MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF SOYBEAN NODULATING RHIZOBIA INDIGENOUS TO ZAMBIA

BY

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DECLARATION

I, **Racheal Kapembwa** hereby declare that all the work presented in this dissertation is my own work and has not been submitted for a degree at this or any other University.

Signature………………………..

Date…………………………...

APPROVAL PAGE

This dissertation of Ms. Rachael Kapembwa is approved as fulfilling part of the requirements for the degree of Master of Science in Integrated Soil Fertility Management by the University of Zambia.

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ABSTRACT

Soybean [*Glycine max* (L.) Merrill] is known for symbiotic nitrogen fixation by rhizobia present in the soil with which it establishes an efficient symbiosis. In Zambia, current rhizobial inoculants used in soybeans production are based on non-indigenous strains; this creates a need to isolate local strains that can be used for the development of local inoculants for soybeans in Zambian soils. This study reports the isolation and characterization of rhizobial isolates from virgin and cultivated soils of the three agro-ecological regions of Zambia. The study was conducted in the greenhouse during the 2013/2014 season. Rhizobia were isolated using the Trap Method; a promiscuous soybean genotype (magoye) was grown for eight weeks in replicates of three arranged in Completely Randomised Design. Morphological and biochemical markers were used to characterize. A total of 61 isolates were isolated on Yeast Extract Mannitol (YEM) agar medium. The isolates were circular, irregular and punctuate in form with convex elevation; entire, undulate and lobate colony margins; with cream, white, yellow, transparent and pink coloured. All isolates produced mucous, were gram negative and rod shaped, a characteristic of rhizobial cells. None of the isolates could tolerate extremes of pH (4 and 9) in growth medium but grew well at pH 6.8. All isolates utilized glucose as a source of carbon. Based on the Bromothymol Blue (BTB) assay, 59 isolates were fast growing while two isolates from cultivated soils of region II were slow growing. The fast growing 59 isolates showed an acidic reaction changing the medium from green to yellow, while the others showed an alkaline reaction. Based on results, the 59 fast-growers could be *Ensifer fredii* or/and *Rhizobium tropici* rather than *Bradyrhizobium*. However, further tests to confirm these findings using ketolactose, genetic characterization and inclusion of reference strains, are still needed and are being recommended here.

DEDICATION

I dedicate this work to my husband Davies Sampa for his immense support,

encouragement and sacrifice and to our children Abigail, Davies Jr. and Rachael.

ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION

1.1 Background

Soybean [*Glycine max* (L.) Merrill.] was introduced in Zambia in the 1930s. The crop is now grown by both small and large scale farmers. It is adapted to regions II and III of Zambia and will grow well wherever maize grows (Miti, 1997). Soybean is a legume that forms root nodules following infection with soybean-nodulating rhizobia, which do symbiotic nitrogen fixation by taking up atmospheric nitrogen through the root nodules. The main soybean-nodulating rhizobia are *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii, and Sinorhizobium xinjiangense,* and *Mesorhizobium tianshanense* have been classified as soybean-nodulating rhizobia (Shiro *et al.*, 2013).

Soybean offers a variety of possible benefits to the production systems, diets, and incomes of its producers. In addition to being a potentially profitable cash crop, the high protein content (about 40%) in soy means it could also contribute to improved nutritional status of its consumers (Dixit *et al*., 2011). Soybean is an economically significant crop because of its flexible use for production of cooking oil, human food, stock feed and other industrial products (Miti, 1997). Soybean production also has potential agronomic benefit of rejuvenating soils. Soybean canopies protect the soil from recurrent erosion, fix atmospheric nitrogen into the soil and decaying root residues improve soil fertility. Soil improvement leads to elevated levels of sustainable agriculture with minimal input requirement.

In Zambia, soybean is mostly used as an industrial crop. It is used in oil production and in products such as soy chunks and soy meal. The by-product (cake) is fed directly to animals or processed in the company of other ingredients into animal feed stock. As an animal feed, soy by-products provide relatively low cost, high quality protein to feed rations. With a livestock revolution in progress in developing countries, including Zambia, industrial demand for soy is likely to increase (Lubungu *et al*., 2013).

Current production of soybean in Zambia is at 214,179 metric tonnes (CSO/MAL, 2013). According to Tefera, (2011), soybean production in Zambia was estimated at 12,000 metric tonnes in 2006. This information shows that the production of soybean had improved by 1,685% in a period of 4 years. Despite the clear benefits of soybean production, production remains limited. In part, this may be linked to the pervasive belief among farmers that soybean markets are unreliable. However, interviews with downstream market actors suggest that there is, in fact, significant unmet demand for soybean in Zambia (Lubungu *et al*., 2013).

Biological Nitrogen Fixation (BNF) is important in farming systems and can be ameliorated by simple and inexpensive inoculation procedures. Extended use of biological fertilizers would reduce the cost of chemical fertilizers ensuring that economic benefits accrue to the farmers while at the same time maintaining soil fertility and sustainability of agro-ecosystems. In Zambia, inoculants are produced by Government facilities essentially to service emerging soybean industries (Hardarson and Broughton, 2003).

While inoculation with *Bradyrhizobia* has been shown to improve nitrogen fixation and subsequent soybean yield (Shiro et al.., 2013), the efficiency of the inoculants maybe poor if the inoculated strains cannot out-compete the indigenous ones or cannot establish an efficient symbiosis with the host plant (Mweetwa et al., 2014). Therefore, a complete understanding of the ecology of indigenous soybean-nodulating rhizobia with respect to their genetic diversity and the environmental factors associated with their localization and dominance in the soil is important. Some soils may be devoid of rhizobia (Hassen *et al*., 2014), contain low numbers of effective strains (Radke, 2003), or have high numbers of ineffective or partially effective strains (Herridge, 2002).

As early as 1961, four indicators necessitating inoculation were outlined as being: the unavailability a related legume in the immediate cropping sequence of the field; unsatisfactory nodulation previously on the same crop; a cropping sequence of a legume followed by non-legume and; land undergoing reclamation (Allen and Allen, 1961). The surest indicator for inoculation is prevailing numbers of rhizobia in the soil. It is better to inoculate when not needed (i.e. over-inoculating) than not and producing N-deficient crops.

In Zambia, current rhizobial inoculants used in soybeans production are based on non-indigenous strains; this creates a need to isolate local strains that can be used for the development of local inoculants for soybeans grown on Zambian soils. The ability to select elite strains indigenous and adapted to prevailing conditions and soybean cultivars relies on the assessment of the diversity of rhizospheric rhizobial communities. There is currently no known information on local strains of rhizobia nodulating soybean in

different soils of Zambia. This paper reports results from a study conducted to isolate and characterize rhizobial strains infecting a common genotype of soybean in Zambia.

1.2 Objectives

1.2.1 Overall Objective

To isolate and characterize Rhizobial strains infecting a common genotype of soybean in Zambia.

1.2.2 Specific objectives

1. To isolate Rhizobial strains infecting a common genotype of soybean in Zambia
2. To characterize isolated Rhizobial strains using morphological and biochemical markers

1.3 Hypothesis

- There is no morphological and biochemical diversity in Rhizobial strains in a soybean genotype grown in different agricultural soil types from different locations in Zambia.

CHAPTER 2: LITERATURE REVIEW

2.1 Soybean as a crop

Soybean is believed to have originated from North Eastern China and was domesticated about 1100 BC. It extended to Southern China, Korea, Japan and other countries in South Eastern Asia and about 100-150 years ago into the United States of America. In Zambia, it is believed to have been introduced in the 1930s (Miti, 1997).

Soybean is an annual legume that belongs to the legume family Fabaceae. It is a strictly self-pollinating legume with 2n = 40 chromosomes (Tefera, 2011). Soybean varieties can either be determinant or indeterminant. Determinate types stop growing in height as soon as flowering begins or shortly thereafter and the terminal bud usually becomes an inflorescence. Stem diameter continues to increase thus determinate plants normally have a somewhat uniform stem thickness over their entire length. On the contrary, indeterminate plants continue growing in stem length throughout much of the pod development period and may double their height after flowering begins, while stem diameter becomes progressively smaller near the tip. Even though stems of determinate varieties frequently terminate in an inflorescence, those of indeterminate varieties do not (Miti, 1997).

Soybean establishes a symbiotic relationship with nodulating bacteria at some stage in the development of the root nodule, the nitrogen fixing organ,. The major soybean nodulating *rhizobia* include *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, and *Sinorhizobium/Ensifer fredii* (Scholla and Elkan, 1984; Kuykendall *et al*., 1992).

