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Isolation and Enumeration of *Bradyrhizobium* Species Dwelling In the Root Nodules of Soybean Plant

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Abstract
Biological Nitrogen fixation is one of the important aspects of organic agriculture gaining considerable attention globally. Information about the number of viable indigenous Bradyrhizobia in soils planted with legumes and their capacity to nodulate is a valuable tool in developing strategies to improve biological nitrogen fixation. Such strategies could potentially lead to increased soybean yields at low cost. This study was conducted to isolate and enumerate Bradyrizobium species dwelling in the root nodule of soybean plant using Bradyrhizobium japonicum selective medium (BJSM). Twenty (20) strains of Bradyrizobium species were isolated from the root nodules of soybean plants harvested from Ahmadu Bello University farm site, located at Bomo district of Sabongari local government area, Kaduna State, Nigeria. This was achieved using the streak method of isolation on BJSM. Ninety percent (18) of these isolates were confirmed as Bradyrizobium species using the plant infection test as they were able to nodulate the roots of soybean plants. The enumeration of the indigenous Bradyrizobium species gave a count ranging from $2.07x10^5$ - $4.0x10^6$ CFU/mL. Thus, the number of Bradyrhizobia obtained in the soil of this study is sufficient to achieve satisfactory results on nodulation and nitrogen fixation.
***Key words**: Soybean, Bradyrhizobium species, Nodulation, Nitrogen fixation.*

INTRODUCTION

Soybean [Glycine max (L.) Merril.] is a leguminous plant of the pea family that grows in tropical, subtropical, and temperate climates. It was domesticated in the 11th century BC around northeast of China and it is believed to have been introduced to Africa in the 19th century by Chinese traders along the east coast of Africa (IITA, 2009). It was introduced to Nigeria in 1908 in Ibadan, Oyo State (Omotayo *et al.*, 2007).

This plant is one of the most economically important legumes that is cultivated worldwide (Hungria and Mendes, 2015). In 2007, more than 216 million tons of soybeans were produced worldwide, of which Africa has about 1.5 million. Nigeria is the largest producer of soybean in sub-Saharan Africa (SSA), followed by South Africa (IITA, 2009).

Soybean as described by (Omotayo *et al.*, 2007) is known as "miracle bean" or the "golden bean" because it is a cheap, protein-rich grain with a high protein content of about 40% which is superior to all other plant foods as it has a good balance of the essential amino acids (Belewu and Belewu, 2007; Hungria and Mendes, 2015). The seeds also contain about 20% oil on a dry matter basis which is about 85% unsaturated and cholesterol-free (Dugje *et al.*, 2009) making it the most important crop for producing edible oil. Generally, it is regarded as a highly versatile and multipurpose agricultural product that has about three hundred and sixty-five (365) applications in the formulation of both human, animal foods and other industrial uses. Therefore, it has a great potential of improving the nutritional status and welfare of poor people in Nigeria (Omotayo *et al.*, 2007; Dianda, 2014).

Bradyrhizobia are bacteria that are members of the group, alpha Proteobacteria. They are gram negative rods with a single subpolar or polar flagellum. They are slow-growing common soil-dwelling bacteria that can form symbiotic relationships with leguminous plant such as soybean where they fix nitrogen in exchange for carbohydrates from the plant (Berrada and Fikri-Benbrahim, 2014).

Biological nitrogen fixation (BNF) is the process where atmospheric nitrogen is converted into plant usable forms such as ammonia by nitrogen fixing organisms using a complex enzyme system known as nitrogenase (Kim and Rees, 1994; Bishnoi, 2015). This process is an efficient source of fixed nitrogen that plays an important role in land remediation (Mohammadi *et al.*, 2012). It is as well an economically beneficial and environmentally friendly alternative to replace the high regimens of chemical fertilizers used in the present day agriculture (Adesemoye *et al.*, 2009; Saturno *et al.*, 2017).

The symbiotic association between leguminous plants and rhizobia has the greatest quantitative impact on the nitrogen cycle. The process of symbiotic nitrogen fixation supplies 50-60% of soybean nitrogen demands (Salvagiotti *et al.*, 2008) but may reach up to 94% for high yields as it requires large amounts of nitrogen (Rodrigues-Navarro *et al.*, 2011; Kinugasa *et al.*, 2012).

