MAKERERE UNIVERSITY

EFFECTIVENESS OF NATIVE RHIZOBIA ISOLATES ON PRODUCTIVITY OF CLIMBING BEANS ON LIMED ACIDIC SOIL OF RWANDA

NGABONZIZA Jean Damascene
BSc. Agric Eng., (ISAE)

A DISSERTATION SUBMITTED TO THE SCHOOL OF AGRICULTURAL SCIENCES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER OF SCIENCE IN SOIL SCIENCE OF MAKERERE UNIVERSITY

APRIL 2014

DECLARATION

This dissertation ***effectiveness of native rhizobia isolates on productivity of climbing beans on limed acidic soil of Rwanda*** is my original work and has never been submitted for a degree in any other university

…… ……

NGABONZIZA JEAN DAMASCENE Date

This thesis has been submitted for examination with our approval as university supervisors

....

Dr. John Baptist TUMUHAIRWE Date

(B.Sc. Agric.; M.Sc. Soil Sc.; PhD)

....

Dr. RUGANZU Vicky Date

(B.Sc. Agric.; M.Sc.; PhD)

DEDICATION

To

Almighty God,

My mother, brothers and sisters

ACKNOWLEDGEMENTS

First and foremost, I thank Almighty God for letting me live to see this thesis through. I am forever indebted to Dr. John Baptist Tumuhairwe and Dr. Vicky Ruganzu for supervising this research work, the useful suggestions, comments and critical feedback. I am grateful to the technical staff in the BNF and Soil laboratory at Makerere University for the contribution to the success of this work

I gratefully acknowledge the Rwanda Agriculture Board (RAB), for granting me permission to pursue the Master course; AGRA Soil Health Program funding.

Last but not least, I am indebted so much to my whole family for their endless support, their solid belief that I can achieve so much. I cannot thank everyone by name because it would take a lifetime but I just want you all to know that you count so much. Had it not been for all your prayers and benedictions; were it not for your sincere love and help, I would never have completed this thesis. Hence thank you all.

God bless you all!

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LIST OF ACRONYMS

ACIAR: Australian Centre for International Agricultural Research

BNF: Biological Nitrogen Fixation

CFU: Colony Forming Unit

EAC: East African Community

FAO: Food and Agriculture Organization

FAOSTAT: Food and Agriculture Organization Statistics

GDP: Gross Domestic Product

GIZ: Gesellschaft für Internationale Zusammenarbeit

ISAR: Institut des sciences Agronomiques du Rwanda

MINAGRI: Ministry of Agriculture and Animal resources

MINECOFIN: Ministry of Finance and Economic Planning

SNFE: Strain Nitrogen fixation efficiency

PABRA: Pan-Africa Bean Research Alliance

RAB: Rwanda Agriculture Board

USAID: United States Agency for International Development

USD: United States Dollar

WFP: World Food Program

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ABSTRACT

Farmers in sub Saharan Africa are financially constrained to afford mineral fertilisers despite the Abuja declaration of 2006 to apply at least 50 kg ha^{-1} of plant nutrients in soil. Whereas P and K can mainly be sourced from the expensive mineral fertilizer, adequate N may be cheaply derived from the atmosphere through biological nitrogen fixation. Yet, climbing bean production in Rwanda is constrained by soil nitrogen and acidity. Thus, the goal of this study was to increase the production of climbing beans using low cost inputs soil management practices. The objectives of the study were to (i) determine the richness of native rhizobia in the acidic soils under different management practices for enhanced BNF; (ii) determine effectiveness of the native rhizobia strains in acidic soils under different management practices on the climbing beans in Rwanda; (iii) determine the effect of liming on biological nitrogen fixation in inoculated climbing beans. A preliminary study was done to isolate native rhizobia strains following standard methodology. The isolated effective native strains $SMP10_4$ and $SMP6_3$ were compared against a Mak bio fixer (from Makerere University) and ISAR strain (Rwanda Agriculture Board). Lime $CaCO_3$ was applied at 0, 2.5 and 4 t ha^{-1} arranged in a completely randomized design in the greenhouse. Dry matter and plant N uptake increased significantly following inoculation and lime application. The isolated native strains $SMP10_4$ and $SMP6_3$ were superior on both climbing bean varieties in increasing total dry matter, nodulation and effectiveness compared to the Mak bio fixer and ISAR strains. Liming at 2.5t ha^{-1} was not significantly different from 4 t h^{-1} on BNF indicator parameters. Therefore, those native isolates and liming at 2.5t ha^{-1} of lime can be recommended for use in low-nitrogen environment as well as procreation materials.

CHAPTER ONE

1.0. Introduction

Agriculture is an important economic activity in Rwanda contributing about 31% of GDP and employing up to 93% of the national population. Several annual and perennial crops are grown including beans, millet, banana, sorghum, soy bean, cassava, potatoes, sweet potatoes, groundnuts, rice and coffee. Common bean (*Phaseolus vulgaris* L.) is an important food and cash crop grown under sole and various intercropping systems (FAO, 2005). Bean continues to fetch high commodity price in Rwanda and increased from 0.24 USD kg^{-1} to 0.75 USD kg^{-1} between 2002 and 2010 (USAID, 2012). It is a major source of protein for low-income families in Sub Saharan Africa, where it contributes about 38% of utilizable protein and 12 to 16% of daily calorific requirements. In eastern African community (EAC), Rwanda is one of the leading producers of bean next to Uganda. Production increased from 0.8MT/ha in 2001 to 0.9 MT/ha in 2010 though this was about a half of Uganda's production (FAOSTAT, 2012).

Rwanda is a hilly, wet and a cool to warm country with elevation ranging between 950 – 2500 m above sea level (asl). There are 12 agro-ecological zones grouped into low, mid and high altitude zones, cool to warm temperatures between 18 and 24^{o}C and annual rainfall of 800 to 2000 mm (MINAGRI, 2009). Rwanda has about 10 different soil types with varied soil properties and production potentials. Beans production in Rwanda cut across the 12-agro ecological zones. The mid-altitude grows both bush and climbing beans while the low and high altitude agro-ecological zones mainly grow climbing beans.

In Rwanda, common bean is among the most consumed food and is grown by about 95% of the farmers countrywide and provides 65% of the protein and 32% of the calorific intake (WFP, 2010). The annual mean production and consumption per capita is 29 kg, minimum and maximum consumption per capita is 22 kg and 35 kg, respectively, (MINECOFIN, 2007). Beans are produced on about 300,000 to 360, 000 ha annually, which translate to about 22 to 25% of total area under crop. Area under climbing beans production is about 100,000 ha whereas the bush and semi-climbing beans cover the rest.

1.2 Problem statement

There has been a decline in bean yield to the extent that farmers do not have sufficient for home consumption alone. The average yield at farm level is about 900 kg ha^{-1} compared potential yield of 5 tha^{-1} for climbing bean. The major factors affecting bean yield are declining soil fertility especially high soil acidity and nitrogen (N) deficiency (Kimani *et al*., 2003). Soil acidity and Al toxicity are prevalent in the south province of Rwanda largely covered by Ferralsols (FAO, 2010). This soil types cover about 65% of the total arable land in Rwanda. Land holding per farmer in Rwanda is very small, an average of about 0.46 ha per household. Therefore, each piece of land is continuously cultivated, sometime even without crop rotation. The effect is continuous nutrient depletion and increases soil acidity. Inorganic fertilizer use is very low (Kelly *et al.*, 2001), at an average of 0.4 kg ha^{-1} due to high cost. Biological nitrogen fixation would be the affordable option but it is currently limited by unfavorable edaphic conditions and a highly variable population of effective indigenous rhizobia population in the soil. Diverse indigenous rhizobia species exist across the different world terrestrial ecologies and present a great potential for

development of native effective inoculants. Rwanda Government has been importing rhizobia inoculants from Uganda and recently formulated her own inoculant but the response on bean has been erratic. Consequently, ISAR produced local rhizobia inoculants but has not produced consistent results across the different Agro-ecological zones in Rwanda (ISAR, 2009) especially those with acidic soils. Furthermore, animal and farmyard manure use is common but insufficient due to limited materials and management could affect the diversity and abundance of the native rhizobia species. Sole cropping of climbing beans is often practiced by about 22% of the farmers. Intercrops with common bean exist at about 15% in banana plantation 43% with bean and about 13% in tubers (Lunze *et al*., 2012).

1.3 Justification of the study

Agriculture in Rwanda is characteristically subsistence and rain-fed agriculture. Under this type of agriculture, it is necessary to enhance BNF as a cheaper than other sources of nutrient N. However, the abundance and diversity of the rhizobia population in soil is influenced by soil amendments such as liming, mineral and organic fertilizers (Anthony *et al*., 2001). Rwanda has acidic soils (Uwizerwa, 2011), which should be mitigated to limit its constraint to BNF. Furthermore, BNF is greatly constrained by acidic soil conditions through P-fixation and other micronutrients that energize and boost the fixation processes respectively, to convert atmospheric nitrogen into utilizable plant form.

The amount of nitrogen fixed by a legume depends on the bacterial strain, host plant, the environment and agricultural management practices (FAO, 2009). Among the common agricultural management practices are liming and fertilization with P and N that importantly affect nitrogen fixation. Phosphorus generates energy for BNF

(Zahran, 1999) and is an essential nutrient required by diazontrophs. BNF is an anaerobic microbiotic process and the enzyme nitrogenase is destroyed by presence of Oxygen. Therefore, a typical health and effective nodule on legumes contains a pink color due to the presence of leghemoglobin, which contains iron (Fe) and molybdenum (Mo) and responsible for Oxygen trapping regulation (Zahran, 1999). Liming acid soils to the pH range of 6.5 - 7 indicates P-requirement for good relationship between phosphorus and nitrogen fixation process (Lindemann *et al.,* 2003). Soil pH plays a role in the growth of rhizobial population and plant roots. Restricted root development reduces the ability of that plant to fix nitrogen, decreasing the time needed for developing nodules to become active and beneficial to the legume plant (FAO, 2009). Therefore, this study aims at improving bean yield through enhancing BNF in acidic soil of Rwanda by use of efficient rhizobia strain inoculum and lime resources.

