biological nitrogen fixation with soybean. Vasilas and Ham (1984) and Sparrow *et al.*(1995) reported significant difference between strains in terms of dry matter production on several legumes. The lower cluster was not significantly different from the control (uninoculated-no fertilized plot) (Figure 4.1) and it can therefore be argument that in this cluster were less effective in forming successful biological nitrogen fixation with soybean. This may be due to their incompatibility with the variety under study. Rubén *et al.*(2012) found out that soybean response to inoculation varies widely between cultivars**.** Milic *et al.* (2002) while studying the activity of nitrogen fixation and nitrogen assimilation enzymes in soybean plants inoculated with *B. japonicum* strains, also reported variability in the performance of different bradyrhizobial strains in terms of dry matter mass. Such variations may be attributed to difference in genomic constitutions of the host or bacteria or both which control symbiosis or there might be more than one affinity group within the legume rhizobia leading to such variation.

4.2.3 Soybean nitrogen content

Significant increase in shoot N was observed in plants inoculated with RACA6 and RANI22 than others and the control (Figure 4.2). However, pots with nitrogen fertilizer treatment showed higher shoot N content compared to other treatments. Nitrogen

fertilizer and these two strains (RACA6 and RANI22) increased total nitrogen content of soybean shoot by 51 and 43% respectively compared to the control.

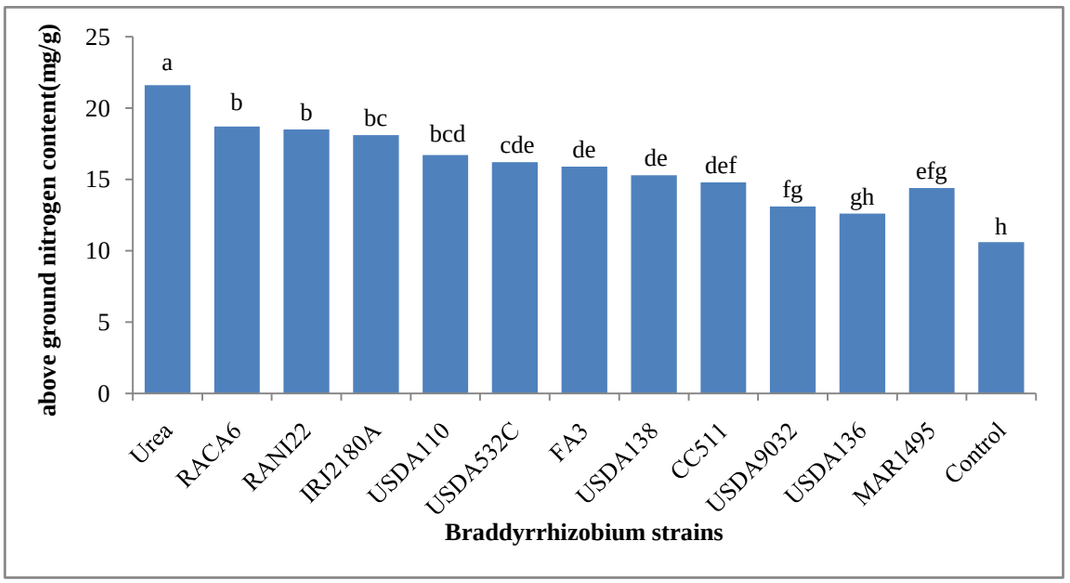

Figure 4.2: Soybean nitrogen content as influenced by inoculation with different bradyrhizobium strains, $p<.001$ and $LSD = 2.073$.

With regard to strains effects on nitrogen content, three clusters were observed, the higher cluster formed by RANI22, RACA6 and IRJ2180A; the middle cluster with USDA110, 532C, FA3 and USDA138 and the lower cluster formed by CC511, USDA9032, USDA136, and MAR1495 (Figure 4.2).

Higher accumulation of nitrogen in shoot N due to inoculation with RANI22, RAC6 and IRJ2180A might be due to the presence of high available nitrogen derived from effective nitrogen fixation. Lukiwati and Simanungkalit (2002) reported significant increase in nitrogen uptake of soybean plants by bradyrhizobial inoculation in sterilized soil. Basu and Bansyopadhyay (1990) and Rashid *et al.* (1999) also reported an increase in nitrogen uptake by plant due to inoculation application. The low to moderate accumulation of nitrogen in soybean leaves due to remaining strains might be attributed to the difference

in their capacity for nitrogen fixation with soybean cultivar under study. Milic *et al.* (2002) while studying the activity of nitrogen fixation and nitrogen assimilation enzymes in soybean plants inoculated with *B. japonicum* strains, reported variability in performance of different bradyrhizobial strains in terms of nitrogen content of soybean varieties. Such variations may be attributed to variations in genomic constitutions of the host or bacteria or both which control symbiosis, or there might be more than one affinity group within the legume rhizobia leading to such variation (Milic *et al*. 2002).

4.2.4 Soybean phosphorus content.

Significant difference ($p<.0001$) between strains in terms of shoot phosphorus content was evident (Figure 4.4). The highest value was recorded with IRJ2180A which was statistically not different from RACA6 and RANI22 but statistically different from the remaining strains, the control and nitrogen fertilizer. The lowest value was recorded in nitrogen fertilizer treatment which was statistically not different from MAR1495, USDA9032, USDA136 and the control.

