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Isolation and Screening of Indigenous Bambara Groundnut (Vigna Subterranea) Nodulating Bacteria for their Tolerance to Some Environmental Stresses

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Abstract Environmental stresses are important limiting factors for crops production. The aim of this experiment is to isolate Legume Nodulating Bacteria (LNB) obtained from root nodules of bambara groundnut (Vigna subterranea L.) plants and evaluate their performance under some environmental constraints. Samples were collected in Cameroon from three location sites of the Humid-forest zone: Logbessou in the Littoral region; Mfoua in the South and Boga in the Centre region. Nodulation of bambara groundnut was examined in plastic bags and root nodules were collected from seedling. After their isolation, the bacteria were confirmed as LNB by re-nodulating Macroptilium atropurpureum. The morphological, cultural and phenotypic characteristics (utilization of carbon, tolerance to salt, pH, aluminium) of isolates were determined. The results obtained were analyzed statistically by ANOVA using the software SPSS analysis version 11.5. Duncan test was used to measure the difference among the means at a level of $p<0.05$. A collection of 18 isolates was obtained on Yeast Extract Mannitol Agar medium. Authentication experiments, confirmed that the majority of the isolates (66.67%) were LNB due to their ability to infect the host plant. Bambara groundnut isolates are different morphologically. Dendrogram of the phenotypic characteristics showed that, below the boundary level of 50% average similarity, isolates fell into at least three distinct groups. All isolates showed fast-growing capacity. Most isolates (66.67%) were able to grow in a medium with pH as low as and Al concentration of 50 µM (58.33 %). Some isolates (50%) showed weak growth capacity at 4% NaCl. The bambara groundnut isolates tested were able to use a broad range of carbohydrates as sole source of carbon. The isolates from the present study may be useful to increase the symbiotic nitrogen fixation in legume.

Keywords: *bambara groundnut, isolation, legume nodulating bacteria, phenotypic characterization*

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1. Introduction

Nitrogen and phosphorus are the most limiting nutrients for crop production in many soils in the tropics (Dogbe et al., 2002). In Cameroon, acid soils cover more than 80% of arable lands (The, 2000). Synthetic fertilizers for improving soil fertility are rarely available to most farmers. In addition, these fertilizers may induce soil acidification and become less efficient after many cropping years (Bado, 2002), leading to a high dependence of soil to N fertilizer for optimum yield (Fening and Danso, 2002). Since fallow practice to restore soil fertility is no longer possible because of land scarcity, there is a need of more efficient practices. Phosphate solubilizing bacteria are one of the alternative to improve plant growth in these soils (Fankem et al. 2014a; Fankem et al. 2014b). *Rhizobium* inoculants significantly improves yield in many leguminous crops and can minimize the use of synthetic nitrogenous fertilizer, which is rather expensive and deteriorates soil properties.

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is the third most important food legume after groundnut (*Arachis hypogea*) and cowpea (*Vigna unguiculata*) in Africa (Bamshaiye et al., 2011). Hillocks et al. (2012) reviewed the contribution of research towards developing the utlisation, market potential and crop improvement of bambara nut. As a nitrogen-fixing legume, bambara also contributes to the maintenance of soil fertility. *Vigna subterranea* possess high crude protein content between 22 and 37% (Adeparusi, 2001; Fasoyiro *et al*., 2006). It is

widely grown in Nigeria and in other African countries like Ghana, Cameroon, Ivory Coast and Togo (Klu et al., 2001). The crop is an indigenous African legume, which is mainly grown for food (Azam-Ali et al., 2001) and nodulates with cowpea-type bradyrhizobia (Dakora and Muofhe, 1997).

Bambara groundnut is a food legume that is under-researched and under-utilized in Africa. Various studies evaluated nitrogen fixation in other legumes (i.e groundnut, soybean, cowpea) in Africa (Belane and Dakora 2009; Pule-Meulenberg and Dakora 2009). However, there are very few on nitrogen fixation in bambara groundnut (Kishinevsky et al. 1996; Nyemba and Dakora, 2010; Mohale et al., 2014). According to Padulosi et al. (2002) the potential of neglected and under-utilized crops such as bambara groundnut could be exploited for overcoming food deficits in the continent. In Cameroon, there is limited research result on the morphological characterization of some local varieties (Ndiang, 2012), on the varietal resistance of *Vigna subterranea* to insect pest in the far North Region (Kouninki *et al*. 2014), and on nitrogen fixation and assessing this species potential as a biofertilizer (Ngakou et al., 2012). Particularly in the humid forest zone, nothing has been done on the nitrogen fixation of bambara groundnut. Dakora and Muofhe (1997) have indicated the potential for increasing yields in bambara groundnut through enhancement of symbiotic nitrogen fixation. According to Yakubu et al. (2010a) bambara groundnut has a high nitrogen-fixing potential provided that an effective *Rhizobium* strain is used and that the plant nutrient requirements other than nitrogen are met. However, environmental constraints, such as soil and salinity, water deficit, phosphorus deficiency and soil pH, recorded in many regions of world, are still the main limiting factors for the productivity of leguminous plants and their symbiotic nitrogen fixation (Faghire et al., 2011; Farissi et al., 2014; Bargaz et al., 2013; Farissi et al., 2013). Inoculation of stress tolerant strains of LNB may enhance the nodulation and nitrogen fixation ability of plants under stress conditions. A study of Ngakou et al. (2012) reported that inoculating bambara groundnut with *Rhizobium* and mycorrhiza significantly improved nodule number and nodulation efficiency by 64 and 80%, the seed weight by 52%, the plant biomass by 30%, as compared to un-inoculated treatment. Nevertheless, the fertility problem can be partly solved through use of efficient rhizobial inoculant, the characteristics of indigenous soil rhizobial strains in different regions are not known. These reflect the need for screening rhizobial isolates which are efficient and adaptable to different soils. Therefore, biological nitrogen fixation should be more exploited to increase nitrogen for bambara groundnut. The objective of this work is to isolate Legume Nodulating Bacteria (LNB) obtained from root nodules of bambara groundnut plants and evaluate their performance under some environmental constraints.

2. Materials and Methods

2.1. Soil Sampling and Analysis

Experiments were carried out in soil samples (2 kg plastic bags) collected according to Swift *et al*. (2001) from three different sites located in two agroecological zone of the humid forest zone of Cameroon, which have no history of *Vigna subterranea* L. inoculation. These sites are: Logbessou in the Littoral region of Cameroon; Mfoua in the South region of Cameroon and Boga in the Centre region of Cameroon. Within each site, soils were collected in the mixed farming. A composite soil sample was a mixture of ten sub-samples all collected from field. Aseptic precautions were taken during sampling and handling of each soil to avoid contamination. Soil sample were taken from sites that had no previous history of LNB inoculation. The composite samples were taken from the top 0 to 20 cm soil depth, from which the un-decomposed plant material were removed by hand. The physico-chemical analysis of the soil was conducted in the Institute of Agricultural Research and Development (IRAD) using standard methods (Anderson and Ingram, 1993).

2.2. Assessment of Nodulation and Growth of *Vigna Subterranea*

Vigna subterranea seeds of the WAK1 variety (Figure 1) obtained from the "Laboratoire de Génétique et d'Amélioration des plantes" at the University of Yaounde I were used for trapping LNB. Nodulation was assessed by growing germinated seed in perforated plastic bags containing more than 2 kg of soil samples. Seeds were surface sterilized in 3.3% calcium hypochlorite solution for 3 min and washed thoroughly with sterile water, then re-suspended in 70% ethanol for 3 min and washed thoroughly with sterile water. The seeds were incubated to germinate in sterile Petri dishes containing sterilized distilled water for 3 days at 28°C. After germination, two seedlings were sown in each plastic bag. One week after germination, the number of plants was thinned to one per plastic bag. Plants were watered and harvested 6 weeks after sowing. The root systems of individual plants were washed separately, their nodules picked and counted. Plant shoot and roots systems were let to dry at 70°C for more than 72 h in an air dry oven and then weighed until constant mass (Athar and Johnson, 1996). Nodule fresh weights, nodule color, and leaf colors (yellow, green) were determined from each plastic bag.

