EFFECTIVENESS OF ACID TOLERANT INDIGENOUS RHIZOBIA AND LIME APPLICATION ON THE PERFORMANCE OF GROUNDNUT IN WESTERN KENYA.

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REG NO.: SC/PGB/008/10

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE MASTER OF SCIENCE DEGREE IN BOTANY (GENETICS) OF THE SCHOOL OF SCIENCE, DEPARTMENT OF BIOLOGICAL SCIENCES, UNIVERSITY OF ELDORET, KENYA.

OCTOBER, 2013

DECLARATION

DECLARATION BY THE CANDIDATE

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DEDICATION

To God almighty for His tender loving care throughout my life. In memory of my beloved mother and to my entire family for their support throughout my academic life. To my mentor Dr. Beatrice A. Were who made it possible for me to climb the academic ladder to this level.

ABSTRACT

Rhizobia inhabit root nodules where they reduce atmospheric nitrogen into a form available to plants. High productivity of grain legumes depends on effective symbiotic nitrogen fixation through successful legume inoculation with an efficient strain of rhizobia. The aim of the study was to identify efficient, acid tolerant rhizobia native to soils of western Kenya that could be used as groundnut (*Arachis hypogeae* L.) inoculants for enhanced crop performance. Rhizobia were isolated from nodules of groundnut collected from various localities of western Kenya. Initial characterization of the rhizobia was done using morphological, microbiological and biochemical features. The response of the isolates to varying pH (3.5, 4.5, 5.5 and 6.8) and aluminium (0.0, 50, 100, 130, 150 and 200 µM) was tested in a basal liquid media. Time taken by each isolate to show visible turbidity in the media was recorded in hours and used as a measure of tolerance to acidity and aluminium stress. Broth cultures of the isolates were used to inoculate groundnut (*Red Valencia*) to assess the ability of the isolates to nodulate groundnut and thereafter cross inoculation to three other varieties (ICGV 9991, ICGV 12991 and ICGVSM99568) was done in the greenhouse. Field evaluation of symbiotic effectiveness of the isolates was done using three isolates and one commercial strain as inoculants. The effect of two different limes (dolomitic lime and calcitic lime) on the yield of rhizobia inoculated groundnut was also assessed at Koyonzo and Ligala. Nodule number and dry weight, nut number per treatment, shoot and grain yield were determined. Recovery of inoculant strains from nodules of field grown plants was then done using resistance of the isolates to various antibiotics, to gauge the competitiveness of the strains. A total of 90 bacterial isolates were obtained from groundnut nodules. Based on their growth on Yeast Extract Mannitol Agar (YEMA) incorporated with Congo red, 15 isolates were found to be very fast growing, 52 fast growing while the remaining 23 isolates were moderate to slow growing. On YEMA incorporated with Bromothymol blue (BTB), 64 of the isolates produced acid while 26 were alkaline producers. Further characterization showed 84 of the isolates to be gram negative rod cells that reduced nitrate to nitrite, hydrolysed urea to ammonia and utilized citrate as a sole carbon source. Screening for tolerance to pH (3.5 - 6.8) showed that 76 isolates tolerated the lowest pH of 3.5. At aluminium concentration of 0-200 µM, 36 of the 76 acid tolerant isolates grew at 130 µM while only 5 strains namely, A6, Biofix, n3, V2 and W1 could withstand 200 µM Al. Among the 36 Al tolerant rhizobia, 3 were slow growing and 33 fast growing. Nodulation test found only 3 (A6, W1, and V2) of the 36 isolates to be the most promising in efficient nitrogen fixation. Significant improvement ($p \leq 0.05$) was observed for nodule number and shoot biomass for cross inoculated plants and this varied with the isolate. In the field experiment, the highest grain yield (1002 kg ha^{-1}) was obtained in the short rain season at Ligala with strain V2 on plots with calcitic lime. At Koyonzo strain V2 again with calcitic lime gave the highest groundnut yield of 879 kg ha^{-1}. There were significant difference for strain x lime, strain x lime x site and strain x lime x site x season. Strains V2 and A6 had no significant difference across sites and seasons. This study has identified effective indigenous rhizobia adapted to acid soils of western Kenya that can be used as groundnut inoculants for enhanced crop performance and grain yield. The findings reported here if adopted will contribute to enhancing groundnut production on acid soils of western Kenya and hence improve livelihoods.

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LIST OF ABBREVIATIONS

Al	aluminium
ANOVA	analysis of variance
B	Boron
BNF	biological nitrogen fixation
Bp	base pairs
BTB	Bromothymol blue
^{0}C	degree Celsius
C	Carbon
Ca	calcium
Co	cobalt
CIAT	Centre International de Agriculture Tropical
cm	centimeter
DNA	deoxyribonucleic acid
dNTP	dinucleotide tri-phosphate
g	gram
GPA	glucose peptone agar
ha	hectare
HCl	hydrochloric acid
hrs	hours
K	potassium
kbp	kilo base pairs
kg	kilograms
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
mo	molybdenum
N	Nitrogen
NaCl	sodium chloride
O	oxygen
P	Phosphorus
PCR	polymerase chain reaction
RNA	ribonucleic acid
RAPD	random amplified polymorphic DNA
Rpm	revolution per minute
S	Sulphur
µl	microlitre
µM	micromolar
W/V	weight by volume
WAE	Weeks after emergence
YEMA	yeast extract mannitol agar
%	percent
KARI -	Kenya Agricultural Research Institute
NIFTAL-	Nitrogen Fixing Tree Association

ACKNOWLEDGEMENT

I wish to gratefully acknowledge the financial support from the Regional University FORUM for capacity building in Agriculture towards my Masters of Science studies.

My special thanks to Chepkoilel University College through School of Science for offering me admission for MSc. I am very grateful to my supervisors, Dr. Beatrice A. Were and Dr. Abigael N. Otinga for their positive criticism and advice throughout the study.

Much thanks to my colleague Janet Ogega for her active involvement in the laboratory and field experiments throughout the study, Mr. Emmanuel Makatiani for sharing ideas and availing reference strains for this study, KARI Kisii through Mrs Rose Okoko for supplying initial groundnut varieties used in the study. To Mr. Richard Nyagwachi am very grateful for the support given during this study and to all my friends thanks for all your support.

Finally, I am very grateful to the two farmers Mr. Bonface Omoto of Ligala and to Mrs. Joyce Angoye of Koyonzo for readily letting part of their land for the field experiments.

CHAPTER ONE

INTRODUCTION

Many families in the sub-Saharan Africa (SSA) experience food insecurity. Arable land in East Africa on which small holder farmers depend for agriculture is increasingly getting degraded through deforestation, soil fertility depletion, soil erosion and water deficit. Hence it is becoming less favorable for cultivation of most staple food crops particularly maize, sorghum, rice and grain legumes including groundnuts (Woomer, et al., 1997). In Kenya the depletion of soil nutrients is particularly high in the densely populated Western and Central provinces. Nitrogen deficiencies in the country and East Africa as a whole greatly affect per capita food production that is low and continues to decline (FURP, 1994). World consumption of fertilizer nitrogen is 88 million tons per year and apart from the consumption of non-renewable energy sources, environmental pollution from fertilizer nitrogen escaping the root zone is high because in many cases nitrogen fertilizers are not used efficiently by crops (Peoples et al., 1994).

Biological nitrogen fixation (BNF), a process that changes inert N_2 to biologically useful NH_3 can be important and integral component of sustainable agricultural systems. BNF from legumes offers more flexible management than fertilizer N because the pool of organic N becomes slowly available to non-legume species (Peoples et al., 1995). In addition to the N_2-fixation, the use of legumes in rotations offers control of cereal pests and diseases (Robson, 1991; Graham and Vance, 2000).

The process of BNF is mediated in nature only by bacteria some of which establish symbiosis with leguminous plants. These elicit the formation of nodules on roots or stems

of their hosts, in which they reduce the atmospheric nitrogen. Symbiotic nitrogen fixation is an important source of nitrogen. Various legume crops and pasture species often fix as much as 200 to 300 kg N ha^{-1} (Peoples et al., 1995). Giller (2001) reported N fixation rates of 1 to 2 kg N ha^{-1} daily in a growing season by most tropical legumes. Therefore by exploring BNF, not only does the system optimize economic returns to farmers but can also minimize the environmental pollutions associated with high N fertilizer use (Bundy and Adraski, 2005).

Soil acidity is a significant problem in agricultural production in many areas of the world and limits legume productivity (Graham, 1992; Correa and Barneix, 1997). Soils in many parts of Kenya are acidic (pH 4.5 – 5.7) and have high levels of exchangeable aluminium (Muok, 1997). Soil acidity adversely affects symbiotic N_2 fixation, limits rhizobia survival and persistence in soils and reduces legumes nodulation (Brockwell et al., 1991). This is because most rhizobia are sensitive to acidity (Odee, 1995). Furthermore, some soils lack rhizobia while others have strains that are unsuitable for the legume of interest to the farmer. Rhizobium that is introduced by inoculation must tolerate local soil factors in order to nodulate and effectively fix N_2 with the legume. Acid soils often harbor adapted rhizobia with varying degrees of effective nitrogen fixation in specific host legumes. Such diverse rhizobia have been characterized for a variety of grain legumes including common bean, soybean and cowpea among others (Keyser and Cregan, 1988; Chen et al., 1991; Meghvansi et al., 2008). It would be necessary to characterize groundnut (*Arachis hypogea* L.) rhizobia native to acid soils in western Kenya to be able to develop suitable inoculants.

Groundnut is capable of biological nitrogen fixation (BNF) by *Bradyrhizobium* (Smartt, 1994). The use of this legume can therefore address the current soil nutrient depletion and

increase crop yields as a natural method of maintaining soil fertility. Groundnuts can also offer advantages for non-N fixing crops by fixing N up to 124 kg ha^{-1} soil N when planted the year prior to these crops (Elkan, 1995).

Apart from being capable of BNF, groundnuts have a number of other uses. For example, they are a primary source of low cost high quality protein and also a valuable source of thymine (B1). In western Kenya, they are important cash crop to the smallholder farmers. The seeds are also ground into a paste known as peanut butter. In southern Nyanza; groundnut sauce is mixed with sour milk to form a delicacy known as ogira that is served with sweet potatoes. Even though groundnuts have a number of uses, the contribution of this crop to BNF has not been explored. For this reason, the endeavors to assess and utilize the BNF potential of this valuable oil crop will help in amending soil N deficiencies as well as increasing its yield in the region.

1.1 Statement of the Problem

Crop production in most smallholder farms in Kenya is characterized by continuous cultivation coupled with low input, which results in reduced soil fertility and productivity. Nitrogen is often the limiting nutrients in these farms. Inorganic N fertilization is needed to alleviate N deficiency. Nitrogen fertilization, however, is costly and therefore out of reach of most resource-poor farmers. Manure from livestock could be used as an inexpensive source of nutrients, but nutrient contents are often low, which requires bulk application to satisfy plant nutrient demand High fixation of N can only be achieved in the presence of efficient rhizobial strains, which can be native to the soil or introduced in-form of commercial inoculants.

Groundnut grows well in soils with pH of about 5.5 – 7.0. The soils of western Kenya where groundnut is cultivated are acidic, posing limitation to the potential production of this valuable oil crop. In acidic soils, nitrogen fixation is hindered as most rhizobia are sensitive to acidity. Hence need to isolate and characterize acid tolerant strains that can be used as inoculants with this legume.

Although groundnuts are popularly sold to earn income and are a source of dietary protein, their yield is low with a continual decline in the recent past. In western Kenya, yield as low as 200-400 kg ha^{-1} has been reported. Although it is well known fact that groundnut is a good source of fixed nitrogen, effort has not been made to study the indigenous rhizobia colonizing roots of this plant. In the present study strains of rhizobia from the groundnut root nodules were isolated their nodulation and nitrogen fixation effectiveness determined.

1.2 Justification

Currently, agricultural legumes account for approximately 35 million metric tons of N_2 fixed as N fertilizer. Increasing BNF of cultivated legumes can have both large financial and environmental impacts. The BNF process can contribute up to 400 kg of N per hectare annually (http: www.fao.org) yet the potential of BNF has not been fully realized in most developing countries. It is therefore essential that the contribution of a particular legume to the BNF system be established. Furthermore, the high consumption rate of N fertilizers under intensive agriculture should be discouraged, because they are expensive and also act as pollutants to water bodies. It is therefore important to focus attention on less expensive, more environmentally friendly and readily available sources of N for sustainable agriculture. One such alternative is the use of biologically fixed nitrogen. Biological

nitrogen fixation (BNF) holds great promise for smallholder farmers in sub-Saharan Africa because it reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops as the bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant. The nitrogen fixed via rhizobia-legume symbiosis has been recommended as a means to sustain traditional agriculture (People et al., 1995; Postgate, 1998). This is because the symbioses are a cheaper and usually more effective agronomic practice for ensuring an adequate supply of N for legume-based crop and pasture production than the application of fertilizer-N.

Inoculation of legumes with rhizobial strains selected for high N_2-fixing capacity can improve nitrogen fixation in agriculture, particularly when local rhizobial strains are ineffective or absent from soils. Enhanced competitive ability of inoculants is a key requirement for successful colonization of plant roots, nodule formation, and subsequent N_2-fixation. Groundnut is among the leguminous crops that can produce root nodules and fix atmospheric nitrogen by symbiotic relationship with cowpea- type rhizobia which predominate in tropical soils (Toomsan et al., 1991). The nitrogen fixing ability in in this crop varies widely depending on groundnut genotypes and rhizobia strains (Wynne et al., 1980). Rhizobia comprise a diverse group of bacteria that acts as the primary symbiotic fixer of nitrogen (Estrada-De Los, 2001). Groundnut is usually nodulated with the slow growing *Bradyhrizobium spp.* and in some cases fast growing rhizobia. These strains are tolerant to varying stress effect and are able to form effective (N_2-fixing) symbioses with their host legumes under salt, heat, and acid stresses, and can sometimes tolerate heavy metal toxicities.

Mandimba (1995) reported that the nitrogen contribution of *A. hypogaea* to the growth of *Zea mays* in intercropping systems could be equivalent to application of 96 kg of fertilizer-N ha^{-1} at a ratio of plant population densities of one maize plant to four groundnut plants. Therefore, exploitation of its high N_2 fixation potential through use of efficient inoculant strains is important for enhanced crop productivity, yields and soil fertility.

This study investigated the diversity and symbiotic effectiveness of indigenous rhizobia from groundnut growing areas of western Kenya characterized by acid soils. The study also assessed the importance of soil pH correction for enhanced field performance of selected acid tolerant rhizobia and groundnut.

1.3 Objectives

1. 3.1 Broad objective

The broad objective of this study was to identify efficient acid tolerant groundnut rhizobia native to soils of western Kenya that could be used as inoculants to enhance groundnut production.

1.3.2 Specific objectives

1. To establish the existence of indigenous rhizobia that can nodulate groundnuts in soils of western Kenya.

2. To find indigenous groundnut rhizobia strains from soils of western Kenya are tolerant to soil acidity and aluminium stress.

3. To determine the nodulation and N- fixation effectiveness of the indigenous rhizobia isolates on groundnuts in a monocrop.

4. To assess the competitive ability of established and selected acid tolerant isolates under calcitic and dolomitic limes at two sites in western Kenya

1.4 Research Hypothesis

There exist diverse acid tolerant rhizobia in the acid soils of western Kenya that can improve the performance of groundnut through nodulation and BNF.

1. There are indigenous rhizobia in soils of western Kenya that can nodulate groundnuts.
2. Indigenous groundnut rhizobia strains from soils of western Kenya vary in tolerance to soil acidity and aluminium stress.
3. To determine the nodulation and N- fixation effectiveness of the indigenous rhizobia isolates on groundnuts in a monocrop.
4. Established and selected acid tolerant rhizobia isolates can compete well against other soil bacteria for nodule occupancy at Ligala and Koyonzo sites in western Kenya when the soils are limed with calcitic or dolomitic lime.

CHAPTER TWO

LITERATURE REVIEW

2.1 Groundnut biology, production and uses

Arachis hypogea L. (peanut, groundnut), is an annual oil seed belonging to the family Papillionacea. It was first domesticated and cultivated in the valleys of Paraguay (Jauron, 1997). It is a herbaceous plant growing 30 to 50 cm tall. The leaves are opposite, pinnate with four leaflets (two opposite pairs, no terminal leaflets), each leaflet 1 to 7 cm long and 1 to 3 cm broad. The flowers are a typical pea flower in shape 2 to 4 cm across, yellow with reddish veining (Putnan, 1991).

Hypogaea means 'under the earth'; after pollination the flower stalk elongates causing it to bend until the ovary touches the ground (Putnan, 1991). Continued stalk growth then pushes the ovary underground where the mature fruit develops into a legume pod. Pods are 3 to 7 cm long, containing 1 to 4 seeds. The domesticated groundnut is an amphidiploid or allotetraploid (Jauron, 1997).

Archeologists have dated the oldest specimens found in Peru to about 7600 years (Putnan, 1991). Cultivation spread as far as Mesoamerica where the Spanish conquistadors found the talcanahuatl (Nahuatl= peanut). The crop was later spread worldwide by Europeans traders (Jauron, 1997).

Groundnuts grow best in light sandy loam soil (Putnan, 1991). They require up to five months of warm weather, and an annual rainfall of 500 to 1000 mm. The pods ripen 120 to 150 days after the seeds are planted (Putnan, 1991; Jauron, 1997). If the crop is harvested

too early, the pods will be unripe. If they are harvested late, the pods will snap off at the stalk and remain in the soil (Jauron, 1997).

Groundnuts can be eaten raw, used in recipes made into solvents and oils, used in makeup's, medicines, textile materials , peanut butter as well as confectionaries such as salted peanut, peanut butter, peanut brittle and plain /roasted shelled nuts (Putnan, 1991; Jauron, 1997; Yao, 2004). Boiled groundnut is eaten as snack in Kenya, United States, India, China and West Africa (Jauron, 1997; Yao, 2004). Groundnut oil is used in cooking, making cosmetics, nitroglycerin, plastics, dyes, paints and insecticides. The flour is used as gluten –free solution, used to make lactose free milk (Yao, 2004). The plant tops are used for hay. Low grade seeds may be sold as a bird feed (Jauron, 1997; Yao, 2004). The groundnut husk or shell can be as a fuel, roughage and energy source or absorbent in livestock feeds, animal litter, mulch, soil conditioner and manure.

2.2 Biological Nitrogen Fixation (BNF)

BNF is the process whereby atmospheric nitrogen (N≡N) is reduced to ammonia with the help of nitrogenase (Bottomley, 1992). This process is controlled by the action of nif genes, which encode enzymes involved in the fixation of atmospheric nitrogen. The primary enzyme encoded by the *nif* genes is the nitrogenase complex that catalyses the conversion of atmospheric nitrogen- N_2 to ammonia which the plant can use for amino acid synthesis. Besides the nitrogenase enzyme, the *nif* genes also encode a number of regulatory proteins involved in nitrogen fixation. These genes are found in both free living nitrogen fixing bacteria and in symbiotic bacteria colonizing various plants. The

expression of the *nif* genes is induced as a response to low concentrations of fixed nitrogen and oxygen (http://ag.arizona.edu/PLP).

2.2.1 Mechanism of BNF

The nitrogenase complex converts atmospheric nitrogen (N_2) to ammonia according to the following equation:

$$\mathbf{N_2 + 8H^+ + 8e^- + 16ATP + 16H_2O \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi}$$

(http://www-saps.plantsci.cam.ac.uk)

The ammonia formed in this process can then be incorporated into amino acids such as Glutamate and to nucleic-acids. The nitrogenase complex contains 2 types of proteins. Component no. 1 is a 220,000 Da protein, formed by 4 subunits containing 28 ions of molybdenum (a rare heavy metal) as co-factors. Component no. 2 is a 70,000 Da molecule formed by 2 subunits that contain 8 atoms of Iron as co-factors. The metal co- factors (Fe and Mo) put the nitrogen in a position in which it is easier to convert it to ammonia. The 2 components together fix atmospheric nitrogen. The nitrogenase complex is very sensitive to oxygen, and is inactivated by O_2 concentration. In order to prevent the oxygen found in the root's environment from reaching the nitrogenase, the plant produces a special form of hemoglobin called leghemoglobin. This protein has a high affinity for oxygen and its binding to the oxygen originated around the root prevents it from reaching the nitrogenase complex. Since nitrogen fixing is a costly process for the plant, utilizing 16 ATPs to fix a single nitrogen molecule, the plant regulates the nitrogenase activity and expression

according to nitrogen availability and oxygen presence (http://www-saps.plantsci.cam.ac.uk).

2.2.2 Genetic control of nitrogen fixation

Nif genes which encode the nitrogenase complex and other enzymes involved in nitrogen fixation have consensus sequences identical among nitrogen fixing bacteria. However, whereas the structure of the *nif* genes is similar, the regulation of the genes varies between different diazotrophes, depending also upon the organism's evolutionary hierarchy.

Activation of the nif gene transcription takes place in times of nitrogen stress (Rigo et al., 2001). In most plants, activation of *nif* gene transcription is done by the nitrogen sensitive NifA protein. When there is insufficient fixed nitrogen available for the plant's use, NtrC which is a RNA polymerase triggers NifA's expression, and NifA activates the rest of the nif genes transcription. If there is a sufficient amount of reduced nitrogen or if oxygen is present, another protein is activated – NifL; NifL inhibits NifA activity resulting in the inhibition of nitrogenase forming. NifL is regulated by glnD and glnK gene products. The nif genes can be found on the bacterial chromosomes, but often occur on plasmids (sym plasmids) with other genes related to nitrogen fixation, such as the nod genes (Eric, 2000; Rigo et al., 2001). There are twenty one *nif* genes with clearly defined functions in nitrogen fixation (Eric, 2000). Regulation of the nif genes expression is done at the transcription level inside the host plant (http://www.asahi-or.wtnb/BNF**).**

2.3 Legume nodules

Legume nitrogen fixation starts with the formation of a nodule. Rhizobia invade the root and multiply within the cortex cells (Marschner, 1995). The plant supplies all the necessary nutrients and energy for the bacteria. Within a week after infection, small nodules are visible with the naked eye (Amerger, 1981). In the field small nodules can be seen 2-3 weeks after planting, depending on legume species and germination conditions (Piha and Munns, 1987). When nodules are young and not yet fixing nitrogen, they are usually white or grey inside (Amerger, 1981). As nodules grow in size they gradually turn pink or reddish in colour, indicating nitrogen fixation has started. The pink or red color is due to activity of leghemoglobin that controls oxygen flow to the bacteria (Tabata, 2000).

Legume nodules that are no longer fixing nitrogen usually turn green, and may actually be discarded by the plant (Marschner, 1995). Pink or red nodules should predominate on a legume in the middle of the growing season. If white, grey, or green nodules predominate, little nitrogen fixation is occurring as a result of an inefficient rhizobial strain, poor plant nutrition, pod filling, or other plant stress (Graham, 1992). Therefore, nodulation and nodule appearance are used as indicators of strain efficiency and symbiotic effectiveness.