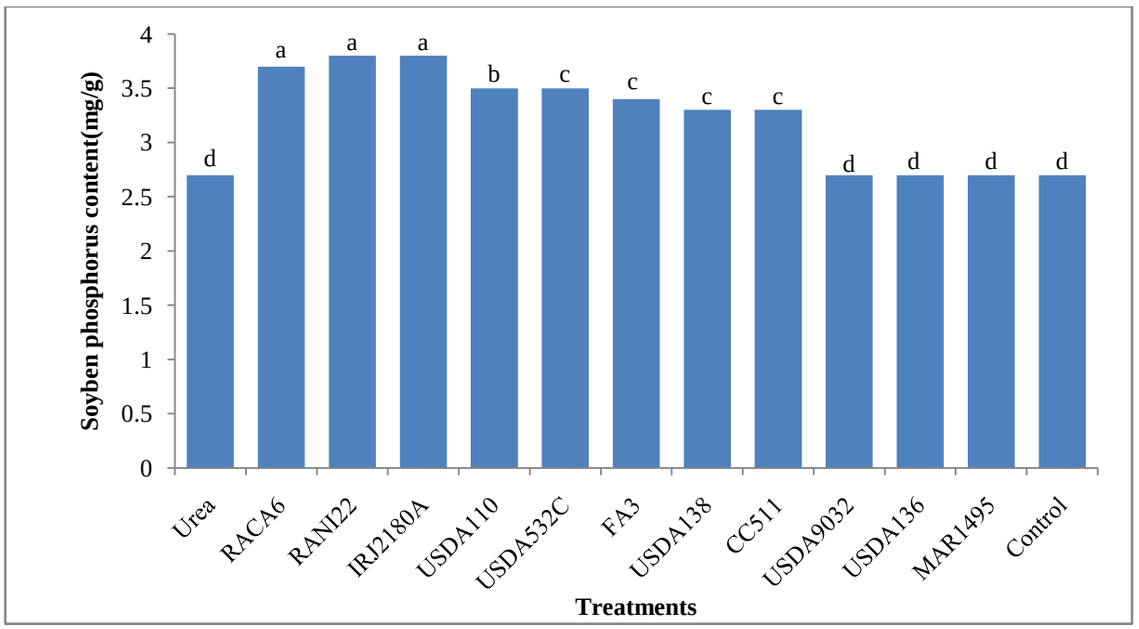

Figure 4.3: Soybean phosphorus content as influenced by inoculation with different bradyrhizobium strains, *P*<.0001 and *LSD*=0.191.

Data presented in Figure (4.4), showed that the experimental treatments were divided into four clusters, the high cluster represented by RACA6, RANI22 and IRJ2180A, the middle cluster formed by USDA110, the lower cluster included 532C, FA3, USDA138 and CC511, and the lowest cluster with nitrogen fertilizer treatment, USDA9032, USDA136, MAR1495, and no input control. We suggested that low phosphorus content in plants inoculated with lowest cluster strains might be due to their inability to solubilize precipitated phosphorus as indicated by an increase in phosphorus content of plants inoculated with IRJ2180A, RANI22 and RACA6. This might also be due to the ineffectiveness of these strains to induce an increased number of roots hairs and lateral roots of soybean, thereby favoring nutrient uptake by exploration of a greater experimental media volume. Biswas *et al*. (2000) reported that rhizobial inoculants may also induce an increased number of roots hairs and lateral roots thereby favoring nutrient

uptake by exploration of a greater soil volume. Biswas (1998) observed that *Rhizobium* inoculation significantly increased uptake of NPK by rice plants compared with uninoculated plants. On the other hand, low phosphorus content in nitrogen fertilizer and control treatments might be due to low phosphorus availability in pot media and the absence of effective microorganisms able to assist plants in solubilizing unavailable phosphorus. Moreover, the difference between strains in accumulating above ground phosphorus content might be due to their potential difference in solubilizing precipitated phosphorus as well as increasing soybean root hairs and lateral roots formation. Chabot *et al* (1996) showed that some rhizobia strains are able to solubilize precipitated P components. Lukiwati and Simanungkalit (2002) reported significant increase in phosphorus uptake of soybean plants by bradyrhizobial inoculation in sterilized soil.

4.2.5. Soybean nodulation

The number of nodules per plant was significantly affected by *B. Japonicum* strains (Table 4.3). In the current study, the strains RANI22, RACA6 and IRJ2180A produced significantly higher number of nodules than others. The strains 1495MAR USDA136, USDA9032, nitrogen fertilizer and the control did not produce any nodule at all. This is an indication that 1495MAR, USDA136 and USDA9032 are not able to form symbiosis with soybean (PK06), as a result of strains incompatibility with soybean variety used in the study.

Table 4.2: Effects of elite bradyrhizobia inoculation on number of nodules and nodules' dry weight per soybean plant

Treatments	**Nodulation (plant^{-1})**	
	Number	**Dry weight (mg)**
RANI22	80.3^{a}	150.0^{ab}
IRJ2180A	69.1^{a}	171.2^{a}
RACA6	63.6^{a}	186.2^{a}
USDA110	43.0^{b}	121.2^{bc}
USDA532c	33.5^{bc}	96.2^{cd}
FA3	21.6^{cd}	65.0^{d}
USDA138	18.3^{cde}	26.2^{e}
CC511	6.5^{ed}	23.6^{e}
MAR1495	0.0^{e}	0.0^{e}
USDA136	0.0^{e}	0.0^{e}
USDA9032	0.0^{e}	0.0^{e}
Urea	0.0^{e}	0.0^{e}
Control	0.0^{e}	0.0^{e}
LSD	19.4	36.7
P	<.0001	<.0001

Thao *et al.* (2002) found a significant interaction between variety and strain on different parameters whereas Munyinda *et al.* (1988) reported a non significant interaction. Since the pot media was autoclaved before use, the absence of nodule in fertilized and control plants might be due to the absence *B. japonicum* that could form symbiotic relation with soybean. Sanginga and Woomer (2009) reported that no nodules imply that the host`s specialized rhizobia are absent from the soil. Kuykendall *et al.* (1996) reported that

soybean plants inoculated with strain TA-11NOD+ were significantly better nodulated than those inoculated with strain I-110ARS. Danso *et al. (*1987) also observed that Nitragin inoculants induced production of more nodules than strain D in soybean. Ramaswami and Oblisami (1986) reported the increase in nodules due to inoculation application. Study on the effect of Rhizobium seed inoculation and nitrogen fertilizer on the growth of green gram, Bengal gram and groundnut revealed no significant nodules in nitrogen fertilizer added and in the control (Sulochana and Gadgi, 2008). Increase in nodules per plant due to application of inoculation in combination with nitrogen fertilizer was also reported by Rashid *et al.* (1999).