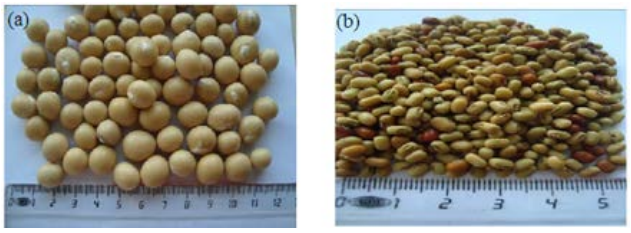

Figure 1. *Vigna subterranea* (a) and *Macroptilium atropurpureum* (b) seeds used in the study

2.3. Isolation of Legume Nodulating Bacteria

LNB were isolated from individual root nodules using standard method as described by Somasegaran and Hoben (1994). Strains were maintained routinely on yeast extract mannitol agar (YEMA) (Vincent, 1970). Root nodules of *Vigna subterranea* were used to isolate legume nodulating bacteria (LNB) using standard method as described by Somasegaran and Hoben (1994); Vincent (1970). Plants were collected from different agroclimatic regions of

Dehradun. Plants were uprooted carefully and nodules were collected from the roots, washed with sterile water followed by surface treatment with 95% alcohol and again with sterile water. The washed nodules were surface sterilized with 0.1% mercuric chloride for 30s and again washed at least 10 times with sterile water to remove the traces of mercuric chloride. The nodules were transferred in culture tube half filled with sterile water for 24h and crushed in a drop of sterile water to obtain a milky bacterial suspension. After serial dilution suspension was streaked on Yeast extract mannitol agar (YEMA) plates supplemented with Congo red to differentiate the bacterial contaminants from LNB.

Plates were incubated at 28°C for 7 days. The purity of the cultures was verified by repeated streaking of a single-colony isolate onto YEMA medium. Each isolate was re-streaked for more purification, and then single colony was selected and streaked on YEMA slants containing Congo red (0.0025 % p/v) and bromothymol blue to ensure purity for further studies. All bacterial isolates were maintained at 4°C on YEMA. After 1 to 3 days of incubation, colonies were obtained. Pure isolates were used for further analysis and all tests were performed. The colony morphology and purity of isolates were examined on YEMA-Congo red plates after an incubation of 5 days at 28°C. Individual colonies were characterized based on their color, shape, colony diameter (Vincent, 1970). Single colonies were also subjected to the Gram staining. The production of acid and alkali was determined in YEM medium with bromothymol blue (25 mg/l) (BTB) plates (Somasegaran and Hoben, 1994). All plates were incubated for 7 days at 28°C. Colour change around growing colonies on YEMA+BTB plates was considered for acid (yellow) or alkali (blue) production by the rhizobial strain.

2.4. Authentication of Isolates

The collected isolates were subjected to authentication test before performing any experiment. All isolates were tested to confirm their ability to nodulate *Macroptilium atropurpureum* under axenic condition. Seeds of *Macroptilium atropurpureum* were scarified and sterilized by immersing in concentrated H_2SO_4 for 5 min, thoroughly rinsed with sterile water. Seeds were distributed on the surface of 0.9% agar plates, then incubated for 1–2 days at 28°C for germination. After germination, seedlings were transferred into sterilized tubes containing autoclaved sand under sterile conditions. Suspension of 18 bambara groundnut isolates grown in YM medium were inoculated onto the roots of plants.. Three days after planting, seedlings were inoculated by adding 1 ml of rhizobial cultures at the middle of the exponential growth phase with about 10^8 cells ml^{-1} for each tube. Seedlings were watered weekly with sterile Jensen nutrient solution of the following composition: H_2O: 1000 ml; K_2HPO_4: 0.2 g; $MgSO_4.H_2O$: 0.2 g, NaCl: 0.2 g; $CaHPO_4$: 1 g; $FeCl_3$. H_2O: 0.14 g; H_3BO_3: 2.86 mg; $MnSO_4$. $4H_2O$: 2.03 mg; $ZnSO_4$. $7H_2O$: 0.22 mg; $CuSO_4$. $5H_2O$: 0.08 mg; Na_2MoO_4. H_2O: 0.09 mg. pH of the nutrient solution was adjusted to 6.5. Pots of uninoculated seedlings were used as controls. Plants were cultivated in controlled growth chambers at 28°C with 16 h in the light and 8 h in the dark. Light intensity was 120 $\mu mol.m^{-2}.s^{-2}$. Six weeks after inoculation, plants were removed from the tubes and the presence or absence of nodules assessed. Only isolates which produced nodules on *Macroptilium atropurpureum* plants were used to stock on YEMA slants and maintained in growth chamber at 28 ± 2°C for further study.

2.5. pH Tolerance of LNB

The ability of the LNB to grow in acidic media was tested by streaking them on YEM plates by adjusting the pH to 3.5, 4.5, 5.5 and 6.5 with HCl or NaOH. All the plates were incubated at 28°C for 7 days and YEM medium plates were used as controls. The effect of pH on the growth of the strains was assessed after 7 days incubation period, by observing the appearance of colonies on solid YEM. All assays were done in duplicate. Growth experiment was also performed in YEM broth. Growth of rhizobial strains was compared at different pH (3.5, 4.5, 5.5 and 6.5) in YEM medium. Hydrochloric acid (HCl) was used to adjust lower pH and NaOH was used to adjust higher pH in medium. All the 12 isolates were multiplied in YEM broth and 1 ml of multiplied rhizobial culture (about 10^8 rhizobial cells/ml) used as standard inoculum introduced into 50 ml YEM broth flask, adjusted to different pH. The inoculated broth were incubated at 28°C and kept at 120 rev. min^{-1} in an incubator shaker. Growth was determined by measuring the optical density (OD) at 470 nm after 5 days of incubation using a spectrophotometer.

2.6. Al Tolerance of LNB

Difference in Al tolerance were investigated by incubation of bacterial cultures in YEM agar containing 50 µM, 100 µM, et 150 µM ($AlCl_3$, $6H_2O$). Control plates were incubated at 28°C. The effect of Al on the growth of the strains was assessed after 7 days incubation period, by observing the appearance of colonies on solid YEM. All assays were done in duplicate. Growth experiment was also performed in YEM broth. The OD was measured after five days of incubation at 470 nm using a spectrophotometer.

2.7. Effects of Salt Concentrations on the Growth of LNB

The ability of the rhizobial isolates to grow in different concentration of salt was tested by streaking them on YEMA medium containing 0%, 1%, 2%, 3%, 4%, and 5% (wt/v) NaCl. NaCl was added to the media before autoclaving. Isolates isolates were inoculated on Petri dishes in duplicate on each NaCl concentration and grown for 7 days. The effect of NaCl concentrations on the growth of the strains was assessed after 7 days incubation, by observing the appearance of colonies on solid YEM. The effect of NaCl concentrations on the growth of the strains was assessed after 5 days incubation period, by determining the absorbance at 470 nm in a mineral liquid medium containing per litre: mannitol: 10 g; K_2HPO_4: 0.5 g; NaCl: 0.1 g; $MgSO_4$, $7H_2O$: 0.2 g; yeast extract: 0.5 g; by adjusting the pH to 6.5. NaCl was added to the media before autoclaving to give the following concentrations. All assays were done in duplicate. The presence or absence of growth was evaluated after the incubation

period, and only isolates presenting positive growth in all replications were considered to show positive growth.

2.8. Use of Carbohydrate as Carbon Source

All isolates were tested for their ability to utilize some carbon sources. The use of different carbon sources was tested on YEMA medium by replacing the mannitol with the carbohydrate. The carbohydrates tested were glucose, glycerol, sucrose, starch, and mannitol. The medium used for this test was a carbohydrate-free medium **(**Somasegaran and Hoben 1994**),** yeast extract was reduced to 0.05 g. l^{-1}. Separately autoclaved sugars were added to the modified YEMA basal medium (depleted of mannitol) to a final concentration of 1 % (w/v). Duplicate plates were used for each different sugar. Two control media were used for comparison, the standard medium containing mannitol and the C-free YMA basal medium. After inoculation, plates were incubated at 28°C for 5 days and visual growth was recorded. All the plates were streaked with the freshly prepared liquid culture of each of the rhizobial isolates, and then incubated at 28°C for 5 days. Growth response of different isolates was recorded positive (visible growth) or negative (no growth**)**. The use of different carbon sources was also tested on YEM broth in which yeast extract was reduced to 0.05 g. l^{-1}. The medium was then inoculated by the addition of exponentially (10^8 cells/ml) growing cultures of the isolates. The inoculated broth were incubated at 28°C and kept at 120 rev. min^{-1} in an incubator shaker. Growth was determined by measuring the OD at 470 nm after 5 days of incubation.

2.9. Statistical Analysis

The statistical experimental result analyses obtained have been done with the help of the ANOVA test while using the statistical package for social sciences (SPSS) analysis software version 11.5. Duncan test was used to measure the difference among the means at a level of $p<0.05$. Isolates were grouped using Pearson correlation through software XLSTAT.