1.4 Objectives

The general objective of this study is to increase yield of climbing beans in the smallholder production systems using low cost soil inputs under soil management practices.

1.5 Specific objectives:

i). Determine the abundance of native rhizobia in the acidic soils under different management practices for enhanced BNF

ii). Determine effectiveness of the native rhizobia strains in acidic soils under different management practices on the climbing beans in Rwanda

iii). Compare the effectiveness of native rhizobia strains and available inocula of climbing bean.

iv). Determine the effect of liming on biological nitrogen fixation in inoculated climbing beans.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview on bean production in Rwanda

Beans rank as the most grown crop in Rwanda to fight hunger and malnutrition. There over 100 varieties released since 1984 (RAB, 2012) covering bush, snap and climbing bean varieties that were bred for high yielding and farmers acceptability amongst other traits. Climbing beans is the second most grown after bush variety in Rwanda and some of the most preferred types are Gasilida (recently released) and Gwinurare (old variety though considered native). The climbing beans are grown for high yield, because they occupy more vertical space than ground space, are well aerated, which reduces incidents of diseases (ISAR, 2009). Climbing beans are adapted to zones ranging from the low to highland but grow minimally in hot and humid climatic zone. Reportedly, climbing beans potentially triple the yield of bush beans in sub-Saharan Africa; exceeding 5 tha^{-1} (Ferris et *al.*, 2002). They require moderate rainfall followed by a dry seasoning interval and is sensitive to extreme soil acidity (pH <4).

Another benefit of climbing beans is enhanced soil fertility improvement. For example, Kenya Agricultural Research Institute (KARI) reported that climbing beans produced 17- 25 t ha^{-1} of leaves, which increases soil organic matter from the high quality biomass that is rich in nitrogen (KARI, 2008). Most varieties of climbing beans are promiscuous legumes capable of symbiotic relationship with many rhizobia strains. Amerger (2001) reported that climbing beans have a high potential for biological nitrogen fixation of up to 125 kg N ha^{-1} than bush beans (35 kg N ha^{-1}) and they can associate with different *Rhizobium* strains in different agro ecological zones. Climbing beans are crawling plants and should be provided with stakes, which is one

of the challenges faced by farmers.

Beans are extremely diverse crops in terms of cultivation methods, uses, the range of environments to which they have been adapted, and morphological diversity (Broughton *et al*., 2003). They thrive best in altitudes between sea level and 3000 m asl. In Rwanda, beans are best suited between altitudes 900 to 2100 m asl (MINAGRI, 2009). At the low altitudes (< 800 m asl), the crop is often stress by high temperatures, which cause poor podding.

It is important to note that social economic factors also determine the decision on beans cultivation. Agriculture and social systems have evolved together; therefore, current state of farming systems is a result of interactions of climate, edaphic, biotic ad social factors. The importance of beans as a crop and as a food is closely associated with human population density (FAO, 2010). Beans production intensity is greatest in area of high population density, where farms are small and there are limited other significant sources of dietary protein (CIAT, 2008). Currently in Rwanda, adoption of improved beans varieties and production technologies are expected to increase in order to meet growing local bean demand despite challenges presented by declining soil fertility, diseases and pests.

2.2. Agro ecological zones suitable for beans production in Rwanda

An agro-ecological zone is a geographic unit, which is homogeneous in terms of climate (rainfall and temperature), topography and soil characteristics (MINAGRI, 2009). Rwanda has been subdivided into 12 agro-ecological zones (Table 1).

Table1: Agro-ecological zones and their characteristics

Zone	Altitude range(m)	Precipitation (mm)	Soils	Agricultural value
Imbo	970-1400	1200	Alluvial	Excellent
Impala	1400-1900	1400	Heavy red basalts	Good
Borders of Kivu lake	1460-1900	1200	Superficiaal loamy-clay	Excellent
Birunga	1600-2500	1500	Volcanic soils	Excellent
Congo-Nile Watershed Divide	1900-2500	1600	Acid Humic soils	Average
Buberuka Highlands	1900-2300	1200	High-altitude lateritics	Good
Central Plateau	1500-1900	1200	Diverse humic soils	Good
Granitic dorsal	1400-1700	1100	Light gravelly soils	Average
Mayaga	1350-1500	1050	Clay soils, schists	Very good
Bugesera	1300-1500	900	Strongly altered clays	Poor
Eastern Plateau	1400-1800	950	Laterites	Average/North; Good/South
Eastern Savana	1250-1600	850	Old, variable/texture	Very poor

Source: MINAGRI (2009)

The major agro ecological zones suitable for bean production are Imbo, Impala, Kivu lake Borders, Buberuka Highlands, Central plateau, eastern plateau (MINAGRI, 2009) due to the favorable climate. The strategic interventions in the high potential areas should focus on intensification based on enhanced use of fertilizer including biofertiliser and liming, high quality seed and improved variety especially for climbing beans. The marginal potential zone will require enhanced use of inputs especially irrigation, fertilizers and water conservation technologies (GIZ, 2011). Moisture deficits severely constrain bean production in semi arid regions of Rwanda, frequently resulting in complete loss when the rain is not well distributed (NISR,

2011). For normal growth, a bean crop requires about 450 mm of rainfall per season (Wortman *et al.,* 1998).

2.3 Factors affecting bean production in Rwanda

Farmers face with several constraints that decrease the yield and commercialization of beans, contributing to food deficit, absence of low-cost protein, and low incomes for both rural and urban populations in Africa (FAOSTAT, 2012). Bean production in Rwanda is constrained by low soil fertility and high acidity, land scarcity, drought, and in some cases, lack of improved seed varieties, inevitably farmers recycle poor and low yielding seed, diseases and pests (Kimani *et al*., 2005). Research and extension has inadequately addressed these challenges. The change is research paradigm has brought breeders in partnership with other scientists to deliver improved seeds and new agricultural innovations (MINAGRI, 2009).

Land size holding per household is progressively reducing constraining bean production due to competing crops to be grown. Small land size has caused crop intensification with a successive soil fertility decline in previously high bean potential producing regions in Rwanda (NISR 2011). Land holding area per family is small (>1 ha) in high production potential areas though it relatively more land moderate and low potential areas, partially due to new land policy resolution in Rwanda and the natural ability to sustain livelihoods (ISAR, 2009). In the high potential areas, the Government of Rwanda has introduced a policy of land consolidation and crop intensification, which will increase bean production among other crops of national interest (MINECOFIN, 2010).

2.4. Soil acidity in Rwanda

Most of the soil in Rwanda are either acidic or border to acidity. The highest acidic soils are found in the Highland, with a pH as low as 3.90 (Nizeyimana *et al*., 1988) and this covers about 2/3rd of the total arable land (Figure 1).

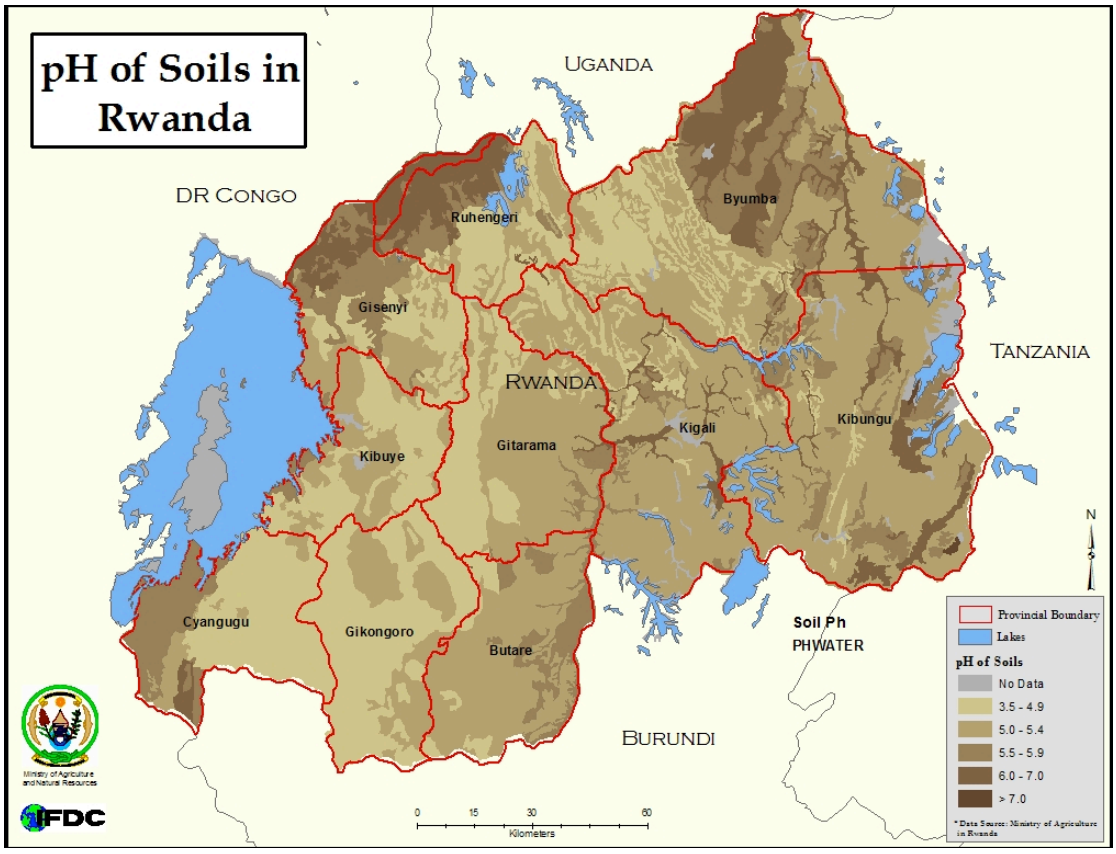

Fig. 1: Soil pH of Rwanda

Like many legumes, common beans prefer well aerated, sufficiently drained soil with a pH of 6.0 to 7.5, the critical pH thresholds being 5.0 and 8.1 (Lunze *et al*., 2007). The minimum organic carbon in the soil should be about 2.4%. Soil pH relates to both soil's capacity to supply nutrients and to its aluminium and manganese toxicity problems (Kimani *et al*., 2010). Common beans are sensitive to high concentration of aluminium, boron and sodium (Horneck *et al*., 2007) but some cultivars show considerable variation in their sensitivity to sodium concentration. Snap or French beans are the most sensitive to high concentrations of aluminum, sodium and boron.