2.3.1 Nitrogen fixation and nitrogen fertilization

Some legumes are better at fixing nitrogen than others. Legumes such as groundnuts, cowpeas, soybeans, and faba beans are good nitrogen fixers and will meet all of their nitrogen needs without that absorbed from the soil (Graham and Vance, 2000). These legumes may fix up to 114 kg of nitrogen per acre and are not usually fertilized. Nitrogen fertilizer is applied to these legumes at planting when grown on sandy or low organic C

soils to nourish the plant before nitrogen fixation starts (Caballero-Mellado and Martinez-Romero, 1999). Such application is done at a low rate of 7 kg ha^{-1}. When large amounts of nitrogen are applied, the plant literally slows or shuts down nitrogen uptake from the soil than to fix it from the air (Martinez-Romero, 2002).

2.3.2 Host specificity and effectiveness of rhizobia

There are roughly 1300 leguminous plant species in the world (Deoliveira, 1990). Of these nearly 10% have been examined for nodulation and 87% found to form nodules. Therefore not all legumes are colonized by rhizobia. Whereas legumes like *Gliricidia sepium* and *Vigna unguiculata* nodulate freely, nodules have never been found on roots of *Cassia siamea* (Castro et al., 1999). Most rhizobia are host specific. For example, rhizobia that nodulates cowpea may not nodulate *Leucaena* and vice versa (Fening, 2002). Leguminous species mutually susceptible to nodulation by a particular group of bacteria constitute a cross-inoculation group (Norris and Date, 1976). Table 1 gives a short list of rhizobia and their hosts to illustrate the grouping of rhizobia

Not all symbioses fix N_2 with equal effectiveness. This means that a given legume cultivar nodulated by different strains of the same species of rhizobia would fix different amounts of nitrogen. Selection of elite strains of rhizobia is based on this observation. Similarly, given strains of rhizobia will nodulate and fix different amounts of N_2 in symbiosis with a range of cultivars of the same plant species (So et al., 1994).

Table 1: A short list of Rhizobium species and their corresponding hosts

Rhizobium species	**Host plants**
Bradyrhizobium japonicum	*Glycine max* (soybean)
Rhizobium fredii	*Glycine max* (soybean)
R. phaseoli	*Phaseolus vulgaris* (common bean)
R. meliloti	*Medicago sativa* (alfalfa)
	Melilotus sp. (sweet clovers)
R. trifolii	*Trifolium* sp.(clovers)
R. Ieguminosarum	*Pisum sativum* (peas)
	Vicia faba (broad bean)
"Cowpea rhizobia" group	*Vigna unguiculata* (cowpea),
	Arachis hypogaea (peanut),
	Vigna subterranea (Bambara groundnut)
	Leucaena sp., *Albizia* sp.,
Azorhizobium caulinodans	*Sesbania* sp. *Sesbania rostrata* (stem nodulating)

Thus, different provenances of a given legume (e.g. *Gliricidia sepium)* can nodulate and fix nitrogen at different levels when they are established in the same field. Also, the free-nodulating *Gliricidia* or promiscuous varieties of soybean can nodulate profusely and fix a great deal of nitrogen depending on the effectiveness of the rhizobial populations present (Sanginga, 2000).

Groundnut is nodulated by a large group of rhizobia classified as cowpea miscellany (Putnan, 1991; Jauron, 1997). Most of the cultivated soils have large population of these rhizobia and ensure normal to abundant nodule formation in the groundnut crop (Jauron, 1997). However, sufficient nodulation does not mean high nitrogen fixation. Some of the nodules are found to be ineffective and do not fix adequate nitrogen required by the groundnut plant (Putnan, 1991). The effective nodules are big and pink and concentrate on the tap root and the top lateral roots. Ineffective nodules are small, green or white and are

distributed throughout the root system. To ensure effective nodules, the groundnut crop has to be provided with highly efficient rhizobia in the vicinity of its root system. This can be achieved by artificially inoculating the seeds with pre-selected effective and efficient strain (Yao, 2004).

2.4 Factors affecting BNF

Interactions between the microsymbiont and the plant are complicated by edaphic, climatic and management factors. A legume – rhizobium symbiosis might perform well in a loamy soil but not in a sandy soil, in the sub humid region but not in the desert or under tillage but not in no-till plots. These factors affect the microsymbiont, the host-plant or both (Svenning et al., 1993).

2.4.1 Edaphic factors

Edaphic factors relate to the soil suitability to support plant life. The six main edaphic factors limiting biological nitrogen fixation are excessive soil moisture, drought, soil acidity, phosphorus deficiency, excess minerals and deficiencies of Ca, Mo, Co and B (Gupta, 1991; Campo, 1998; Carpena, 2000).

Excessive moisture and water logging prevent the development of root hairs and sites of nodulation (Hardason et al., 1989). Waterlogging also interfere with diffusion of O_2 in the root system of plants.

Drought reduces the number of rhizobia in soils, and inhibits nodulation as well as N_2 fixation. Prolonged drought will promote nodule senescence **(**Sprent and Zahran, 1988**)**. Deep-rooted legumes exploiting moisture in lower soil layers can continue fixing N_2 when

the soil is drying. Mycorrhizal infection has also been found to improve tolerance of plants to drought (Sprent and Sprent, 1989). For example, *Acacia auriculiformis* inoculated with the ectomycorrhizal *Baletus suillus* is tolerant to low or excess moisture stress (Brockwell et al., 1982). Survival of *Rhizobium* under water logged conditions is reduced leading to poor nitrogen fixation (Yao, 2004). Groundnut is grown during the long and/ or short rains in certain parts of Kenya. This means that there is possibility of the crop experiencing excess or insufficient soil moisture depending on the season of growth, with subsequent effect on association with rhizobia as well as nitrogen fixation.

Soil acidity and related problems of Ca deficiency, aluminum or manganese toxicity adversely affect nodulation, N_2 fixation and plant growth (Carter et al., 1995). Low soil pH negatively influences crop production either directly or indirectly, mostly by limiting plant growth and development. At low soil pH nodulation and nodule development are strongly depressed through the inhibition of genetic activity (Alva et al., 1990).

At pH below 5.5, toxic aluminium species become abundant in the soil solution. These can block the sites where calcium is normally taken in on the membranes of young roots. Aluminum also interferes with the metabolism of phosphorus containing compounds essential for energy transfer (ATP) and genetic coding (DNA) and restricts cell wall expansion (Brady and Weil, 2002).

In legumes, aluminum restricts entry of growth promoting substances like cytokinins into the root tissues hence curtailing tissue proliferation and eventual root nodule formation (Troeh and Thompson, 1993).

Soil acidity could also be accompanied by manganese toxicity. This interferes with metabolic processes of the plant by causing iron antagonism. As a result of soil acidity, molybdenum becomes unavailable in the soil and this restricts the performance of leguminous plants (Dilworth et al., 2001). Research on the identification of symbioses adapted to acid soil should focus on the host plant, because effective rhizobia adapted to soil acidity can be found naturally and some could also be produced through genetic manipulations. Both soil acidity and Al toxicity limit nodulation, N_2 fixation and growth of groundnut (Franco and Munns, 1982). Therefore, rhizobia that are introduced by inoculation must tolerate these local soil factors in order to nodulate and effectively fix N_2 with the host legume like groundnut.

Phosphorus deficiency is widespread place in tropical Africa and reduces nodulation, N_2 fixation and plant growth (Graham and Vance, 2000). Cultivation of plant genotypes adapted to low-P soils can be good strategy to overcome this soil constraint (Almandras and Bottomley, 1987). Rhizobial P deficiencies occur when there is a low available P in the soil and rhizosphere, especially under acidic conditions, where dissolved phosphorus salts may be precipitated in the presence of aluminum (Sessitsch et al., 2002). Slow-growing strains of rhizobia appear more tolerant to low P levels than the fast-growing rhizobia (Taurian et al., 2002). The role of mycorrhizal fungi in increasing plant P uptake with beneficial effects on N_2 fixation has been reported (Stevenson, 1999). Dual inoculation with effective rhizobia and mycorrhizal fungi has been reported to have synergistic effects on nodulation and N_2 fixation in low P soils (Sprent and Sprent, 1989). P-solubilizing microorganisms, particularly of the genera *Bacillus, Penicillium,* and *Aspergillus* can solubilize rock phosphate and organically bound soil P that constitutes 95 -

99% of the total phosphate in soils. However, the use of these microorganisms alongside rhizobial inoculants is not widespread. The use of local rock phosphate has been recommended, particularly in acid soils, as an inexpensive source of P **(**Smithson et al., 2003).

Some reports show nodulation response to K under field conditions (Almandras and Bottomley, 1987). However, other investigators consider the K effect to be indirect, acting through the physiology of the plant (Stevenson, 1999).

Mineral N is another soil component that inhibits both the *Rhizobial* infection process and N_2 fixation. Inhibition of the infection process probably results from impairment of the recognition mechanisms by nitrates, while nitrogen fixation failure occurs due to the diversion of photosynthates toward assimilation of nitrates (Gupta, 1991).

Some strains of rhizobia, and particularly stem-nodulating *Azorhizobium caulinodans*, fix N_2 actively even when plants are growing in high-N soils, for example, in the presence of 200 kg fertilizer N ha^{-1} (Palanipappan et al., 1997). However, in most cases, 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 the overall N_2 fixation of root nodule forming legumes. The amount of this starter N must be defined in relation to available soil N (Msrivani, 2009). It is estimated that a well nodulated groundnut under normal growth conditions at soil pH of 4.3-6.8 is capable of fixing about 180 kg N ha^{-1} (Pimratch et al., 2008). Hence it is a cheaper and cost effective way for adequate nitrogen supply and this can be higher when the crop is inoculated with the more effective rhizobial strains.

Various microelements (Cu, Mo, Co, B) are necessary for N_2 fixation. Some of these are components of nitrogenase for example Mo. These micronutrients could become unavailable in saline or acidic soils (Zahran, 1999), a situation that depresses nitrogen fixation.

2.4.2 Climatic factors

The two important climatic determinants affecting BNF are temperature and light.

Extreme temperatures affect N_2 fixation adversely. This is because N_2 fixation is an enzymatic process. However, there are differences among symbiotic systems in their ability to tolerate high (>35°C) and low (<25°C) temperatures (Sanginga, 2000).

The availability of light regulates photosynthesis, upon which biological nitrogen fixation depends. Photosynthetic rate affects nodule metabolism directly. This is demonstrated by diurnal variations in nitrogenase activity (Chelule, 2007). Very few plants can fix N_2 under shade (Gupta, 1991**)**. In alley farming if hedgerows are not weeded, or if trees are planted with food crops, their nitrogen fixation and growth will be reduced due to shading (So et al., 1994). Early growth of legume trees is slow and in addition they cannot compete successfully for light.

Shading has been reported to greatly affect field grown groundnut (Castro et al., 1999). When these legumes are continuously shaded their overall capacity to fix N is likely to be impaired since growth and photosynthesis will be limited. Castro et al. (1999) reported that groundnuts that experienced prolonged shading during their growth had fewer nodules and lower N_2 fixation per plant.

2.4.3 Biotic factors

Among biotic factors, the absence of the required rhizobia species constitutes the major constraint to the nitrogen fixation process. The other limiting biotic factors include excessive defoliation of host plant, crop competition, insects and nematodes (Sanginga, 2000).

Inoculation of legumes **is** important if specific and effective rhizobia are absent in the soil, or if they are present in low numbers. Inoculation introduces the rhizobia into that soil to ensure proper nodulation and nitrogen fixation. Where there are specific and effective rhizobia in a sufficient number, there will be no need to inoculate the legume. In agrisystems, whenever one is not sure of the presence and effectiveness of the native rhizobia, it could be necessary to inoculate the legume with an ideal strain (Fening, 2002).

An accurate relative effectiveness trial will provide more precise information. The trial consists of growing the legume with and without fertilizer N while controlling all other limiting factors (Lodeiro, 2001). When the rhizobia in a soil are capable of colonizing and nodulating a legume but poorly effective, they constitute a barrier to the successful exploitation of rhizobia inoculants (Gupta, 1991**)**. Introduced rhizobia must therefore be more aggressive and competitive as nodulators than the native strains in order to nodulate the legume first. Inoculant rhizobia usually persist in the soil for long periods, particularly when the host is cultivated frequently or is permanent. Persistence of a strain is desirable because it obviates the need for inoculation in subsequent years, assuming inoculant strains maintain their original effectiveness (Mc Dermot, 1990).

Inoculation with rhizobia is usually recommended for newly introduced legumes (Wacek and Triplett, 1994). Most positive responses to inoculation are confined to crops which have specific requirements for rhizobia, including *Leucaena leucocephala* and American varieties of soybean (Gupta, 1991**)**. Groundnuts have been reported to show positive response to inoculation in acid soils (Brown, 2004). Indigenous legumes seldom respond to inoculation with introduced rhizobia because they nodulate with resident strains, even if these native rhizobia are not the most effective ones (Wange, 1989). Therefore it is important to evaluate native strains for effective nodulation and N-fixation.

Defoliation, crop competition and pests affect the performance of crops. For instance, pruning and lopping decreases the photosynthetic ability of legumes. It impairs N_2 fixation and can lead to nodule decay (Montanez et al., 1995). For perennial legumes, nodule decay sheds a high number of rhizobia into the root zone. When new roots develop in subsequent vegetative cycles, nodulation of the legume is expected to improve (Sanginga, 2000).

Intercropping legumes with non-leguminous crops can result in competition for water and nutrient and shading. The competition can limit N_2 fixation negatively (Chelule, 2007). However, it has been shown that pasture legumes are weak competitors for soil N if grown with grasses (Gupta, 1991). If this finding can be extrapolated to cereals, then it follows that in a soil which is deficient in N the cereal crop will absorb most of the mineral N. This will compel the legume to fix more N than in a situation in which it is growing alone, provided other factors, such as light and water, are not limiting.

Insects and nematodes have also been reported to interfere with nodule formation, development and functions by damaging the roots and rarely form nodules (Fening, 2002).

2.5 Tolerance of rhizobia to soil acidity and aluminium stress

It has been estimated that over 50% of the world potentially arable land are acidic (Bot et al., 2000) posing a worldwide limitation to crop production. In developing countries like Kenya where food production is critical, acid soils occur up to 13% (Kanyanjua et al 2002). Aluminium toxicity as a result of acidity is a major problem in acid soils and it has been known for long that many plant species including legumes show great variability in response to aluminium stress (Bona et al., 1993). Aluminium in soil occurs as insoluble alumino-silicates and oxides (Hoekenga et al., 2003). As the soil pH drops below 5, Al^{3+} is solubilized into the soil solution (Taylor, 1995). This form of aluminium appears to be the most rhizotoxic aluminium species (Kinraide, 1991; Kinraide and Parker, 1990).

Soil acidity and aluminium toxicity generally reduces nodulation in legumes by directly affecting the host plant. Aluminium binds to the charge sites on the cell wall surface thus reducing movement of water and mineral nutrients through the cell wall interstices (Blamey and Dowling, 1995). High soil acidity also affects nodulation, N_2 fixation and plant growth. Mugwe et al. (2007) attributed poor nodulation of legumes to soil acidity, low soil P and lack of adequate indigenous rhizobia in Chuka, Eastern Kenya.

2.5.1 Screening for acid tolerance and BNF effectiveness

The ability of the rhizobia to nodulate crops in acid soil is not well studied. In general, most rhizobial strains which nodulate tropical legumes do not grow in culture below pH of 4.0 (Castro et al., 1999). Yet some soils in Kenya have lower pH range than this (Kanyanjua et al., 2002). Therefore, it is important to screen such soils for presence of rhizobia that can do well under the acidic conditions since the indigenous rhizobia population size is an important factor in symbiotic nitrogen fixation.

The variability for acid tolerance among strains of rhizobia presents an opportunity for selection of naturally occurring acid tolerant strains (Bogino et al., 2006). These strains may be matched with a suitable variety of host plants to improve nodulation and dinitrogen fixation in acid soils. It is worth noting that rhizobia's acid tolerance does not automatically mean it is effective in dinitrogen fixation (Guene et al., 2004**)**. Furthermore, the selected acid tolerant rhizobia strain must be able to compete favorably with the native rhizobia for the limited nodulation sites on the roots to be able to colonize nodulate and fix atmospheric nitrogen.

Studies with *Bradyrhizobium japonicum* show that a strain which gave satisfactory nodulation in greenhouse plants, when introduced into the field was unable to compete favorably with less effective native rhizobia (Meghvansia et al., 2008). Therefore, it is necessary to test the selected acid tolerant strains for their N fixation effectiveness before recommending them for use as inoculants.

Acid tolerant rhizobia in different parts of the world for L*eucaena* has come up with some commercial strains including TAL 582 (Australia), TAL1145 (NifTAL, Hawaii), and TAL1887 (Malaysia). But even with this selected acid tolerant strains, experiments show that some of the strains are less effective in certain soils (Sanginga et al., 1989). This points to the need to select appropriate rhizobia for localities with different soil types.

2.5.2 Screening for tolerance of rhizobia to aluminium

Soil acidity increases solubility of iron, aluminium and manganese and causes precipitation of soluble phosphates in the soil. Aluminium concentration in mineral soil solutions are usually below 1 mg per litre, equivalent to about 37 µM Al at pH 5.5, but rises sharply and

reaches toxic levels at pH 5 (Marschner, 1995; FAO, 1984). Aluminium toxicity is considered the most severe component of stress in acidic soils. Aluminium has impacts on growth and survival of legumes as well as rhizobia (Rowell, 1988). Soil acidity and aluminium toxicity have been reported to be the major limiting factors to nodulation in Phaseolus vulgaris (Franco and Munns, 1982).

Strains of *Rhizobium* (Vargas and Graham, 1988) and Bradyrhizobium (Graham, 1992) that were resistant to aluminum (50 μM) at low pH (<5.0) were identified; however, rhizobia from clover were sensitive to these conditions (Wood et al., 1988). Absorbed Al may bind to DNA of both sensitive and tolerant strains but DNA synthesis by the tolerant strains of *R. loti* was not affected. However, Richardson et al. (1988) found that 7.5 μM Al depressed *nod* gene expression at low pH (4.8). Therefore, for acid soils with high Al content, improvement could be achieved by manipulating the plant rather than the rhizobia (Taylor et al., 1991).

Screening rhizobia for tolerance to Al is commonly done *In Vitro* at pH 4.5 and an Al concentration of 50 μM. Most rhizobia are sensitive to Al at this concentration. Hence screening rhizobia for Al tolerance at low pH enables selection for acid-Al effective rhizobia isolates that can enhance N-fixing potential of the groundnut crop. This is supported by studies of Muok (1997) in which selected acid-Al tolerant rhizobia from the laboratoty showed the best N-fixing ability in the field with tree legumes. In another study, Chemwetich (2004) while screening indigenous *Rhizobium* for acid tolerance to improve nodulation in common beans also reported significant improvement on nodule biomass for plants inoculated with acid-Al tolerant strains isolated from the laboratory.

2.5.3 Lime and its effects on groundnut and rhizobia

Liming is one of the most cost effective methods of slowing the effects of acidification and reducing the toxic effects of metals especially aluminium, copper, cadmium, lead, nickel and zinc in the soil (Paramananthan, 2000). Lime contains Ca which gets adsorbed to the soil colloids, joining the existing Ca pool in soils thus alleviating Ca deficiencies (Paramananthan, 2000). Similarly, liming increases the soil pH with a concomitant reduction in Al and Mn toxicities. Limes containing Mg also provide this nutrient to the soil thereby alleviating its deficiency. Due to lime application, Ca/Al ratio in the soil solution is expected to increase substantially. According to Shamshuddin et al. (1991), a Ca/Al ratio > 79 is required for good growth of maize and groundnut. It appears that at a high rate of lime application (> 4 t/ha), the ratio approaches this value. Hence, there is justification to apply lime to ameliorate acid soils, such as ferralsols and acrisols common in western Kenya. At this rate of application of lime, Shamshuddin et al. (1998) found that the ameliorative effects would last more than 4 years. If groundnut is grown alone, then 1 tone of lime is sufficient to supply enough Ca and Mg to the growing crop. This is because unlike maize, groundnut is moderately tolerant to soil acidity. Therefore, it would be a viable practice to apply a small dosage of lime on soils cultivated with groundnut. It has been demonstrated that groundnut benefits from lime application through increased availability and uptake of nutrients namely N, P, K, S Ca and Mg (Ranjit et al., 2007).

Although it is possible to correct soil acidity by direct liming (Lulandana and Hall, 1991), the manner in which liming affects growth and performance of rhizobia is still not fully understood (Paramananthan, 2000). Lime application was found to increase the survival and persistence of *Sinorhizobium* in acid soils (Andrade et al., 2002). The calcium supplied by lime may perform a variety of functions in both the host legume and its microsymbiont

rhizobia. It is an essential component in symbiotic N_2 fixation and nodule formation in legumes. Ca-deficiency in legumes has been found to depress the Ca content of nodules, impairing nitrogen fixation due to inadequate calcium for nodule structure and/or function (Graham, 1992). Calcium is implicated in plant-rhizobia signaling and host recognition, leading to root hair infection and nodule formation (Bonilla and Bolaños, 2009). Ca increases the activity of *Nod* genes and is required for optimal colonization of the host root system.

Lime application and acid tolerant efficient rhizobia are useful for improved symbiosis and legume performance in acid soils. This has been demonstrated in several studies (Zahran 1999, Guo et., 2010). Kisinyo et al. (2012) showed that combined use of lime, acid tolerant rhizobia inoculants and P fertilizer enhanced the performance of *Sesbania sesban.* Therefore, it would be necessary to test the effect of liming on groundnut response to inoculation with acid tolerant rhizobia.

2.6 Genetic diversity of rhizobia

It is important to identify rhizobial strains with useful agronomic properties, including those having high efficiency in nodulation and dinitrogen fixation or tolerance to soil acidity and toxic aluminum concentrations. In ecological and agronomic studies, one may want to know the persistence or survival of released strains in the soil or to identify a particular strain in root nodules. However, rhizobial identification is generally difficult, given that strains having different physiological properties may often be morphologically indistinguishable in culture and under a microscope. The official classification of the genus

Bradyrhizobium as presented in Bergey's manual of systematic Bacteriology (Jordan, 1984), considers only phenotypic features and mol% G+C.

For a long time, genotyping has been done by various methods such as DNA (rRNA) nucleotide sequence analysis, amino acid sequence analysis, DNA: DNA hybridization, DNA: rRNA hybridization (Iteman et al., 2000). In the past, molecular markers such as Restriction Fragment Length Polymorphism (RFLP), fingerprinting for repetitive sequences in the genome RNA oligonucleotide cataloguing and mol % guanine plus cytosine (G+C%) of total DNA have been used (Rossum et al., 1994). However, for low complex DNA like plasmid, 16S-23S rDNA amplification and analysis should be used. This is because the ribosomal intergenic spacer region (IGS) located between the 16S rRNA and 23S rRNA genes shows a high degree of length and sequence variation and holds potential for intraspecies discrimination (Gürtler and Stanisich, 1996; Buchan et al., 2001; Sadeghifard et al., 2006). Indeed, its sequence variability has been successfully exploited to edit probes and primers allowing species or subspecies discrimination when applied on clinical or environmental isolates (Glennon et al., 1996; Rachman et al., 2004; Valcheva et al., 2007). To date, the internal spacer regions (ISR) to design strain-specific PCR primers and develop tools for environmental detection of bacteria has been successfully applied only for rhizobia inoculants (Tan et al., 2001).

