EFFICACY AND COMPETITIVENESS OF INDIGENOUS BRADYRHIZOBIA STRAINS ON SOYBEAN (*GLYCINE MAX (L.) MERR.*) PRODUCTIVITY IN MALAWI

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Egerton University

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DECLARATION AND RECOMMENDATION

Declaration

I declare that this is my original work and has not been presented in this or any other University for any other award.

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Recommendation

This work has been submitted with our approval as University supervisors

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ABSTRACT

Soybean is an important cash crop with a growing demand in Malawi. Its ability to fix nitrogen has been exploited through the use of rhizobia inoculants to supplement indigenous rhizobia and improve soil fertility. Previous research has however shown instances whereby inoculation has had no yield response which suggests a need to fill the knowledge gap on the nitrogen fixation efficiency of indigenous rhizobia. The main objective of this study was to contribute to increased soybean yields through the use of indigenous rhizobia strains which are efficient in nitrogen fixation. 170 isolates of indigenous rhizobia were obtained from the 4 agro-ecological zones of Malawi and grouped into 19 presumptive species based on a binary matrix of tolerance to acid and salt stressed environments and antibiotic resistance patterns. The Shannon's species diversity index analysis yielded a low index of 2.48 with a high evenness of 0.8 showing very few evenly distributed species of rhizobia, 90.5% of which were less stress-resilient than the reference strain USDA110. The top 5 most resilient strains were evaluated for BNF potential on 3 common soybean varieties. All the strains were found to perform either at par or better than control treatments strain in Shoot Dry Weight ($P < 0.001$). The top 3 most effective strain-variety combinations were evaluated for their symbiotic efficiency in three soil types. Significant differences were found in the combinations in different soil types ($P<0.001$) with MAL_120 to Tikolore interaction having the highest symbiotic efficiency (87.71%). All other interactions had less efficiency than the reference interaction (USDA 110-Makwacha). These results led to the recommendation of MAL_120 and MAL_117 for field tests and genetic fingerprinting to ascertain its genotypic uniqueness. The overall conclusion was that the abundance of indigenous rhizobia in Malawi's soils which affect the success of introduced strains. Differences in symbiotic efficiency of variety-strain combinations based on soil mineralogy show that recommendation of inoculation should be complimented by strain-variety compatibility tests.

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LIST OF ACRONYMS AND ABBREVIATION

AEZ……..… Agro-Ecological Zone

ANOVA……... Analysis of Variance

BNF…….. Biological Nitrogen Fixation

BTB…….. Bromothymol Blue

CFU……..... Colony Forming Unit

CIAT……..…. Centre for International Tropical Agriculture

CR……..... Congo red

CRD……. Completely Randomized Design

DARS……... Department of Agricultural Research Services

IITA……. International Institute of Tropical Agriculture

LSD…….. Least Significant Difference

MASL……..… Meters above Sea Level

MICERN……..…… Microbiological Resource Center

MMT…… Million Metric Tonnes

MPN……. Most Probable Number

N2Africa……. Nitrogen Fixation to Africa

NGO……. Non Governmental Organization

PCR…….. Polymerase Chain Reaction

SSA……. Sub-Saharan Africa

TSBF……...…… Tropical Soils Biology and Fertility Institute of CIAT

USAID…… United States Agency for International Development

YMA……..…… Yeast Mannitol Agar

CHAPTER 1

Introduction

1.1 Background Information

Soybean is an important cash crop in Malawi with its production on a steady increase since the early 1990's. Tinsley (2009) reported a growing demand for soybean in Malawi. This is mainly as a cash crop, with very little village-based consumption. Data from FAOSTAT (2012) shows that the total output of soybean grain in Malawi increased from 40,000 metric tons to 80,000 tons between 2002 and 2010. Production requirements are favourable for farmers since soybean is able to nodulate and fix nitrogen through a symbiotic association with bacteria of the Bradyrhizobia species in the soil. this reduces the need for inorganic nitrogen fertilizers in soybean and other legumes, however, a low proliferation of nitrogen fixing bacteria in most soils necessitates supplementation which is mainly done during planting through the inoculation of soybean seeds with compatible Bradyrhizobia species.

Although a relatively old product, rhizobia inoculants are facing a challenge to achieve adoption in Malawi due to erratic supply mechanisms, perceived lack of efficacy and lack of farmer awareness. Until the late 1980s and early 1990s, seed-applied peat-based inoculants dominated the commercial inoculant market (Dakora *et al*., 2008). The earliest records on inoculation activities were from 1951 when inoculation trials at Bvumbwe Research Station indicated the benefit of Rhizobia inoculant to soybean (Davis, 1982). Subsequent trials in the 1960s and 1970s at Chitedze Agricultural Research Station (ARS) demonstrated dramatic increases in soybean yields due to the application of locally produced inoculants containing indigenous rhizobia strains (Khonje, 1989). Commercially available inoculants produced by the Microbiology Section of Chitedze ARS, Lilongwe, were available starting from 1975. Inoculants for several pasture and grain legumes were produced and sold in 50 g packets (Khonje, 1989). Lack of extensive follow-up research and public-domain information on advances in this area has rendered almost all of the progress made subject to neglect and limited research has left a gap in information and expertise in this sector.

1.2 Statement of the Problem

Very little research has been dedicated to studying the characteristics and effectiveness of these indigenous rhizobia species in causing nodulation and successful nitrogen fixation. Previous research has suggested that although inoculants have generally been shown to have an effect on the performance of soybean, there have been instances whereby uninoculated soybean has been shown to perform at par or even better than inoculated soybean (Soko, 2001). Such results suggest the availability of effective indigenous strains of rhizobia in the soil. So far, there has been very little documentation of studies in Malawi that have characterized and evaluated the rhizobia germplasm from the different AEZsof Malawi or evaluated the need to inoculate the soils with rhizobia in order to improve yields. Recommendations made to farmers to use inoculants are based on general nitrogen deficiency symptoms in plants with the assumption that inoculation will directly result into yield improvement which is sometimes not the case (Soko, 2001). There is a knowledge gap on the nitrogen fixation efficiency of indigenous rhizobia species and how these may affect the performance of recommended strains like the USDA 110 *(Bradyhizobium japonicum*) which may help to explain the ineffectiveness of inoculants in some soils (Bala *et al.*, 2011).

1.3 Objectives

The main objective of this study was to contribute to increased soybean yields through the use of indigenous rhizobia strains which are efficient in nitrogen fixation.

The specific objectives of the study were as follows:-

1. To characterize the diversity of indigenous rhizobia strains in acidity, antibiosis and salinity tolerance ranges
2. To determine the nitrogen fixation potential of indigenous rhizobia strains through their effect on shoot weight accumulation in market available soybean varieties Makwacha, Tikolore and Nasoko.
3. To establish the nitrogen fixation efficiency of indigenous rhizobia strains to soybean variety combinations against recommended combinations in potted soil.

1.4 Hypotheses

This research tested the following three hypotheses:

1. H_0: There is low diversity in the tolerance on indigenous rhizobia species in acidity, antibiosis and salt tolerance.
2. H_0: There are no significant differences in contribution of different rhizobia strains to shoot weight accumulation in different soybean varieties.
3. H_0: There are no significant differences in the nitrogen fixation efficiency of different rhizobia strains in different soybean plants grown under different soil conditions.

1.5 Justification

The potential use of *Bradyrhizobium* in improvement of soybean yields in Malawi has been observed as early as 1970 under research management (Khonje, 1989). Under smallholder farmer management in Malawi, inoculation has also been shown to work in both promiscuous and specific released varieties (Thuita *et al*., 2012). It has however been noted that on farmer field evaluations in Malawi, the effect of inoculation is more evident in promiscuous varieties than in older specific varieties which suggests the effect of variety on the efficacy of inoculation (Thuita *et al*., 2012). Intermediate soybean lines tested in five different locations of Chitedze, Makoka, Baka, Kandiyani and Bembeke in central and southern Malawi showed self nodulation without inoculation in all locations and a significant difference ($P<0.05$) in the interactions between the soybean genotype and the nodule count to suggest that abundance of indigenous rhizobia and a diversity in their ability to nodulate different soybean lines (Soko, 2001). Effective inoculation in the field has been observed in both peat-based and liquid inoculants in Lisasadzi, Biloti, and Tembwe in Malawi where inoculation was seen to significantly increase the shoot dry weight of soybean (Dakora *et al*., 2008). Chilimba *et al*. (2008) reported response failure to inoculation in Nyala and 1SCS1 in four sites, this was reportedly due to the promiscuity of the Nyala variety which interacted better with the indigenous rhizobia than the introduced strain. The same results were observed by Musiyiwa *et al*. (1998). The abundance and efficacy of indigenous rhizobia is a major factor in soybean inoculation response (Singleton and Tavares, 1986). This body of literature suggests the availability of indigenous rhizobia in the soil that have competitive advantage in colonization of soybean roots and nitrogen fixation at par with introduced strains.

Inoculants in Malawi are sourced from Chitedze Research station which acquires its rhizobia strains from nodule trapping of soybean inoculated with imported *Bradyrhizobium*

japonicum (Bala *et al*., 2011). Chitedze inconsistently uses harmonized quality control protocols which ensure the availability of at least 10^6 colony forming units of bacteria in all inoculants after curing (Bala *et al*., 2011). The level of quality assurance is meant to make sure that there are enough bacteria for successful inoculation and nodulation so as to narrow down and non-response to efficacy constraints.

These scenarios presented an opportunity to conduct a definitive probe on the diversity of indigenous rhizobia characters that affect survival in the soil and colonization of soybean roots which relate to the symbiotic efficiency hence the nitrogen fixation efficiency. Comparisons between indigenous rhizobia and recommended strains as well as nitrogen fertilization would also be useful in assessing the effectiveness of inoculation. Conclusions derived from this study would be used as a platform for advocating for improvements in locally produced inoculant products. The results will also help to form a basis for establishing and enforcing quality control standards for local inoculant production and importation for the certifying authorities. It will also provide a platform for further research towards understanding the diversity of rhizobia in Malawi and how this can be integrated in localized recommendations for soil fertility management.

1.6 Scope and Limitation of the Study

This study was based in Malawi and the nodule collection was done in a stratified random sampling technique based on AEZs and areas of widespread adoption of soybean as a crop. The initial sample size was 200 nodules from the four AEZs out of which 170 isolates were successfully cultured. Each zone contributed a proportion of the 170 nodules based on its coverage (land size) and its importance in soybean production. Throughout this paper, rhizobia were characterized "presumptively". Although the morphological characterization is reliable and widely adopted, PCR-based genetic fingerprinting is required to definitively characterize any individual strain, a technique that was not at the disposal of the researcher due to its funding requirements. The study also assumed that all sampled nodules contain indigenous rhizobia since they were sampled from fields with no known history of inoculation. The evaluation of elite strains for symbiotic efficiency in different soil types and different soybean varieties was done in a greenhouse environment. This was to eliminate all sources of variation that may impact the performance of different rhizobia (*ceteris paribus*). This enabled the selection of rhizobia strains based on their true phenotypic expression with

adequate nutrient supply and without competition. Outside the scope of this research, multi-locational field trials in different biophysical conditions are required in order to finally recommend a specific strain of rhizobia for release as an agricultural technology. The limitation of this research is to screen out these potential indigenous strains for their maximum phenotypic expression in non-restrictive environments.

1.7 Definition of Terms

Species Richness: The total number of species of a particular organism found in a community or sample (Shannon & Weaver, 1949).

Shannon's Evenness: a measure of biodiversity in a community which quantifies how equal the community's species composition is numerically (Shannon & Weaver, 1949).

Shannon's Exponent: A transformation of the nonparametric Shannon's diversity index which reflects the true species diversity of the index (Shannon & Weaver, 1949).

Rarefaction: A technique used to estimate species richness in a community or sample based on the probability of finding a new individual of the same species within the community or sample (Chao *et al.*, 2014)

CHAPTER 2

Literature Review

2.1 The Soybean Crop

Soybean is the most important and most cultivated oilseed crop in the world grown in more than 50 countries (Chianu *et al.*, 2009). In sub Saharan Africa, soybean is a relatively new crop to farmers. By the year 2000, 1.1% of the total 73 million ha planted with soybean in the world was is SSA (Chianu *et al.*, 2009). In 2008, worldwide soybean production was 230 MMT. Hartman *et al*. (2011) predicts that future production of soybean is expected to expand due to expanding area of production and higher yield. By 2011, 6% of the world's arable land was subtended by a soybean crop (Hartman *et al*., 2011).

According to Chianu *et al.* (2009), the soybean plant can grow to 60-120 cm within a maturity period of 3 to 6 months and has the potential to fix up to 150 kg N ha^{-1} annually and is a key component of most crop rotation systems. A typical soybean pod contains three or four round cream yellow or black seeds and the pod itself is hairy. Chianu *et al.* (2009) explain that soybean is best grown as a pure stand but also does relatively well in strip and rotation systems mostly for its nitrogen fixation potential and the subsequent benefits it imparts on the other crops in the cropping system.

Soybean productivity is affected by a number of biotic and abiotic constraints. Hartman *et al.* (2011) identifies extremes of nutrients, temperature and moisture as the main abiotic factors that are likely to directly affect the yield of a soybean crop. Indirect effects identified are increases in pathogen and pest attack whose effects are made more pronounced by the abiotic stresses. Biotic stresses however tend to be geographically and environmentally restricted (Hartman *et al.*, 2011). Soybean is also affected a lot by salinity in arid and semi-arid areas. According to Katerji *et al.* (2003), soybean's sensitivity to salinity causes deterred root development, leaf chlorosis and reduced plant vigour and yield. Soil pH is also a constraint in production as it reduces the ability of nitrogen fixing bacteria in forming a symbiosis with the soybean plant (Mensah *et al.*, 2006). Rhizobium that is able to tolerate high salinity and pH stress can hence be ideal for maximizing the effectiveness of the symbiosis. In most heavily weathered soils such as those that pre-dominate Malawi, soil pH is an ever present stress with salinity also becoming a challenge with the increasing aridity

(MoA&Ir, 2005). It is hence the purpose of this research to identify some indigenous rhizobium strains that are adapted to this stress and have the potential to give comparable or better results in nitrogen fixation potential as market available strains.

