

Morphological and Biochemical Characterization of Soybean Nodulating Rhizobia Indigenous to Zambia

Rachael Kapembwa¹, Alice M. Mweetwa¹, Munsanda Ngulube¹ & Jones Yengwe¹

¹ Department of Soil Science, School of Agricultural Sciences, University of Zambia, Lusaka

Correspondence: Alice M. Mweetwa, Department of Soil Sciences, School of Agricultural Sciences, University of Zambia Box 32379, Lusaka. Tel: 260-971-803-918. E-mail: alicemweetwa@yahoo.com

Received: April 5, 2016 Accepted: May 3, 2016 Online Published: June 27, 2016

doi:10.5539/sar.v5n3p84

URL: <http://dx.doi.org/10.5539/sar.v5n3p84>

Abstract

Soybean [*Glycine max* (L.) Merrill] is known for 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 paper reports the isolation and characterization of rhizobial isolates from virgin and cultivated soils of the three agro-ecological regions of Zambia. Rhizobia were isolated using the Trap Method and characterized using selected morphological and biochemical markers. A total of 61 isolates were isolated on Yeast Extract Mannitol (YEM) agar medium. Isolates varied in colony form, color, margin and texture. From the 61 isolates from the three regions, 87 % were circular, 8 % irregular and 5 % punctuate in form with 100 % convex elevation. The isolates had 88% entire, 10% undulate and 2 % lobate colony margins with different colors – 56 % cream, 24 % white, 11 % yellow, 5 % transparent and 3 % pink. Transparent colonies were peculiar to Region I and III while pink colonies were peculiar to Region III. 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 the 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.

Keywords: *Bradyrhizobia*, Rhizobia, *Ensifer*, Soybean, Zambia

1. Introduction

Soybean [*Glycine max* (L.) Merrill.] is an important legume crop that was introduced to Zambia in the 1930s, but has remained an insignificant crop among smallholder farmers and thus is grown mostly by commercial farmers. The crop is now grown by both small and large scale farmers. It is adapted to agro-ecological regions II (800 – 1000 mm of rainfall) and III (above 1000 mm of rainfall) of Zambia and will grow well anywhere maize grows (Miti, 1997). Following successful infection with soybean-nodulating rhizobia, soybean forms root nodules where rhizobia symbiotically fix atmospheric nitrogen. The main soybean-nodulating rhizobia are *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Sinorhizobium xinjiangense*, *Mesorhizobium tianshanense*, *Ensifer fredii*, and *Rhizobium tropici* (Shiro et al., 2013). Of these, *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* are both slow growing while *Ensifer fredii*, and *Rhizobium tropici* are fast growing (Li et al., 2011).

Soybean offers a variety of possible benefits to crop production systems, diets, and incomes of its producers. In addition to being a potentially profitable cash crop, the high protein content (about 40%) in soybeans means it could also contribute to an improved nutritional status of its consumers (Dixit et al., 2011). Soybean is an economically significant crop because of its wide use for production of cooking oil, human food, stock feed and several 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. This soil improvement leads to elevated levels of sustainable agriculture with minimal input requirement.

In Zambia, soybean is mostly grown for industrial purposes; for example, in the production of edible oils, soy chunks, soy milk, and soy meal. The by-product (soy cake) can be fed directly to animals or processed with other ingredients into animal feed stock. With an increase in animal population in Zambia, industrial demand for soybeans is likely to increase because it provides a cheaper source of high quality protein in feed rations (Lubungu, Burke, & Sitko, 2013). Current production of soybean in Zambia is estimated at 214,179 metric tons (CSO/MAL, 2013). This shows an increase of more than 17 times from what Tefera (2011) reported in 2006.

Biological Nitrogen Fixation (BNF) is important in farming systems and can be ameliorated by simple and inexpensive inoculation procedures. Extended use of biological fertilizers maintains soil fertility of agro-ecosystems, thereby reducing the cost of chemical fertilizers borne by farmers. In Zambia, inoculants are produced by Government facilities essentially to service emerging soybean industries (Hardarson & 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, Bopape, Rong, & Seane, 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 of a related legume in the immediate cropping sequence of the field, previous unsatisfactory nodulation of the same crop, a cropping sequence of a legume followed by non-legume and land undergoing reclamation (Allen & Allen, 1961). The surest indicator for inoculation is the prevailing numbers of rhizobia in the soil. It is better to over-inoculate than to produce 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. We isolated rhizobial strains infecting Magoye, a common promiscuous genotype of soybean in Zambia and characterized these isolated strains by using morphological and biochemical markers.