New bean varieties that were bred to grow at low soil Phosphorus and Nitrogen, and tolerate high aluminium and manganese toxicity were recently released in eastern Africa (Lunze *et al.*, 2007; Kimani *et al.*, 2010).

2.5 Fertilizer use for bean production in Rwanda.

Rwanda is the most populous country in East Africa with a population density of 416 per km^2 (NISR, 2012). Consequence of high population especially in the high bean potential producing areas, farmers on average own 0.46 ha. This has led to continuous cultivation and depletion of plant nutrients, particularly nitrogen and phosphorus (Kelly *et al*., 2000; FAO, 2010). Soils in bean producing areas are deficient in major nutrients and micronutrients requiring fertilizer application. Past surveys in high potential areas in Rwanda revealed soil nutrient deficiencies particularly deficient in Nitrogen and Phosphorus (Kelly *et al.*, 2000; FAO, 2010).

However, inconsistent results have been reported from fertilizer trials (ISAR, 2009) but more pronounced responses have been noted for phosphorus than nitrogen on cereal crops. In general, there is low fertilizer use by smallholder farmers (Kelly *et al.*, 2000). However, productivity of common bean in this region of Rwanda is unlikely to improve significantly without application of fertilizers and appropriate crop management practices. Soils with high fertility, applying 20 – 30, 30 – 35 and 20 - 30 kg N, P and K kg ha^{-1}, respectively is recommended (Kelly *et al*., 2001). It is important to note the rate of recommended N can be more than achieved through biological nitrogen fixation under climbing beans. The amount of N fixed may reach 60 to 120 kg ha^{-1} in favorable soil conditions (Kelly *et al.*, 2000). The rates of inorganic fertilizers are progressively increased with decreasing soil fertility. The commonly used mineral fertilizers in Rwanda include diammonium phosphates

(DAP), triple phosphate (TSP), and 8:16:8 N: P: K compound fertilizers (Kelly *et al.*, 2001). DAP fertilizer is widely applied in commercial maize production and may be used for beans at the rate of 200 kg ha^{-1}. However, when used for a long time, DAP increases soil acidity. Alternating with less acidifying compound fertilizer (NPK) or straight fertilizers (TSP) can reduce acidity problems (Kimani *et al*., 2010).

2.6 Production and Consumption trends of beans in Rwanda

Comparatively, bean production dominated by climbing bean shares the largest part of arable land, up to 25% in Rwanda. Production of beans rose from 200,000 Mt in 2001 to 330,000 Mt in 2010 and an overall bean consumption increase of 130.000 Mt per year over same period. Consumption increased to 29 kg/capita partly due to population growth rate, rising from 8.2 million in 2000 to 10.8 million in 2010 (NISR, 2011).

Whereas bean consumption steadily increased, area under bean production fluctuated with a peak at 385,000 ha in 2002 but later dropped to about 314,000 ha in 2010 (ISAR, 2011). This was attributed to severe drought in 2007, 2008 and 2009 that affected most of the bean producing regions. Earlier survey indicated that beans are planted on 300,000 to 360,000 ha annually (ISAR, 2009). This is about 22-25% of total area under crops and is the largest compared to the other crops. Out of the area occupied by beans, the climbing beans cover about 100,000 ha.

Gasilida is the variety commonly grown by farmers in high land including south province potentially yielding 4.0 - 4.5 t ha^{-1}. The variety matures in 90 - 96 days, and it tolerates low soil fertility (RAB, 2012). Another common variety is Ngwinurare, which originated from CIAT but improved by ISAR, tolerant to which is also low soil fertility, with grain yield of 2 - 3 kg $acre^{-1}$ in 88 - 94 days (CIAT, 2008).

2.7 Biological Nitrogen Fixation (BNF) and its importance in Agriculture

Biological nitrogen fixation (BNF) is the process by which atmospheric nitrogen gas (N_2) is converted into forms usable by the plant. The atmospheric gas contains about 78% diatomic nitrogen yet most soils are deficient of nitrogen limiting plant growth and crop yield. This gas can be harnessed by diazotrophic microorganisms for plant use through BNF. BNF is a biochemical process catalyzed by nitrogenase complex enzymes found in some groups of bacteria (Lindemann *et al.*, 2003). Most of these bacteria are symbiotic such as. *Rhizobium* spp, *Bradyrhizobium* spp, *Sinorhizobium* spp and *mesorhizobium* spp. There are also free living diazotrophs such *Azotobacter* spp, *Azospirillum* spp and *Cyanobacteria* spp. However, symbiotic diazotrophs are the more efficient and fix higher quantities of nitrogen. Thus they are the most applicable in most agricultural system where legumes than free living group (Peoples *et al*., 1995; Vitousek *et al*., 2002). The free living diazotrophs are more relevant in natural grassland ecosystems.

In legume production systems, BNF is very important because it provides low cost N-source for plants and can supplement fertilizer nutrients sources. Apparently, beans farmers who maximize the amount of nitrogen obtained from the atmosphere via BNF ably to reduce fertilizer costs while maintaining soil fertility and high yields (Peoples *et al*., 1995; Vitousek *et al*., 2002; Lindemann *et al.,* 2003).

2.7.1 Mechanism of biological nitrogen fixation

Biological nitrogen fixation can be represented by the following equation, in which two molecules of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen ions):

$N_2 + 8H^+ + 8e- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi$ (Nail, 2008)

This reaction is performed exclusively by prokaryotes (the bacteria and archaea), using an enzyme complex nitrogenase. This enzyme consists of two proteins - an iron protein and a molybdenum-iron protein. The reactions occur while N_2 is bound to the nitrogenase enzyme complex. The Fe protein is first reduced by electrons donated by ferredoxin. Then the reduced Fe protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to $H_2N\text{-}NH_2$, and this in turn is reduced to $2NH_3$ (Lindemann *et al*., 2003).

Depending on the type of microorganism, the reduced ferredoxin, which supplies electrons for this process, is generated by photosynthesis, respiration or fermentation. The Biological Nitrogen Fixation agents are classified according to their area activity in agricultural or natural system.

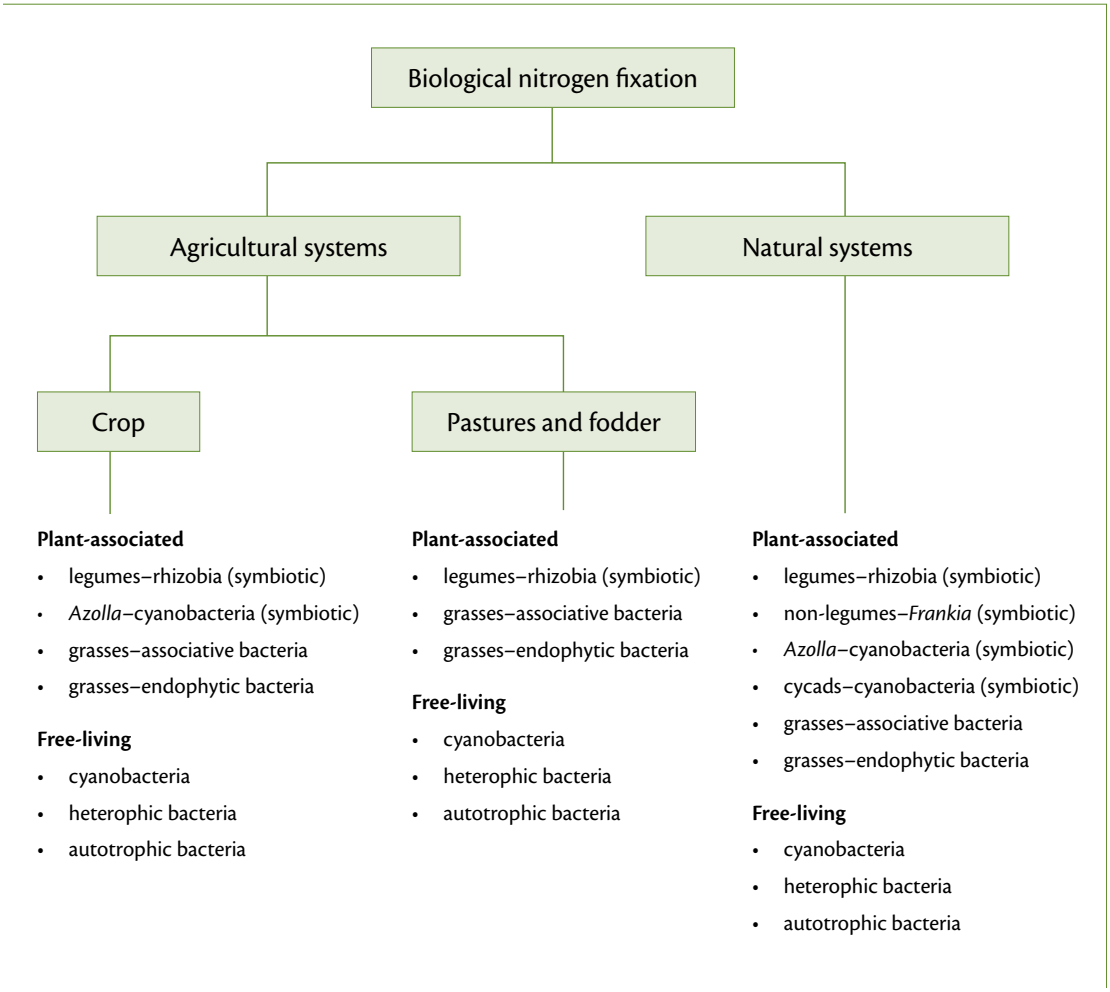

Fig.2: The biological N_2-fixing agents in agricultural and natural systems (Source: ACIAR 2008)

2.7.2 Edaphic factors affecting Biological Nitrogen Fixation

Different strains and species of nitrogen fixers live in the soil and their ability to fix nitrogen is influenced by a number of factors including soil acidity, salinity, soil temperature, moisture, fertility (nutrient deficiencies), soil structure, concentrations of calcium and boron (Kellman, 2008). Presence of other soil microorganism like bacteria, fungi and protozoa reduces nitrogen fixation by causing competition with the nitrogen-fixers.