Soybean is well adapted to Malawi's climatic conditions ranging from warm to hot low lying areas of the Lowland AEZ with marginal rainfall of less than700mm to the highlands of Nyika and Vipya Plateaus with more than 2,000 mm rainfall; and soil types ranging from sandy loams to heavy clay soils with sufficient drainage (MoA&Ir, 2005). Currently average farmers' yields are very low ranging from 400 to 1,000 kg per hectare against an average yield potential of 4,500 kg per hectare that can be obtained with good crop management (MoA&Ir, 2005). Soybean in Malawi is generally attacked by soybean rust which has been known to lead to total crop failure in some instances but is otherwise generally disease free. The main pests that attack soybean are termites and aphids which can be easily controlled by the use of pyrethroids like Cypermethrin (MoA&Ir, 2005). The most widely available released varieties include Makwacha, Nasoko, Ocepara, Tikolore (IITA, 2011) and Magoye with Makwacha and Nasoko being the most widely adopted (Kapalasa, 2012).

2.2 Soils in Malawi

The location of Malawi shows that it has tropical and sub-tropical environments. These are aggressive soil-forming environments where the soils formed have low inherent fertility because their clay components are predominantly1:1 (*Kaolinite*) as opposed to 2:1 (iron oxides),both of which are inactive materials; and low content of rock minerals which can yield nutrients when they break down (FAO, 1999). Soil organic matter should therefore be the expected determinant of the fertility of these soils. The most prevalent soils in the country are the red soils (i.e. *Ferrisols*, *Ferruginous* soils and *Ferralitic* soils) which occur on the highlands and medium plateau physiographic units (MoA&Ir, 2005). They are highly weathered and hence have low inherent soil fertility. According to (MoA&Ir, 2005), of the *Ferritic* and *Ferallitic* soils, *Ferralsols* are most dominant and prevalent in the *Kasungu* and Kawinga plains and parts of the Highlands. *Lixisols* can be found dominant in *Mchinji*, *Dedza*, *Mzimba* south-west, *Kasungu* and *Kawinga* plains and much of central and northern Malawi. *Fluvisols* on the other hand are found in much of the lowland AEZ, *Phalombe*, the Lakeshore plains and the *Lilongwe* plain.

2.3 Rhizobia Classification and Characterization

Rhizobia generally refer to alpha-proteobacteria of the genus *Rhizobium* which has the general characteristic of being able to enter into nitrogen-fixing symbiotic relationships with leguminous plants (Valerie &Sharon, 1999). The first recorded isolation and description of this genus of bacteria was reported in 1888 by Beijerinck who described that isolations of bacteria from root nodules of plants had led to the establishment that they were the causative agent of nitrogen fixation in legumes. Beijerinck named these bacteria "*Bacillus Radiciola*" (Kaisa *et al.,* 1996). The genus name was changed a year later to *Rhizobium* with only one species identified, the *Rhizobium leguminosarium* (Tindall, 2008).

According to Tindall (2008), as interest into the nitrogen fixation phenomenon increased in the 20th century, more nitrogen fixing symbiotic bacteria were found and later classified into the family *Rhizobiacaea* which to date contains the genera *Rhizobium, Mesorhizobium, Ensifer* and *Bradyrhizobium*. *Ensifer* has recently gained prominence over *Sinorhizobium* as the latter was found to be simply a recombination of the former (Tindall, 2008). The genera *Rhizobium* and *Bradyrhizobium* are particularly known to be the nitrogen fixing bacteria (Sofie *et al.*, 2011). The genus *Bradyrhizobium* was created by dividing the genus *Rhizobium* into two genera of nitrogen fixing bacteria based on their growth characteristics on yeast mannitol. Fast growing (2 to 3 incubation days) and acid producing bacteria are classified as *Rhizobium* (Valerie & Sharon, 1999) while the slow growing strains (8 days) typically alkalize their environment and are classified into the *Bradyrhizobium* genus (Samrudhi *et al.*, 2013).At the time the *Bradyrhizobium* genera were classified, there was only one species, the *Bradyrhizobium japonicum* which was primarily in the soybean cross inoculation group (CIAT, 1988; Willens, 2006). To date 16 species of *Bradyrhizobium* have been accepted on the official List of Prokaryotic Names with Standing Nomenclature (LPSN) namely *betae, canariense, cytisi, daqingense, denitrificans, diazoefficiens, elkanii, huanghuaihaiense, iriomotense, japonicum, jicamae, lablabi, liaoningense, oliotrophicum, pachyrhizi,* and *yuanmingense* (LPSN, 2013).

Pinton (2008) discusses that the rhizobia-legume symbiosis is affected by the same environmental factors that affect all microorganisms in the soil such as water stress, pH stress and competition. Pinton (2008) further states a hypothesis that symbiotic interaction does not start with a carbon/nitrogen exchange, which takes place only in already developed nodules,

but with the supply of essential trace elements (like Molybdenum and Iron) by the host plants to the rhizobia in a competition-limiting environment. Aluminum, manganese toxicity and phosphorus deficiency also affect symbiotic effectiveness. Like other species of bacteria, Rhizobia are characterized according to phenotypic expressions in culture. Of particular importance in agriculture is its ability to tolerate low pH and high salinity environments.

Competition dynamics in the soil determine the efficacy of rhizobia to survive in its environment (Bohlool *et al.*, 1992). Altering of nutrient levels (*i.e.* P) ultimately changes these competition dynamics and equilibrium states. Salinity is an important factor that affects the survival of *Rhizobium* in the soil and one that is most likely to be of utmost importance in the semi-humid and semi-arid regions of Africa at large and Malawi in particular. In salinity tests by Keneni *et al.* (2010) on indigenous and exotic strains, it was shown that *Rhizobium* from both sources was not able to survive NaCl concentrations higher that 5%. In addition to salinity, Samrudhi *et al.* (2013) state that the other factors affecting symbiosis are the soil pH, nutrient deficiency as well as mineral and heavy metal toxicity, temperature extremes while singling out salinity as a particularly important condition limiting rhizobium growth due to its effects on plant growth hence on the symbiosis although it is noted that *Rhizobium* have shown a degree of tolerance to salinity. Keneni *et al.* (2010) discuss the importance of using salinity tolerant strains as their enhancement on the nodulation is better than other strains. The authors illustrate the importance of pH in the rhizobium-legume symbiosis where exotic rhizobia were not able to survive pH levels below 5.5 while the indigenous ones were able to form nodules in a pH range of 4 - 7 which suggested more robustness on the part of the indigenous strains. Mensah *et al.* (2006) report the optimum pH level of cowpea rhizobia which is in the same cross-inoculation group as soybean to be in the range of 6 - 7. Salinity and pH tolerance are of particular interest for this research and will be used to group indigenous rhizobium strains into presumptive species and estimate a Shannon diversity index.

One Rhizobium strain is distinguished from another in culture by its morphological characteristics and reactions. Somasegaran & Hoben (1994) explain the aspects of distinction of rhizobia as they form in culture, the size, colour, gram reaction as well as its characteristic growth in a range of media *e.g.* acidic or alkaline media. Rhizobia morphology is often described by the type of shape their colonies form. Vertical colony shapes can range from flat, domed and conical. When growing below the surface of the agar, colonies are typically

shaped like a lens (Somasegaran & Hoben, 1994; Woomer *et al.*, 2011).In terms of colour and texture, colonies are typically opaque white or they may be milky to watery-translucent. The variation in growth rate is such that the fast-growing rhizobia typically take 3 - 5 days to produce visible colonies on agar with colony size of about 1mm while the slow-growing ones usually take 5 - 7 days with colonies of 4 - 5 mm (Somasegaran & Hoben, 1994; Woomer *et al.*, 2011).

The Gram stain test also helps to presumptively filter rhizobia from other types of bacteria. Woomer *et al.* (2011) explains that Gram-positive organisms retain the crystal violet stain after treating with iodine and washing with alcohol, and appear dark violet after staining while gram-negative organisms lose the violet stain retain a red coloration of *Safranin* which is used as a counter-stain. Rhizobia are typically gram negative. Another way of characterizing bacteria is using serological methods (Swift & Bignell, 2001) where the rhizobia acting as an antigen is responded to by an antibody cultured/produced in rabbits (Somasegaran & Hoben, 1994; Woomer *et al.*, 2011). Sessitsch *et al*. (2002) report that *Rhizobium leguminosarum* strains have been shown to produce bacteriocins — antibiotics that are active against closely related strains or species. The author notes that this is a mechanism deployed for reducing competition for root colonization. To survive, competing rhizobia have to develop differential resistance to antibiotics therefore intrinsic antibiotic resistance is an important genetic marker that has been used to recognize different groups or rhizobia, some of which are considered members of separate species (Dupuy *et al*., 1994). Keneni *et al.* (2010) used growth media amended for different concentrations of *Streptomycin, Chloramphenicol, Rimfampenicillin, Oxytetracycline, Penicillin* and *Tetracycline* to establish antibiotic resistance patterns of indigenous and exotic rhizobia species as part of characterization which would enable the evaluation of "exotic" rhizobia's competitiveness to the indigenous rhizobia. This study found that indigenous rhizobia were more resistant to the said antibiotics than the "exotic" strains. According to Sessitsch (2002), different rhizobia species also utilize different carbon sources in the rhizosphere as part of manipulation of competition dynamics. Scientists have also used these properties to characterize rhizobia. PCR techniques are also used to produce the genetic fingerprint of bacteria for definitive characterization and species identification (Swift & Bignell, 2001).

2.4 Inoculation and Biological Nitrogen Fixation in Legumes

Nitrogen fixation through the Rhizobium-legume symbiosis is an important source of nitrogen in marginalized farmsteads in terms of size, income and distance from markets (Giller, 2001). Such farmsteads which are not able to provide sufficient nitrogen to their soils sometimes find themselves with BNF as the only feasible source of nitrogen. The significance of nitrogen fixation is put into perspective when consideration is taken of the potential quantities of fixed nitrogen per hectare of different legumes. As reported by AABNF (2001), soybean has the potential to fix up to 150 kg/ha nitrogen in semi-arid to humid places although its current observed BNF level is only 50 kg/ha. In sub-humid places, BNF in soybean can go up to 70 - 80 kg/ha against the current 50 kg/ha. Pigeon peas have a similar potential to soybean while *Phaseolus* beans have a lower potential of 70 kg N/ha and peanuts at about 80 kg N/ha.

Pigeon peas, peanuts and soybean all currently have observed BNF inputs of less than 60 kg/ha (Giller, 2001). These BNF potential figures are much more than many African farmers apply to their legume farms and if these potentials could be reached, they could substantially reduce the need for fertilizer (AABNF, 2001). Research consequently has to be channeled into driving the currently observed BNF on African farms towards these potentials. Giller (2001) reports that the impact of agricultural research on improving the contribution of nitrogen to the productivity of smallholder farms in the tropics is still a meager maximum of 5 kg Nha^{-1} yr^{-1}. BNF technologies are hard to recommend in Malawi because of the uncertainty of the need-to-inoculate status of the soils and the diversity or competitiveness of already present rhizobium species in the soils in question. This research contributes towards filling the gap of information of the presumptive composition of soybean-nodulating *Bradyrhizobium* species in different Malawian soils so as to aid in future research and strain selection for inoculant production to maximize the chance of success upon inoculation.

During the 20^{th} century, scientists found out that there was legume group-*Rhizobium* specificity in nodule formation which led to the development of the cross inoculation group concept where one rhizobia species from one plant in a cross-inoculation group is supposed to nodulate all other plants in the group (Willens, 2006). Although there were inconsistencies, cross inoculation groups were the primary means of classifying rhizobia taxonomically before their metabolic and morphological characters were fully understood as distinguishing

factors. Developments in genetic engineering in the 1980s have brought the ability to classify rhizobia further based on genetic markers (Willens, 2006) and to date, PCR techniques are routinely used in the characterization of rhizobia. These developments have led to the increase in the number of distinguishable genera and species of the *Rhizobiacaea* family.

Cross inoculation groups of rhizobia are particularly important in the production of rhizobia-based inoculants for legumes since they aid in strain selection for the inoculants. Some rhizobia are able to form effective nodules on a broad range of hosts while some are specific to certain hosts. The same scenario happens in plants whereby some are able to associate with many rhizobia strains while some also nodulate with specific strains. Plant cultivars that form nodules with a broad range of rhizobia strains are generally called promiscuous cultivars (Elkan & Upchurch, 1997). *Bradyrhizobium japonicum* species seem to confine to nodulation with soybean while other *Bradyrhizobium* species seem to belong to the cowpea cross inoculation group which contains such grain legumes as pigeon pea (*Cajanus cajan*) and peanuts (*Arachis hypogaea*) among others (Elkan & Upchurch, 1997). *Phaseolus* beans are associated with *Rhizobium leguminosarium biovar phaseoli* (Elkan & Upchurch, 1997).

2.5 The Effects of Rhizobia on Various Agronomic Parameters

There has been a lot of research on rhizobia inoculation in African legumes. Much research has been directed to rhizobia that associate with pasture legumes, a result of wider adoption in forage legume inoculants than food legume inoculants. One of the most widely studied beneficial plant-microbe interactions is the symbiotic relationship between legumes and *Rhizobium* spp (Lupwayi *et al.*, 2012). The amount of nitrogen fixed by a legume crop varies widely depending on the legume genotype, *Rhizobium* strain and the soil environment (Ojiem *et al.,* 2007). Various grain, green manure and fodder legumes have demonstrated that greater nitrogen fixation potential occurs under medium rainfall than under high or low rainfall in western Kenya (Lupwayi, 2012). Furthermore, Lupwayi (2012) reports that experimentation by the Center for International Tropical Agriculture (CIAT) has shown that the highest biomass yield in all the legumes occurred under medium rainfall compared to high rainfall or low rainfall. Ojiem *et al.* (2007) also observed declining nitrogen fixation with declining soil fertility status, for example, a 44% decrease in nitrogen fixed by grain legumes under low fertility relative to that fixed under high fertility was observed. Results

from agronomic studies on maize-bean intercropping systems and their influence on N_2 fixation in Malawi showed that applying P fertilizer consistently increased nitrogen fixation and grain yield (Snapp *et al.*, 1998).

Quoting various sources, Giller *et al.* (1997) summarized the amounts of nitrogen fixed by grain legumes in southern Africa ranged from 11 to 201 kg N ha^{-1} for sole cropped cowpea, 9 to 125 kg N_2 ha^{-1} for intercropped cowpea, 2 to 58 kg Nha^{-1} for sole-cropped common bean, and 0 to 71 kg Nha^{-1} for intercropped common bean. With these figures and the estimated hectares for their respective crops, it can be estimated that about 500 and 150 million kg N were fixed by cowpea and common bean, respectively, in southern African soils in 2007. Including other grain legumes, a total of about 720 million kg nitrogen were fixed, representing 14% of the global estimate of 5000 million kg nitrogen fixed by grain legumes excluding soya bean (Herridge *et al.*, 2008).