2. Materials and Methods

2.1 Site description and Soil Sampling

Zambia is divided into three agro-ecological regions largely based on the average amount of rainfall received annually. The study was conducted on soils collected from selected sites in all the agro-ecological regions of Zambia. Soil samples were collected from Mambwe in agro-ecological region I (less than 800 mm of annual rainfall) located at S13° 15'22.6" and E031° 55'08.3"; Chipata in agro-ecological region II (between 800 mm and 1000 mm of rainfall annually) at S13° 38'49.8" and E032° 34'22.3"; and Luanshya in agro-ecological region III (greater than 1000 mm of rainfall annually) at S13° 11'45.5" and E028° 20'49.4". All the fields from which soil samples were collected had soybean grown on them in the previous growing season (2012/13). Additionally, adjacent fields that had never been cultivated (virgin) were identified and soils were collected from them likewise.

Soil sampling was done following the procedure outlined by Barker and Pilbeam (2007). Soil samples were randomly collected from various points to a depth of 20 cm. These soil sub-samples were then thoroughly mixed to make composite samples. Six composite samples were collected from each location in total (virgin and cultivated). Five kilograms of soil from each composite sample was then air-dried and sieved (2 mm) for characterization.

2.2 Soil Characterization

Soils were initially characterized for texture, soil reaction, total nitrogen, soil organic carbon, and available phosphorus. Soil texture was determined by the Hydrometer method according to Day (1965). Soil reaction (pH) was measured in 0.01 M CaCl₂ using a soil: solution ratio of 1:2.5 using glass-calomel electrodes connected to a

pH meter (Van Reeuwijk, 1992). Total nitrogen, available phosphorus and soil organic carbon (SOC) were determined using the Kjeldahl (Bremner & Mulvaney, 1982), Bray 1 (Olsen & Sommers, 1982) and Walkley and Black chromate reduction (VanRanst, Verloo, Demeyer, & Pauwel, 1999) methods, respectively.

2.3 Isolation, Morphological and Biochemical Characterization of Rhizobia

2.3.1 Rhizobia Isolation

Rhizobia were isolated from soils using the Trap method (Dubey & Maheshwari, 2006). A promiscuous soybean genotype (Magoye) was grown in pots containing five (5) kg of soil collected from the different agro-ecological regions as previously described. Pots were set up in the greenhouse in a completely randomized design (CRD) with three replicates. Five (5) healthy nodules of soybeans were collected from each plant for rhizobia isolation at eight (8) weeks after planting. Nodules were first washed thoroughly with tap water, followed by a rinse with sterile distilled water. Nodules were then immersed in 95% ethanol for 5-10 seconds and in 4 % Sodium hypochlorite for 3 minutes and then finally rinsed five times with sterile distilled water. Each sterilized nodule was then transferred into a sterile test tube containing 1 ml sterilized distilled water. Using a sterile glass rod, each nodule was crushed, after which a loopful of the crushed nodule material was streaked on the Yeast Extract Mannitol (YEM) agar. YEM was prepared from 10 g mannitol, 0.5 g di-potassium hydrogen phosphate (K_2HPO_4), 0.2 g Magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 0.1 g Sodium chloride (NaCl), 1g yeast extract and 15g agar suspended in 1 litre distilled water (adjusted to pH 6.8 using 1 M HCl and 1 M NaOH before the addition of the agar). The plates were then incubated for 48-72 hours in the dark in an incubator at 27 °C. Single colonies were selected and re-streaked on the same medium; this process was repeated until pure single colonies were obtained.

2.3.2 Morphological Characterization

Isolated colonies were characterized on the basis of colony morphology-form, elevation, margin and colour (Aneja, 1996) and on the cell shape (Bisset, 1959).

