ASSESSMENT OF THE ABUNDANCE AND EFFECTIVENESS OF COWPEA [*Vigna unguiculata* (L) Walp] RHIZOBIA IN SOILS FROM DIFFERENT FIELDS IN CHIWOSYA EXTENSION PLANNING AREA, MCHINJI DISTRICT

MSc. (AGRONOMY) THESIS

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UNIVERSITY OF MALAWI

BUNDA COLLEGE OF AGRICULTURE

APRIL, 2016

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A THESIS SUBMITTED TO THE FACULTY OF AGRICULTURE IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN AGRONOMY

UNIVERSITY OF MALAWI

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APRIL, 2016

DECLARATION

I, Esnart Nyirenda Yohane, declare that this thesis is a result of my own original effort and work, and that to the best of my knowledge, the findings have never been previously presented to the University of Malawi or elsewhere for the award of any academic qualification. Where assistance was sought, it has been accordingly acknowledged. All sources of information have been fully acknowledged.

Esnart Nyirenda Yohane

Signature: _____

Date: _____

CERTIFICATE OF APPROVAL

We the undersigned, certify that this thesis is a result of the author's own work and that to the best of our knowledge, it has not been submitted for any other academic qualification within the University of Malawi or elsewhere. The thesis is acceptable in form and content, and that satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate through an oral examination held on 27th January 2016.

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Date: _____

DEDICATION

I dedicate this work to my beloved parents (Mazwell and Florence Nyirenda) for working tirelessly to shape me to be where I am today. I am really proud of you. May the good Lord grant you long life.

ACKNOWLEDGEMENTS

Firstly, let me thank God the Almighty for taking me through this study. I have experienced God's hand in my life. The Lord is good all the time.

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ABSTRACT

A study was conducted in 2013 at Chitedze Agricultural Research Station to assess the abundance and effectiveness of cowpea rhizobia in soils from different fields and their nodulation capacity by different cowpea genotypes using native cowpea strains. The soils from four fields (Groundnut, Cowpea, Maize and Virgin/non cropped) were used as inoculums including Malawi cowpea inoculant and Australian cowpea inoculant and no inoculation in experiment 1. Highly significant differences (P=0.001) in *Rhizobium* numbers were observed among the fields. The inoculums from cowpea field and virgin land gave higher *Rhizobium* numbers (342 and 217 cfu/g of soil respectively) such that nodulation capacity was higher in plants inoculated with inoculums from cowpea field and virgin land. There significant differences in colony growth, reaction to bromothymol blue and reaction on congo red (P=0.03 P=0.001 and P=0.001 respectively) such that five presumptive strains (CZS1, CZS2, CZS3, CZS4, CZS5) were selected from cowpea field and virgin land including MG5013 (check) were evaluated with the following cowpea genotypes; IT00K-126-3 and IT97K-390-2, Sudan-1, IT82E-16 and Mkanakaufiti in a factorial experiment. There was significant interaction on nodule color and plant nitrogen content (P=0.009 and P=0.001 respectively). Genotype IT00K-126-3 was compatible with CZS2 and Mkanakaufiti and Sudan-1 were compatible with CZS4 since had nodules with dark pink color and gave 4.8% and 3.6% plant N content respectively. This means CZS2 and CZS4 can be considered for production of inoculant for specific cowpea genotypes/varieties.

Key words: Cowpea, nodulation and rhizobia

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BNF	Biological Nitrogen Fixation
BSc	Bachelor of Science
BTB	Bromothymolblue
CFU	Colony forming units
$COSO_4$	Cobalt Sulphate
CR	Congo Red
$CUSO_4$	Copper Sulphate
CZS	Chitedze Strain
DARS	Department of Agricultural Research Services
EPA	Extension Planning Area
EtoH	Ethanol
$FeSO_4$	Ferrous Sulphate
g	Grams
Ha	Hactares
H_2SO_4	Sulphuric Acid
HCl	Hydrochloric Acid
K_2HPO_4	Potassium Hydrogen Phosphate
Kg	Kilogram
$MgSO_4.7H_2O$	Magnesium SulphateHeptahydrate
MG	Malawi Government

Ml	Millilitre
$MnSO_4$	Manganese Sulphate
N	Nitrogen
$Na2M_OO_4$	Sodium Molybdate
NaCL	Sodium Chloride
NaOH	Sodium Hydroxide
NSO	National Statistical Office
Ph	Power of Hydrogen
T	Temperature
TSP	Triple superphosphate
$ZnSO_4$	Zinc Sulphate

CHAPTER 1

INTRODUCTION

1.1 Background information

Cowpea [*Vigna unguiculata* (L) Walp] is of major importance to the livelihoods of millions of people in the developing countries of the tropics (Singh *et al.*, 1997). From the production of this crop, rural families derive food, animal feed and cash. Fresh tender leaves, immature pods and fresh peas are consumed as vegetables, while several snacks and main dishes are prepared from the dry grain. All plant parts that are used for food have been found to be nutritious, providing protein, vitamins and minerals; but of these the grain contains the highest at 23-25% protein (Singh *et al.*, 1997). Like other legumes, cowpea fixes atmospheric nitrogen (N) through Biological Nitrogen Fixation (BNF), a symbiotic association between soil dwelling bacteria, commonly known as rhizobia, and legume host plants. This symbiosis results in nitrogen replenishment as evidenced in many experimental findings that have illustrated increasing soil N levels following cowpea cultivation (Thies *et al*., 1995; Mulongoy and Ayanaba, 1985). It has been estimated that cowpea can fix up to 200 kg N under field conditions (Giller, 2001). However, for cowpea to provide an adequate supply of N through BNF, grain legumes require rhizobia to be provided to the host plant either through the presence of effective native rhizobia, or through inoculation.

Inoculation is the process of introducing commercially prepared sources of rhizobia to the legume plants to promote nitrogen fixation. This usually is done by applying inoculum

directly to the seed prior to planting, or by metering the inoculum into the seed furrow during planting. If the legume crop was grown in the field previously, there is a good chance that the soil already contains the appropriate rhizobial species for nodulation (Mothapo, 2011). Commercial inoculants are composed of rhizobial strains selected for maximum fixation potential. However, even when efficient strains are introduced into the soil, there is no guarantee that these strains will compete well with native strains for entry into plant roots (Erker and Brick, 2011). Thus, the competitive potential of introduced strains has to be verified.

1.2 Problem statement and justification of the study

Nitrogen and Phosphorus are major nutrient constraints in most soils of Malawi (Kumwenda *et al*., 1996). Legumes can provide substantial inputs of N from biological nitrogen fixation hence may not need to be supplied with N where the soils are good. In areas where the soils are bad there is need for starter N.; however, phosphorus, sulfur, molybdenum and iron are needed for BNF. Availability of these nutrients is below optimum requirement in most Malawi soils due to poor soil fertility management (Sakala *et al*., 2000), which has affected cowpea production in Malawi. Currently, if accessible, farmers have to use synthetic fertilizers to improve soil fertility (Mkandawire *et al*., 1997). However, chemical fertilizers are expensive to most smallholder farmers hence there is need to identify less expensive alternative methods of improving soil fertility. One of the ways to improve soil nitrogen levels is through use of legumes (Kamanga *et al*., 2013). However, successful nodulation and effective nitrogen fixation requires soils to have adequate population density and effective native rhizobia or use of inoculants

(Woomer *et al.*, 1997). Cowpea inoculation has been used worldwide in situations where soil fertility is degraded and where effective rhizobia are absent or in insufficient numbers in the soil (Appanu *et al.,* 2008). In Malawi, most farmers do not apply cowpea rhizobia when planting cowpea due to limited access to inoculants, such that farmers rely solely on native rhizobia populations for cowpea nodulation and BNF. Despite this dependence on native rhizobia, little is known about native soil rhizobia that associate with cowpea in Malawi. There is need to understand population diversity, effectiveness, and efficiency of cowpea-nodulating rhizobia, followed by selection of elite strains that can be used for inoculant production. Currently, the cowpea strain that is used for producing cowpea inoculant was collected 10 years ago and its effectiveness is questionable due to such a long stay. The goal of the study described here was to assess the abundance and effectiveness of rhizobia in different in soils from diverse fields collected in Chiwosya Extension Planning Area, Mchinji district, and to evaluate cowpea genotypes using a range of selected cowpea rhizobia strains.

1.3 Study objectives

1.3.1 Main objective

The main objective of the study was to assess the abundance and effectiveness of cowpea rhizobia in soils from diverse fields and evaluate nodulation capacity by distinct genotypes/varieties using native cowpea strains.

1.3.2 Specific objectives

The specific objectives of the study were to:

i. Evaluate the effectiveness of native rhizobia from soils of varying fields to nodulate cowpea

ii. Determine competitive native rhizobia strains that could be used as effective cowpea inoculants

iii. Evaluate distinct cowpea genotypes for nodulation capacity using competitive cowpea native rhizobia strains

1.4 Study hypotheses

The following were the study hypotheses:

i. Nodulation capacity and rhizobia population would vary with the field history

ii. Rhizobia effectiveness would vary with the field history

iii. Nodulation capacity of cowpea genotypes is associated with distinct rhizobia strains

CHAPTER 2

LITERATURE REVIEW

2.1 Legume production in Malawi

Legumes commonly grown in Malawi include groundnut (*Arachis hypogaea*), Bambara ground- nut (*Vigna subterranea*), common bean (*Phaseolus vulgaris*), soybean (G*lycine max*), Cowpea (*Vigna unguiculata* [L.] Walp) and pigeon pea (*Cajanus cajan*) (Kamanga *et al*., 2010). According to Rusike *et al*. (2013), common bean is very important for cash, food security, nutrition and gender equity. Common bean is one of the crops relied by farmers on a daily basis for food as a relish and for cash. The crop is grown country wide.

Groundnut (*Arachis hypogaea*) is among the major valuable grain legume crops, with tremendous contributions to improving household food security, nutrition, soil health and fertility in Malawi. It is mostly grown for food and cash. Over 25% of the Malawi's agricultural cash income among smallholder farmers is being realized from groundnut (Monyo and Kananji, 2013).

Soybeans are also very important because there is a high demand resulting from the urban demand for edible oil and soybean cake for poultry feeds. This permits farmers to generate high cash incomes (Rusike *et al*., 2013).

Cowpea is fifth important legume crop that comes after common beans, groundnuts soybean and pigeon pea. The grains contain 25% protein, and several vitamins and minerals. The crop tolerates drought, performs well in a wide variety of soils, and being a

legume, it fixes N. It is grown mainly by small-scale farmers in marginal areas where it is often cultivated with other crops as it tolerates shade. It also grows and covers the ground quickly, which helps preventing soil erosion. It is, therefore, an important crop for farm incomes, food security, nutrition and natural resource management especially in the low lying areas of Malawi where the production of cowpea is high.

2.2 Legume role in cropping systems

Maize (*Zea mays*) is a staple food crop in Malawi. However, smallholder farmers' yields average less than a ton ha^{-1} due in part to low soil fertility, with N in particular being the most limiting nutrient (Mhango *et al*., 2008).Other than maize, legumes are the main components of maize-based systems in Malawi. A wide range of legumes are grown in the country, either as monocrops or in association with maize. The main benefit of cropping system diversification with legumes is soil fertility improvement. Through the cultivation of grain legumes, smallholder farmers are able to increase soil nitrogen through the process of BNF which enables legumes to utilize atmospheric N. However, the choice of the legume will significantly influence the benefits derived from diversification. Long-duration legumes, such as pigeon pea, are biologically superior at fixing significant amounts of N, enhancing P availability and yields of subsequent cereal crops, compared to short-duration legumes such as groundnuts (Giller and Cadish, 1995).

The soil fertility benefits of legume depend on the ratio of legume to cereal, the duration of legume biomass production and residue management (Kamanga, 2002). The integration of the legumes requires consideration of the competitive effect of relay or

intercropped legumes within maize-dominated systems for water and nutrient availability between the legume and the main crop (Kamanga, 2002). Legumes such as cowpea, pigeon pea, mucuna and soybean have minimally competitive growth traits, such as late-season branching patterns and deep taproots that minimize intra-row competition (Snapp *et al*., 2010).

2.3 Cowpea production and utilization

Cowpea (*Vigna unguiculata* [L.]Walp) was domesticated in Southern Africa later spreading to East Africa, West Africa and Asia (Ng and Maréchal, 1985). Cowpea is an important economic crop in many developing countries because of its high protein content, adaptability to different soil types, improving soil fertility through BNF, weed suppression, and drought tolerance characteristics as such it is often grown in marginal areas and is able to perform well (Kimiti and Odee, 2010).