Furthermore, additional species of soybean nodulating *rhizobia* have been extensively discussed in the literature owing to the complexity of their taxonomical classification (Xu *et al*., 1995; Tan *et al*., 1997; Peng *et al*., 2002; Hungria *et al*., 2006; Vinuesa *et al*., 2008). Soybeans that nodulate effectively with diverse indigenous rhizobia are considered as promiscuous, and the characteristic promiscuity (Kuneman et al., 1984). Thus, promiscuous genotypes of soybean form symbiotic association with available Rhizobium strains in the soil and thus fix atmospheric nitrogen whilst non-promiscuous genotypes need specific rhizobial strains to fix nitrogen from the air (Tefera, 2011).

2.2 Importance of Soybean

Soybean is the world's leading oilseed crop. Of the eight major oilseeds traded in world markets (soybean, cottonseed, peanut, sunflower, rapeseed, flaxseed, copra and palm kernel), soybean's production has been twice that of every other oilseed since 1970 (Smith and Huyser, 1987). The estimated composition of soybean is 40% protein, 21% oil, 34% carbohydrate and 5% ash (Scott and Aldrich, 1983).

Soybean has become well-known as the plant that will help feed the world's present and future population and aid to solve world protein deficiency. In Zambia, the soybean is mostly used as an industrial crop. It is used in oil production and in products such as soy chunks and soy meal. The by-product (cake) is fed directly to animals or processed with other ingredients into animal feed stock. As an animal feed, soy by-products provide relatively low cost, high quality protein to feed rations. With a livestock revolution underway in developing countries, including Zambia, industrial demand for soy is likely to increase (Delgado *et al*., 1999). The seeds can be processed into soy milk, which is an

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outstanding source of protein for babies, especially those just weaned from breast-feeding (Maingi *et al*., 2006). Though soybean is not usually boiled and eaten unlike other legumes such as beans, cowpeas, or groundnuts, the seeds can be processed into soy flour is frequently mixed with other ingredients to form nutritious rich protein mixes. The growing demand of soy offers significant opportunity for smallholder farmers to improve their cash base. Soy production also has prospective agronomic benefit of rejuvenating soils. Soybean canopies protect the soil from recurrent erosion, fix atmospheric nitrogen into the soil and decaying root residues improve soil fertility. Soil improvement leads to higher levels of sustainable agriculture with minimal input requirement (Lubungu *et al*., 2013).

2.3 Current production of soybean in Zambia

Cultivation of soybean crop in Zambia has been popularized through various programmes by governments and non-governmental bodies. These efforts have helped to create awareness to small scale farmers of the importance of soybean as a crop for improving their financial standing.

Soybeans are cultivated in nearly all the parts of Zambia. The Eastern Province leads the country in smallholder soybean production in almost every harvest season (Table 1). Statistics show that, from 2001 to 2010, 42% of all soy produced by Zambian smallholders was grown in the Eastern Province. Other provinces with sizable production include Central and Northern Provinces (Lubungu *et al*., 2013).

Table 1: Smallholder Soybean Production over Time by Province

Province	Year									
	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
	Soy Production in metric tonnes					*mt x (1000)				
Central	0.9	1.4	2.4	3.5	4.3	4.9	1.4	4.8	10.6	6.9
Copperbelt	0.06	0.35	0.19	0.44	0.26	0.28	0.17	0.38	1.2	0.18
Eastern	3.5	2.2	2.3	8.3	8.9	5.5	5.1	6.7	7.8	7.3
Luapula	0.07	0.04	0.07	0.04	0.16	0.2	0.16	0.25	0.17	0.09
Lusaka		0.04	0.04	0.17	0.46	0.16	0.03	0.15	0.11	0.18
Northern	1.1	1	2.5	0.7	4.4	1.8	1.9	2.6	5.1	2.1
N.western	0.08	0.04	0.11	0.03	0.1	0.31	0.26	0.55	0.61	0.55
Southern	0.01	0.02	0.09	0.2	0.04	0.03	0.04	0.39	0.47	1.1
Western	0.01	0	0	0	0.03	0.03	0.02	0.01	0.04	0.09
All Zambia	5.8	5.1	7.7	8.5	18.6	13.1	9.1	15.1	26.2	18.5

Source: http://www.iapri.org.zm 2013

In Zambia, the total soybean production ranges from about 2,000 to 9,000 tonnes depending on the season; average yields have remained less than one tonne per hectare. Whereas yields have increased moderately over time, changes in total soy production in Zambia appear to be driven primarily by changes in the area planted to soy and the number of farmers growing it rather than improvement in yield.

2.4 Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) is an inexpensive and environment friendly alternative source of nitrogen in which atmospheric nitrogen (N_2) is transformed into biologically active nitrogen that can be utilized by plants (Zahran, 1999). Rhizobium by means of their ability to fix nitrogen in symbiosis with legumes, play a fundamental role in the Nitrogen supply to the ecosystem.

Symbiotic nitrogen fixation plays a central role in the production of million tons of total biological nitrogen. The significance of these microbes is evident from the fact that although a total of 100 million metric tons of synthetic nitrogen is produced per year, nitrogen fixing microbes yearly converts about 200 million tons of nitrogen to ammonia (Glazer and Nikaido, 2007).

The symbiosis involving soybean and *Bradyrhizobia/ Ensifer* is a well ordered system and goes through many steps, beginning at the root surface and resulting in a N_2 fixing nodule (Vincent, 1980). The host plant provides photosynthates as a source of energy, and the bacteria reduce atmospheric nitrogen to ammonia (N_2 to NH_4) which is exported to plant tissues for ultimate protein synthesis. The efficiency of symbiotic BNF is clearly

dependent on the mutual compatibility of both partners, and is influenced by a number of environmental factors (Vincent, 1980; Sprent and Minchin, 1983).

George *et al*., (1988) found that soil N availability at different sites determined the relative contribution of symbiotic N_2 fixation, regardless of crop duration and total N accumulation by different varieties. Therefore, available soil N has a large influence on BNF. In addition soybean cultivar, bradyrhizobial strain, root nodule position, and crop management practices also influence the amount of N fixed (Eaglesham *et aI*., 1983; Buttery and Dirks, 1987; Danso *et aI*., 1987; Herridge and Holland, 1987; George *et aI*., 1988; Hardarson *et aI*., 1989).

2.5 Inoculation of Soybean seed

Soybean is not an indigenous crop to Africa (Okogun and Sanginga, 2003); it either needs inoculation with specific effective strains of *Bradyrhizobium japonicum* or to be fertilized with nitrogen fertilizers to grow well. In Africa, small-scale farmers are resource poor and cannot afford the nitrogen fertilizer to address limiting soil nitrogen. Unfortunately many of the smallholder farmers in Africa also fail to procure inoculants that can provide an alternative to nitrogen fertilizers for soy bean production. (Kaleem, 2002).

Soybean-nodulating bacteria are found over wide regions of the world, and their genetic diversity may reflect geographical and climatic differences as well as host diversity. Some strains of *Bradyrhizobium japonicum* effectively fix atmospheric nitrogen and are expected to be an effective bacterial inoculant. Inoculation of soybean seeds with an effective inoculant can increase soybean yield, but field-indigenous rhizobia frequently

compete with the inoculant (Yamakawa *et al*., 2003). Most research results indicate that rhizobium inoculation is a promising fertilizer because it is cheap, easy to handle and improves plant growth (Nishita and Joshi, 2010; Sulochana and Prashanth, 2012).

There are two types of inoculants that can be used: seed or soil applied. The seed applied inoculant is more effective once mixed with water to form slurry which is used to coat the seed. This must be done as close to planting as possible, preferably within several hours. On the other hand, the soil-applied inoculate may be easier to apply than the seed-applied inoculant, but it is more expensive on a per unit area basis. Therefore, seed applied inoculant is generally the method of choice for most growers (Thelen and Schulz, 2011).

The technology of using inoculants is cumbersome and difficult to apply by the farmers (Hornetz *et al*., 2000). They face problems in acquirement and storage of inoculants because cooling facilities are not readily available. Such requirements place constraints on the farmer's capability to use inoculants, as a result, soybean crops grown by farmers in Zambia receive no inoculants and little or no commercial nitrogen fertilizer (Lubungu *et al*., 2013).