2.3.3 Biochemical Characterization

Biochemical characteristics included: the ability of the strains to utilize glucose as a carbon source, Gram stain reaction, growth rate (fast vs. slow), mucous production and pH tolerance. 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 colour change from green to blue or yellow as indicators of alkali or acid production, respectively, arising from the utilization of glucose. Gram staining was conducted to confirm that the isolates were gram negative, a characteristic of rhizobia (Bisset, 1959; Somasegaran & Hoben, 1985). 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). Mucous production assessment was based on the appearance of isolates on YEM agar plates after the incubation of 7 days at 27 °C.; isolates were scored based on the amount of mucous (exo-polysaccharides) they had produced during the incubation period (Sayyed, Jamadar, & Patel, 2011). To analyse the tolerance of isolates to extremes of pH, YEM agar media were prepared with pH adjusted to 4.0, 6.8 and 9.0. After inoculation, the plates were kept at 27 °C and growth was observed after 72 hours.

3. Result

3.1 Selected Soil Characteristics and Classification

The texture of the soils collected from regions I and II were sandy loam while those from region III were loamy sand (Table 1).

Soils from Mambwe were classified as Fluvisols, which in the Food Agriculture Organisation (FAO) World Reference Base (WRB) 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 fertility.

Critical values for key soil parameters are a rough guide on the fertility status. Depending on the concentration determined through laboratory analysis, the critical value might indicate whether the soil is able to supply sufficient quantities of a nutrient for crop growth (Fairhurst, 2012). Critical values also differ from crop to crop. The soil reaction (pH value) was 6.0, 5.8 and 4.8 for soils from region I, II and III, respectively (Table 1). The other parameters, SOC, available P and total N were below the critical levels in all the three regions. In this study,

SOC in regions I, II and III was 47%, 72% and 55% less than the critical level of 1.5%, respectively (Table 1). Available P was 53%, 47% and 25% lower than the critical level of 15 mg/kg soil, for regions II, III and I, respectively. For all regions, N content was 67% lower than critical value.

3.2 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. The isolates were designated 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).

Table 1. Properties and classification of soil from selected sites of agro-ecological I, II and III regions of Zambia

Soil Collection Site	Soil Characteristics					Soil Classification
	pH	SOC (%)	Total N (%)	Available P (mg/kg)	Texture	Soil type (FAO)
Region I (Mambwe)	6.0	0.79	0.04	11.2	Sandy loam	Fluvisols
Region II b(Chipata)	5.8	0.42	0.04	7	Sandy loam	Alisols
Region III (Luanshya)	4.8	0.67	0.04	8	Loamy sand	Acrisol
Critical levels	4.5*	1.5*	0.12*	15*		

3.2.1 Morphological Characteristics of Rhizobial Isolates

The colonies obtained from region I were all circular in form, all convex in elevation, with entire or smooth and undulate margins with cream, yellow, white or transparent colors (Table 2). Isolates obtained from region II varied from irregular, punctuate to circular in form, all colonies had convex elevation with entire, smooth margins to lobate margins. The colors of isolates were observed that is cream, yellowish and white (Table 2).

Table 2. Rhizobial colony morphological characteristics of isolates from cultivated and virgin soils of regions I, II and III

	Form	Elevation	Colour	Margin
Region I	All circular	All convex	cream, white, yellow, transparent	Entire, undulate
Region II	Irregular, punctuate, circular	All convex	cream, white, yellow	Entire, lobate
Region III	Irregular, punctuate, circular	All convex	cream, white, yellow, transparent, pink	Entire, undulate

In the case of colonies obtained from 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 different colors of isolates were observed that is cream, yellow, white, transparent and pink (Table 2).

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 peculiar to Region I and III while pink colonies were peculiar to Region III.

3.2.2 Glucose-C Utilization, Gram Stain Reaction, Growth Rate, Mucous Production and Tolerance to pH Extremes of Rhizobial Isolates

After incubating the plates containing BTB at 27 °C for 72 hours, 97 % of the isolates showed an acidic reaction, changing the medium colour from green to yellow while 3 % of the isolates changed the colour of medium from green to blue showing an alkaline reaction (Table 3; Figure 1A), an indication that all isolates could utilize glucose as a carbon source.

Table 3. Gram stain and acid/alkali reaction of the isolates from agro-ecological regions I, II and III

Region I			Region II			Region III		
Culture ID (Region I)	Gram stain	Acid/alkali production*	Culture ID (Region II)	Gram stain	Acid/alkali production*	Culture ID (Region III)	Gram stain	Acid/alkali production
MC1RI	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 production changed colour of plate from green to yellow; alkaline production from green to blue.