As a sustainable alternative for improving nitrogen fixation without the use of inoculants, scientists at IITA Ibadan have bred promiscuous soybean varieties which can nodulate freely and effectively with indigenous *Bradyrhizobium* populations in Nigerian soils. These soybean varieties were reported to have an increased potential to fix nitrogen over most other lines (IITA, 1996). Muhammad (2010) hence did a study to assess: (i) The response of an early maturing promiscuous soybean cultivar (*TGX 1485*) released by IITA to four rhizobia inoculants and (ii) The influence of increasing phosphorus rates on growth and yield of *TGX 1485*. The four rhizobia inoculants were found to increase the grain yield and other parameters investigated over those of the control. Except for the nodule number that was significantly depressed by N fertilization among treatments, one strain produced yields that were comparable with those of the plots supplied with 60 kg N ha^{-1}. On the other hand, as phosphorus rates increased the parameters investigated also increased. These results are indicative of the potential of rhizobia inoculants in improving farmer yields per unit piece of land.

In Kenya, the country's agricultural research institute (KARI) has published a milliard research papers on the performance of grain legumes subjected to rhizobia inoculants in its annual proceedings publications and other international journals. Efforts have been concentrated on evaluating the effect of rhizobia inoculation on the grain yield of target legumes like *Phaseolus* beans and soybean and these trials have brought some mixed results

into the limelight. For example, Mbugua *et al.* (2010) reports that a trial was conducted in 2007 during long and short rains at KARI-Thika and on farmers' fields in *Kirinyaga* and *Maragua* districts in Central Kenya to investigate the effect of three commercial *Rhizobium* strain inoculants and triple superphosphate (TSP) fertilizer on yield of new dry bean lines. TSP fertilizer consistently increased bean yields during the long and short rain seasons at the research site (Thika) and on farmers' fields in *Kirinyaga* and *Maragua* districts. Mbugua *et al.* (2010) further explain that compared to fertilizer effects on yield, inoculation effects were not as consistent and were of much lower magnitude. Most of the bean lines had their highest yield where both fertilizer and *Rhizobium* inoculants were applied.

Wasike *et al.* (2010) conducted a trial where they aimed at determining the infectiveness and competitiveness of two selected rhizobia isolated from Eastern and Western Kenya to nodulate two promiscuous and one specific nodulating soybean local variety *Nyala,* grown in soils derived from three AEZs with no known previous history of soybean cultivation. The varieties were grown in each of three previously autoclaved and non-autoclaved soils. In all the soils, variety *Nyala* was well nodulated with both indigenous strains (*TSBF531* and *TSBF442*) isolated from promiscuous soybean varieties. The results further showed that the nodulation of promiscuous varieties by these strains was higher in the three soils irrespective of sterilization. Wasike *et al.* (2010) also explain that in non-autoclaved soils, strain *TSBF531* was highly competitive in nodules of all varieties except one and it was competitive in all soils. In all autoclaved and non-autoclaved soils, *USDA110* reference and commercial strain was not at all identified in any nodules analyzed of all the varieties. The paper concluded that a potential exists for the use of indigenous strains such as *TSBF531* and *TSBF442* as inoculants to replace strain *USDA110* strain. This gives a strong case for further evaluation of indigenous strains to explain the lack of effectiveness of commercial strains in some instances.

2.6 The Effect of Edaphic Factors on Rhizobia

The effectiveness of the symbiotic relationship between soybean and rhizobia is affected by nutrient stress within the soil. Phosphorus (P) is one such nutrient that is required in high quantities for BNF (Vance *et al*., 2002). Phosphorus is needed to service the plant with much needed ATP for nitrogenase activity with several studies showing that there is a higher concentration of P in the nodules than shoots in actively fixing plants (Vance *et al*., 2002;

Zahran, 1999). With this in mind, it is expected that different soils will respond differently to different rhizobia strains based on their inherent available P. Zahran, (1999) reports that inherent soil acidity affects the availability of essential nutrients like P and Ca. In addition, increased acidity is often accompanied by the fixing or excess release of other nutrients whose deficiencies may reduce BNF like the case of molybdenum or may affect the host and hence its ability to contribute to the symbiosis like the case on manganese toxicity in acidic soils. These findings strengthen the premise that the inherent mineralogy of the soil affect the success of a symbiosis. Malawi's soil map is prevalent with heavily weathered *Ferralsols, Lixisols* and *Fluvisols* (MoA&Ir, 2005). For any rhizobia strain recommendation, it is important to assess them in these heterogenous edaphic conditions without the interference of indigenous adapted rhizobia. Such a study would require to be conducted in potted soils with very low MPNs of indigenous rhizobia or sun-dried long enough to eliminate any indigenous rhizobia.

CHAPTER 3

Materials and Methods

The study involved 4 phases of exploration and testing; nodule recovery and preservation; isolation and purification; Characterization and authentication and Evaluation of the efficiency of the rhizobia strains in plant growth trials.

3.1 Site Description

Nodules were collected from the four agro-ecological zones (AEZs) of Malawi namely; (1) The Plateaux (Middle attitude) which makes up three quarters of Malawi at elevations of 750-1300 MASL (NSO, 2000). The topography is flat rolling with deep well drained soils in some parts and poorly drained sands or clays in others. SADC (2008) reports that this zone covers *Blantyre, Chiradzulu, Dowa, Kasungu, Lilongwe, Machinga, Mchinji, Mzimba, Zomba, Chitipa and Dedza*. A part of this agro-ecological zone (referred to as *Kasungu – Lilongwe* Plain) is Malawi's breadbasket. The farming population is estimated at around 1.5 million households (SADC, 2008). The zone covers an extensive portion of the country's total land size (75%) and the majority of soybean production; (2) The low attitude areas (200-760 MASL) which are the Lakeshore and upper Shire valley, has calcimorphic soils. Rainfall ranges from 600 to 800 mm annually and the areas are characterized by very fertile alluvial soils and high average temperatures (SADC, 2008).

Soybean production within the low altitude zone is prevalent in *Salima* district but is on the increase in *Nkhatabay* and *Rumphi* district where it overlaps with the *Kasungu* plain (part of the Plateaux zone) (SADC, 2008). The agricultural population in the zone is about 0.8 million households and the zone forms about 20% of Malawi's land mass; (3) The lower Shire valley (<200 MASL) with coarse textured alluvials and colluvials with *Vertisols* dominant with pockets of saline soils forming about 8% of the total land area (NSO, 2000). SADC (2008) reports that this area receives less than 600 mm of rain annually and is not suitable for rain-fed farming of most crops grown in Malawi.

In the Lower Shire zone, Sorghum and Millet are the commonly grown crops and dry land agriculture is favoured with cotton production and livestock rearing taking priority while irrigated agriculture mainly consists of sugarcane, rice and banana production (SADC, 2008).

There is very little soybean production with the most notable being done through irrigated seed multiplication, the zone has an agricultural population of 0.2 million households. (4) The highlands (1000-1500 *masl*) which have a humid environment with *Latosols* dominating the area (NSO, 2000; Chinsinga, 2011). The zone receives over 1,200mm of rainfall annually with low average temperatures and has an agricultural population of approximately 0.8 million households (SADC, 2008). Some types of crops for these areas are different from most of the other ecological zones and include Irish potatoes, wheat, coffee and tea (SADC, 2008). Soybean adoption is substantial in *Ntcheu* district within the zone. This heterogeneity gives a premise of different vegetation and soil properties and hence a premise of different species of rhizobia.

3.2 Nodule Recovery and Preservation

Soybean nodules were collected from each of the 4 AEZs of Malawi in accordance with its size while targeting districts that have the most soybean production (each AEZ being a strata of the stratified sampling procedure representative of the soil and climatic heterogeneity that it imposes). The nodules within each AEZ were systematically sampled from uninoculated farmer-managed soybean plots with no known history of inoculation to maximize the chance that only indigenous rhizobia are sampled. The farmer-managed soybean plots were sampled in a judgemental non-probability sampling procedure based on areas of importance in soybean cultivation and no history of rhizobia inoculation. There were 150 nodules collected from the plateux zone with Dowa, Kasungu, Lilongwe, Mchinji, and Dedza each contributing 30 nodules due to the high prevalence of soybean production in these areas. The lower shire contributed 20 nodules from uninoculated research managed trials in Chikhwawa district. 25 nodules were sampled from the highland zone in Ntcheu district which is the only stand-out district in the zone with substantial soybean production while the lakeshore region contributed 20 nodules from Salima district. Nodule recovery was done in June 2013. In each district, the allocated number of nodules was collected from at least 3 randomly selected farms from which the nodules were recovered from randomly selected plants.

Nodule recovery was done using procedures for nodule recovery and preservation as described in Woomer *et al.* (2011). This entails that for each plant sampled; a circle with a radius of approximately 15 cm around the plant was cut out to a depth of at least 20 cm with a

spade then slowly lifted out. The soil was carefully removed from the roots while avoiding detaching any roots then the whole plant was placed in a plastic bag. The soil around each plant was then gently washed with water and nodule distribution recorded. The nodules were cut off from the roots and preserved by desiccation in vials of silica gel in readiness for laboratory processing. The spade was sterilized with 95% alcohol between samples. For each vial, the physical location and GPS coordinate were collected and each vial was uniquely coded for tracking purposes. The cluster collection zones by GPS coordinate have been illustrated in *Figure 1*.

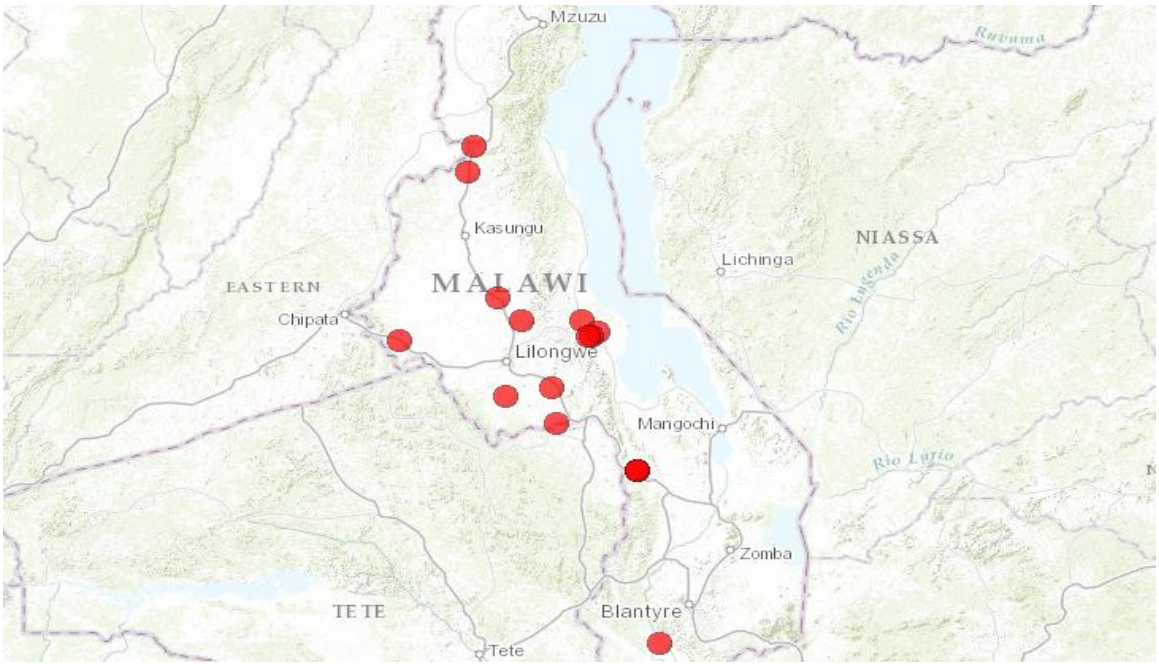

Figure 1: *Map showing rhizobia sampling clusters (dots) across major soybean growing areas in Malawi. Scale 1:2000000*

3.3 Isolation and Characterization

The second phase of the experimentation was done at the Chitedze Microbiology laboratory within the Department of Agricultural Research Services in Lilongwe, Malawi. This activity was implemented from May 2013 to July 2013. Nodules recovered and preserved from the first phase were rehydrated and subjected to 95% ethanol to break their surface tension. To sterilize them, each of the nodules was then transferred to a 3% (v/v) solution of sodium hypochlorite, and soaked for 2-4 min after which they were rinsed in five changes of sterile water using sterile forceps. To ensure an aseptic procedure, the forceps were sterilized by dipping in alcohol and flaming. All glassware and petri dishes used were

also steam-sterilized in an autoclave. Nodules were crushed using a sterile glass rod and the resultant suspensions of all effective (brown colour inside) nodules was streaked on Yeast Mannitol Agar (YMA) plates containing Bromothymol Blue (BTB) or Congo Red (CR). BTB and CR are indicators to detect the acid/base forming properties of the isolates and ascertain contamination or lack thereof respectively (Somasegaran & Hoben, 1994).

The formulation of the broth and its subsequent agar plates for each media has been elaborated in *Appendix 1*. The cultures were incubated at 28 °C for 8 days and observed everyday for colony growth and emergence of contaminants. As a presumptive test, rhizobia do not generally absorb CR in a dark incubation environment while most other bacteria show red colouration from CR absorption in the dark (Somasegaran & Hoben, 1994). Absorption of CR from the Petri dish proves that the colonies are not rhizobia. A blue color indicative of an alkaline reaction on BTB should be obtained with slow-growing *Bradyrhizobium* spp. A yellow color (acid) reaction is usually produced by the fast-growing *Rhizobium* species. Woomer *et al*. (2011) further explain that these tests only give presumptive surety of the isolated organism being rhizobia but the definitive test for rhizobia is a nodulation test on a host legume.