One of the major troubles in inoculation technology with soybean is the establishment of an introduced inoculant strain of *Bradyrhizobium japonicum* in the nodules of soybean grown in soils which have indigenous populations of *Badyrhizobia* (Ham, 1976, 1980; Tang, 1979; Vest *et al.*. 1973). Preceding inoculation and continued cropping of soybean confer a formidable advantage in numbers and environmental adaptation to the

indigenous population in competition with the introduced strains. Thus, when the indigenous strains dominate the nodules, response to inoculation is not observed (Kapusta and Rouwenhorst, 1973; Kvien *et al.*. 1981; Ge and Xu, 1982).

In Zambia, the conventional soybean varieties (Kaleya, Santa Rosa, and Tunia) require the bacteria, *Bradyrhizobium japonicum* to form nodules and fix atmospheric nitrogen. These soybean varieties therefore must be inoculated before planting. The inoculated *Bradyrhizobium* may remain viable in the soil under favourable conditions although annual inoculation is recommended. Promiscuous varieties that are capable of effective nodulation by indigenous soil *Bradyrhizobia* do not necessarily require inoculums. However, they can be inoculated with *Bradyrhizobium japonicum* if and when available. An example of Promiscuous varieties is Magoye and Hernon 147 (Miti, 1997).

Zambia Agriculture Research Institute (ZARI) is the sole producer of inoculum in Zambia. ZARI has developed a powder inoculum which overcomes a few of the limitations of liquid inoculum (specifically the need for cold storage), but this in turn introduces the need for training farmers on the re-hydration and application of the input (Lubungu *et al.*, 2013). Other inoculants available commercially are based on non-indigenous strains.

2.6 Morphological and Biochemical characteristics of Rhizobia

One of the ways of describing Rhizobia is according to their growth in solid media. Morphological and biochemical characters; size, shape, colour, texture of colonies and the ability to alter the pH of the medium are generally stable characteristics useful in

defining strains or isolates. Typical morphological colony characteristics, when grown on standard yeast-mannitol medium, may include; form, elevation, colour and margin (Somasegaran and Hoben, 1985). Biochemical characterization can be very helpful in confirming that isolates are rhizobium species. Among the important biochemical tests is the glucose assay which can be performed to determine the capability of micro-organisms to utilize glucose as a sole carbon source for growth (Singh *et al*., 2008). Biochemical characterization plays an important role to differentiate between fast and slow growers of Rhizobium species (Singh *et al*., 2013).

There is currently no known information on local strains of *Rhizobia* nodulating soybean in different soils of Zambia. The present study reports the isolation of *Rhizobial* isolates from soybean plants and their characterization on the basis of morphological and biochemical characters. The importance of this information is that it will lead to the documentation of indigenous strains and as foundation for further study to determine whether the strains are high nitrogen fixers.

2.7 Information gap

Nutrient improvement of soils by nitrogen fixing symbiotic bacteria present in legumes has been known for centuries (Zsbrau, 1999). Large quantities of rhizobia in the soil have been shown to be able to facilitate cultivation of legumes as it reliefs the use of nitrogenous fertilizers in legumes (Deb *et al*., 2015). However, indigenous rhizobia can form barriers to the establishment of introduced, more efficient inoculants in nodules of intended host plants. There is evidence of widespread suboptimal efficiency of indigenous strains with legumes. But it is generally argued that indigenous populations

are greatly adapted to their local soil environments and may form additional effective symbioses than commercial inoculants isolated from a distant and unrelated soil environment (Gandee *et al*., 1999). Thus, selection of indigenous strains with high nitrogen fixing capacity, adapted to a range of environmental conditions at a specific site, is a significant strategy to maximize legume production (Sajjad *et al.*, 2008).

CHAPTER 3: MATERIALS AND METHODS

3.1 Site Description

Soil samples for the isolation of soybean-nodulating rhizobia were obtained from three agro-ecological zones of Zambia during the 2013/2014 growing season. Soil samples were collected from Mambwe (S13^o 15'22.6" and E031^o 55'08.3") in region I, Chipata (S13^o 38'49.8" and E032^o 34'22.3") in region II and Luanshya (S13^o 11'45.5" and E028^o 20'49.4") region III.

3.2 Soil sample collection

Soil sampling was done following a procedure described by Barker and Pilbeam (2007). Surface litter was removed from three randomly selected places in each location, and samples approximately 35kg collected, to a depth of 20 cm. Samples were then homogenized to make composite samle. Six composite samples were collected that is from each site one sample from virgin soils and another from soybean cultivated soils. Five kilograms from each composite sample was air-dried and ground to pass through a 2 mm sieve. The soil samples were stored until they were analyzed.

3.3 Initial Soil characterization

Soils were characterized in triplicate for texture, soil reaction, electrical conductivity, total nitrogen, organic carbon, exchangeable bases and available phosphorus.

3.3.1 Determination of texture

Texture was determined using the hydrometer method. Air dried soil (50 g) was weighed and put in a dispersing cup to which 50 ml calgon and approximately 500ml of distilled

water was added (Bashour and Sayegh, 2007). The mixture was stirred for 5 minutes using an electric stirrer. The suspension was then transferred into a 1 liter sedimentation cylinder and filled to the mark using distilled water. The suspension was mixed using a plunger. The hydrometer was lowered carefully and hydrometer reading was taken at 40 seconds to give the sand content. Temperature readings were also done at the same time. Hydrometer and temperature readings were done after 8 hours to determine the clay content of the sample.

3.3.2 Determination of soil reaction (pH)

Soil reaction was determined the method of Mclean (1982). Ten grams of air-dried soil was weighed into 50 ml plastic bottles followed by the addition of 25 ml $CaCl_2$ resulting in soil: solution ratio of 1:2.5. The solution was shaken for 30 minutes on a mechanical shaker and allowed to equilibrate. The pH of the solution was determined using the pH meter.

3.3.3 Determination of electrical conductivity

Following the procedure of Mclean (1982), fifteen grams of air dried soil was weighed into 100 ml plastic bottles and then 75 ml of distilled water was added. The mixture was shaken for 1 hour and then the suspension was filtered. This was followed by the measurement of electrical conductivity of the filtrate using the electrical conductivity meter. Results were recorded in μS.

3.3.4 Determination of Total nitrogen

The method of Bremner and Mulvaney (1982) was used to determine total nitrogen. A

gram of air dried soil was weighed and put in the digestion tube followed by 35g of mixed catalyst and 10 ml concentrated sulphuric acid. The digestion tubes were placed in a preheated digestion block and samples heated to 410 ^{o} C for 45 minutes. Samples then removed and placed on a stand to cool in the fume hood for 15 minutes. The digest was carefully transferred into another clean micro-kjeldahl flask (750 cm^{3}). 20 mls boric acid indicator solution was added into 250 ml Erlenmeyer flask which was then placed under the condenser of the distillation apparatus. Then 10 ml of 10 M NaOH was poured into the distillation flask gently, and then the kjeldahl flask was attached quickly to the distillation apparatus. Distillation was done for 15 minutes and then the distillate was collected for titration. Finally, the distillate was titrated with 0.01 M HCl.

The following formula was used to calculate % N:

% N = (Vs – Vb) x Molarity of Acid x Dilution Factor x 14.01 g / mol x 100

Weight of oven dry soil used (g)

Where Vs = Titer, Volume of standard acid for samples (ml);

Vb = Titer, Volume of standard acid for blank (ml)

% N = (Vs – Vb) x liter x 0.01 mol / liter x 10 x 14.01 g/ mol x 100

Weight of oven dry soil used (g)

= [(Vs – Vb) / 1000] liter x 0.01 mol / liter x 14.01 g/ mol x 100

1g

% N = (Vs – Vb) x 0.1401

3.3.5 Determination of Soil organic matter

Soil organic matter was determined using the Walkley and Black (1999) method. A

gram of air dried soil was weighed into an Erlenmeyer flask to which 10 ml of 1 N potassium dichromate was added. Concentrated sulphuric acid (20 ml) was then added using an automatic pipette. The mixture was then left for 30 minutes under the fume hood to allow for digestion. After the digestion 150 ml of distilled water and 10 ml of phosphoric acid were dispensed into the Erlenmeyer flask and the contents were mixed by swirling. Two drops of Diphenylamine indictor were added. To determine the soil organic content, the reaction was titrated with ferrous sulphate solution to an end point indicated by green color. The volume of ferrous sulphate solution used for to the end point was used to calculate the percent carbon as follows:

4 X $\underline{\text{(Volume FeSO}_4 \text{ used for the blank} - \text{Volume of FeSO}_4 \text{ used for the sample)}}$

Volume FeSO$_4$ used for the blank

Organic matter % (% OM) was calculated using the formular:

% C X 2

3.3.6 Determination of available Phosphorus

Bray 1 method of Olsen and Sommers (1982) was used to determine available Phosphorus. Soil (2.5g) was weighed into clean polyethylene bottles to which 25 ml of the extracting solution was dispensed and the mixture shaken for one minute. The mixture was then filtered through filter paper several times until a clear filtrate was obtained and 5 ml of the filtrate was pipetted into a 50 ml micro beaker. 10 ml of reagent B was dispensed and 35 ml of water was added. The solution sample was left for 10 minutes to react before reading on a spectrophotometer at 882 nm.