A common approach to improve symbiotic nitrogen fixation and legume productivity has been the reliance on superior or very effective exotic Bradyrhizobia strains as inoculants (Emmanuel *et al.*, 2008) since many soils are deficient in the number and quality of indigenous rhizobia to enhance biological nitrogen fixation (Abdullahi *et al.*, 2013). In order to address the problem of *Bradyrhizobium* inocula as regards to the lack of local production and difficulties in the importation of inoculants in the 1970s, the soybean-breeding program at the International Institute of Tropical Agriculture (IITA), Nigeria, developed soybean genotypes, designated TGx (tropical glycine cross) to nodulate effectively with indigenous *Bradyrhizobium* spp. populations (Santos *et al.*, 2019). However, nitrogen deficiency and poor yields will be experienced whenever Bradyrhizobia that effectively nodulate this soybean genotypes are not available in adequate numbers (Chianu *et al.*, 2011). As such, obtaining sufficient knowledge on the population characteristics of indigenous Bradyrhizobial populations in soils as well as determining their nodulation capacity will be valuable for developing strategies to improve BNF and thus increase soybean yields at low cost (Abaidoo *et al.*, 2007). Imrana (2017) reported nodulation of promiscuous soybean with indigenous Bradyrhizobia in the Northern Guinea savannah zone of Nigeria. This study was, therefore, aimed at isolating and enumerating *Bradyrhizobium* species dwelling in the root nodules of soybean plant.

MATERIALS AND METHODS

Collection of Soil Sample

Soybean plants were harvested from five sites without history of inoculation (one plant from each site) on Ahmadu Bello University farm located at Bomo district of Sabongari local government area, Kaduna state, Nigeria. This was done in September, 2018 as this month is the harvesting period of soybean in Nigeria. The samples were placed in sterile sampling bags and transported at ten o'clock in the morning to the laboratory in the Department of Microbiology, Ahmadu Bello University Zaria. The soil attached to each of the harvested plants was gently removed by shaking and 500 g of each of the five soil subsamples were pooled together and mixed thoroughly as one composite soil sample; the sample was then sundried for one day, crushed and passed through the mesh of a 2mm sieve for physicochemical analysis. The sieved sample was stored in plastic bag at room temperature until required for further analysis.

Sample of river sand was also collected from river Kubani flowing through Ahmadu Bello University, Zaria in September, 2018. Sample was obtained using a sterile hand trowel, placed in clean sampling bags and transported to the laboratory in the Department of Microbiology, Ahmadu Bello University, Zaria. The river sand collected was dried under the sun, passed through the mesh of a 4mm sieve for planting and sterilized by autoclaving (Imrana, 2017).

Determination of Physicochemical Properties of the Soil

The soil particle size was determined by the Hydrometer method and the soil texture was classified using the United States Department of Agriculture (USDA) soil textural class (Okalebo *et al.*, 2002). Soil pH was measured in a 1:2.5 soil: water ratio using a pH meter as described by Okalebo *et al.* (2002). Total Nitrogen was determined by the Kjeldahl method (Machido, 2010); available phosphorous by the Bray and Kurtz P-1 method (Okalebo *et al.*, 2002); organic carbon by the method of Walkley-Black (Okalebo *et al.*, 2002) and water holding capacity by the method described by Pawar and Shah (2009). Exchangeable bases were extracted with ammonium acetate solution 1N (NH_4OAc) (Okalebo *et al.*, 2002), exchangeable potassium and sodium were determined using flame photometer (Okalebo *et al.*, 2002) while exchangeable calcium and magnesium were determined by the titration method (Pawar and Shah, 2009). The cationic exchange capacity was determined by 1N ammonium acetate saturation method (Pawar and Shah, 2009) and the exchangeable acidity was by the titration method (Okalebo *et al.*, 2002).

Determination of Total Viable Counts of Bradyrhizobia in the Soil Sample

This was carried out following the pour plate technique as described by Woomer *et al.* (2011). Ten grams of the soil sample was weighed into conical flask containing 90mL of sterile distilled water. The sample was mixed with the diluent by shaking thoroughly and a ten-fold dilution was made up to dilution of 10^{-6}. Using a sterile pipette an aliquot of 1mL was transferred aseptically from each of the dilutions to sterile petri dish in duplicate. An already prepared sterile molten medium (15

mL) (*Bradyrhizobium japonicum* selective medium) (Tong and Sadowsky, 1994) was poured into each inoculated petri dish and swirled gently for even mixing. The agar was allowed to set and the dishes were incubated in an inverted position at 28°C for 10days in an incubator.