In Malawi, cowpeas are grown country-wide; however, they tend to predominate in warmer, drier areas with low rainfall such as Shire valley, Bwanje valley, lake shore areas, Phalombe plains and dry plateau areas of Machinga (Government of Malawi, 2007). In these areas cowpea does well since itisa drought tolerant crop capable of maintaining growth even under dry conditions. Ng and Marechal (1985) attributes the crop's drought tolerance to its deep rooting, making it most popular in semi-arid regions of the tropics where other food legumes do not perform well. Cowpea is also shade-tolerant and fast growing, characteristics that support its cultivation as an intercrop with cereals (maize or sorghum) and root crops (Ng and Marechal 1985).In Malawi, cowpea is

primarily cultivated in an intercropped system where it is planted together with other crops like maize and cowpea in one field, unlike mono-cropping whereby cowpea is planted alone in one field. The reasons for intercropping are labour reduction, profit maximization, risk minimization against crop failure, soil conservation, weed control and balanced nutrition (Shetty *et al*., 1995).

Cowpea production levels are less than 500 kg ha^{-1}compared to potential yield of 1500 - 2500 kg ha^{-1}in Malawi, due to severe attacks from insect pests and diseases, declining soil fertility, unreliable rainfall and lack of availability of seeds of improved varieties (Kananji, *personal communication*, 20-02-12). However, lack of availability of seeds of improved varieties is most crucial. There are only three cowpea varieties that have been released and recommended for cultivation in Malawi (Sudan-1, IT82E-16 and Mkanakaufiti). However, efforts are underway to increase its production through release of more varieties and seed of the released varieties available. (Kananji, *personal communication*, 20-02-12).Cowpeas are mostly consumed as a relish, a vegetable dish often served with *Nsima,* a thick paste made from maize flour, which is Malawi's main staple food. In this case, cowpea grains are consumed when green or dry. The grains can also be soaked, boiled and mashed into thick paste locally known as *Chipere.* Green or dried cowpea leaves are also consumed as relish, known locally as *Chitambe* or *Ntambe*. Fresh cowpea grains are also boiled and eaten as a snack (*Makata*).

2.4 Biological nitrogen fixation (BNF)

2.4.1 Biological nitrogen fixation process

Takishima *et al*., (1989) defines biological nitrogen fixation as the reduction of nitrogen gas to ammonia. The process requires sixteen molecules of ATP and a complex set of enzymes to break the nitrogen bonds so that it can combine with hydrogen. The fixed nitrogen is made available to plants by the death and lysis of free nitrogen fixing bacteria or from the symbiotic association of some nitrogen fixing bacteria with plants (Chenn, 1999). Soil contains many types of microorganisms, such as bacteria, actinomycetes, fungi, algae, among others. Amongst soil bacteria, a unique type known as rhizobia has a beneficial effect on the growth of legumes by biologically fixing otherwise-unavailable atmospheric N into a form plants can use for growth and development, when they are in association with legume plants (Chenn, 1999). Rhizobia can live either as saprophytic organisms in the soil or in association with host legumes by forming plant-derived growths on the roots, known as nodules. The legumes begin the nodule formation process by communicating with compatible rhizobia through release of compounds called flavonoids from their roots, which in turn trigger the production of *nod* factors by the bacteria (Hutton, 2010). When the *nod* factor is sensed by the root a number of biochemical and morphological changes take place, triggering cell division in the root cortex to create the nodule. The root hair growth is then redirected to wind around the bacteria multiple times until it fully encapsulates one or more bacteria. The encapsulated bacteria divide multiple times, forming a microcolony. From this microcolony, the bacteria enter the developing nodule through a structure called an infection thread, which

grows through the root hair into the basal part of the epidermis cell, and onwards into the root cortex; they are then surrounded by a plant-derived membrane and differentiate into bacteroids that fix nitrogen(Watanabe, 2000). Sets of genes in the bacteria control different aspects of the nodulation process. Specificity genes determine which *Rhizobium* strain infects which legume. Even if the strain is able to infect a legume, the nodules formed may not be able to fix nitrogen. It is only the effective strains that induce nitrogen fixing nodules. Effectiveness is governed by Nod genes for nodulation. Biological nitrogen fixation process plays a significant role in improving the fertility and productivity of low-N soils because it provides continuous supply of nitrogen for plant growth (Lindemann and Glover, 2003). The direct availability of the fixed N to the host plant allows it to grow in environments that are low in N and also reduces losses from denitrification, volatilization and leaching. Studies have shown that grain legumes fix about 15 – 210 kgNha-1 seasonally in Africa (Dakora and Keya, 1997).

2.4.2 Factors affecting biological nitrogen fixation

Legumes can obtain nitrogen from three sources: soil nitrogen, native rhizobia, and rhizobia introduced as inoculants. In most cases, legumes will obtain some of their N from the soil, even if they fix high amounts of N (Mpepereki and Makonase, 1995). The amount of nitrogen fixed by legume plants depends on the abundance and longevity of the root nodules, the effectiveness of rhizobia within the nodules, and the level of available soil nitrogen (Singleton *et al*., 1990). Nodulation and nitrogen fixation are affected by a number of factors including soil pH, soil moisture, temperature, mineral nutrients among others.

Excessive moisture and / or water logging are one of the factors that affect nodulation and nitrogen fixation. It also prevents the development of root hair and sites of nodulation, and interferes with a normal diffusion of O_2 in the root system of plants. *Sesbania rostrata* and *Aeschynomene* sp. can actively fix N under these conditions because they are located on the plant stems, rather than on the roots (Mohammadi *et al*., 2012).Water stress is also known to reduce the number of rhizobia in soils, and inhibits nodulation and N fixation. Prolonged drought will promote nodule decay, hence affecting nitrogen fixation (Graham, 1992). Deep-rooted legumes exploiting moisture in lower soil layers can continue fixing N when the surface soil is drying (Zahran, 1999).

Soil pH is another factor that affects nitrogen fixation. Mohammadi *et al*. (2012) reported that low soil pH is generally accepted as an indicator of conditions under which some other soil properties may limit crop growth rather than as a primary cause of poor growth. In addition to the direct effects of soil acidity, growth of legumes may be reduced indirectly through depression of nodulation and nitrogen fixation. Extremes of soil pH affect nodulation and BNF in different ways. There is a range of effects of soil pH on rhizobia, but generally few rhizobia grow and survive well below pH values of 4.5 to 5.0 (Hungria and Vargas, 2000). Acidity also depresses the growth of the legume plant and the infection process. This effect is most likely due to both a disruption of signal exchange between macro- and micro-symbionts and depression of nodulation genes and excretion of *nod* factor in the rhizobia (Singleton *et al*., 1990).Soil acidity also limits rhizobial growth and existence in the soil. Fast-growing rhizobia are generally considered

more sensitive than bradyrhizobia. Failure to nodulate is also common in acid soils not only because of lowered numbers of rhizobia but also because acidity affects attachment (Andrade, 2002). Brockwell *et al.* (1991) reported a nearly 10^{-3}decrease in the number of *S. meliloti* rhizobia in soils with a pH<6 compared to those with a pH>7.0, hence affecting nodulation.

Mineral nitrogen inhibits the rhizobia infection process and also inhibits N fixation in the sense that it is less expensive for the plant to use N from the soil than to fix N (Mohammadi *et al*., 2012). On the other hand, less energy is used for the plant to take up soil N than to fix N. As a general principle, nitrogen fixation goes up as soil nitrogen goes down, and the *vice versa*. Given high levels of nitrogen in the soil, plants may not form nodules at all, or they may reduce or cease nitrogen-fixing activity in the nodules already formed (Mohammadi *et al*., 2012).Addition of large quantities of nitrogen fertilizer inhibits N fixation, but low doses (<30 kg N ha^{-1}) of nitrogen fertilizer can stimulate early growth of legumes and increase their overall N fixation. The amount of this starter N must be defined in relation to available soil nitrogen (Singleton *et al.,* 1990).

Extreme temperatures also negatively affect N fixation. Since N fixation is an enzymatic process, enzyme activity can be strongly affected by temperature fluctuations. Singleton *et al.* (1990) reported that soil temperature range of 25ºC – 30ºC is preferred for nodulation and nitrogen fixation. However, Mohammadi *et al.* (2012) reported that the influence of temperature on rhizobia appears to be both strain and soil dependent.

2.4.3 Role of some mineral nutrients on N fixation

2.4.3.1 Phosphorus

Phosphorus is the major required macro nutrient in nitrogen fixation. The nutrient is used in numerous molecular and biochemical plant processes particularly in energy acquisition, storage and utilization. Nodulation and nitrogen fixation are strongly influenced by phosphorus availability. Phosphorus is an essential ingredient for *Rhizobium* bacteria to convert atmospheric nitrogen (N_2) into an ammonium (NH_4) from useable form by plants. *Rhizobium* are able to synthesize the enzyme nitrogenase which catalyses the conversion of nitrogen to two molecules of ammonia (NH_3). Phosphorus becomes involved as an energy source when 16 molecules of adenosine triphosphate (ATP) are converted to adenosine diphosphate (ADP) as each molecule of nitrogen is reduced to NH_3). The ATP is generated during the process of photosynthesis when light energy is transformed and stored in the form of ATP for later use by the plant. Nitrogen fixing plants such as legumes have an increased requirement for phosphorus due to need for nodule development and signal transduction. When legumes receive inadequate supply of phosphorus they may suffer nitrogen deficiency (Jakobsen, 1985). Soil phosphorus is classified into two broad groups, organic and inorganic. Organic phosphorus is found in plant residues, manures and microbial tissues. Soils low in organic matter may contain only 3% of their total phosphorus in the organic form, but high-organic-matter soils may contain 50% or more of their total phosphorus content in the organic form (Weisnay, *et al*., 2013). Inorganic forms of soil phosphorus consist of apatite (the original source of all phosphorus), complexes of iron and aluminum

phosphates, and phosphorus absorbed onto clay particles. The solubility of these phosphorus compounds as well as organic phosphorus is extremely low, and only very small amounts of soil phosphorus are in solution at any one time (Weisnay, *et al*., 2013).

2.4.3.2 Boron

Boron is most required trace element in nitrogen fixing plants. A study by Bolanos *et al.* (1996) in the effect of boron on rhizobium-legume cell surface interaction and nodule development in Pea, it was reported that in boron deficient plants, the number of rhizobia infecting the host cells and the number of infection threads were reduced and the infection threads developed morphological aberrations showing lack of the covalently bound hydroxyproline/proline rich proteins which contribute to oxygen barrier preventing inactivation of nitrogenase and associated decrease in nitrogen fixation.

2.4.3.3 Zinc

Zinc is a micronutrient needed in small amounts by crop plants, but its importance in crop production has increased in recent years. Weisany *et al.* (2011) reported that zinc application on plants exposed to salinity stress caused a noticeable enhancement of photosynthesis, water use efficiency, mesophyll efficiency and quantum yield compared with plants exposed to salinity stress alone. Weisany *et al*. (2013) also reported that lipid peroxidation and hydrogen peroxide concentration under salinity treatments significantly reduced as a result of zinc application.

2.4.3.4 Iron

Iron (Fe) is required for several key enzymes of the nitrogenase complex as well as for the electron carrier ferredoxin and for some hydrogenases. A particular high iron requirement exists in legumes for the heme component of hemoglobin. Therefore, in legumes iron is required in a greater amount for nodule formation than for host plant growth (Weisany *et al*., 2013). The single most abundant protein that the plant host makes in the nodule is leghaemoglobin, an iron protein. In the bacteria, nitrogenase and nitrogenase reductase contain FeS clusters and the former has the cofactor FeMoCo at the active site for N2 reduction. Further, bacteroids have a very high respiratory demand, requiring abundant cytochromes and other electron donors, each with their own Fe centers (Delgado, *et al*., 1998). Although iron deficiency did not significantly affect shoot growth, it severely depress nodule mass and particularly leghemoglobin content, number of bacteroids and nitrogenase activity.

2.4.3.5 Cobalt

Cobalt is essential for nitrogen fixing microorganisms. Cobalt has been shown to be essential for symbiotic nitrogen fixation by legumes and non-legumes. The role of cobalt in nitrogen fixation is essentially attributed to its role as a cofactor of cobalamine (Vitamin b6) which functions as a coenzyme involved in nitrogen fixation and nodule growth. Cobalt is also required as a part of a bacterial enzyme complex such that its deficiency affects nodule development and function (Weisany, *et al*., 2013).