The amount of phosphorous in the samples was calculated by subtracting the blank from the obtained result and multiplying by the dilution factor to get the Phosphorous levels in the sample.

P = (Reading–Blank)* Total dilution factor

3.3.7 Determination of Exchangeable Bases

The method by Thomas (1982) was used to determine Exchangeable bases. Ten grams of air -dried soil was put into a 100 ml plastic bottle to which 50 ml of 1 M ammonium acetate (NH_4O_{ac}) solution at pH 7 was then added. The mixture was shaken for 30 minutes; the suspension was filtered through filter paper and the filtrate collected in a micro beaker. Where necessary the sample was diluted with Lanthanum or Strontium chloride for determination of magnesium and calcium to suppress interference from phosphates and read using Atomic –Absorption spectrophotometer 285.2 nm and 422.7 nm wavelength respectively. For potassium and sodium, dilution was done with water if levels were very high and read using emission or flame photometer at 766.5 nm and 585.2 nm respectively.

The concentration of cations (c mol / kg) was determined using the formula below:

C mol / kg = <u>Reading (mg / l) X Volume of extract (l) X Dilution factor</u>

Mass of soil (Kg) X Equivalent weight of cation (mg / cmol)

3.4 Isolation of Rhizobia from soils

The Trap Method (Dubey and Maheshwari, 2006) was used to isolate rhizobia from soils collected from the cultivated and virgin fields described in 3.2. A promiscuous soybean

genotype (Magoye) was used as a host plant. Soybean was grown in five (5 kg) of soil per pot in the green house. Each soil type was replicated three times and all the pots arranged in a Completely Randomised Design. After growth for 8 weeks, nodules were collected from the plants and rhizobia isolated on Yeast Extract Mannitol (YEM) agar.

3.4.1 Preparation of Yeast Extract Mannitol (YEM) agar

YEM agar was prepared according to Dubey and Maheshwari (2006). Mannitol (10 g/l), 0.5 g/l di-potassium hydrogen phosphate (K_2HPO_4), 0.2 g/l Magnesium sulphate ($MgSO_4.7H_2O$), 0.1 g/l Sodium chloride (NaCl), 1g/l yeast extract were suspended in a litre of distilled water. Before adding 15g of agar, pH was adjusted to 6.8 using 1 M HCl and 1 M NaOH. The mixture was then heated on hot plate to dissolve the agar completely. To sterilize the medium, it was autoclaved for 15 minutes at 0.15 pressure (121^oC). After cooling to about 50 ^oC, approximately 20 ml of medium was dispensed into sterile petri dishes.

3.4.2 Isolation of Rhizobia from root nodules

The method by Somasegaran and Hoben (1985) was used to isolate rhizobia from root nodules. A sterile towel was spread under the Lamina Flow on which the cleaned nodules were cut from the root system using sterile razor and forceps. Nodules were then washed with 95% ethanol for 5-10 seconds followed by sodium hypochlorite for 3 minutes. Nodules were then rinsed five times with sterile distilled water. Each Nodule was then transferred into a sterilized test tube containing 1 ml of sterilized distilled water. Using sterile glass rods, nodules were crushed and a loopful of crushed nodule material was streaked on the YEM agar plates. The plates were then incubated upside

down for 48-72 hours in an incubator at 27$^{\text{o}}$C. The colonies were restreaked until single colonies were obtained.

3.5 Morphological Characterization of rhizobial isolates

After incubation for 48-72 hours at 27$^{\text{o}}$C, isolated colonies were characterized on the basis of colony form, elevation, margin and color using the morphological keys (Aneja, 2003). Cell shape was determined by viewing gram stained cells under a Brightfield light and microscope (Bisset, 1959). The size, shape and color of colonies are generally stable characteristics useful in defining strains or isolates.
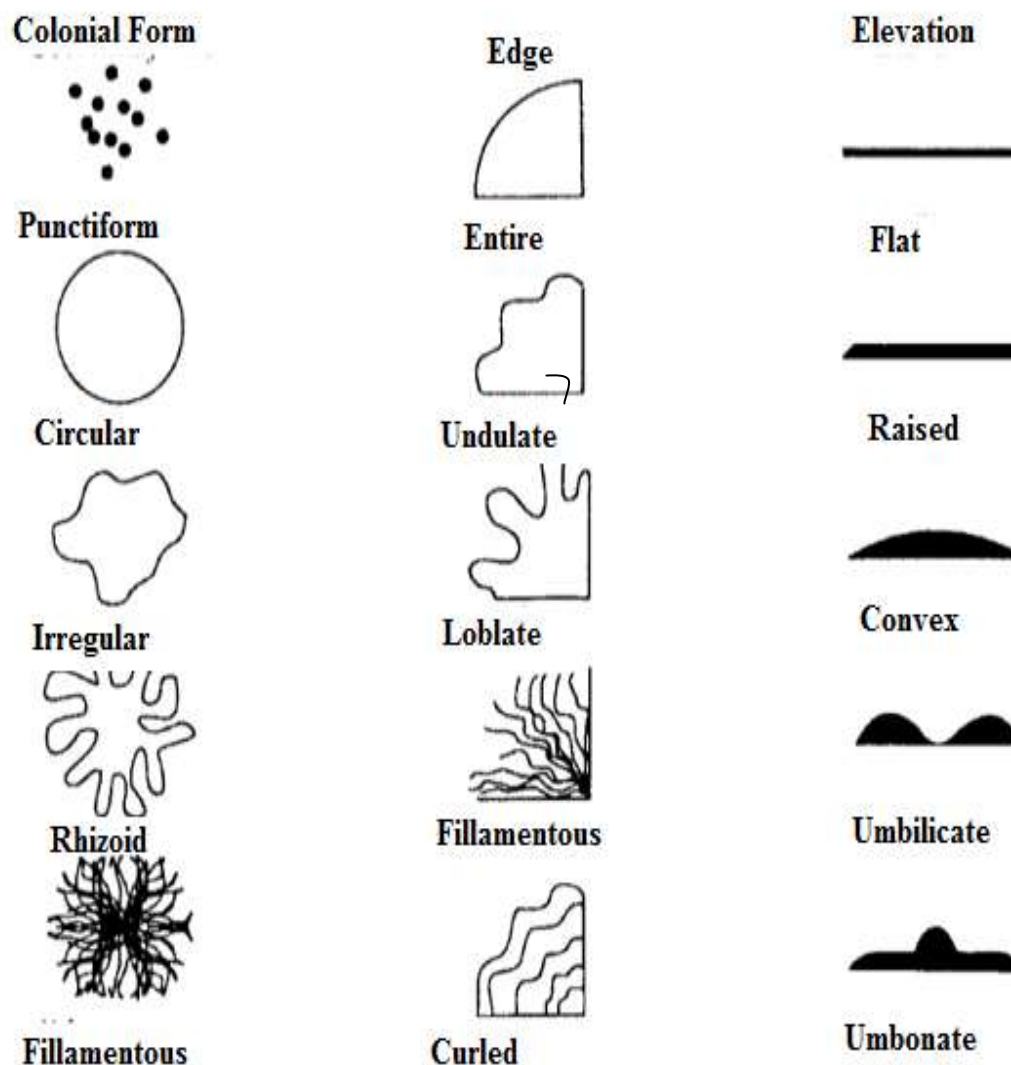
Figure 1: Colonial characteristics [source: Tiwari *et al*., 2009]

3.6 Biochemical Characterization

Isolates were characterized for their ability to utilize glucose as a carbon source, growth

rate (fast vs. slow), Gram stain reaction, mucous production and tolerance to extreme of

pH. Acid or alkali production from glucose indicates glucose-C source utilization. This

assay was conducted by replacing mannitol in the YEM with glucose (10 g/l) and adding

an indicator for pH change, Bromothymol Blue (BTB; 0.025 g/l). Plates were then

incubated to observe color from green to blue or yellow as indicators of alkali or acid production, respectively, arising from the utilization of glucose. To determine whether isolates were fast or slow growing, YEM agar was supplemented with BTB; isolates were classified as fast if they turned the medium yellow and slow growers if they turned the medium blue (Chen et al., 2001).