The 61 rhizobial isolates were all gram negative and rod shaped as revealed by Gram's staining technique (Table 3; Figure 1B). Exo-polysaccharide (mucous) production of the isolates after 7 days of incubation is indicated in Table 4. All 61 isolates produced mucous, although the extent of production varied from low, intermediate to high. A higher percentage of isolates were high and intermediate mucous producers.

Table 4. Mucous production of rhizobial isolates from agro-ecological regions I, II and III

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

+ indicates extent of mucous production after 7 days growth of isolates.

Rhizobial growth was good on medium at pH 6.8 (27 °C) (Figure 1C), however, this was not the case for pH 4.0 and 9.0 at the same temperature.

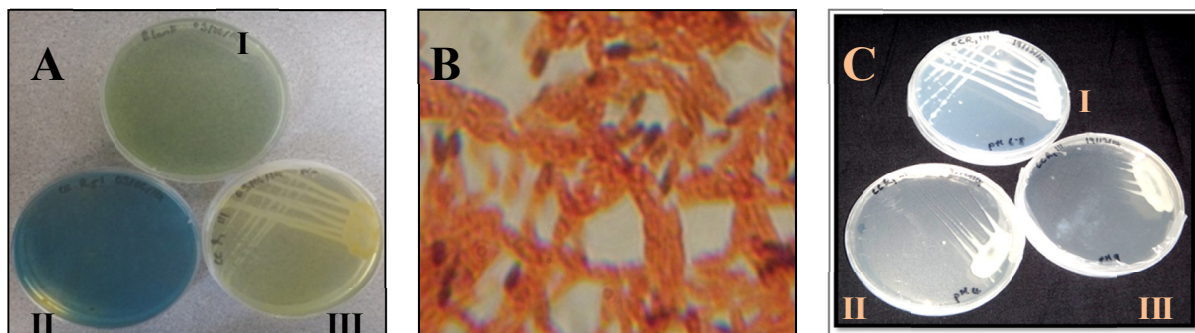


Figure 1. Glucose-C utilization, Gram stain reaction, and tolerance to pH extremes of rhizobial isolates. **A.** Acidic and alkaline reaction on BTB plates. (I). Uninoculated plate-green (II). Alkaline reaction-blue. (III). Acidic reaction-yellow. **B.** Gram stain reaction; reddish colour indicating Gram-negative bacteria. **C.** Growth of isolates on plates with pH 6.8 (I), pH 4 (II) and pH 9 (III); normal growth on pH 6.8 plates and limited growth at pH 4 and 9

4. Discussion

The description of Fluvisols, Alisols and Acrisols for the soils obtained from selected sites of region I, II and III corroborates with the soil characteristics that were obtained in this study. A fertile, medium-textured loam soil usually is best although soybean can be produced on a wide range of soil types. Soybean can be produced in the soil from the selected sites of this study as they all fall within the loamy soil class. The pH of the soil in the three regions was above the critical value of 4.5 indicating a moderately fertile soil. Low pH is often considered a constraint to crop production, however, this is generally true where crops sensitive to low pH are grown or low pH is associated with Aluminium (Al) toxicity (Fairhurst, 2012). 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, Draeger, & Ferrey, 1994). The pH in all the three regions was lower than the optimum pH for the growth of rhizobia which is around neutral pH 7 (Danso, Hera, & Douka, 1987; Kaur, Sharma, Kaur, & Gill, 2012). The pH of soils in region III, especially, may lead to little or no growth of rhizobia particularly *Bradyrhizobium* strains which are sensitive to acid soils. SOC, available P and total N were below the critical levels in all the three regions. The low SOC in these soils indicate a need to adopt practices that would improve soil organic matter build-up in the fields. For all regions, the low N content also indicates a need to add N to increase crop productivity.

Isolates obtained in this study had varied morphological characteristics. Colors of the isolates in this study included cream, white, yellow, transparent and pink. Previously, pink (Gachande & Khansole, 2011), white-opaque (Li et al., 2011), white (Kaur et al., 2012), and translucent (Deka & Azad, 2006; Kaur et al., 2012) isolates have been obtained from soybean nodules. In this study, yellow colonies were also observed; such colonies have been said to occur but are rather uncommon (Somasegaran & Hoben, 1985). The observed elevation and margins have also been previously observed by others (Deka & Azad, 2006). Earlier work has suggested that colony morphology is of significance as it differentiates strains according to their ability to fix nitrogen (Mathis, Israel, Barbour, Jarvis, & Elkan, 1986).