Nodule dry weight was significantly ($p<.0001$) affected by *B. japonicum* strains (Table 4.2). RACA6 and IRJ2180A produced higher nodule dry weight per plant (186.25mg and 171.25mg plant^{-1} respectively). RANI22 also produced statistically higher nodule dry weight (150mg) but not at first position as for nodule number. Given the absence of nodule formation on plants inoculated with the strains MAR1495, USDA136 and USDA9032 as well as those with nitrogen fertilizer and uninoculated-no fertilized control; these treatments were automatically eliminated for nodule dry weight parameter. The least nodule dry weight was recorded with CC511 and USDA138 was statistically at par CC511. This is in agreement with the report of Lindermann and Ham (1978) who observed that nodule dry weight was significantly dependent on *B. japonicum* strain. Tamiru Solomon *et al.*(2012) also reported significant effect of rhizobial strains on nodule dry matter. The high nodule dry weight recorded with the strains RACA6, RANI22 and IRJ2180A and the low nodule dry weight recorded with CC511 and

USDA138 might be the result of high and low nodules number respectively. Rubén *et al*. (2012) reported that nodule number and nodule dry weight are positively correlated. However, the strain 532C and USDA110 felled in between the two extreme groups, indicating their moderate performance. The high nodule dry weight recorded with the strain RACA6, RANI22 and IRJ2180A might be the result of effective biological nitrogen fixation as affected by effective compatibility of these three strains with the variety PK06 used in this study. The establishment and functioning of an effective symbiosis in soybean-bradyrhizobia system is dependent on host-strain compatibilities for nodulation, effectiveness and efficiency of N2 fixation, and competitiveness for nodule occupancy (Vest *et al.*, 1973; Evans *et al*., 1980; Cregan *et al.*, 1989a). Kadiata *et al.* (2012) also reported that rhizobial strains may vary in their symbiotic effectiveness.

4.3. Evaluation of elite bradyrhizobia for their symbiotic effectiveness with soybean under field conditions.

4.3.1 Soybean chlorophyll content

The leaf chlorophyll content was significantly ($p<.001$) affected by *B. japonicum* strains. The highest value was recorded with RANI22 for measurements taken 4WAP and 6WAP and similar to IRJ2180A 8WAP. Relative to the control, these strains significantly increased leaf chlorophyll content by 40-47% for measurements taken 6WAP and 8WAP. The lowest value was recorded in the control which was statistically similar to MAR1495, USDA136 and USAD9032. Nitrogen fertilizer also increased significantly the total leaf chlorophyll content by 36%, 36%, and 40% for measurements taken at 4WAP, 6WAP and 8WAP respectively relative to the control.

Table 4.3: Chlorophyll content during the growth period of soybean inoculated with different bradyrhizobium strains

Treatments	Chlorophyll Content		
	4WAP	**6WAP**	**8WAP**
RANI22	38.9a	46.1a	53.6a
IRJ2180A	37.3 b	44.1b	53.6a
RACA6	35.8 c	41.6c	50.0b
Urea	35.6cd	41.5c	46.9c
USDA110	35.1d	40.6d	44.7d
532C	33.3e	37.1e	42.6e
FA3	32.5f	35.3f	38.7f
USDA138	30.8g	33.8g	37.9f
CC511	25.9h	28.7h	32.1g
MAR1495	24.7i	27.7i	30.6h
USDA9032	24.3i	27.2i	30.2h
USDA136	24.1i	27.1ij	29.9h
Control	22.8j	26.3k	28.3i
LSD	0.6	0.7	0.8
P	<.0001	<.0001	<.0001

The significant increase in leaf chlorophyll recorded with RANI22 and IRJ2180A might be a result of available nitrogen derived from successful biological nitrogen fixation with soybean variety under study. Qrbanly *et al.* (2006) found a positive correlation between the nitrogen and chlorophyll content of leaves, mainly due to presence of nitrogen in the structure of chlorophyll molecules. The strains, USDA9032, USDA136 and MAR1495 were statistically similar to the control for all measurements taken. This indicated the inability of these strains to form efficient biological nitrogen fixation with the soybean variety PK06 used in the current study. Anjum and Rauf (2006) also reported that beneficial rhizobia bacteria may influence the physiological growth conditions of leguminous plants by increasing chlorophyll contents in leaves. Eutropia and Ndakidemi (2013) showed that rhizobial inoculation significantly increased total leaf chlorophyll content in both glasshouse and field experiment. Many other studies have also shown that

B. japonicum increased chlorophyll content in legume plant that finally ending up in improved plant growth (Malome, 2001; Montanaro, 2005). Nitrogen fertilizer, USAD110, 532C, FA3, USDA138 and CC511 formed different groups in between the most effective treatments (RANI22, RACA, and IRJ2180) and the most ineffective ones(MAR1495, USDA9032, USDA136 and the control uninoculated no fertilized treatment). The fact that nitrogen fertilizer did not appear at first position as for the screenhouse experiment indicated that not all the nitrogen applied was available to the plant. A given quantity might have been lost through various processes in the soil such as leaching, etc. Manochehr *et al.* (2008) found that 100% fertilizer showed significantly more chlorophyll index than control.

4.3.2 Soybean above ground biomass.

Soybean shoot dry matter accumulated per plant was significantly affected (P<.0085) by *B. Japonicum* strains and nitrogen fertilizer added as compared to the control. Maximum dry weight per plant (23.7 g) was observed with nitrogen fertilizer treatment which was statistically not different from RANI22 (20.9 g), and RACA6 (20.3g) and of which were not statistically different from IRJ2180A (18.4g) and USDA110 (17.8g). Lowest dry weight was recorded in the control (5.6g) which was statistically not different from CC511 (8.4 g) MAR1495 (6.8 g), USDA9032 (6.2g) and USDA136 (5.9g).