Whereas 16S rRNA sequence analysis is a powerful tool for inferring inter- or intrageneric relationships, due to the strong conservation of its nucleotide sequence across species and genera, the 16S–23S internally transcribed sequence (ITS) region, which shows a faster rate of evolution, provides information concerning intraspecific relationships (Gürtler and

Stanisich, 1996). Therefore, the use of ITS in phylogenetic studies is limited to detecting recently diverged species and to the typing of bacteria.

One of the molecular methods which are utilized in taxonomic studies of organisms including bacteria is the restriction fragment length polymorphism (RFLP) (Domezas et al., 1991). When this method is used to study the rDNA region, PCR-amplified products of either the 16S rRNA gene or the IGS are digested using restriction endonucleases and separated by agarose or polyacrylamide gel electrophoresis and scored for fragment size. The method is highly reproducible and is commonly used to explore variability among bacteria (insert appropriate refs similar to the work you did in UG recently).

Williams et al. (1990) developed molecular techniques for bacterial identification based on the property of the DNA known as Randomly Amplified Polymorphic DNA (RAPD). This method utilizes polymerase chain reaction (PCR) based DNA amplification that depends on 10 base pair (bp) random/ arbitrary primers. The method is highly discriminative and has abundant markers capable of distinguishing even closely related individuals (Gudu et al., 1993). In some cases RAPD analysis has been used in combination with sequencing of the hyper variable region of 16S rRNA in order to properly define genetic diversity in rhizobia (Young et al., 1991).

2.7 Cropping System

Cropping system design is not a mechanical or automatic process; it must be developed from an understanding that agricultural practices, that are to be evolved and adopted

successfully across an ecological zone, in which a minor adjustment of the system should provide the prospect of exploiting any potential to the optimum **(**Simpson 1976**).**

Intercropping legumes with non-legumes has proved to be beneficial for subsistence farmers in the tropics and subtropics which are limited by low crop productivity and inflexible land tenure systems (Abiadoo, 1987). Megueni et al. (2006) outlined advantages of intercropping systems such as profit and resources maximization, built-in balanced nutritional supply of energy and also an improvement in soil fertility. Ngo Nkot (2009) reported the system provided greater stability toward higher yield. The individual yield of forage legumes and companion crops are generally lower with intercropping than in monocrops. The decrease in biomass production has been attributed to competition for light, moisture and nutrients. Several workers (Willey, 1979; Reddy and Willey, 1979; Baker and Yusuf, 1976) considered light to be the most important factor in competition, particularly when the crops are of different durations. At the same time, higher soil nutrient removal in intercropped systems has been reported (Fraga and Salcedo, 2004) and soil fertility may decrease more quickly (Bado, 2002). Lindström et al. (2010) reported no increase in N-fixing ability by legumes when intercroppoed with non legumes. Cropping systems may affect soil functioning along with diversity and occurrence of beneficial micro-organisms (Nwaga et al., 2010). Therefore, in order to determine BNF effectiveness of groundnut inoculated with different rhizobia isolates, groundnut was grown as a monocrop to reduce competition between crops and to maximize N-fixing ability of the crop.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant Materials and Chemicals

Complete groundnut plants were collected at nodulation from different groundnut growing areas of western Kenya to isolate and characterize root nodule rhizobia. All the chemicals for culture media were procured from Lobachemie. One kilogram of groundnut seeds per variety (*Red Valencia*, ICGV 9991, ICGV 12991 and ICGVSM 99568) was obtained from Kenya Agricultural Research Institute (KARI) Kisii station.

3.2 Nodule Collection and Preservation

At least ten groundnut seedlings were uprooted carefully from the farmer's field and each seedling packed in a separate bag and carried to the laboratory for further assessment. In the laboratory roots were washed carefully in under a gentle stream of running water. The nodules were removed randomly from each seedling by severing the roots 0.5 cm on either side of nodules attachments to facilitate handling. The nodules were then cleaned superficially and blotted dry on a paper towel before placing them in a container with anhydrous calcium chloride. The nodules were separated from the desiccant by placing a layer of cotton wool below and above. The container was then tightly closed and kept in the refrigerator.

3.3 Isolation of rhizobia from root nodules

The rhizobia were isolated on Yeast Extract Mannitol Agar (YEMA). The medium was prepared according to Somasegaran and Hoben (1994) (Appendix1a). The medium was

placed on a magnetic stirrer to form a homogeneous mixture and pH adjusted to 6.8 by adding 1M HCl or1M NaOH and then agar. Either 0.25mg Congo red or 0.5mg Bromothymol Blue (BTB) (Appendix 1b) per litre of medium was added from stock solutions before autoclaving at 121^0C for 20 minutes. The medium was allowed to cool to about 50^0C and then poured into sterile Petri dishes and allowed to solidify overnight in a laminar flow hood.

3.3.1 Isolation Procedures

Five healthy nodules from every collection unit (single seedling) section 3.3 were selected randomly and rehydrated by soaking in sterile distilledwater for two hours. The nodules were sterilized by immersion for 10 seconds in 95% ethanol, then for 2 minutes in 5% solution of sodium hypochlorite for further surface sterilization. The nodules were removed from the sterilant and rinsed in six changes of sterile distilled water.

Individual nodules were crushed in a drop of sterile distilled water under aseptic conditions to give a turbid suspension. Using a sterile inoculation loop, a loopful of squashed nodule was streaked on the surface of a YEMA plate. The loop was flamed after every streak to avoid cross inoculation of plates. The streak was done in a way as to progressively dilute the suspension to a stage where isolated colonies could be produced (serial dilution). The plates were incubated in an inverted position at 28^0C until colonies appeared. Observation and careful examination was done every day to detect the progress of the cultures and presence of any contamination. A single colony of rhizobia was picked from every plate and restreaked to purify. Where multiple infection was suspected, serial dilution was done according to Somasegaran and Hoben (1994) to separate the strains.

3.3.2 Growth Characteristics

Growth on Congo red was used to classify the strains as very fast if the colonies appeared within 12 hours, fast growers for those which appeared between 48-72 hours, moderate 96-120 hours and slow for those that took more than 120 hours. The reaction with BTB was also recorded, classifying them as acid producing if the colonies turned BTB from green to yellow or as alkaline producing if they turned BTB from green to blue.

3.3.3 Morphological assays

The morphological traits evaluated were colony morphology and mucus production. Mucus production was based on type, elasticity and appearance whereas colony morphology included diameter, form, transparency and colour (Aneja, 2003). Gram staining reaction was performed as per the standard gram's procedure (Somasegaran and Hoben, 1994) to visualize cell morphology and type of staining under a light microscope.

3.4 Metabolic tests

3.4.1 Determination of nitrate reduction

Nitrate reduction test was performed to determine the isolates ability to reduce nitrate to nitrite. Nitrate broth (1 g KNO_3, 5 g Peptone, 3 g beef extract) was inoculated with suitable rhizobia culture and then it was incubated at 28 ^{0}C for 48 hours. Three drops of reagent: (2 g zinc chloride, 4 g starch, 2 g Potassium iodide, distilled water 1litre), and 1 drop of dilute sulphuric acid (1 acid:3H_2O) was dispensed into a porcelain plate after which one drop of nitrate broth culture was transferred to it. Observation was made for color change and in cases where there was no change in color 1g of powdered zinc metal was added to the

medium on the porcelain plate and observation for colour change from blue to colourless observed.

3.4.2 Determination of Urease production (urea hydrolysis)

Urease production test was performed to determine if the isolates were capable of reducing urea to ammonia. Urea broth medium (20 g urea, 0.1 g yeast extract, 9 g KH_2PO_4, 9.5 g K_2HPO_4, 0.01 g phenol red, 1 litre distilled water and of pH 6.8) was inoculated with rhizobial culture and the culture incubated at 28^0C for 48 hours. Colour change from blue to colourless was taken to be positive for the test and where the blue colour was retained was taken to be negative result

3.4.3 Citrate utilization by rhizobia

Citrate utilization test was done to determine the ability of the isolates to utilize citrate as the only source of carbon and ammonia as nitrogen. Simmon's citrate agar medium (2 g sodium citrate, 0.2 g $MgSO_4$, 1 g $(NH_4)H_2PO_4$, 1 g K_2HPO_4, 5 g NaCl, 0.08 g BTB, 15 g Agar, distilled water 1litre, pH 6.8) was used to prepare slants which were inoculated by stabbing to the base of the slant and thereafter streaking the surface. The tubes were then incubated at 28^0C for 48 hours. A sign of citrate utilization was observed in colour change from green to blue.

3.5 Screening of Rhizobium for Acid-Aluminum stress

The aim of this experiment was to select acid-Aluminium tolerant isolates that are effective in nitrogen fixation.

3.5.1 Screening for tolerance to acidity

Two media containing the basal solution of Kyser and Munns (1979) but with galactose and arabinose, at 5 g/L each, instead of mannitol was used to test the response of rhizobia to acid-stress. The basal solution contained (µM): 300 $MgSO_4$; 300 CaCl2; 10 FeEDTA; 10 KCl; 1 $MnCl_2$; 0.4 $ZnSO_4$; 0.1 $CuCl_2$; 0.02 Na_2MoO_4; 0.001 $Co(NO_3)_2$. The pH was adjusted using HCl or filter-sterilized 0.5M NaOH to a range of pH values 3.5, 4.5, 5.5 and 6.8. The medium was autoclaved and left to cool after which 1.8 g/l of sodium glutamate, 5 µM KH_2PO_4 and 1.5 mM KCl were added as filter-sterilized solution. Seven mililitres (7ml) of the medium was dispensed into universal bottles. Starter culture was prepared by aseptically scrapping a loopful of each culture and transferring it to 10 ml YEM broth in a universal bottle. The broth culture was placed on an orbital incubator shaking at 110 rpm at a temperature of 28^oC and left for two days to attain a cell density of about 10^9 cell/ml (Somasegaran and Hoben, 1994). A 0.1 ml aliquot of each isolate was drawn from the starter culture and introduced into the bottles representing each pH treatment in three replicates.

The bottles were placed in an orbital incubator at 28^oC and agitated at 110 rpm. The experiment was observed for visible turbidity on a 12-hour interval, over a period of four days. The number of hours taken by each isolate to form visible turbidity was recorded and used as a criterion to decide whether an isolate is tolerant to that particular pH. Those which formed visible turbidity within 96 hours were regarded as tolerant to the test pH.

3.5.2 Screening for tolerance to soluble Aluminium

Only rhizobia strains identified as acid tolerant were tested for tolerance to aluminium. The experiment was conducted in a defined basal solution (Kyser and Munns, 1979) (section

3.5.1). A pH of 4.5 was used in combination with four aluminium concentrations tested (0, 50, 100, and 130 µM or 200 for some isolates. The Al was added as filter-sterilized solution of 5 mM $AlK(SO_4)_2.12H_2O$ to the medium. The medium was dispensed in 7ml aliquot into universal bottles. A 0.1 ml of pre-cultured rhizobia prepared as described earlier (section 3.5.1) was introduced to each bottle. The cultures were incubated under similar conditions (section 3.5.1) and observed at a 12-hour interval for visible turbidity. After five days, the cultures were removed from the shaker and the number of days taken to achieve visible turbidity recorded.

3.6 Distinguishing Rhizobia from Agrobacterium

3.6.1 Growth on YEMA-Congo red medium

On Congo red incorporated YEMA medium (Appendix 1b), rhizobia stand out as white, translucent, glistening, elevated and comparatively smaller colonies with entire margins in contrast to red stained colonies of agrobacteria. Putative rhizobia isolates were streaked on YEMA-Congo red as previously described (section 3.3.2) and incubated at 28^0C for 2 days. Colony morphology and colour were recorded.

3.6.2 Hofer`s alkaline broth

Growth in Hofer`s alkaline broth was used to distinguish the isolates because agrobacteria grow at higher pH of up to 11, while rhizobia are unable to do so. The medium consisted of: 0.5 g K_2HPO_4; 0.2 g $MgSO_4$; 0.1 g NaCl; 0.05 g CaCO; 0.1 g yeast extract; 10 g manitol, and 1 litre water. The pH was adjusted to 11.0 by adding 28 ml of NaOH and 1 ml of 0.6% thymol blue.

3.6.3 Growth in Lactose agar

The growth of isolates in Lactose agar was also used to distinguish the isolates. This is because agrobacteria utilize lactose to form an oxidized product ketolactose, through the activity of the enzyme ketolactase while rhizobia do not.

Benedict`s reagent was prepared by dissolving 173 g sodium citrate and 100 g anhydrous sodium carbonate in 600 ml distilled water; 17.3 g crystalline copper sulphate was dissolved in 100 ml distilled water.

The latter solution was then added to the former with constant stirring; the mixture filtered and made up to 1000 ml with distilled water. The reagent was poured over agar medium containing lactose (10 g/ litre) on which the isolated nodule bacteria were growing. The formation of yellow coloration due to copper II oxide (Cu_2O) indicates the presence of agrobacteria in the culture plate.

3.6.4 Temperature assay

Agrobacteria grow up to a temperature of 30^0C whereas rhizobia can tolerate higher temperatures up to 44^0C. To analyze the effect of temperature variation on the growth of isolates, YEMA medium was prepared with pH 6.8 and after inoculation; the plates were incubated at 28^0C and 37^0C separately. Growth of the isolates at 37^0C was used to rule out the presence of agrobacteria in the culture.

3.6.5 Glucose peptone agar (GPA)

GPA assay was performed to determine which isolates could utilize glucose as the sole carbon source for growth. GPA medium comprising 40 g/L glucose, 5 g/L peptone, 15 g/L

agar, pH 6.8 was inoculated with bacterial culture, incubated at 28^{o}C and growth of colonies was observed.

3.7 Nodulation test

This test was done to verify that the isolates selected for acid-Al tolerance had the capacity to nodulate groundnut and carry out effective nitrogen fixation. All the Al tolerant isolates were tested by selecting a sample of twelve clean undamaged and uniform sized seeds of *Red Valencia* groundnut. The selected seeds were washed with detergent then sterilized using 10 ml of 1% JIK solution for 10 minutes, rinsed with sterile distilled water eight times for 10 minutes. The seeds were left to imbibe in sterile distilled water for 30 minutes after which they were plated aseptically onto in 0.8% (w/v) water agar in Petri dishes observing aseptic conditions. The seeds were germinated in an incubator at 28^{0}C for two days. The pre-germinated seeds were transplanted into growth pouches, having attained a radical length of 1-2 cm. One seedling was grown per pouch. Pouches were prepared by placing cotton wool in a polythene bag measuring 12.5 by17.5 cm before sealing.

Sterile N-free nutrient solution prepared according to Somasegaran and Hoben (1994) (Appendix 2) was dispensed into the growth pouches through a small hole. The seedlings were left to grow for three days before inoculation with the isolates selected for acid-Al tolerance. The seedlings were raised in a growth chamber for six weeks with constant monitoring and addition of the N-free media to maintain a favorable nutrition and humidity for the plant growth. At six weeks the experiment was terminated. The seedlings were removed from the growth pouches, roots checked for nodulation. The nodules were examined for effective nitrogen fixation by slicing through them using a surgical blade.

3.8 Effectiveness of Rhizobial Isolates to Cross Inoculation

To determine the symbiotic effectiveness of the individual isolates (nodulation and nitrogen fixation efficiency) with different groundnut cultivars, each of the four selected Al tolerantrhizobial strains was tested on four groundnut varieties (ICGV 9991, ICGV 12991, ICGVSM 99568 and *Red Valencia)* all obtained from KARI Kisii. The growth media used was one kilogram sand which was sieved, washed and sterilized before transfer to polythene bags measuring 12.5×17.5 cm.

Pre-germinated seeds that were prepared as described earlier (section 3.7) were planted in each bag before sealing. Inoculations were done in triplicate by adding 1 ml (10^9cell/ml) of the broth culture prepared as described in (section 3.5.1). Unlike the other experiments, this inoculation was done on the same day as planting. Two controls consisting of –N and + N uninoculated seedlings were included for every variety and strain. The +N control treatment was added as described earlier (section 3.7). The experiment was set in a completely randomized design in a greenhouse and maintained for 8 weeks. N-free media was added regularly to maintain the sand at or near field capacity. The experiment was terminated 8 weeks after planting. Data was collected on the plant height, number of nodules, total nodule fresh weight, shoot dry weight and nodule dry weight per plant. SPSS was used to analyze the data and the data subjected to analysis of variance (ANOVA). Where there was significant difference ($P \leq 0.05$), the means was separated by Duncan's multiple Range Test (DMRT).

3.9 Field Evaluation of Symbiotic Effectiveness of Isolates

The aim of this experiment was to determine the nodulation and N- fixation effectiveness of the isolates on groundnuts in a monocrop system with or without lime application.

3.9.1 Experimental site

Field experiments were conducted at Koyonzo and Ligala in western Kenya. Koyonzo (0^0 25' N, 35^0 04'E) Matungu district and Ligala (0^0 03'N, $34^0$25'E) Ugenya district (GOK, 1997).

Matungu District receives an annual rainfall of range between 1250 mm and 1800 mm. The area has two rainy seasons; the long rains occur from March to June and the short rains from August and October. The mean annual temperature varies between 21^0C and 25^0C. The soils are of well drained and deep to very deep, and are dark brown acrisols (GOK, 1997).

Ugenya District receives a bimodal rainfall pattern, with long rains occurs from March to June with the peak in April. Short rains fall from September to November with peak in October. The annual rainfall ranges between 800 and 2000 mm. The mean temperature varies between 27^0C and 30^0C. The soils are well drained, deep and friable. The predominant soils at the research sites were nitisols (GOK, 1997).

3.9.2 Initial site characterization

Soils were sampled for chemical analysis to assess the overall nutrient status of the sites at the onset of the experiment. For each experimental site soil samples were taken at a depth of 0-15 cm in a random distribution along a zig-zag line (W transformation) across the direction of the fields using a soil auger. Each soil sample was kept in a labeled plastic bag in which it was dried and stored until the chemical analysis was conducted.

Soil chemical analysis was done on properties which are known to affect rhizobial growth. Soil pH, nitrogen, phosphorus, carbon, and particle size were analyzed. Detailed procedures for soil analysis are presented in Appendix 3. Soil pH was measured using a glass electrode in a suspension of 5.0 g soil in 10 ml water after equilibrating for 60 min. Organic carbon was determined using the Nelson and Sommers (1975) oxidation method. Soil extraction for available P was done using the bicarbonate solution (0.5 M $NaHCO_3$ at pH 8.5) method (Olsen and Dean, 1965). Particle size analysis was carried out according to the procedure of Okalebo et al. (2002).

3.9.3 Experimental design, treatments and crop husbandry

The field experiments were conducted for two consecutive seasons: long rains crop from April to August 2011 (2011 LRs) and short rains crop from August to December 2011 (2011 SRs). The groundnut was *Red Valencia* variety which was planted as a sole crop. The first season and second season crops were grown in three different blocks on the same field. The ploughing was done by tractor until a fine tilth was achieved. A Randomized Complete Block Design (RCBD) with a split plot arrangement of treatments was used in both seasons (Figure 1). The size of each experimental plot was 5.0 x 4.5 m with an inter-plot spacing of 1 m and an inter-sub plot spacing of 0.5 m. Two different limes; calcitic lime, dolomitic lime was applied in each block. Lime was applied at the rate of 2 t ha^{-1}. The treatments; no rhizobia inoculation, rhizobia inoculation, starter-N fertilization (34 kg N ha^{-1}) and control (0 kg N ha^{-1}) were assigned to the individual subplots within a plot and each treatment were replicated three times. Three rhizobial isolates (W1, A6 and V2) obtained from the laboratory isolation in this study and one commercial strain (Biofix) obtained from MEA ® Limited Stores in Eldoret was used to inoculate groundnut seeds.

Peat-based inoculants containing gum arabic adhesive were used. At planting, that is four weeks after liming, phosphorous was applied to all plots in form of Triple Super Phosphate (TSP) at 26 kg ha^{-1}, Potassium in form of Murate of Potash (MOP) at 60 kg ha-^{1} and Nitrogen in form of Calcium Ammonium Nitrate (CAN) at 34 kg N ha^{-1}. MOP fertilizer was broadcasted on all plots before planting rows were dug. TSP fertilizer was banded along crop rows to a depth of 15 cm to avoid direct contact with seeds while CAN was broadcasted on N treated plots. The seeds were sowed at a spacing of 75 x 15 cm. The experimental fields were hand weeded.

	0	N		3	4		2	1
	2	4		1	0		3	N
R1	3	1		2	N		4	0
	Lime 1				Lime 3			Lime 2
R2	0	N		3	4		2	1
	2	4		1	0		3	N
	3	1		2	N		4	0
	Lime 2				Lime 1			Lime 3
R3	0	N		3	4		2	1
	2	4		1	0		3	N
	3	1		2	N		4	0
	Lime 3				Lime 2			Lime 1

Figure 1: Randomized Complete Block Design layout for the nine treatments in different experimental sites

Treatment key

0- no rhizobia inoculant; N-nitrogen in form of CAN

1- 3-indigenous rhizobia inoculants 4-commercial rhizobia inoculant (Biofix)

Lime 1-dolomitic lime; Lime 2-calcitic lime; Lime 3-control (no lime)

R1 –replication 1; R2- replication 2; R3-replication 3

3.9.4 Data collection and analysis

Sampling to determine number of nodules, nodule fresh weight, and nodule dry weight for all the treatments was conducted at 6 weeks after emergence (WAE) in both seasons. Three plants were carefully uprooted from each experimental plot by digging 15 cm around the plant using a spade to ensure that nodules did not remain in the soil. The roots were washed with clean tap water to remove all attached soil. The nodules along the tap root and four top lateral roots were detatched from the roots, active nodules were checked by slicing open big nodules, the nodules were then counted, their fresh weights taken, and then oven dried at 60°C for 48 hours after which the dry weights were determined. At physiological maturity, 60 plants were harvested from each experimental plot, excluding the outer rows and the outer guard plants in each row and the pods shelled. The grains were sun-dried and weighed.The shoots of the harvested plants were chopped into small sizes after cutting out the roots and oven dried in a paper bags at 60°C for 72 hours before determining their dry weight. Statistical analysis of the data was carried out using the SAS software version 11 (2009). Analysis of variance (ANOVA) was performed using the general linear models procedure (GLM). Treatment means were separated using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. Regression analysis was used to establish the relationship between pairs of variables.

3.10 Intrinsic Antibiotic Resistance

The aim of this experiment was to confirm that the inoculants used at planting were actually the ones that nodulated the groundnuts in the experimental field. The identity of inoculant strains in the nodules after the field experiment was determined by testing for their characteristic intrinsic antibiotic resistance (IAR). The IAR test was conducted using

specific antibiotics as described by Ladha and So (1994). Resistance and sensitivity of rhizobial isolates against designated antibiotics was determined by spot inoculation on YEMA supplemented by seven different antibiotics: kanamycin (100 µg/ml), gentamycin (250 µg/ml), streptomycin (100 µg/ml), chloramphenicol (100 µg/ml), ampicillin (250 µg/ml), nalidixic acid (50 µg/ml), polymyxin-B sulphate (50 µg/ml). The concentrations used with the antibiotics acted as the final solution used for this experiment after adjusting the pH of every solution to 6.8. The solutions were sterilized and poured in sterile petri dishes and left to solidify. A marker pen was used to partition the petri dish into two. One part of the petri dish was inoculated using the initial isolate (before taking to the field) while the second part of the petri dish was inoculated with the isolate recovered from the nodules after the field experiment. The petri dishes were incubated at 28^0C for four days and the colony numbers and morphologies noted after every 12 hours of incubation.