Isolated typical colonies were recovered from the cultures and re-streaked on YMA following which observations were made on the growth characteristics of the colonies in different media amendments to characterize the rhizobia strains. Characteristics assessed included the growth (ability to form distinctive colonies along the streak lines within 8 days) in amended media containing 1.17% (11.69 g/L) and 1.75% (17.53 g/L) NaCl saline mixtures prepared as 200 mol/m^3 and300 mol/m^3 NaCl solutions respectively, another set of media was adjusted to pH 4.0 and 5.0. This was done to filter out a workable number of salt and acid tolerant strains. The growth rate and survival of rhizobium strains is expected to be reduced with the addition of NaCl in these ranges as noted by Keneni *et al*. (2010) and Singleton & Tavarez (1986) where free living rhizobia strains exhibited reduced growth in salinity stress above 300mol/m^3 and 120mol/m^3 respectively. Although pH tolerance is an important trait, rhizobia often have a higher tolerance to acidity than their corresponding plant hosts (Zahran, 1999). For this reason, a pH tolerant rhizobia has to be classified according to its ability to tolerate the lowest possible pH of the host like soybean which has a pH tolerance range of 5.0 to 6.0 (Zahran, 1999). All strains able to tolerate the acidity treatment of pH 5.0 were considered as *acid tolerant* strains for the purpose of the Shannon

diversity index. This phase of experimentation produced data on morphology and growth characteristics, salinity tolerance and low pH tolerance. Each combination of characters was then used as a presumptive grouping/species of rhizobia which constituted the richness parameters of the Shannon-Weaver index calculation to predict the diversity of the species in the sampled AEZs. The computation of the Shannon-Weaver index has been elaborated in *Section 3.6*.1All the presumptive strains cultured were then further characterized for their tolerance to antibiotics. The strains were tested for antibiotic resistance in nutrient media supplemented with a concentration series of 0.00015, 0.00016 and 0.000175 mol/$m^3$of each of tetracycline, streptomycin and penicillin antibiotics according to Milicic (2006).

3.4 Authentication of Rhizobia Strains

This third step involved authenticating the ability of the strains in forming nodules on Soybean. This was done to ensure that the strains being handled are genuinely able to associate and form nodules with their target legumes. This activity was done in July 2013 by inoculating each of the presumptive rhizobia strains to pre-germinated soybean seedlings in Leonard jars filled with sterile sand as media with the *Broughton and Dilworth* nutrient solution under greenhouse conditions. The formulation of the *Broughton and Dilworth* nutrient solution was as outlined in *Appendix 3*. The test was done in a greenhouse at the Chitedze Soils and Microbiology Department in Lilongwe Malawi. The seedlings were allowed to grow for 5 weeks to allow for nodule development. Rhizobia strains which fail to develop a symbiosis (by formation of nodules) were discarded and those that develop nodules were promoted into the BNF potential test.

3.5 Plant Growth Promotion Tests

The second test for BNF potential was a Split-Plot Completely Randomized Design (CRD) greenhouse experiment with three replications as modeled in Lawson, (2010). The trial investigated the BNF potential of the 5 strains selected from *Section 3.3* that also pass the authentication test of *Section 3.4* against uninoculated and nitrogen-supplied controls. The varieties in use are as indicated in *Table 1* and the trial layout is illustrated in *Table 2*. For this purpose, soybean seeds were grown under greenhouse conditions for 5 weeks in Leonard jars. Surface sterilized pre-germinated seeds of soybean were transferred aseptically to 750 ml size Leonard jars using sterile sand as media with five seeds being planted per jar. Woomer *et al.* (2011) recommends application of a minimum of 1 ml broth per soybean seed in a Leonard

jar. To minimize the chances of failed inoculation, 2 ml broth was applied per seed hence every jar was inoculated with 10 ml of bacterial culture of the authenticated indigenous Rhizobium strains. There were two uninoculated treatments with one being supplied with 70 $mgkg^{-1}$ N (105 $kgha^{-1}$) applied as a 0.05% KNO_3 (w/v) solution (Somasegaran & Hoben, 1994).

Table 1: *Soybean varieties used in the second and third evaluation tests*

Soybean Cultivar	Characteristics
Makwacha	Promoted by Seedco. Most common among farmers due to seed size. Specific
Nasoko	Public domain variety from DARS, Specific, Common among farmers
Tikolore (TGx 1740-2F)	IITA bred variety, released in 2011. Currently recommended variety in Malawi, Promiscuous and responsive to indigenous rhizobia, under promotion by IITA and USAID

Table 2: *Split-plot layout for the first plant growth promotion test.*

Variety	**Treatments Structure and Randomization**						
Rep 1							
MAKWACHA	Rh4	Rh2	N-Contr	Rh5	No Rh	Rh3	Rh1
TIKOLORE	N-Contr	Rh3	No Rh	Rh2	Rh1	Rh4	Rh5
NASOKO	Rh5	Rh1	Rh4	Rh3	Rh2	No Rh	N-Contr
Rep 2							
NASOKO	Rh1	Rh2	N-Contr	Rh4	No Rh	Rh3	Rh5
MAKWACHA	Rh4	No Rh	N-Contr	Rh3	Rh2	Rh5	Rh1
TIKOLORE	Rh2	Rh5	No Rh	N-Contr	Rh1	Rh4	Rh3
Rep 3							
TIKOLORE	Rh2	Rh3	Rh4	N-Contr	Rh1	No Rh	Rh5
MAKWACHA	Rh3	Rh2	No Rh	Rh5	N-Contr	Rh4	Rh1
NASOKO	Rh5	Rh2	N-Contr	No Rh	Rh1	Rh3	Rh4

Rh denotes rhizobium strain, N-Contr denotes Nitrogen control treatment

The plants were thinned to 2 uniform plants in each jar after 5 days (Woomer *et al.*, 2011). Upon harvest, data was recorded on shoot weight, total dry matter, and nodule number. This data was used to screen out the top 3 effective strain-variety combinations in terms of total shoot biomass accumulation in comparison with the control which were then promoted to the final test of BNF potential against the USDA110-Makwacha known strain-variety combination in potted field soils under greenhouse conditions. Analysis of data was done by ANOVA.

The second plant growth promotion experiment (third test, Objective 3) was carried out in a screenhouse at IITA Malawi Research Station, Chitedze, Lilongwe between August and September 2013. The experimental design was a split-plot CRD with three replicates as modeled in Lawson, (2010). There were 6 treatments comprised of 3 Strain-Variety combinations, and the Makwacha-USDA110 reference combination, a Makwacha-uninoculated control and a Makwacha-nitrogen control. Soil samples of the three dominant soil types of Malawi, *Ferralsols, Lixisols* and *Fluvisols* (MoA&Ir, 2005) were collected and analysed for total N, extractable P and exchangeable K then used as main plots in the CRD in order to assess response in the different soil types. *Ferralsols* and *Lixisols* are particularly heavily weathered with dominant 1:1 clays (FAO, 1999). The 1:1 dominance gives them a low CEC and hence are expected to have low concentrations of Ca and other cations. Their high acidity is expected to cause fixation of both P and Ca. With this in mind, these soils are expected to respond differently to the *Fluvisols* in BNF from rhizobia inoculation.

Fluvisols (originating from alluvial deposition) are prevalent along the rift valley on the east of Lake Malawi which runs throughout the northern and central regions of Malawi from the many tributary rivers of the lake. *Fluvisols* are generally more fertile that *Ferralsols* and *Lixisols* due to the alluvial deposits and are more suitable for agricultural production (FAO, 1999). These soils are hence expected to offer a less stressful environment to favour BNF symbiosis. Soybean seeds were grown in clay pots (diameter; 17.5 cm) filled up with 3kg of field soil collected from fields with no known history of inoculation. The soils were sun dried for 7 days after which analysis for pH, N, P and K was done. The seeds were pre-germinated in 3-4 days in distilled water petri dishes before the actual sowing and setup of the experiment.

Three healthy seeds were sown per 3 kg soil pot at 5 cm depth and inoculated with their particular strain. Each pot was treated with 10 ml of liquid inoculant of the 4 treatment combinations. All plants were watered with sterilized distilled water every 2 days. The layout of the trial was as shown in *Table 3*. Plants were harvested after 8 weeks and observations recorded on number of nodules, shoot dry matter content and total shoot nitrogen. Total shoot nitrogen was assessed by the Kjeldahl method at the Chitedze Research Station Soil Laboratory. Symbiotic Efficiencies were calculated both for shoot and soil N fixation as described in *Section 3.6.*4. All the data was analyzed by ANOVA in GENSTAT14 statistical software in accordance with the model illustrated in *Section 3.6.4*. The means were separated using Tukey's test and orthogonal contrast analysis was done to compare the performance of the three promising combinations against (a) the nitrogen control, (b) the Bradyrhizobium japonicum and Makwacha combination and (c) the uninoculated control.

Table 3: *Treatment arrangement and layout for strain evaluation*

Soil	Variety-Strain Combination and Randomization					
Ferralsols	N-Control	Jap+Mak	Comb 3	No-Rh	Comb 1	Comb 2
Lixisols	Jap+Mak	Comb 1	No-Rh	Comb 2	Comb 3	N-Control
Fluvisols	Jap+Mak	Comb 2	Comb 1	N-Control	No-Rh	Comb 3
Lixisols	N-Control	Jap+Mak	Comb 3	No-Rh	Comb 1	Comb 2
Fluvisols	Jap+Mak	Comb 1	No-Rh	Comb 2	Comb 3	N-Control
Ferralsols	Jap+Mak	Comb 2	Comb 1	N-Control	No-Rh	Comb 3
Fluvisols	N-Control	Jap+Mak	Comb 3	No-Rh	Comb 1	Comb 2
Lixisols	Jap+Mak	Comb 1	No-Rh	Comb 2	Comb 3	N-Control
Ferralsols	Jap+Mak	Comb 2	Comb 1	N-Control	No-Rh	Comb 3

"comb" denotes strain-variety combination, "jap+mak" denotes USDA110 to Makwacha combination

All strains collected were preserved for future referencing and testing as part of the Malawi *Rhizobium* germplasm.

3.6 Statistical Analysis

3.6.1 Shannon Diversity Index

For *Objective 1*, the Shannon-Weaver Diversity index (Shannon and Weaver 1949) was used to predict the diversity of the parameters analyzed in the respective AEZs. The Index is stated as :-

$$\boldsymbol{H = -\sum_{i=1}^{s}[Pi(ln\,Pi)]}$$

Where:

H is the Shannon diversity index

$\mathbf{P_i}$ is the fraction of the entire population made up of the i^{th} rhizobium species

S is the number of presumptive rhizobium species encountered (*R*ichness)

Ln in a natural logarithm

3.6.2 Rarefaction Generation

Rarefaction curves are used to estimate the maximum number of species that would be found in a community if sampling size was standardized at a specific level across the target population (Chao *et al*., 2014).In a search for unique species, a simple rarefaction curve can give information as to the extent with which the sampling effort covered the available population, hence validate the sample size or advise for a larger or more diverse sample. Rarefaction typically calculates the probability of selecting the same type of individual from a random sample as the sample size increases (Chao *et al*., 2014). Therefore, in less diverse communities with a dominant species, the curve is expected to steepen from right to left as the sample size decreases. The curve remains a straight line as sample size increases if the population is very diverse with no dominant species.

Rarefaction curves were generated from all the presumptive species found using the rarefaction species accumulation curves function in GENSTAT 14 by plotting the sample size against the expected number of species. Expected number of species in a sample of size *n* is calculated by:

$$E(S_n) = S - (\frac{1}{C(n,N)}) \times \sum\nolimits_i \{C(n, N - N_i)\}$$

Where:

N_i is the number of individuals in species *i* of the unrarefied sample

C(*n*, *N*) is the number of combinations of *n* from *N*

C(*n*, *N*-N_i) is the number of combinations of *n* from *N*-N_i. Heck *et al*. (1975).

3.6.3 Nitrogen Fixation Potential Analysis

Nodule weight, nodule number, total shoot nitrogen and above ground plant biomass data were collected in the Leonard jars and potted soil experiment. An analysis of variance was done on all the shoot biomass and nitrogen content in both stages in order to test the significance of the treatments. All the treatment means were separated using Tukey's test and conclusions made on the most symbiotically efficient strains from the highest ranking treatment means after means separation. Correlation coefficients were calculated between nodule weight and total nitrogen as well as nodule weight and shoot biomass (Woomer *et al.*, 2011).

As explained in *Section 3.5*, the first plant growth promotion experiment was done in a CRD Split-plot design in conformity to the model:

$$\mathbf{Y_{ijk} = \mu + \alpha_i + \eta_{ki} + \beta_j + \alpha\beta_{ij} + \varepsilon_{kij}}$$

Where:

$\mathbf{Y_{ijk}}$ is the shoot biomass associated with the j^{th} isolate on the i^{th} variety in the k^{th} replicate.

$\boldsymbol{\mu}$ is the overall mean shoot biomass

$\boldsymbol{\alpha_i}$ is the effect of the i^{th} main plot (variety)

$\boldsymbol{\eta_{ik}}$ is the random error associated with the i^{th} main plot (variety) in the k^{th} replicate

$\boldsymbol{\beta_j}$ is the effect of the j^{th} subplot (isolate)

$\boldsymbol{\alpha\beta_{ij}}$ is the effect of the interaction between the i^{th} variety and the j^{th} isolate

$\boldsymbol{\varepsilon_{ijk}}$ is the random error associated with the j^{th} split-plot (isolate) in the k^{th} replicate

3.6.4 Symbiotic Efficiency Analysis

The second plant growth promotion test was done in CRD Split-plot design in conformity to the model:

$$\mathbf{Y_{ijk} = \mu + \alpha_i + \eta_{ki} + \beta_j + \alpha\beta_{ij} + \varepsilon_{kij}}$$

Where:

$\mathbf{Y_{ijk}}$ is the symbiotic efficiency associated with the j^{th}variety-strain combination on the i^{th}soil order in the k^{th} replicate.

$\boldsymbol{\mu}$ is the overall mean symbiotic efficiency

$\boldsymbol{\alpha_i}$ is the effect of the i^{th} main plot (soil)

$\boldsymbol{\eta_{ik}}$ is the random error associated with the i^{th} main plot (soil) in the k^{th} replicate

$\boldsymbol{\beta_j}$ is the effect of the j^{th} subplot (variety-strain combination)

$\boldsymbol{\alpha\beta_{ij}}$ is the effect of the interaction between the i^{th}soil and the j^{th}variety-strain combination

$\boldsymbol{\varepsilon_{ijk}}$ is the random error associated with the j^{th} split-plot (variety-strain combination) in the k^{th} replicate

Total symbiotic efficiency was determined using the formula (Beck *et al.*, 1993):

$$\boldsymbol{S.E.}(\%) = \left(\frac{\boldsymbol{A}}{\boldsymbol{B}}\right) * \mathbf{100}$$

Where, S.E. = symbiotic effectiveness, A = the amount of nitrogen in the plant isolate inoculated, B = the amount of nitrogen in the nitrogen control.