3. Results

3.1. Physico-chemical Characteristics of Soils

Soil pH of the three sites ranged from 4.13 at Mfoua to 5.72 at Logbessou, while soil organic matter varied from 1.97% at Mfoua to 2.39% at Logbessou. The total mean soil N value of the soil sample was low, 0.80% and 0.13% at Logbessou and Boga respectivily. The available P content was < 50. The results of the soils analyzes of the three studied sites showed that the soils all the sites are acidic, with low nutrients. The results of the soils analyzes of the three studied sites showed that the soils of Boga, Logbessou and Mfoua are clay-loam, sandy texture and loamy respectivily.

3.2. Bambara Groundnut Nodulation

The spontaneous nodulation of bambara groundnut indicates the presence, in the three soils, of native populations of bacteria able to colonize the roots. Results on nodulation showed that Logbessou had the highest ($P \leq 0.05$) nodule number at an average of 102 nodules per plant. It differed significantly from Mfoua at $P \leq 0.05$, which had averages of 28 nodules per plant. Logbessou had the nodule fresh weight (1.9g / plant) and was significantly different from the other two sites at $P \leq 0.05$. The dry weight of the air system is significantly higher in Logbessou (3.5 g), Boga (1.1 g) and Mfoua (1.1 g) that there were no significant differences. Dry weight of root system, fresh and dry weight of the plant are significantly higher at Logbessou compared to other sites.

3.3. Isolation, Purification and Authentication

Rhizobial isolates were obtained from all sampling sites. A total of 18 isolates were isolated from root nodules of *Vigna subterranea* collected from the three sites and purified. The 18 rhizobial isolates from root nodule's plants were tested for their ability to nodulate roots (authentication as LNB). The effective nodulation observed with all rhizobial isolates clearly indicated that 12 isolates were able to to nodulate *Macroptilium atropurpureum*. It was noted that the nodules were pink, indicating leghemoglobin content, and the leaves of the nodulated plants were dark-green, while uninoculated unfertilized control plants were yellow without nodules. White nodules were also recorded. Six isolates out of the 18 test isolates did not form nodules and were eliminated from further consideration. A total of 12 isolates were confirmed as LNB after the authentication test in tubes experiment using sterile sandy soil under controlled environmental conditions. Amongst these isolates, 02 were obtained from Boga (VsBo3, VsBo4), 07 (VsLo1, VsLo2, VsLo3, VsLo4, VsLo5, VsLo6, VsLo7) were from Logbessou and 03 (VsMf4, VsMf5, VsMf7) from Mfoua. Rhizobial isolates were nomenclatured so as to indicate the name of the legume (Vs-*Vigna subterranea*), the site of origin (Bo-Boga, Lo-Logbessou, Mf-Mfoua) and isolate numbers (numeric figure given in the end).

3.4. Morphological Characteristics of LNB

Differences between isolates were verified using some morphological parameters. All isolates were subjected to Gram staining and microscopic observation showed that the studied isolates were all Gram negative and did not absorb Congo red upon incubation in dark room in 3-**7** days time. General microscopic view of the isolates showed them to be Gram negative in nature. Except VsLo1 (rod-shaped), the majority of the cells are coccus (Table 1). Colonies usually cream or white with circulair shape, convex with diameters ranging from ≤ 2 to ≥ 2 mm at the age of 3 days in medium Yeast Extract Mannitol Agar (YEMA). These isolates showed the capacity to acidify the culture medium after one to three days of incubation at 28°C.

LNB isolates from bambara groundnut were considered as fast-growing strains with growth occurring 72 h after incubation. Twelve isolates were fast growing (appearance of colonies 1 to 3 days after incubation). The colonies are circular, only VsLO3 is oval, convex and flat. The isolates did not absorb red colour when cultured in YEMA containing congo red medium. All the isolates have acidified the YMA + bromothymol blue after 24 h incubation (the indicator turned yellow). LNB isolated from Bambara groundnut were characterized on YEMA

Congo red solid medium by comparing the elevation, aspect, edge, surface and colour of colonies (Table 1). The elevation was convex for eight of the isolated colonies and flat for four isolates. The edges of colonies were irregular, except for four colonies from Logbessou and Mfou soil which had irregular edge. Regarding the color of colonies, except VsBo4 and VsLo1 (white color), the remaining isolates displayed milky appearance in 3 days of incubation.

Table 1. Growth, colony characteristics and cell morphology of isolates from bambara groundnut

Isolates	Shape of the colony	Color	Elevation	Edge of the colony	Colony size (mm)	Growth	Shape of the cell	Gram stain	pH reaction	YEMA-BBT
VsBo3	Circulair	Creamy	Convex	Irregular	≤ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsBo4	Circulair	White	Flat	Irregular	≤ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsLo1	Circulair	White	Flat	Irregular	≤ 2 mm	Fast	Rod	Negative	Acid	Yellow
VsLo2	Circulair	Creamy	Convex	Irregular	≥ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsLo3	Oval	Creamy	Convex	Regular	≥ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsLo4	Circulair	Creamy	Flat	Irregular	≤ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsLo5	Circulair	Creamy	Convex	Irregular	≤ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsLo6	Circulair	Creamy	Convex	Irregular	≤ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsLo7	Circulair	Creamy	Convex	Regular	≤ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsMf4	Circulair	Creamy	Flat	Irregular	≥ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsMf5	Circulair	Creamy	Convex	Regular	≤ 2 mm	Fast	Coccus	Negative	Acid	Yellow
VsMf7	Circulair	Creamy	Convex	Regular	≤ 2 mm	Fast	Coccus	Negative	Acid	Yellow

The dendrogram based on the morphological characteristics of the colonies, placed these isolates in three distinctive groups separated at 50% similarity level (Figure 2). Group 1 was the largest, with eight isolats (VsMf4, VsLo4; VsLo5, VsBo3, VsLo6, VsLo7, VsMf5, VsMf7) origninating from different locations. These isolates are fast growing. Their colony diameter was ≤ 2 mm after 3 days on YEMA at 28°C. All the isolates in group 1 produced acid in YEMA, and are creamy, coccus, negative, yellow. Group II consisted of two fast-growing isolates (VsLo2 and VsLo3**)** originating from one soil (Logbessou). All isolates in group II are creamy, convexe, fast growth, coccus, negative and acid producing bacteria. Their colony diameter was ≥ 2 mm after 3 days on YEMA at 28°C. Isolates belonging to group III consisted also of two fast-growing isolates (VsBo4 and VsLo1**)** originating from two different soils (Logbessou and Boga). These isolates were characterized by white and circulair, flat and rregular colonies. Their colony diameter was ≤ 2 mm after 3 days on YEMA at 28 °C. VsLo1 and VsBo4 had diverse origin but clusered together in the dendrogram.

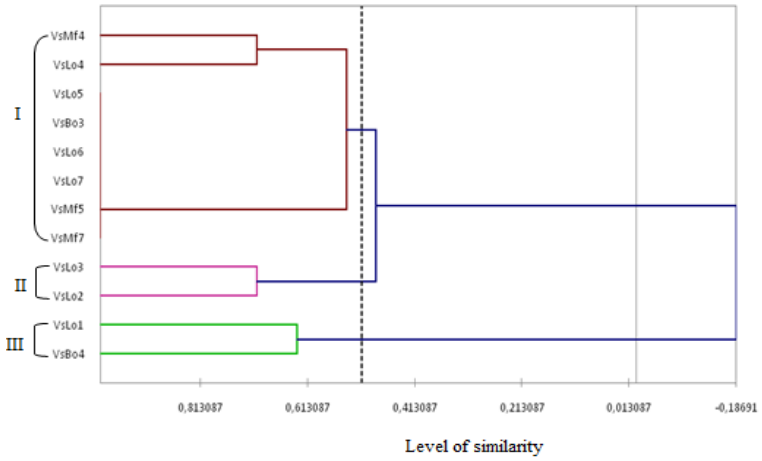

Figure 2. Dendrogram showing the phenotypic similarities among the bambara groundnut isolates obtain from nodules

3.5. pH Tolerance

When evaluating the complementary phenotypic characteristics, it was observed that all 12 rhizobial isolates evaluated were able to grow in YMA media with the pH adjusted to 6.5, while approximately 91.67% of these bacteria were able to grow in YMA media with the pH adjusted to 5.5; 66.67 % were also able to grow in a medium with the pH decreased to 3.5 and 4.5 (Figure 3). Isolates tested showed significant differences in their ability to grow in a medium with the pH 3.5 to 6.5. Four rhizobial isolates VsBo4, VsLo1, VsLo4 and VsMf4 did not show any growth at pH 3.5 and 4.5. Isolate VsBO4 was able to grow only on the medium adjusted to pH 6.5, whereas isolates VsLo1, VsLo4 and VsMf4 were able to grow at pH of the medium adjusted to 5.5 and 6.5.