2.4.4 Assessment of nodulation and nitrogen fixation potential

Nodulation is defined as the capacity of the plant to produce nodules whereas nitrogen fixation is the potential of the plant to fix nitrogen (Peoples *et al*., 1995). The plant's total potential to fix nitrogen is therefore evaluated by assessing nodulation and plant growth characteristics. In most cases, a healthy-looking legume plant in the field does not give a true reflection of healthy nitrogen fixation underground; hence, there is need to assess the nodulation. Nodulation assessment is normally done during early flowering when the nitrogen fixation rates are generally at maximum (Zaychuk, 2006). Nodulation is assessed by examining the roots of the plants and assigning a score to the nodules based on a number of different criteria, including root nodule number, mass, color, distribution and longevity of the nodule population and visual assessment scores using a scale of 0-5 whereby 5 means greater than five clusters of pink pigmented nodules, 3, three to five clusters of predominantly pink nodules, 1, less than three clusters of nodules or whitish/greenish nodules, 0, no nodules (Unkovich *et al*., 2008).

In terms of plant growth, assessment of nitrogen fixation is based on the plant vigor. Zaychuk (2006) utilized a system where assessment criteria were based on plant and growth vigor using a scale of 1-5. Plants that are green and vigorous are given a score of 5, plants that are green and relatively small are given a score of 3, plants with slightly chlorotic or less green are given a score of 2 and plants that are very chlorotic are given a score of 1. Combined, nodule and plant values can offer valuable insights into total nodulation and nitrogen fixation capacity of the plant-rhizobia relationship.

Nitrogen fixation can also be assessed via nodule internal color. Nodules that are actively fixing nitrogen have pink or red color, showing the presence of the oxygen carrier, leghaemoglobin, which is essential for nitrogen fixation. Nodules with white, greenish or dark colors are indicative of ineffective nodulation and might correlate with low nitrogen fixation rates (Unkovich *et al.*, 2008). This is supported by Woomer (1997), who used a scale of 1-5 in assessing the nitrogen fixation by legumes by looking at the internal color of the nodules whereby white color was represented by a score of 1, brown color a score of 2, green color a score of 3, light pink a score of 4 and strong pink/red a score of 5. Nodule position is also crucial when assessing nitrogen fixation in legume plants since it has been observed that nodules that concentrate on the crown are more efficient in terms of nitrogen fixation than those that are concentrated in the lateral position. Zaychuk (2006) used a scale of 1-3 whereby 3 was mainly crown nodulation, 2 was a combination of crown and lateral nodulation and 1 was mainly lateral nodulation. A mean nodule score of 4-5 across a treatment group represents excellent nodulation and excellent potential for nitrogen fixation, a mean score of 3-4 indicates good nodulation and good potential for nitrogen fixation, a mean score of 2-3 represents fair nodulation and nitrogen fixed may not be sufficient to supply the demand of the crop while a mean score of 0-2 indicates poor nodulation and probably little or no nitrogen fixation. The rating differs from Zychuk (2006) who reported that scores for each parameter are added and then nodulation and nitrogen fixation assessment is based on the total scores, not the mean. A total score of 11-13 means effective nodulation and good nitrogen fixation potential, a

total score of 7-10 means less effective and low nitrogen fixation and finally total score of 1-6 means unsatisfactory nodulation and poor nitrogen fixation.

2.5 Native rhizobia

Already-existing soil rhizobia, referred to as native or indigenous rhizobia, are strains that have naturalized in the soil following introduction through past inoculation, or *via* wind or seed transport. Native rhizobia are important in legume BNF and play a significant role in growth and yield of most leguminous crops, especially where inoculants are unavailable (Woomer *et al*., 1997). The population of native rhizobia in any soil can be very diverse, in terms of species, and can comprise many distinct strains within each species. Silva and Uchida (2000) reported that some soils have high populations of native rhizobia that are compatible with the legume crop. If the crop's requirement of BNF can be met by these native rhizobia, inoculation may not be necessary to increase yield. Large native rhizobia populations often occur when legume crops are grown in the same field for many crop cycles or when crops have been previously inoculated and the rhizobia persist. Bushby (1993) reported that previous legume cropping increases native rhizobial populations through release of the rhizobia following nodule senescence. This is in agreement with Thies *et al.,* (1995) who observed significant increases in bradyrhizobia species population densities only in response to cropping with the homologous host legume, which suggests enrichment of soil populations to be host-specific. However, Abaidoo *et al.,* (2006) reported contrasting findings of low population sizes of *Bradyrhizobium* sp. (cowpea), *Bradyrhizobium* sp. (TGx), and *B. japonicum* (Clark) identified in locations previously cropped with their specific hosts. In this case, native

Bradyrhizobia populations appear to be related to soil management and environmental factors such as soil temperature, moisture and pH. Ngokota *et al.,* (2008) also reported similar results in a study of groundnut-nodulating rhizobia in Cameroon, where highest diversity was found in sites with no history of groundnut cultivation. Higher rhizobia populations have also been shown to be supported in soils with higher organic matter content. This is in agreement with Bushby (1993) who reported that fields receiving good fertility management, such as manure and fertilizer application, have higher rhizobial numbers and diversity. Rhizobia populations have also been shown to increase under field moist soil conditions and where the temperature ranges from 28 to 31^{o}C (Mwenda *et al*., 2011).

2.5.1 Cowpea native rhizobia

Cowpea, *Vigna unguiculata* (L.)Walp is known to be a promiscuous crop in that it establishes a symbiotic association with a wide variety of *Bradyrhizobium* species (Sellschop, 1962). Rhizobia associating with cowpea are also of special significance to nitrogen fixation because they are able to form nodules on a broad spectrum of tropical legumes. In optimal environments, well-nodulated cowpeas can derive 90% of their nitrogen needed for maximum yield from an effective symbiosis (Eaglesham *et al.*, 1977). The yield and nodulation of cowpea, however, are reported to be seldom increased by inoculation. This is thought to be due, in part, to the typically high population sizes of native bradyrhizobia already present in tropical soils (Danso and Owiredu, 1988; Kang, 1977). Cowpea cultivation can enhance native soil rhizobia populations such that other legumes nodulated by *Bradyrhizobium* sp like soybean, can also benefit. Simply, the act

of cowpea cultivation is known to stimulate proliferation of rhizobia in a field (Mulongoy and Ayanawa, 1985). While many fields do contain resident native cowpea rhizobia strains, if cowpea is being introduced for the first time in the area, inoculation with commercial rhizobia may ensure sufficient rates of biological nitrogen fixation and hence increase yield.

Similar to other rhizobia, cowpeas symbiont population size and diversity are affected by factors such as soil management and environmental conditions (soil moisture, temperature, pH among others). This is supported by a study carried out by Kimiti and Odee (2010) in semi-arid eastern Kenya who reported that native rhizobia population counts varied with the time of soil sampling, nutrient input and cowpea variety used. Higher populations were recorded when cowpea plants were inoculated with soils collected at the time of crop harvesting as compared to soils collected at the time of cowpea planting. Rhizobia counts also varied with variety and inputs applied. For example, addition of triple superphosphate (TSP) (15 kg ha^{-1}), and manure (2.5 t ha^{-1}+ TSP 15 kg ha^{-1}) to the cowpea crop increased native soil rhizobia populations under the cowpea genotype IT95K-52-34 (coded by M14) by 23% above control (soils collected at planting). Soils collected from manured treatments reduced rhizobia counts under M14 by about 35% below the rhizobia counts at the start of the season. These findings were in contrast to the observation made by Zengeni *et al.* (2006), where addition of 10 t ha^{-1} of manure enhanced rhizobia populations in the soil.

The study reported herein, therefore, sought to (i) evaluate the capacity of native rhizobia from soils of diverse fields to nodulate cowpea (ii) determine competitive native rhizobia strains that could be used as effective cowpea inoculants and (iii) evaluate distinct cowpea genotypes for nodulation capacity using competitive cowpea native rhizobia strains. The guiding hypotheses were that (i) nodulation capacity and rhizobia population diversity would vary with the field history (ii) rhizobia effectiveness would vary with field history (iii) nodulation capacity of cowpea genotypes is associated with distinct rhizobia strains.

CHAPTER 3

MATERIALS AND METHODS

3.1 Description of study area

The research was conducted in a glasshouse and soil microbiology laboratory at Chitedze Agricultural Research Station. The station is located at the latitude of 13º 59' S, and longitude of 33º 31'E, at the elevation of 1100m above sea level. The mean daily temperatures for the glasshouse for the first experiment (rhizobia trapping) were 20ºC (min) and 38ºC (max), and the daily temperature for the second experiment (nitrogen fixation by cowpea genotypes using rhizobia strains) were 14ºC (min) and 31ºC (max) (Appendix 3 and 4, respectively).

3.2 Soil field collection

Soil for this experiment was collected from Chiwosya Extension Planning Area, in Mchinji district where farmers benefited from McKnight cowpea project. The soil was collected from: 1) Cowpea field, 2) Groundnut field, 3) Maize field and 4) Non-cropped/virgin land. Selection of fields was based on their past history. For instance, the cowpea and groundnut fields, the target fields included those with cowpea or groundnut as a pure stand. For the maize field, the target was identified in which maize was continuously grown as monocrop for more than three seasons. In case of virgin land/ non-cropped, the target was the field that was newly opened up for cultivation. Soils were collected from a depth of 0 – 15cm using an Eldelman soil auger. Five soil samples per field were collected along a transect and were bulked into one composite soil sample. To

avoid contamination the auger was cleaned by removing all the soil sticking into the auger and then sterilized by placement in sodium hypochlorite solution for 4 minutes and rinsing in five changes of sterilized distilled water between fields (Vincent, 1970).

3.3 Soil chemical analysis

Sub-samples of soil were air-dried, grounded and sieved through a 2 mm sieve before analyzing for chemical soil properties.

3.3.1 Determination of total Nitrogen

Total nitrogen was determined using the Kjeldahl method (Motsara and Roy, 2008). The following procedure was followed:1 g of soil sample was weighed and placed in a Kjeldahl flask and 0.7 g of copper sulphate, 1.5 g of K_2SO_4 and 30 ml of H_2SO_4 were added. These were heated gently until frothing ceased. Thereafter, the solution was boiled briskly until it was clear and then digestion was continued for at least 30 minutes. The flask was then removed from the heater and cooled, and 50 ml of water was added and transferred to a distilling flask.20–25 ml of standard acid (0.1M HCl or 0.05M H_2SO_4) was added in the receiving conical flask so as to have an excess of at least 5 ml of the acid. 2–3 drops of methyl red indicator were added. This was followed by addition of 30 ml of 35%NaOH in the distilling flask in such a way that the contents do not mix. The contents were heated to distil the ammonia for about 30–40 minutes then removed the receiving flask and rinsed the outlet tube into the receiving flask with a small amount of distilled water. This was followed by titration of excess acid in the distillate with 0.1M NaOH.

The following calculation was used to determine total N:

$$\%N = 1.401 \frac{[(V1M1 - V2M2 - (V3M1 - V4M2)]}{W} \text{ x df} \quad (1)$$

Where:

V1 =Milliliters of standard acid put in receiving flask for samples

V2 =Milliliters of standard NaOH used in titration

V3 = Milliliters of standard acid put in receiving flask for blank

V4 = Milliliters of standard NaOH used in titrating blank

M1 =Molarity of standard acid

M2 = Molarity of standard NaOH

W = Weight of sample taken (1 g)

Df= dilution factor of sample (if 1 g was taken for estimation, the dilution factor will be 100).

Note: 1 000 ml of 0.1M HCl or 0.05M H2SO4 corresponds to 1.401 g of N

3.3.2 Determination of Phosphorus (Mehlich-3 Extraction)

Available phosphorus was analyzed using Mehlich 3 method. 25 ml of the Mehlic-3 extractant were added to 2.5g soil in centrifuge tubes. The tubes were shaken for 10 minutes, rested for 5 minutes then centrifuged. Samples were filtered by passing them through the funnels fitted with filter paper. Thereafter, 1 ml of both the extractant and standards were pipetted into glass vials and 8 ml of the MR working solution were added to samples and standards. After 30 minutes, the absorbance was read at the wavelength of 880nm.