3.6.1 Preparation of Yeast Extract Agar (YEA) with Bromothymol Blue (BTB) indicator

Preparation of this media was similar to that of YEM agar. In YEA with BTB, instead of 10 g mannitol, 10 g glucose, 0.025 g BTB indicator was added, giving rise to a green colour. Arising changes in colour were due to pH changes after incubation at $27^{o}C$ for 72 hours at, the pH change was scored on the basis of the color change of the medium (Chen *et al*., 2001).

3.6.2 Gram stain reaction

Gram staining was conducted to confirm that the isolates were gram negative, a characteristic of rhizobia (Bisset, 1959; Somasegaran and Hoben, 1985). Gram-staining was carried out according to the procedure of Somasegaran and Hoben (1985). A loopful of pure culture thinly spread on a glass slide was prepared. The smear was air dried then heat fixed. Crystal violet dye was droped onto the slide and left before running off. Then the smear was then flooded with iodine solution for a minute. The iodine solution was drained off and the cells were decolourized with 95% ethanol for 30 seconds. The smear was washed with water and blot dried carefully. Then a counter stain with safranin was dropped onto the smear. Finally the smear was rinsed with water; air dried and observed

under a brightfield compound microscope at magnification; 10 x ocular lens and 100 x oil immersion objective (Tiwari et al., 2009).

3.6.3 Assessment of Mucus production

Isolates were scored based on the amount of mucous (exo-polysaccharides) they had produced during the 7 day incubation period at 27 °C (Sayyed *et al*., 2011).

3.6.4 Assessment of tolerance to pH extremes

To analyze tolerance of isolates to extremes of pH, YEM agar media were prepared with pH adjusted to 4.0, 6.8 and 9.0 using 1 M HCl and 1 M NaOH. After inoculation, the plates were kept at 27°C and growth was observed after 72 hours.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Selected soil characteristics

The pH for the soils of Regions I, II and III were 6.0, 5.8 and 4.8 respectively. The critical pH for crop production is 4.5 (Fairhust, 2012). In this case the pH of the soils from the selected sites of Region I, II and III were above the critical level and could therefore be considered as moderately fertile.

Table 2: Properties and classification of soil from selected sites of agro-ecological Regions I, II and III of Zambia

Source of Soil	Soil Characteristics					Soil Classification
AER District	pH 0.01M $CaCl_2$	Organic C%	Total N%	P mg/kg	Textural Class	(FAO) 2007
Region I Mambwe	6.0	0.79	0.04	11.2	Sandy loam	Fluvisols
Region IIa Chipata	5.8	0.42	0.04	7.0	Sandy loam	Alisols
Region III Luanshya	4.8	0.67	0.04	8.0	Loamy sand	Acrisol
Critical levels	4.5*	1.50*	0.12*	15.0*		

In both tropical and temperate soils, soil acidity is known to limit symbiotic nitrogen fixation by negatively affecting the survival and persistence of rhizobia in soils, and by directly decreasing the plants ability to form effective nodules (Graham *et al*., 1994). While the pH of the soil in selected sites was above the 4.5 critical level for crop production, it was below the optimum 7.0 for the growth of rhizobia (Danso *et al*., 1987; Kaur *et al*., 2012). Low pH is often considered a constraint to crop production. This is generally only true, however, where crops sensitive to low pH are grown or low pH is associated with Aluminium (Al) toxicity and crops sensitive to Al toxicity are included

in the cropping system or rotation (Fairhust, 2012). According to Taylor *et al*. (1991) acidity has more severe effects on rhizobia multiplication than low P-conditions. Phosphorous is most critical essential element after nitrogen in influencing plant growth and production throughout the world. Amongst the more significant functions and qualities of plants on which phosphorous has an important effect include –

1) Photosynthesis

2) Nitrogen fixation

3) Crop maturation – flowering and fruiting including seed formation

4) Root development

5) Protein synthesis

Thus, calculation of the available phosphorous present in the soil is essential (Pawar, 2009). Available P was 53%, 47% and 25% lower than the critical level of 15 mg/kg soil, for regions II, III and I, respectively. Extractable P content of greater than 15mg/kg is considered high (Okalebo, 2007). The low P availability in acid soils results when free Al-oxides bind native and applied P into a form unavailable to plants (Liao et al., 2006).For all the regions I, II and III, the low available P content also indicates a need to add P to increase crop production.

Soil organic matter (SOM) ranged from 0.42-0.79 %; below the critical limit of 1.5 %. Carbon is the chief element present in soil organic matter, comprising from 48 to 58% of the total weight (Nelson and Sommers, 1982). SOM is a very active and important portion of the soil. It is the nitrogen reservoir, it furnishes large portions of the soil phosphorus and sulphur, protects soil against erosion, supplies the cementing substances

for desirable aggregate formation, and it loosens the soil to provide better aeration and water movement in heavy soils (Nelson and Sommers, 1982). The low SOC in these soils indicate a need to adopt practices that would improve soil organic matter build-up in the fields. Soils with an organic C of 1.5 to 3.0% would be considered to have moderate fertility, while extractable P content of greater than 15mg/kg is considered high (Okalebo, 2007).

Nitrogen is an essential plant nutrient and is available to the plant in the ammonium or nitrate form. In soil, nitrogen is present in many forms which can be transformed to ammonium or nitrate (Tel and Jansen, 1992). The determination of total nitrogen in soils is intended to estimate the C/N ratio in the soil rather than to determine the nitrogen requirement of soils and crops. As one of the difficulties presented in Total N analysis of soils is inadequacy of knowledge concerning the forms of N present (Bremner and Malvaney, 1982). For all regions, N content was 67% lower than critical value, the low available N content also indicates a need to add N to increase crop production.

The term soil texture denotes the precisely measured distribution of particle sizes and the proportions of the different size ranges of particle sizes and the proportions of the different size ranges of particles composing a given soil. As such, soil texture is a fundamental attribute of the soil and the one most often used to characterize its physical makeup (Hillel, 2004). The relative proportion of the textual fractions in a particular soil determines its soil texture. The textural triangle (Figure 2) is used to determine the soil texture class.

The texture of the soils collected from regions I and II were sandy loam while those from region III were loamy sand (Table 2). Deep and well drained soils, varying in texture from sandy, sandy loams to clay loams are recommended for growing soybeans. Heavy clays as well produce good quality soybean crops, provided they are well drained and germination is not impended by soil capping (Miti, 1978). Soybean can be produced in the soil selected in the study as they fall within the loamy soil class. As with most other crops, good drainage is important when growing soybean on fine textured soils. The ultimate classification of the soil is made when analytical data are available. It is recommended that procedures for soil analysis (Van Reeuwijk, 2006) be followed in determining chemical and physical characteristics.
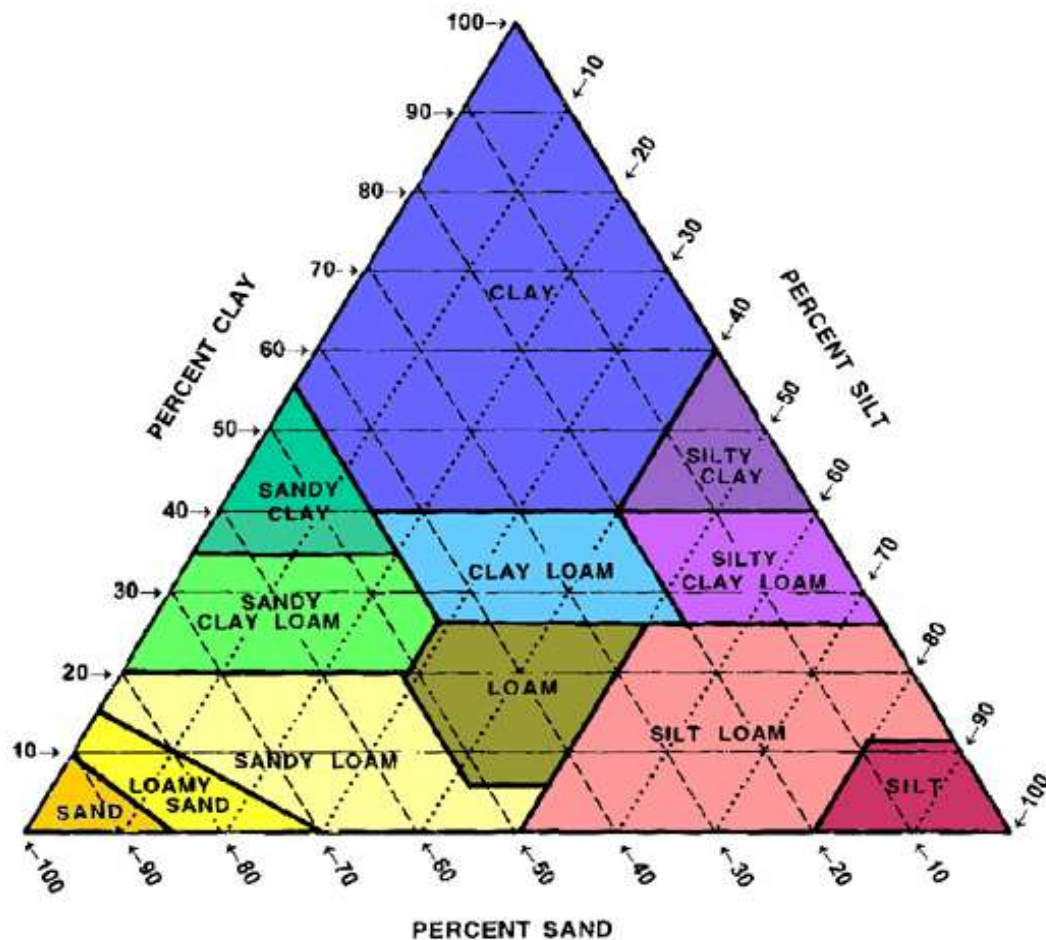
Figure 2: Textural triangle Soil Textural Triangle - Based on the triangle, a loamy soil has 40% sand, 20% clay and 40% silt. A sandy loam has 60% sand, 10% clay and 30% silt [Source: U.S.D .A.]