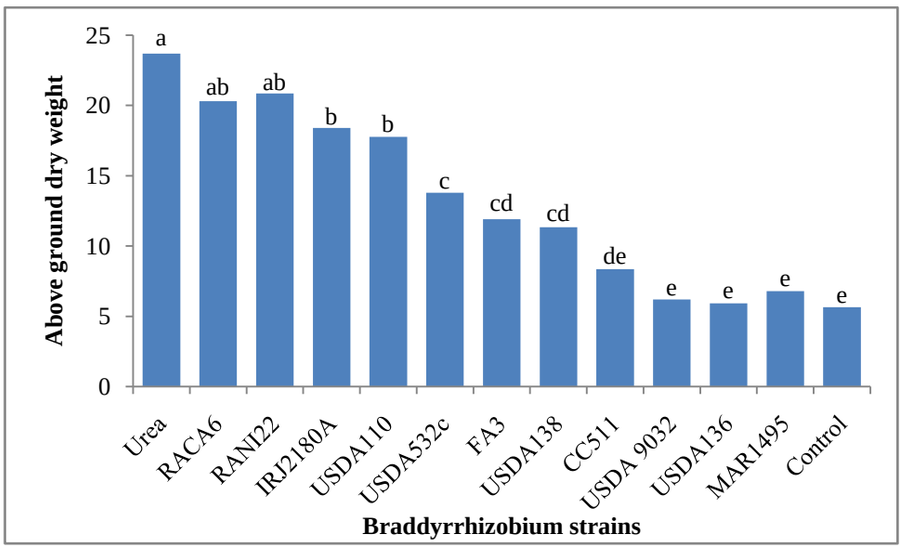

Figure 4.4: Above ground biomass of soybean as influenced by inoculation with different bradyrhizobium strains, $p<0.05$ and LSD=3.5687

The significantly higher above ground dry matter accumulation induced by nitrogen fertilizer might be due to the presence of available nitrogen in the plant rhizosphere leading to high biomass formation. Sulochana and Prashanth (2008) observed significant effect of nitrogen fertilizer on above ground dry weight of green gram, Bengal gram and groundnut as compared to the control. Based on the above ground biomass, the experimental strains would be categorized into three clusters regarding their effects. The higher cluster formed by RANI22, RAC6, IRJ2180A, and USDA110, the middle cluster formed by USDA532c, FA3, and USDA138 and the lower cluster formed by CC511, MAR1495, USDA9032, and USDA136. The lower cluster was statistically not different from the control and the higher cluster not statistically different from nitrogen fertilizer treatment. The middle cluster felled in between the two. The difference between strains in accumulating above ground dry matter might therefore be the result of different strain potential for biological nitrogen fixation with soybean. The higher cluster strains might

be the most efficient in biological nitrogen fixation with soybean variety under study while the lower cluster strains might be less efficient. The efficiency of symbiotic BNF is markedly dependent on the mutual compatibility of both partners, and is influenced by a number of environmental factors (Vincent, 1980; Sprent and Minchin, 1983). Tamiru Solomon *et al*. (2012) reported significant difference between strains in terms of soybean dry matter production. Vasilas and Ham (1984) and Sparrow *et al*. (1995) have also reported similar findings on several legumes.

4.3.3 Soybean nitrogen.

RANI22, IRJ2180A, RACA6, USDA110 and nitrogen fertilizer (Figure 4.5) showed higher quantity of nitrogen content compared to other treatments. Nitrogen content was increased by 69, 69, 69, 66, and 63% by RANI22, IRJ2180A, RANCA6, Urea and USDA110, respectively, relative to the control.

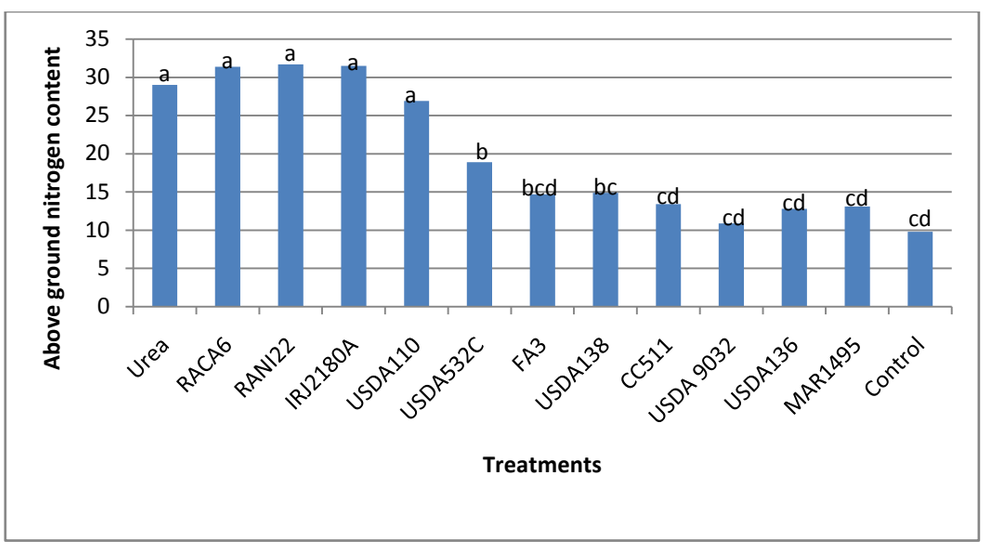

Figure 4.5: Soybean nitrogen content as influenced by inoculation with different bradyrhizobium strains, $p<.0001$ and $LSD= 4.36$

