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Isolation and molecular characterization of domestic *Bradyrhizobium* species from soybean roots in the savannah soil of Nigeria

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Abstract. *The identification of effective indigenous strains of Bradyrhizobia could lead to the development of efficient and affordable inoculants for improving soil fertility. This can also promote nitrogen fixation in smallholder farming systems of Nigeria and as well make the use of nitrogen fertilizers unnecessary. This study was conducted to characterize and evaluate the nodulating properties of indigenous Bradyrhizobium species in soybean plant. A total of 18 strains were isolated using Bradyrhizobium japonicum selective medium (BJSM) from the root nodules of plants harvested from five sites on Ahmadu Bello University farm, Zaria Kaduna State, Nigeria. These isolates were evaluated for nodulating potential in a screen house using soybean (TGx 1448-2E) as a test crop. The total number of nodules, percentage effective nodules, nodule fresh and dry weights, shoot fresh and dry weight and plant nitrogen content were assessed. The nodules produced by the isolates showed high percentage effectiveness with isolate A4 having significantly higher nodule dry weight (78.00 mg) than the rest of the test isolates. The soybean inoculant, BIOFIX used in this study performed poorly having a nodule dry weight of 6.0 mg. There was positive and highly significant correlation between the nodule dry weight, shoot dry weight and plant nitrogen content (r=0.740, 0.641, 0.616, respectively) at p<0.001. Sequence analyses were carried out on the high performing isolates from each site of sampling and the result obtained using the NCBI Database showed similarity of these isolates with reference strains belonging to the genus Bradyrhizobium (A4- Bradyrhizobium japonicum RV9, B2- Bradyrhizobium guangdongense CCBAU 51649, C1- Bradyrhizobium sp. UFLA05-149, D3- Bradyrhizobium sp. B918 and E3- Bradyrhizobium sp.UFLA05-149). A 16srRNA phylogenetic tree constructed with the sequences obtained grouped the isolates without any close reference strain. However, isolate B2 showed close affiliation with Bradyrhizobium guagdongense with 95% sequence identity. Based on the effectiveness of these five strains of Bradyrhizobium, it is suggested that they can be used as potential candidate for inoculants production.*

Keywords: genus *Bradyrhizobium*, nodulation, 16SrRNA, Nigeria, soybean

Introduction

Nitrogen is one of the most abundant elements in the atmosphere, it is highly inert and unavailable to growing plants and thus plants can only utilize it in its reduced form (Wagner, 2011; Bishnoi, 2015). Plants acquire these forms of nitrogen through the addition of fertilizer or manure to soil, their release during organic matter decomposition, the conversion of atmospheric nitrogen into the compounds by natural processes such as lightning, and biological nitrogen fixation (Wagner, 2011).

Soybean plants require high amount of nitrogen, however, most of the soils used for its production in Nigeria are inherently of low fertility (Machido et al., 2011). This is because of the continuous depletion of total nitrogen content by processes such as leaching, denitrification, volatilization and removal of crop residues from the land for alternative uses (Yakubu et al., 2010; Machido et al., 2011). These factors necessitated the addition of supplemental nutrients to the soil which include the use of inorganic fertilizer to ensure optimal crop growth and profitability (Larry**,** 2017). However, the long-term and large-scale use of inorganic fertilizer has led to environmental degradation (Abby, 2015). In addition, most low income farmers cannot

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afford these inorganic fertilizers; as such they tend to plant legumes without any major external input, thus obtaining low grain yields. Under such conditions, soybeans depend on symbiosis with microorganisms from genus *Bradyrhizobium* for nitrogen fixation to partially or fully meet their N requirement (Hungria and Kaschuk, 2014). Unfortunately, most of the indigenous Bradyrhizobial strains cannot meet all the N requirements of soybeans even when promiscuous ones are planted (Sanginga et al., 1996) leading to limitation in soybean production.

A common approach to improve symbiotic nitrogen fixation and legume productivity has been the reliance on superior or very effective exotic Bradyrhizobial strains as inoculants (Emmanuel et al., 2008). The use of these Bradyrhizobial inoculants is limited due to the high cost of the culture, and poor performance due to lack of adaptation to local agro-climatic conditions or negative microbial interactions (Ouma et al., 2016). In order to proffer a solution to this problem, it is imperative to identify indigenous Bradyrhizobial strains that are as effective in nitrogen fixation as the commercially available inoculants (Emmanuel et al., 2008; Ojo et al., 2015).