CHAPTER FOUR

RESULTS

4.1 Growth of isolates in Congo red and Bromothymol blue (BTB)

A total of 90 bacteria isolates were obtained from groundnut root nodules collected at various localities in western Kenya (Table 2). These isolates were designated as rhizobia based on their growth on the standard medium YEMA, colony characteristics and cell morphology. Based on growth on YEMA with Congo red as an indicator, 15 isolates were very fast growing, 52 fast growing whereas the remaining 23 isolates moderate to slow growing rhizobia. When the isolates were further cultured on YEMA plates containing Bromothymol Blue (BTB), 64 formed mucoid yellow colonies after two days of incubation. The remaining 26 isolates produced blue colonies (Plate 1: Table 3). This indicated acid and alkali production, respectively.

Table 2: Sites of nodule collection from various parts of western Kenya, and the respective culture codes assigned

Sites	Culture codes	Isolates							
Rabango	A	A1	A2	A3	A4	A5	A6	A7	A8
Koyonzo	B	B1	B2	B3	B4	B5	B6	B7	
Ugunja	C	C1	C2	C3	C4	C5	C6		
Bumala	D	D1	D2	D3	D4	D5			
Matayos	E	E1	E2	E3	E4				
Harambee	F	F1	F2	F3	F4	F5	F6	F7	F8
Ulafu	G	G1	G2	G3	G4	G5	G6	G7	
Awelo	H	H1							
Korinda	I	I1							
Bungoma central	L	L1	L2	L3	L5	L6			
Kiminini	M	M1	M2	M3	M4				
Malaba	N	N1	N2	N3					
Serem	Q	q4							
Khwisero	S	S1	S2	S3	S4	S5	S6	S7	S8
Musoli	T	T1	T2	T3	T4	T5	T6		
Shikunga	U	U8							
Emasatsi	V	V2							
Vijalo	W	W1							
Agalo	X	X1	X4						
Shivagala	Y	Y1							
Malava	Z	Z1							
Serene	B	b1							
Awendo	G	g1	g5	g6					
Port Victoria	H	h1	h4						
Oyugis	N	n2	n3						

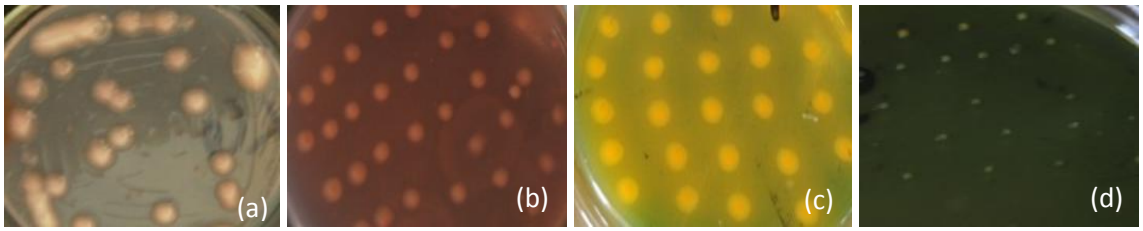

Plate 1: Growth characteristics of rhizobia on YEMA with Congo red or BTB
(a) Fast growing (b) slow growing on Congo red, (c) acid producer (d) alkali producer on BTB. (Source: Author, 2013)

4.2 Morplogical Assays

Colony and cell morphology of the isolates was variable (Plate 2, Table 3). Fast growing isolates formed globose or domed shape colonies reaching full growth after 3 to 4 days of incubation at 28 ^{0}C whereas slow growing colonies achieved good growth within 6 to 7 days of incubation under the same conditions. Fast growing isolates displayed large colonies of 3-4 mm diameter whereas slow growing isolates formed smaller colonies with diameters ranging between 1 and 2 mm. All the colonies were white with smooth entire margins. Microscopic examination of the isolates showed they were gram negative rod shaped cells.

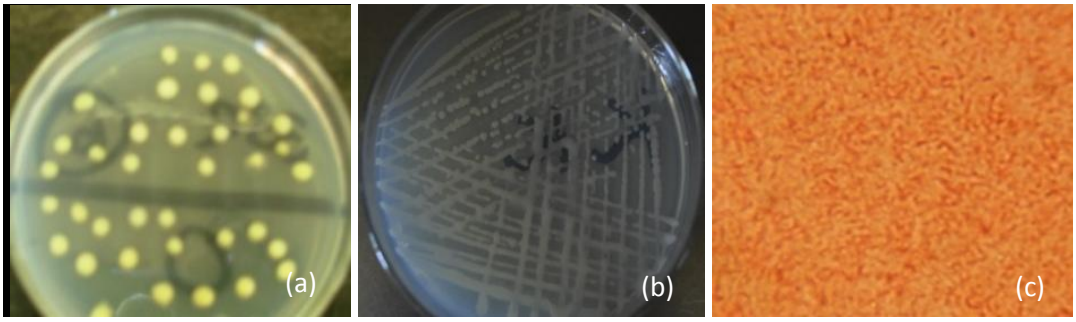

Plate 2: Colony morphology of fast and slow growing rhizobial isolates (a,b) and gram negative rods of rhizobial cells (c). (Source: Author, 2013)

Table 3: Colony and cell morphology of 84 rhizobial isolates cultured on YEMA at 28^0C for seven days

medium	Growth		Colony morphology				Cell morphology
Isolate	**Congo red**	**BTB**	**Diameter**	**Shape**	**Elevation**	**Consistency**	**Grouping**
A1	S	B	2mm	Semi globose	Raised	Mucoid	chains
A2	VF	A	4 mm	Domed	pulvinate	Mucoid	chains
A3	VF	A	4 mm	Domed	pulvinate	Mucoid	chains
A4	F	A	3 mm	Globose	pulvinate	Mucoid	chains
A5	F	A	3 mm	Globose	pulvinate	Mucoid	chains
A6	F	A	3 mm	Globose	pulvinate	Mucoid	chains
A7	F	A	3 mm	Globose	Raised	Mucoid	chains
A8	S	B	2 mm	Semi globose	pulvinate	Mucoid	chains
B1	F	A	3 mm	Globose	pulvinate	Mucoid	chains
b1	F	A	3 mm	Globose	Raised	Mucoid	chains
B2	F	A	3 mm	Globose	Raised	Mucoid	chains
B3	VF	A	4 mm	Domed	Raised	Mucoid	chains
B4	F	A	3 mm	Globose	pulvinate	Mucoid	Single
B5	S	B	2 mm	Semi globose	pulvinate	Mucoid	chains
B6	F	B	3 mm	Globose	Raised	Mucoid	chains
B7	S	B	2 mm	Semi globose	Raised	Viscous	chains
C1	S	B	2 mm	Semi globose	Raised	Mucoid	chains
C2	S	B	2 mm	Semi globose	Raised	Mucoid	paired
C3	S	B	2 mm	Semi globose	Raised	Viscous	chains
C4	S	B	2 mm	Semi globose	Raised	Mucoid	chains
C5	F	A	3 mm	Globose	Raised	Mucoid	chains
C6	F	A	3 mm	Globose	Raised	Mucoid	chains
D1	VF	A	4 mm	Domed	Raised	Mucoid	chains
D2	S	A	2 mm	Semi globose	Raised	Viscous	chains
D3	F	A	3 mm	Globose	Raised	Viscous	chains
D4	VF	A	4 mm	Domed	Raised	Mucoid	chains
D5	F	A	3 mm	Globose	pulvinate	Viscous	chains
E1	S	A	2 mm	Semi globose	Raised	Mucoid	chains

E2	F	A	3 mm	Globose	Raised	Mucoid	chains
E3	F	A	3 mm	Globose	pulvinate	Viscous	chains
E4	F	B	3 mm	Globose	Raised	Mucoid	chains
F1	VF	A	4 mm	Domed	Raised	Mucoid	Single
F2	F	A	3 mm	Globose	Raised	Mucoid	chains
F3	VF	A	4 mm	Domed	Raised	Mucoid	chains
F4	VF	A	4 mm	Domed	Raised	Mucoid	Single
F5	S	B	2 mm	Semi globose	Raised	Mucoid	chains
F6	S	B	2 mm	Semi globose	Raised	Mucoid	chains
F7	F	A	3 mm	Globose	Raised	Mucoid	chains
F8	VF	A	4 mm	Domed	Raised	Mucoid	chains
G1	VF	A	4 mm	Domed	Raised	Mucoid	chains
g1	F	A	3 mm	Globose	Raised	Viscous	Single
G2	S	B	2 mm	Semi globose	Raised	Viscous	chains
G3	S	B	2 mm	Semi globose	Raised	Mucoid	chains

A-acid producers, B-alkali producers, F- fast growing rhizobia, S-slow growing rhizobia, VF- very fast growing rhizobia.

Table 3 continued
Colony and cell morphology of 84 rhizobial isolates cultured on YEMA at 28^0C for seven days

	Growth medium		Colony morphology				Cell morphology
Isolate	**Congo red**	**BTB**	**Diameter**	**Shape**	**Elevation**	**Consistency**	**Grouping**
G4	F	A	3 mm	Globose	Raised	Mucoid	chains
G5	F	A	3 mm	Globose	Raised	Mucoid	chains
g5	F	A	3 mm	Globose	Raised	Mucoid	Single
G6	S	B	2 mm	Semi globose	Raised	Mucoid	chains
g6	F	A	3 mm	Globose	Raised	Mucoid	chains
G7	VF	A	4 mm	Domed	pulvinate	Mucoid	chains
H1	F	A	3 mm	Globose	Raised	Mucoid	paired
h1	VF	A	4 mm	Domed	Raised	Mucoid	Single
h4	F	A	4 mm	Domed	Raised	Mucoid	Single
I1	S	B	2 mm	Semi globose	pulvinate	Viscous	chains
L3	F	B	3mm	Globose	Raised	Mucoid	chains
L5	F	B	3 mm	Globose	Raised	Mucoid	chains
L6	F	A	3 mm	Globose	Raised	Viscous	chains
M1	S	B	2 mm	Semi globose	Raised	Mucoid	chains
M2	VF	A	4 mm	Domed	Raised	Viscous	chains
M3	F	A	4 mm	Domed	Raised	Mucoid	chains
M4	S	B	2 mm	Semi globose	Raised	Mucoid	chains
N1	S	B	2 mm	Semi globose	Raised	Mucoid	chains
N2	S	B	2 mm	Semi globose	pulvinate	Mucoid	chains
n2	F	B	3 mm	Globose	Raised	Mucoid	paired
N3	F	A	3 mm	Globose	Raised	Mucoid	chains
n3	F	A	3 mm	Globose	Raised	Mucoid	paired
q4	F	A	3 mm	Globose	Raised	Mucoid	single
S1	F	A	3 mm	Globose	Raised	Mucoid	single
S2	F	A	3 mm	Globose	pulvinate	Mucoid	single
S3	F	A	3 mm	Globose	Raised	Mucoid	chains
S4	F	A	3 mm	Globose	Raised	Mucoid	single
S5	F	A	3 mm	Globose	Raised	Mucoid	paired
S6	F	A	3 mm	Globose	Raised	Mucoid	chains
S7	F	A	3 mm	Globose	Raised	Mucoid	chains
S8	F	A	3 mm	Globose	Raised	Mucoid	paired
T4	F	A	3 mm	Globose	Raised	Mucoid	chains
T5	F	A	3 mm	Globose	Raised	Mucoid	paired

T6	F	A	3 mm	Globose	Raised	Mucoid	paired
U8	S	A	2 mm	Semi globose	Raised	Mucoid	chains
V2	F	A	3 mm	Globose	Raised	Mucoid	chains
W1	F	A	3 mm	Globose	Raised	Mucoid	paired
X1	S	B	2 mm	Semi globose	Raised	Mucoid	single
X4	F	A	3 mm	Globose	Raised	Mucoid	single
Y1	F	A	3 mm	Globose	Raised	Mucoid	chains
Z1	F	A	3 mm	Globose	Raised	Mucoid	single

A-acid producers, B-alkali producers, F- fast growing rhizobia, S-slow growing rhizobia, VF- very fast growing rhizobia. The six isolates (L1, L2, L4, T1, T2 and T3) excluded from the table were found to be gram positive.

4.3 Tolerance of Rhizobia to Acid-Aluminium stress

4.3.1 Selection of acid tolerant strains

Results on the growth response of eighty four (84) isolates of rhizobia to decreasing pH are presented in Table 4. The results revealed variability in acid tolerance among the isolates. This could be clearly detected at pH 3.5. At this pH, eight isolates showed turbidity after 12 hours, five after 24 hours, two after 36 hours, sixteen after 48 hours, six after 60 hours, ten after 72 hours and twenty six after 84 hours of culture incubation. The isolates N2, N1, A1, A4, A8, B7, C1, C2, C3, D5 and E1 did not show growth at this pH after 4 days of incubation.

At pH 4.5, fourteen isolates showed turbidity after 12 hours, two after 24 hours, six after 36 hours, twelve after 48 hours, eighteen after 60 hours, fourteen after 72 hours and ten after 84 hours in culture. A1, A8, B7, C1, C2, C3, N1and N2 did not show visible turbidity at this pH. All the isolates except B7, C1 and C3 grew at pH 5.5. Among those that could withstand this pH, fourteen isolates showed turbidity after 12 hours, four after 24 hours,

ten after 36 hours, twenty nine after 48 hours, seven after 60 hours, four after 72 hours and thirteen after 84 hours of incubation. Growth was observed in all the isolates tested in medium with a pH of 6.8. At this pH, fourteen isolates showed turbidity after 12 hours, seven after 24 hours, twenty four after 36 hours, eighteen after 48 hours, four after 60 hours, one after 72 hours and sixteen after 84 hours of incubation. From this experiment, 76 of the 84 isolates tested could be considered as acid tolerant at the pH 4.5, routinely used to screen rhizobia for tolerance to low pH. The 8 isolates that showed normal growth at pH 3.5 were regarded as highly tolerant to acidity.

Table 4: Growth of 84 groundnut rhizobia isolates to low pH in liquid basal media incubated at 28 ^{0}C

	Medium pH			
Isolate	**3.5**	**4.5**	**5.5**	**6.8**
A1	-	-	84 hrs	84 hrs
A2	12 hrs	12 hrs	12 hrs	12 hrs
A3	12 hrs	12 hrs	12 hrs	12 hrs
A4	-	60 hrs	48 hrs	36 hrs
A5	24 hrs	24 hrs	24 hrs	24 hrs
A6	24 hrs	24 hrs	24 hrs	24 hrs
A7	72 hrs	48 hrs	36 hrs	36 hrs
A8	-	-	84 hrs	84 hrs
B1	84 hrs	60 hrs	48 hrs	36 hrs
b1	48 hrs	36 hrs	24 hrs	24 hrs
B2	72 hrs	72 hrs	48 hrs	36 hrs
B3	12 hrs	12 hrs	12 hrs	12 hrs
B4	84 hrs	72 hrs	48 hrs	36 hrs
B5	84 hrs	72 hrs	60 hrs	36 hrs
B6	84 hrs	60 hrs	48 hrs	48 hrs
B7	-	-	-	84 hrs
C1	-	-	-	84 hrs
C2	-	-	84 hrs	84 hrs
C3	-	-	-	84 hrs
C4	84 hrs	84 hrs	84 hrs	84 hrs
C5	60 hrs	60 hrs	48 hrs	48 hrs
C6	84 hrs	72 hrs	72 hrs	60 hrs
D1	12 hrs	12 hrs	12 hrs	12 hrs

D2	84 hrs	72 hrs	60 hrs	60 hrs
D3	84 hrs	72 hrs	48 hrs	48 hrs
D4	12 hrs	12 hrs	12 hrs	12 hrs
D5	-	84 hrs	72 hrs	72 hrs
E1	-	12 hrs	12 hrs	12 hrs
E2	72 hrs	60 hrs	48 hrs	36 hrs
E3	84 hrs	60 hrs	48 hrs	36 hrs
E4	48 hrs	48 hrs	48 hrs	48 hrs
F1	24 hrs	12 hrs	12 hrs	12 hrs
F2	60 hrs	60 hrs	60 hrs	48 hrs
F3	24 hrs	12 hrs	12 hrs	12 hrs
F4	24 hrs	12 hrs	12 hrs	12 hrs
F5	36 hrs	36 hrs	24 hrs	24 hrs
F6	48 hrs	48 hrs	36 hrs	36 hrs
F7	48 hrs	48 hrs	36 hrs	36 hrs
F8	12 hrs	12 hrs	12 hrs	12 hrs
G1	12 hrs	12 hrs	12 hrs	12 hrs
g1	48 hrs	36 hrs	36 hrs	24 hrs

Note: hrs represents time taken (hours) by each isolate to form visible turbidity in a liquid medium; - means the isolate did not show visible turbidity at that particular pH after four days of incubation

Table 4 continued

Growth of 84 groundnut rhizobia isolates to low pH in liquid basal media incubated at 28 ^{0}C

	Medium pH			
Isolate	**3.5**	**4.5**	**5.5**	**6.8**
G2	84 hrs	84 hrs	84 hrs	84 hrs
G3	84 hrs	84 hrs	84 hrs	84 hrs
G4	84 hrs	60 hrs	48 hrs	48 hrs
G5	84 hrs	60 hrs	48 hrs	36 hrs
g5	84 hrs	72 hrs	48 hrs	36 hrs
G6	84 hrs	84 hrs	84 hrs	84 hrs
g6	72 hrs	72 hrs	60 hrs	48 hrs
G7	12 hrs	12 hrs	12 hrs	12 hrs
H1	60 hrs	60 hrs	48 hrs	36 hrs
h1	48 hrs	12 hrs	12 hrs	12 hrs
h4	60 hrs	60 hrs	48 hrs	48 hrs
I1	84 hrs	84 hrs	84 hrs	84 hrs
L3	84 hrs	72 hrs	60 hrs	48 hrs
L5	72 hrs	60 hrs	60 hrs	48 hrs
L6	72 hrs	72 hrs	72 hrs	60 hrs
M1	84 hrs	84 hrs	84 hrs	84 hrs

M2	48 hrs	12 hrs	12 hrs	12 hrs
M3	36 hrs	36 hrs	36 hrs	36 hrs
M4	48 hrs	36 hrs	36 hrs	36 hrs
N1	-	-	84 hrs	84 hrs
N2	-	-	84 hrs	84 hrs
n2	72 hrs	60 hrs	48 hrs	48 hrs
N3	84 hrs	60 hrs	48 hrs	36 hrs
n3	48 hrs	48 hrs	48 hrs	48 hrs
q4	84 hrs	72 hrs	48 hrs	48 hrs
S1	48 hrs	48 hrs	48 hrs	48 hrs
S2	48 hrs	48 hrs	48 hrs	36 hrs
S3	48 hrs	48 hrs	36 hrs	36 hrs
S4	72 hrs	60 hrs	48 hrs	48 hrs
S5	72 hrs	48 hrs	36 hrs	24 hrs
S6	72 hrs	60 hrs	48 hrs	36 hrs
S7	48 hrs	48 hrs	48 hrs	48 hrs
S8	84 hrs	72 hrs	60 hrs	60 hrs
T4	60 hrs	60 hrs	48 hrs	48 hrs
T5	60 hrs	60 hrs	48 hrs	36 hrs
T6	48 hrs	48 hrs	48 hrs	36 hrs
U8	84 hrs	84 hrs	84 hrs	84 hrs
V2	48 hrs	36 hrs	36 hrs	24 hrs
W1	48 hrs	48 hrs	36 hrs	36 hrs
X1	84 hrs	84 hrs	84 hrs	84 hrs
X4	84 hrs	72hrs	72 hrs	60 hrs
Y1	84 hrs	72 hrs	48 hrs	48 hrs
Z1	84 hrs	84 hrs	48 hrs	36 hrs

Note: hrs represents time taken (hours) by each isolate to form visible turbidity in a liquid medium; - means the isolate did not show visible turbidity at that particular pH after four days of incubation

4.3.2 Selection for aluminium tolerant strains

Seventy six (76) isolates that grew well at pH 4.5 were all subjected to four Al concentrations (0, 50, 100 and 130 µM) together with the commercial strain Biofix (Table 5). At 0 µM, fourteen (14) isolates and Biofix showed turbidity after 12 hours, two (2) after 24 hours, six (6) after 36 hours, twelve (12) after 48 hours, eighteen (18) after 60 hours, fourteen (14) after 72hours and ten (10) after 84 hours. At 50 µM, five isolates (G6, Z1, X1, I1 and U8) were completely inhibited and showed no sign of growth after four days of

incubation. Among the remainder, one (1) isolate showed turbidity after 12 hours, one (1) isolate after 24 hours of incubation, six (6) isolates after 36 hours, thirteen (13) isolates and Biofix after 48 hours, eleven (11) after 60 hours, eighteen (18) after 72 hours and twenty one (21) after 84 hours of incubation.

At 100µM Al, six (6) isolates and Biofix showed turbidity after 48 hours of incubation, twelve (12) isolates showed turbidity after 60 hours of incubation, eight (8) isolates showed turbidity after 72 hours of incubation, ten (10) isolates after 84 hours, whereas all the remaining forty (40) isolates did not show turbidity even after four days of incubation.

At the critical Al levels of 130 µM; which is used for screening rhizobia; two isolates showed turbidity after 48 hours of incubation, four isolates and Biofix after 60 hours, ten isolates after 72 hours, and nineteen isolates after 84 hours. In some instances turbidity of the medium was observed on the same day of incubation. In such cases, more frequent observations were done to ascertain that the turbidity was not as a result of contamination.

Table 5: Growth of 76 acid tolerant groundnut rhizobia strains screened at pH 4.5 for aluminium tolerance at 0, 50, 100, 130 µM Al.