Isolation of Indigenous *Bradyrhizobium* species from the Root Nodules of Soybean Plant

Bradyrhizobium japonicum selective medium (BJSM) was used for the isolation. Healthy nodules were selected from the harvested plants and surface sterilized by immersing them in 95% ethanol for 10 seconds, transferred to 3% (v/v) solution of sodium hypochlorite for 2 min and rinsed six times with sterile distilled water. After surface sterilization, noodles were sliced open with a sterile razor blade and isolate were made from those with characteristic pink or red interior (Gyogluu *et al.*, 2018). They were crushed with a pair of blunt tipped forceps in 5ml of normal saline in a sterile petri-dish. One loopful of the nodule suspension was streaked onto plates of BJSM and the plates were incubated at 28°C for 10days in an incubator (Tong and Sadowsky, 1994). Small white colonies that grew after ten days were aseptically subcultured onto the surface of freshly prepared Yeast Extract Mannitol Agar (YEMA) slants and stored at room temperature for further use (Woomer *et al.*, 2011). These presumptive Bradyrhizobial isolates based on their cultural properties were subjected to plant infection test (nodulation test) explained in the next subheading below as it is indicated that an isolate cannot be properly regarded as *Bradyrhizobium* until its identity had been confirmed through nodulation test on soybean (Imrana, 2017).

Authentication of the Isolates as *Bradyrhizobium* species

Healthy seeds of a soybean variant (TGX1448-2E) of similar size and colour were selected for pre-germination. Seeds were surface sterilized by immersing them in 95% alcohol for 10 seconds to remove waxy material and trapped air. They were immersed in 3% sodium hypochlorite solution for 3 minutes in a large sterile petri-dish; rinsed six times with sterile distilled water and left in the water for four hours until they were fully imbibed (Woomer *et al.*, 2011). The seeds were again rinsed twice with sterile distilled water and pre-germinated by transferring them aseptically with forceps to the surface of large sterile petri-dish containing moistened cotton wool and incubated at room temperature until the radicles were 0.5 to 1.0 cm long for easy transfer during planting (Woomer *et al.*, 2011).

The presumptive Bradyrhizobial isolates were grown in sterile Erlenmeyer flasks (50 mL) containing 25 mL of freshly prepared yeast-mannitol broth. The inoculated culture media were incubated at room temperature on a rotary shaker for a period of three (3) days (Woomer *et al.*, 2011).

Planting was done in the screen house at the Department of Microbiology, Ahmadu Bello University, Zaria. Plastic cups with a depth of 10.7 cm and diameter 6.2 cm, were punctured at the base with three or more holes and used for planting the seeds. A Whatman filter paper was placed at the bottom of each cup to prevent excess water from draining out. Four hundred grams of autoclaved soil was added to surface sterilized cups and saturated with sterile plant nutrient solution prior to sowing seeds. Three equally spaced holes were made in the soil to a depth that will accommodate seeds one centimetre below the surface. Pre-germinated seeds were picked with sterile forceps and one seed in each hole were placed with the radicle entering first (Woomer *et al.*, 2011; Imrana, 2017).

Nitrogen-free nutrient solution (20 mL) was supplied to each plant once every week and the plants were replenished with sterile distilled water on a daily basis. Uninoculated controls with nitrogen (positive control) and without nitrogen (negative control) were included, for the uninoculated control with nitrogen, nitrogen was applied as 5mL of 0.05 % potassium nitrate (KNO_3) (w/v) solution once every week (Howienson and Dilworth, 2016).

At eleven days after planting, the plants were inoculated with the culture yeast-mannitol broth using a fresh pipette for each isolate. At 30days after planting, harvesting was carried out. The soil was washed off the plants using a gentle stream of water and the presence or absence of nodules was noted and recorded accordingly (Woomer *et al.*, 2011).

RESULTS

Physicochemical properties of the soil

The physicochemical analysis of the soil in this study indicated that the soil is sandy loamy in texture and slightly acidic (6.44). For the exchangeable cations, the level of potassium (0.072 Meq/100g) was found to be very low, sodium (0.696 Meq/100g) and magnesium (1.67 Meq/100g) were moderate and the calcium (2.60 Meq/100g) content was low. The total Nitrogen (0.034%) and organic carbon (0.379%) were found to be low while the available phosphorus (11.46 mg/kg) was at moderate level.