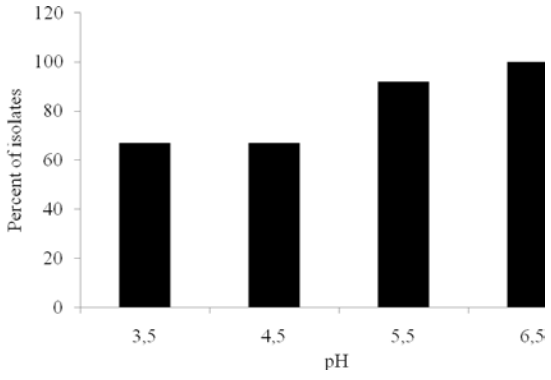

Figure 3. Tolerance of bambara groundnut nodulating bacteria to different pH in solid medium

Seven isolates (VsBo3, VsLo2, VsLo3, VsLo5, VsLo6, VsMf5 andVsMf7) were able to grow in all the various media adjusted to pH 3.5 to 6.5. At pH 3.5, eight isolates were able to survive as maximum value (0.33) of OD was observed for VsLo7. Maximum value (1.66) of OD at pH 6.5 was observed for VsMf4 (Figure 4). A considerable increase of the OD with an increase in pH is observed. The inhibitory effect of pH on the growth of the LNB is clearly visible at pH 3.5 where only eight isolates are able to survive with a maximum OD (0.33) for VsLo7. At pH 6.5, all isolates showed optimal development (100%). Tolerance to acidity was variable depending on the tested isolate: we noticed a decrease in tolerance from 6.5 to 3.5. Isolates produced acid in YMA containing BTB. In culture medium, the VsMf4 isolate strongly acidified the medium (2.1 units), while this acidification is lower for VsLo3, VsLo6, VsLo7 and VsMf7 (1.1 unit). No isolate from nodules absorbed Congo red.

3.6. Aluminium Tolerance

Generally, the higher concentration of aluminium has higher toxicity to the bacteria. Therefore, the number of isolates tolerant to aluminium decreased when aluminium concentration increased. All isolates (100%) grew at 0 µM Al. About 58.33% of isolates were tolerant to 50 µM of aluminium. Amongst these isolates, 01 (14.28%) was obtained from Boga, 04 (57.14%) were from Logbessou and 02 (28.57 %) from Mfoua. None of the isolates grew on YEMA supplemented with 100 µM Al.

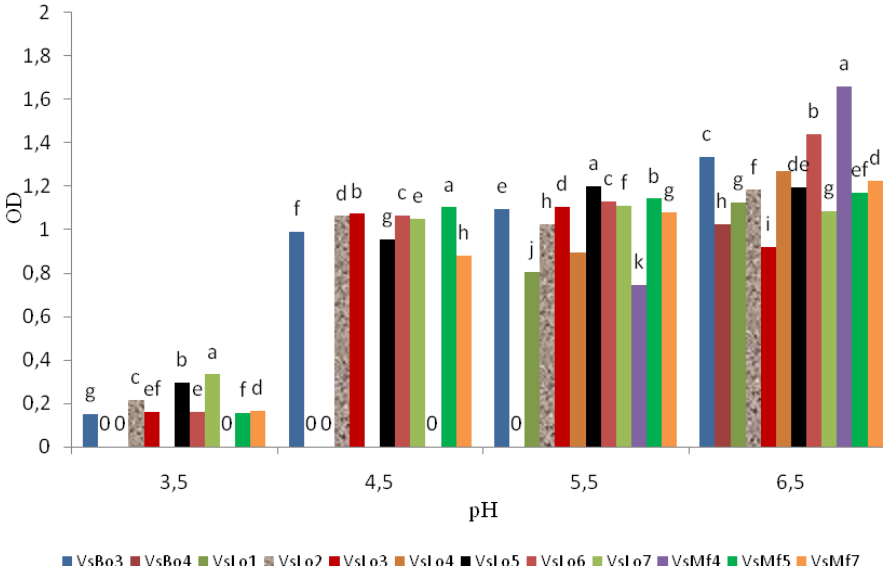

Figure 4. Effect of pH on growth of bambara groundnut nodulating bacteria

Seven isolates (VsBo3, VsLo2, VsLo3, VsLo5, VsLo6, VsMf5 and VsMf7) were able to tolerate 50 µM Al (Figure 5). Conversely, there was no rhizobial isolate able to tolerate 100 and 150 µM Al. The isolates ranked in the following order for aluminium tolerance: VsMf5 > VsLo5 > VsLo2 > VsLo3 > VsBo3 > VsLo6 > VsMf7. VsMf5 is the most tolerant isolate to aluminium toxicity with OD significantly high compared to other isolates. Isolate VsMf7 is the most sensitive to aluminum (OD = 0.05). The results obtained in liquid medium show that the VsMf5 isolate which is the most tolerant at pH 4.5 is also more tolerant of aluminum. Similarly the VsMf7 isolate at pH 4.5 is also more sensitive to aluminium toxicity.

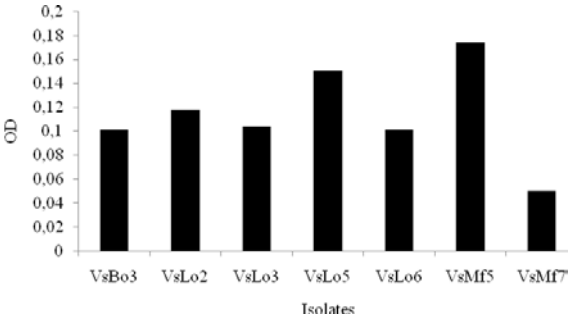

Figure 5. Growth of bambara groundnut nodulating bacteria at 50 µM Al

3.7. Tolerance of Rhizobial Isolates to Salinity

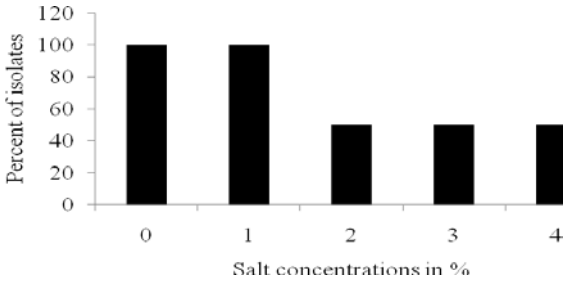

Figure 6. Tolerance of bambara groundnut nodulating bacteria to different concentrations of NaCl

The tolerance of rhizobial isolates to different levels of NaCl in solid medium is presented in Figure 6. All isolates grow in the medium without salt (0% NaCl). The VsMf4 isolate presents significant growth with an OD of 1.66 significantly higher compared to other isolates. Isolate VsLo3 has the lowest growth (OD = 0.87). All isolates grow in the medium without salt (0% NaCl). The VsMf4 isolate presents significant growth with an OD of 1.66 significantly higher compared to other isolates. The VsMf4 isolate presents significant growth with an OD of 1.66 significantly higher compared to other isolates. Isolate VsLo3 has the lowest growth (OD = 0.87). Except

for isolates Vslo6 and VsMf4, which had their greatest OD values at 0% NaCl, all the isolates strains had maximum OD at 1% NaCl. The OD of all isolates was affected significantly by salt concentration, in that the OD decreased with increasing salinity after 1% (Figure 7). VsLo2 (OD = 1.95) is more tolerant than all other isolates. VsLo6 is the most sensitive isolate with an OD = 1.11. At 2% NaCl, growth of all isolates was inhibited. VsLo5 is the most tolerant with a OD = 0.80, while VsLo6 (OD = 0.65) is the most sensitive. At 3% NaCl, VsBo3 (OD = 0.52) was the most salt tolerant isolate and VsLo6 (OD = 0.23) the most sensitive. A significant difference was observed among all isolates. At 4% NaCl, isolate VsBo3 is the most tolerant, while VsLo6 (OD = 0.12) is the most sensitive to this concentration of NaCl. With 5% NaCl concentration, no isolate was developed. Indeed, at 3% and 4% NaCl concentration, the percentage of survival was 50%. However, at 4% of NaCl, the strains become more sensitive to the high concentration of salinity.