CHAPTER 4

Results and Discussion

4.1 Results

This chapter presents the results of the qualitative and quantitatively analyzed data for the study as well as a discussion of the results. The results and discussion are presented under the following subsections drawn from the objectives of the study.

- Diversity of characters of rhizobia samples
- BNF potential of rhizobia strains and their interaction with recommended soybean varieties
- The symbiotic efficiency of the strain-variety combinations in soil

4.1.1 Diversity of Characters of Rhizobia Samples

The pre-selected conditions for grouping the bacteria samples gave rise to 19 possible character combinations in a binary matrix as presented in *Table 4*. These character combinations were based on resilience to adverse media conditions therefore the binary matrix made it possible to sort the rhizobia from the most resilient to least resilient. Out of the top 10 most resilient samples, 9 were from the Plateux region and 1 from the Lakeshore region. Out of these 9 samples, 4 came from *Lilongwe* district, 3 from *Kasungu* and 2 were from *Mchinji* district. The one sample from the Lakeshore AEZ came from *Salima* District. The first 9 samples were able to survive both pH 4 and pH 5 media. The *MAL_120* (*sp17* group) sample was only able to survive pH 5 media but was the only sample out of the 170 collections to survive both 200 mol/m^3 and 300 mol/m^3 of salt and pH 5 at the same time. Ultimately, the top 10 consists of three possible species based on the binary matrix (species 19, 18 and 17). The top 3 most resilient were from species 19 with the next 6 being from *sp 18* and the final strain from *sp 17*.

As presented in *Table 4*, the majority of the samples recorded character combination sp4 with 37 samples. This grouping was characterized by no growth in pH 5 and pH 4 media, ability to withstand only the initial 200 mol/m^3 salt concentration, ability to withstand only the initial 0.00015 mol/m^3 of penicillin and tetracycline and the ability to withstand up to

0.00016 mol/m^3 of streptomycin antibiotic. This character set was ranked 16^{th} out of the 19 combinations by resilience.

Table 4: *Characteristics of the 19 discovered binary combinations recorded as sp1 to sp19.*

Code	**pH**		**Salinity (**mol/m^3**)**		**Tetracycline (***10^{-5} mol/m^3**)**			**Streptomycin (***10^{-5} mol/m^3**)**			**Penicilin (***10^{-5} mol/m^3**)**			**Count**
	4	**5**	**300**	**200**	**15**	**16**	**17.5**	**15**	**16**	**17.5**	**15**	**16**	**17.5**	
sp1	-	-	-	+	+	-	-	+	+	-	+	-	-	8
sp2	-	-	-	+	-	-	-	+	-	-	+	-	-	2
sp3	-	-	-	+	+	-	-	+	-	-	+	-	-	13
sp4	-	-	-	+	+	-	-	+	+	-	+	-	-	37
sp5	-	-	-	+	+	+	-	-	-	-	+	-	-	4
sp6	-	-	-	+	+	+	-	+	-	-	+	-	-	27
sp7	-	-	-	+	+	+	-	+	-	-	+	+	-	3
sp8	-	-	-	+	+	+	-	+	+	-	+	-	-	16
sp9	-	-	-	+	+	+	-	+	+	+	+	-	-	23
sp10	-	-	-	+	+	+	+	+	-	-	+	-	-	6
sp11	-	-	-	+	+	+	+	+	+	-	+	-	-	3
sp12	-	-	-	+	+	+	+	+	+	-	+	+	-	3
sp13	-	-	+	+	+	-	-	+	-	-	+	-	-	3
sp14	-	-	+	+	+	-	-	+	+	-	+	-	-	3
sp15	-	+	-	-	+	+	-	+	+	-	+	-	-	3
sp16	-	+	-	+	+	+	-	+	+	-	+	-	-	6
sp17	-	+	+	+	+	+	-	+	+	+	+	-	-	1
sp18	+	+	-	-	+	+	+	+	-	-	+	-	-	6
sp19	+	+	-	+	+	+	+	+	+	-	+	-	-	3
USD A 110	-	+	-	+	+	+	-	+	+	-	+	-	-	1

*"+" denotes resilient, "-" denotes susceptible***.**

The binary matrix as adopted from Workalemahu, (2009) made it possible that a more resilient sample would always register a higher number of 1's than a less resilient one. By arranging the experimental treatments (*i.e.* Acidity, Salinity, *e.t.c*) from the highest to lowest dose, it was possible to take a combined binary figure that would get higher as the sample tolerated a higher dose of the treatment. This made it possible to rank the samples from the most resilient to the least resilient. The 170 species successfully cultured at this stage registered 19 possible binary combinations and hence 19 possible ranks. *Species 19* was the most resilient while species 1 was the least. The market available species (USDA110) was also tested and it had the characters of the fourth most resilient species fitting in with the *sp 16* group along with 6 other samples. This group consisted of two samples from the highland AEZ, two from the plateux, one from the lakeshore zone and one from the lower shire. Out of the 170 samples, only 10 samples recorded a higher tolerance to adverse conditions than the USDA110 of which 9 were authenticated as rhizobia.

Table 5: *Shannon's Indices of Diversity for the 19 possible binary combinations in both the whole country and the different AEZ.*

Zone	Shannon's Index	Shannon's Exponent	Shannon's Evenness
Plateux	2.56	12.89	0.87
Lakeshore	2.08	8.08	0.71
Highlands	1.89	6.6	0.64
Lowlands	1.23	3.42	0.42
Whole Country	2.48	11.95	0.84

Out of the 170 samples successfully cultured, 107 were collected from the Plateux zone. This is the only zone in which all of the 19 binary groupings were represented giving it the highest species richness of 19 species. The high species richness meant that the Plateux region would have a higher diversity index than the other zones as can be seen in *Table 5*. The large number of samples collected in this zone however can be suspected to give it a sampling advantage that pushes its diversity index. This can only be eliminated if rarefaction curves show that the other AEZs were also adequately sampled. The Plateux forms 75% of the total land area of Malawi while the Lakeshore zone represents 10% and 8% is divided

among the highland and Lowlands zones (NSO, 2000). The Lakeshore zone had 19 samples successfully tested which fell into 10 binary groupings. *Table 6* shows the dominance of *sp 4* and *sp 6* in all AEZ with a combined total of 64 samples forming 37% of the sample size.

Table 6: *A summary of the prevalence of each binary grouping (presumptive species) across the country and within each AEZ.*

Code	Lakeshore	Plateux	Highland	Lowland	Total
sp1	1	5	0	2	8
sp2	0	2	0	0	2
sp3	2	6	5	0	13
sp4	3	19	3	12	37
sp5	0	4	0	0	4
sp6	5	18	2	2	27
sp7	1	2	0	0	3
sp8	3	8	4	1	16
sp9	1	14	7	1	23
sp10	0	5	1	0	6
sp11	0	2	1	0	3
sp12	0	3	0	0	3
sp13	1	2	0	0	3
sp14	0	3	0	0	3
sp15	0	3	0	0	3
sp16	1	2	2	1	6
sp17	0	1	0	0	1
sp18	1	5	0	0	6
sp19	0	3	0	0	3
Totals	**19**	**107**	**25**	**19**	**170**

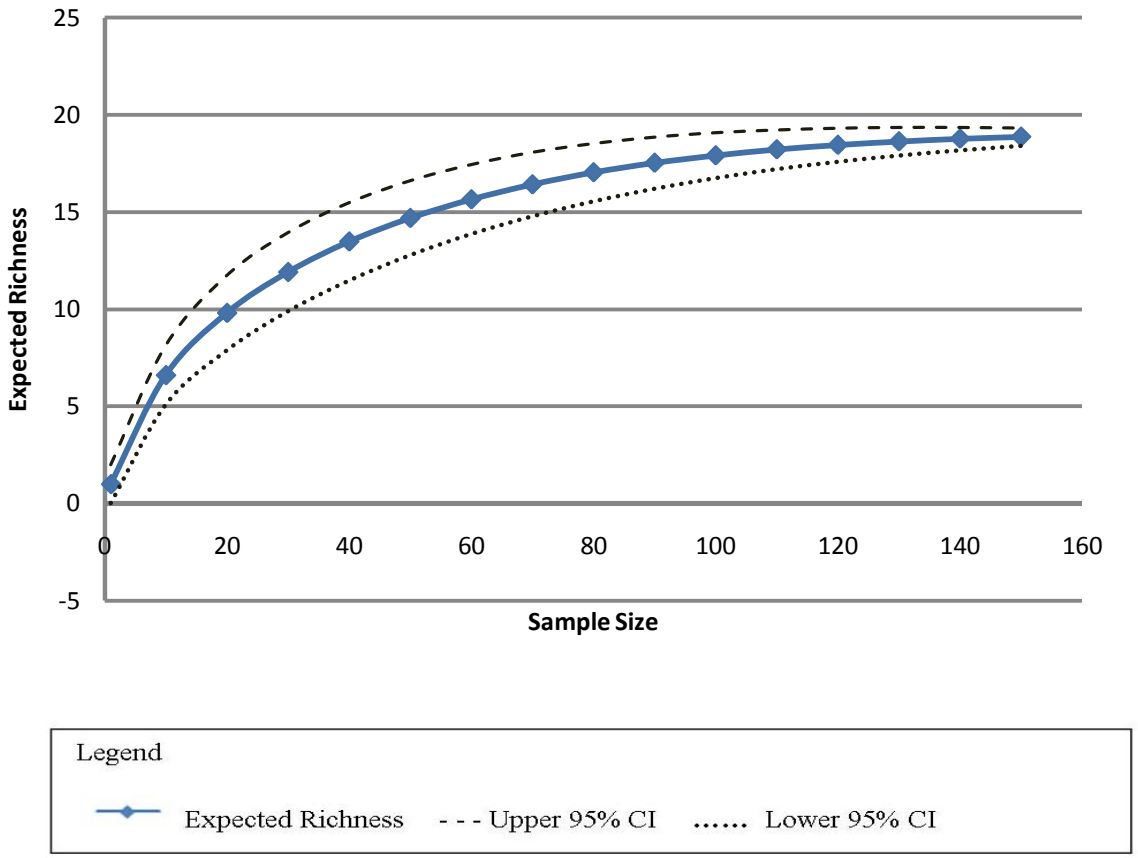

Figure 2: *Rarefaction curve for the 19 presumptive rhizobia species found in 170 samples across 4 AEZ in Malawi*

Figure 2 shows the rarefaction curve for expected species richness for all 170 samples across all the four AEZs in bold trend line with dotted plots above and below to illustrate the upper and lower 95% confidence intervals. The species richness increases rapidly between the first and fiftieth sample then slowly becomes asymptote as more previously identified species are found with increasing sample size. The curve becomes asymptote after 150 samples with a species richness of 19 species. There is no increase in species richness after 150 samples which confirms the adequacy of the overall sampling effort. *Figure 3 a-d* shows the rarefaction curves for the samples within each of the four AEZs. The plateux and highlands AEZs curves become completely asymptote while those for the lowlands and lakeshore areas have an incomplete asymptote curve to show the likelihood of finding more combinations with increased sample size.

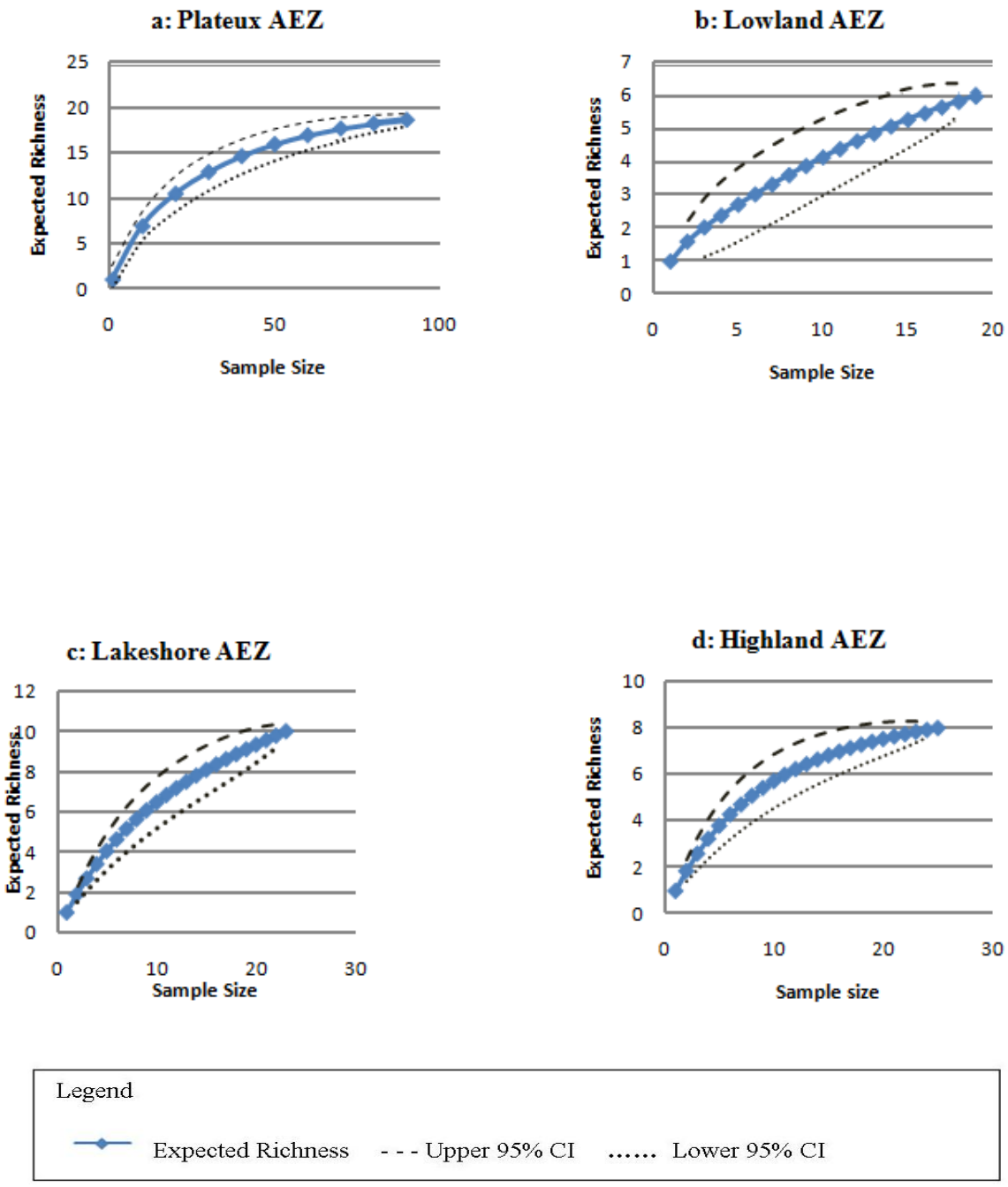

Figure 3: a-d, *Rarefaction curves for all the presumptive species in each AEZ of Malawi.*