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 associated with utilization of mostly hexoses and pentoses but with limited utilization of disaccharides and sugar alcohols. (Wagner, Skipper, & Hartel, 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, Keyser, & Bohlool, 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.

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, Srivastava, & Sharma, 2010; Somasegaran & Hoben, 1994). Typically, fast-growing types are said to have

average generation times of 2 to 4 h and tend to depress the pH of the medium, while the slow-growers have average regeneration times of 6 h (Sadowsky et al., 1983). *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*, the species that typically nodulate soybean have 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), we speculate that the 59 isolates are in fact *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.

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, 1959). 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.

Growth of rhizobia in soils is sensitive to pH; pH has in fact been shown to limit survival and persistence in soils. The results of this study show normal growth at pH 6.8, but limited growth at pH 4 and 9. These results are similar to what has already been shown by others (Kaur et al., 2012) 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 *Bradyrhizobium*, 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 tolerate 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 our study, both fast- and slow-growers could not tolerate extremes of pH, placing them in the same group with respect to this attribute.

The ability to produce a variety of exo-polysaccharides (gum or mucous) 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 mucous production of all the isolates. The mucous 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). Mucous produced by rhizobia has been shown to consist of glucose, maltose, rhamnose, galactose and other glucans. In rhizobia infected plant nodules, the mucous has been shown to accumulate between the peribacteroid membrane and the symbiosome membrane filling up most of the symbiosome volume (Streeter, Salminen, Whitmoyer, & Carlson, 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 mucous are completely dependent on the genotype of the microorganism.

In conclusion, morphological characteristics varied among the 61 isolates obtained from the three agro-ecological regions of Zambia. The colours of colonies were translucent, cream, white, pink and the uncommon yellow. Pink colonies were peculiar to region III. The elevation of all the colonies was convex. Biochemically, all Rhizobia were Gram-negative, mucous 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* or/and *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 and are being recommended here.

Acknowledgments

The authors thank Alliance for Green Revolution in Africa (AGRA) for the financial support towards Rachael Kapembwa's MSc research. We are also grateful for the support from Mr Muma Chashi, the research assistant and Ms. Charity Nachalwe the technician in the Soil Microbiology Lab of the University of Zambia.

References

- Allen, E. K., & Allen, O. N. (1961). The Scope of Nodulation in the Leguminosae. In: Recent Advances in Botany. *Proceedings of the Ninth International Botanical Congress*. University of Toronto, (Vol. 1, pp. 585-588).