3.3.3 Determination of Organic Matter and Organic Carbon

The percent organic matter and organic carbon were analyzed using the Walkley and Black method as described by Anderson and Ingram (1993). The following procedure was used: 1 g of the soil sample was weighed into a 500 ml Erlenmeyer flask followed by the addition of 10 ml of potassium dichromate using the pipette and the contents were mixed by swirling. Thereafter, 20 ml of sulphuric acid were added and the flask was gently rotated for 1 minute in order to mix up the solution. The mixture was left for 30 minutes then 200 ml of water were added into the mixture and mixed thoroughly. This was followed by addition of 1 ml of diphenylamine indicator. The mixture was then titrated with 0.5N ammonium ferrous sulphate until the mixture turned dark green. Two blanks were then made without soil addition in order to standardize the dichromate. The amount of carbon was determined from the standard titre and percent organic carbon was calculated using the following formula:

$$\%OC = \frac{M \text{ x } 0.39 \text{ x } mcf \text{ x } (V1 \text{ x } V2)}{S} \quad (2)$$

Where:

M = Molarity of ferrous sulphate solution

V1 = ml of ferrous sulphate solution

V2 = ml of ferrous sulphate solution required for blank

S = Weight of air dry sample in grams

mcf = moisture correcting factor (100 + % moisture)/100

% SOM = % OC x 1.72

A factor of 1.72 was obtained from the division of 100% by 58% and is universally used in converting organic carbon values to organic matter values. The 58% represents an average value of organic carbon in humus obtained in 1900s. This factor is universally used in converting values of organic carbon to soil organic matter values (Anderson and Ingram, 1993).

3.3.4 Determination of soil pH

Soil pH was determined in water as described by Motsara and Roy (2008). The following procedure for measuring soil pH was used: a pH meter was calibrated using two buffer

solutions, one buffer with neutral pH (7.0) and another buffer of pH 4.The buffer solutions were put in the beakers. The electrodes were then inserted alternately in the beakers containing the two buffer solutions, and then the pH was adjusted. 10.0 g of soil sample were placed into a 50 ml beaker and 20 ml of water (suspension medium) was added and shaken for 30 minutes, left to stand for 30 minutes and shaken again for 2 minutes. Soil pH was recorded on the calibrated pH meter and the soil pH values were determined by immersing the glass electrode in the soil sample suspension. The electrode was washed with distilled water after every reading.

3.4 Experiment 1: Assessment of abundance and diversity of rhizobia in inoculums from different cropping fields

The goal of experiment I was to assess the abundance and diversity of rhizobia in different in soils from diverse fields collected in Chiwosya Extension Planning Area, Mchinji district.

3.4.1 Seed and Seedling Management

3.4.1.1 Pre-germination

Cowpea seeds used for rhizobia trapping were surface sterilized by immersing them in 70% ethanol for 1 minute, then in 4% sodium hypochlorite solution for 3 minutes, and thereafter rinsed in six times with sterilized distilled water (Vincent, 1970). The sterilized seeds were placed in sterile paper towels layered in petri dishes using a sterile applicator stick. Distilled water was poured over the seeds to be imbibed, then the seeds were covered with another layer of paper towel and a petri dish lid placed on top, and

kept at room temperature. After two days, seeds that germinated were placed in a refrigerator at 4^{o}C to reduce further growth until planting.

3.4.1.2 Sowing and management

Medium sized clay Pots were filled with sterilized sand and placed in a glasshouse. Cotton wool was used to plug the holes at the bottom of the pots to avoid heavy drainage. Pots, river sand and cotton wool were sterilized by autoclaving at 121^{o}C for 20 minutes. Thereafter, the pots full of sand were washed with boiled water to remove nitrogen. After the water drained, the pots were covered with aluminium foil to avoid contamination from aerosols. During planting, three holes were made using a sterile stick around a perimeter of each pot and one pre-germinated seedling was placed in each hole using a pair of forceps and the seedlings were covered with sand. After a day of planting, each seedling was inoculated using the soil inoculum. The pots were covered with plastic wrap for up to three days when the seedlings were almost 2cm high then 1ml was applied to the roots of each plant using a new pipette tip. After two weeks, the seedlings were thinned to one in each pot. Plants were also provided with 90 ml of both N-free solution and distilled water every day in alternate way. The plants were harvested after 45 days.

3.4.2 Treatments and experimental design

SUDAN-1, a variety commonly grown by smallholder farmers was used as the cowpea test variety.

The treatments were as listed below;

Treatment	Description
T1	Inoculum from groundnut field
T2	Inoculum from cowpea field
T3	Inoculum from maize field
T4	Inoculum from virgin land
T5	No inoculation (negative check)
T6	Malawi cowpea inoculums (Positive check)
T7	Australian cowpea inoculum (Positive check)

In total, there were seven treatments, replicated five times in a complete randomized design (CRD)

3.4.3 Data collection and analysis

The following response variables were assessed: nodulation (nodule numbers, nodule color rating, nodule position and plant vigor rating), plant height at harvest, plant nitrogen and phosphorus content, rhizobia population density, rhizobia isolation and characterization of cultures.

3.4.3.1 Nodulation assessment

Growth vigor and nodule position included a visual assessment using a rating system as described by Zaychuk (2006).The plants showing green color and vigorous growth were given a score of 5, Less vigorous plants with green color were given a score of 3, slightly chloritic plants were given a score of 2, and plants that were very chlorotic were given a score of 1. Plants with both crown and lateral nodulation were given a nodule position score of 3, those with only crown nodulation were given a score of 2 and finally plants with only lateral nodulation were given a score of 1.

Nodule color was assessed by removing nodules from the root system and slicing them to observe the color. Pink/red color was considered to have active nodulation because of the presence of leghemoglobin while brown, white or green were considered non-effective due to absence of leghemoglobin. A scale of 1-5 was used to assess nodule color where 1 represented white color, 2 represented green color, 3 represented brown, 4 represented light pink/red and 5 represented dark pink/red (Singleton *et al*., 1992).

After nodule color, plant vigor and nodule position assessments, the scores for the three parameters were summed to find the total score, thereby determining nodulation effectiveness, whereby a total of 11-13 meant effective nodulation, a total of 7-10 meant less effective nodulation and a total of 1-6 meant unsatisfactory nodulation (Zaychuk, 2006).

3.4.3.2 Plant nitrogen and phosphorus determination

The plant samples (shoot and root biomass) inoculated with inoculums from diverse fieldswere oven dried at 70^{o}C for 24 hours to remove the water from the plant tissue to stop enzymatic reactions and to stabilize the sample. After drying, samples were ground to 1.0mm particle size to ensure homogeneity. Total nitrogen and phosphorus were determined using Kjeldal digests, which involved the digestion of plant material with a mixture of potassium sulphate and concentrated sulphuric acid with finely powdered selenium metal as the catalyst. The digest was analysed for nitrogen as indo-phenol blue in the Technicon Auto analyzer II. Phosphorus was determined colorimetrically as reduced phosphomolybdate using the Technicon Autoanalyzer II (Chirimba, 2007).

3.4.3.3 Rhizobia isolation and characterization

Roots were washed thoroughly to remove sand. Two nodules were collected from each plant by cutting the root about 0.5 cm on each side of the nodule using forceps to reduce the risk of damaging the nodule. A total of 14nodules were collected from across the experiment, representing all the treatments. Undamaged nodules were immersed intact for 1min in 70% ethanol (to break the surface tension and to remove air bubbles and microorganisms from the tissue) using a pair of forceps and then transferred into a 4%(v/v) solution of sodium hypochlorite for 3 min (Vincent, 1970). The nodules were then rinsed in six changes of sterile water using sterile forceps for transferring. Forceps were surface sterilized with ethanol (ETOH) and then flamed.

Surface sterilized nodules were crushed using a pair of blunt-tipped forceps in a large drop of sterile water in a petri-dish; thereafter, one loopful of the nodule suspension was streaked onto a yeast-mannitol agar (YMA) plate containing Congo Red (CR) or bromothymol blue (BTB).The isolates were characterized on yeast extract mannitol mineral salts agar (YEMA) media containing bromothymol blue or Congo red. The fast growing rhizobia took 3-5 days to grow while slow growers took 7-10 days. A score of 1-3 was used to characterize colonies in BTB, whereby 1= acid forming (yellow), 2= non-reactive (green) and 3= basic (blue). Colonies of slow growing rhizobia were characterized by the blue coloration, indicating alkaline reaction in BTB, while yellow color indicated acid reaction produced by fast growing rhizobia. In CR, a score of 1-4 was used, whereby 1=non-absorbent, 2=partly absorbent, 3= centre absorbent and 4= fully absorbent. Typical rhizobia colonies show little or no absorption (Bala *et al*., 2011).

3.4.3.4 Rhizobium population density

Seeds of cowpea (*Vigna unguiculata* [L.] Walp) were surface sterilized in 70% ethanol for 1 min, 4% sodium hypochlorite for 3 min and then 6 times rinses with sterile water. The seeds were pre-germinated in sterile paper towel layered inside the sterile petri dishes and then transferred into pots after germination. Seedlings were inoculated with 1 ml of soil dilution using a10-fold dilution series (10^{-1} to 10^{-6}) after one week starting with the most diluted. The following procedure was used for making a soil dilution: 10 g of sub-sample was placed into 90 ml of water in a cylinder to form the 10^{-1} dilution, and was shaken for 10 minutes on a wrist action shaker. Thereafter, 1.0 ml of the 10^{-1} was transferred to a tube containing 9.0 ml of diluent using a sterile pipette, forming the 10^{-2}

dilution. The tube was shaken well for 10 minutes. With a new pipette tip, 1.0 ml of the 10^{-2} dilution was transferred to another tube containing 9.0 ml of diluent to form the 10^{-3} dilution. With a new pipette tip, 1.0 ml of the 10^{-3} dilution was transferred to another tube containing 9.0 ml of diluent to form the 10^{-4} dilution. With a new pipette tip, 1.0 ml of the 10^{-4} dilution was transferred to another tube containing 9.0 ml of diluents to form a 10^{-5} dilution up until 10^{-6}.The pots were replicated four times at each dilution level. Plants growing in the pots were provided with 90 ml of both N-free solution and distilled water every day but in alternate way. Pots were scored for the presence or absence of nodules after 28 days from inoculation. The presence of a single nodule in a pot meant a positive score then thereafter number of rhizobia cells were estimated using most probable number tables as described by Woomer, (1994).

3.4.3.5 Gram staining

The following reagents were prepared; Crystal violet solution (Crystal violet-10 g, Ethyl alcohol-100 ml, Ammonium oxalate-4 g, distilled water-400 ml); Iodine solution (Iodine-1 g, Potassium iodide-2 g, Ethyl alcohol-25 ml, Distilled water-100 ml); Iodinated alcohol (Iodine solution (b)- 5ml, Ethyl alcohol-95 ml) Counter stain (2.5% safranin in ethyl alcohol-10 ml, distilled water-100 ml). Gram stained smear were prepared with a loop full of a selected bacterium and spread over on a slide in a drop of water and allowed to dry in air (Graham and Parker 1964). The slide was dried in the vicinity of the flame and allowed to cool and then stained with crystal violet solution for 1 min followed by rinsing with water and removal of excess water, the slide was then flooded with iodine solution followed by decolourized with iodinated alcohol for one minute, for 5 min the

slide was washed in water, drained and counterstained with safranin. Finally the slide was washed in water, drained and air dried and observed under oil immersion (Vincent 1970).

All the collected data except data on characterization were subjected to one – way analysis of variance (ANOVA) using the GenStat 16th Edition Computer Package. Means were separated by multiple comparison tests using Fisher's Least Significance Difference (LSD) at $P \leq 0.05$.

3.5 Experiment 2: Assessment of nodulation capacity and effectiveness by cowpea genotypes in response to different rhizobia strains

The experiment was designed to address hypothesis 3 of the research, that is, nodulation capacity and effectiveness of cowpea genotypes is associated with distinct rhizobia strains. The effective strains selected from the first experiment were used to inoculate different cowpea genotypes in order to test the cowpea genotype response to the strains in terms of nodulation capacity.

3.5.1 Treatments and experimental design

Two pre-released cowpea genotypes (IT00K-126-3 and IT97K-390-2) and three released cowpea varieties (Sudan-1, IT82E-16 and Mkanakaufiti) were used as legume hosts. The pre-released genotypes were in the third year of evaluation by the Department of Agricultural Research Services (DARS), with predicted release based on their superiority in terms of yield and disease resistance. These genotypes were evaluated for their performance in BNF using five presumptive strains: CZS1, CZS2, CZS3, CZS4, and

CZS 5, and MG5013 (Malawi released cowpea inoculant). These strains were selected from virgin land and cowpea fields basing on plant effective nodulation, nitrogen and phosphorus content, biomass yield and rhizobia characterization from experiment1.

The experiment was a two-way factorial experiment. The first factor was cowpea genotype with 5 treatment levels and the second was cowpea strains with 6 treatment levels, making a 5 by 6 treatment structure and a total of 30 treatments. The treatments were replicated three times in a complete randomized design, making a total of 90 observations.