4.1.2 BNF Potential of Rhizobia Strains and their Interaction with Recommended Soybean Varieties

Table 7: *Mean Shoot Dry weight (g), Nodule of the 21 variety-strain combinations from the potted sand greenhouse trial.*

	Nitrogen	MAL117	MAL120	MAL28	MAL17	MAL24	-RH	Mean
Makwacha	0.65	0.64	0.53	0.44	0.31	0.35	0.32	**0.46**
Nasoko	0.67	0.47	0.54	0.44	0.32	0.43	0.36	**0.46**
Tikolore	0.65	0.56	0.63	0.38	0.43	0.4	0.37	**0.49**
Mean	**0.66**	**0.56**	**0.57**	**0.42**	**0.35**	**0.39**	**0.35**	
F-Prob	0.036							
CV %	13.6							

Table 8: *Mean Number of Nodules of the 21 variety-strain combinations from the potted sand greenhouse trial.*

	Nitrogen	MAL117	MAL120	MAL28	MAL17	MAL24	-RH	Mean
Makwacha	0	20	22	7	5	5	0	**8.43**
Nasoko	0	9	17	29	25	15	0	**13.57**
Tikolore	0	20	31	7	5	17	0	**11.43**
Mean	**0.00**	**16.33**	**23.33**	**14.33**	**11.67**	**12.33**	**0**	
F-Prob	0.24							
CV %	57							
R^2 (against SDW)	0.52							

Table 9*: Mean Nodule Dry Weight (g) per plant of the 21 variety-strain combinations from the potted sand greenhouse trial.*

	Nitrogen	MAL117	MAL120	MAL28	MAL17	MAL24	-RH	Mean
Makwacha	0	0.036	0.021	0.028	0.001	0.027	0	**0.02**
Nasoko	0	0.028	0.029	0.027	0.024	0.024	0	**0.02**
Tikolore	0	0.021	0.038	0.022	0.028	0.029	0	**0.02**
Mean	**0.00**	**0.03**	**0.03**	**0.026**	**0.02**	**0.03**	**0**	
F-Prob	<0.001							
CV %	23.3							
R^2 (against SDW)	0.57							

Table 10: *Shoot Dry weight separation of means for the top 3 variety-strain combinations from the greenhouse Leonard jar trial.*

Treatment	SDW (g)	Nodule Count	NDW (g)
N-trt Nasoko	0.67^{a}	0	0^{a}
N-trt Tikolore	0.65^{ab}	0	0^{a}
N-trt Makwacha	0.65^{ab}	0	0^{a}
MAL_117 Makwacha	0.64^{abc}	20	0.036^{bc}
MAL_120 Tikolore	0.63^{abc}	31	0.038^{c}
MAL_117 Tikolore	0.56^{abcd}	20	0.021^{b}
MAL_120 Nasoko	0.54^{abcde}	17	0.029^{bc}
F Prob	**0.036**	**0.241**	**<0.001**
CV (%)	**13.6**	**57**	**23.3**
R (against SDW)		**0.52**	**0.57**

Means followed by the same letters are not significantly different.

Table 11: *Mean Shoot Dry Weight of soybean treated with different rhizobia strains and percentage yield increase against the control uninoculated treatment.*

Strain	Mean SDW (g)	% Yield Increase (Against Control)
N-Trt	0.66^{c}	186.73
MAL_120	0.57^{bc}	161.09
MAL_117	0.55^{b}	157.59
MAL_28	0.42^{a}	119.62
MAL_24	0.39^{a}	111.71
MAL_17	0.36^{a}	101.91
Control	0.35^{a}	-
F-Prob	**<0.001**	**-**
CV (%)	**13.6**	**-**

Means with the same letters are not significantly different.

4.1.3 The Symbiotic Efficiency of the Strain-Variety Combinations in Different Soil Types

Table 12 shows the response of the three chosen combinations from *Section 4.1.2* in various indicators of BNF tried to potted field soils. It can be seen that there is a significant difference in the symbiotic efficiency of each of the combinations in potted field soils at $P<0.05$. *Table 13* emphasizes this efficacy difference as it illustrates a significant difference in symbiotic efficiency between the different soils used as main plots. *Fluvisols* and *Lixisols* were the highest in symbiotic efficiency using Tukey's test with *Ferrasols* being the least efficient. Comb2 was highest in symbiotic efficiency, having been able to contribute to the shoot up to 87% of the amount that the nitrogen control was able to contribute. This combination did better than the popular Makwacha-USDA110 control showing its potential for an alternate/substitute strain to the market available *Bradyrhizobium japonicum*.

Table 12: *Symbiotic Efficiency (SE), Nodule Dry Weight (NDW), Shoot Dry Weight (SDW) and Soil Nitrogen Fixation Efficiency (SNFE) of different soybean variety to rhizobia strain combinations observed in 3 different soil types.*

Treatment	SE (%)	NDW (g)	SDW (g)	SNFE (%)
no-rh	52.63[c]	0[a]	0.039[a]	6.47[a]
comb3	61.83[c]	0.039b[b]	0.62[bc]	72.91[b]
comb1	62.71[c]	0.034[b]	0.52[ab]	83.85[b]
jap+mak	77.82[b]	0.043[b]	0.73[c]	88.34[b]
comb2	87.71[ab]	0.042b[b]	0.73[c]	95.73[b]
N-control	100[a]	0[a]	0.77[c]	100[b]
F-prob	**0.001**	**0.001**	**0.01**	**0.01**
CV	**7.1**	**10.1**	**12.1**	**15.4**
R (with SDW)	**0.7**	**-**	**0.9**	**0.4**

Means followed by the same letter are not significantly different.

Table 13: *Performance of Soybean-Rhizobia symbiosis in Fluvisols, Ferralsols and Lixisols.*

Soil	SE (%)	NDW (g)	SDW (g)	SNFE (%)
Fluvisol	78.36[b]	0.02[a]	0.69	75.11
Ferralsol	68.11[a]	0.03[b]	0.54	85.61
Lixisol	74.88[ab]	0.03[b]	0.65	62.94
F-prob	**0.013**	**0.174**	**0.003**	**0.049**
CV	**13.7**	**31.7**	**20**	**36**

Means followed by the same letters are not significantly different.

Results from ANOVA presented in *Table 14* show that there were highly significant difference in S.E, NDW, SDW and SNFE when the three combinations were contrasted to the non-nitrogen control and the nitrogen control. This is expected for the NDW because the means of the *N-control* and the *No-Rh* treatments are zero since they contained no rhizobia.

Table 14: *ANOVA results for SE, NDW, SDW and amount on N fixed. Probability values for each of the analysed contrasts.*

Contrast	Yield Aspect			
	SE	**SDW**	**NDW**	**N_Fixed**
N X ALL	***	***	***	***
NoRh X ALL	***	***	***	***
JapMak X ALL	NS	0.038	NS	NS

*** denotes that p < 0.001, NS denotes non significance at P<0.05.

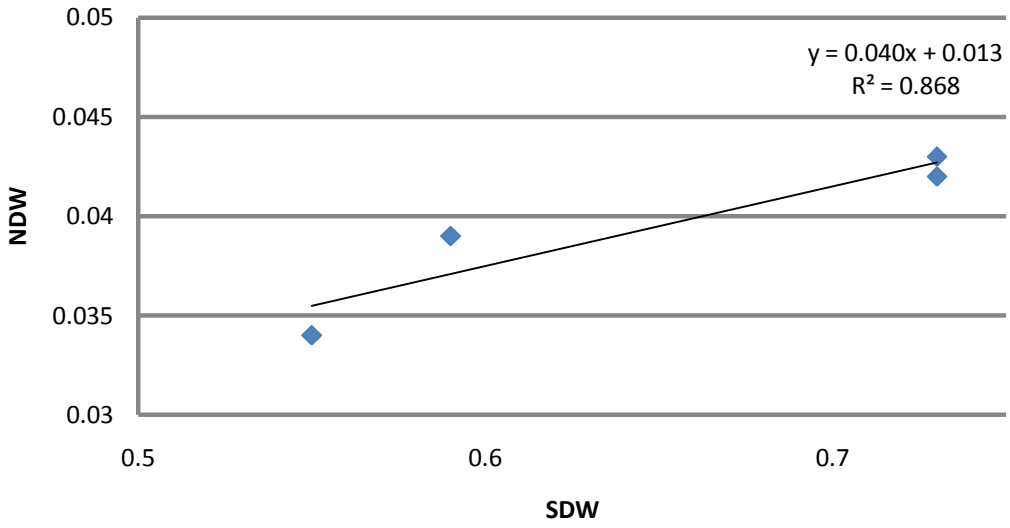

Figure 4: *Relationship between nodule and shoot dry weights in soybean inoculated with various rhizobia isolates.*

Table 15: *Characteristics of the soils used in the potted field soil experiment.*

	Soil Type		
Test	**Ferralsol**	**Lixisol**	**Fluvisol**
Total N (mgkg^{-1})	4.28	9.01	12.73
Extractable P (mg/kg)	22	8	19
Exchangeable K (mg/kg)	583	321	651
pH (CaCl2)	4.6	5.1	5.1

The differential response of the different symbiotic partnerships to the soil mineralogy suggest that blanket recommendations for use of rhizobia inoculants on legumes will not necessarily lead to consistent yield increases. This research is however not conclusive as to whether only the initial N aspect of the soil affects the symbiosis. It is important to assess the extent to which all edaphic factors like phosphorus concentration affect symbiotic efficiency in these combinations to find out if they should affect the choice of rhizobia strain.

4.2 Discussion

4.2.1 Diversity of Characters of Rhizobia Samples

The salinity binaries in *Table 4* are consistent with results from Workalemahu (2009) who found that the most sensitive rhizobia were not able to grow above 1.5% NaCl. However, most rhizobia in their trial was able to grow between 1-2% NaCl to show considerable adaptation to salinity by the rhizobia in the Tigray highlands of northern Ethiopia where the study was conducted. Keneni *et al*. (2010) also found that rhizobia of northern Ethiopia were able to withstand up to 5% NaCl while the exotic ones never went beyond 2%. Samrudhi *et al*. (2013) further acknowledge that rhizobia develop tolerance to salinity locally which means that rhizobia from more saline soils will likely be more tolerant than exotic strains and more adoptable for survival in their particular environment. In terms of pH, Keneni *et al*. (2010) found that native rhizobia were able to show satisfactory growth up to pH4 while the exotic rhizobia were only able to form colonies above pH 5.5 which was an indication of competitive advantage of the native species over introduced rhizobia. This relates with the results in *Table 4* where 9 of the 170 samples were able to grow at pH4 and 19 were able to grow at pH5 while the USDA110 was not able to grow at pH4. Evans *et al*. (1989) found that rhizobium species from temperate regions exhibited low multiplication below pH6-5 which is consistent with the growth inhibition exhibited by the USDA110 at pH4. These results suggest that top 9 pH resilient samples would likely have competitive advantage over acid-intolerant exotic strains like USDA110 in acidic environments, which is consistent with assessments by Auraj & Sasson (1992). Out of the top 9 samples, only the top 3 were able to survive 200mol/$m^3$of salt but none of them survived the 300 mol/m^3 concentration which shows a lower adaptation to salinity than those evaluated by Workalemahu *et al*. (2010) in northern Ethiopia. The survival of the top 9 samples in pH4 suggests their suitability for use in inoculants as they can adopt to the acidic *Ferralsols* that dominate Malawi (MoA&Ir, 2005) and have competitive advantage over other indigenous

rhizobia since the optimum pH of most rhizobia is usually neutral or slightly alkaline (Niste *et al*., 2013) and are sensitive to acidic environments (Ali *et al*., 2009).

Table 5 shows the Shannon diversity assessment of the 170 strains tested in this research. The overall diversity index is 2.48.The highest diversity within the AEZs was found in the Plateux region where soybean is mostly grown in Malawi (MoA&Ir, 2005) with an overall Shannon's index of 2.56. Despite having less successfully tested samples than the Highlands zone which had 25 samples, this zone had the second highest diversity index of 2.08 while the Highlands have a diversity of 1.89 as seen in *Table 5*. This is because the 25 samples from the Highlands fell into only 8 binary groupings. These results agree with research by Parr *et al*. (2013) that the diversity of rhizobia in Malawian soils sampled at Ekwendeni in the Plateux region were affected by the soil's cropping history. According to Parr *et al*. (2013), findings indicate that the diversity of rhizobia is higher in areas where soybean is commonly grown and very low in soybean virgin lands. These findings are also consistent with those of Palmer & Young (2000) who studied genetic diversity differences in *Rhizobium leguminosarium* populations between arable and grass soils from farms in Yorkshire, United Kingdom using the Shannon Diversity Index and found that Chromosomal types, repC profiles. In all comparisons between arable and grasslands within Yorkshire farms, arable land samples had higher diversity indices. In one farm (*High Mowthorpe)*, the Shannon index was 3.46 on arable sections and 0.28 on grassland. Even in *Askan Bryan* farm where overall diversity was low, the arable section had a higher Shannon index of 1.58 while the grassland had 0.52. Our results show the highest diversity in the Plateux region and Lakeshore region where soybean is mainly grown in Malawi than the Highlands and Lowland zones which is consistent with the conclusions of Palmer & Young (2000) that rhizobia diversity can be affected by differences between land management regimens. As expected, the Lowland zone had the lowest diversity index of 1.23 and a richness of 6 species.

In naturally occurring ecosystems, a well calculated Shannon's diversity index typically ranges between the low and high limits of 1 and 4 (Shannon & Weaver, 1949). The low species richness and diversities discovered in these results show that there might be ecosystem dominance of very few genetically unique species within the targeted area range. *Table 4* gives evidence that 154 out of the 170 samples are less resilient than *Bradyrhizobium japonicum* strain (USDA110) representing 90.5% of the sample size. This suggests that response to inoculation with soybean rhizobia can be expected since introduced species can

be expected to persist in the presence of competitors. The resilience trend in *Table 4* suggests that USDA110 is better placed to withstand antagonism from antibiotic-producing and soil acidizing fungi and other bacteria than most of the indigenous rhizobia hence better placed to colonize seedling roots and establish symbiosis before lethal antibiotic dosages.