Above ground nitrogen accumulation parameter divided the experimental treatments into two clusters. The higher cluster formed by nitrogen fertilizer, RACA6, RANI22, IRJ2180A,and USDA110, and the lower cluster formed by FA3, USAD138, CC511, USDA90321, USDA136, MAR1495, and the control (no input). The strain USDA532c formed the break between the two. This means that the two experimental controls divided the strains into two groups with USDA532c, the interception. Nitrogen fertilizer group involved four strains (RANI22, IRJ2180A, RACA6, and USDA110) and the uninoculated-no fertilized group included 6 strains (USAD138, FA3, CC511, MAR1495, USDA136, and USDA9032). The high nitrogen accumulation due to high cluster`s treatments might be the result of high nitrogen availability in the plant rhizosphere either due to available nitrogen from nitrogen fertilizer or derived from effective biological nitrogen fixation. Soybean, like other nodulated legumes, utilizes two sources of N for its growth; mineral N in the soil and atmospheric N fixed in nodules (Keyser and Fudi, 1992). The N requirement of soybean can be met by both mineral N assimilation and symbiotic N_2fixation. Although each N input system has independent pathways and control points, the soybean plant under almost all field conditions will use both systems, and these systems are interdependent (Harper, 1987). Basu and Bansyopadhyay (1990) and Rashid *et al.* (1999) reported an increase in nitrogen uptake by plant due to inoculation application. The lower cluster strains ranging on uninoculated- no fertilized side might be the results of ineffective biological nitrogen fixation of these strains with soybean. Moreover, the low accumulation of nitrogen in this cluster might be due to low available nitrogen in the plant rhizosphere mainly due to low soil nitrogen in the experimental field and the inability of strains of this cluster to supply nitrogen from

biological nitrogen fixation. Kadiata *et al.* (2012) reported that rhizobial strains may vary in their symbiotic effectiveness.

4.3.4 Soybean phosphorus content.

There were no significant difference ($p>0.05$) between treatments in terms of shoot phosphorus content (Figure 4.6). This observation might be due to availability of phosphorus in the experimental field since 60Kg TSP were blanket applied in all the plots before planting.

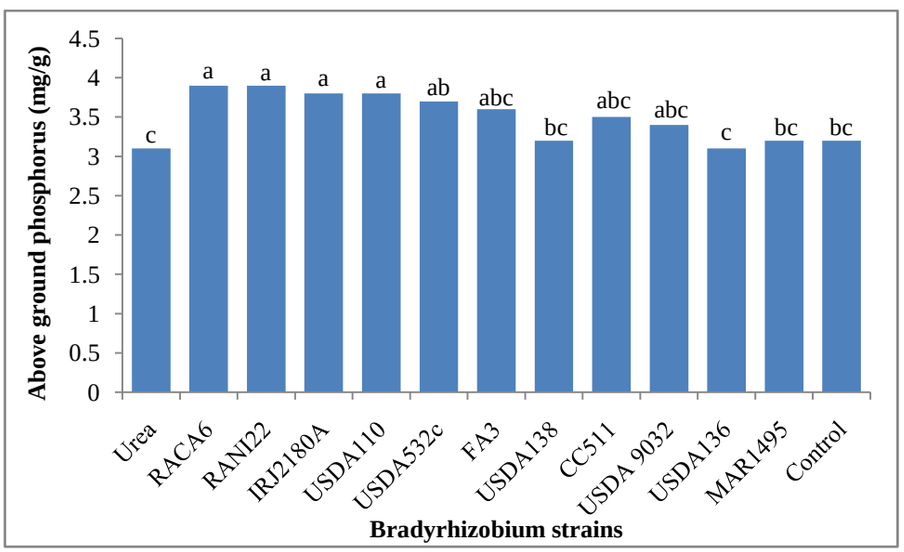

Figure 4.6: Soybean phosphorus content as influenced by inoculation with different bradyrhizobium strains, $p>0.05$ and $LSD= 0.534$

Though all experimental plants accumulated statistically equal amount of phosphorus in their above ground biomass, plants inoculated with RACA6, RANI22, IRJ2180A and USDA110 accumulated relatively higher phosphorus compared to other treatments in the experiment. This might be the result of the greater ability of these strains to induce an increased number of roots hairs and lateral roots leading to high volume of soil exploited

by the plant. Biswas *et al*. (2000) reported that rhizobial inoculants may also induce an increased number of roots hairs and lateral roots thereby favoring nutrient uptake by exploration of a greater soil volume. Eutropia and Ndakidemi (2013) also reported that rhizobia bacteria may stimulate plant growth and ultimately increased the uptake of macronutrients in plant tissues. Inoculation of legumes with *Rhizobium* increase rhizospheric microflora viz. acid producers and phosphate solubilizers causing more available phosphorus (Lipman and Conybeare, 1936). The potential of some Rhizobia bacteria to stimulate nutrient uptake in different legumes tissues also has also reported by several works (Makoi *et al.* 2013; Yahya-Abadi, 2008; Mehrpouyan, 2012).

4.3.5. Soybean nodulation

The number of nodules per plant (Table 4.5) was significantly affected by *B. Japonicum* strains ($p<.0001$). The highest number was recorded with RANI22 (58) and lowest with USDA9032 which was statistically similar to CC511, USDA136, MAR1495 and control. Nitrogen fertilizer did not produce any nodule at all. The absence of root nodules in nitrogen fertilizer treatment might be the result of enough available nitrogen to support nitrogen requirement of the plant. This is also an indication that 100Kg/ha split applied in this particular treatment met the target of nitrogen control plot.