3.5.2 Sowing and seedling management

The activities under this sub-section were similar to experiment 1 (refer to sub-section 3.4.1.2.)

3.5.3 Data collection and analysis

Collected data included nodule color, nodule number, nodule position, plant height, above and below ground fresh biomass, above and below dry biomass and nitrogen and phosphorus content. Description on how the data was collected is as in experiment 1 except for above and below ground biomass.

3.5.3.1 Above and below ground biomass

When the plants were due for harvesting (45 days after planting), and plant height was determined from the base of the stem to the tip using a ruler. As for biomass, shoots were separated from the root system at the shoot base at the time of harvesting. The roots were washed by dipping in the distilled water to remove the sand. Fresh shoot and root biomass were weighed separately using a digital scale. After weighing, the fresh biomass for shoot and root were dried in the oven at 80^{o}C for two days and then reweighed to find the dry weights.

The collected data were subjected to a two – way analysis of variance (ANOVA) using GenStat, 16th Edition. The following statistical model as described by Gomez and Gomez (1984) was used in the analysis.

$$Y_{ij} = \mu + r_i + t_i + (rt)_{ij} + \varepsilon_{ij} \quad (3)$$

Where: Y_{ij}= Observed yield value treated with i^{th} cowpea genotype and j^{th}cowpea rhizobia strain.

μ = Overall mean

r_i = Main effect of i^{th} cowpea genotype

t_j = Main effect of j^{th} cowpea rhizobia strain

$(rt)_{ij}$ = Simple effect (interaction) of i^{th} cowpea genotype and j^{th}cowpea rhizobia strain

ε_{ij} = Residual error

Nodule color and nodule position scores data were transformed to square roots before subjected to analysis of variance. Means were separated by multiple comparison tests using Fisher's Least Significance Difference (LSD) at $P \leq 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Experiment 1: Assessment of abundance and diversity of rhizobia in inoculums from different cropping fields

4.1.1 Soil chemical properties

Soil pH readings showed that all the fields were within the range of moderately acidic (Table 4.1). However, the soil pH for virgin and cowpea fields (6.0 and 6.1) was within a desirable range for nodulation and nitrogen fixation. Singleton *et al.,* 1990 reported that the optimal pH range for nodulation and nitrogen fixation is 6.0- 6.8 since acidic soils are known to affect infection process due both disruption of signal exchange between macro and micro symbionts and depression of nodulation genes and excretion of nod factor in the rhizobia. The temperatures for the soils from cowpea, maize, groundnuts fields and virgin land ranged from 27^{o}C- 31^{o}C (Table 4). Singleton *et al.,* 1990, reported that the optimal temperature range for nodulation and nitrogen fixation is 20^{o}C–30^{o}C. Higher temperatures, depresses root hair formation reducing the sites for nodulation and affect adherence of bacteria to hairs and it also affect nodule development and functioning (Singleton *et al*., 1990).The results also showed that there was a slightly higher temperature (31^{o}C) in maize field. This means that nodulation capacity, rhizobia population and nitrogen fixation would be affected if soils from these fields could be used as a growth media or inoculum for rhizobia trapping. The results also showed that virgin land had slightly higher organic matter (OM), organic carbon (OC) and nitrogen content indicating that land use system induced effect on soil organic matter, soil organic matter

and total nitrogen. This suggests that these soil chemical properties were intimately connected. The results could be attributed to higher residue or biomass accumulation in the virgin land as compared to the cultivated land. Chibsa and Asefa Ta'(2009) reported that large amounts of mineralizable N accumulate in the virgin land (grassland or forest) as compared to cultivated land due to higher residue accumulation. Soils collected from cowpea fields and virgin land had very high phosphorus content (Table 4.1).This could be attributed to the soil pH. Previous studies have shown that phosphorus availability in most soils is greatest when the soil pH is in the range of 6-7 (Weisany *et al*., 2013).Adequate soil P content helps in root development, photosynthesis process, translocation of sugars and other functions that influence nitrogen (N) fixation in legume plants (Weisany *et al*., 2013).

Table 4.1 Chemical soil properties for soils from groundnut, cowpea, maize fields and virgin land collected in Chiwosya EPA

Soils from different fields	pH	Temperature	%OC	%OM	%N	P (ug/g)
Groundnut	5.7 SD± 0.79	30 SD± 1.48	0.40 SD± 0.22	0.69 SD± 0.37	0.04 SD± 0.01	19.5 SD± 3.12
Cowpea	6.1 SD± 0.17	27 SD± 1.79	0.42 SD± 0.09	0.72 SD± 0.17	0.04 SD± 0.01	78.4 SD± 12.99
Maize	5.6 SD± 0.56	31 SD± 2.59	0.65 SD± 0.13	0.89 SD± 0.34	0.1 SD± 0.03	15.6 SD± 2.33
Virgin land	6.0 SD± 0.72	27 SD± 2.88	1.40 SD± 0.11	2.41 SD± 0.19	0.12 SD± 0.01	52.1 SD± 20.32
Optimal range	**6.0-6.8**	**20-30**	**0.88-2.35**	**1.5 - 4.0**	**0.12- 0.20**	**19-33**

4.1.2 Rhizobium population dynamics in soils from diverse cropping fields

Highly significant differences (P=0.001) in *Rhizobium* numbers were observed among the fields (Fig.4.1). Soil inoculums derived from the following cropping fields and inoculants containing the most rhizobia from greatest to least were: cowpea field> virgin land > Malawi inoculant > maize field and Australian inoculant being statistically indistinguishable from each other, producing almost no nodules. All soils collected from the measured fields contained rhizobia cells with varying numbers. A total of 343cfu/g of soil rhizobia were reported in inoculums from cowpea fields followed by virgin land (217

cfu/g of soil), out-performing the positive controls of Malawi cowpea inoculant and Australian cowpea inoculant, with rhizobial numbers of 112cfu/g of soil and 1cfu/g of soil, respectively. Malawi cowpea inoculant reported lower rhizobia population than inoculum with a previous history of cowpea possibly due to the inoculant strain (MG5013) being developed some 10 years earlier and sub-culturing of the strain is not frequently done such that there is a possibility that the strain has developed mutation. On the other hand, the storage conditions for the strain are not all good due to the power problem experienced in Malawi, hence affecting the performance of the strain. Plants inoculated with the Australian cowpea inoculant (Nodulaid) showed almost no nodulation, possibly indicating adverse environmental conditions during transportation or storage, such as high heat. The variation among the cropping fields in population sizes of rhizorbial (*i.e.* 0-343cfu/g of soil) observed in this study could be attributed to differences in soil pH. The initial soil characteristics indicated that soils from cowpea field and virgin land were in the optimal pH range for the survival of rhizobia hence higher population as compared to soils from other fields. The low soil pH in some fields adversely affect both survival of rhizobia and nodulation process in legumes since nodules are known to be stronger sinks of phosphorus than roots, shoots and leaves (Graham, 1992).Fenning and Danso (2002) reported similar findings that soil pH significantly influence the numbers of indigenous rhizobia. Similar findings have been reported by Abaidoo *et al.* (2006) who reported variation in population sizes of rhizobia cells ranging from 0-10^4 cells g^{-1}in 63 soils collected from Africa, including Malawi due to variation in pH, soil temperature among others. On the other hand, the higher rhizobia number observed in virgin land could be attributed to shading from the tree canopy that reduces heating that could kill the

rhizobia. Increases in rhizobia numbers in forest soils have been reported (Mpepereki and Makonese, 1995), suggesting that virgin land that is mostly forested, like those sampled here, may have distinctly different, and higher, rhizobia populations, than cultivated fields. De Fatima Loureiro *et al*. (2007)also reported high rhizobia diversity in no-till systems as compared to conventional tillage systems. The higher rhizobia number on cowpea cropping system could be attributed to accumulation of rhizobia from the previous crop. Chemining'wa and Vessey (2006) reported higher rhizobia population and nodulation in fields previously grown with peas than fields without pea cultivation history.

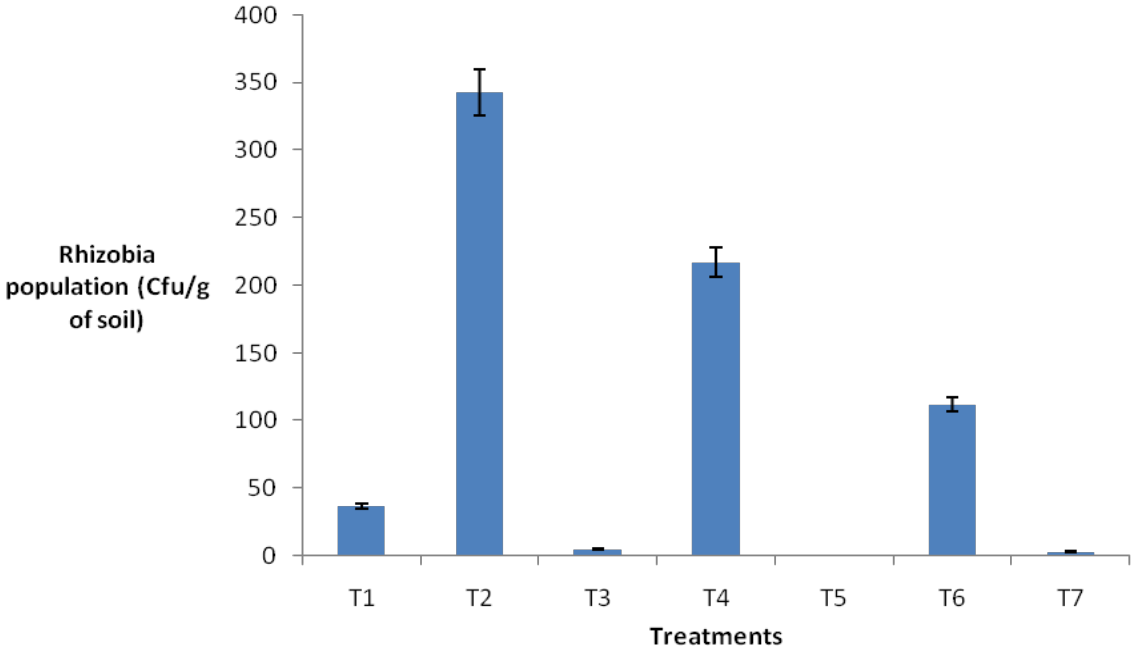

Figure 4.1 Most Probable Number counts of *Rhizobium* species from soil inoculums from various cropping fields

TI= Inoculum from groundnut fields, T2= Inoculum from cowpea fields, T3=Inoculum from maize fields T4= Inoculum from virgin land, T5= No inoculation, T6= Inoculum from Malawi cowpea inoculant, T7= Inoculum from Australian cowpea inoculant

4.1.3 Nodulation assessment

Significant differences (P=0.001) were reported in number of nodules per plant and total nodule score among the treatments (Table 4.2). Plants inoculated with Malawi cowpea inoculant gave highest number of nodules (83) followed by plants inoculated with inoculum from cowpea field (47) and then plants inoculated with inoculum from virgin land (38). With respect to the nodule color, position and plant vigor that gave the total

score, plants inoculated with inoculum from cowpea fields and virgin land gave a total score of 13 followed by plants inoculated with Malawi cowpea inoculant, with a total score of 10 (Table 4.2). Despite that plants inoculated with Malawi cowpea inoculants gave highest number of nodules as compared to plants inoculated with inoculums from cowpea field and virgin land, there was an indication of effective nodulation in the plants that were inoculated with inoculum from cowpea field and virgin land and there was less effective nodulation in plants inoculated with Malawi cowpea inoculant by looking at the total scores (Zaychuk, 2006). The effective nodulation in plants inoculated with inoculum from cowpea fields, could be attributed to accumulation of rhizobia from the previous cowpea crop as indicated in rhizobia population (Fig. 1). Chemining'wa and Vessey (2006) also reported higher rhizobia population and nodulation with fields previously grown with peas than fields without pea cultivation history. The higher nodulation for virgin land could be attributed to no tillage since de Fatima Loureiro *et al*.(2007) reported higher rhizobia diversity in no-till systems compared to conventional tillage systems. The less effective nodulation in plants inoculated with Malawi cowpea inoculant could be attributed to lower rhizobia population (Fig. 1) since the strain has over stayed hence high chances of mutation.