The combined rarefaction curve for all the 170 samples validates the adequacy of the sample size in projecting the species richness across the four AEZs. It can be suggested that there is very little diversity across the country in the characteristics of the available rhizobia species in terms of their ability to withstand agriculturally important effects of soil pH, Salinity and antibiotic resistance, this is expected due to the relative newness of soybean as a crop in Malawi in terms of adoption (Tinsley, 2009) which entails underdeveloped mutations in the indigenous rhizobia species hence low diversity as asserted by Parr *et al*., (2013). This may be important in justifying the recommendation for use of a single superior inoculant across many sites. As it can be seen in *Table 4* and explained earlier, the reference rhizobia strain (USDA110) has a resilience similar to sp16 which is ranked as 4th most resilient, better than 90% of all the samples. This shows that USDA110 may be able to withstand fungal and bacterial antagonism as well as adverse abiotic stress of acidity and salinity better than most indigenous species, making the reference strain a good inoculant strain. The complete curvature of the rarefaction curve in *Figure 2* shows that it is unlikely that more species would be found within the limits of the sampling area that would be more or less resilient than what has already been discovered.

The story is slightly different within the four AEZs. Since sampling was done based on the AEZ size and importance of soybean as a crop, the plateux region had the biggest sample size and hence was inherently expected to have a larger diversity of rhizobia and a more complete rarefaction curve as can be seen in Figure 3(a).The lowlands in Figure 3(b) however produce an incomplete rarefaction partly because of the sample size but also because of the very low evenness of species distribution as seen in *Table 5*. A larger sample size in this region would likely lead to a complete rarefaction curve. The curvature of the upper 95% confidence interval band shows that reducing the sampling error (i.e. increasing the sampling size) would likely result in a complete rarefaction curve with a comparable number of species.

4.2.2 BNF Potential of Rhizobia Strains and their Interaction with Recommended Soybean Varieties

There were significant differences found in the mean dry biomass weight at $P<0.05$ to prove that the effect of the combination of rhizobia strain and soybean variety can have significant effects on the amount of nitrogen fixed within the plant. It must be noted that Tikolore variety (*TGx-1740-2f* IITA line) has been reported to be a highly promiscuous variety (IITA, 2011) Makwacha and Nasoko are specific varieties (Kapalasa, 2012). The interaction of these varieties with the type of rhizobia strain applied thereby should form the basis of whether the inoculation would be effective or not. *Table 7* shows the mean shoot dry weight (SDW) at six weeks recorded for each variety-strain combination.

The highest dry matter yielding treatment combinations were those of Nitrogen fertilizer and each variety. Nasoko responded best to fertilizer treatment, yielding the highest mean shoot dry matter content of 0.67 g with Makwacha and Tikolore yielding means of 0.65 g and 0.64 g respectively (*Table 7*). The lowest yielding treatments as expected were the nitrogen "starved" control treatments for each variety. The lowest yield however was from the *MAL_17* to Makwacha treatment combination posting a mean SDW of 0.313 g (Table 7). This suggests incompatibility of the strain variety combination which if encountered leads to non-fixation of nitrogen and performance similar to non-fertilized treatments as observed by CIAT, (1988). This is confirmed by the low mean nodule weight of 0.001 g. Woomer *et al*., (2011) report that nodule weight is a suitable indicator of rhizobia symbiotic activity and shoot dry weight's has a high correlation to nodule weight making it a suitable proxy for assessing the efficiency of the strain-variety symbiosis. These statements are consistent with those of Somasegaran & Hoben (1994). The same can be said about the *MAL_17* to Nasoko and the *MAL_24* to Makwacha treatments which recorded yields similar to the control plots suggesting specificity of these two varieties. In similar SDW results, Appunu *et al*. (2008) observed that host cultivars and inoculation treatment as well as interactions had significant ($P< 0.05$) effect on nodulation, plant growth and seed yield. Soybean cultivars differed in the average nodule dry weight per plant induced. Careful scrutiny of this data shows that the 10 least SDW yielding combinations all yielded less than 0.5 g mean SDW and were dominated by the three strains *MAL_17*, *MAL_24* and *MAL_28* in random combination with the three varieties. This suggests strain weakness in nitrogen fixation rather than incompatibility of the varieties with these strains. This information means there should be a significant difference in

the performance of the strains themselves as can be confirmed in *Table 7* as having a P-Value of <0.001.

Rhizobia inoculation was shown in this trial to be able to increase the amount of shoot nitrogen by between 101% and 161% from the uninoculated control depending on the strain used. There were significant differences in the mean SDW as separated by Tukey's test and presented in *Table 6*. This ascertains that strains may have different symbiotic efficiencies which is in line with findings by Appunu *et al*. (2008) where a trial of different rhizobia strains against different cultivars of soybean showed that the strain used had a significant effect ($p<0.05$) on the SDW. Similar impact of inoculation on SDW has also been reported to be significant at $P<0.05$ in Zanjan Province by Mehrpouyan (2011) where SDW was increased in inoculated treatments and led to a resultant average yield increased of 43% compared with the non-inoculated treatments. Abaidoo and Van Kassel, (1989) also reported higher SDW in inoculated soybeans with symbiotic efficiencies of up to 84%. The effect of the soybean variety used in the inoculation on the symbiotic capacity of the strain is of more importance for this research because of its implication on choice of strain to use during inoculant preparation for the recommended varieties in Malawi. The significance of this host-rhizobia interaction is also recognized by Israel *et al*. (1986) who found that there was significant variation in the nitrogen-fixation capabilities of four North American cultivar-strain symbioses. Rhizobia strain USDA 110 fixed 3.7, 39.1, 4.6, and 57.3 times more nitrogen than did rhizobia strain USDA 191 with soybean cultivars *Pickett* 71, *Harosoy* 63, *Lee*, and *Ransom* as host plants, respectively ($P <0.05$). With one unimproved Peking cultivar however, USDA 191 fixed 3.3 times more nitrogen than did the USDA 110. This Host-Cultivar effect is further consistent with the findings of Muthuri *et al*. (2014) who also explored the effect of the cultivar on symbiotic effectiveness of strains USDA 191 and USDA 110 in greenhouse trials. It was found that USDA 191 was more effective in nitrogen fixation in *TGx* variety than in *Gazelle* variety while *Bradyrhizobium japonicum* was more effective in Gazelle variety than in *TGx* variety in both symbiotic effectiveness and SDW.

4.2.3 The Symbiotic Efficiency of the Strain-Variety Combinations in Different Soil Types

Table 12 shows that *comb2* (*MAL_120* to Tikolore) ranks high in all the other indicators of BNF namely Shoot Dry Weight, Nodule Dry Weight and Soil Nitrogen Fixation Efficiency than *comb1* (*MAL_117* to Makwacha) and *comb3* (*MAL_117* to Tikolore). This combination

consistently performed either superiorly or at par with the known combination and control treatments. The *USDA110* to Makwacha combination was only able to surpass combination 2 in nodule dry weight albeit not by a statistically significant difference. Similar results were found in a study by Waswa *et al*. (2014) in Kenyan clay soils in a greenhouse environment with findings showing that native strains outperformed the USDA110 ($P<0.05$) in SDW by 27% suggesting the occurrence of indigenous strains that may prove suitable for inoculant production. Similar results have also been observed in other legumes like *Phaseolus Vulgaris* in Turkish soils by Karacas and Uyanoz (2012) where indigenous strains outperformed ($P<0.05$) the international industry standard strain (CIAT899) in symbiotic efficiency and were recommended for inoculant trials.

There was a very high correlation (0.9) between the shoot dry weight and nodule dry weight to concur with the statements from Woomer e*t al.* (2011) and a moderately high correlation was also found between the shoot symbiotic efficiency and the nodule dry weight as observed in *Figure 4*. There was however no significant correlation found between the Nodule Dry Weight and the amount of nitrogen fixed in the soil after post-harvest soil N analysis.A significant difference is also expected for the SDW and SE especially in the N-control due to the supply of nitrogen as compared to the test combinations and the *No-Rh* treatment. However comparison of the three combinations with the USDA110 to Makwacha combination shows no significant differences for all these traits except SDW. This lack of significance particularly for SE shows that there is relatively comparable percentage of nitrogen accumulation per plant but the significance of the SDW affirms that the USDA110 to Makwacha combination assimilates more nitrogen into its tissue matter which translated into significantly higher mean shoot biomass than comb1 and comb3 as can be seen in *Table*12. This concurs with findings by Appunu *et al*. (2008) who established the effect of host specificity and the significance of the host-strain interaction at $P<0.05$. Waswa *et al*. (2014) also found that in potted soil, all native rhizobia isolates nodulated promiscuous soybean but only 46% of them nodulated specific soybean. This further proves the differential performance of isolates depending on their host cultivar as observed in this research. The lack of significant difference with comb2 causes the significance of this contrast to be low at 0.038 but this essentially means that comb2 performs at par with the reference strain-variety combination in SDW accumulation and significantly higher than the other 2 combinations.

Table 13 ascertains that the soil type (hence mineralogy) affects the level of BNF possible in a symbiotic partnership. It can be seen that the above-ground statistics of shoot dry weight and symbiotic efficiency are both affected by the type of soil in which the symbiosis occurs. *Fluvisol*s and *Lixisols* both perform better than *Ferralsols* as illustrated in *Table 13*. This effect however can be attributed to their higher level of organic matter and lower level of weathering compared to the *Ferralsols* (FAO, 1999). In an experiment by Delic *et al* (2011), soil type was also shown to significantly influence the efficacy of rhizobia-legume symbiosis. *Fluvisols* were found to produce better SDW than *homofluvisols* after inoculation. Inoculation was able to increase grain yield by 23-53% in the *Fluvisols* while the increase was by 11-28% in the *Homofluvisols*. Similarly in SDW, there was a significant difference between the two soils with *Fluvisols* producing an average 10 tons per hactare against 8 tons in the *Homofluvisols*. This confirms the effect of soil type on the efficiency of the rhizobia symbiosis which is also suggested by Parr *et al*. (2013) who suggests that edaphic factors like soil pH, inherent nitrogen and organic matter as influenced by the soil formation may affect the efficiency of the legume-rhizobia symbiosis.

Initial total N soil analyses using Kjeldahl showed that the *Fluvisols* and *Lixisols* had respective higher contents of nitrogen of 12.73 and 9.01 $mgkg^{-1}$than the 4.28 $mgkg^{-1}$ found in the *Ferralsols* as illustrated in *Table 15*. The higher content of starter N can also be a contributing factor to the better performance on the symbiotic partnerships (Woomer *et al*, 2011). *Ferralsols* had the highest soil nitrogen symbiotic efficiency of the three soils. This can be attributed to the low initial N in the *Ferralsols* which enabled the effect of the symbiotic partnerships to be more pronounced than the other soils types. This also explains the poor F-probability value of 0.049 for this statistic. In line with *Objective 3*, these results show that soil mineralogy significantly affects the amount of nitrogen fixed in a symbiosis.

CHAPTER 5

Conclusion and Recommendations

5.1 Conclusions

5.1.1. Diversity of Rhizobia

This research shows that Malawi has indigenous rhizobia which exhibit a wide diversity in tolerance to acidity, salinity and antibiotics some of which are more resilient than the USDA110. The results of this research show that any rhizobia strain that can survive a pH of 5 and salinity of 300 mol/m^3 as well as 0.00016 mol/m^3 of streptomycin, penicillin and tetracycline will generally have a better chance of survival than many of the indigenous rhizobia.

5.1.2 Nitrogen Fixation Potential of Indigenous Rhizobia Strains

It can be summarized from this research that the efficacy and competitiveness of indigenous soybean-rhizobia symbioses in nitrogen fixation is affected by the soybean variety involved in the symbiosis by way of its promiscuity or specificity and the soil and rhizobia strain characteristics. This research has been conclusive about the significance of the selective nature of rhizobia on the variety of soybean with which it forms symbiotic partnerships and the importance of host-strain relationship which affects the nitrogen fixation potential.

5.1.3 Nitrogen Fixation Efficiencies in Soils

Soil mineral characteristics have been shown in this research to hold clues as to why some potentially effective symbiotic partnerships might fail despite adequate rhizobia inoculation. The mineralogy and fertility of soil affect the potential of a symbiosis by affecting other edaphic factors like the acidity of the rhizosphere and the release of nitrogen into the root area which are essential determinants of symbiosis.

5.2 Recommendations

5.2.1 Diversity of Rhizobia

Recommendations are made for follow-up expanded rhizobia diversity prospecting studies and continual characterization of germplasm to better understand the diversity of Malawi's indigenous rhizobia population which may help in shaping recommendations for inoculation.

5.2.2 Fixation Potential of Indigenous Rhizobia Strains

The findings show that it is important for the soybean industry to move towards developing variety-specific inoculation recommendations based on established and evaluated symbiotic relationships which may prove invaluable in reducing the instances of non-response and incompatibility to inoculation.

5.2.3 Nitrogen Fixation Efficiencies in Soils

This research confirms the need for extensive evaluation of Malawian soils' nitrogen deficiencies and their need-to-inoculate status. These studies are recommended to help explain or anticipate non-response to inoculation and help to formulate policy on the adoption of rhizobium technologies as a sustainable soil fertility management strategy.

The results of the two plant growth promotion trials in this research show that two strains of rhizobia and their respective soybean variety combinations are able to compete either at par or better than the available combination of Makwacha and *Bradyrhizobium japonicum*. These two combinations are MAL_117 to Makwacha (*comb1*) and MAL_120 to Tikolore (*comb2*). *Comb2* performed higher than the control (Makwacha-USDA110) in most of the target statistics while *Comb1* performed lower than the control but not with a significant difference. This prompts further testing of these combinations in multi-location field factorial experiments where soil type, variety and strain can be evaluated elaborately over several growing seasons to find out if *MAL_117* and *MAL_120* can be recommended for release as rhizobia inoculant strains. It is also recommended to characterize these strains for their genetic fingerprints to ascertain that unique strains are being dealt with.

CHAPTER 6

6.1 References

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6.2 Appendices

Appendix 1: Preparation of Yeast-Carbon Source Broth (YMB). Adopted from Woomer *et al*. (2011).