Table 4.1: Effects of elite bradyrhizobia inoculation on number of nodules and nodules' dry weight per soybean plant

Treatments	**Nodulation (plant^{-1})**	
	Number	**Dry weight (mg)**
RANI22	58.1a	389.5a
RACA6	43.4b	259.0ab
IRJ2180A	41.6b	348.0a
USDA110	32.1bc	184.5bc
USDA532c	21.0cd	48.0cd
FA3	13.6de	39.0cd
USDA138	8.8de	26.8cd
USDA136	5.7e	14.5d
CC511	4.9e	9.5d
Control	4.8e	7.5d
MAR1495	4.2e	8.5d
USDA 9032	3.0e	5.3d
Urea	0.0e	0.0d
LSD	12.8	151.7
P	<.0001	0.0313

It is well established that increasing levels of mineral N in the rhizosphere inhibit soybean nodule formation and functioning (Harold and Fudi, 1992).Zahran (1999) reported that high concentrations of available nitrogen (nitrate and ammonium) inhibit nodule formation, number of infection sites in the root, nodule development, N fixation in pre-existing nodules, and nitrogenase activity. The low number of root nodules recorded in the control, might be an indication that indigenous rhizobia were present in the experimental field but not effective to form efficient symbiosis. Given the fact that CC511, USDA9032, USDA136 and MAR1495 produced statistically equal number of nodule per plant at par with uninoculated-no fertilized control, indicated that these strains are either not able to form symbiotic relation with soybean (PK06), as a result of strains incompatibility with soybean variety used in the current study or unable to compete with

the indigenous rhizobia population. Hartmann *et al.* (1998) and Bromfield *et al.* (1995) found that poorly competitive strains may nodulate even if another highly competitive strain is present but in lesser numbers. Thao *et al.* (2002) found a significant interaction between variety and strain on different parameters whereas Munyinda *et al.* (1988) reported a non significant interaction. Kuykendall *et al.* (1996) reported that soybean plants inoculated with strain TA-11NOD+ were significantly better nodulated than those inoculated with strain I-110ARS. Danso *et al.* (1987) also observed that Nitragin inoculants induced production of more nodules than strain D in soybean. Study on the effect of Rhizobium seed inoculation and nitrogen fertilizer on the growth of green gram, Bengal gram and groundnut revealed no significant nodules number in nitrogen fertilizer added and in the control (Sulochana and Prashanth, 2008). Increase in nodules per plant due to application of inoculation in combination with nitrogen fertilizer also has been reported by Rashid *et al.* (1999).

Nodule dry weight was significantly ($p<.0313$) affected by *B. japonicum* strains (Table 4.5). RANI22 and IRJ2180A produced higher nodule dry weight per plant (389.5mg and 348.0mg plant^{-1}, respectively). RACA6 also produced statistically high nodule dry weight (259 mg) but not at the first position. A part from nitrogen fertilizer which did not produce any nodule, the least nodule dry weight was recorded with USDA9032 (5.3mg) which was statistically not different from USDA136 (14.5mg), CC511 (9.5mg), control (7.5mg), MAR1495 (8.5mg). The lowest nodule dry weight recorded with the strain USDA136, CC511, MAR1495, USDA9031 might has been resulted from low nodule formation as indicated by number of nodule in this experiment. Rubén *et al*. (2012)

reported that nodule number is positively correlated with nodule dry weight. The fact that these strains were equally compared to control uninoculated- no fertilized treatment indicated their inability to form effective symbiosis with soybean. Many factors might be contributed to their ineffectiveness, such as strain-host compatibility, environment conditions, and inability to compete with indigenous microbial population. The efficiency of symbiotic BNF is markedly dependent on the mutual compatibility of both partners, and is influenced by a number of environmental factors (Sprent and Minchin, 1983; Vincent, 1980).

The establishment and functioning of an effective symbiosis in soybean-bradyrhizobia system is dependent on host-strain compatibilities for nodulation, effectiveness and efficiency of N_2 fixation, and competitiveness for nodule occupancy (Cregan *et al*. 1989a; Evans *et al*., 1980; Vest *et al.*, 1973). The higher nodule dry weight recorded with RANI22, RACA6, IRJ2180A and USDA110 might be due to the effective nodule formation as a result of high association of these strains with soybean for biological nitrogen fixation. These findings are in agreement with the report of Lindermann and Ham (1978) who observed that nodule dry weight was significantly dependent on *B. japonicum* strain. Tamiru *et al.* (2012) also reported significant effect of rhizobial strains on nodule dry matter.

4.4. Effect of elite bradyrhizobium strain's on indigenous AMF root colonization under field conditions.

The infection rate with indigenous AMF was not significantly affected by inoculation with *B. Japonicum* strains ($p>.005$) or addition of nitrogen fertilizer. Hyphae were

recorded in all the root at 100%, arbuscules 72-80% and vesicles appeared only 1-5% in some roots.

Table 4.2: Effect of elite bradyrhizobium strains on indigenous AMF root colonization under field conditions

Strains	**Indigenous mycorrhizal root colonization**		
	(%)Hyphae	**(%)Arbuscules**	**(%)Vesicles**
Urea	100	80	4
CC511	100	78	4
MAR1495	100	78	3
USDA138	100	78	3
USDA136	100	77	1
IRJ2180A	100	76	1
Control	100	76	1
FA3	100	76	1
RACA6	100	76	1
USDA110	100	75	1
USDA532c	100	73	5
USDA 9032	100	73	0
RANI22	100	72	4
LSD	0	5.7	4.1
P	.	>0.05	>0.05

The presence of hyphae in all treatments indicated that symbiosis between soybean and indigenous AMF was established and not dependent on any of the experimental treatments. Goss *et al.* (2006) observed no significance difference in soybean root colonization by indigenous AMF between plants inoculated or not with *B. Japonicum*, indicating that both inoculated and uninoculated plants have equal capability of hosting indigenous AMF and the presence of *B. Japonicum* did not affect the establishment of fungal symbionts. Katsunori *et al.*(2011), also reported that arbuscular mycorrhizal fungi (AMF) are one of the most important soil microorganisms, forming symbiotic associations with terrestrial plant roots of most species, including soybean. Table (4.6)

also showed that roots were highly colonized with arbuscules (>70%) and rarely with vesicles (0-5%). Thus, out of 30 roots pieces examined for AMF colonization per experimental treatment more than 70% contained arbuscular structures and less than 6% vesicles. This is an indication that soybean was mostly colonized by Gigasporaceae sp. The family Gigasporaceae is characterized by the absence of vesicles (Morton and Benny, 1990). Antunes *et al.* (2006), while evaluating the effect of two AMF life strategies on the tripartite symbiosis with *Bradyrhizobium japonicum* and soybean confirmed the absence of vesicles in plants inoculated with spores of *Gigaspora. margarita*.