Table 4.2 Nodule assessment of cowpea plants inoculated with different sources of inoculums

Treatment	Number of nodules	Total nodule scores
T1	32e	6d
T2	47b	13a
T3	33d	5e
T4	38c	13a
T5 (Negative check)	0g	2f
T6 (Positive check)	83a	10b
T7 (Positive check)	2f	5e
P-value	**0.001**	**0.001**
LSD	**0.38**	**0.62**
CV (%)	**3.1**	**8.7**

TI= Inoculum from groundnut fields, T2= Inoculum from cowpea fields, T3=Inoculum from maize fields T4= Inoculum from virgin land, T5= No inoculation, T6= Inoculum from Malawi cowpea inoculant, T7= Inoculum from Australian cowpea inoculants, LSD= Least Significant Difference, CV = Coefficient of Variation. Means followed by the same letter are not significantly different

4.1.4 Nutrient content (%) and height (cm) of plants inoculated with inoculum from varying sources

Significant differences (P=0.001) were reported on percent nitrogen content, percent phosphorus and plant height (P=0.02) among the treatments (Table 4.3). Higher mean

nitrogen content (2.8%, 2.6% and 2.03% respectively) was reported in plants inoculated with inoculum from virgin land, cowpea fields and Malawi cowpea inoculant. The results on plant height showed that plants inoculated with inoculum from cowpea field, virgin land, maize field and Malawi cowpea inoculant were taller (>20 cm). Shortest plants (14.3 cm) were reported in plants inoculated with the Australian inoculant which was comparable to the negative check. The findings on the nitrogen content showed that the plants inoculated with inoculum from virgin land, cowpea fields and Malawi cowpea inoculant fixed adequate nitrogen that was utilized for the growth of plants since the amount of nitrogen nitrogen fixed were within the optimal range (2.0-2.5 %) for plant growth as described by Motsara and Roy 2008. The nitrogen that was fixed by the plants are directly used by the plants for growth such that the more nitrogen fixed by plant, the more the plant nitrogen content and the less the nitrogen fixed by the plant, the less the plant nitrogen content (Lindemann and Glover, 2003). Vollmann *et al.* (2011) reported similar findings that nodulating soybean lines had a significantly large leaf size and higher chlorophyll content, increased number of pods and increased seed weight as compared to non-nodulating lines. The results on the phosphorus content, could be attributed to the soil phosphorus content in the initial soil chemical properties (Table 4.1)whereby the soils from cowpea fields and virgin land reported highest values of phosphorus content hence resulting in the higher (0.45 and 0.43%) plant phosphorus content (Table 4.3) which was comparable to Malawi cowpea inoculant (check). In addition, the plants inoculated with inoculum from cowpea field and virgin land gave higher plant nitrogen content meaning that the plants had enough phosphorus for effective nodulation. This is in agreement with Weisany *et al.* (2013) who reported that

nitrogen fixing plants have an increased requirement for phosphorus for nodule development and nitrogen fixation such that when there is inadequate supply of phosphorus, plants suffer nitrogen deficiency. The present findings give room for selection of better rhizobia types from the diverse fields for cowpea inoculant production to replace the current Malawi cowpea inoculant.

Table 4.3 Mean nutrient content and plant height (cm) of cowpea inoculated with soil inoculums collected from varying fields

Cropping system	**Nitrogen (%)**	**Phosphorus (%)**	**Plant height (cm)**
T1	1.46b	0.31b	20.04b
T2	2.60a	0.43a	22.62b
T3	1.60b	0.33b	27.78a
T4	2.80a	0.45a	24.30a
T5 (Negative check)	0.59c	0.14c	10.04c
T6 (Positive check)	2.03a	0.39a	23.86a
T7 (Positive check)	0.08c	0.29b	14.3c
P-value	**0.001**	**0.001**	**0.001**
LSD	**0.83**	**0.10**	**4.70**
CV (%)	**35.4**	**24.3**	**18.2**

TI= Inoculum from groundnut fields, T2= Inoculum from maize fields, T3=Inoculum from cowpea fields T4= Inoculum from virgin land, T5= No inoculation, T6= Inoculum from Malawi cowpea inoculant, T7= Inoculum from Australian cowpea inoculants, LSD= Least Significant Difference, CV = Coefficient of Variation. Means followed by the same letter are not significantly different

4.1.5 Rhizobia characterization

There significant differences in colony growth, reaction to BTB and reaction on CR (P = 0.03 P = 0.001 and P = 0.001 respectively) (Table 4.4). The results indicated that the colonies from the strains extracted from nodules obtained from plants inoculated with inoculums from cowpea field and virgin land started growing within 1-5 days and the rest of treatments they starting growing within 7-10 days. In terms of reaction on bromothymol blue (BTB) yeast mannitol agar (YMA) and Congo red YMA, the colonies from the strains extracted from nodules obtained from plants inoculated with inoculums from cowpea field and virgin land were producing a yellow reaction with bromothymolblue YMA, linked to acid formation and partial absorbance in Congo red as compared to other treatments. The results means that the colonies from the strains extracted from nodules obtained from plants inoculated with inoculums from cowpea field and virgin land were fast growing rhizobia since studies have shown that distinct colonies of fast-growing rhizobia begin to appear within 3-5 days, while those of slow-growers require 7-10 days to appear. In additional, typical rhizobia colonies show little or no CR absorption and produces yellow color in BTB, indicating acid reaction. Colonies of slow-growing rhizobia are characterized by a blue coloration in BTB, which indicates alkaline reaction on BTB (Bala, 2011). Previous studies have shown that the bacteria that nodulate cowpea have been routinely considered as belonging to the miscellaneous group "cowpea" or *Bradyrhizorbium* species comprising of a large number of slow growing strains capable of nodulating several species of herbaceous legumes Saleena *et al*. (2001). However, present findings contrast the current knowledge that cowpea nodulating

rhizobia belong to the genus *Bradyrhizobium* species because they were observed to be fast growing belonging to *Rhizobium spp*. Nonetheless, these findings are in agreement with Chagas Jr. *et al*. (2013) who reported that 55 % of cowpea isolates obtained from different regions in the Cerrado in Brazil showed rapid growth in culture medium, indicating that the strain capable of cowpea nodulation goes beyond the genus *Bradyrhizobium* species. Similarly, Zhang *et al*. (2007) reported that fast growing rhizobia are also capable of inducing nodules on cowpea.

It was also noted that after conducting gram staining, the bacteria cells were rods in chains and dense clumps that stained gram negative as indicated by the faint pink red color of the rod membrane walls. Inside the rods, there were small rounded bodies of granules (three to five rod cells that stained dark purple. These rods were wider and more irregular in shape. Many of the rods were curved. These characteristics confirmed that colonies were of *Rhizobium spp*. Basing on the findings on rhizobia characterization, five best strains (CZS1, CZS2, CZS3, CZS4 and CZS5) were selected that were used in experiment 2.

Table 4.4 Rhizobia characterization of strains isolated from cowpea nodules inoculated with different sources of inoculum

Treatment	Colony growth		Reaction on BTB		Reaction on CR	
	1-5 days	**7-10 days**	**1-5 days**	**7-10 days**	**1-5 days**	**7-10 days**
T1	1	2	3	3	3	2
T2	2	2	1	1	2	2
T3	1	2	3	3	3	2
T4	2	2	1	1	2	2
T5 (Negative check	1	1	3	3	1	1
T6 (Positive check)	1	2	3	3	3	2
T7 (Positive check)	1	2	3	2	3	2
P-value	**0.03**	**0.03**	**0.001**	**0.001**	**0.001**	**0.001**
$LSD_{(0.05)}$	**0.22**	**0.29**	**0.62**	**0.61**	**0.37**	**0.31**
CV(%)	**3.1**	**2.8**	**22.6**	**26.1**	**12.7**	**16.8**

Growth rate: 1=no growth, 2=growth, Colony characteristics on Congo Red YMA: 1=non-absorbent, 2=partly absorbent, 3=centre absorbent, 4=fully arbsorbent. Reaction on bromothymolblue YMA: 1=acid forming, 2=non-reactive (green), 3= basic (blue)TI= Inoculum from groundnut fields, T2= Inoculum from cowpea fields, T3=Inoculum from maize fields T4= Inoculum from virgin land, T5= No inoculation, T6= Inoculum from Malawi cowpea inoculant, T7= Inoculum from Australian cowpea inoculants, LSD= Least Significant Difference, CV = Coefficient of Variation. Means followed by the same letter are not significantly different, BTB= Bromothymolblue, CR= Congo red

4.2 Experiment 2: Assessment of nodulation capacity and effectiveness by cowpea genotypes in response to different rhizobia strains

4.2.1 Plant height (cm)

There was no significant interaction (P=0.120) between rhizobia strain and genotype on plant height (Table 4.5). Plant height was significantly (P=0.001) affected by rhizobia strain as compared to cowpea genotype (P=0.167). Two strains, CZS5 and CZS4, significantly out-performed the check strain (MG 5013) with the mean height of 20.9cm and 19.9 cm, respectively, whilst CZS1 (16.0 cm) was comparable to the check strain (16.5cm). The present findings agree with Oyatokun *et al.* (2013) who reported that cowpea varieties that were inoculated with rhizobia strains produced taller plants than cowpea varieties that were not inoculated. It is an unexpected result that CZS5 and CZS4 were shown to out-perform cowpea inoculants in terms of nitrogen fixation, suggesting that improved strain selection might be possible for cowpea inoculant production and replace the current cowpea inoculant.

Table 4.5 Mean plant height (cm) for cowpea genotypes inoculated with presumptive rhizobia strains

	Genotype					
Strain	**IT00K-126-3**	**IT82E-16**	**IT97K-390-2**	**MKANAKAUFITI**	**SUDAN**	**Mean**
CZS1	12.9	18.0	18.3	15.8	14.9	**16.0b**
CZS2	16.6	13.9	14.2	18.0	14.4	**15.4b**
CZS3	14.5	16.6	16.2	16.0	14.2	**15.2b**
CZS4	16.9	16.1	24.5	24.6	17.5	**19.9a**
CZS5	20.9	19.0	21.1	21.1	22.6	**20.9a**
MG5013 (check)	17.9	14.9	16.1	16.1	18.2	**16.5b**
Mean 16.6		**16.4**	**18.4**	**16.1**	**16.9**	
P-value						
Strain	**0.001**					
Genotype	**0.167**					
str x genotype	**0.120**					
LSD$_{(0.05)}$						
Strain	**2.36**					
Genotype	**2.16**					
str x gen	**5.29**					
CV (%)	**18.6**					

CZS= Chitedze strain, MG5013 = Malawi Government strain 5013, LSD= Least Significant Difference,CV = Coefficient of Variation. Means followed by the same letter are not significantly different

4.2.2 Above ground biomass yield (g)

The variety-strain interaction showed a significant response (P=0.015) on the above ground biomass yield. However, the main effects of genotypes and rhizobia had no

significant (P=0.211 and P=0.769, respectively) effect on above ground biomass yield (Table 4.6). Mkanakaufiti inoculated with CZS4 gave the highest (2.53 g) above ground dry biomass yield followed by Sudan inoculated with CZS4 with an above ground dry biomass yield of 1.69 g. The results from the present study support the idea that specific strains could be recommended for different common cowpea varieties in order to increase the nitrogen fixation and yields. This is in agreement with Solomon *et al.* (2012), who reported that an increase in the above ground biomass yield after rhizobial inoculation is known to increase yields of several legumes by way of increasing the nodulation and the biomass of shoot and root. Similarly, Hadad *et al*. (2012) also reported significant increase in tissue dry weight, root dry weight and nodulation following inoculation of cowpea with rhizobia strains. From this study, there is a clear indication that CZS4 is a better cowpea strain that can be used to inoculate Mkanakaufiti and Sudan-1 so as to increase above ground biomass that is used in photosynthesis hence resulting into higher grain yield.