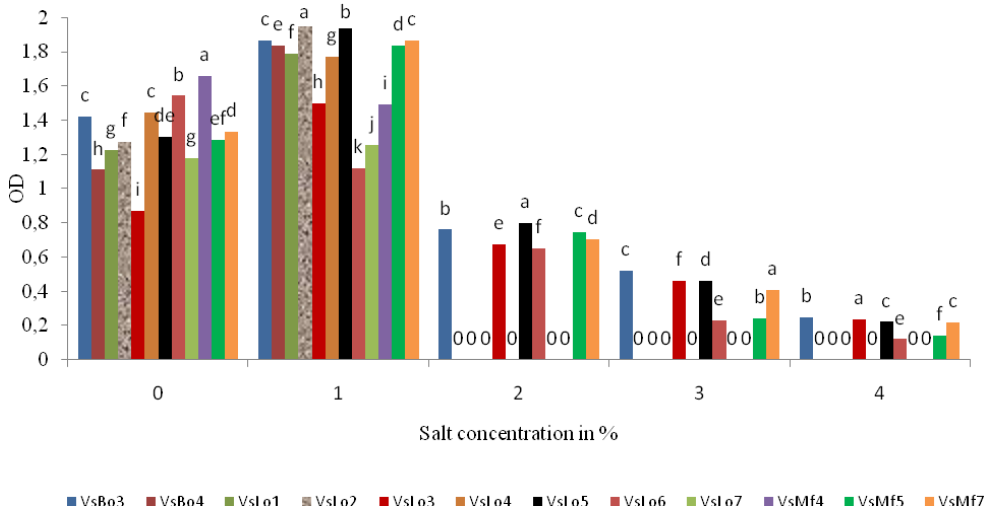

Figure 7. Growth of bambara groundnut isolates at different concentrations of NaCl

3.8. Study under Different Carbon Sources

Growth of the isolates was studied in different carbon sources in medium by replacing mannitol. Results indicate that all strains grew under different carbon sources. All isolates degraded tested sugars: glucose (monosaccharide), sucrose (disaccharide), starch (polysaccharide), mannitol and glycerol (polyols) (Figure 8). The data obtained using YEMA were confirmed by the results obtained using broth cultures. The intensities of utilization varies from one carbon source to another with the isolate tested. Any of the rhizobial isolates tested showed significant differences in their ability to utilize different carbohydrates as a sole carbon source.

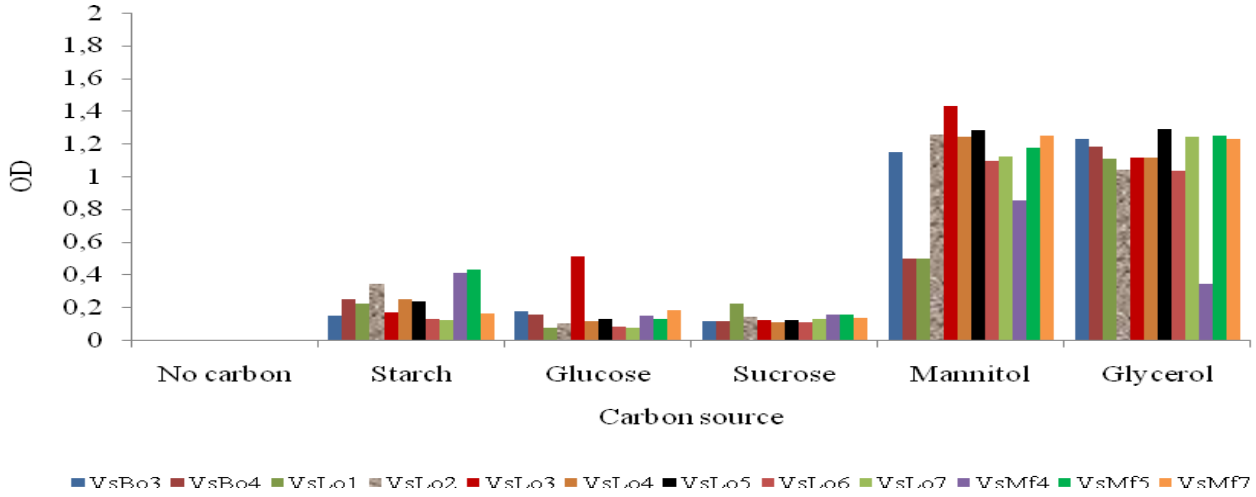

Figure 8. Growth of bambara groundnut isolates on carbohydrate enriched culture medium

Isolates using all the sugars tested with a preference for mannitol and glycerol for most isolates. Isolate VsLo3 showed maximum growth in glucose compared to other isolates, with an OD of 0.51 with a significant difference from the other isolates. Isolate VsMf5 (OD = 0.43) and VsMf4 (0D = 0.41) showed maximum growth in starch compared to other isolates. Isolate VsLo3 showed maximum growth in glucose compared to other isolates, with an OD of 0.51 with a significant difference from the other isolates. Isolate VsLo5 (OD = 1.30) assimilated

more glycerol than other isolates. The least growth of strains was observed in sucrose medium. Response of different carbohydrates: mannitol, glycerol, glucose, sucrose and starch on the growth of rhizobial isolates differed considerably and profuse growth was offered in mannitol and glycerol, poor to moderate growth in glucose, sucrose and starch. For example, for the VsLo3 isolate tested, the following order can be based on the capacity to assimilate carbon substrates: mannitol > glycerol > glucose > sucrrose > starch. In the absence of carbohydrate there was no growth at all.

4. Discussion

Nodulation of leguminous crops by *Rhizobium* largely depends on the presence of a specific and compatible strain in soil for a particular legume. All plants from bambara groundnut developed nodules in all soils studied. There was a significant difference in the nodulation of *Vigna subterranea* between soils obtained from Logbessou and Boga compared to Mfoua. Logbessou had the highest mean nodulation of 102 nodules per plant and plants appeared greener as compared to those in soils from Mfoua. There was no significant difference on mean nodulation between Logbessou and Boga. Bambara groundnut grown on soils from Mfoua had the lowest mean nodulation. These results showed that, although native bambara isolates occure in the soils, the nodulation potential of different sites may vary greatly. Factors like soil pH and mineral composition of the soil, the number of LNB could also have contributed to the low nodulation in the soil from Mfoua.

There was no history of bambara groundnut inoculation in the soils use in this study. The spontaneous nodulation of bambara groundnut indicates the presence of native populations of bacteria able to colonize the roots in the three soils. The natural occurrence of LNB populations able to form symbiosis with bambara groundnut (Mbenou, 1992) and peanut (Ngo Nkot et al., 2011) growing in soils of Cameroon has been reported. Egbe et al. (2013) also observed spontaneous nodulation of bambara groundnut grown in Southern Guinea Savanna of Nigeria. Ample populations of nodulating bacteria are common in the soils of the regions where the legume species are native. On the other hand, nodulation frequently fails when a legume species is planted outside its original region (Bala et al., 2003). Bambara groundnut is an indigenous african leguminous crop cultivated in tropical regions since the seventeenth century. The centre of origin of bambara groundnut is probably north – eastern Nigeria and northern Cameroon. The bambarra groundnut has been reported to be nodulated in various geographical areas (Allen and Allen, 1981). Presence or absence of LNB in the soils was also reported by Ciani and Diriye (1995); Ngo Nkot et al. (2011); Costa et al. (2014).

The ability of forming root nodules with the host plant is the basic test to confirm the purification and assignment of strains to group of LNB. A total of 12 bacterial isolates showed the ability to nodulate *Macroptilium atropurpureum* under bacteriologically controlled conditions. Six of the isolates failed to nodulate *Macroptilium atropurpureum*. Similarly, Farissi (2014) identified 17 isolates from alfalfa nodule that failed to be authenticated as root nodule bacteria. Johnston and Beringer (1976) reported that a non-rhizobial contaminant can occur in soybean nodules.

Both morphological observations of the cells or colonies indicated that the tested bacteria are Gram-negative bacteria. All isolates produced colonies on agar plate from diameters ranging from ≤ 2 to ≥ 2 mm. Differences in colony morphology of soybean nodulating bacteria have also been reported by Singh et al. (2013). Colony size variation has been utilized as the primary way for differentiating rhizobial isolates of common bean (Muthini et al. (2014), cowpea (Mpepereki et al., 1997), peanut (Yang et al., 2005) and other diverse legumes.