Earlier, FAO (1999), reported edaphic, climatic and management practices that interactions between symbiotic microorganisms and the plant. Edaphic factors related

to soil excessive soil moisture, drought, soil acidity, phosphorus deficiency, excess mineral nitrogen and deficiency of Calcium, Molybdenum, Cobalt, and Boron (Lupwayi *et al*., 1998; Musoni, 2008). Waterlogging within the soil inhibits the development of root hairs and places nodulation sites because they interfere with normal diffusion of Oxygen in the root system. Nitrogen fixation by legumes is greatly reduced by excessive soil moisture >25% (FAO, 1999). Similarly, drought decreases the number of rhizobia in the soils and reduces nodulation and nitrogen fixation capacity (MINAGRI, 2007). Moisture stress affects nodulation through reduced rhizobial motility in the soil, curtails rhizobia multiplication in the rhizosphere, and reduces formation of infection threads and retards nodule development. Fluctuation in soil water can also greatly affect the rate of N_2 fixation because of inadequate aeration in saturated soil and reduced water availability in dry soils at critical times during development and growth. Optimum soil moisture for symbiotic N_2 fixation occurs at near field capacity (Kellman, 2008).

Soil pH is also an important factor to consider during BNF and the optimum is between 6.0 and 7.0, which provides favourable conditions for plant nutrient uptake. Where the pH is acidic such as in most of Rwanda, it is advisable to lime (MINAGRI, 2009). Generally, if soil pH is less than 6.0, and where it is economically feasible, liming may increase soil pH and hence enhance biological nitrogen fixation (Lunze *et al*, 2012). Acidic soil conditions also lead to P-fixation. When phosphorus requirements for the plant are not satisfied, nodule formation and functioning are adversely affected (Lynd *et al*., 1984). Reportedly, symbiotic plants need higher rates of phosphorus fertilization than mineral nitrogen fed plants (Cassman, 1981).

2.7.3 Effect of fertilizer and soil nitrogen on BNF

The nitrogen content of the soil influences nodulation and biological nitrogen fixation. The amount of nitrogen a fixing plant can access determines whether it is stimulating or detrimental to N_2 fixation (Hansen, 1994). Availability of adequate inorganic nitrogen in the rhizosphere (>30kg N ha^{-1}) generally inhibit N fixation (Vance *et al*., 1988), by limiting the development of the *Rhizobium*-legume symbiosis (Dazzo and Truchet, 1884; Kijne, 1992) and reducing N fixation in already formed nodules (Beringer *et al*., 1988). However if plant available nitrogen is restricted, legumes tend to undergo a 'N hunger period' (Pate and Layzell, 1990), which can retard development and may reduce yield. Small amounts of starter N tend to increase BNF (Becker *et al*., 1991) but further increases in soil N decrease BNF, which may approach zero at high levels of soil N (George and Singleton, 1992; Sanginga *et al*., 1996).

2.7.4 Overview on Rhizobia

Rhizobia belong to the domain (a pseudo kingdom) of bacteria, phylum of proteobacteria and class α-proteobacteria. They are gram-negative bacteria with low Guanine-cytosine content, therefore unable to withstand high temperatures. Rhizobia belong to the order rhizobiales, family of rhizobiaceae and genus *Rhizobium* (Imhoff, 2006). *Rhizobium* spp are fast growing aerobic bacteria and therefore easy to process their inoculum. Rhizobia are symbiotic nitrogen fixers, when living homologous with leguminous plants as hosts (Leigh, 2002). Under nitrogen-limiting conditions, legumes form root nodules, in which the rhizobia are hosted by intracellular process. Within the nodules, rhizobia find the suitable conditions for reducing atmospheric nitrogen into ammonia (Kellman, 2008). Nodule formation requires the reprogramming of differentiated root cells to form a primordium, from which a

nodule can develop. When rhizobia have colonized the root surface of their host, they induce morphological changes in the epidermis (Biseeling *et al*., 2002). These morphological changes are preceded by the induction of certain genes in a broad region of the epidermis.

2.8 Status of soil fertility in Rwanda

Most tropical soils are known to have low soil fertility including those in Rwanda. The country is dominated by Ferralsol covering about 65% (MINAGRI, 2009; ISAR, 2009). Characteristically, Ferralsols have low pH, nitrogen and available phosphorus concurrent high Al toxicity (FAO, 1997). Soil acidity and mineral nutrient deficiencies are a major constraint limiting legume nitrogen fixation and yield in climbing beans in Rwanda (ISAR, 2009). The soils are deficient of calcium, which could limit *Rhizobium* cell multiplication and growth. Low calcium in the legume rhizosphere limited rhizobia multiplication (Paudyal *et al*., 2007). Incidentally low Ca is also associated with low soil pH, further suppressing low rhizobia population, availability of phosphorus and plant growth. In this case, application of lime in acidic conditions enhances the growth of rhizobia hence a high nitrogen fixation index.

Application of large quantities of fertilizer N inhibits N_2 fixation, but low doses (<30 kg N ha^{-1}) of fertilizer N can stimulate early growth of legumes and increase their overall N_2 fixation. The amount of the starter N dose must be defined in relation to available soil N, such that it is reduced under high soil N content. Reduction of BNF due to soil fertility will often be related to either an excess of soil nitrates or a deficiency of some essential nutrient limiting plant growth and development. In general, excess soil nitrate levels will depress BNF (Kellman, 2008).

2.9 Soil fertility management in Rwanda

The major soil fertility management practices in Rwanda include application of organic and inorganic fertilizers, liming and sometimes fallowing. Application of organic fetilisers cuts across all the agro-ecological zones and is largely constrained by availability of the organic materials (GIZ, 2011) Application of inorganic fertilizer is limited to largely commercial crops and rice field. Inorganic fertilizers commonly applied in Rwanda include Urea, NPK, DAP, TSP (Kelly, 2000). Mineral fertilizers are expensive and therefore least used by the subsistence farmers, who are the majority in Rwanda. Liming is practiced in the agro-ecological zones of Kivu lake boarders, Congo-Nile watershed divide, Buberuka Highland, Granitic Ridges where the pH is acidic (MINAGRI, 2009). Rhizobia inoculum (ISAR) is also increasingly being adopted by farmers for both soybean and bean.

2.9.1 Agronomic practices for beans in Rwanda

Chrispeels and Savada (2003) noted that 40% of the potential yield of a given crop is genetically controlled while 60% is attributed to management (seed quality, fertilizer, pesticide spray, weeding). This is very relevant to bean production in Rwanda. High yielding climbing beans that are new in Rwandan cropping systems are equally demanding more in terms of management. Due to population pressure (about 340 people/km^2) and shrunk household land of less than one hectare, fallow is impossible. Rotations are rare and restricted to potato and maize. Here beans gain from residue fertilizer applied to the previous crops. Mineral fertilizers application is extremely low, at 0.4kg ha^{-1} (Lunze *et al*, 2012). Animal and farmyard manure practices are more common but in adhoc and insufficient rates. Sole cropping stands at 22%, but these are mostly for the climbing types. Intercrops are more common with banana 15%, maize

43% and with tubers 13% but also to a lesser extent with coffee and fruit trees (Lunze *et al*., 2012). Less than 10% of the farmers use improved quality seed. There are also some farmers who broadcast bean seed thus affecting planting and staking densities, weeding and may increase incidences of pests and diseases.

CHAPTER THREE

MATERIALS AND METHODS

This study was comprised of laboratory and greenhouse experiments to isolate and study the effect of lime and rhizobia inoculum application on climbing bean performance at Makerere University and Rwanda Agricultural Board (RAB) between 2011 and 2012.

3.1 Study area

Different sites of acidic soils were selected in South Province of Rwanda near the Nyungwe National Park, around Gasaka, Kitabi, Gatare and Akanyirandori at $2^{o}30'16"$ S $29^{o}29'40"$E (2093 msl) and $2^{o}28'54"$S $29^{o}27'58"$E (2147m) of Nyamagabe District with altitude lying between 2300 and 2700 m above sea level. This district is situated in Southwest of the Southern Province. Karongi and Ruhango Districts border it in the North, Nyanza and Huye districts on the East, Nyaruguru District in the South, Rusizi and Nyamasheke districts in West. The soils from these sites were characterized. Rhizobia isolation process was conducted in BNF Laboratory at Makerere University. The greenhouse was established at Rubona Research Station (Southern Zone) in Rwanda.