Soils from Mambwe (Table 2) were classified as Fluvisol, which in the Food Agriculture Organisation (FAO) World Reference Base for Soil Resources is a genetically young soil in alluvial deposits (IUSS Working Group WRB, 2007). The soils that were collected from Chipata were classified as Alisols which are moderately weathered but strongly leached acid soils; while the soils collected from Luanshya were classified as

Acrisols which are highly weathered, strongly leached and of poor soil fertility. The description of Fluvisols, Alisols and Acrisols above well fits in the soil characteristics that were obtained in this study (Table 2).

4.2 Isolation and characterization of rhizobial isolates

A total of 61 isolates from soybean nodules were obtained, of these 22, 17 and 22 were from region I, II and III, respectively. And these isolates were used for further analysis. The isolates were labeled using a combination of the site (district name) abbreviation, abbreviation for soil (V, Virgin: C, Cultivated), nodule number (1-5), replication number (RI to RIII) and letters A to C depending on the number of different colonies on the initial YEM agar plates to be streaked (e.g., for Mambwe district, MV1RI, MC1RI,MC1RII A).

4.2.1 Morphological Characteristics of rhizobial isolates

Rhizobia can be described according to their growth in solid and liquid media. The colonies obtained in region I were all circular in form, all convex in elevation, with entire or smooth and undulate margins with cream, yellow, white and transparent colours (Table 3).

The form, elevation, color, margin of colonies and the ability to alter the pH of the medium are generally stable characteristics useful in defining strains or isolates. Rhizobia isolates usually have discrete, round colonies varying from flat to domed and even conical shape (form and elevation) on agar surface. Colonies may be white-opaque or they may be milky- to watery-translucent. The opaque colony growth is usually firm

with little gum, whereas the less dense colonies are often gummy and soft. Red or pink and yellowish colonies occur, but are not common. Colonies usually have a smooth margin (Somasegaran and Hoben 1985).

Table 3: Rhizobial colony morphological characteristics of isolates from cultivated and virgin soils of regions I, II and III

AER Margin	District	Form	Elevation	Colour	
Region I	Mambwe	All circular	All convex	Cream, white, yellow, transparent	Entire, undulate
Region II	Chipata	Irregular, punctuate, circular	All convex	Cream, white, yellow	Entire, lobate
Region III	Luanshya	Irregular, punctuate, circular	All convex	Cream, white, yellow, transparent, pink	Entire, undulate

The colonies obtained in region II varied from irregular, punctuate to circular in form, all colonies had convex elevation with entire, smooth margins to lobate margins and different colored cream, yellowish and white (Table 3).

In the case of colonies obtained in region III, colony morphology varied from circular, irregular to punctuate, all colonies had a convex elevation with entire or smooth margins to undulate margin and cream, yellow, white, transparent and pink in colouration (Table 3).

From the 61 isolates from the three regions, 87 % were circular, 8 % irregular and 5 % punctuate in form with 100 % convex elevation. The isolates were 88 % entire, 10 % undulate and 2 % lobate margins with different colors − 56 % cream, 24 % white, 11 %

yellow, 5 % transparent and 3 % pink. Transparent colonies were perculiar to Region I and III while pink colonies were perculiar to Region III. According to Somasegaran (1985) pink and yellowish isolates occur, but are not common.

Similarly Gachande and Khansole (2011), isolated *Rhizobium japonicum* and *Bradyrhizobium japonicum* colonies, which were circular in shape with whitish pink color on Congo Red Yeast Extract Mannitol Agar (CRYEMA) medium. ln general, the colonies were circular, convex, whitish pink and glistering with entire margin. Deka and Azad (2006) also obtained colonies on YEMA medium that were circular as well as irregular, with entire margins, convex elevation and colors included translucent colored isolates. Earlier work has suggested that colony morphology is of significance as it differentiates strains according to their ability to fix nitrogen (Mathis,*et al*., 1986).
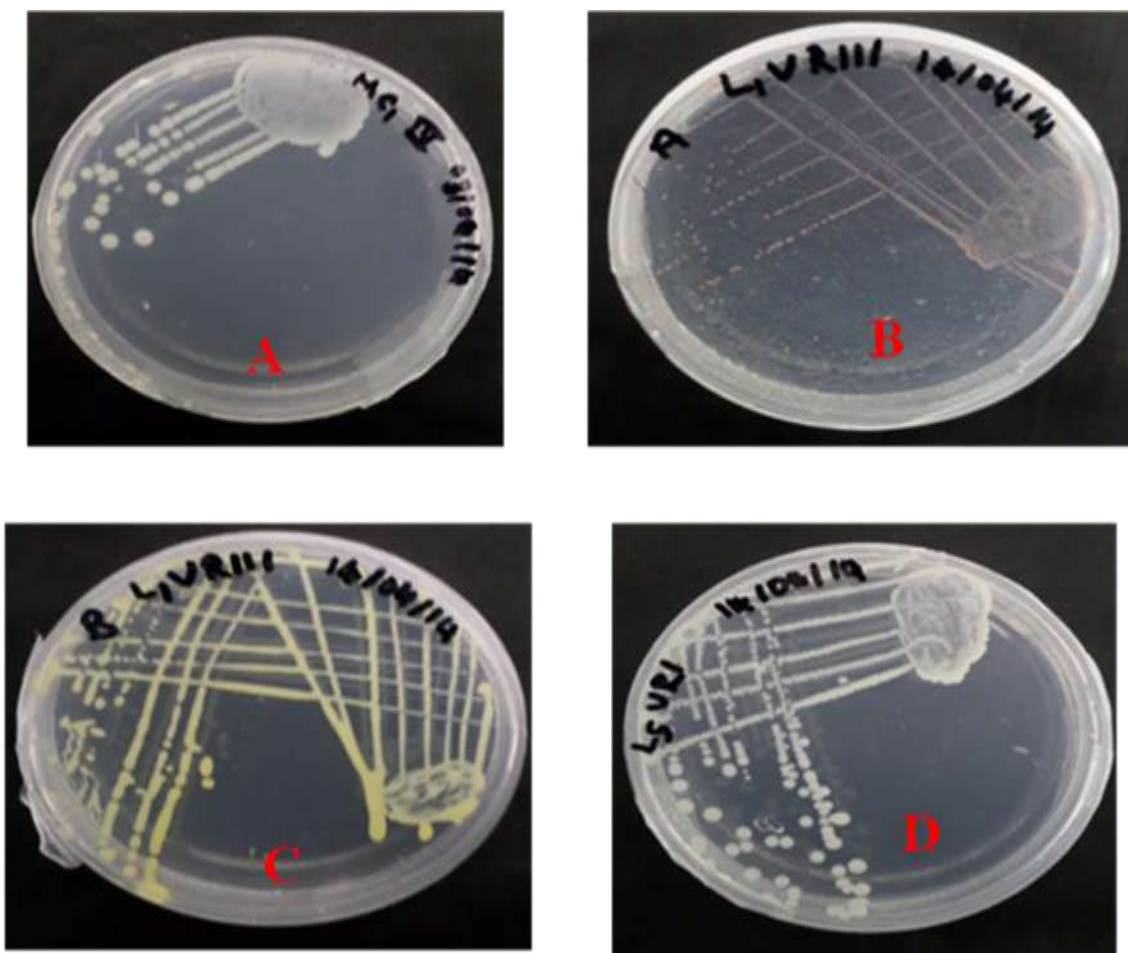
Figure 3: Rhizobial colonies on Yeast Extract Mannitol Agar after 48 hours of incubation at 27 ^{o}C. A: Cream colonies from soils of Region I. B: Pink colonies from soils of Region III. C: Yellow colonies from soils of Region III. D: Cream colonies from soils of Region III.