The exchangeable acidity (0.50 Meq/100g) was low and the cationic exchange capacity (2.86 Meq/100g) was very low (Table 1). These ratings are according to United States department of agriculture standard (USDA, 1993).

Population of *Bradyrhizobium* species in the soil sample

The plate count of the soil sample revealed an average population of $2.1x10^6$ CFU/mL ranging from $2.07x10^5$ - $4.0x10^6$ CFU/mL (Table 2).

Occurrence and Confirmation of *Bradyrhizobium* species in soil sample

A total of twenty (20) strains (four from each of the five plants) presumptively identified as *Bradyrhizobium* species based on their cultural properties were isolated. Colonies were small and whitish in colour on *Bradyrhizobium japonicum* selective medium (BJSM) (plate I). Ninety percent (18) of the twenty (20) isolates tested positive to nodulation (plate II) while the rest ten percent (2) tested negative (Table 3).

Plate I: Colonies of Bradyrhizobium sp. isolated in tis study growing on the surface of *Bradyrhizobium japonicum* selective medium

Plate II: Nodules formed in roots of soy bean plant after inoculation with *Bradyrhizobium* sp. isolated in this study.

Table 1: Physicochemical properties of soil used for isolation of Bradyrhizobia

Property	Value	Ratings (USDA Standard)			
		Very low	Low	Moderate	high
Particle size distribution (%)					
Clay (%)	5				
Silt (%)	38				
Sand (%)	57				
Textural class	Sandy loam				
pH(H_2O)1:2.5	6.44				
Water holding capacity (%)	31.2				
Total Nitrogen (%)	0.034	-	0-0.15	0.15-0.20	> o.20
Organic Matter (%)	0.653	-	< 2.0	2.0-3.0	> 3.0
Organic Carbon (%)	0.379	-	< 1.0	1.0- 1.5	> 1.5
Available P (mg/kg)	11.46	-	0-10	10-20	> 20
Exchangeable Bases (Meq/100g)					
K	0.072	< 0.2	0.2 -0.3	0.3-0.6	0.6-1.2
Na	0.696	< 0.1	0.1-0.3	0.3-0.7	0.7-2.0
Ca	2.60	< 2	2-5	5-10	10-20
Mg	1.67	< 0.3	0.3-1.0	1.0-3.0	3.0-8.0
Exchangeable Acidity (H^++Al^{3+}) (Meq/100g)	0.50	< 0.5	0.5-1.0	1.0-1.5	1.5-2.5
Cation Exchange Capacity (Meq/100g)	2.86	< 6	6-12	12-25	25-40

Source of ratings: (USDA, 1993).

Table 2: Population of *Bradyrhizobium* in the soil

Source of Inoculants	Population of *Bradyrhizobium*	
	Range (CFU/mL)	Mean (CFU/mL)
Soil	$2.07x10^5$-$4.0x10^6$	$2.10x10^6$

Table 3: Capacity of the Isolates to Form Nodules on Soybean (TGX1448-2E).

Isolate Code	Nodulation ability
AF1	+
AF2	+
AF3	+
AF4	+
AF5	+
AF6	+
AF7	+
AF8	-
AF9	+
AF10	+
AF11	+
AF12	-
AF13	+
AF14	+
AF15	+
AF16	+
AF17	+
AF18	+
AF19	+
AF20	+
PC	-
NC	-

Key: AF-------Ahmadu Bello University Farm, AF1-AF20------Isolates obtained from the root nodules of harvested plants, PC---------Plant fertilized with KNO_3, NC-------- Plant without inoculant nor fertilized, + ----------Positive to nodulation, - ----------Negative to nodulation

DISCUSSION

The soil's textural class of sandy loam may have accounted for its low clay and silt content. This class of soil texture enables high rate of water infiltration and moisture loss through evaporation and erosion resulting to the low level of water holding capacity recorded (Umeri *et al*., 2017). The soil pH was 6.44, this slight acidity of the soil is a feature common to the soils of the savannah zone and it is within the pH range (5.5-7.0) that is optimum for the release of plant nutrient (Sharu *et al*., 2013). This pH value is also favourable for the growth and survival of the indigenous population of *Bradyrhizobium* spp. (Howieson and Dilworth, 2016). The addition of ammonium (NH_4) fertilizer and the decomposition of organic matter could result in the acidic nature of soils. This is because the hydrogen ions produced by these processes displace calcium, magnesium and potassium from the surface of the soil particles that are then leached from the upper regions of the soil profile by water moving downward through the soil especially soil of high sand fraction (Umeri *et al*., 2017). This accounts for the low content of potassium and calcium in the soil of this study. The moderate level of sodium and magnesium contents is in line with the work of Adekiya *et al*. (2019) who also reported a moderate magnesium content, low potassium level and low to medium range of CEC.