	Aluminium concentration			
Isolate	**0µM**	**50 µM**	**100µM**	**130µM**
A2	12 hrs	12 hrs	48 hrs	60 hrs
A3	12 hrs	24 hrs	48 hrs	60 hrs
A4	60 hrs	72 hrs	-	-
A5	24 hrs	48 hrs	60 hrs	72 hrs
A6	24 hrs	36 hrs	48 hrs	48 hrs
A7	48 hrs	60 hrs	72 hrs	84 hrs
B1	60 hrs	84 hrs	-	-
b1	36 hrs	60 hrs	72 hrs	72 hrs
B2	72 hrs	84 hrs	-	-
B3	12 hrs	48 hrs	60 hrs	72 hrs
B4	72 hrs	72 hrs	84 hrs	84 hrs
B5	72 hrs	84 hrs	84 hrs	-

B6	60 hrs	72 hrs	84 hrs	84 hrs
Biofix	12 hrs	48 hrs	48hrs	60 hrs
C4	84 hrs	84 hrs	-	-
C5	60 hrs	72 hrs	-	-
C6	72 hrs	72 hrs	84 hrs	84 hrs
D1	12 hrs	48 hrs	60 hrs	84 hrs
D2	72 hrs	72 hrs	-	-
D3	72 hrs	84 hrs	-	-
D4	12 hrs	48 hrs	60 hrs	72 hrs
D5	84 hrs	84 hrs	-	-
E1	12 hrs	36 hrs	60 hrs	84 hrs
E2	60 hrs	72 hrs	-	-
E3	60 hrs	72 hrs	-	-
E4	48 hrs	60 hrs	-	-
F1	12 hrs	48 hrs	72 hrs	84 hrs
F2	60 hrs	84 hrs	-	-
F3	12 hrs	60 hrs	-	-
F4	12 hrs	48 hrs	60 hrs	72 hrs
F5	36 hrs	48 hrs	48 hrs	60 hrs
F6	48 hrs	72 hrs	-	-
F7	48 hrs	72 hrs	-	-
F8	12 hrs	36hrs	48 hrs	60 hrs
G1	12 hrs	36 hrs	60 hrs	72 hrs
g1	36 hrs	48 hrs	60 hrs	72 hrs
G2	84 hrs	84 hrs	-	-
G3	84 hrs	84 hrs	-	-

Note: hrs represents time taken (hours) by each isolate to form visible turbidity in a liquid medium; - means the isolate did not show visible turbidity at the specific Al level after four days of incubation.

Table 5 continued
Growth of 76 acid tolerant groundnut rhizobia strains screened at pH 4.5 for aluminium tolerance at 0, 50, 100, 130 µM Al.

	Aluminium concentration			
Isolate	**0µM**	**50 µM**	**100µM**	**130µM**
G4	60 hrs	72 hrs	-	-
G5	60 hrs	72 hrs	-	-
g5	72 hrs	84 hrs	-	-
G6	84 hrs	-	-	-
g6	72 hrs	84 hrs	-	-
G7	12 hrs	48 hrs	72 hrs	84 hrs
h1	12 hrs	36 hrs	60 hrs	84 hrs
H1	60 hrs	84 hrs	-	-
h4	60 hrs	72 hrs	-	-
I1	84 hrs	-	-	-
L3	72 hrs	84 hrs	-	-
L5	60 hrs	72 hrs	84 hrs	84 hrs
L6	72 hrs	84 hrs	-	-
M1	84 hrs	84 hrs	-	-
M2	12 hrs	48 hrs	72 hrs	84 hrs
M3	36 hrs	60 hrs	72 hrs	84 hrs
M4	36 hrs	48 hrs	60 hrs	72 hrs
n2	60 hrs	84 hrs	-	-
N3	60 hrs	72 hrs	84 hrs	84 hrs
n3	48 hrs	48 hrs	60 hrs	72 hrs
q4	72 hrs	84 hrs	-	-
S1	48 hrs	60 hrs	72 hrs	84 hrs
S2	48 hrs	60 hrs	-	-
S3	48 hrs	60 hrs	-	-
S4	60 hrs	84 hrs	-	-
S5	48 hrs	60 hrs	72 hrs	84 hrs
S6	60 hrs	84 hrs	-	-
S7	48 hrs	60 hrs	84 hrs	84 hrs
S8	72 hrs	84 hrs	84 hrs	84 hrs
T4	60 hrs	72 hrs	84 hrs	84 hrs
T5	60 hrs	72 hrs	84 hrs	84 hrs
T6	48 hrs	60 hrs	-	-
U8	84 hrs	-	-	-
V2	36 hrs	36 hrs	48 hrs	48 hrs
W1	48 hrs	48 hrs	60 hrs	72 hrs
X1	84 hrs	-	-	-
X4	72 hrs	84 hrs	-	-
Y1	72 hrs	72 hrs	-	-
Z1	84 hrs	-	-	-

Note: hrs represents time taken (hours) by each isolate to form visible turbidity in a liquid medium; - means the isolate did not show visible turbidity at the specific Al level after four days of incubation.

Table 6: Growth of 36 groundnut rhizobial isolates grown at pH 4.5 for 4 days in a basal medium containing galactose and arabinose

	Aluminium concentration			
Isolate	**0 µM**	**130 µM**	**150 µM**	**200 µM**
A2	12 hrs	60 hrs	84 hrs	-
A3	12 hrs	60 hrs	72 hrs	-
A5	24 hrs	72 hrs	84 hrs	-
A6	24 hrs	48 hrs	48 hrs	60 hrs
A7	48 hrs	84 hrs	-	-
b1	36 hrs	72 hrs	84 hrs	-
B3	12 hrs	72 hrs	84 hrs	-
B4	72 hrs	84 hrs	-	-
B6	60 hrs	84 hrs	-	-
Biofix	12 hrs	60 hrs	60 hrs	72 hrs
C6	72 hrs	84 hrs	-	-
D1	12 hrs	84 hrs	84 hrs	-
D4	12 hrs	72 hrs	84 hrs	-
E1	12 hrs	84 hrs	84 hrs	-
F1	12 hrs	84 hrs	-	-
F4	12 hrs	72 hrs	84 hrs	-
F5	36 hrs	60 hrs	72 hrs	-
F8	12 hrs	60 hrs	84 hrs	-
G1	12 hrs	72 hrs	84 hrs	-
g1	36 hrs	72 hrs	84 hrs	-
G7	12 hrs	84 hrs	-	-
h1	12 hrs	84 hrs	-	-
L5	60 hrs	84 hrs	-	-
M2	12 hrs	84 hrs	84 hrs	-
M3	36 hrs	84 hrs	-	-
M4	36 hrs	72 hrs	84 hrs	-
N3	60 hrs	84 hrs	-	-
n3	48 hrs	72 hrs	72 hrs	84 hrs
S1	48 hrs	84 hrs	-	-
S5	48 hrs	84 hrs	84 hrs	-
S7	48 hrs	84 hrs	-	-
S8	72 hrs	84 hrs	-	-
T4	60 hrs	84 hrs	-	-
T5	60 hrs	84 hrs	-	-
V2	36 hrs	48 hrs	60 hrs	60 hrs
W1	48 hrs	72 hrs	84 hrs	84 hrs

Note: hrs represents time taken (hours) by each isolate to form visible turbidity in a liquid medium; - means the isolate did not show visible turbidity at the given Al level after four days of incubation.

Thirty six (36) isolates that tolerated 130 µM Al levels were further tested at an Al of 200 µM. The isolates showed varied tolerance with only five (5) isolates A6, Biofix, n3, V2 and W1 growing at this Al level (Table 6). All the remaining thirty one (31) isolates did not show visible turbidity at this Al level even after four days of incubation.

At the Al concentration of 200 µM, A6 and V2 showed turbidity after 60 hours of incubation, W1 and n3 showed turbidity after 84 hours of incubation whereas Biofix showed turbidity after 72 hours of incubation.

Generally, the response of isolates to low pH and increasing Al concentration varied among isolates suggesting differences in isolate tolerance. Very fast and fast growing isolates exhibited greater tolerance, forming visible turbidity in the growth media within 48 hours of incubation, than did the slow growing isolates that formed visible turbidity after 84 hours of incubation. All the 36 isolates that were tolerant to pH 4.5 and Al concentration of 130 µM can be considered for selection as tolerant to acidity and Al.

4.4 Metabolic Identification of Rhizobia

Of the eighty four (84) isolates, only thirty six (36) were identified as tolerant to both acidity and Al stress. These were further subjected to selected biochemical tests viz nitrate reduction, urea hydrolysis and citrate utilization.

4.4.1 Nitrate reduction test

Nitrate reduction test was positive for all the isolates (Plate3; Table 7). Eleven isolates readily reduced nitrate to nitrite, which was indicated by colour of the reaction mixture changing from brown to blue then colourless (Plate 3) within 48 hours after incubation.

However, the remaining twenty five isolates changed the reaction mixture to blue but required addition of zinc dust for the complete to occur.

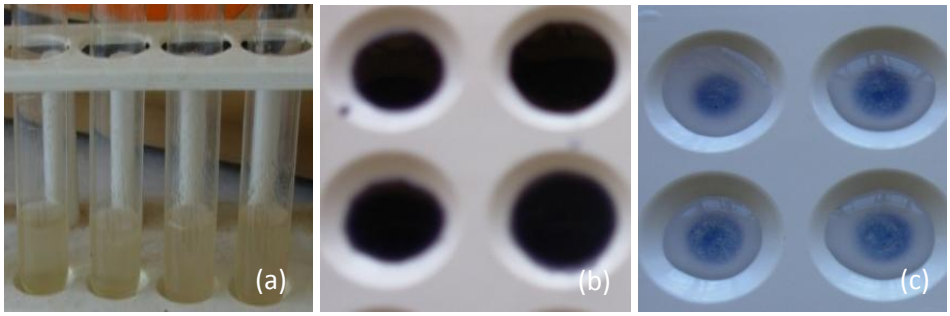

Plate 3: Nitrate reduction by rhizobia isolates tested in broth. (Source: Author, 2013)

Nitrate broth before inoculation (a); porcelain plates showing colour change to blue and colourless (b, c) indicating nitrate reduction to nitrite.

4.4.2 Citrate utilization test

Out of the thirty six (36) isolates tested, thirty two (32) showed the ability to utilize citrate in Simon's citrate agar medium test (Table 7; Plate 4) two days after incubation. Only four (4) isolates namely; F2, F5, A1 and A8 failed to elicit a colour change in the culture medium, indicating their inability to catabolize citrate.

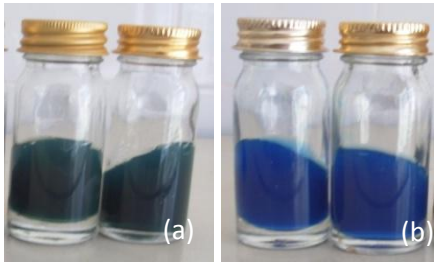

Plate 4: Slant cultures showing colour change of the Simon's citrate agar medium from green (a) to blue (b) after two days of culture in response to selected groundnut rhizobia isolates. (Source: Author, 2013)

Table 7: Selected metabolic properties of 36 acid-aluminium tolerant groundnut rhizobia isolates nodulating groundnuts in western Kenya

Isolate	Nitrate reduction test		Citrate utilization test		Urease test	
	Initial colour	Final colour	Initial colour	Final colour	Initial colour	Final colour
A2	colourless	Blue	Green	Green	Blue	Colourless
A3	colourless	Blue	Green	Blue	Blue	Colourless
A5	colourless	Blue	Green	Blue	Blue	Colourless
A6	colourless	Blue	Green	Blue	Blue	Colourless
A7	colourless	Blue	Green	green	Blue	Colourless
b1	colourless	Blue	Green	Blue	Blue	Colourless
B3	colourless	Blue	Green	Blue	Blue	Colourless
B4	colourless	Blue	Green	Blue	Blue	Colourless
B6	colourless	Blue	Green	Green	Blue	Colourless
Biofix	colourless	Blue	Green	Blue	Blue	Colourless
C6	colourless	Blue	Green	Blue	Blue	Colourless
D1	colourless	Blue	Green	Green	Blue	Colourless
D4	colourless	Blue	Green	Blue	Blue	Colourless
E1	colourless	Blue	Green	Blue	Blue	Colourless
F1	colourless	Blue	Green	Blue	Blue	Colourless
F4	colourless	Blue	Green	Blue	Blue	Colourless
F5	colourless	Blue	Green	Blue	Blue	Colourless
F8	colourless	Blue	Green	Blue	Blue	Colourless
G1	colourless	Blue	Green	Blue	Blue	Colourless
g1	colourless	Blue	Green	Blue	Blue	Colourless
G7	colourless	Blue	Green	Blue	Blue	Colourless
h1	colourless	Blue	Green	Blue	Blue	Colourless
L5	colourless	Blue	Green	Blue	Blue	Colourless
M2	colourless	Blue	Green	Blue	Blue	Colourless
M3	colourless	Blue	Green	Blue	Blue	Colourless
M4	colourless	Blue	Green	Blue	Blue	Colourless
N3	colourless	Blue	Green	Blue	Blue	Colourless
n3	colourless	Blue	Green	Blue	Blue	Colourless
S1	colourless	Blue	Green	Blue	Blue	Colourless
S5	colourless	Blue	Green	Blue	Blue	Colourless
S7	colourless	Blue	Green	Blue	Blue	Colourless
S8	colourless	Blue	Green	Blue	Blue	Colourless
T4	colourless	Blue	Green	Blue	Blue	Colourless
T5	colourless	Blue	Green	Blue	Blue	Colourless
V2	colourless	Blue	Green	Blue	Blue	Colourless
W1	colourless	Blue	Green	Blue	Blue	Colourless

4.4.3 Urea hydrolysis test

All the isolates were able to reduce urea into ammonia. This was deduced from the change in colour of the medium from blue to colourless (Table 7) after 48 hours of culture incubation.

4.5 Distinguishing Rhizobia from Agrobacterium

Out of the thirty six (36) isolates in Table 5, four (4) isolates (V2, A6, n3 and W1) together with the commercial inoculants (Biofix) tested for tolerance to of 200 µM Al were considered for verification of identity and function. This is because the two genera have some common morphological features and can both grow on YEMA, the semi-selective medium used for bacteria isolation in the present study.

4.5.1 Temperature variation assay

All the five (5) isolates, the cells were able to grow at 37^0C as well as 28^0C on YEMA at pH 6.8. At 37 ^{0}C the colonies appeared after 12 hours of incubation whereas at 28 ^{0}C growth was observed after 24 hours of incubation.

4.5.2 Glucose peptone agar (GPA) assay

All the isolates grew on the GPA media (Plate 5) 12 hours after incubation and with the colonies reaching full size 24 hours after incubation. The isolates which were incubated at 28^0C grew faster in GPA compared to their growth on YEMA-Congo red medium at the same temperature.

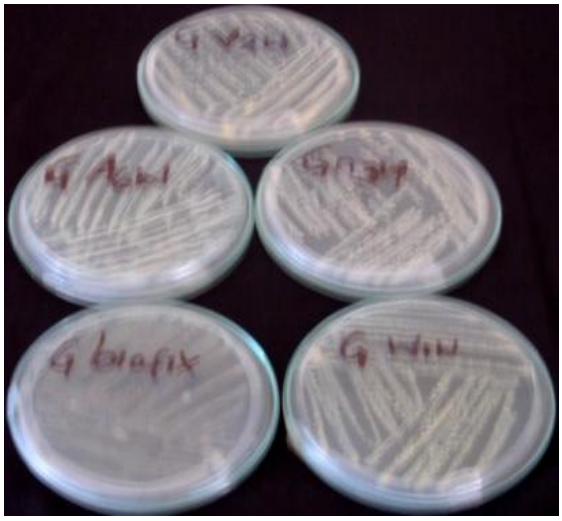

Plate 5: Growth of five rhizobial isolates on a glucose peptone agar media. (Source: Author, 2013)

4.5.3 Growth on YEMA- Congo red

Congo red assists in the recognition of rhizobia amongst other kinds of bacteria. This is because rhizobia absorb the dye weakly whereas agrobacteria take it up strongly. In this study all the five isolates did not absorb Congo red in YEMA plates even after 48 hours of incubation.

4.5.4 Growth on Hofer's alkaline broth

Some rhizobia have been reported to tolerate alkaline pH depending on the pH of soils they originated from. In this study none of the five isolates exhibited detectable growth in Hofer's alkaline broth even after four days of incubation.

4.5.5 Growth on lactose agar

All the five isolates grew on lactose agar. When Benedict's solution was poured over the lactose agar cultures, there was no colour change indicating absence of agrobacteria among the isolates.

4.6 Authentication test

Four (4) of the five Al tolerant groundnut rhizobia isolates caused nodulation of groundnut (*Red Valencia*) (Table 8). The nodulated groundnut plants had abundant nodules. However, even a large number of nodules did not mean that they were actively fixing nitrogen. Active nodules were found to be pink to dark red inside. White color indicated that the nodule was not yet active. Light red indicated that little nitrogen fixation was taking place as was observed in strain W1 (Table 8).

Table 8: Cross section of nitrogen fixing nodules sampled from rhizobia inoculated groundnut seedlings after six weeks of growth.

Isolate	Mean nodule number	Nodule color	Cross section of nodule
A6	39	Pink-dark red	
W1	27	White-Pink	
Biofix	33	Pink-dark red	
V2	42	Pink-dark red	
n3	0	-	-

Note: Nodules were counted from three groundnut plants per rhizobia treatment (n=3)

Some of the nodules were found to be ineffective and did not fix adequate nitrogen required by the groundnut plants. The effective nodules were big (2-3 mm) and occured along the tap root and the top lateral roots as shown in Plate 7. Ineffective nodules were comparatively smaller. They were distributed throughout the root system. The

effectiveness of nodulation and availability of nitrogen to plants resulted in dark green foliage and better growth of plants as shown in Plate 6.

Plate 6: Set up for testing nodulation effectiveness test in polythene pouches with cotton wool support showing dark green foliage for nodulated nitrogen fixing plants. (Source: Author, 2013)

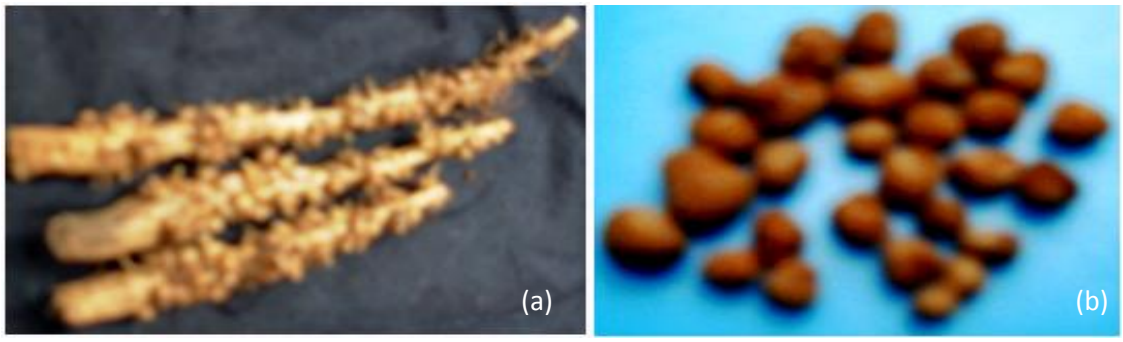

Plate 7: Distribution of effective nodules along tap root of the groundnut plant and their size differences. (Source: Author, 2013)

4.7 Response of Four Groundnut Varieties to Inoculation with Three Rhizobia Isolates and Biofix

The three (3) isolates (A6, V2, W1) and Biofix effectively nodulated all the test groundnut varieties except the variety ICGVSM 99568 which failed to form nodules when inoculated with W1. In all cases where there was effective nodulation the isolates showed varying degree of relative effectiveness and groundnut growth improvement. However, mean nodule number, nodule fresh weight and shoot dry weight were not statistically significant among treatments (Table 9). The plants supplied with N showed vibrant growth at the initial stages that subsided after 4 weeks of growth. Inoculated plants on the other hand showed poor initial growth characterized by pale green leaves but this changed at 5 weeks of growth when they started showing vibrant growth and the leaves turned dark green. Non-inoculated plants that were not supplied with N showed poor growth exhibited in pale green foliage throughout the experimental period. The variety *Red Valencia* supplied with nitrogen had the highest mean plant height while all the four groundnut varieties that were neither inoculated nor supplied with nitrogen elicited the least mean plant height.

Table 9: Relative effectiveness of four rhizobial strains to enhance performance of four groundnut varieties

Treatment	Variety	Mean plant height (cm)	Mean nodule number (mg)	Mean nodule fresh weight (mg)	Mean shoot dry weight (g)
Biofix	ICGV-9991	32.0bc	50a	0.83a	0.47a
	ICGVSM-99568	33.33bc	51a	0.63a	0.60a
	ICGV-129991	32.67bc	58a	0.69a	0.57a
	Red Valencia	41.0abc	70a	0.93a	0.88a
A6	ICGV-9991	29.33bc	56a	0.97a	0.78a
	ICGVSM-99568	30.67bc	54a	0.67a	0.67a
	ICGV-129991	31.33bc	58a	0.67a	0.79a
	Red Valencia	46.67abc	68a	0.88a	0.97a
V2	ICGV-9991	32.67bc	56a	0.78a	0.71a
	ICGVSM-99568	33.33bc	45a	0.71a	0.67a
	ICGV-129991	33.0bc	59a	0.67a	0.75a
	Red Valencia	37.33abc	64a	0.83a	0.67a
W1	ICGV-9991	39.0ab	55a	0.73a	0.51a
	ICGVSM-99568	38.33ab	-	-	0.76a
	ICGV-129991	33.67ab	50a	0.60a	0.50a
	Red Valencia	40.33ab	65a	0.60a	0.63a
Nitrogen	ICGV-9991	40.33ab	40a	0.53a	0.37a
	ICGVSM-99568	36.33ab	13a	0.23a	0.33a
	ICGV-129991	35.67ab	41a	0.54a	0.43a
	Red Valencia	44.67a	58a	0.71a	0.76a
control (-N)	ICGV-9991	24.33bcd	28a	0.47a	0.29a
	ICGVSM-99568	27.67bcd	20a	0.44a	0.29a
	ICGV-129991	29.83bcd	28a	0.49a	0.23a
	Red Valencia	36.0abcd	50a	0.47a	0.56a

Note: Means followed by the same letters in a column are not significantly different from each other at $P < 0.05$ according to Duncan's Multiple Range Test.

4.8 Nodulation and N- fixation Effectiveness of four Rhizobia isolates on Groundnuts in a Monocrop

4.8.1 Soil properties

Results for initial site characterization are presented in Table 10. Most of the parameters analyzed varied between the two sites. Soil pH was generally low across both sites with Ligala having a pH of 4.63 and Koyonzo 5.20. Both sites had low total N contents with values of 0.06 and 0.08 % for Ligala and Koyono, respectively. Available P by the Olsen extractable method was 2.2 and 9.0 mg kg $^{-1}$ for Ligala and Koyonzo, respectively. Soil type varied from sand clay loam at ligala site to clay loam sand at Koyonzo site. Hence under normal agricultural practice the soils would not be suitable for groundnut growing. The sites were therefore identified as good for this study.

Table 10: Selected chemical and physical characteristics of top soil (0-15cm) at Ligala and Koyonzo

Site	Ligala	Koyonzo
pH	4.63	5.20
%C	1.26	1.32
%N	0.06	0.08
Olsen P (mg kg^{-1})	2.2	9.0
%Sand	66.1	16.2
%Clay	23.4	56.7
%Silt	12.4	17.9
Textural Class	Sandy clay loam	Clay loam sandy

4.8.2 Response of groundnut to rhizobia inoculation and liming

Generally, for all parameters measured (Tables 11 and 12), groundnut performance varied across sites and seasons ($p < 0.05$).