4.2.2 Glucose-C utilization, growth rate, Gram stain reaction, mucous production and tolerance to pH extremes of rhizobial isolates

Freshly prepared YEM agar plates containing bromthymol blue at pH of 6.8 are green in color. Slow-growing rhizobia show an alkaline reaction in this medium, turning the dye

blue. Fast-growing rhizobia show an acid reaction, turning the medium yellow (Somasegaran and Hoben 1985).

The species of the genus *Rhizobium* have been separated into fast and slow growing types based on their rate of growth and their effect on the acidity of the YEM under laboratory conditions (Saeki et al., 2005; Sharma *et al*., 2010; Somasegran and Hoben, 1994). Typically, fast-growing types are said to have average generation times of 2 to 4 hrs and tend to depress the pH of the medium, while the slow-growers have average regeneration times of 6 hrs (Sadowsky et al., 1983). *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*, the species that typically nodulate soybean has been classified as slow growers. The results of this study indicate that 59 out of 61 isolates from the three agro-ecological regions were fast growers, while only 2 from region II cultivated soils were slow-growers. Based on this observation and the knowledge that soybean are also nodulated by *Ensifer fredii and Rhizobium tropici* which are fast growers (Li *et al*., 2011; Kaur *et al*., 2012), it is possible that the 59 isolates are infact *Ensifer fredii* and/or *Rhizobium tropici* rather than *Bradyrhizobium*. Without molecular characterization, this cannot be stated with certainty but beckons further characterization of the isolates using DNA extraction and sequencing tools. The ability to grow fast has been implicated in the establishment of nodules and the subsequent nitrogen-fixing abilities of the organism. Based on the acid/alkaline reaction results on medium supplemented with glucose, all the isolates were able to utilize glucose as a carbon source. Just like tolerance to extremes of pH, carbon source utilization has ecological significance when considering development of inoculants. Generally, fast growing rhizobia have been shown to have a wider range of utilization of carbon sources than-slow growers. Slow-growers have been

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associated with utilization of mostly hexoses and pentoses but with limited utilization of disaccharides and sugar alcohols (Wagner *et al*., 1995). Slow-growers tend not to possess the catabolic enzymes for disaccharides. Fast growers on the other hand, can utilize disaccharides, cellobiose, sucrose and other such compounds not utilized by slow-growers (Sawdosky *et al*., 1983). Previously it has been shown that both fast- and slow-growers are able to utilize glucose as a carbon source, this corroborates with the findings in this study.

Table 4: Gram stain and acid/alkali reaction of the isolates

	Region I			Region II			Region III	
Culture ID	Gram stain	Acid/alkali production*	Culture ID	Gram stain	Acid/alkali Production	Culture ID	Gram stain	Acid/alkali production
MC1 RI	Negative	Yellow	CC1RI	Negative	Yellow	LC1RI A	Negative	Yellow
MC1 RI B	Negative	Yellow	CC2RI	Negative	Yellow	LC3RI	Negative	Yellow
MC5 RI	Negative	Yellow	CC3RI	Negative	Blue	LC4RI	Negative	Yellow
MC2RII	Negative	Yellow	CC5RI	Negative	Blue	LC1RII	Negative	Yellow
MC5RII	Negative	Yellow	CC1RII	Negative	Yellow	LC4RII	Negative	Yellow
MC1RIII	Negative	Yellow	CC3RII	Negative	Yellow	LC5RII	Negative	Yellow
MC2RIII A	Negative	Yellow	CC5RII	Negative	Yellow	LC1RIII A	Negative	Yellow
MV1RI	Negative	Yellow	CC1RIII	Negative	Yellow	LC3RIII	Negative	Yellow
MV2RI B	Negative	Yellow	CV3RI	Negative	Yellow	LC5RIII	Negative	Yellow
MV5RI	Negative	Yellow	CV1RII	Negative	Yellow	LV1RI	Negative	Yellow
MV1RII	Negative	Yellow	CV3RII	Negative	Yellow	LV2RI	Negative	Yellow
MV2RII	Negative	Yellow	CV5RII	Negative	Yellow	LV3RI	Negative	Yellow
MV4RII A	Negative	Yellow	CV1RIII	Negative	Yellow	LV4RI	Negative	Yellow
MV4RIII A	Negative	Yellow	CV5RIII A	Negative	Yellow	LV5RI	Negative	Yellow
MC4RI B	Negative	Yellow	CVRIII	Negative	Yellow	LV1RII A	Negative	Yellow
MC4RI A	Negative	Yellow	CV5RIII B	Negative	Yellow	LV3RII	Negative	Yellow
MC1RI A	Negative	Yellow	CV4RI	Negative	Yellow	LV4RII	Negative	Yellow
MC3RIII B	Negative	Yellow				LV1RIII A	Negative	Yellow
MV4RII C	Negative	Yellow				LV4RIII	Negative	Yellow
MV5RIII	Negative	Yellow				LV1RIII B	Negative	Yellow
MV2RII A	Negative	Yellow				LC1RI B	Negative	Yellow
MC1RII B	Negative	Yellow				LC1RIII B	Negative	Yellow

*Based on acid/alkaline production on YEM agar + BTB at pH 6.8, 27°C
-Acid producton changed color of plate from green to yellow; alkaline production from green to blue
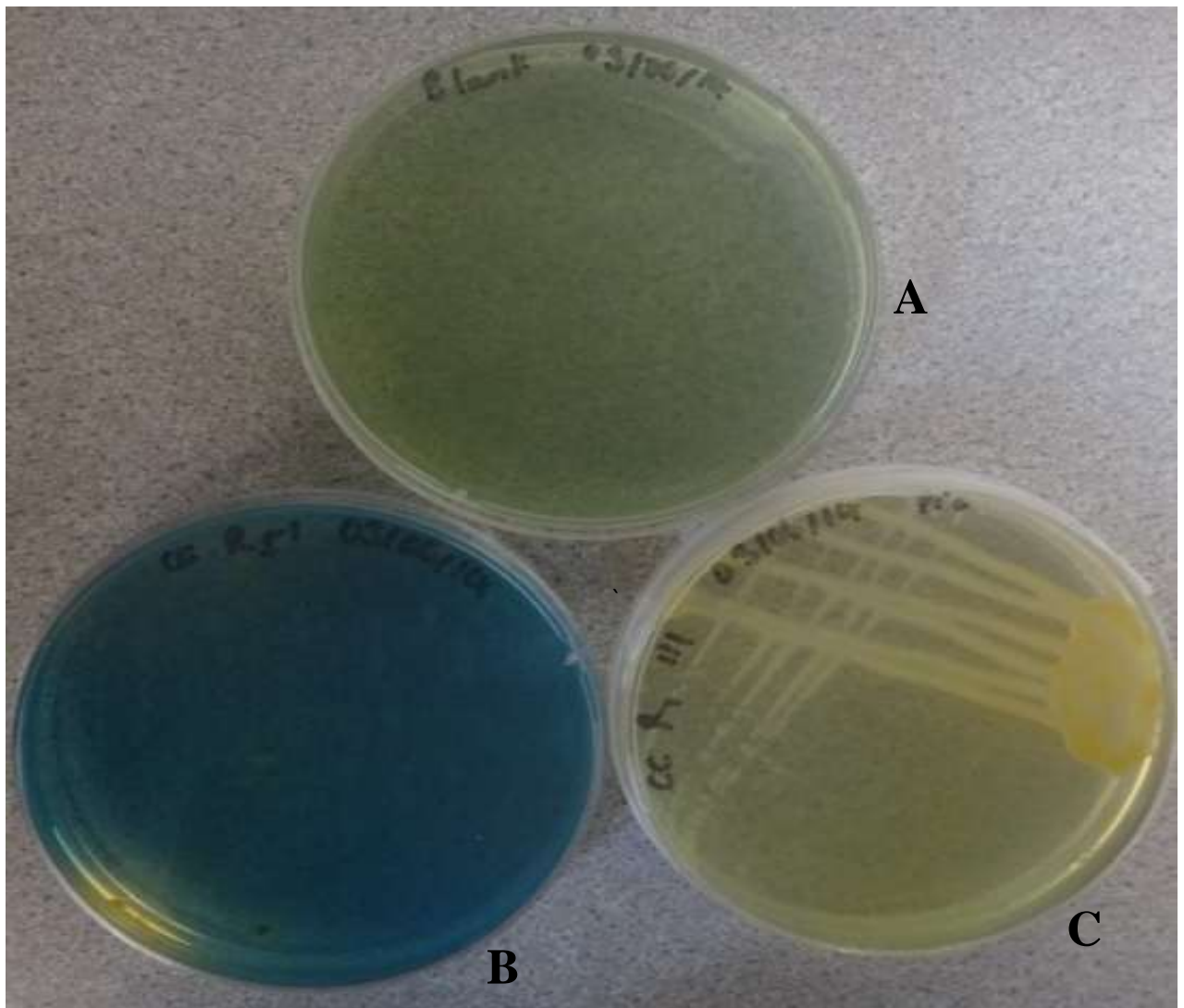
Figure 4: Acid/ alkaline production of rhizobial cells on Bromothymol Blue Yeast Extract Glucose Agar after 72 h of incubation at 27°C. A: Green plate-Control neutral pH 6.8; B: Blue plate-Alcohol production; C: Yellow plate-Acid production