Isolates from bambara groundnut were considered as fast-growing strains with growth occurring 72 h after inoculation. Similar results were obtained by Costa et al. (2014) on pigeonpea, Singh et al. (2013) on soybean, Ngakou et al. (2009) on bambara groundnut, cowpea and soybean. According to Somasegaran and Hoben (1994), the indicator dye bromothymol blue is green in YEMA with pH 6.8. In this study, all isolates changed the color of YEMA supplemented with BTB to yellow during the first 5 days of incubation indicating they are acid producers and hence possible to categorize them as fast-growers (Jida et al., 2011). The pH of the medium during the growth of isolates was changed from 6,5 to 4,4, thus showing the production of acid. The reduction of the medium pH is an common characteristic for fast growing rhizobial isolates. Similar findings were made by Baoling *et al*. (2007). Fast-growing bacteria able to acidify the culture medium have been isolated from peanut nodules (Torres-Júnior et al., 2014; Lyra et al., 2013) and from other tropical legumes such as cowpea (Leite et al., 2009) and pigeonpea (Fernandes Júnior et al., 2012; Costa et al., 2014)). LNB with a higher tolerance to acidity will be of great impacts in acidic soil conditions in the field. Recently, Freitas et al. (2014) observed that all the isolates obtained from nodules *M. paraibana* and *M. tenuiflora* grown in the soils from Serra Talhada, Santa Terezinha and Remígio also had rapid development and acidified the medium, some of them did not modify the YMA culture medium. According to Buendia-Clavenia et al. (1994), some of the claimed advantages of using fast-growing strains for grain legume inoculation include the facility of commercial production, easier establishment in soil, and displacement of indigenous strains.

All 12 isolates did not absorb the Congo red as it has been described by Jordan (1984) for the LNB. Similarly Shetta *et al.* (2011); Vishal and Abhishek, 2014) mentioned that *Rhizobium* strains failed to absorb congo red stain in the YEMA containing congo red. According to Somasegaran and Hoben (1994), the Congo red absorption is distinctive character of LNB with only few exceptions.

The grouping of isolates did not correlate with the geographical origin since isolates from the same origin were included in different group and isolates from diverses origin were found in the same group. The fact that isolates of the same origin were included in different group and isolates from diverses origin were found in the same group has been demonstrated previously (Rai et al., 2012; Torres-Jùnior et al., 2014). The dendrogram based on the morphological characteristics of the colonies, placed the bambara groundnut isolates in three distinctive groups separated at 50% similarity level. All these isolates

are fast growing. Apart from sharing phenotypic characteristics, these isolates may differs genetically. Most of the colonies had a cream color (91,67%) but there were also white (8,33%) colonies. Freitas et al. (2014) reported that white and creamy colonies also predominated among the isolates from *M. tenuiflora* and *M. paraibana* but orange and green colonies were also present. White, creamy or translucent colonies are commonly formed by bacteria associated with wild tree legumes such as *Acacia* spp. (Wolde-Meskel et al., 2004).

All isolates were able to grow on 1% NaCl but were unable to grow on higher concentrations, thus showing that the isolate was sensitive to the salt. Similar results were also reported by Vishal and Abhishek (2014), Maâtallah et al. (2002). Zahran (1999) reported that increasing salt concentration might have adverse affect on rhizobial population as a result of direct toxicity and indirectly by osmotic stress. In the case of high salt resistance VsBo3; VsLo3, VsLo5, VsLo6, VsMf5 and VsMf7 survived at 4% NaCl. Cheriet et al. (2014) reported that LNB from *Medicago ciliaris* L. root nodules survive at 4% NaCl. Küçük et al. (2006) also reported that strains obtained from root nodules of beans could survive at 4% NaCl. This ability of growth of the native rhizobial strains in high concentrations of sodium chloride solution can give high competitive value in the rhizosphere to survive and nodulate the host plants particularly at high concentrations of salt in the soil. The high salt tolerance of some rhizobial strains was associated to their ability to limit adverse effects caused by accumulation of protective organic osmolytes such as amino acids (proline, betaine and glutamate) or carbohydrates (Vriezen et al., 2007). According to Essendoubi *et al.*, (2007), accumulation of glutamate and mannosucrose were the osmolytes of the halotolerant rhizobial strains.

LNB appear to be varying in their pH tolerance in nutrient broth. In the current investigation, at pH 3.5, the isolates showed very poor growth. Appunu et al. (2009) reported that eight acid tolerant bradyrhizobia strains, isolated from nodules of indigenous cowpea plants were able to grow at pH 3.5. These findings are similar to what has been observed in the current study. Rai et al. (2012) also reported fast growing isolates able to grow at pH 4. There was considerable increase in OD values with increasing pH to 6.5. Somewhat similar to this, Rodrigues *et al.* (2006) quoted that the pH 6.5-7.0 is the most optimum pH for the growth of root nodulating bacteria.

None of the isolates grew in controls lacking carbon sources. This result is in agreement with those found by Missbah El Idrissi and Abdelmoumen (2008) on the the ability of LNB to utilize carbon sources 2008). Any growth in the absence of carbon indicates the importance of carbon on rhizobial survival. The twelve bambara groundnut nodulating bacteria use a large variety of carbohydrates as sole carbon and energy sources. Utilization of carbon source is one it is one of the important criteria to be considered as plant growth promoting bacteria. Similar observations were also reported in bean rhizobial isolates (Hungria and Vargas (2000). This results also coincided with reports on some rhizobial isolates from chickpea (Maâtallah *et al*. 2002), most of the isolates were able to catabolize a large variety of carbon sources including D-fructose, sorbitol, mannitol, trehalose, and cellobiose. Also, Kücük *et al*. (2006); Zahran et al. (2012) tested the ability of rhizobial isolates to utilize a variety of carbon sources, all isolates were able to grow well in the presence of D-fructose, D-galactose, D-glucose, D-mannitol, and sucrose. As reported van Rossum et al. (1995), fast-growing LNB were able to grow on a large variety of carbon substrates whereas slow-growing LNB were more limited in their ability to use diverse carbon sources. Isolates prefer mannitol and glycerol. According to Arias and Martı´nez-Drets (1976), glycerol is one of the most universal carbon sources for both fast-growing and slow-growing LNB. Zabaloy and Gomez (2005) used the carbon source utilization as one of the taxonomic markers to discriminate rhizobial isolates. Glucose, sucrose and starch were slowly metabolized by all the isolates. Elsheikh and Wood (1989) observed good growth of fast growing *Rhizobium* in the above carbohydrates.

5. Conclusion

Various soils of the humid forest zone of Cameroon contain LNB able to nodulate bambara groundnut. The study revealed that these bacteria are different morphologically. Isolates that are more tolerant to acid pH, aluminium and utilize wide sources of carbon were isolated in this study. The results obtained from Gram staining, growth on solid and liquid medium confirming the standard cultural and morphology characteristics of *Rhizobium* sp. These LNB are diverse, as shown from phenotypic characterization. Further studies are needed to characterize major factors involved such as LNB density; genetic diversity and select tolerant strains adapted to others environmental conditions of the humid forest zone of Cameroon. Investigations on these isolates could provide additional information on their symbiotic effectiveness with the aim of identifying very effective indigenous nitrogen fixing strains for local production of a specific bambara groundnut inoculant.

References

[1] Adeparusi EO (2001). Effect of processing on some minerals, anti-nutrients and nutritional composition of African yam bean. J. Sustain. Environ. 3:101-108.

[2] Allen ON, Allen EK (1981). *The Leguminosae.* A Source *Book of* Characteristics, Uses and Nodulation, The University of Wisconsin Press, 812 p.

[3] Anderson, JM, Ingram JS (1993). *Tropical soil biology and fertility*: *a hand book of methods*. 2nd ed. C.A.B. International Wallingford, U.K. 171 p.

[4] Appunu C, Reddy LML, Reddy CVCM, Sen D, Dhar B (2009). Symbiotic diversity among acid-tolerant bradyrhizobial isolates with cowpea. J. A. S. 4 (3): 126-131.

[5] Arias A, Martı´nez-Drets G (1976). Glycerol metabolism in *Rhizobium*. Can. J. Microbiol. 22 (2): 150-153.

[6] Athar M, Johnson AD (1996) Nodulation, biomass production and nitrogen in alfalfa under drought. J. Plant Nutr. 19: 185-199.

[7] Azam-Ali SN, Sesay A, Karikari SK, Massawe FJ, Aguilar-Manjarrez J, Brennan M, Hampson KJ (2001). Assessing the potential of an underutilised crop-a case study using bambara groundnut. Exp. Agric. 37: 433-472.

[8] Bado BV (2002). Rôle des légumineuses sur la fertilité des sols ferrugineux tropicaux des zones guinéennes et soudaniennes du Burkina Faso. PhD thesis, Université Laval, Laval, Canada.