3.2 Laboratory experiment

3.2.1 Soil sampling

Soil samples were taken from farmer fields previously subjected to the following management practices had been applied: (i) Farm Yard Manure, (ii) inorganic fertilizer, (iii) lime after two seasons of its application and (iv) without any soil input. These fields characteristically had pH below 5, previously under beans production but with no history of *Rhizobium* inoculation. After selecting a management practice

field, soils were randomly collected from the top 0 – 20 cm and a composite sample per field obtained through quartering procedure. There were three replicates per management practice field. About 500g of composite soil sample were collected and stored in cooler boxes and later used in Laboratory experiments to isolate native rhizobia. Next samples were carried in the Cooler box to the laboratory and immediately stored in the refrigerator at 4^{o}C, and later used to isolate and enumerate native rhizobia

Another set of sample from the same soil was obtained for physical and chemical characterization. This set of the soil samples was air dried, ground and screened through a 2 mm sieve. The physical and chemical parameters analysed were soil pH, organic matter, texture, total N, available P and exchangeable bases (K, Mg, Na, Ca and Mn), electrical conductivity (EC) following Okalebo *et al*. (2002) methods. The analyses were performed in the soil science chemical analytical laboratory at Department of Agricultural Production, Makerere University.

3.2.2 Rhizobia isolation and enumeration

Bacteria presumed rhizobia from the study soil samples were isolated and enumerated using dilution plate technique (Somasegaran and Hoben, 1994). The bacterial growth medium was Yeast extract mannitol agar (YEMA). Constitutively, YEMA contained 0.3 g of yeast extact, 1.0 g of mannitol, and 15g of agar plus 100 ml of buffer all made up to 1L of distilled water. Two 1-L media were made, one modified with 10 mL Congo red (CR) while another had 5 mL bromothymol blue (BTB) indicators. The media were sterilized in an autoclave set at 121^{o}C, pressure 15 pa for 15 minutes. On cooling the media were aseptically poured into sterile plates and left to solidify.

Subsequently, 10g of each test soil was aseptically introduced into a separate bottle containing 90 ml of sterile water (Fig.3). The contents were agitated on an orbital shaker for two minutes. From the suspensions, tenfold dilutions were made up to 10-5(Fig.3) following Somasegaran and Hoben (1994) protocol. Starting with the highest dilution 2ml-aliquot were aseptically drawn with sterile graduated pipettes and 0.5 ml portions were separately dispensed onto sterile Yeast extract mannitol agar (YEMA) plates either modified with Congo red or Bromothymol blue indicators as illustrated in Figure 3. Using a sterile L-shaped spreader, the inocula were uniformly distributed on the plates. On drying, the plates were placed inverted in an aerated incubator set at 25oC, for three days.

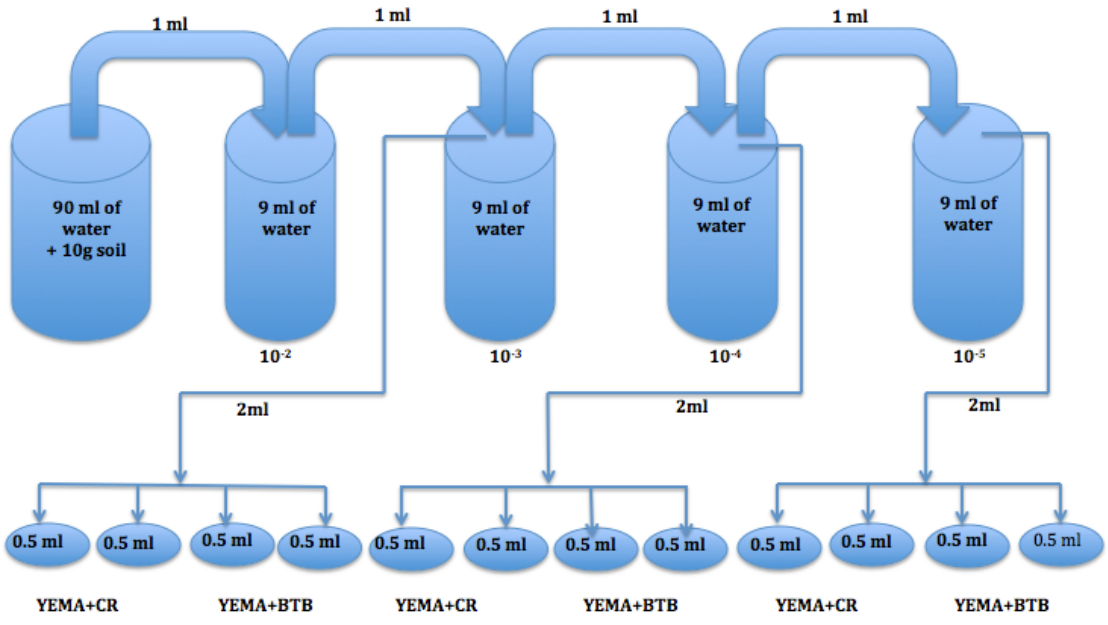

Fig 3: An illustration of the dilution techniques used for isolation and enumeration of soil rhizobia

The media used was Yeast Extract Mannitol Agar (YEMA) containing 15 gL^{-1} of agar, 1 $gL^{-1}$of Mannitol, 0.3 g of Yeast Extract, 100 mlL^{-1} of Buffer. Two litres, 1 separate each of the growth media were prepared. In the first litre, 5mLBromothymol

blue (BTB) and in second litre, 10 mL of Congo red were added. The media was sterilized at 121^{o}C for 15 minutes as described in the protocol (Somasegaran and Hoben, 1994). Using sterile pipettes, 0.5 mL were spread on the media in a petri-dish using a spreader sterilized by dipping it in ethanol and burnt, starting with plates of the lower dilutions to the higher dilutions. The plates were labeled with respective sample dilutions and placed in the aerobic incubator at 25^{o}C for 3 days for growth.

3.2.3 Sub culturing

Colonies were counted using digital colony counter machine (Stuart Scientific colony counter, UK) and cells per gram of soil computed. Colonies of exhibiting likely rhizobia characteristics were sub cultured on fresh YEMA sterile media to obtain pure colonies from which inoculum was been developed. Gram staining (Somasegaran and Hoben, 1994) was done as a confirmatory test to identify cells that are gram negative (*Rhizobium* cells are a characteristically gram negative). Repeated sub-culturing produced pure culture which were kept on the agar slants and was stored in the refrigerator a 4^{o}C for inoculum production.

3.2.4 Gram staining

Isolates were characterized as *rhizobia* strains in the laboratory using gram staining technique where rhizobia cells are gram negative due to their cell membrane nature that decolorizes when alcohol is applied and on counter staining, the pink color remains. Using a sterile wear loop a thin smear was made on the glass slide for the various bacterial slants provided and heat fixed. Staining the smears with solution of crystal violet for one minute was done and later washed lightly with water, then flooded with iodine to fix the purple color within the cell membrane. The iodine was drained immediately, and then was flooded again with iodine for one minute. Iodine was washed and the smear decolorized with 95% alcohol for 40 seconds and flooded

with water to remove the alcohol. Counter staining was done using Safranin solution for 1 minute for cells that would have lost the purple color to be gain the pink color. The slides were washed and blotted to dry. The slides were observed under microscope at a magnification of X40. Smears of pink cells were recorded and the slants stored, this procedure has helped us to separate the non-rhizobia bacterial slants.

3.2.5 Authentication and enumeration of native Rhizobia in soil

All selected isolated on the slants were used to develop liquid inocula that were dispensed into the pouches in the growth chamber. This was to test the efficacy of the isolates from the soils. Each pouche was supplied with 30 ml of N free nutrient stock solution containing Ca, P, Fe, Mg, K, Mn, B, Zn, Cu, Co, and Mo (Broughton and Dilworth, 1970), After 21 days, nodules developed on the bean plants were collected and crashed for production of the liquid inoculum. The inocula were used in the screen house for evaluating N-Fixation potential of the rhizobia obtaining under the different management practices.

The numbers of native rhizobia present in soils under different agricultural management practices, nodulating *Phaseolus vulgaris*, were assessed using the Most-Probable-Number (MPN) method, plant infection technique as described by Somasegaran and Hoben (1994). Bean seeds were surface sterilized with 95% of ethanol and in 3% (v/v) solution of sodium hypochlorite. Repeatedly they were rinsed in sterile distilled water, germinated, and stored on sterilized petri dishes containing filter paper with moisture content of 45%. Good healthy grown seedlings with similar size and radicle length were aseptically transferred into sterile growth pouches aseptically (one/pouche). After one week of growth, the pouches were rearranged on the rack.

At this stage, two effective isolates, which were laboratory coded $SMP10_4$ and $SMP6_3$ were inoculated on the test climbing bean varieties (Ngwinurare and Gasilida) in four replicates. The inoculants used contained different rhizobia cell concentrations ranging between$10 - 10^{10}$. There was also one control (without inoculants) pouch per replicate. After 28 days, nodulation was assessed and the number of rhizobia was calculated from the following formula:

m= number of nodulated plants

d=Lowest dilution (first unit used in the tabulation)

v=Volume of aliquot applied to plant

The MPN per gram of inoculant is: MPN=$\frac{mxd}{v}$

3.3 Screen house activities

3.3.1 Experimental design and treatments

A greenhouse experiment was conducted to assess the effectiveness of native rhizobia isolated in comparison to the existing types of inoculums on two climbing bean varieties under different liming levels. The experimental treatments included: (i) two climbing beans varieties Gasilida (improved) and Ngwinurare (local) commonly planted in the Southern province, (ii) four types of *Rhizobium* inoculants (ISAR-*Rhizobium*, Mak-Biofixer, $SMP10_4$ and $SMP6_3$ and (iii) three levels of lime $CaCO_3$ (0, 2.5 and 4) t ha^{-1}. The experimental design was Complete Randomized Design with three replicates. Each treatment received basal rate of 16 kg P ha^{-1}.