Gram staining is a bacteriological laboratory technique used to differentiate bacterial species into two large groups (gram-positive and gram-negative) based on the physical properties of their cell walls (Madigan *et al*., 2004). Gram-positive organisms retain the

crystal violet stain after treating with iodine and washing with alcohol, and appear dark violet after staining. Gram-negative organisms lose the violet stain after treating with iodine and washing with alcohol but retain the red coloration of the counter-stain, safranin (Somasegaran and Hoben, 1985). While rhizobia are commonly reported to be Gram negative, non-spore forming and rod-shaped, earlier work suggested that they can present as weakly Gram-positive bacilli forming occasional refractile spores (Bisset, 1958). This suggests possible pleomorphism for rhizobia making it possible for them to assume this form under certain culture conditions and the Gram-negative, rod-form with polar flagella, under different culture conditions. The results of the current study indicated gram-negative and rod-shaped cells as revealed by Gram's staining technique (Figure 5). This characteristic is typical of *Rhizobia*.

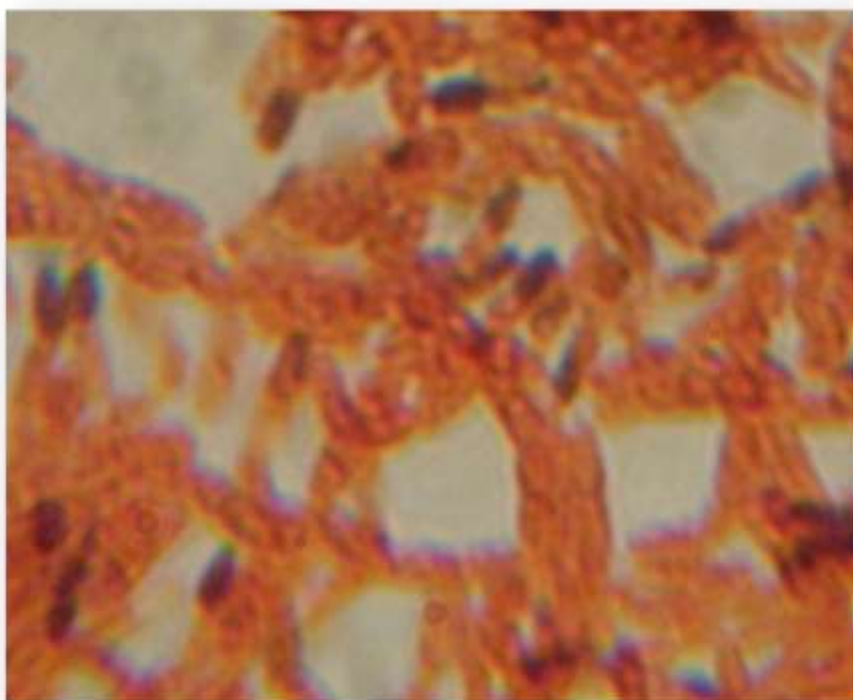Figure 5: Gram stain of rhizobial cells: showing gram negative cells

The ability to produce a variety of exo-polysaccharides (gum or mucus) is a characteristic of a very large number of microorganisms. This characteristic has been associated with and is characteristic of rhizobia. Results of this study indicated mucus production of all the isolates. The mucus plays a critical role in maintaining minimum moisture in the immediate environment of the microorganisms. Through the high moisture holding capacity, it prevents desiccation, and serves as a potential source of energy under conditions of paucity (Sayyed *et al*., 2011). Mucus produced by rhizobia has been shown to consist of glucose, maltose, rhamnose, galactose and other glucans. In rhizobia infected plant nodules, the mucus has been shown to accumulate between the peribacteroid membrane and the symbiosome membrane filling up most of the symbiosome volume (Streeter *et al*., 1992). The ability to produce the mucous *in-vitro* is an indication that this process is independent of the plant host and that the kind of polysaccharides making up the mucus are completely dependent on the genotype of the microorganism.

All 61 isolates produced mucus, although the extent of production differed (Table 6). Mucus production was from low, intermediate to high. Our results collaborate with Kaur *et al*. (2012) who obtained yellow rhizobial isolates that produced high amounts of mucus after 2 days of incubation. The ability to produce mucous is a characteristic of *Rhizobia spp*.

Table 5: Mucus production of the isolates

	Region I	Region II	Region III
High +++	50%	70.59%	22.73%
Intermediate ++	50%	17.65%	77.27%
Low +	-	11.76%	-

+ Low to high mucus production of the isolates

Growth of rhizobia in soils is sensitive to pH; pH has infact been shown to limit survival and persistence in soils. The results of this study show normal growth at pH 6.8 (Figure 6), but limited growth at pH 4 and 9. These results are similar to what has already been shown by others (Kaur *et al*., 2012) showed that the best rhizobial growth is in media with pH around neutral. On the other hand, Sadowsky *et al*. (1983) have shown that slow-growing rhizobia such as *Bradyrhizobia*, can have a high level of tolerance to acid conditions (pH 4.5) while fast-growers can tolerate alkaline conditions of pH 9 and 9.5 in growth media. This observation *in-vitro* agrees with what has been observed in China that *Ensifer* (acid producers, fast-growers) are dominant in alkaline-saline soils while *Bradyrhizobium japonicum* (alkaline producers, slow-growers) in acid soils (Yan *et al*., 2014). Both of these studies, indicate that fast-growers are relatively more alkali tolerant and acid sensitive than slow growers. This attribute is particularly important when considering strains of rhizobia to be included in the development of inoculants suited for use in different regions with differing soil reaction. From this study, both fast- and slow-growers could not tolerate extremes of pH, placing them together with respect to this attribute.

Figure 6: Growth of isolates on YEM agar media at A: pH 6.8; B: pH 4; and C: pH 9

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Morphological characteristics varied among the 61 isolates obtained from the representative of three agro-ecological regions of Zambia. The colors of colonies were translucent, cream, white, pink and yellow. Pink colonies were peculiar to region III. The elevation of all the colonies was convex. Biochemically, all Rhizobia were Gram-negative, mucus producing and acid producing with only 2 producing alkali. Isolates were fast growing with two slow growing, able to utilize glucose as a carbon source and unable to tolerate extremes of pH at 27 °C. Based on results, the 59 fast-growers could be *Ensifer fredii* and/or *Rhizobium tropici* rather than *Bradyrhizobium*. However, further tests to confirm these findings using ketolactose, genetic characterization and an inclusion of reference strains, are still needed.

5.2 Recommendation

Further tests to confirm the isolates using ketolactose, genetic characterization and an inclusion of reference strains are being recommended.

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