[9] Bala A, Murphy PJ, Osunde AO, Giller KE (2003). Nodulation of tree legumes and the ecology of their native rhizobial populations in tropical soils. Appl. Soil Ecol. 22:211-223.

[10] Bamshaiye OM, Adegbol, JA, Bamishaiye EI (2011). Bambara groundnut: an Under-Utilized Nut in Africa. Advances in Agricultural Biotechnology 1: 60-72.

[11] BaoLing H, ChengQun L, Bo W, LiQin F (2007). A rhizobia strain isolated from root nodule of gymnosperm *Podocarpus macrophyllus*. Science in China Series C-Life Science 50: 1-6.

[12] Bargaz A, Faghire M, Farissi M, Drevon JJ, Ghoulam C (2013). Oxidative stress in the root nodules of *Phaseolus vulgaris* L. is induced under conditions of phosphorus deficiency. Acta Physiol. Plant. 35: 1633-1644.

[13] Belane AK, Dakora FD (2009). Measurement of N_2 fixation in 30 cowpea (*Vigna unguiculata* L. Walp.) genotypes under field conditions in Ghana using ^{15}N natural abundance technique. Symbiosis 48: 47-57.

[14] Buendia-Claveria AM, Rodriguez-Navaro DN, Santamaria-Linaza C, Ruiz-Sainz JE, Temprano-Vera F (1994). Evaluation of the symbiotic properties of *Rhizobium fredii* in European soils. Syst. Appl. Microbiol. 17: 155-160.

[15] Cheriet D, Ouartsi A, Chekireb D, Babaarbi S (2014). Phenotypic and symbiotic characterization of rhizobia isolated from *Medicago ciliaris* L. growing in Zerizer from Algeria. Afr. J. Microbiol. Res. 8 (17): 1763-1778.

[16] Ciani M, Diriye FU (1995). Presence of rhizobia in soils of Somalia. Worl J. Microbiol. Biotechnol. 11:615-617.

[17] Costa, FM, Schiavo JA, Brasi MS, Leite J, Xavier GR, Fernandes-Jr PI (2014). Phenotypic and molecular fingerprinting of fast growing rhizobia of field-grown pigeonpea from the eastern edge of the Brazilian Pantanal. *G*enet. Mol. Res. 13 (1): 469-482.

[18] Dakora FD, Muofhe LM (1997). Nitrogen fixation and nitrogen nutrition in symbiotic bambara groundnut (*Vigna subterranean* (L.)Verdc.) and Kerting's bean (*Macrotyloma geocarpum* (Harms) Marech et Baud.). In Heller J, Begemann F, Mushonga J (Eds.) Bambara Groundnut *Vigna Subterranea* (L.) Verdc: Proceedings of the Workshop on Conservation and Improvement of Bambara Groundnut (*Vigna Subterranea* (L.) at Harare, Zimbabwe. Bioversity International, pp 72-77.

[19] Dogbe W, Fening JO, Kumaga FWK, Danso SKA (2002). Maximizing the benefits of using mucuna on farmers'mixed farming. Trop. Sci. 42: 87-91.

[20] Egbe OM, Godwin Adu Alhassan GA, Ijoyah M (2013). Nodulation, Nitrogen Yield and Fixation by Bambara Groundnut (*Vigna Subterranea* (L.)Verdc.) Landraces Intercropped with Cowpea and Maize in Southern Guinea Savanna of Nigeria. Agricultural Science 1: 15-28.

[21] ElSheikh EAE, Wood M (1989) Response of chickpea and soybean rhizobia to salt: Influence of carbon source, temperature and pH. Soil Biol. Biochem. 21: 883-887.

[22] Essendoubi M, Brhada F, Eljamali, JE, Filali-Maltouf A, Bonnassie S, Georgeault S, Blanco C, Jebbar M (2007). Osmoadaptative responses in the rhizobia nodulating acacia isolated from south-eastern Moroccan Sahara. Environ. Microbiol. 9 (3): 603-611.

[23] Faghire M, Bargaz A, Farissi M, Palma F, Mandri B, Lluch C, Tejera García NA, Herrera-Cervera JA, Oufdou K, Ghoulam C (2011). Effect of salinity on nodulation, nitrogen fixation and growth of common bean (*Phaseolus vulgaris)* inoculated with rhizobial strains isolated from the Haouz region of Morocco. Symbiosis 55: 69-75.

[24] Fankem H, Tchuisseu Tchakounte GV, Ngo Nkot L, Nguesseu Njanjouo G, Nwaga D, Etoa FX (2014a). Maize (*Zea mays*) growth promotion by rock-phosphate solubilising bacteria isolated from nutrient deficient soils of Cameroun. Afr. J. Microbiol. Res. 8 (40): 3770-3579.

[25] Fankem H, Ngo Nkot L, Nguesseu Njanjouo G, Tchuisseu Tchakounte GV, Tchiaze Ifoué A V, Nwaga D (2014b). Rock phosphate solubilisation by strains of *Penicillium* spp. Isolated from farm and forest soils of three ecological zones of Cameroon. Am. J. Agric. For. 2 (2): 25-32.

[26] Farissi M, Ghoulam C, Bouizgaren A (2013). Changes in water deficit saturation and photosynthetic pigments of alfalfa populations under salinity and assessment of proline role in salt tolerance. Agric. Sci. Res. J. 3: 29-35

[27] Farissi M, Bouizgaren A, Aziz F, Faghire M, Ghoulam C (2014). Isolation and screening of rhizobial strains nodulating alfalfa for their tolerance to some environmental stresses. Pacesetter J. Agric Sci. Res. 2 (2): 9-19.

[28] Fasoyiro SB, Ajibade SR, Omole AJ, Adeniyan ON, Farinde EO (2006). Proximate, minerals and anti-nutritional factors of some underutilized grain legumes in south western Nigeria. Nutr. Food Science 36: 18-23.

[29] Fening JO, Danso SKA (2002). Variation in symbiotic effectiveness of cowpea bradyrhizobia indigenous to Ghanaian soils. Appl. Soil Ecol. 21: 23-29.

[30] Fernandes-Jr PI, Lima AA, Passos SR, Gava CAT (2012). Phenotypic diversity and amylolytic activity of fast growing rhizobia from pigeonpea [*Cajanus cajan* (L.) Millsp.]. Braz. J. Microbiol. 43: 1604-1612.

[31] Freitas ADS, Borges WL, Andrade MMM, Sampaio EVSB, Santos CERS, Passos SR, Xavier GR, Mulato BM, Lyra MCCP (2014). Characteristics of nodule bacteria from *Mimosa* spp grown in soils of the Brazilian semiarid region. Afr. J. Microbiol. Res. 8 (8): 788-796.

[32] Hillocks RJ, Bennett C, Mponda OM (2012). Bambara nut: A review of utlisation, market potential and crop improvement. Afr. Crop Sci. J. 20 (1): 1-16.

[33] Hungria M, Vargas MAT (2000). Environmental factors affecting nitrogen fixation in grain legumes in the tropics with an emphasis on Brazil. Field Crops Res. 65: 151-164.

[34] Jida M, Assefa F (2011). Phenotypic and plant growth promoting characteristics of *Rhizobium leguminosarum bv. viciae* from lentil growing areas of Ethiopia. Afr. J. Microbiol. Res.5: 4133-4142.

[35] Johnston AWB, Beringer JE (1976). Pea root nodules containing more than one *Rhizobium* species. *Nature* 264:502-504.

[36] Jordan DC (1984). Family III. *Rhizobiaceae*. In: Krieg NR, Holt JG. (Eds.) Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, pp 234-242.

[37] Kishinevsky BD, Zur M, Friedman Y, Meromi G, Ben-Moshe E, Nemas C (1996). Variation in nitrogen fixation and yield in landraces of bambara groundnut (*Vigna subterranea* L.). Field Crop. Res. 48 (1): 57-64.

[38] Klu GYP, Amoatey HM, Bansa D, Kumaeja FK (2001). Cultivation and use of African yam bean (*Sphenostylis stenocarpa* ex A Rich) in the Volta region of Chana. J. Food Technol. Africa 6: 74-77.

[39] Kouninki H, Sobda G, Nukenine NE (2014). Screening of Bambara groundnut (*Vigna subterranea)* lines for *Callosobruchus maculatus* resistance in the Far North Region of Cameroon. Journal of Renewable Agriculture 2 (1): 18-22.

[40] Küçük C, Kivanc M, Kinaci E (2006). Characterization of *Rhizobium* sp. Isolated from Bean. Turk. J. Biol. 30: 127-132.