4.8.2.1 Number and Dry Weight of Nodules

Rhizobia inoculation in the limed soils significantly ($p < 0.05$) improved nodule number per plant in both seasons compared to the treatment combinations without lime (Table 11, 12). Similarly, application of nitrogen in the limed plots improved nodulation in both seasons and at both sites. The highest nodule numbers were obtained at the Ligala site in the 2011 short rain season in the treatment with dolomitic lime and control, the lowest was recorded for during the same season in Koyonzo in the control treatment that did not receive lime. Nodule number showed significant interaction ($p < 0.05$) of lime x strain at both sites in each season. This was most pronounced for V2, A6 and Biofix, which showed improved performance under dolomitic lime treatment. Strain x site, strain x season and site x season were significant at ($p < 0.05$) for nodule number with interaction between Ligala site x dolomitic lime x strain A6 x long rain season giving the best performance across all treatments (appendix 4a and 4b).

Table 11: Effect of soil liming and rhizobia inoculation on Red Valencia groundnut at Ligala during the 2011 long and short rain seasons

LIME	Rhizobia treatment	2011 Long Rain Season					2011 Short Rain Season				
		NN	NDW (mg)	NUT NO	SDW (g)	GY kg ha^{-1}	NN	NDW (mg)	NUT NO	SDW (g)	GY (kg ha^{-1})
Calcitic lime	V2	32.3^{c}	56.0^{a}	85.3^{a-d}	44.7^{c}	901^{a}	33.7^{c}	57.0^{a}	162.0^{ab}	196.7^{a}	1002^{a}
Calcitic lime	W1	48.3^{b}	34.0^{e}	64.3^{a-d}	40.0^{cd}	808^{b}	35.3^{c}	47.0^{ab}	146.3^{bc}	96.7^{b}	754^{bc}
Calcitic lime	A6	44.7^{b}	33.0^{c}	94.3^{a-c}	25.3^{cd}	865^{ab}	22.0^{d}	43.0^{b}	196.0^{ab}	182.7a	901^{a}
Calcitic lime	Biofix	24.0^{d}	32.0^{c}	78.0^{a-d}	27.3^{cd}	589^{cd}	17.3^{e}	45.0^{ab}	156.0^{b}	163.3^{a}	614^{cd}
Calcitic lime	Nitrogen	36.7^{c}	28.0^{de}	87.3^{cd}	52.7^{c}	589^{cd}	45.7^{b}	33.0^{c}	147.7^{bc}	83.3^{b}	614^{cd}
Calcitic lime	Control	48.0^{b}	34.0^{c}	78.0^{a-d}	35.0^{cd}	523^{d}	25.0^{d}	43.0^{b}	125.0^{c}	106.0^{ab}	543^{d}
Dolomitic lime	V2	53.3^{a}	46.0^{ab}	64.3^{a-d}	23.7^{cd}	789^{bc}	24.0^{d}	45.0^{ab}	209.7^{ab}	117.7^{ab}	803^{b}
Dolomitic lime	W1	45.7^{b}	26.0^{de}	77.0^{cd}	43.0^{c}	689^{c}	37.3^{c}	35.0^{c}	134.3^{bc}	87.7^{b}	657^{c}
Dolomitic lime	A6	48.0^{b}	36.0^{bc}	76.0^{a-d}	43.3^{c}	764^{bc}	26.0^{d}	37.0^{bc}	176.3^{ab}	104.7^{ab}	875^{ab}
Dolomitic lime	Biofix	35.7^{c}	37.0^{bc}	56.0^{a-d}	33.7^{cd}	527^{d}	27.0^{d}	34.0^{c}	83.3^{cd}	64.7^{bc}	547^{d}
Dolomitic lime	Nitrogen	45.3^{b}	33.0^{c}	94.0^{c}	44.3^{c}	517^{d}	34.0^{c}	42.0^{b}	132.0^{bc}	137.3^{ab}	590^{cd}
Dolomitic lime	Control	37.3^{c}	37.0^{bc}	61.7^{a-d}	35.7^{cd}	497de	54.0^{a}	45.0^{ab}	197.0^{ab}	93.7^{b}	532^{d}
No lime	V2	42.7^{b}	28.0^{de}	71.7^{b-d}	32.7^{cd}	476de	21.7^{d}	31.0^{c}	206.7^{ab}	153.0a	493de
No lime	W1	39.0^{c}	45.0^{ab}	71.7^{cd}	36.0^{cd}	465de	21.7^{d}	38.0^{bc}	187.0^{ab}	102.7^{ab}	534^{d}
No lime	A6	43.7^{b}	37.0^{bc}	63.7^{cd}	42.3^{c}	467de	25.0^{d}	34.0^{c}	205.7^{ab}	141.3^{a}	543^{d}
No lime	Biofix	23.7^{d}	41.0^{b}	85.7^{b-d}	34.3^{cd}	476de	43.3^{b}	46.0^{ab}	225.3^{a}	123.7^{ab}	541^{d}
No lime	Nitrogen	36.3^{c}	35.0^{c}	86.0^{cd}	33.7^{cd}	441^{e}	44.7^{b}	49.0^{ab}	194.0^{ab}	94.0^{b}	546^{d}
No lime	Control	21.3^{d}	26.0^{de}	63.3^{b-d}	25.0^{cd}	461de	25.3^{d}	32.0^{c}	112.3^{a-c}	88.7^{b}	523^{d}
Overall Mean		**41.22**	**37.7**	**75.5**	**36.3**	**602.4**	**31.3**	**40.8**	**166.5**	**118.8**	**645.1**
SE		**4.8**	**2.0**	**3.1**	**2.7**	**2.9**	**2.6**	**2.1**	**2.5**	**2.3**	**2.7**
CV%		**11.7**	**8.7**	**4.1**	**7.5**	**5.1**	**8.3**	**3.4**	**1.5**	**1.9**	**1.6**

Note: Means in a column followed with same letters are not significantly different at 95% level of probability: n=54; NN- Nodule Number, NDW- Nodule Dry Weight, Nut no.- Nut Number, SDW- Shoot Dry Weight, GY- Grain Yield

Table 12: Effect of soil liming and rhizobia inoculation on Red Valencia the groundnut at Koyonzo during the 2011 long and short rain seasons

Lime	Rhizobia treatment	2011 Long Rain Season					2011 Short Rain Season				
		NN	NDW (mg)	Nut no.	SDW (g)	GY (kg ha^{-1})	NN	NDW (mg)	Nut no.	SDW (g)	GY (kg ha^{-1})
Calcitic lime	V2	16.7^{e}	53.0^{a}	85.3^{a}	114.0^{ab}	765^{bc}	26.7^{d}	51.0^{a}	46.0^{c}	103.7^{ab}	879^{ab}
Calcitic lime	W1	25.7^{b-d}	48.0^{a}	77.7^{ab}	112.3^{ab}	674^{c}	24.0^{d}	39.0^{bc}	33.3^{cd}	127.0^{a}	698^{c}
Calcitic lime	A6	34.3^{c}	44.0^{b}	67.0^{b}	103.3^{ab}	769^{bc}	23.0^{d}	41.0^{b}	34.0^{cd}	122.0^{a}	824^{b}
Calcitic lime	Biofix	34.3^{c}	42.0^{b}	77.7^{ab}	136.7^{a}	579^{cd}	24.0^{d}	39.0^{bc}	36.3^{cd}	84.3^{b}	679^{c}
Calcitic lime	Nitrogen	26.0^{d}	51.0^{a}	73.7^{ab}	133.0^{a}	537^{d}	23.7^{d}	34.0^{c}	32.0^{d}	131.7^{a}	611^{cd}
Calcitic lime	Control	25.0^{d}	41.0^{b}	59.7^{bc}	126.7^{a}	521^{d}	25.0^{d}	25.0^{cd}	35.0^{d}	97.0^{b}	534^{d}
Dolomitic lime	V2	33.0^{c}	42.0^{b}	74.7^{ab}	15.7^{d}	672^{c}	24.0^{d}	40.0^{b}	51.7^{bc}	116.0^{ab}	768^{bc}
Dolomitic lime	W1	33.7^{c}	45.0^{ab}	68.0^{b}	26.3^{cd}	603^{cd}	22.0^{d}	37.0^{bc}	34.3^{cd}	93.0^{b}	621^{cd}
Dolomitic lime	A6	53.7^{a}	48.0^{a}	44.3^{c}	44.7^{c}	679^{c}	21.7^{d}	32.0^{c}	45.3^{c}	77.3^{bc}	709^{c}
Dolomitic lime	Biofix	37.3^{c}	35.0^{c}	56.3^{bc}	47.3^{c}	521^{d}	17.7^{e}	27.0^{cd}	34.7^{cd}	102.3^{ab}	564^{cd}
Dolomitic lime	Nitrogen	32.3^{c}	45.0^{ab}	54.7^{bc}	31.3^{cd}	532^{d}	15.0^{e}	34.0^{e}	33.3^{d}	155.3^{aa}	549^{d}
Dolomitic lime	Control	35.7^{c}	37.0^{bc}	72.3^{ab}	33.7^{cd}	501^{d}	18.7^{e}	43.0^{b}	26.0^{de}	75.7^{b}	508^{d}
No lime	V2	47.3^{b}	32.0^{c}	73.7^{ab}	85.0^{b}	467de	41.0^{b}	21.0^{d}	41.0^{c}	102.3^{ab}	492de
No lime	W1	20.3^{d}	39.0^{bc}	57.3^{bc}	71.3^{bc}	478de	24.3^{d}	21.0^{d}	32.0^{cd}	123.7^{a}	560^{cd}
No lime	A6	54.0^{a}	43.0^{b}	69.0^{b}	83.7^{b}	471de	32.3^{c}	25.0^{d}	34.0^{cd}	123.0^{a}	505^{d}
No lime	Biofix	36.3^{c}	23.0^{d}	67.0^{b}	80.0^{b}	487de	26.7^{a-c}	26.0^{cd}	31.7^{d}	102.7^{ab}	541^{d}
No lime	Nitrogen	42.7^{b}	36.0^{bc}	57.0^{bc}	72.3^{bc}	432^{e}	16.3^{e}	30.0^{e}	23.3^{de}	133.0^{a}	531^{d}
No lime	Control	19.7^{e}	21.0^{d}	56.7^{bc}	73.7^{b}	450de	13.0^{e}	25.0^{ed}	29.3^{d}	105.7^{ab}	493de
Overall mean		**33.8**	**40.3**	**66.2**	**77.3**	**563.2**	**23.3**	**32.7**	**35.2**	**109.8**	**614.7**
SE		**2.1**	**2.0**	**3.1**	**2.3**	**5.8**	**2.2**	**1.8**	**2.6**	**1.9**	**1.8**
CV%		**6.3**	**9.3**	**4.8**	**3.0**	**4.6**	**9.5**	**6.7**	**7.3**	**1.7**	**3.8**

Note: Means in a column followed with same letters are not significantly different at 95% level of probability:n=54; NN- Nodule Number, NDW- Nodule Dry Weight, Nut no.- Nut Number, SDW- Shoot Dry Weight GY- Grain Yield

Rhizobia inoculation also improved nodule biomass significantly ($p < 0.05$) in both seasons regardless of the site (Table 11, 12). The highest nodule dry weight (57 mg/plant) was recorded at Ligala during the 2011 short rain season in plots where strain V2 and calcitic lime were combined. The control without lime treatment recorded the least nodule dry weight of 21 mg/plant in Koyonzo during the LRs. Generally, nodule biomass varied with season and site with Koyonzo giving the highest biomass (40.3 mg plant^{-1}) during the LRs and Ligala giving the highest (40.8 mg plant^{-1}) biomass during the SRs. Similarly, lime x strain interaction was significant ($p < 0.05$) at each site in both the long and short rain seasons. The isolates response to calcitic lime was similar to that observed in the treatment without lime but varied under dolomitic lime when compared based on nodule biomass production and showed similar trend at either site. Nodule dry weight showed significant interaction ($p < 0.05$) of site x strain x season. This was best for Ligala site x strain V2 x short rain season (appendix 4b).

4.8.2.2 Nut Number

The number of nuts per plant varied significantly ($p < 0.05$) among treatments. Nut number was highest (225.3 nuts per plant) in Biofix inoculated plants without liming during the SRs at Ligala and lowest at Koyonzo site during the same season with the treatment combining N and no lime (23.3 nuts per plant). Significantly ($p < 0.05$) higher nut numbers was recorded for Ligala in the short rains compared to the long rain season whereas at Koyonzo the number was reduced by nearly 50% in the short rain season. Interaction between lime and rhizobia inoculant strain for nut number was not significant at Ligala in both seasons and at Koyonzo during the short rains. During the long rains dolomitic lime seemed to suppress nut production especially in the plots inoculated with A6. Generally,

liming influenced strain performance with respect to nut production. Significant interaction ($p < 0.05$) for strain x season, strain x site, season x site and strain x lime x season x site was also observed. Ligala site x strain V2 x short rain season x no lime showed the best interaction for this parameter.

4.8.2.3 Shoot Dry Weight

There was significant variation ($p < 0.05$) in shoot dry weight among treatments. Shoot dry weight increased greatly during the short rain season at both sites across the treatments. Shoot dry weight was highest (196.7 g per plant) at Ligala in the short rain season in treatments with calcitic lime and strains V2 and lowest for the same strain and dolomitic lime at Koyonzo (15.7 g per plant). Lime x strain interaction for shoot dry weight was not significant at ligala during the long rains. However, in the short rains it was significant ($p < 0.05$) with dolomitic lime application apparently reducing shoot dry weight in plants inoculated with Biofix. The interaction was also significant at Koyonzo in both cropping seasons and was most evident for strain A6. At this site, the shoot dry weight was generally reduced in treatments combining dolomitic lime and inoculants. The interactions involving strain x season, strain x site, season x site and strain x lime x season x site were all significant. Strains V2 x calcitic lime x Koyonzo site x short rain season interaction gave the best overall shoot dry wweight.

4.8.2.4 Grain Yield

Groundnut grain yield was significantly ($p \leq 0.05$) improved with both innoculation and application of lime. The control treatment with no lime gave less than 500 kg of grain per ha for both seasons at the Koyonzo site. Similarly in Ligala, this treatment gave only 461

and 523 kg ha^{-1} grain for the LRs and SRs, respectively. The grain yield varied significantly among rhizobia strains and lime treatments at the two sites in both seasons. The highest grain yield of 1002 kg ha^{-1} was obtained at Ligala during the 2011 short rains in the treatment where rhizobia strain V2 was combined with calcitic lime (Table 11). At Koyonzo, strain V2 with calcitic lime also gave the best groundnut yield of 879 kg ha^{-1} (Table 12). These yields represent 84.5% and 64.6% increases respectively, above the control. Lime x strain interaction was significant ($p \leq 0.05$) for grain yield at both experimental sites in the two cropping seasons. There was grain yield increase for all the rhizobia inoculants under liming. This was most noticeable for V2 and A6, which evidently to performed better calcitic lime plots.

Generally at Ligala site had better crop performance in long rains with calcitic lime and strain V2 compared to short rains, dolomitic lime and other strains and the controls (Appendix 4). At the Koyonzo site, long rains, calcitic lime and strain A6 treatments showed better results compared to short rains, dolomitic lime and other strains and controls. On the basis of the parameters assessed and the results obtained in this study, V2 and A6 were the most superior isolates compared to Biofix. The isolate W1 also gave good results compared to the commercial inoculant Biofix.

4.9 Recovery of Inoculant Strains from Root Nodules of Field grown plants

The recovery of groundnut rhizobia showed that most (89 to 94 %) of the tested isolates exhibited high resistance to ampicillin and polymixin (Table 13). In the presence of chloramphenicol, 78% of isolates recovered from field – grown inoculated plants were resistant. Out of all tested isolates 82% were resistant to the seven antibiotics. The original inoculant strains showed 100% resistance to each of the antibiotics. Based on intrinsic antibiotic resistance, there was better recovery of inoculant strains from Koyonzo than from Ligala.

Table 13: Percent recovery of rhizobia isolates from nodules of inoculated plants grown at Ligala and Koyonzo estimated using antibiotic resistance

Isolate	Str	Kan	Gen	Chl	Pol	Rif	Amp
A6 original	100	100	100	100	100	100	100
A6 Ligala	40	55	25	75	50	40	75
A6 Koyonzo	90	95	100	95	100	75	95
Biofix	100	100	100	100	100	100	100
Biofix Ligala	45	20	50	65	75	55	75
Biofix Koyonzo	100	100	90	100	100	80	75
Control	75	70	50	85	85	60	100
Nitrogen	80	70	50	65	85	60	100
V2 original	100	100	100	100	100	100	100
V2 Ligala	40	40	50	40	60	55	100
V2 Koyonzo	70	100	60	80	100	85	80
W1 original	100	100	100	100	100	100	100
W1 Ligala	20	20	25	45	60	30	100
W1 Koyonzo	95	100	100	85	95	80	100

Str-Streptomycin, Kan- Kanamycin, Gen- Gentamycin, Chl- Chloramphenicol, Pol-Polymymixin, Amp- Ampicillin, Rif- Rifampicin

The lowest recovery from inoculated plants was registered for W1 and V2 at Ligala and Koyonzo, respectively. In contrast, the highest recovery from inoculated plants was registered for V2 at Ligala and for W1 at Koyonzo site. The isolates recovered from nitrogen fertilized and control plots showed 70% resistance to the antibiotics. These

isolates native to the experimental fields show high similarity to those used as inoculants in this study and are worth considering in future studies. The summarized main activities and key results of this study are illustrated in figure 2.

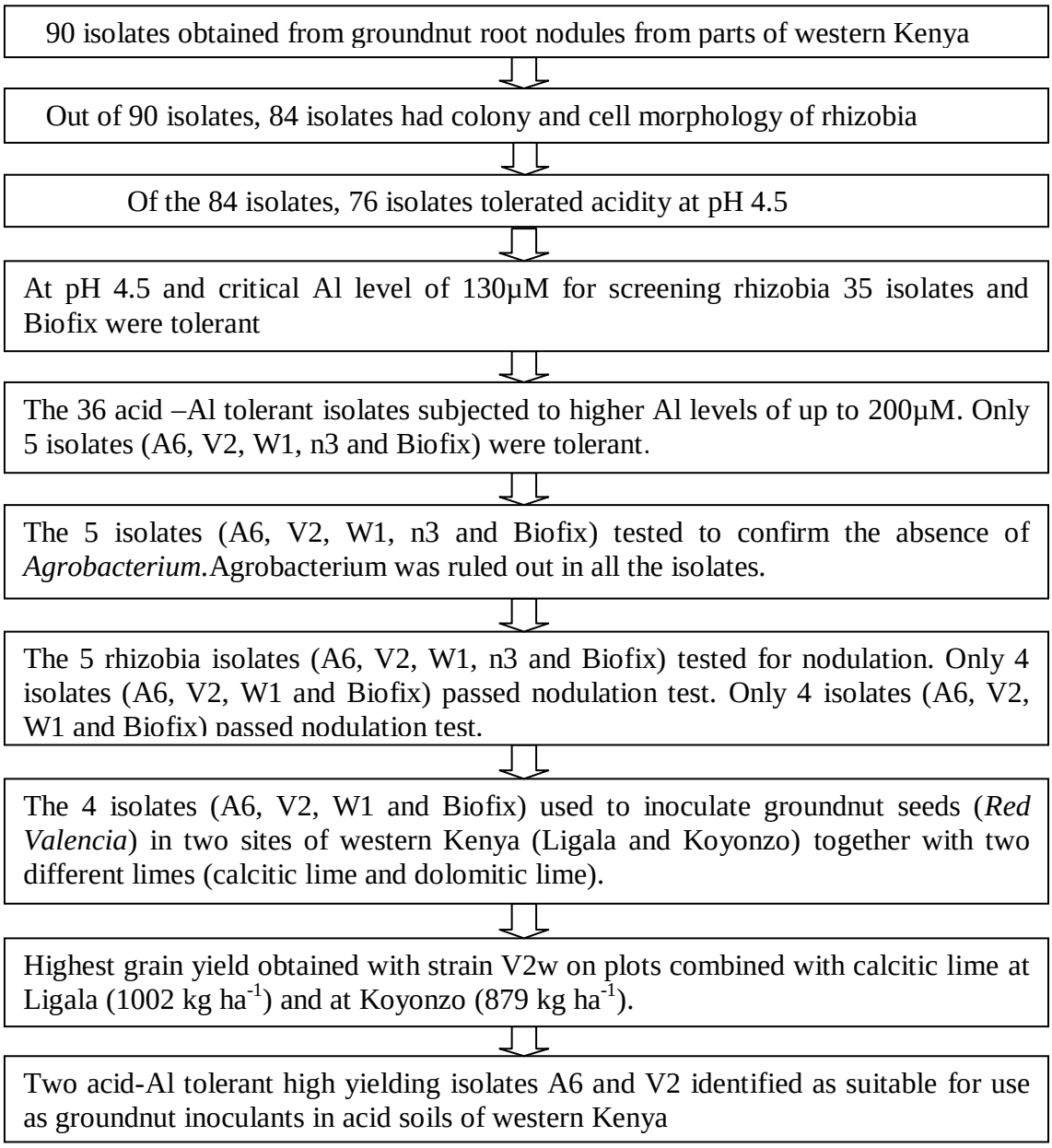

Figure 2: Steps to identifying two superior acid-Al tolerant indigenous groundnut rhizobia from western Kenya.

CHAPTER FIVE

DISCUSSION

5.1 Isolation and Characterization of Rhizobia

The fact that many isolates were obtained in this study confirms that there is a broad diversity of naturally occurring groundnut rhizobia in the acid soils of western Kenya. Rhizobia colonies appeared white, translucent, gummy, glistening, elevated and small with entire margins. These findings were in agreement with those of Saeki et al. (2005) and Sharma et al. (2010) who used YEMA-Congo red medium for categorizing indigenous soybean root nodulating fast and slow growing rhizobia. The colony diameter of the isolates ranged from 2 to 4 mm indicating the isolates belonged to different growth categories. According to Jordan (1984), such characteristics are common among fast growing rhizobia in general. The current study did not estimate the natural population of rhizobia from every field of collection. However, Odee et al. (1995) showed that rhizobial populations in Kenyan soils varied from place to place. Using growth on Congo red and BTB, colony and cell characteristics alone, isolates can be classified as independent strains but this has proved difficult. In many cases it is not possible to tell accurately the morphological differences in cultures because many of the features are shared by different strains making it almost impossible to precisely distinguish among them. Therefore, in using growth, colony and cell morphologies alone; genetically unique strains may be grouped together while they are actually different. That is why metabolic characterization was also performed in this study.

5.2 Tolerance of Rhizobia to Acidity and Aluminium stress

5.2.1 Tolerance to acidity

The present study identified seventy six indigenous isolates that were tolerant to acidity, with some withstanding low pH of up to 3.5. To the best of our knowledge nobody has achieved this for groundnut rhizobia and specifically so in western Kenya. Muok (1997), working on three leguminous trees was also able to isolate five rhizobia strains tolerant to soil pH of 3.5. Karanja and Wood (1988) isolated slow growing *Rhizobium phaseoli* that could tolerate soil pH of 4.0. Similarly, Harun et al. (2009) found that lentil nodulating rhizobia can grow well at acidic pH as low as 4.

This study and others demonstrate the existence of native rhizobia that are highly tolerant to soil acidity. Such strains are well adapted to survive the harsh environment created by the prevalent soil acidity in most arable lands and would be useful under those conditions. Forty two out of the seventy six acid tolerant strains did not show visible turbidity within the expected two days, but continued growing and finally showed visible turbidity four days later. This delayed turbidity is an indication that the strains moderately tolerated that particular pH but their growth rate was greatly reduced.