3.3.2 Pot and Seed preparation

Plastic buckets of 5 L size were purchased from Kenpoly plastics Company (Kenya). They were perforated at the bottom with four holes and six holes on sides to avoid water logging and allow aeration. The buckets were labeled according to treatments

before they were packed with soil. Gravel was arranged at the bottom of the pots followed by 3 cm thickness of sand and 5 kg of soil. Lime was manually mixed with the 5 kg soil before being added into the buckets. *Rhizobium* inoculants were dressed on the seeds to increase the chances of bacteria and plant root interaction. High quality seed certified of climbing bean varieties (obtained from Rwanda Agricultural Board at Rubona Research Station) was inoculated with the test inocula. Agricultural lime was obtained from fertilizer dealers in Rwanda.

Gasilida and local variety seeds were inoculated with test-rhizobial strains. Following a manual obtained from the Soil Science Unit at Makerere University. Briefly, a sugar based sticker was applied on seeds followed by thoroughly mixing the wet seeds with solid media inoculant till uniformly coated. Thereafter the seed were immediately planted in the soil within the buckets. Treatments without rhizobia were planted first to avoid cross contamination. Five seeds of each variety were planted for respective treatment buckets at 4 cm soil depth.

3.3.3 Experimental maintenance

Plants were thinned to four plants per pot one week after seed germination. All pots were placed on plastic plates to collect the leachate. To avoid nutrient loss from the soil, leachate was routinely returned to the respective bucket. Watering to maintain field capacity was done three times a week using separate glass tubes to minimize soil compaction and contamination while providing adequate moisture to the plant. The amount of water needed was determined by carrying out a procedure of field capacity on the soils by Somasegaran and Hoben (1994) method. Weeds were manually removed to avoid weed competition with bean for soil nutrient.

3.3.4 Data collected

Data collected included plant height, diameter, leaf area, plant biomass, nodule count, nodule weight, total N and assessing the pH after liming. Destructive sampling for nodulation and plant analysis was done. Two plants per pot were removed and the following parameters were measured: leaf area, number of effective and non-effective nodules, plant biomass, leaf area, plant diameter, plant height. At full podding state (when they were no more flowers available per plant), plants were harvested for above biomass determination. Biomass was determined after drying in oven at 75^{o}C for 72 h. Thereafter, biomass was ground for analysis of total nitrogen following Kjeldhal procedures described in Okalebo *et al*. (2002). Strain effectiveness (SE) was assessed according to the equation proposed (Purcino *et al.,* 2000)

SE= $(\frac{Inoculated\ plant\ DM}{Un-Inoculated\ DM}\ x\ 100)$ and Nitrogen fixing efficiency being classified as:

DM: Dry matter

i. Ineffective <35%,
ii. Moderately effective 35- 50%,
iii. Effective 50-80% and
iv. Highly effective >80%

Strain Nitrogen Fixation efficiency

This efficiency is calculated using empirical formula more used in nitrogen fixation assessment. Refer to (Otabbong et al., 1992 and Valverde et Al., 1997) the formula is set as follow:

SNFE%=⦉(N uptake in strain treatment- N uptake in Control)/(N uptake in control)⦊*100

3.3.5 Data analysis

The data were recorded in Excel sheet**,** processed and analyzed for variance (ANOVA) by using GENSTAT (Version 14) software. The means were separated using Fisher's protected LSD at 5% significance level. A regression analysis was done on the number of effective nodule and total nitrogen in the plant biomass to assess the contribution of BNF.

CHAPTER FOUR

RESULTS

4.1 Characteristics of soil according to management practices

Soils used for the study were acidic, pH ranged between 3.8 and 4.8 in natural and limed fields, respectively. This was fairly acidic for bean production and rhizobia to biologically fix nitrogen. Soil analysis showed that the texture was either sandy clay loam or sandy loam, hence fairly aerated soils, which is favourable for BNF. Available phosphorus as well as the cations in soil (Table 2) were low.

Table 2: Characteristics of soils obtained from Nyamagabe, southern Rwanda

Practices	pH	N	OM	P (Bray I)	K	Ca	Mg	Na	Sand	Clay	Silt
		%		mg kg^{-1}	Cmol kg^{-1}				%		
Natural	3.8	0.26	6.8	1.71	0.10	0.4	0.19	0.14	48	21	31
Manure	4.3	0.29	7.5	3.77	0.15	3.3	1.00	0.04	51	28	21
Mineral	4.3	0.13	2.8	4.21	0.29	2.3	0.66	0.04	44	40	16
Lime	4.8	0.29	7.6	4.08	0.18	1.4	0.42	0.05	55	20	25
Critical values	5.5-6.5	0.20	5.2	15.00	0.40	4.0					

4.2 Changes in soil pH following lime application

Soil pH significantly ($p < 0.05$) increased following lime application. Importantly, liming at 2.5 tha^{-1} sufficiently raised soil pH from about 4.0 to almost 6.0, contrary 4.0 tha^{-1} had no added advantage (Fig. 4).

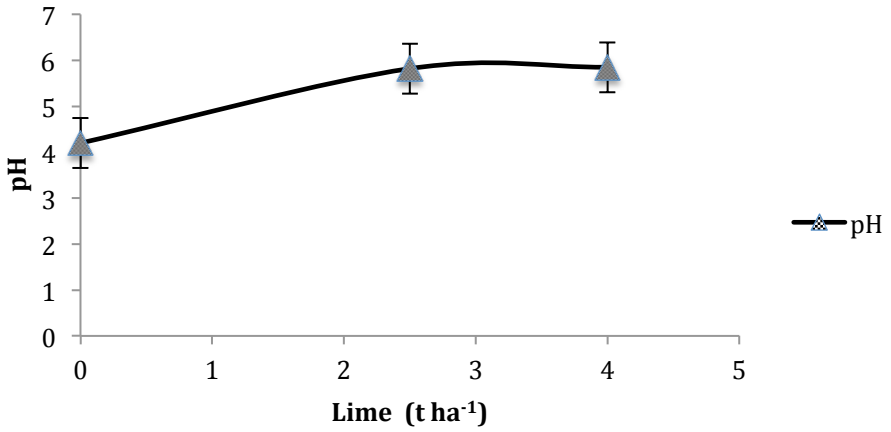

Fig. 4: Change in pH following application of lime an acidic soil of Rwanda

4.3 Abundance of rhizobia in soil and effective strain

There was a significant ($p < 0.05$) variability of rhizobia population in soils obtained from the different land management practices. Land that was fertilized with organic fertilizers (manure) had most population of rhizobia followed by areas that had received inorganic fertilizer and the least population was noted under natural environment (Table 3). Similarly, the highest effective strains were noted under the fertilizer management practices especially where organic matter had been added (Table 3). There was high population of effective strains for Gasilida compared to Ngwinurare bean varieties.

Table 3: Number of native rhizobia from different agricultural management practices on acidic soils in southern Rwanda

		Number of rhizobia cells (cell/ g of soil)	
Practices	**CFU**	**Ngwinurare($x10^4$)**	**Gasilida($x10^3$)**
Organic matter	2.67×10^5	37	49
Inorganic fertilizer	3.55×10^4	28	32
No inputs added	1.10×10^5	0	0
Lime	4.20×10^4	0	0
$LSD_{(0.05)}$		9	11

4.4 Interaction of lime and Rhizobia on bean total dry matter

The total dry matter (TDM) of climbing bean varieties was significantly ($p < 0.05$) increased by application of lime and Rhizobia inoculation. For Gasirida variety, the highest TDM was obtained in the pots that received 2.5t ha^{-1} of lime for all inocula except SMP104 at 4 t ha^{-1} (Table 4). The highest TDM for variety Ngwinurare was observed in pots that had not received any lime for all the inocula except Mak-bio-fixer. Without lime, Gasirida variety responded more to Mak-bio-fixer than other rhizobia inocula used in this study. Of the isolated strains, SMP 10_4 had consistently higher TDM than $SMP6_3$.

Table 4: Interaction of rhizobia and lime on dry matter (g $plant^{-1}$) of climbing bean varieties grown on acidic soils of southern Rwanda

Variety	**Inoculant**	**Lime rate (tha^{-1})**			**LSD (0.05)**
		0	**2.5**	**4**	
Ngwinurare	O input	1.822	1.694	1.062	0.307
	ISAR	2.501	1.508	2.505	
	Mak-Biofixer	1.350	1.814	1.962	
	$SMP6_3$	1.810	1.472	1.794	
	$SMP10_4$	1.738	2.961	1.778	
Gasilida	O input	1.942	1.309	1.415	
	ISAR	0.635	2.288	1.781	
	Mak-Biofixer	2.162	2.244	2.224	
	$SMP6_3$	1.606	1.897	1.697	
	$SMP10_4$	1.560	3.416	3.906	
CV%=13.9					

The ISAR inoculant significantly increased total dry matter of Gasilida following lime application with the highest value obtained at lime rate of 2.5 t ha^{-1} (Table 4). On the contrary, Mak-Bio-Fixer increased TDM of Ngwinurare bean variety following liming of soil. The isolate $SMP10_4$ increased TDM in all bean varieties following lime application (Table 4). Isolate $SMP6_3$ displayed an erratic TDM increased on both bean varieties following lime application. Without liming, inoculation with ISAR and Mak-Bio-Fixer inoculations caused highest TDM both test-varieties. After liming, isolate $SMP10_4$ consistently produced the highest TDM on the two bean varieties (Table 4).

4.5 Effect of liming on effectiveness of *Rhizobium* strains

Liming significantly ($p < 0.05$) increased effectiveness of all rhizobia inoculants used in this study. ISAR inoculant exceeded the minimum of 35% without any liming compared to the other inoculants under acidic conditions (Table 5). Generally, rhizobia isolates $SMP10_4$ and $SMP6_3$ had superior effectiveness compared to Mak-Bio-Fixer and ISAR at both lime rates used in this study. It is noteworthy, inoculation was important because non-inoculated soil had ineffective nodules, indicating a low BNF. Pot that received no input had significantly the least effective nodules just like the pots without lime. Further nodule effectiveness due to inoculation was observed after liming at 2.5 and 4.0 t ha^{-1} for Gasilida and Ngwinurare varieties respectively (Table 5).