[41] Leite J, Seido SL, Passos SR, Xavier GR, Rumjanek NG, Martins LMV (2009). Biodiversity of rhizobia associated with cowpea cultivars in soils of the lower half of the São Francisco River Valley. R. Bras. Ci. Solo 33: 1215-1226.

[42] Lyra MCCP, Freitas ADS, Silva TA, Santos CERS (2013). Phenotypic and molecular characteristics of rhizobia isolated from nodules of peanut (*Arachis hypogaea* L.) grown in Brazilian Spodosols. Afr. J. Biotechnol. 12: 2147-2156.

[43] Maâtallah J, Berraho E, Sanjuan J, Lluch C (2002). Phenotypic characterization of rhizobia isolated from chickpea (*Cicer arietinum*) growing in Moroccoan soils. Agronomie, 22: 321-329.

[44] Mbenoun LE (1992). Characterization of *Bradyrhizobium* sp of cowpea and bambara groundnut isolated from diverse agro-ecologic zones of Cameroon. MSc. dissertation, University of Yaounde, 65 p.

[45] Mbenoun LE (1992). Caractérisation de *Bradyrhizobium* sp. du niébé et du poids bambara isolés de diverses zones agroécologiques du Cameroun. Mémoire de maîtrise, Université de Yaoundé. 65 p.

[46] Missbah El Idrissi M, Abdelmoumen H (2008). Carbohydrates as carbon sources in rhizobia under salt stress. Symbiosis 46: 33-44.

[47] Mohale KC, Belane AK, Dakora FD (2014). Symbiotic N nutrition, C assimilation, and plant water use efficiency in Bambara groundnut (*Vigna subterranea* L. Verdc) grown in farmers fields in South Africa, measured using ^{15}N and ^{13}C natural abundance. Biol. Fertil. Soils 50: 307-319.

[48] Mpepereki S, Makonese F, Wollum AG (1997). Physiological characterization of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. Symbiosis 22: 275-292.

[49] Muthini M, Maingi JM, Muoma JO, Amoding A, Mukaminega D, Osoro N, Mgutu A, Ombori O (2014). Morphological assessment and effectiveness of indigenous rhizobia isolates that nodulate *P. vulgaris* in water hyacinth compost testing field in Lake Victoria Basin. Br. J. Appl. Sci. Tech. 4 (5): 718-738.

[50] Ndiang Z, Bell JM, Missoup AD, Fokam PE, AmougouAkoa (2012). Etude de la variabilité morphologique de quelques variétés

de voandzou (*Vigna subterranea* (L.) Verdc) au Cameroun. Journal of Applied Biosciences 60: 4410-4420.

[51] Ngakou A, Megueni C, Ousseni H, Massai A (2009). Study on the isolation and characterization of rhizobia strains as biofertilizer tools for growth improvement of four grain legumes in Ngaoundéré-Cameroon. Int. J. Biol. Sci. 3 (5): 1078-1089.

[52] Ngakou A, Ngo Nkot L, Doloum G, Adamou S (2012). Mycorrhiza-*Rhizobium*-*Vigna subterranea* dual symbiosis: impact of microbial symbionts for growth and sustainable yield improvement. Int. J. Agric. & biol. 14 (6): 915-921.

[53] Ngo Nkot L, Nwaga D, Ngakou A, Fankem H, Etoa FX (2011). Variation in nodulation and growth of groundnut (*Arachis hypogaea* L.) on oxisols from land use systems of the humid forest zone in southern Cameroon. African Journal of Biotechnology 10 (20): 3996-4004.

[54] Nyemba RC, Dakora FD (2010). Evaluating N_2 fixation by food grain legumes in farmers' fields in the three agro-ecological zones of Zambia, using ^{15}N natural abundance. Biol. Fertil. Soils 46:461-470.

[55] Padulosi S, Hodgkin T, Williams JT, Haq N (2002). Underutilized crops: trends, challenges and opportunities in the 21st Century. In: JMM Engels, VR Rao, AHD Brown, MT Jackson (eds) Managing plant genetic diversity. Wallingford, UK: CAB International Publishing; Rome: International Plant Genetic Resources Institute (IPGRI), pp 323-338.

[56] Pule-Meulenberg F, Dakora FD (2009) Assessing the symbiotic dependency of grain and tree legumes on N_2 fixation for their N nutrition in five agro-ecological zones of Botswana. Symbiosis 48: 68-77.

[57] Rai R, Dash PK, Mohapatra T, Singh A (2012). Phenotypic and molecular characterization of indigenous rhizobia nodulating chickpea in India. Indian J. Exp. Biol. 50: 340-350.

[58] Rodrigues CS, Laranjo M, Oliveira S (2006). Effect of heat and pH stress in the growth of chickpea mesorhizobia. Curr. Microbiol. 53 (1): 1-7.

[59] Shetta ND, Al-Shaharani TS, Abdel-Aal M (2011). Identification and characterization of *Rhizobium* associated with woody legume trees grown under Saudi Arabia condition. Am. Eurasian J. Agric. Environ. Sci. 10 (3): 410-418.

[60] Singh SK, Jaiswal, SK, Akhouri Vaishampayan, Dhar B (2013). Physiological behavior and antibiotic response of soybean (*Glycine max* L.) nodulating rhizobia isolated from Indian soils. Afr. J. Microbiol. Res. 7 (19): 2093-2102.

[61] Somasegaran P, Hoben HJ (1994) Handbook for Rhizobia. Methods in Legume-Rhizobium Technology. New York: Springer-Verlag, pp 240-58.

[62] Swift MJ, Bignell DE, Huang SP, Cares JE, Moreira F, Pereira EG, Nwaga D. Holt JA, Hauser S (2001). Standard methods for assessment of soil biodiversity and land use practice. *In* The ASA Review Meeting 1999, ASB Project, Bogor, Indonesia, ICRAF, Vol 1, 40 p.

[63] The C (2000). Identification of heterotic groups for acids soil on some maize varieties in Cameroon. INCO 1 and 2 Meeting, June 2000. Yaoundé, Cameroon.

[64] Torres-Júnior CV, Leite J, Santos CERS, Fernandes-Júnior PI, Zilli JE, Rumjanek NG, Xavier GR (2014). Diversity and symbiotic performance of peanut rhizobia from Southeast region of Brazil. Afr. J. Microbiol. Res. 8 (6): 566-577.

[65] van Rossum D, Schuurmans FP, Gillis M, Muyotcha A, van Verseveld HW, Stotthamer AH, Boogerd FC (1995). Genetic and phenotypic analysis of *Bradyrhizobium* strains nodulating Peanut (*Arachis hypogae* L.) roots. Appl. Environ. Microbiol. 61: 1599-1609.

[66] Vincent, JM (1970). A manual for practical study of root nodule bacteria. IBP Handbook No. 15, Blackwell Scientific Publishers, Oxford, 164p.

[67] Vishal KD, Abhishek C (2014). Isolation and characterization of *Rhizobium leguminosarum* from root nodule of *Pisum sativum* L. J. Acad. Indus. Res. 2: 464-467.

[68] Vriezen JAC, de Bruijn JF, Nusslein K (2007). Responses of rhizobia to desiccation in relation to osmotic stress, oxygen, and temperature. Appl. Environ. Microbiol. 73: 3451-3459.

[69] Wolde-Meskel, E., Berg T., Peters N.K. and Frostegard, A. 2004. Nodulation status of native woody legumes and phenotypic characteristics of associated *Rhizobia* in soils of southern Ethiopia. *Biol. Fert. Soils*. 40: 55-66.

[70] Yakubu H, Kwari JD, Ngala AL (2010). N_2 fixation by grain legume varieties as affected by rhizobia inoculation in the sandy loam soil of sudano-sahelian zone of North Eastern Nigeria. Nig. J. Basic Appl. Sci. 18 (2): 229-236. Yang JK, Xie FI, Zhou Q, Zhou JC (2005). Polyphasic characteristics of bradyrhizobia isolated from nodules of peanut (*Arachis hypogaea*) in China. Soil Biol. Biochem. 37: 141-153.

[71] Zabaloy MC, Gómez MA (2005). Diversity of rhizobia isolated from an agricultural soil in Argentina based on carbon utilization and effects of herbicides on growth. Biol. Fert Soils 42: 83-88.

[72] Zahran HH (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol. Mol. Biol. Rev. 63 (4): 968-989.

[73] Zahran HH, Abdel-Fattah M, Yasser MM, Mahmoud AM, Bedmar EJ (2012). Diversity and environmental stress responses of rhizobial bacteria from Egyptian grain legumes. Aust. J. Bas. Appl. Sci. 6 (10): 571-583.