Table 4.6 Above ground biomass (g) for cowpea genotypes inoculated with presumptive rhizobia strains

	Genotype					
Strain	**IT00K-126-3**	**IT82E-16**	**IT97K-390-2**	**MKANAKAUFITI**	**SUDAN**	**Mean**
CZS1	0.13	0.28	1.21	0.29	0.16	**0.41**
CZS2	1.30	0.96	0.11	0.08	1.44	**0.78**
CZS3	0.12	0.50	0.29	1.40	0.76	**0.62**
CZS4	0.09	0.03	0.63	2.53	1.69	**0.90**
CZS5	1.07	1.03	0.53	0.29	0.01	**0.58**
MG5013 (check)	0.11	0.04	0.14	0.01	0.48	**0.68**
Mean	**0.47**	**0.47**	**0.56**	**0.77**	**0.68**	
P-value						
Strain	**0.211**					
Genotype	**0.79**					
str x gen	**0.015**					
LSD$_{(0.05)}$						
Strain	**0.59**					
Genotype	**0.54**					
str x gen	**1.33**					
CV (%)	**34**					

CZS= Chitedze strain, MG5013 = Malawi Government strain 5013, LSD= Least Significance Difference, CV = Coefficient of Variation. Means followed by the same letter are not significantly different

4.2.3 Below ground biomass yield (g)

Below ground dry biomass yield was significantly affected by rhizobia strain (P=0.045) and not by cowpea genotype (P=0.537). However, strain-genotype interaction did not

have a significant effect (P=0.293) on below ground biomass (Table 4.7). Similar findings on lentil varieties and bacterial strains were reported by Ghanem (2009). All the new strains reported higher below ground dry biomass yield as compared to the check strain(MG5013), which gave the lowest (0.06g) value. The highest mean biomass yield (0.18 g) was reported by CZS2 followed by CZS4 with a mean biomass yield of 0.15g.The lower biomass yield observed in MG5013 (check strain) could mean that the inoculant is losing its effectiveness. The reason could be that the strain has over-stayed without being sub-cultured such that there is a possibility of it undergoing mutation hence affecting biological nitrogen fixation thereby requiring replacement with new strains. The results indicated that the plants that were inoculated with CZS2 and CZS4 produced vigorous roots with more and big nodules that resulted into higher below biomass yield as compared to plants inoculated with MG5013 (check) and other strains. Ghanem (2009) reported similar findings in his study which showed that below ground biomass was not significantly affected by the main effect of variety of lentil. Despite the fact that his findings differ with the present results on the bacterial strain, he also reported non-significant effect of bacterial strain on the below ground biomass. From these findings, two putative strains, CZS2 and CZS4 seem to be promising to replace MG5013.

Table 4.7 Below ground biomass yield (g) for cowpea genotypes inoculated with presumptive rhizobia strains

	Genotype					
Strain	**IT00K-126-3**	**IT82E-16**	**IT97K-390-2**	**MKANAKAUFITI**	**SUDAN**	**Mean**
CZS1	0.19	0.23	0.003	0.01	0.18	**0.10b**
CZS2	0.20	0.28	0.08	0.16	0.06	**0.16a**
CZS3	0.07	0.14	0.06	0.02	0.21	**0.10b**
CZS4	0.06	0.09	0.28	0.28	0.05	**0.15a**
CZS5	0.23	0.11	0.16	0.15	0.15	**0.14a**
MG5013 (check)	0.01	0.05	0.1	0.09	0.08	**0.06c**
Mean	**0.11**	**0.11**	**0.1**	**0.11**	**0.12**	
P-value						
Strain	**0.045**					
Genotype	**0.537**					
str x gen	**0.293**					
LSD$_{(0.05)}$						
Strain	**0.09**					
Genotype	**0.08**					
str x var	**0.19**					
CV (%)	**32.1**					

CZS= Chitedze strain, MG5013 = Malawi Government strain 5013, $LSD_{(0.05)}$= LSD= Least Significance Difference, CV = Coefficient of Variation. Means followed by the same letter are not significantly different

4.2.4 Nodule number

A highly significant interaction (P=0.001) was observed between strains and genotypes (Table 4.8) in the mean number of nodules of cowpea plants inoculated with different

rhizobia strains. The cowpea variety IT00K-126-3 inoculated with CZS2 gave the highest number (50) of nodules followed by IT82E-16 inoculated with CZS4 (42). It was also observed that some genotypes inoculated with different strains like IT00K-1-126-3 and Sudan-1 inoculated with CZS1 strain, IT82E-16 and IT97K-390-2 inoculated with CZS2 strain, IT00K-126-3, Mkanakaufiti and Sudan -1 inoculated with CZS3 strain, IT82E-16 and IT97K-390-2 inoculated with CZS4 strain and IT00K-126-3 and Sudan-1 inoculated with MG5013 (check strain) reported a mean of zero nodules, meaning that the combination between some genotypes and strains were not compatible to form nodules. No significant differences (P=0.341) were observed amongst the genotypes on number of nodules. These findings differ with Ghanem (2009) who reported that the number of nodules per plant was significantly affected by varieties of lentils and peas and not rhizobia strains. The positive interaction between rhizobia strains and cowpea varieties on nodule formation shows that there is strain-variety specificity hence the need to identify the effective bacterial strain for each variety of cowpea in order to exploit full potential of nitrogen fixation. Similar results were reported by Hafeez *et al.* (2009) and Solomon *et al.* (2012) on legume varieties and rhizobial strains. However, Ghanem (2009) reported no significant interaction between pea and lentil varieties and rhizobia strains on the number of nodules. The results from this study have clearly shown that the best strain-genotype combinations are IT00K-126-3 inoculated with CZS2 and IT82E-16 inoculated with CZS4.

Table 4.8 Nodule number for cowpea genotypes inoculated with presumptive rhizobia strains

	Genotype					
Strain	**IT00K-126-3**	**IT82E-16**	**IT97K-390-2**	**MKANAKAUFITI**	**SUDAN-1**	**Mean**
CZS1	0	34	19	1	0	**11**
CZS2	50	0	0	39	6	**19**
CZS3	0	25	16	0	0	**8**
CZS4	15	42	26	0	32	**23**
CZS5	2	0	0	14	9	**5**
MG5013 (check)	0	1	1	3	0	**1**
Mean	**11**	**17**	**10**	**10**	**8**	
P-value						
Strain	**0.074**					
Genotype	**0.341**					
str x gen	**0.001**					
LSD$_{(0.05)}$						
Strain	**9.17**					
Genotype	**10.05**					
str x gen	**22.47**					
CV (%)	**31.7**					

CZS = Chitedze strain, MG5013 = Malawi Government strain 5013, LSD=Least Significance Difference, CV = Coefficient of Variation. Means followed by the same letter are not significantly different

4.2.5 Nodule position

Strain-genotype interaction had a significant effect (P=0.001)on the nodule position of the plants. However, the main effect of strain and genotype had no significant effect

(P=0.640 and P= 0.577, respectively) (Table 4.9). IT00K-126-3 inoculated with CZS2 and IT82E-16 inoculated with CZS5 reported a mean position of 3 indicating that the nodules were located on both crown and lateral. On the other hand, IT82E-16 inoculated with CZS1,Mkanakaufiti and Sudan-1 inoculated with CZS2, Mkanakaufiti and Sudan-1inoculated with CZS4 and IT00K-126-3 and IT97K-390-2 inoculated with CZS5 gave a mean score of 2 showing that the roots had nodules positioned on the crown only. MG5013 (check) inoculated to IT82E-16, Mkanakaufiti, IT97K-390-2 and Sudan-1 resulted into a mean score of 1 meaning the nodules were positioned on the lateral part of the roots. From the results, it means that the plants which had a mean score of 2 and 3 were able to fix nitrogen as compared to those nodules on the lateral position as reported by Zaychuk (2006). Similar findings were reported by Bhagwat *et al.* (1981), who reported that the position of the uppermost nodule in a legume is a sensitive indicator of factors that affect rate at which infections are initiated after rhizobial inoculation. The findings show that the following strains CZS1, CZS2, CZS4 and CZS5 were better off in terms of nitrogen fixation if inoculated to different cowpea genotypes as compared to MG5013 due to their crown nodulation, hence recommended to be used for cowpea inoculants production in Malawi replacing MG5013.

Table 4.9 Nodule position for cowpea genotypes inoculated with presumptive rhizobia strains

	Genotype					
Strain	**IT00K-126-3**	**IT82E-16**	**IT97K-390-2**	**MKANAKAUFITI**	**SUDAN-1**	**Mean**
CZS1	0	2	1	1	0	**1**
CZS2	3	0	0	2	2	**1**
CZS3	0	1	1	0	0	**1**
CZS4	1	0	0	2	2	**1**
CZS5	2	3	2	0	0	**1**
MG5013 (check)	0	1	1	1	1	**1**
Mean	**1**	**1**	**1**	**1**	**1**	
P-value						
Strain	**0.640**					
Genotypes	**0.577**					
str x gen	**0.001**					
LSD$_{(0.05)}$						
Strain	**0.49**					
Genotypes	**0.45**					
str x gen	**1.11**					
CV (%)	**25.9**					

CZS = Chitedze strain, MG5013 = Malawi Government strain 5013, LSD=Least Significance Difference, nodule position: 0= no nodules 1= lateral nodulation, 2=crown nodulation, 3= crown and lateral nodulation, CV= Coefficient of Variation

4.2.6 Nodule color

There was a significant interaction (P=0.009) between strains and genotypes (Table 4.10) in the mean nodule color of cowpea inoculated with different rhizobia strains. The results showed that both genotypes and strains had an influence on nodule color. The cowpea genotype IT00K-126-3 inoculated with CZS2 and Mkanakaufiti and Sudan-1 inoculated with CZS4 out-performed the other combinations of strains and genotypes (mean score of 5) with nodules having a strong pink color. On the other hand, Mkanakaufiti inoculated with CZS1, Sudan-1 inoculated with CZS2, IT82E-16 and IT97K-390-2inoculated with CZS5 reported a mean score of 3 and had light pink nodule interiors. However, significant differences (P=0.001) were also reported amongst strain mean nodule color score, with CZS4 having nodules with a mean score of 3. The results show that the nodules with strong pink color had good nitrogen fixing ability due to the presence of leghemoglobin, which is responsible for active nitrogen fixation while the light pink color indicates average nitrogen fixation as was also reported by Zaychuk (2006) and Bala *et al.* (2011). The results also suggest that the best strain-genotype combinations for optimal nitrogen fixing ability were IT00K-126-3 inoculated with CZS2 and Mkanakaufiti and Sudan-1 inoculated with CZS4 since these strain CZS2 was able to infect IT00K-126-3 and CZS4 was able to infect Mkanakaufiti and Sudan-1 and produced effective nodules that were capable of fixing nitrogen. This further supports the idea that specific inoculants can be produced using CZS2 and CZS4 strains.

Table 4.10 Mean nodule color for cowpea genotypes inoculated with presumptive rhizobia strains

	Genotype					
Strain	**IT00K-126-3**	**IT82E-16**	**IT97K-390-2**	**MKANAKAUFITI**	**SUDAN-1**	**Mean**
CZS1	0	2	2	3	0	**1**
CZS2	5	0	0	1	3	**2**
CZS3	0	2	2	0	0	**1**
CZS4	3	0	0	5	5	**3**
CZS5	1	3	3	0	0	**1**
MG5013 (check)	0	4	0	2	2	**2**
Mean	**2**	**2**	**2**	**2**	**2**	
P-value						
Strain	**0.001**					
Genotype	**0.317**					
str x gen	**0.009**					
$LSD_{(0.05)}$						
Strain	**0.51**					
Genotype	**0.46**					
str x gen	**1.14**					
CV (%)	**35.3**					

Nodule color: 0= no nodules 1= white 2=green 3=brown 4=light pink 5= dark pink, CZS = Chitedze strain, MG5013 = Malawi Government strain 5013, SE= Standard error, CV = Coefficient of Variation. Means followed by the same letter are not significantly different

4.2.7 Plant nitrogen content

A highly significant interaction (P=0.001) between strains and genotypes was observed on total plant nitrogen content (Table 4.11). On average, cowpea genotype IT00K-126-3 inoculated with CZS2, Mkanakaufiti and Sudan-1 inoculated with CZS4 out-performed other combinations of strains and varieties, with a mean of 4.8% nitrogen content, followed by Mkanakaufiti inoculated with CZS4, with a mean of 3.6% nitrogen content. The results strongly suggest that the best strain-variety combinations that had good nitrogen fixing ability wereIT00K-126-3 inoculated with CZS2 and Mkanakaufiti, Sudan-1 inoculated with CZS4 and Mkanakaufiti inoculated with CZS4.This means that CZS2 and CZS4 strains can be selected for production of inoculants for specific varieties so as to increase nitrogen fixation, nitrogen up-take and use by the plants hence maximizing cowpea production in Malawi. On the other hand, the combinations that had very low nitrogen content, such as CZS3 inoculated to IT00K-126-3 and Mkanakaufiti (0.4%) suggest that there was incompatibility between strains and genotypes. The results could be attributed to highest number of nodules reported in the combination of CZS2 and IT00K-126-3 and CZS4 in combination with Mkanakaufiti and Sudan-1 (Table 4.8) and effective nodules that were produced by these combinations (Table 4.10). This means that CZS2 and CZS4 were ably infected to the specific genotypes such that more effective nodules that resulted in fixing large amount of nitrogen used up by the plants hence resulting into higher plant nitrogen content.