Table 5: Effect of lime and inoculation on effectiveness (%) of *Rhizobium* strains

Variety	Inoculant	Lime rate (tha^{-1})			LSD (0.05)
		0	2.5	4	
Ngwinurare	O input	0.00	2.23	4.74	2.733
	ISAR	36.93	41.83	42.74	
	Mak-Biofixer	22.41	39.45	39.75	
	$SMP6_3$	12.15	61.06	55.64	
	$SMP10_4$	13.20	55.25	51.60	
Gasilida	O input	0.00	9.08	5.95	
	ISAR	36.86	67.28	66.57	
	Mak-Biofixer	29.42	71.05	68.78	
	$SMP6_3$	21.17	61.41	64.53	
	$SMP10_4$	22.09	76.93	75.29	
CV%=6.2					

4.6 Interactive effect of rhizobia, lime and variety on plant tissue total N

Lime significantly ($p < 0.05$) increased total nitrogen in bean biomass. It was noted that without any input, liming increased tissue total N for Ngwinurare variety whereas the response on Gasilida variety was inconsistent (Table 6). On the other hand isolate $SMP6_3$ had the highest plant tissue total N without liming. Mak bio fixer inoculum recorded the highest total N was realised when soil was limed at 2.5 and 4.0 t ha^{-1} for Ngwinurare and Gasilida varieties, respectively (Table 6). The ISAR isolated strains, $SMP6_3$ and $SMP10_4$ inoculants showed inconsistent increase in total bean-biomass nitrogen within the bean biomass following liming. A regression done (Appendices1 and 2) showed that the number of effective nodules positively correlated to the total biomass nitrogen in biomass ($p < 0.05$) though it accounted for about 10% variability.

Table 6: Effect of rhizobia and lime on plant tissue total N (%) in climbing bean varieties grown on acidic soils from southern Rwanda

Variety	Inoculant	Lime rate (tha^{-1})			LSD (0.05)
		0	2.5	4	
Ngwinurare	O input	1.875	2.552	2.173	0.468
	ISAR	2.875	2.500	3.345	
	Mak-Biofixer	2.246	3.132	2.872	
	$SMP6_3$	3.058	2.898	3.034	
	$SMP10_4$	2.652	3.005	3.295	
Gasilida	O input	2.702	2.508	2.734	
	ISAR	2.357	1.928	2.085	
	Mak-Biofixer	1.974	3.083	3.540	
	$SMP6_3$	2.175	2.813	2.432	
	$SMP10_4$	2.005	2.684	2.145	
CV%=15.6					

4.6.2.Effect of rhizobia and lime on SNFE in climbing bean varieties grown on acidic soils from southern Rwanda

Strains and lime significantly ($p < 0.05$) increased total nitrogen fixation efficiency. It was noted that without any input, ISAR strains increased N- fixation efficiency for Ngwinurare variety whereas the response on Gasilida variety was unreliable (Table 7). On the other hand isolate $SMP10_4$ had the highest N-Fixation efficiency at 2.5 t ha^{-1}. Mak-biofixer inoculum recorded constant in N-fixation efficiency when soil was limed at 2.5 and 4.0 tha^{-1} for Ngwinurare variety, respectively (Table 7). The ISAR isolated strains; $SMP6_3$ and $SMP10_4$ inoculants showed consistent increase the efficiency in N-fixation.

Table 7: Effect of rhizobia and lime on SNFE

Variety	Inoculant	Lime rate (tha^{-1})			LSD (0.05)
		0	2.5	4	
Ngwinurare	O input	0.0	64.6	-31.9	47.07
	ISAR	117.8	15.7	147.7	
	Mak-Biofixer	-10.9	70.8	71.7	
	$SMP6_3$	63.1	26.3	64.0	
	$SMP10_4$	37.9	164.4	77.2	
Gasilida	O input	0.0	-33.8	-25.0	
	ISAR	-69.9	-14.7	-27.0	
	Mak-Biofixer	-17.3	36.3	86.0	
	$SMP6_3$	-31.9	3.3	-18.4	
	$SMP10_4$	-38.5	80	62.7	

CHAPTER FIVE

DISCUSSION

5.1 Soil characteristics and liming effect on soil acidity

Soils from the study area were characteristically acidic with pH ranging between 3.8 and 4.8 across all the current soil fertility management practices against the agricultural range of 5.5 – 6.5 (Okalebo *et al*., 2002). This part of Rwanda with acidic soil is sparsely inhabited and less used for agriculture. Soil acidity is a major constraint to agricultural production, which once alleviated; makes it a high potential area with average rainfall 1600 mm per annum. Current research thrust in this area focused on plant breeding with less consideration on soil health. Notably, even areas that were limed are still acidic (Table 4) indicating that the rate of lime used is insufficient. Liming at a rate of 2.5 t ha^{-1} was sufficient to raise the pH to within the agricultural range (Fig. 2). Liming introduces Ca^{2+} and the OH^- that neutralize the H^+ released from the active and exchangeable acidity by AL^{3+} ions. Secondly, lime increased on the concentration of Ca^{2+}, which is known to be essential for biological nitrogen fixation (Andrade *et al*., 2002).

From the characterization results, nitrogen and organic matter are above the critical values indicating high agricultural potential. The concentration of cations is low as expected for acidic soils. Therefore, enhancing Biological Fixation in this area will require use of infective and effective native rhizobia, which was the objective of this research. Alternatively, liming could be considered and this study also determined the appropriate lime rate to reduce soil acidity to level pH>5.6 tolerated by plants (Mbonigaba *et al*., 2009). Available soil phosphorus is very low (Table 1) as expected in most acidic soils. This could be attributed to AL-phosphate complexes formed

under acidic conditions rendering phosphorus unavailable to plants. Low soil P concentration reportedly inhibits symbiotic nitrogen fixation (Zahran, 1999). About 16 ATP moles/mole NH_3 formed are required and P is an essential component of ATP. In this study, after realizing that the available P was low, a basal rate of 16 kg P ha^{-1} to mitigate its limitation on biological nitrogen fixation process was added. The soil texture (Table 1) indicates a well aerated soil, which is ideal for growth of the aerobic rhizobia cells.

5.2 Abundance of rhizobia in soil and effective strains

The rhizobia abundance significantly changed under the different agricultural management practices, with highest population occurring within soil that received organic amendments (Table 3). This could be attributed to organic matter added serving as source of energy for the microorganisms. Secondly, beans are often grown in the Agroecological zone from where soil was picked, possibly explaining the abundance of the legume nodulating rhizobia in these soils. The population noted in the different land management practices falls within or above the range reported for other acidic soils (e.g. Anyango *et al*., 1995; Andrade *et al*., 2002).

It was noted that where mineral fertilizers were added, there was low soil organic matter and rhizobia population. Incidentally, there were no effective native strains under the limed and no agricultural management land. Several agricultural practices, such as crop rotation, continuous cropping and tillage, stimulate variations in microbial communities in soil (Lupwayi *et al*., 1998) but specific microbial group may respond differently. Higher levels of soil nitrogen in the soil apparently decrease the abundance of rhizobia in the soil (Palmer & Young, 2000). Soil amendments such as lime, organic and inorganic fertiliser influence rhizobia abundance (Anthony *et al*.,

2001). However, it should be noted that rhizobia occurred under all land management practices within acidic soils as high as pH 3.8 in Rwanda. In earlier studies, Anyango *et al*. (1995), and Mwenda *et al*. (2011) isolated bean nodulating rhizobia at pH of <4.5 and 3.2 – 4.9, respectively, in the Kenya highland.

From the most probable number results (Table 3), it was noted that natural and organic matter agricultural management practices are poor in isolates population. Soils with the effective strains were used to isolate native rhizobia. Strains coded $SMP10_6$ and $SMP6_3$ were isolated from soils where lime and mineral fertilizers, respectively had been added. In greenhouse, these two native rhizobia isolates exhibited high dry matter yield and total nitrogen content than the currently used strains from Makerere University and ISAR (Fig. 3). This indicated that these native strains have higher potential of improving crop yield in the regions from where the soils were picked. This could be attributed to local adaptability of the native strains especially the acidic conditions. Previously, abundance of rhizobia and the existence of different rhizobial types were influenced by soil acidity (Murphy *et al*., 2002).

Soil acidity reduces the persistence and effectiveness of introduced rhizobia strains (Coventry and Evan, 1989). Similarly, Kellman 2008 reported abundance of rhizobia in soil and on plant roots due to the soil acidity which directly inhibited colonization, nodulation and infectiveness. Variable ability of rhizobia strains in different acidic soil were observed, with only 5% of the strains able to nodulate and persist under acidic environments (Carter *et al*., 1995). Further, Thies *et al*. (1991) showed that native rhizobia population affected inocula response competitiveness and effectiveness on the plant host within the soil.

5.3 Interaction of lime and Rhizobia on bean dry matter yield

The two climbing bean varieties used in this study variably responded to application of lime and rhizobia inoculums. While Gasirida required lime application up to 2.5 t ha^{-1} and rhizobia strains for a significantly high TDM, Ngwinurare performed well without any liming even where rhizobia inocula were added (Table 4). Noteworthy these climbing bean varieties were bred to tolerate soil acidity (PABRA, 2007) and this explains the high TDM without liming (Table 4). Indeed, Ngwinurare has been grown in this region (where soils used in the greenhouse were picked) for a long period of time and now adopted as a traditional variety.