The soil was found to be low in total nitrogen and organic carbon but moderate in available phosphorus. Machido *et al*. (2011) indicated low level of organic carbon, total nitrogen and phosphorous as a common feature of the soils found in the semi-arid zone of Nigeria, hence their inherent low fertility. The sandy texture of the soil could have also played a role in the washing away of nutrients such as Nitrogen and Phosphorus. This finding is supported by the work of Imrana (2017) who reported a sandy loam soil texture, low content of organic carbon and total nitrogen in the soil samples from Zaria, Kaduna State. Abubakar and Ado (2016) also reported a slightly acidic to moderately acidic pH, low carbon and nitrogen content in the soils across three agro-ecological zones of Nigeria (Northern Guinea, Southern Guinea and Sudan Savannah zones). The low level of cationic exchange capacity (CEC) can be related to the inherent sandy nature of the soil containing a low weatherable mineral reserve necessary for nutrient recharge and a small capacity for carbon storage (Shehu *et al*., 2018). These ratings are according to USDA (1993).

The soil's average Bradyrhizobial count of $2.1x10^6$ CFU/ mL which range from $2.07x10^5$ to $4.0x10^6$ CFU/mL is against the work of Ojo *et al*. (2015) who reported Rhizobial counts of $4x10^6$ – $1.4x10^7$ cells/g of soil in three different locations in the rain forest zone of Nigeria using the plant infection most probable number MPN method. However, it has been indicated that a soil population density of at least 10^3 *Bradyrhizobium japonicum* cells g^{-1} is required for maximizing nodule numbers on seedling tap roots, and efficient N_2 fixation to satisfy the Nitrogen demand of soybean (Atieno *et al*., 2012). Phanuel *et al*. (2015) has also reported that in the evaluation of effect of *Bradyrhizobium japonicum*m population on nodulation in some Ghanaian soils, the soil with the highest Bradyrhizobial count of $6.0x10^3$ cells /g of soil also produced the highest number of nodules. Thus, the Bradyrhizobial population observed in the soil of this study is sufficient to achieve satisfactory results on nodulation and nitrogen fixation.

A total of twenty (20) isolates were obtained from the root nodules of the plants using *Bradyrhizobium japonicum* selective medium (BJSM). Since the development BJSM by Tong and Sadowsky (1994), several researchers such as Pongdet *et al*. (2015), Rouws *et al*. (2014), among others have reported its use. The need to confirm the identity of the isolates as Bradyrhizobia through plant infection test cannot be over emphasized as it is indicated that an isolate cannot be properly regarded as *Bradyrhizobium* until its identity had been confirmed through nodulation test on soybean (Imrana, 2017). Ninety percent of the twenty (20) isolates tested positive to nodulation while the remaining ten percent tested negative (Isolate number AF8 and AF12). This supports the work of Chibeba *et al*. (2017) where eighty-seven (87) out of one hundred and five (105) isolates obtained from soybean nodules collected in Mozambique were able to nodulate both promiscuous and non-promiscuous cultivars. It has been indicated by Tong and Sadowsky (1994), that there is no direct correlation between the nodulation ability of an isolate and its ability to grow on the selective medium. This was obtained in the work that soybean-nodulating *Rhizobium fredii* was unable to grow on the medium since the medium is selective for *Bradyrhizobium* species but 98% of the randomly selected colonies recovered from a soil sample on BJSM were able to nodulate soybean plants (Tong and Sadowsky, 1994). The ten percent non-nodulating isolates obtained could be opportunistic bacteria that found their way into the nodules during the root infection process and have the ability to utilize the medium (Ibanez *et al*., 2009). They could also be other species of *Bradyrhizobium* that do not nodulate soybeans.

twenty (20) isolates were obtained from the root nodules of the plants and 90% (18) of these isolates were able to cause nodule formation in soybean plants.

CONCLUSION

The number of Bradyrhizobia in the soil of this study ranged from $2.07x10^5$- $4.0x10^6$ CFU/mL with an average of $2.10x10^6$ CFU/mL, a total of

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