4.5. Effect of Walungu peat on *B. Japonicum* inoculant under different storage conditions.

Selected physical chemical properties of peats are shown in Table 4.7. Standard America peat can hold up to 21% more moisture than Walungu peat but amendment of the same can reduce the difference by 4.3%. The table also shows that Walungu peat was 2% more dried than standard American peat. Walungu peat was moderately acidic (4.5) while standard America peat was close to neutral (6.14). Organic carbon was almost equal in both America and walungu peat (88.9% and 87.2% respectively).

Table 4.3: Selected physical chemical properties of peats.

Peat type	*WHC (%)	Moisture content (%)	pH	Organic carbon (%)
APT ground	33.33	4.3	6.14	89
Walungu peat not limed	11.85	2.1	4.50	87
Walungu peat limed	16.67	2.1	6.14	87

*: water holding capacity

Figure 4.8 shows that during the investigation period, initial *B. japonicum* population was gradually increasing and decreasing depending on the storage temperature, sterilization conditions and the peat type. Therefore, two months after storage (investigation period) at 4^{o}C and 15^{o}C, sterile standard APT and Walungu peat limed were equally effective in increasing the survival of initial *B. japonicum* population (5x10^{8}) by 77% and 67% respectively while at laboratory temperature, sterile standard america peat and Walungu peat limed increase initial rhizobial population by 23% and 25% respectively. Without sterilization both standard America peat and walungu peat limed decrease initial rhizobial density by at least 97% in all the storage temperatures.

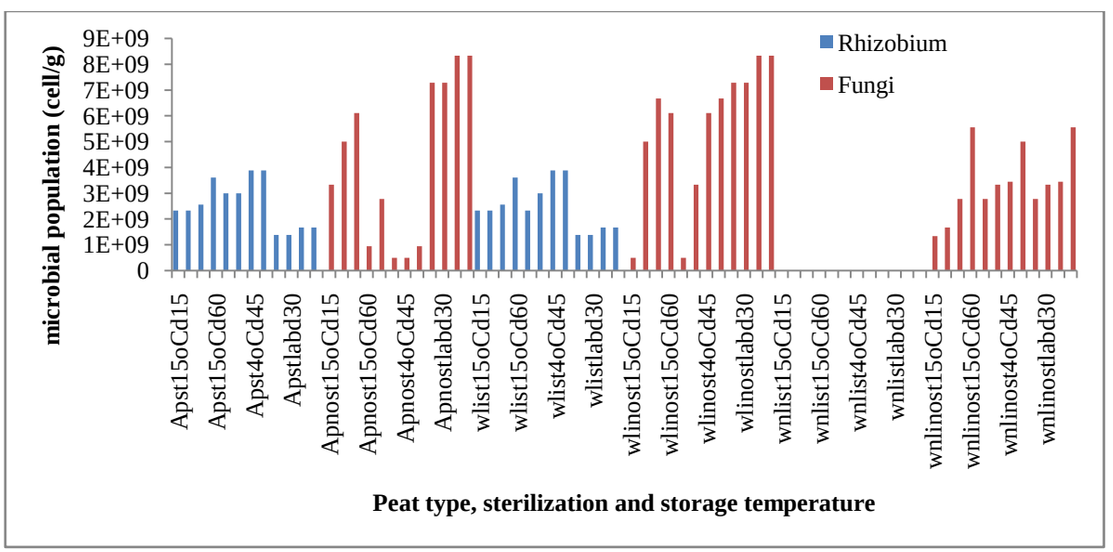

Figure 4.7: The effects of local peat and sterilization on the survival of *B. japonicum*, USDA110 under different storage temperatures.

With storage at 4^{o}C and 15^{o}C no difference were observed between sterile standard America peat and sterile walungu peat limed. Thus, at the end of investigation period (two months), both sterile standard and sterile local peat limed equally increased initial bradyrhizobium by 77% and 67% respectively. This means that if refrigerated facilities

are available standard America peat may be substituted with local peat amended with 41% lime. Temprano *et al.* (2002) reported that the initial population in sterile peat inoculants kept at 4^{o}C increased over the first 4 weeks, then leveled off to about 10^{10} Rhizobia and remained almost unchanged until the end of the assay (32 weeks). However, stored at laboratory temperature, sterile standard America peat increased initial population by 23% while sterile local peat increased it by 25%. Since, the initial population did not decrease in both standard and Walungu peats, though the increase is low compared to refrigerated conditions, good bradyrhizobium inoculants can still be produced under laboratory conditions. This may be an alternative way of providing farmers with good inoculants in a situation where refrigerated facilities are not available. However, in unsterile conditions, after the investigation period, both standard APT and walungu peat limed decrease initial rhizobial density by at least 97% in all storage temperatures. This might be due to the fact that, the inoculants were highly contaminated with fungi (Figure4.8) which may have restricted *Bradyrhizobium* cells growth on the plate during the investigation. Since *Bradyrhizobium japonicum*, USDA110 used in this investigation is a slow-growing rhizobia, when plate count was performed, fungi grew overnight on the plate and by the time *bradyrhizobium* should have grown, fungi were already overspread on the plate preventing *bradyrhizobium* to grow. Thus, even if high population of *bradyrhizobium* may have been in the inoculants, plate count method performance was challenged by fungi growth. Date and Roughley (1977) state that inoculants prepared with non-sterile peat may contain 100-fold fewer rhizobia than those made with sterilized peat, because mortality of rhizobia increased in unsterilized peat and the difference increases during storage. There is also evidence that certain slow-growing