Constituents

- Mannitol/Glycerol/Sucrose/Lactose 10.0 g
- K_2HPO_4 0.5 g
- $MgSO4.7H_2O$ 0.2 g
- NaCl 0.1 g
- Yeast Extract 0.5 g
- Distilled Water 1.0 liter

1. Add carbon source and salts to 1L distilled water

2. Dissolve under continuous stirring

3. Adjust pH to 4 with 0.1 N HCl

4. Autoclave at 121^0 C for 15 min

Appendix 2: Preparation of Saline Yeast Mannitol Agar (YMA)

Procedure the same as for normal yeast mannitol broth but:-

1. Adjust pH to 6.8 with 0.1N HCl
2. Adjust salinity to three levels (200mM, 300mM)
    a. For 200mM, add 11.59g NaCl to 1L of water
    b. For 300mM, add 17.54g NaCl to 1L of water

Appendix 3: *Broughton and DilworthN-free Nutrient Solution Constituents, adopted from Woomer et al. (2011)*

Stock Solution	Element	M	Form	MW	g/l	M
1	Ca	1000	$CaCl_2 \cdot 2H_2O$	147.03	294.1	2.0
2	P	500	KH_2PO_4	136.09	136.1	1.0
3	Fe	10	Fe-citrate	355.04	6.7	0.02
	Mg	250	$MgSo_4 \cdot 7H_2O$	246.5	123.3	0.5
	K	250	K_2SO_4	174.06	87.0	0.5
	Mn	1	$MnSO_4 \cdot H_2O$	169.02	0.338	0.002
4	B	2	H_3BO_3	61.84	0.247	0.004
	Zn	0.5	$ZnSO_4 \cdot 7H_2O$	287.56	0.288	0.001
	Cu	0.2	$CuSO_4 \cdot 5H_2O$	249.69	0.100	0.0004
	Co	0.1	$CoSO_4 \cdot 7H_2O$	281.12	0.056	0.0002
	Mo	0.1	$Na_2MoO_2 \cdot 2H_2O$	241.98	0.048	0.0002

For each 10 liters of full strength culture solution, take 5.0 ml each of solutions 1 to 4, then add to 5.0 liters of water, then dilute to 10 liters. Use 1N NaOH to adjust the pH to 6.6-6.8. For plus N control treatments, KNO_3 (0.05%) is added giving an N concentration of 70 $mgkg^{-1}$.

Appendix 4**:** *Analysis of Variance GENSTAT OUTPUTS*

1. Objective 2: BNF Potential CRD SPLIT-PLOT GENSTAT ANOVA Output

Analysis of variance

Variate: SDW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Strain	6	0.772016	0.128669	31.53	<.001
Variety	2	0.011051	0.005525	1.35	0.269
Strain.Variety	12	0.104194	0.008683	2.13	0.036
Residual	42	0.171400	0.004081		
Total 62	1.058660				

2. Objective 3: Symbiotic Efficiency. CRD SPLIT-PLOT GENSTAT output

Analysis of variance

Variate: S_E%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
treatment	5	14497.25	2899.45	30.34	<.001
soil	2	976.35	488.17	5.11	0.011
treatment.soil	10	1256.50	125.65	1.31	0.260
Residual	36	3440.19	95.56		
Total 53	20170.29				

Appendix 5: Mapping information for all successfully cultured bacterial samples

Code	Latitude	Longitude	District	Agro zone
MAL01	-14.17493	34.08058	Dedza	plateux
MAL02	-14.17493	34.08058	Dedza	plateux
MAL03	-14.17493	34.08058	Dedza	plateux
MAL04	-14.17493	34.08058	Dedza	plateux
MAL05	-14.17493	34.08058	Dedza	plateux
MAL06	-14.17493	34.08058	Dedza	plateux
MAL07	-14.17493	34.08058	Dedza	plateux
MAL08	-14.17493	34.08058	Dedza	plateux
MAL09	-14.17493	34.08058	Dedza	plateux
MAL10	-14.17493	34.08058	Dedza	plateux
MAL100	-16.07785	34.82576	Chikwawa	lower shire
MAL101	-16.07785	34.82576	Chikwawa	lower shire
MAL102	-16.07785	34.82576	Chikwawa	lower shire
MAL103	-14.44451	34.11157	Lilongwe	plateux
MAL104	-14.44451	34.11157	Lilongwe	plateux

MAL105	-14.44451	34.11157	Lilongwe	plateux
MAL106	-14.44451	34.11157	Lilongwe	plateux
MAL107	-14.44451	34.11157	Lilongwe	plateux
MAL108	-14.44451	34.11157	Lilongwe	plateux
MAL109	-14.44451	34.11157	Lilongwe	plateux
MAL11	-14.79926	34.67134	Ntcheu	highlands
MAL110	-14.44451	34.11157	Lilongwe	plateux
MAL111	-14.44451	34.11157	Lilongwe	plateux
MAL112	-14.44451	34.11157	Lilongwe	plateux
MAL113	-14.44451	34.11157	Lilongwe	plateux
MAL114	-14.44451	34.11157	Lilongwe	plateux
MAL115	-14.44451	34.11157	Lilongwe	plateux
MAL116	-14.44451	34.11157	Lilongwe	plateux
MAL117	-14.44451	34.11157	Lilongwe	plateux
MAL118	-16.07785	34.82576	Chikwawa	lower shire
MAL119	-14.44451	34.11157	Lilongwe	plateux
MAL12	-14.79926	34.67135	Ntcheu	highlands
MAL120	-14.44451	34.11157	Lilongwe	plateux
MAL121	-14.44451	34.11157	Lilongwe	plateux
MAL122	-14.44451	34.11157	Lilongwe	plateux
MAL123	-16.07785	34.82576	Chikwawa	lower shire
MAL124	-14.23916	33.75805	Lilongwe	plateux
MAL125	-14.23916	33.75805	Lilongwe	plateux
MAL126	-14.23916	33.75805	Lilongwe	plateux
MAL127	-14.23916	33.75805	Lilongwe	plateux
MAL128	-14.23916	33.75805	Lilongwe	plateux
MAL129	-14.23916	33.75805	Lilongwe	plateux
MAL13	-14.79926	34.67135	Ntcheu	highlands
MAL130	-14.23916	33.75805	Lilongwe	plateux
MAL131	-14.23916	33.75805	Lilongwe	plateux
MAL132	-14.23916	33.75805	Lilongwe	plateux
MAL133	-16.07785	34.82576	Chikwawa	lower shire
MAL134	-16.07785	34.82576	Chikwawa	lower shire
MAL135	-13.79098	34.35822	Salima	Lakeshore
MAL136	-13.79098	34.35822	Salima	Lakeshore
MAL137	-13.79098	34.35822	Salima	Lakeshore
MAL138	-13.79098	34.35822	Salima	Lakeshore
MAL139	-13.79098	34.35822	Salima	Lakeshore
MAL14	-14.79926	34.67135	Ntcheu	highlands
MAL140	-13.79098	34.35822	Salima	Lakeshore
MAL141	-13.79098	34.35822	Salima	Lakeshore
MAL142	-13.79098	34.35822	Salima	Lakeshore
MAL143	-13.79098	34.35822	Salima	Lakeshore
MAL144	-13.79098	34.35822	Salima	Lakeshore
MAL145	-16.07785	34.82576	Chikwawa	lower shire

MAL146	-16.07785	34.82576	Chikwawa	lower shire
MAL147	-13.79098	34.35822	Salima	Lakeshore
MAL148	-13.79098	34.35822	Salima	Lakeshore
MAL149	-14.80057	34.67009	Ntcheu	highlands
MAL15	-14.79926	34.67135	Ntcheu	highlands
MAL150	-12.56785	33.50145	Kasungu	plateux
MAL151	-12.56785	33.50145	Kasungu	plateux
MAL152	-12.56785	33.50145	Kasungu	plateux
MAL153	-12.56785	33.50145	Kasungu	plateux
MAL154	-12.56785	33.50145	Kasungu	plateux
MAL155	-12.56785	33.50145	Kasungu	plateux
MAL156	-12.56785	33.50145	Kasungu	plateux
MAL157	-12.56785	33.50145	Kasungu	plateux
MAL158	-12.56785	33.50145	Kasungu	plateux
MAL159	-13.76221	34.39699	salima	Lakeshore
MAL16	-14.79926	34.67135	Ntcheu	highlands
MAL160	-16.07785	34.82576	Chikwawa	lower shire
MAL161	-16.07785	34.82576	Chikwawa	lower shire
MAL162	-13.76221	34.39699	salima	Lakeshore
MAL163	-13.76221	34.39699	salima	Lakeshore
MAL164	-13.76221	34.39699	salima	Lakeshore
MAL165	-13.76221	34.39699	salima	Lakeshore
MAL166	-16.07785	34.82576	Chikwawa	lower shire
MAL167	-13.79945	34.33675	salima	Lakeshore
MAL168	-16.07785	34.82576	Chikwawa	lower shire
MAL169	-16.07785	34.82576	Chikwawa	lower shire
MAL17	-12.56785	33.50145	Kasungu	plateux
MAL170	-34.28342	13.67883	salima	Lakeshore
MAL18	-14.80057	34.67009	Ntcheu	highlands
MAL19	-14.80057	34.67009	Ntcheu	highlands
MAL20	-14.80057	34.67009	Ntcheu	highlands
MAL21	-14.80057	34.67009	Ntcheu	highlands
MAL22	-14.80057	34.67009	Ntcheu	highlands
MAL23	-14.80057	34.67009	Ntcheu	highlands
MAL24	-12.37645	33.54578	Kasungu	plateux
MAL25	-12.37645	33.54578	Kasungu	plateux
MAL26	-12.37645	33.54578	Kasungu	plateux
MAL27	-12.37645	33.54578	Kasungu	plateux
MAL28	-12.37645	33.54578	Kasungu	plateux
MAL29	-12.37645	33.54578	Kasungu	plateux
MAL30	-12.37645	33.54578	Kasungu	plateux
MAL31	-12.37645	33.54578	Kasungu	plateux
MAL32	-12.37645	33.54578	Kasungu	plateux
MAL33	-12.37645	33.54578	Kasungu	plateux
MAL34	-12.37645	33.54578	Kasungu	plateux

MAL35	-12.37645	33.54578	Kasungu	plateux
MAL36	-12.37645	33.54578	Kasungu	plateux
MAL37	-12.37645	33.54578	Kasungu	plateux
MAL38	-12.37645	33.54578	Kasungu	plateux
MAL39	-12.37645	33.54578	Kasungu	plateux
MAL40	-13.50818	33.70929	Dowa	plateux
MAL41	-13.50818	33.70929	Dowa	plateux
MAL42	-13.50818	33.70929	Dowa	plateux
MAL43	-13.50818	33.70929	Dowa	plateux
MAL44	-13.50818	33.70929	Dowa	plateux
MAL45	-13.50818	33.70929	Dowa	plateux
MAL46	-13.50818	33.70929	Dowa	plateux
MAL47	-13.50818	33.70929	Dowa	plateux
MAL48	-13.50818	33.70929	Dowa	plateux
MAL49	-13.50818	33.70929	Dowa	plateux
MAL50	-13.50818	33.70929	Dowa	plateux
MAL51	-13.50818	33.70929	Dowa	plateux
MAL52	-13.50818	33.70929	Dowa	plateux
MAL53	-13.50818	33.70929	Dowa	plateux
MAL54	-13.50818	33.70929	Dowa	plateux
MAL55	-13.50818	33.70929	Dowa	plateux
MAL56	-13.67343	33.86805	Dowa	highlands
MAL57	-13.67343	33.86805	Dowa	highlands
MAL58	-13.67343	33.86805	Dowa	highlands
MAL59	-13.67343	33.86805	Dowa	highlands
MAL60	-13.67343	33.86805	Dowa	highlands
MAL61	-13.67343	33.86805	Dowa	highlands
MAL62	-13.67343	33.86805	Dowa	highlands
MAL63	-13.67343	33.86805	Dowa	highlands
MAL64	-13.67343	33.86805	Dowa	highlands
MAL65	-13.67343	33.86805	Dowa	highlands
MAL66	-13.67343	33.86805	Dowa	highlands
MAL67	-13.67343	33.86805	Dowa	highlands
MAL68	-13.82456	33.02679	Mchinji	plateux
MAL69	-13.82456	33.02679	Mchinji	plateux
MAL70	-13.82456	33.02679	Mchinji	plateux
MAL71	-13.82456	33.02679	Mchinji	plateux
MAL72	-13.82456	33.02679	Mchinji	plateux
MAL73	-13.82456	33.02679	Mchinji	plateux
MAL74	-14.17493	34.08058	Dedza	plateux
MAL75	-14.17493	34.08058	Dedza	plateux
MAL76	-14.17493	34.08058	Dedza	plateux
MAL77	-14.17493	34.08058	Dedza	plateux
MAL78	-14.17493	34.08058	Dedza	plateux
MAL79	-14.17493	34.08058	Dedza	plateux

MAL80	-14.17493	34.08058	Dedza	plateux
MAL81	-13.82456	33.02679	Mchinji	plateux
MAL82	-13.82456	33.02679	Mchinji	plateux
MAL83	-13.82456	33.02679	Mchinji	plateux
MAL84	-13.82456	33.02679	Mchinji	plateux
MAL85	-13.82456	33.02679	Mchinji	plateux
MAL86	-13.82456	33.02679	Mchinji	plateux
MAL87	-13.82456	33.02679	Mchinji	plateux
MAL88	-13.82456	33.02679	Mchinji	plateux
MAL89	-13.82456	33.02679	Mchinji	plateux
MAL90	-13.82456	33.02679	Mchinji	plateux
MAL91	-13.82456	33.02679	Mchinji	plateux
MAL92	-13.82456	33.02679	Mchinji	plateux
MAL93	-13.82456	33.02679	Mchinji	plateux
MAL94	-13.82456	33.02679	Mchinji	plateux
MAL95	-16.07785	34.82576	Chikwawa	lower shire
MAL96	-16.07785	34.82576	Chikwawa	lower shire
MAL97	-16.07785	34.82576	Chikwawa	lower shire
MAL98	-16.07785	34.82576	Chikwawa	lower shire
MAL99	-16.07785	34.82576	Chikwawa	lower shire

Appendix 6: List of Publications

Journal: International Journal of Research in Agricultural Sciences.

Paper Tracking ID: IJRAS-183.

Paper Title: Evaluation of the Efficacy and Competitiveness of Indigenous Rhizobia on Soybean (*Glycine Max (L.) Merr*.) Nodulation, Nitrogen Fixation and Growth.