Table 4.11 Mean Plant Nitrogen content (%)for cowpea genotypes inoculated with presumptive rhizobia strains

	Genotype					
Strain	**IT00K-126-3**	**IT82E-16**	**IT97K-390-2**	**MKANAKAUFITI**	**SUDAN-1**	**Mean**
CZS1	0.9	2.4	2.4	3.6	0.4	**1.9**
CZS2	4.8	1.3	0.6	1.5	3.2	**2.1**
CZS3	0.4	2.4	2.5	0.4	0.6	**1.3**
CZS4	3.7	0.6	0.5	4.6	4.8	**2.8**
CZS5	2.5	3.7	3.7	0.4	0.4	**2.1**
MG5013 (check)	0.5	4.1	0.5	1.8	2.4	**1.9**
Mean	**2.1**	**2.2**	**1.7**	**2.0**	**2.0**	
Significance						
Strain	**0.001**					
Genotype	**0.649**					
str x gen	**0.001**					
$LSD_{(0.05)}$						
Strain	**0.34**					
Genotype	**0.31**					
str x gen	**0.71**					
CV (%)	**23.2**					

CZS = Chitedze strain, MG5013 = Malawi Government strain 5013, LSD=Least Significance Difference, CV = Coefficient of Variation. Means followed by the same letter are not significantly different

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The main aim of the study was to assess abundance and effectiveness of cowpea rhizobia in soils from different fields and assess capacity of nodulation by different genotypes using native cowpea strains. The following conclusions have been drawn from the findings of this study:

a. Inoculums from cowpea field and virgin land were observed to have high rhizobia population for cowpea nodulation

b. Rhizobia strains isolated from inoculums from cowpea fields and virgin land were more effective in terms of nodulation capacity hence can be used for the production of inoculants to replace the strain (MG5013) that is currently used for cowpea inoculant production.

c. There was compatibility between the genotypes and strains (IT00K-126-3 with CZS2, Sudan-1 and Mkanakaufiti with CZS 4) in terms of nodulation capacity and effecctivess suggesting that strains can be considered for production of inoculants for specific cowpea genotypes/varieties.

5.2 Recommendations

The following recommendations have been drawn from the study:

a. Fields where cowpea was previously grown and a newly open land do not require rhizobia inoculation of the plants.
b. Further study should be done on molecular characterization of the superior isolates obtained from soils from different fields collected in Mchinji district in order to determine the phylogenetic relationship of the isolates with other rhizobial species and determine *nod* and *nif* genes of the strains capable of improving both nodulation and nitrogen fixation.
c. Further evaluation of the presumptive rhizobia strains under field conditions should be done before they are considered for commercial inoculant production so as to validate the findings.
d. Breeders should consider nitrogen fixation as one of the important parameters in legume breeding so as to select varieties or breed for high nitrogen fixation with selected proven superior rhizobia strains as those identified presently.

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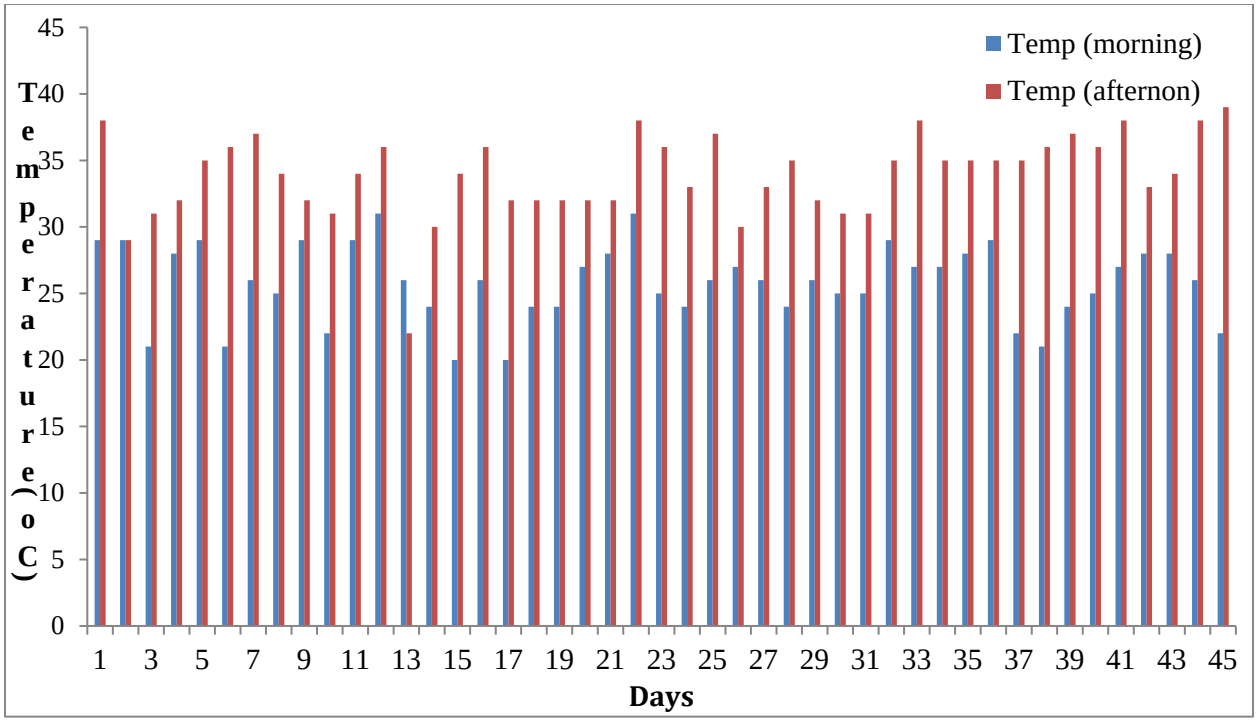
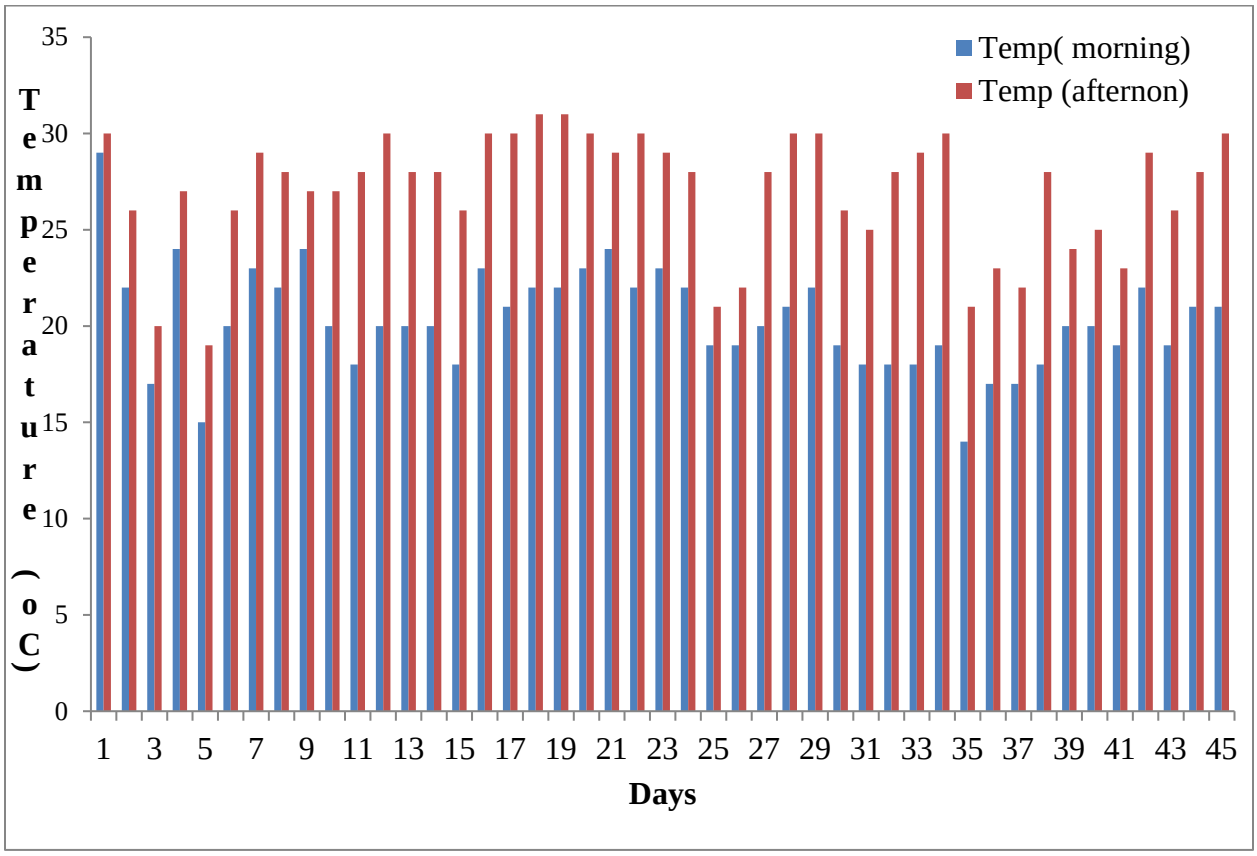
APPENDICES

Appendix 1: Daily temperature (^{0}C) recordings for two experiments conducted at Chitedze Research Station

Appendix 1a: Glasshouse temperature recordings (January-March) for experiment 1



Appendix 1 b: Glasshouse temperature (^{0}c) recordings (End May-June) for experiment 2



Appendix 2: Preparation of growth media and nutrient solutions that were used for the experiment

Appendix 2a: Preparation of Yeast-Mannitol Broth (YMB)

Dissolve following in distilled water to prepare 1 L of YMB, Mannitol 10 g, $K_2HPO_4$0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl, 0.1 g, Yeast Extract 0.5 g then adjust pH to 6.8 and autoclave at 121^0C for 20 min

To prepare Yeast-Mannitol Agar (YMA) 15 g agar was added prior to autoclaving

Appendix 2b: Preparation of plant growth nutrient solution (minus nitrogen)

Make the following stock solutions:

1. 12.3 g / L of $MgSO_4.7H_2O$

2.6.8 g / L of KH_2PO_4

3. 17.5 g / L of K_2SO_4

4. 2.5 g / L of Fe-EDTA

5. Trace element solution (store at 4^0C) H_3BO3 0.464 g/L,NA_2MoO_4 0.018 g/L, $ZnSO_4$ 0.539 g/L, $MnSO_4$ 0.042 g/L, $CoSO_4$ 0.141 g/L, $CuSO_4$ 0.125 g/L

6. 2.04 g/L of $CaSO_4$ agitated solution

Appendix 2c: Preparation of nutrient stock solution

The nutrient stock solution is prepared by mixing 250 mL of each stocks 1-4 and 2 ml of trace element solution (5).

The nutrient solution for watering is handled in 2 litre glass screw-capped (schott) bottles.

Add 200 ml of Nutrient Stock solution to 1600 ml of de-ionised water and autoclave. When cooled add 200 ml of autoclaved $CaSO_4$ solution (6) (well agitated) to make 2 litres.

Appendix 3: Analysis of variance for different variables

Appendix 3a: Analysis of variance for percent nitrogen in experiment 1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	6	33.1844	5.5307	13.5	<.001
Residual	28	11.4698	0.4096		
Total	**34**	**44.6542**			

Appendix 3b: Analysis of variance for number of nodules in experiment 1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	6	308.69544	51.44924	2160.75	<.001
Residual	28	0.6667	0.02381		
Total	**34**	**309.36214**			

Appendix 3c: Analysis of variance for nodulation assessment in experiment 1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	6	23.98281	3.99714	71.55	<.001
Residual	28	1.56424	0.05587		
Total	**34**	**25.54705**			

Appendix 3d: Analysis of variance for nodule number in experiment 2

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Variety	4		870.3	217.6	1.16	0.341
Strain	5		2020	404	2.15	0.074
Variety.Strain	20		18090.4	904.5	4.81	<.001
Residual	53	-7	9973.2	188.2		
Total	**82**	**-7**	**29880.7**			

Appendix 3e: Analysis of variance for nodule color in experiment 2

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Variety	4		1.3707	0.3427	0.65	0.635
Strain	5		12.2106	2.4421	4.61	0.005
Variety.Strain	7	-13	13.6293	1.947	3.67	0.009
Residual	22	-38	11.6667	0.5303		
Total	**38**	**-51**	**22.359**			

Appendix 3f: Analysis of variance plant height (cm) in experiment 2

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Variety	4		70.35	17.59	1.68	0.167
Strain	5		439.87	87.97	8.39	<.001
Variety.Strain	20		312.39	15.62	1.49	0.12
Residual	58	-2	607.89	10.48		
Total	**87**	**-2**	**1351.67**			