Large variations were observed among fast-growing rhizobial isolates with regard to growth in relation to pH of the medium. The pH 4.5 was selected as the standard for screening rhizobia for acid tolerance. This is because at this pH the isolates grew without restriction. This observation was in agreement with that of Harun et al. (2009).

5.2.2 Tolerance to aluminium stress

Out of seventy six acid tolerant isolates, thirty six isolates tolerated high Al levels of up to 130 µM usually used as a cut-off for rhizobia (Harun et al., 2009) and were considered Al tolerant. Of the thirty six Al tolerant rhizoba isolates, five isolates A6, W1, V2, n3 and Biofix withstood the highest aluminium concentration of 200 µM and could therefore be considered as very tolerant to Al stress. Such isolates if also effective in nitrogen fixation and are competitive with the naturally occurring rhizobia could be good candidates for groundnut inoculant production for acid soils especially in the study areas.

Selection for Al tolerance greatly reduced the number of tolerant isolates compared to selection for acidity tolerance. It was noted in this study that the isolates showing tolerance to lower pH levels were also highly tolerant to toxic Al concentrations. This observation was in agreement with that of Muok (1997). This implies that truly acid tolerant rhizobia could be identified through indirect selection for tolerance to aluminium toxicity.

The most tolerant were fast growers and acid producers. The only alkaline producing slow growers with appreciable tolerance to Al (50-100 µM) were C4, G2, G3 and M1. The fast-growing strains of rhizobia have generally been considered less tolerant to acidity than the slow growing strains of *Bradyrhizobium* (Graham et al., 1994). However, some strains of the fast-growing rhizobia, such as, *R. loti* and *R. tropici*, are highly acid tolerant (Cooper et al., 1985; Graham et al., 1994). Recent reports, however, support the existence of acid-tolerant fast-growing strains, since both fast- and slow-growing, and tolerant strains that nodulate *Vigna unguiculata* have been isolated (Mpepereki et al., 1997 Graham et al. (1994) reported that the outer membrane properties played a major role in the acid tolerance among rhizobia.

All acid-Al tolerant strains showed appreciable turbidity with the liquid media. The turbidity was observed as a result of tolerant strains using sodium glutamate as the sole source of carbon and nitrogen, leading to their growth and accumulation of ammonia in the media (Ayanaba and Munns, 1982).

Physiological concentrations of glutamate are known to favor ammonia production whereas higher levels as used in the media for this investigation favor aspartate synthesis (Jones, 1965). The visible turbidity method used to assess tolerance worked well and gave a trend that could be used to segregate the isolates into tolerant and less tolerant. This method was advantageous as it is cheap and rapid to use.

5.3 Metabolic Characterization

The ability to carry out various enzymatic processes is an important biochemical characteristic feature of rhizobia. Three enzymatic activities studied were nitrate reductase, urease and citrase. Nitrate reductase and urease functions influence nodulation and nitrogen fixation in legumes.

5.3.1 Nitrate reduction

All the thirty six Al tolerant isolates were able to reduce nitrate to nitrite. Certain bacteria use nitrate in place of oxygen as an external terminal electron acceptor. In the beginning, nitrate can easily be reduced to nitrite. In aerobic bacteria, oxygen is first used to prevent nitrate reduction and then nitrate is utilized (Maheshwari, 2006). The nitrate may further give rise to nitrogen, ammonia and nitrogen dioxide (NO_2). This enzyme reaction is catalyzed by nitrate reductase as given:

$$NO_3^- + 2H + 2e^- \rightarrow NO_2^- + H_2$$

For the nitrate reduction test performed in this study, there were twenty five isolates with which no blue colour developed directly while eleven others produced the typical positive reaction from a total of thirty six isolates. Where the blue colouration failed to develop initially, two possible explanations can be sought. Firstly, it is possible that there was no reduction of nitrate to nitrite or secondly, all the nitrite so formed must have been further converted to other products such as nitrous oxide, nitric oxide (Schuur, 2011). In such situations zinc dust was added to each reaction that appeared negative for blue color; if it turned colorless then that meant that the rhizobia reduced nitrate. The nitrite formed subsequently converted to ammonia and the culture was therefore positive for nitrate reduction (Maheshwari, 2006). Gachande and Khansole (2011), suggested rapid nitrate utilization by slow growing root nodule bacteria. In this study among the eleven isolates that directly reduced nitrate to nitrite were A6, W1, V2, n3 and Biofix, which are fast growing. In this respect, the present study is at variance with the view of Gachande and Khansole (2011). Rapid conversion of nitrate is reportedly evident in active cultures which utilize oxygen quickly since in the presence of oxygen nitrate cannot be utilized (Maheshwari, 2006).

Experiments have demonstrated that NO_3^- inhibits nodule formation on legumes primarily as a root-localized effect rather than as a function of whole-plant N nutrition (Eaglesham, 1989; Abdel-Wahab et al., 1996). The inhibitory effect of exogenous nitrate on N_2 fixation has variously been attributed to a direct competition between nitrate reductase and nitrogenase for reducing power (Stephens and Neyra, 1989) or to the fact that nitrite (a by-product of nitrate reductase activity) inhibits the function of nitrogenase and

leghemoglobin (Becana and Sprent, 1987). In addition, it has been found that the decline in total nitrogenase activity upon exposure to nitrate is independent of the N_2-fixing efficiency of the symbiosis (Blumenthal et al., 1997).

Nitrate reductase positive strains of rhizobia can sufficiently infect host legume roots and establish effective symbiosis even in the presence of nitrate levels that would often inhibit these processes (Luciñski et al., 2002). Therefore, positive nitrate reductase activity is an important characteristic in isolates with potential use as inoculants.

5.3.2 Urea hydrolysis

Urea is a nitrogenous by-product of amino acid breakdown. A variety of bacteria producing the enzyme urease degrade urea into ammonia and carbon dioxide.

$$\underset{\text{urea}}{NH_2CONH_2} + H_2O \rightarrow \underset{\text{ammonium carbamate}}{NH_2COONH_4} \rightarrow 2NH_3 + CO_2\uparrow$$

In this way they can utilize urea as a source of energy and assimilable nitrogen to sustain them. The isolates tested in the present study were all able to reduce urea into ammonia. Having passed this presumptive test, they were considered to be rhizobia. These findings are similar to Gachande and Khansole (2011) as reported for soybean rhizobia. The ability of both the fast and slow growing rhizobia to utilize urea is well-documented (Thies et al., 1995). In a study to increase groundnut yield, Lanier et al. (2005) reported a positive urea hydrolysis by groundnut nodulating rhizobia.

Urea converts rapidly to ammonia in the soil by hydrolysis. If applied properly, urea is as effective as other N sources in common use losses (Engelstand & Russell, 1975). Urea appears quite attractive as N source as it allows extensive legume nodulation relative to NO_3^-. The use of urea may allow increased N utilization from fertilizer source without a concomitant decrease in symbiotic N_2 fixation, providing that inter conversion of urea to NO_3^- under field conditions can be inhibited. Hence, these urea hydrolyzing isolates when used as inoculants can give promising yield by making the N in applied Urea more easily available for seedling growth prior to their initiating symbiotic nitrogen fixation. Excess urea if allowed to accumulate in the rhizosphere could inhibit root hair colonization and establishment of effective symbioses. For instance, it has been found that application of urea (90 kg of N ha^{-1}) to soybean plants suppressed nodulation by curtailing the enrichment of *Bradyrhizobium* spp. on the host plant (Thies et al., 1995). This situation could be avoided with urea hydrolyzing rhizobia as those characterized in this study.

5.3.3 Citrate Utilization

All except four of the thirty six acid-aluminium tolerant isolates were able to utilize citrate as sole source of carbon and energy. These included both the fast growing and slow growing isolates. The results agree with Graham and Parker (1964) who found that utilization of citrate as sole source of carbon was restricted to slow-growing rhizobia and that fast-growing rhizobia were able to grow on a large variety of carbon substrates whereas slow-growing rhizobia were more limited in their ability to use diverse carbon sources. Citrate utilization is commonly used in rhizobia characterization because the rhizobia utilize it as one of the starting products of metabolism. The citrate test medium also contains inorganic ammonium salts, which are utilized as sole source of nitrogen.

Use of citrate involves the enzyme citrase, which breaks down citrate to oxaloacetate and acetate (Beishir, 1991). Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline pH (Beishir, 1991) observed as a colour change of medium incorporated with a pH indicator like BTB.

5.4 Distinguishing Rhizobia from Agrobacteria

5.4.1 YEMA-Congo Red

The four highly acid-Al tolerant isolates and Biofix in this study showed distinct characteristics of rhizobia on Congo red as white, translucent and elevated. These results were in harmony with those of Baljinder et al. (2008). However, the isolates were turning yellow when left to grow for five days after the appearance of the colonies. This was also reported in previous studies of Boaling et al. (2007). In their study, Baoling and others found that first growing rhizobia turned yellow after five days of growth as a result of mucoid acid production. This could also be the case in the present study.

5.4.2 Hofer's alkaline broth

In this study no growth was observed with Hofer's alkaline solution which had a pH of 11, indicating that none of the isolates was *Agrobacterium*. These results were in harmony with previous studies (Gao et al., 1994; Kucuk et al., 2006; Baoling et al., 2007). An important attributefor the growth of the organism is pH of the growth media. Slight variations in pH of media may have enormous effect on the growth of the organism.

Rhizobia have been reported to show optimal growth at a pH of 6-7 whereas agrobacteria can grow in a pH range of 4-11 (DeVries et al., 1980; Baoling et al., 2007).

5.4.3 Lactose agar

All the isolates grew in lactose agar indicating that they were able to use lactose as the sole carbon source. These findings contrasted with those of Hunter et al. (2007) who reported fenugreek rhizobia isolates were unable to grow on lactose. Other studies have reported lactose utilization by rhizobia The isolates in this study did not produce ketolactose, a compound that gives yellow coloration in Benedict's test. These findings were in agreement with previous studies (Sadowasky et al., 1983; Mahaveer et al., 2010; Sharma et al., 2010; Gachande and Khansole 2011). Failure to form the yellow colour indicates the absence of agrobacteria. Agrobacteria utilize lactose to form the reduced product 3-ketolactose, through activity of the enzyme ketolactase. Rhizobia do not have this enzyme and therefore cannot produce 3-ketolactose.

5.4.4 Temperature Assay

Temperature has a great effect on rhizobial growth and symbiotic performance (Zahran, 1999). All the four examined isolates and Biofix were able to grow at between 28 ^{0}C and 37 ^{0}C. This ruled out *Agrobacterium* since none of the isolates was inhibited at temperatures above 30 ^{0}C, which do not favour agrobacteria (Zehran, 2000). Rhizobia grow well at a temperature range of 25-44 ^{0}C (Zehran, 2000), with an optimum of 29.4 ^{0}C (Baoling et al., 2007). The high temperature optima of these isolates may be beneficial for its application in temperature stressed conditions. The symbiotic performance of different rhizobial strains under temperature stress has been correlated with their ability to grow in pure culture at elevated temperature (Hungaria, 2000).

5.4.5 Glucose Peptone agar (GPA)

Glucose is one of the carbon sources commonly utilized by rhizobia. The ability of all the isolates to grow on glucose peptone agar was an indication that the isolates were indeed rhizobia. Kucuk et al. (2006) reports GPA test as a confirmatory test for rhizobia since rhizobia readily use glucose as a source of carbon as its first choice. Rhizobia poorly utilize peptone as a nitrogen source compared to agrobacteria which breaks it down very fast (Saeki et al., 2005).

5.5 Authentication

All the five isolates tested except n3 caused effective nodulation of groundnut as observed from the pink-red colour of the nodules cross section after six weeks of growth. Isolate n3 however was quite similar to rhizobia in colony and cell morphologies. If this isolate was indeed rhizobia then it is possible that it was a mutant that had lost its nodulation ability. Muok (1997) similarly reported isolation of rhizobia mutants that could not nodulate common agroforestry tree legumes like *Sesbania* and *Calliandra.* It is known that the ability to cause nodulation and nitrogen fixation is controlled by Nod genes and Nif genes (Long 1992; Anyango et al., 1995; Rigo et al., 2001). It is possible that this isolate is indeed a Nod mutant that has lost one or all its Nod genes. Such nod mutants have been reported (Long, 1992) and are unable to infect prospective hosts and establish symbioses. Molecular studies of nod-strains may contribute to my added understanding of the nodulation process.

5.6 Effectiveness of Rhizobia Isolates to Cross Inoculate Groundnut Varieties

Plant height did not vary significantly when the different varieties of groundnut were inoculated with the four isolates but great variation was observed in the controls and nitrogen treated plants. Nitrogen fertilized plants were the tallest. Nitrogen promotes internode elongation and hence increased plant height (Gopal, 1992). Therefore, for proper establishment of plants in N depleted soils before BNF derived N is availed, a little N supply in the beginning is necessary.

All the four rhizobia isolates showed great variability in their capacity to nodulate four groundnut varieties. Generally, all of the isolates formed more than 30 nodules per plant on the roots of their host. Harun et al. (2009) also reported that lentil rhizobia nodulated their host very well with different level of infectivity. Out of the four isolates in the current study three isolates did not vary significantly in nodule number and nodule fresh weight while W1 failed to nodulate the groundnut cultivar ICGVSM 99568. This could be attributed to either a narrower host specificity of the isolate or the cultivar seed coat antagonizing the rhizobia before infection. However, since rhizobia inoculant in this study was introduced after groundnut germination it may be concluded that isolate W1 a narrow host range. Chen et al. (2003) found that different groundnut cultivars varied in their ability to nodulate when inoculated with a given *Bradyrhizobium* strain. Differences among cultivars in terms of nodule weight and numbers and the ability of symbiotic N_2 fixation have also been reported earlier (Wynne et al. 1983).

Shoot dry weight varied across cultivars and isolates with the non-inoculated controls recording the least weight although this was not statistically significant. Similarly, Ayala

(1997) observed that groundnut inoculated with rhizobia had significantly increased shoot dry weight over the uninoculated control.

The improvement of nodulation in inoculated plants indicates that the isolates were effective in nitrogen fixation. These isolates can therefore be used for production of broad spectrum inocula which can be used for inoculation of diverse groundnut cultivars.

The correlation between the increase in shoot dry matter and the number or the dry weight of nodules was not significant ($p>0.05$). Similarly, Maâtallah et al. (2002) has also reported that there was no positive correlation between the increase of shoot dry matter and the number or dry weight of nodules although it has long been demonstrated that the dry matter yield was rather correlated with the nodule leghaemogblobin concentration than with the number or the dry weight of nodules (Dudeja et al., 1981). Generally there was good response of groundnut cultivars to inoculation in the greenhouse. The plants were assessed 6 weeks after planting and inoculation which gave them enough time for allocation of photosynthates to the nodules for N fixation (Larcher, 1995) and hence for the difference observed.

5.7 Response of Groundnut to Rhizobia Inoculation in a Monocrop

The soils from the selected sites were acidic. The soil pH 5.2 is below the critical values for optimum growth of rhizobia and groundnut which have been given as 6.0-6.8 and 5.5-7.0 respectively (Somasegaram and Hoben 1985). Soil acidity reduces growth of groundnuts, the rhizobia symbiont and nitrogen fixation. The effect may be indirect by increasing the level of toxic elements such as aluminium and manganese in soil solution and causing deficiency of mineral nutrients (Brady and Weil, 2002). The amount of N in

the experimental site was low. Therefore the soils require supplement nitrogen or acid tolerant effective rhizobia strains for optimum groundnut growth and production. Olsen P values were very low according to standard soil test classification (Estaban, 2000). These low values would therefore respond to P fertilizer application (Okalebo et al., 2002). Low phosphorus concentration in soils adversely affects growth of rhizobia, limits nodulation and nitrogen fixation (Beck and Munns, 1984) therefore BNF and the host rhizobia are expected to be low in these sites. Hence need to use rhizobia strains that are tolerant to acidity and low P concentration (Morals and Ramirez, 1988) or supplement soil with P as was done for this study using Triple Super Phosphate (TSP). Following the general guidelines of Okalebo et al. (1993) % C of 1.26 and 1.32 in Ligala and Koyonzo respectively in these soils would be rated as having a low (0.5-1.5%) organic carbon content. The low soil organic carbon in these soils could adversely affect production of groundnut since it is especially sensitive to soil drying. This could be so since soil organic carbon content influences a number of chemical and physical properties including soil aggregration, moisture holding capacity and cation exchange capacity (Fitz Patrick, 1986). It is therefore clear that organic carbon content of soils serves as an indicator for soil degradation. Hence in this experiment, Ligala site which had sand clay loam soil would be good for groundnut growth because groundnut is frequently grown sand soils sensitive to soil drying (Sinclair, 1991). Koyonzo soils would not be good for groundnut growth under sustainable agriculture because the soil type of clay loam sand is prone to high water retention that affects nodulation in groundnut (Venkateswarlu et al., 1991).

5.7.1 Number and Dry Weight of Nodules

Rhizobia inoculation had significant improvement ($p \leq 0.05$) on nodule numbers in both seasons, suggesting that the inoculant rhizobia had very good competitive ability to nodule occupancy. Thies et al. (1991) showed that inoculation of eight leguminous crops growing in soils containing 10 to 100 indigenous rhizobial cells g^{-1} soil increased the number of nodules per plant. Establishment of inoculant strains in soils with substantial populations of indigenous rhizobia is considered difficult and response to inoculation unlikely (Houngnandan et al., 2000; Bloem and Law, 2001; Abaidoo et al., 2007). The rhizobia strains in the soils compete during nodulation. Some of them may be effective and others less or completely ineffective (Monsalud et al., 1989). Effective strains can sometimes be more competitive in nodulation than ineffective strains. However, there is varied competition among effective strains to occupy nodulation sites on the root surface. Many of the effective rhizobia are reported to be poor competitors (Franco and Vincent, 1976). The strain difference in the ability to compete and occupy nodulation sites in field-grown plants could affect speed of nodulation (Oliveira and Graham, 1990). In this study the positive control (+N treatment) improved mean nodule number at Ligala in both the long and short rain seasons of 2011 while at Koyonzo the same response under this treatment was observed in the long rains only. High levels of inorganic N, especially nitrate-N, have been shown to suppress nodulation of legumes (Chemining'wa and Vessey, 2006). However, under soils low in mineral N, a moderate dose of N has been demonstrated to stimulate seedling growth and subsequently N_2-fixation (Goi et al., 1993). Inorganic N is required by legume plants during the 'nitrogen hunger period' for their nodule development, shoot and root growth before the onset of N_2-fixation process (Hansen,

1994). In both sites and seasons nodulation was improved in N treated plots by application of 34 kg N ha^{-1} possibly because the initial soil N level of 0.06-0.08% which is far below the optimal (0.2%) was not adequate during the nitrogen hunger period.

Nodule biomass increased significantly due to rhizobial inoculation in both seasons suggesting that the inoculant strains were possibly more effective than indigenous strains. Difference in the nodule biomass for the plants inoculated with different isolates suggests difference in effectiveness among the isolates.

5.7.2 Shoot Dry Weight

Rhizobia inoculation improved groundnut shoot biomass. Similar work by Musandu and Ogendo (2002) in low-N sites in western Kenya reported lack of response of the common bean to inoculation with rhizobia. However, Maina (1999) reported significant increases in shoot dry weight inGLP24 and other bean cultivars inoculated with local strains ao *Rhizobium*. It can therefore be concluded that the inoculants used in this study were effective in nitrogen fixation in groundnuts and competed appropriately for the nodulation sites under field conditions, contributing to improved plant growth and dry matter accumulation.

5.7.3 Groundnut grain yields

Grain yield varied with inoculant strain. Groundnut plots inoculated with strains V2 recorded the highest yield in both sites. This could be attributed to varying tolerance to acidity of this isolates since the two experimental sites had varying soil acidity. Ligala soil was sandy clay loam and highly acidic (pH 4.63) compared to Koyonzo site with clay sandy loam soil and less acidic (pH 5.20). It is therefore evident that isolate V2 can tolerate

high acidity levels compared to other isolates tested in this experiment and is very competitive in nodule occupancy.

The basis for differences in pH tolerance among strains of *Rhizobium* and *Bradyrhizobium* is still not clear (Graham et al., 1994), although several workers have shown that the cytoplasmic pH of acid-tolerant strains is less strongly affected by external acidity (Chen et al., 1993). The failure of legumes to nodulate under acid-soil conditions is common, especially in soils of pH less than 5.0. The inability of some rhizobia to persist under such conditions is one cause of nodulation failure (Carter et al., 1995). Another reason could be genetic differences among the isolates as interactions between legume species and microsymbionts have been demonstrated to be highly specific (Qiang et al., 2003).

V2 had the highest grain yield over the two sites and seasons (819 kg ha^{-1}). Strain W1 produced the lowest grain yield compared to the other strains over the two seasons in the two sites (700 kg ha^{-1}). This suggests that the isolate may not be suitable for use as an inoculant in the study sites.

The highest yield was obtained with a combination of inoculation with strain V2 and calcitic lime application. Similar response was also reported in groundnut by Simbajon and Duque (1987), with greater yields obtained recorded in treatments involving rhizobia inoculation and calcitic lime. Doddamani (1975) and Patil and Ananthanaryana (1989) also obtained better yields of groundnut when acid soils were limed. Lime raises the pH of acid soils and supplies calcium, an essential nutrient.

Furthermore calcitic lime has Ca which is essential for cell development and nitrogen metabolism. Since groundnut has a high demand for calcium, it responds well to increasing calcium supply (Bheemaiah and Ananthanrayana, 1984; Prasad et al., 1983). Studies have demonstrated that supply of Ca^{2+} through lime significantly increased plant growth and productivity. Richardson et al. (1988) reported that Ca^{2+} (10 mM) increased *nod* gene expression activities of clover plants 5- to 10-fold at pH 4.5 to 5.2. Hartley et al. (2004) also observed that lime application increased nodulation and yield of *Serradella* (*Ornithopus compressus*). The beneficial effects of liming on nodulation and plant growth most likely resulted from the enhanced conditions for seedling growth and nodulation. Interestingly, a study by Phillips et al. (1999) has also reported that rhizobia inoculants can stimulate growth and final yield of leguminous plants. The results from this study clearly demonstrate that inoculation had significant effects on yield and all the other yield components (nodule number, nodule weight, nut number and shoot weight) assessed in this study.

The higher biomass and grain yield obtained with inoculation indicates that the rhizobia technology is efficient in supplying N to legumes as inorganic-N fertilizer and a better option for resource-poor farmer who cannot afford to purchase expensive inputs. It is well established that leguminous plants in partnership with *Rhizobium* have the ability to convert the atmospheric nitrogen into usable forms (Ndakidemi et al., 2006).

The application of rhizobia and lime interacted significantly in such a way that the grain yield of groundnut was increased. Therefore, from this study, it is clear that inoculation

with rhizobia is important and plays crucial role in improving plant growth and increasing the grain yield of groundnut in the study sites. It may then be suggested that the observed benefits were due to ability of these treatments to improve the nutrition on N (from rhizobia) and Ca (from lime). It is well established that acidic soils have low capability to support plant growth and are deficient in N and Ca (Ndakidemi and Semoka, 2006).