- Aneja, K. R. (1996). Experiments for Microbiology, Plant Pathology and Tissue Culture. WishwaPrakashan, New Age International (P) Limited, New Delhi. pp. 190-212.
- Barker, A. V., & Pilbeam, D. J. (2007). Handbook of Plant Nutrition. Taylor and Francis Group, LLC. London.
- Bisset, K. A. (1959). Some characters of rhizobium strains from tropical legumes. *Journal of General Microbiology*, 20(1), 89-90. <http://dx.doi.org/10.1099/00221287-20-1-89>
- Bremner, J. M., & Mulvaney, C. S. (1982). Nitrogen-Total. In: Page, A. L., Miller, R. H., & Keeney, D. R. (Eds.). *Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties* (2nd Ed.). ASA, SSA, Madison, Wisconsin, USA.
- Chen, W. M., Laevens, S., Lee, T. M., Coenye, T., Vos, P. D., & Mergeay, M., et al. (2001). *Ralstonia taiwanensis* sp nov. isolated from root nodules of mimosa species and sputum of a cystic fibrosis patient. *International Journal of Systematic & Evolutionary Microbiology*, 51(Pt5), 1729-1735. <http://dx.doi.org/10.1099/00207713-51-5-1729>
- CSO/MAL. (2013). *Crop Forecast Survey*. Central Statistics Office (CSO). Lusaka.
- Danso, S. K. A., Hera, C., & Douka, C. (1987). Nitrogen fixation in soybean as influenced by cultivar and rhizobium strain. *Plant & Soil*, 99(1), 163-174. <http://dx.doi.org/10.1007/BF02370163>
- Day, P. R. (1965). Particle fractionation and particle size analysis. In Clark, C. A. (Eds.) *Methods of Soil Analysis, Agronomy 9, part1*, 545-567, Am. Soc. Agron. Madison, Wis, U.S.A.
- Deka, A. K., & Azad, P. (2006). Isolation of rhizobium strains: cultural and biochemical characteristics. *Legume Research - An International Journal*, 29(3), 209-212.
- Dixit, A. K., Antony, J. I. K., Navin, K., Sharma, N. K., Rakesh, K., & Tiwari, R. K. (2011). Soybean Constituents and their Functional Benefits. In Tiwari, V., & Mishra, B. (Eds.) *Opportunity, Challenge, and Scope of Natural Products in Medicinal Chemistry*. Kerala, India: Research Signpost.
- Dubey, R. C., & Maheshwari, D. K. (2006). *Practical Microbiology*. S. Chand and Company Ltd. 7361, Rans Nagar, New Delhi.
- Fairhurst, T. (2012). *Handbook for Integrated Soil Fertility Management*. Africa Soil Health Consortium. Nairobi.
- Gachande, B. D., & Khansole, G. S. (2011). Morphological, cultural and biochemical characteristics of rhizobium japonicum syn and bradyrhizobium japonicum of soybean. *Bioscience Discovery Journal*, 2, 1-4.
- Graham, P. H., Draeger, K. J., Ferrey, M. L., Conroy, M. J., Hammer, B. E., & Martinez, E., et al. (1994). Acid ph tolerance in strains of rhizobium, and bradyrhizobium, and initial studies on the basis for acid tolerance of rhizobium tropici, umr1899. *Canadian Journal of Microbiology*, 40(3), 198-207. <http://dx.doi.org/10.1139/m94-033>
- Hardarson, G., & Broughton, W. L. (2003). *Maximizing the use of Biological Nitrogen Fixation in Agriculture*. Kuwer Academic Publishers and Food and Agriculture Organisation of the United Nations International Atomic Energy Agency.
- Hassen, A. I., Bopape, F. L., Rong, I. H., & Sean, G. (2014). Nodulation efficacy of bradyrhizobium japonicum strain wb74 on soybean (glycine max l. merrill) is affected by several limiting factors. *African Journal of Microbiology Research*, 8(20), 2069-2076. <http://dx.doi.org/10.5897/ajmr2014-6709>
- Herridge, D. (2002). Inoculants and Nitrogen Fixation of Legumes in Vietnam. ACIAR Proceedings 109e.
- IUSS Working Group WRB. (2007). *World Reference Base for Soil Resources 2006, First Update 2007*. World Soil Resources Reports No. 103. FAO, Rome.
- Kaur, K., Sharma, P., Kaur, N., & Gill, B. S. (2012) Phenotypic and Biochemical Characterization of *Bradyrhizobium* and *Ensifer* spp. Isolated from Soybean Rhizosphere. *Bioscience Discovery*, 3(1), 40-46.
- Li, Q. Q., Wang, E. N., Chang, Y. L., Zhang, Y. Z., Zhang, Sui, X. H., Chen, W. F., & Chen, W. X. (2011). *Ensifer sojae* sp. Nov., isolated from root nodules of *Glycine max* grown in saline-alkaline soils. *International Journal of Systematic and Evolutionary Microbiology*, 61(8), 1981-1988. <http://dx.doi.org/10.1099/ijms.0.025049-0>
- Lubungu, M., Burke, W. J., & Sitko, N. J. (2013). *Analysis of the Soya Bean Value Chain in Zambia's Eastern Province*. Working Paper 74. Indaba Agricultural Policy Research Institute (IAPRI). Lusaka. Retrieved April 1, 2016, from <http://www.iapri.org.zm/research-reports/draft-working-papers>