rhizobium strains for example those from cowpea, soybean and Lotononis, may survive poorly in a non-sterile peat that satisfactorily supports survival of fast- growing strains (Roughley and Vincent, 1967, Vincent, 1968). Walungu not limed decreased initial rhizobial density by at least 99.8% in both sterile and non-sterile conditions and at the three different storage temperatures. Poor survival of *B. Japonicum* in walungu peat not limed might be attributed to low pH (4.5) which is detrimental for rhizobial survival. Burton (1965), reported that most *Rhizobium* strains grow well at 6.0-7.0 and peat carrier are usually adjusted to pH 6.5-7.0.

CHAPTER5: CONCLUSION AND RECOMMENDATIONS

5.1. Conclusions

This study carried out in the International Institute of Tropical Agriculture (IITA) was to evaluate symbiotic effectiveness of Bradyrhizobium isolate for soybean inoculant production, their effect on indigenous AMF root colonization and the suitability of Walungu peat/ DR. Congo for inoculant production. Based on the findings, the strains RANI22, RACA6 and IRJ2180A were found to be most efficient in Biological Nitrogen Fixation under different soybean growth conditions. Indigenous AMF were no-responsive to Bradyrhizobium inoculation, and Walungu peat limed met the international standard of 10^9 cells rhizobium g^{-1} under sterile-refrigerated conditions.

In the screenhouse, the strains RANI22, IRJ2180A and RACA6 were effective in improving most of the parameters under study (chlorophyll, above ground dry weight, above ground nitrogen and phosphorus contents, nodule number and dry weight). Similarly, nitrogen fertilizer led to an increase in some of the parameters measured especially chlorophyll and nitrogen content but not significantly different from one or another of the three strains. USDA9032, USDA136 and 1495MAR were similar to the control (uninoculated- no fertilized plot) in all the parameters under study in screenhouse indicating their inability to form efficient biological nitrogen fixation with soybean variety PK06 used in the current study. However, compared to other treatments, the strains RANI22, RACA6 and IRJ2180A showed greater ability to form efficient biological nitrogen fixation with soybean variety PK06 as indicated by the improvement of all the parameters under study.

In the field experiment, RANI22, RACA6 and IRJ2180A were found to be most effective in improving soybean growth parameters under study. These strains also showed great ability to compete with indigenous population for BNF of soybean as indicated by nodules number and dry weight as compared to the controls and other strains under study. The strains USDA9032, USDA136 and 1495MAR were similar to the control (uninoculated- no fertilized plot) in all the parameters indicating their inability to form effective BNF with soybean variety under study.

None of *B. japonicum* strains used in the current study or nitrogen addition affected significantly the infection rate of indigenous AMF. All the plants were colonized by AMF, as indicated by the presence of hyphae in all the investigated roots. However, more than 70% presented arbuscular structures and less than 6% vesicles, indicating high colonization of soybean with Gigasporaceae sp.

Walungu peat limed and America peat were equally effective in increasing the growth and survival of *B. japonicum* (USDA110) over a period of two months. Higher population density was recorded in sterile condition at 4^{o}C and 15^{o}C (storage temperature) with an increase of 97% and 67% respectively compared to the initial population of $5x10^{8}$. Walungu not limed decreased initial rhizobial density by at least 99.8% in both sterile and non-sterile conditions and at the three different storage temperatures. Thus, Walungu peat not amended is not a suitable carrier for *Bradyrhizobium* inoculant production.

5.2. Recommendations

Since the strains RANI22, IRJ2180A and RACA6 mostly performed very well in both screenhouse and field conditions, inoculation of soybean with these three strains may be recommended to farmers for soybean performance and inoculant production in DR. Congo and Nigeria. Given, the no- responsiveness of indigenous AMF to *Bradyrhizobium* inoculation, dual inoculation of soybean with the three most effective *B. japonicum* strains (RANI22, RACA6 and IRJ2180A) and some AMF strains must be imperatively carried out. Walungu peat amended with 4.1% lime is a suitable alternative carrier to APT for good quality inoculant production under sterile- refrigerated (4^{o}C and 15^{o}C) conditions.

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Appendix 1: Screenhouse experimental layout

R1	R2	R3	R4
CC511	USDA138	USDA9032	IRJ2180A
USDA110	Urea	USDA136	FA3
RACA6	RANI22	USDA138	532C
532C	FA3	USDA110	1945MAR
Urea	1945MAR	RACA6	USDA9032
RANI22	USDA136	Control	USDA138
USDA9032	UDSA 110	IRJ2180A	CC511
FA3	532C	1945MAR	RANI22
USDA136	IRJ2180A	Urea	Control
USDA138	RACA6	532C	RACA6
1945MAR	Control	CC511	USDA136
IRJ2180A	USDA9032	RANI22	USDA110
Control	CC511	FA3	Urea

Appendix 2: Field experimental layout

Appendix 3: Peat experimental layout

	Apt		Wlg no limed		Wlg limed	
	sterile	no sterile	sterile	no sterile	sterile	no sterile
R1	4	4	4	4	4	4
	15	15	15	15	15	15
	20	20	20	20	20	20
	Apt		Wlg no limed		Wlg limed	
	sterile	no sterile	sterile	no sterile	sterile	no sterile
R2	4	4	4	4	4	4
	15	15	15	15	15	15
	20	20	20	20	20	20
	Apt		Wlg no limed		Wlg limed	
	sterile	no sterile	sterile	no sterile	sterile	no sterile
R3	4	4	4	4	4	4
	15	15	15	15	15	15
	20	20	20	20	20	20