Therefore, this study has demonstrated a significant advantage of combining the two treatments together for enhanced crop performance. It has also shown that the performance of acid tolerant rhizobia could be enhanced by liming. However the requirement and type of lime vary with the strain of rhizobia used.

5.8 Intrinsic Antibiotic Resistance

Great variation was observed among the isolates with respect to their intrinsic antibiotic resistance pattern with the tested antibiotics. The isolates recovered from the nodules of the groundnut plants grown in the experimental field with different treatments showed great morphological resemblance to the initial isolates. In this study all the initial isolates used as inoculants were fast growing just like the recovered strains. The recovery of isolates from the field was lower in Ligala site 41% compared to Koyonzo site 72%. The difference in recovery from sites could only mean that the isolates differed in their ability to tolerate acidity since Ligala site was more acidic (pH 4.63) and had low P, N and C compared to Koyonzo site (pH 5.20) It has long been noted that soil acidity constrains symbiotic N_2 fixation in both tropical and temperate soils (Munns 1986) by limiting the survival and persistence of rhizobia in soils and reducing nodulation (Brockwell et al., 1991). Hence rhizobia with a higher tolerance to acidity like the ones used in this study usually but do not always perform better under acidic soil conditions in the field (Graham et al., 1994).

Padmanabham et al. (1990) studied the IAR pattern of fast growing rhizobia and found lower IAR than that of slow growing bradyrhizobia. According to Kremer and Peterson (1982), the intrinsic resistance to antibiotics can be used for the identification of rhizobial strains that occupy nodules in studies designed to evaluate the ecological competitiveness. However, there was variation on the IAR pattern with site and isolate. This could only mean that the isolates had great genetic variability generated through recombination in the field that could lead to acquisition or loss of resistance to some of the antibiotics.

Abaidoo et al. (2002) suggested that the antibiotic resistance profiling can be used as a simple means of assessing genetic variability and grouping of a large number of *Bradyrhizobium* species. Representative isolates from each group can then be selected for further characterization. In addition, the pattern of antibiotics resistance has been used to identify diversity among strains of rhizobia. As a result, it could be used as supplementary diagnostic character for different rhizobial strains (Amarger et al., 1997).

This study achieved its broad objective of identifying efficient acid tolerant rhizobia native to soils of western Kenya that would be used as groundnut inoculants for enhanced crop performance and grain yield. Two superior isolates A6 and V2 could be recommended to farmers for use as groundnut inoculants.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

i. There exists a great diversity of native groundnut nodulating rhizobia in the acid soils of western Kenya. Such strains are well adapted to survive the harsh environment created by the prevalent soil acidity

ii. Symbiotic effectiveness of rhizobia varies with site and strain. Four of the acid-Al tolerant strains isolated and evaluated in this study were found to effectively nodulate four different groundnut varieties. This implies that they could be useful in production of inocula for a wide range of groundnut cultivars.

iii. The rhizobia isolates A6 and V2 together with calcitic lime showed great promise to increase groundnut performance in the low fertility acid soils of western Kenya. These strains have potential for conversion to bio-fertilizer forms that can be commercialised upon further testing across a wider geographical area for stable performance.

6.2 Recommendations

1. The strains identified in this study for groundnut inoculation need to be assessed for their competitive ability to nodule occupancy in diverse sites in order to fully determine the biodiversity inherent in Kenyan soils and to select more competitive and efficient adapted strains at each site for potential development of inoculants in order to optimize BNF and thus increase groundnut yield at low cost.

2. During this study the methods used for characterizing and distinguishing rhizobial strains were morphological, biochemical and symbiotic. However these traditional methods of rhizobial characterization frequently fail to identify strains within species. Hence such kinds of study should be substantiated by PCR based molecular methods so as to obtain better understanding of microbial diversity and strain identification.
3. There is need to screen more groundnut cultivars with the rhizobia isolates to establish their symbiotic and nitrogen fixation effectiveness. This is because potential nitrogen fixation is influenced by genotypes of the host plant as well as of the microsymbiont. Strain n3 that failed to nodulate groundnut need to be subjected to molecular tests to establish its failure to nodulate. This will help in the understanding of symbiotic interaction between groundnut and rhizobia.
4. The two groundnut rhizobia (V2 and A6) identified in this study as acid-Al tolerant can therefore be recommended for adoption as inoculants for groundnut in western Kenya regions.

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APPENDICES

Appendix Ia: Composition of Yeast Mannitol Agara

Ingridients	g/l
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Yeast extract or	0.5
Mannitol	10

Appendix Ib: Dyes Incorporated in YEMA Congo red (CR)

Required concentration in YEMA =25 µg ml^{-1}

Stock solution: 0.25g of CR was dissolved in 100ml sterile water.

10ml stock solution was added per litre of YEMA before autoclaving.

Bromothymol blue (BTB)

Required concentration in YEMA =25 µg ml^{-1}

Stock solution: 0.5g BTB was dissolved in 100ml ethanol. 5ml stock solution was added per litre of YEMA before autoclaving.

"Adopted from Somasegaren and Hoben, (1994).

Appendix 1c: Composition of Gram Stains[a]

Crystal violet solution

Crystal violet	10 g
Ammonium oxalate	4 g

Ethanol	100 mL
Distilled water	400 mL
Iodine	1 g
Potassium iodide	2 g
Ethanol	2.5 mL
Distilled water	100 mL
95%ethanol	
Absolute ethanol	95 mL
Distilled water	5 mL
Counterstain	
2.5%safranin	
(2.5 g in 100 mL ethanol)	
Distilled water	100 mL

“Adopted from Somasegaran and Hoben, (1994)

Appendix II: N-free medium for Grain Legumes (Somasegaran and Hoben 1994)

Stock solution	Nutrient	Form	g/1	Vol.of stock solution/10Lof medium(ml)
A	Ca	$CaCl_2.2H_2O$	294.1	5
B	P	KH_2PO_4	136.1	5
C	Fe	Fe-citrate	6.7	5
	Mg	$MgSO_4.7H_2O$	123.3	
	K	K_2SO_4	87.0	
	Mn	$MnSO_4.H_2O$	0.338	
D	B	H_3BO_3	0.247	5
	Zn	$ZnSO_4.7H_2O$	0.288	
	Cu	$CuSO_4.5H_2O$	0.100	
	Co	$CoSO_4.7H_2O$	0.056	
	Mo	$Na_2MoO_2.H_2O$	0.048	

To prepare 10 Litres of the nutrient solution, 5.0 ml of each stock solution A to D was added to about 5 litres of distilled water, and then diluted to 10 litres. 1M NaOH was used to adjust pH to 6.8.

Appendix IIIa: Determination of soil pH

The soil pH was determined by adding 25ml of distilled water to 10g of soil (<2 mm) in a beaker and the suspension stirred for 10 minutes and then stirred again for 2 minutes. The soil pH was then measured using a glass electrode on a pH meter (Okalebo et al, 2002).

Appendix IIIb: Determination of total organic carbon in the soil

Organic carbon was determined using the Nelson and Sommers (1975) oxidation method. This method involved complete oxidation of soil organic carbon using acid (H_2SO_4) and potassium Dichromate solution. The excess (unused) dichromate was determined by back titration using standard ferrous ammonium sulphate. Thus to determine organic carbon, 0.3g of fine soil (60 mesh) was weighed into block digester tubes. 5ml of potassium dichromate and 7.5ml of H_2SO_4 was added to each tube. The tubes were placed in a pre heated block digester at 145-150' C for 30 minutes, after which they were removed and cooled. The digests were then transferred quantitatively into 100ml conical flasks and 0.3ml ferroin indicator added, and then titrated with standard ferrous ammonium sulphate solution. The end point was noted through a color change from greenish to brown and the titre volume recorded. The blank correction was made by subtracting the sample reading from the mean of the two reagent blanks (T)

$$\textbf{Organic carbon} = \frac{\textbf{T x 0.2 x 0.3}}{\textbf{Sample weight (0.3)}}$$

Appendix IIIc: Soil particle size analysis

Soil particle analysis was done using the procedure of sedimentation that involves the dispersion of soil particles into constituents using sodium hexametaphosphate (calgon) solution and subsequent sedimentation of particles. Sedimentation allows the particles to settle to the bottom of the cylinder according to size, density and the viscosity of the fluid (Stokes law). After 2 hours 50 g of air-dried soil (<2 mm) was weighed into a 500ml beaker, 10 ml of calgon was added after the soil had been saturated with distilled water and

the mixture allowed to stand for ten minutes. The suspension was then quantitatively transferred into a string cup where further dispersion was done using an electric high speed stirrer for two minutes. The suspension was then transferred in to a graduated cylinder and topped with distilled water up to the 1130ml mark. These contents were covered well and inverted ten times and a hydrometer inserted and the first reading taken at 40 seconds (H_1). Then the contents were inverted again ten times and allowed to stand for two hours and the hydrometer left in the cylinder. A second hydrometer reading (H_2) was taken at 2 hour timing. Temperature reading was taken concurrently with both hydrometer readings.

Calculation

$$\% \text{ sand} = \left(\frac{50.0 - H_1}{50}\right) \times 100$$

$$\% \text{ clay} = \frac{H_2 \times 100}{50}$$

% silt = 100 (% sand + % clay)

A textural triangle was used to assign the textural class of the soil

Appendix IIId: Available phosphorus

Soil extraction for available P was done using the bicarbonate solution (0.5 M NaHCO3 at pH 8.5) method (Olsen et al, 1954). The bicarbonate extractant decreases the concentration of Ca as CaCO3 in the calcareous, alkaline and neutral soils containing calcium phosphates. The result is an increase of the P concentration in the solution. In acid soils containing Al and Fe phosphates, P concentration in the solution increases as the pH rises.

Precipitation reactions in acid and calcareous soils are reduced to a minimum because the concentration of Al, Fe and Ca remain at low levels in this extractant solution. P was then measured calorimetrically using a spectrophotometer after the development of a blue colored phosphomolybdate complex.

Colometric P measurements

The available P was determined by adding 10ml of each P standard solution (0, 0.5, 1, 2.5, 5.0, 7.5, 10.0 and 12.5 ppm P), sample filtrate and reagent blanks into 50 ml volumetric flasks. To suppress the interference of fluorides and sulphates, 5ml of 0.8 M boric acid was added into each flask. 10 ml of ascorbic acid reducing agent was added and the flasks topped using distilled water to the 50ml mark and shaken well. After 1 hour, the absorbance was read at 880 nm (Murphy and Riley, 1962). Concentration of P ppm P in soil = concentration of P in solution x 100.

Appendix IIIe: Digestion procedure for total N and P in plants and soil

The principle involved in the digestion of plant and soil materials is oxidation of the organic material into soluble N and P components (NH_4 and phosphate) in H_2SO_4 /Se/$LiSO_4$/H_2O_2 digestion mixture. Hence, 0.3g of dry ground plant material (20 meshes) or soil was weighed into a dry and labeled digestion tube and 4.4 ml of the digestion mixture was added including two reagent blanks for each batch of samples. The mixture was then digested slowly on a block digester up to a temperature of 360' C for three hours until the solution is clear and allowed to cool. It was then quantitatively transferred into 50ml volumetric flasks and topped to the mark with distilled water and transferred into 75 ml storage bottles. The mixtures were used to determine both total P and N.

Determination of total N from sample digests

It was done using the colometric method. In a clean set of 50 ml volumetric flasks 0, 5, 10, 15, 20 and 25 ml of the standard solution was added. (100 µg NH4 +/ml). 0.2 ml of the sample was pipetted using a micropipette into clearly marked test tubes. 5 ml of the reagents N1 (made by dissolving 34g of sodium salicylate, 25g of sodium citrate and 25g of sodium citrate in about 750 ml of distilled water). 0.12g of sodium nitroprusside was then added and shaken well and topped to make 1000ml with distilled water and allowed to stand for fifteen minutes. Then five ml of reagent nitrogen (prepared by dissolving 30g of NaOH in 750 ml of distilled water) was added and well shaken. Absorbance was read at 655 nm after standing for one hour for color development.

N% = (C x W) x 0.01

Where C = corrected concentration (µg/ml)

W = weight of sample

Determination of total P

Zero, 1, 2, 3, 4, 5 and 6 ml of the standard 10 ppm P working solution was pipetted into 5o ml volumetric flasks. 5ml of the plant digests was separately pipetted including the blanks into the volumetric flasks. 10 ml of ascorbic acid was added and topped with distilled water and shaken well. Contents were allowed to stand for one hour for the molybdenum-ascorbic acid blue color development and the absorbance read at 880nm using a spectrophotometer. A graph of absorbance against standard P concentrations was constructed.

The % P will be calculated as follows for 5 ml aliquots

$$\%P \text{ in sample} = \frac{C \times 0.05}{W}$$

Where C = blank corrected concentration for sample solution in ppm P

W= weight of sample taken (0.3g)

Appendix IIIf: Colorimetric determination of nitrate

The determination of nitrate consist of an extraction in 0.5 M K_2SO_4 followed by the colometric estimate of NO_3N in the extractant. To 10g of fresh soil, 20ml of the extractant were added and shaken for 30 minutes at 60 rpm. Filtering was done using Whatman No. 42 filter paper. Using a micropipette, 0.5 ml of the standard solution and samples including blanks were pipetted into marked test tubes. One ml of salicylic acid solution (4M NaOH) was added, mixed well and left to stand for one hour for color development. Absorbance was then read at 410nm. A graph of absorbance against standard concentration was drawn and after blank correction, amount of nitrate was calculated as:-

NO_3N (µg/g soil) = (C x V)/W

Where:

C = corrected concentration (µg/ml)

V = extractant volume (ml)

W = weight of sample (g)

Appendix IV: Field performance of rhizobia inoculated groundnut at Koyonzo and Ligala in two rain seasons of 2011

Appendix IVa: Table of means showing overall crop performance across sites and seasons

Variable	Parameter Scored				
	NN	**NDW(mg)**	**Nut No.**	**SDW (g)**	**GY (kg ha^{-1})**
Ligala site	36.25^{a}	42.98^{a}	120.97^{a}	77.51^{b}	**870^{a}**
Koyonzo site	28.53^{b}	24.21^{b}	50.70^{b}	93.54^{a}	**816^{b}**
Season1	37.5^{a}	21.93^{b}	70.8^{b}	56.8^{b}	**810^{b}**
Season 2	27.3^{b}	45.3^{a}	100.83^{a}	114.3^{a}	**811^{a}**
Calcitic lime	31.56^{b}	32.8^{c}	86.6^{b}	101.9^{a}	**970^{a}**
Dolomitic lime	33.85^{a}	33.5^{b}	81.6^{c}	68.7^{c}	**803^{b}**
No lime	31.76^{b}	34.6^{a}	89.4^{a}	85.99^{b}	**804^{b}**
Strain V2	33.03^{b}	35.31^{a}	97.67^{a}	92.08^{a}	**819^{a}**
StrainW1	31.44^{c}	33.83^{b}	81.94^{d}	79.97^{c}	**700^{e}**
Strain A6	35.69^{a}	34.39^{ab}	92.14^{b}	91.19^{a}	**813^{b}**
Biofix	28.94^{d}	33.47^{b}	82.33^{d}	83.36^{b}	**801^{c}**
Nitrogen	33.22^{b}	32.19^{c}	84.58^{c}	91.83^{a}	**770^{d}**
Control	32.0b^{c}	32.39^{c}	76.36^{e}	74.69^{d}	**680^{f}**
Grand mean	**32.39**	**33.6**	**85.84**	**85.52**	**805**
SE±	**3.1**	**1.8**	**2.9**	**2.3**	**3.6**
%CV	**9.7**	**5.9**	**3.3**	**2.7**	**3.6**

Note: Means in a column followed with same letters are not significantly different at 95% level of probability: n=108; NN- Nodule Numbe, NDW- Nodule Dry Weight, Nut no.- Nut Number, SDW- Shoot Dry Weight, GY- Grain Yield

Appendix IVb: ANOVA for the effect of rhizobia inoculation on groundnut performance at the Ligala site

Dependent Variable: Nodule Number

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Season	1	2670.0	2670.0	177.1	<.0001

		8	8	7	*
Lime	2	860.22	430.11	28.54	<.0001*
season*Lime	2	548.67	274.33	18.20	<.0001*
Strain	5	1962.97	392.59	26.05	<.0001*
season*Strain	5	2045.64	409.13	27.15	<.0001*
Lime*Strain	10	3516.56	351.66	23.33	<.0001*
season*Lime*Strain	10	6358.11	635.81	42.19	<.0001*
Error	60	904.22	15.07		
Corrected Total	95	18966.47			

R-Square	Coeff Var	Root MSE	NN Mean
0.952269	10.70912	3.882057	36.25000

* Significantly different

Dependent Variable: Nodule Dry Weight

Source of variation	DF	Sum of squares	Mean Square	F Value	Pr > F
Season	1	44896.33	44896.33	10661.4	<.0001*
Lime	2	1393.91	696.95	165.50	<.0001*
season*Lime	2	690.06	345.03	81.93	<.0001*
Strain	5	317.97	63.59	15.10	<.0001*
season*Strain	5	1831.22	366.24	86.97	<.0001*
Lime*Strain	10	6530.09	653.01	155.07	<.0001*

	10	2571.72	257.17	61.07	<.0001*
season*Lime*Strain					
Error	60	252.67	4.21		
Corrected Total	95	58483.97			

R-Square	Coeff Var	Root MSE	NDW Mean
0.995687	4.774380	2.052099	39.25

* Significantly different

Dependent Variable: nut number

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Season	1	223678.01	223678.01	28327.0	<.0001*
Lime	2	5950.89	2975.44	376.82	<.0001*
season*Lime	2	8076.96	4038.48	511.44	<.0001*
Strain	5	12350.31	2470.06	312.81	<.0001*
season*Strain	5	9698.82	1939.77	245.66	<.0001*
Lime*Strain	10	28312.56	2831.26	358.55	<.0001*
	10	21219.37	2121.94	268.73	<.0001*
season*Lime*Strain					
Error	60	473.78	7.90		
Corrected Total	95	309760.7			

R-Square	Coeff Var	Root MSE	nutno Mean
0.998471	2.322876	2.810035	120.9722

* Significantly different

Dependent Variable: Shoot Dry Weight

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
season	1	183768.75	183768.75	29050.1	<.0001*
Lime	2	6485.24	3242.62	512.59	<.0001*
season*Lime	2	6149.06	3074.53	486.02	<.0001*

Strain	5	13502.82	2700.57	426.90	<.0001*
season*Strain	5	16240.97	3248.19	513.47	<.0001*
Lime*Strain	10	13507.09	1350.71	213.52	<.0001*
season*Lime*Strain	10	15110.39	1511.04	238.86	<.0001*
Error	60	379.56	6.33		
Corrected Total	95	255143.88			

R-Square	Coeff Var	Root MSE	SDW Mean
0.998513	3.244953	2.515139	77.50926

* Significantly different

Dependent Variable: Grain yield

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Season	1	373003.79	373003.79	46973.4	<.0001*
Lime	2	3671.24	1835.62	231.16	<.0001*
season*Lime	2	4460.24	2230.12	280.85	<.0001*
Strain	5	14380.60	2876.12	362.20	<.0001*
season*Strain	5	3970.60	794.12	100.01	<.0001*
Lime*Strain	10	28867.65	2886.77	363.54	<.0001*
season*Lime*Strain	10	5194.87	519.49	65.42	<.0001*
Error	60	476.44	7.94		
Corrected Total	95	434025.43			

R-Square	Coeff Var	Root MSE	GY Mean
0.998903	2.422098	2.817932	623.75

* Significantly different

Appendix IVc: ANOVA for the effect of rhizobia inoculation on groundnut performance at Koyonzo site

Dependent Variable: Nodule Number

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Season	1	2976.75	2976.75	632.85	<.0001*
Lime	2	541.06	270.53	57.51	<.0001*

season*Lime	2	1040.17	520.08	110.57	<.0001*
Strain	5	2233.19	446.64	94.95	<.0001*
season*Strain	5	1318.31	263.66	56.05	<.0001*
Lime*Strain	10	2346.83	234.68	49.89	<.0001*
season*Lime*Strain	10	541.28	54.13	11.51	<.0001*
Error	60	282.22	4.70		
Corrected Total	95	11279.81	4781.17		

R-Square	Coeff Var	Root MSE	NN Mean
0.975115	7.602423	2.168802	28.52778

* Significantly different

Dependent Variable: Nodule Dry Weight

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
season	1	942.23	942.23	262.95	<.0001*
Lime	2	776.69	388.34	108.37	<.0001*
season*Lime	2	2330.35	1165.18	325.17	<.0001*
Strain	5	1472.49	294.50	82.19	<.0001*
season*Strain	5	799.38	159.88	44.62	<.0001*
Lime*Strain	10	1902.76	190.28	53.10	<.0001*
season*Lime*Strain	10	626.87	62.69	17.49	<.0001*
Error	60	215.00	3.58		
Corrected Total	95	9065.77			

R-Square	Coeff Var	Root MSE	NDW Mean
0.976431	7.818000	1.892969	36.50

* Significantly different

Dependent Variable: nut number

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
season	1	26009.04	26009.04	3114.16	<.0001*
Lime	2	978.57	489.29	58.58	<.0001*
season*Lime	2	790.35	395.18	47.32	<.0001*
Strain	5	3151.41	630.29	75.47	<.0001*
season*Strain	5	428.52	85.70	10.26	<.0001*
Lime*Strain	10	790.87	79.09	9.47	<.0001*
season*Lime*Strain	10	1950.43	195.04	23.35	<.0001*
Error	60	501.11	8.35		
Corrected Total	95	36550.73			

R-Square	Coeff Var	Root MSE	nutno Mean
0.985582	5.699696	2.889957	50.70370

* Significantly different

Dependent Variable: Shoot Dry Weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
season	1	28551.26	28551.26	6472.58	<.0001*
Lime	2	41567.36	20783.68	4711.66	<.0001*
season*Lime	2	29160.25	14580.12	3305.32	<.0001*
Strain	5	6132.52	1226.50	278.05	<.0001*
season*Strain	5	8422.41	1684.48	381.87	<.0001*
Lime*Strain	10	3133.32	313.33	71.03	<.0001*
season*Lime*Strain	10	8662.43	866.24	196.38	<.0001*
Error	60	264.67	4.41		
Corrected Total	95	125894.22			

R-Square	Coeff Var	Root MSE	SDW Mean
0.997898	2.245383	2.100265	93.53704

* Significantly different

Dependent Variable: Grain Yield

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
season	1	165440.08	165440.08	8930.19	<.0001*
Lime	2	573.69	286.84	15.48	<.0001*
season*Lime	2	5105.72	2552.86	137.80	<.0001*
Strain	5	21785.49	4357.10	235.19	<.0001*
season*Strain	5	9557.19	1911.44	103.18	<.0001*
Lime*Strain	10	18281.76	1828.18	98.68	<.0001*
season*Lime*Strain	10	16108.17	1610.82	86.95	<.0001*
Error	60	1111.56	18.53		
Corrected Total	95	237963.73			

R-Square	Coeff Var	Root MSE	GY Mean
0.995330	4.944697	4.304175	588.96

* Significantly different