- Mathis, J. N., Israel, D. W., Borbour, W. M., Jarvis, B. D. W., & Elkan, G. H. (1986). Analysis of the Symbiotic Performance of *Bradyrhizobium japonicum* USDA 110 and Its Derivative I-110 and discovery of a New Mannitol-Utilizing, Nitrogen-Fixing USDA 110 Derivative. *Applied and Environmental Microbiology*, 53(1), 75-80.
- Miti, M. J. (1997). *Zambia Seed Technology Handbook* Sweden, Berlings, 1.
- Mweetwa, A. M., Malama, M., Mulilo, X., Ngulube, M., Banda, J. S. K., Kapulu, N., & N'gandu, S. H. (2014). Response of cowpea, Soya Beans and Groundnuts to Non-Indigenous Legume Inoculants. *Sustainable Agriculture Research*, 3(4), 84-95. <http://dx.doi.org/10.5539/sar.v3n4p84>
- Olsen, S. R., & Sommers, L. E. (1982). Phosphorus. In: Page, A. L., Miller, R. H., & Keeney, D. R. *Methods of Soil Analysis, Part 2, 2nd edition*, Agron. Monogr 9. ASA and ASSA, Madison WI. pp. 403-430.
- Radtke, M. L. (2003). Environmental influences of *Bradyrhizobium* sp. Survival and nodulation potential on peanut (Master's thesis, Texas Tech University, Lubbock, Texas). Retrieved April 4, 2016, from <https://repositories.tdl.org/tdl-ir/handle/2346/19500>
- Sadowsky, M. J., Keyser, H. H., & Bohlool, B. B. (1983). Biochemical Characterization of Fast- and Slow-Growing Rhizobia that Nodulate Soybeans. *International Journal of Systematic Bacteriology*, 33(4), 716-722. <http://dx.doi.org/10.1099/00207713-33-4-716>
- Saeki, Y., Ai, K., Hara, T., Suzuki, K., Yamakawa, T., & Nguyen, M. T., et al. (2005). Phylogenetic analysis of soybean-nodulating rhizobia isolated from alkaline soils in vietnam. *Soil Science & Plant Nutrition*, 51(7), 1043-1052. <http://dx.doi.org/10.1111/j.1747-0765.2005.tb00143.x>
- Sayed, R. Z., Jamadar, D. D., & Patel, P. R. (2011). Production of Exo-polysaccharide by *Rhizobium* sp. *Indian Journal of Microbiology*, 51(3), 294-300. <http://dx.doi.org/10.1007/s12088-011-0115-4>
- Sharma, M. P., Srivastava, K. & Sharma, S. K. (2010). Biochemical characterization and metabolic diversity of soybean rhizobia isolated from Malwa region of Central India. *Plant Soil & Environment*, 56(8), 375-383.
- Shiro, S., Matsuura, S., Saiki, R., Sigua, G. C., Yamamoto, A., & Umehara, Y., et al. (2013). Genetic diversity and geographical distribution of indigenous soybean-nodulating bradyrhizobia in the united states. *Applied & Environmental Microbiology*, 79(12), 3610-3618. <http://dx.doi.org/10.1128/AEM.00236-13>
- Somasegaran, P., & Hoben, H.J. (1985). *Methods in Legume- Rhizobium Technology*. Springer-Verlag, New York, Inc. pp. 1-450.
- Somasegaran, P., & Hoben, H. J. (1994). *Handbook for Rhizobia Methods in Legume- Rhizobium Technology*, springer Verlag, New York, USA. pp. 58-101.
- Streeter, J. G., Salminen, S. O., Whitmoyer, R. E., & Carlson, R. W. (1992). Formation of novel polysaccharides by bradyrhizobium japonicum bacteroids in soybean nodules. *Applied & Environmental Microbiology*, 58(2), 607-613.
- Tefera, H. (2011). Breeding for Promiscuous Soybeans at IITA, Soybean - Molecular Aspects of Breeding. In Dr. Aleksandra Sudaric (Ed.), Tech ISBN: 978-953-307-240-1. Retrieved April 1, 2016, from <http://www.intechopen.com/books/soybean-molecular-aspects-of-breeding/soybean-molecular-aspects-of-breeding-for-promiscuous-soybeans-at-iita>
- Van Reeuwijk, L. P. (1992). *Proceedings for soil Analysis* (3rd Ed.) International soil Reference and Information Center (ISRIC), Wageningen, the Netherlands.
- Wagner, S. C., Skipper, H. D., & Hartel, P. G. (2011). Medium to study carbon utilization by bradyrhizobium strains. *Canadian Journal of Microbiology*, 41(7), 633-636. <http://dx.doi.org/10.1139/m95-085>
- Yan, J., Han, X. Z., Li, Y., Wang, E. T., Xie, Z. H., & Chen, W. F. (2014). Abundance and Diversity of Soybean-Nodulating Rhizobia in Black Soil Are Impacted by Land Use and Crop Management. *Applied and Environmental Microbiology*, 80(17), 5394-5402. <http://dx.doi.org/10.1128/AEM.01135-14>

Copyrights

Copyright for this article is retained by the author(s), with first publication rights granted to the journal.

This is an open-access article distributed under the terms and conditions of the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/3.0/>).