The response of both bean climbing varieties to rhizobial inoculation was unpredictable. This could be attributed to the presence of effective native strains observed and reported in this study (Table 3). The abundance of indigenous rhizobia of 10^4 and 10^5 effectively masked the effect of external inocula. Note that the ISAR and Mak-bio-fixer inoculums formulations contained about 10^8 (as stated on the containers and not validated in this study).

For effective biological nitrogen fixation, the edaphic conditions should be favourable for the *rhizobia*. Under stressful conditions, the bacteria divert energy to survival (Schlegel and Jannasch, 2006) and for *Rhizobia*; it means less N will be biologically fixed. It is known that BNF is favoured by pH above 5.0 and when the acidity stress is alleviated by liming, the strains biologically fixed N increasing plant biomass but it was more significant with the isolate strains. Apparently, ISAR has the most superior rhizobia inoculums for the Ngwinurare (local) variety as Mak bio fixer is for Gasirida (improved) variety. However, the native strains competed favourably comparable to

ISAR and Mak bio fixer on plant biomass (Table 4), effectiveness (Table 5) and total nitrogen (Table 6). Implicitly, these are the most adaptable, effective and suitable strain for the Rwanda, from where the soils were picked. Diversity of rhizobia in different soil conditions affects the effectiveness of strain on leguminous host (Sharma, 2012). Earlier, Brauer *et al*. (2002) reported that the highest nodulation and dry matter accumulation after rhizobia inoculation was realized when liming increased pH to 6.5 and nitrogen fixed was positively correlated with biomass produced.

5.4 Effect of liming on effectiveness of *Rhizobium* strains

Lime significantly ($p < 0.05$) increased effectiveness of the four rhizobia strains used in this study. The ISAR inoculant recorded effectiveness above the minimum of 35% (Table 5) indicative of acid tolerance. Improved performance of these strains following liming presumes alleviated acidity stress was hence the bacteria used their energy to biological nitrogen fixation. *Rhizobia* isolate $SMP10_4$ and $SMP\ 6_3$ exhibited superior effectiveness compared to Mak-Bio-Fixer and ISAR at both lime rates used. By inference, legume inoculation would expectedly increase effectiveness and BNF.

This study has further shown that the improved variety (Gasilida) responded more to inoculation than the local variety. Low dry matter attained in non-limed treatments could also be attributed to direct pH stress on the plant, limiting nutrient uptake and subsequent dry matter accumulation. The suitable pH range for growing common beans is 5.6 to 6.5 (Musoni, 2008; Lunze *et al*., 2012). A linear relationship between pH and liming rate up to 8 t ha^{-1} was reported in the acidic soils of Rwanda (Yamoah, 1992). The low pH was mainly contributed by the exchangeable acidity attributed to high Al concentration in the soil. Earlier, Ibekwe *et al*. (1997) had noted that high

concentrations of heavy metals and Aluminum reduced *Rhizobium* population is soils and consequently low nodulation and the symbiotically fixed nitrogen. A regression of effective nodulation accounted for only about 10% variability in tissue nitrogen concentration indicating that there are other yet unknown factors that influence N accumulation within the plant.

Enhancing biological nitrogen fixation should consider the genetics of plant and rhizobia strain, environmental conditions such as soil pH, moisture and temperature stress as well as management. Liming of acidic soils is still necessary for increased BNF even when native strains are applied as earlier reported that soil acidity constrains symbiotic N fixation (Munns, 1986). Liming increases the survival and persistence of rhizobia in soil (Andrade *et al*., 2002), which are pertinent issues to enhanced BNF. Apparently plant grown in pots that never received lime had nil effectiveness (Table 3), possibly due to pH stress on the rhizobia strains. It was earlier reported that high soil acidity restricted *R. leguminosum* population and nodulation (Richardson *et al*., 1988). Acidic conditions appear to restrict root colonization by the rhizobia hence affecting nodulation. In another study, raising pH to 6 did not increase nodulation in acidic soils (Ibekwe *et al*., 1997) attributed to low effective rhizobia population.

5.5 The interactive effect of rhizobia, lime and bean variety on tissue total N

The improved climbing bean variety (Gasilida) was the more nodulated than the local variety climbing bean (Ngwinurare). Thus the farmer would benefit more from biological nitrogen fixation when they plant the improved variety on these acidic soils. However, the regression analysis (Appendices1 and 2) showed a weak correlation in this study between nodulation and plant tissue total N. Therefore, effective nodulation did not directly translate to more N within the plant. This could perhaps partly be attributed to adequate soil N and organic matter (Table 1). BNF is a crisis pathway when a leguminous plant experiences low N stress (Vance *et al*., 1988, Kijne, 1992).

The tissue total N dominance by $SMP6_3$ and $SMP10_4$ was no surprise since the same strain was the most effective and had significant response to other biological nitrogen fixation parameters observed in this study. This showed the resilience of the native strains to tolerate acidic soils comparable to ISAR inoculums. The pot without inputs also showed considerable quantity of plant tissue N. Possibly originated from the soil. Basing on soil parameters, the main challenges to bean production in Rwanda are acidity and P-deficiency (Table 1). Importantly, P is an essential element for BNF (Musoni, 2008), suggesting that there could be low BNF in these soils despite the high abundance of native rhizobia strains (Table 3). As a result, the regression analysis (Appendices 1 and 2) showed a direct correlation between effective nodulation and plant tissue N but explaining about 10% of the variability. Another factor that could explain part of the remaining variability is effect of P on BNF. In this study, a basal P at 40 kg ha^{-1} may not have been adequate.

Ngwinurare had a high tissue total N when inoculated with ISAR stain without liming attributed to the genetic soil acidity stress tolerance of plants and rhizobia strains to tolerate acidic stress. The model of increasing BNF considers these two factors as well as environmental stress and agricultural management. Earlier studies have indicated that ISAR strains are and Ngwinarure climbing bean varieties are best adapted to acid conditions (Musoni, 2008; CIAT, 2010).

The evaluation of lime interactions with different rhizobia strains under greenhouse conditions is rather difficult due to the complication of the soil-plant-microorganism factor interactions (Andrad, 2002). Despite several studies involving various *Rhizobia* strains in different acidic soils, few have explained interactions on more than two bean varieties (Giller, 1997). Maintaining rhizobial diversity could be a key to ensuring resilience to further environmental stress or disturbance, as appears to be the case for other organisms (Giller, 1997). Climbing bean Ngwinurare is relatively tolerant to soil acidity than Gasilida but this character does not necessarily lead to high and effective biological nitrogen fixation. The rhizosphere acidity and habitat of *Rhizobium* cell could be the major factor to explain strain effectiveness as noted by Ibekwe *et al*. (1997). However, this study has demonstrated that the acidic soil of Rwanda contains high abundance of native effective rhizobia strains for climbing bean varieties Ngwinarure and Gasilida.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATION

6.1 Conclusion

The study objectives were to (i) determine the richness of native rhizobia in the acidic soils under different management practices for enhanced BNF; (ii) determine effectiveness of the native rhizobia strains in acidic soils under different management practices on the climbing beans in Rwanda; (iii) determine the effect of liming on biological nitrogen fixation in inoculated climbing beans; iv) to compare effectiveness of native *Rhizobia* isolates and available inoculums of climbing bean. From the results, it is concluded that:

i). The acidic soil of Rwanda contains high abundance of native effective rhizobia strains to the magnitude of minimum 10^4 across all types of land management for climbing bean varieties Gasilida and Ngwinarare.

ii). Effective isolates on both local and improved climbing bean varieties were only obtained from areas where fertilizers (both organic and inorganic) had been applied.

iii). Two native effective isolates $SMP10_4$ and $SMP6_3$ were recovered from the soil. Both isolates compared favourably with the available inoculums ISAR and Mak fix. Indeed $SMP10_4$ had superior response on most of the measured parameters.

iv). Liming at 2.5 t ha^{-1} is sufficient to increased effectiveness all rhizobia strains

6.2 Recommendations

This study stopped at greenhouse level, the stability and effectives of the isolates $SMP10_4$ and $SMP6_3$ from this study should be tested in the field and scored under different agriculture management practices before massive inoculums production for sale is undertaken.

6.3 Areas for further studies

i). It is suggested that further characterization of isolates $SMP10_4$ and $SMP6_3$ be carried using both cultural and molecular methods to identify these isolates.

ii). These isolates can be tested on other bean varieties grown in Rwanda to confirm their potential BNF across the regions and bean varieties.

iii). Evaluate the relative contribution of liming and P fertiliser on BNF in acidic soils of Rwanda both on climbing and bush beans.

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APPENDICES

Appendix 1: Regression analysis

Response variate: Nod_count
Fitted terms: Constant, Plant_N_g_kg_1

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	1	2308.	2308.4	7.84	0.006
Residual	178	52427.	294.5		
Total	179	54735.	305.8		

Percentage variance accounted for 3.7
Standard error of observations is estimated to be 17.2.

Message: the following units have large standardized residuals.

Unit	Response	Residual
117	84.0	2.97

Message: the following units have high leverage.

Unit	Response	Leverage
19	28.0	0.032
161	36.0	0.039
162	34.0	0.032

Estimates of parameters

Parameter	estimate	s.e.	t(178)	t pr.
Constant	18.26	5.87	3.11	0.002
Plant_N_g_kg_1	0.611	0.218	2.80	0.006

Correlations between parameter estimates

Parameter	ref	correlations	
Constant	1	1.000	
Plant_N_g_kg_1	2	-0.976	1.000
		1	2

300 RGRAPH [GRAPHICS=high; CIPLOT=yes]

Appendix 2: Regression analysis on plant N and effectiveness %

Response variate: Plant_N_g_kg_1
Fitted terms: Constant, Effective_nodules_%

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	1	265.	265.35	7.98	0.005
Residual	178	5916.	33.24		
Total	179	6182.	34.54		

Percentage variance accounted for 3.8
Standard error of observations is estimated to be 5.77.