Soybean is becoming one of the most cultivated grain legumes in sub-Saharan Africa where Nigeria is rated to be the largest producer, since it has the greatest potential of producing the cheapest source of food protein and other essential nutrients for farm households (IITA, 2009; Rao and Reddy, 2010).

Identifying indigenous *Bradyrhizobium* species that are effective in nodulation as inoculants will increase the yield of this crop as well as assisting the low income farmers. In addition, for sustainable soybean production in Nigeria, a better understanding of the diversity/phylogeny of the native soybean Bradyrhizobia is required (Gyogluu et al., 2018). Dianda et al. (2014) have shown that indigenous Bradyrhizobia have the potential of improving soybean production. This study was, therefore, aimed at characterizing indigenous *Bradyrhizobium* species and evaluating their nodulating properties in soybean plant.

Material and methods

Samples collection

Soybean plants were harvested from five sites without history of inoculation (one plant from each site) on Ahmadu Bello University farm, Zaria Kaduna State, Nigeria. This was done in September, 2018 as this month is the harvesting period of soybean in the country. The samples were placed in sterile sampling bags labeled A-E and transported at ten o'clock in the morning to the laboratory in the Department of Microbiology, Ahmadu Bello University, Zaria.

A Sample of river sand was also collected from river Kubanni flowing through Ahmadu Bello University (Figure 1), Zaria in September, 2018. The 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 4 mm sieve for planting and sterilized by autoclaving (Imrana, 2017).

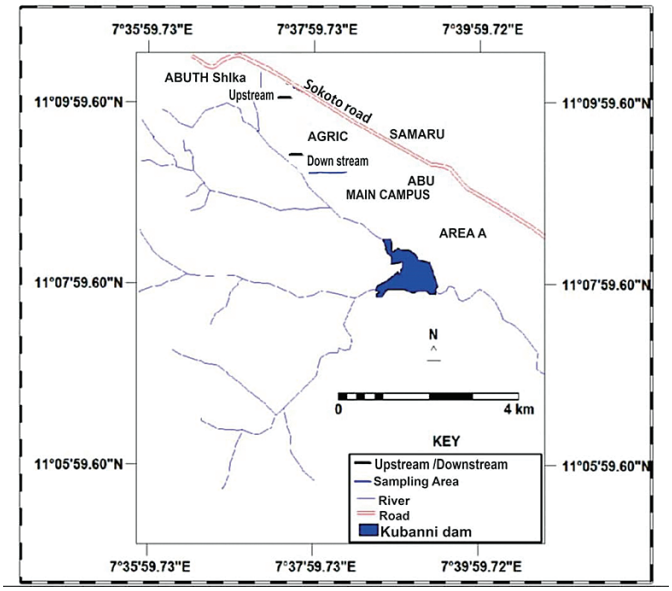

Figure 1. River Kubanni flowing through Ahmadu Bello University (ABU) showing the sampling area

(Source: Adapted and modified from Zaria South West topographic map, Yusuf and Shuaib, 2012)

Isolation of Bradyrhizobium species

A selective medium for *Bradyrhizobium* species, *Bradyrhizobium japonicum* selective medium (BJSM) was used for isolation. Healthy nodules were selected from each of the harvested plants and surface sterilized by immersing them in 95% ethanol (JHD, China) for 10 seconds, transferred to 3% (v/v) solution of sodium hypochlorite for 2 min and rinsed six times with sterile distilled water. After that, nodules were sliced open with a sterile razor blade and isolates 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 5 ml 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 10 days 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 until required for further use (Woomer et al., 2011). BJSM is a mineral salt medium of the following constituents: 125 mg Na_2HPO_4, 250 mg Na_2SO_4, 320 mg NH_4Cl, 120 mg $MgSO_4.7H_2O$, 10 mg $CaCl_2$, 4 mg $FeCl_3$,

20 g agar, and 1 litre of distilled water. The medium was supplemented with 1 g Yeast agar, 1 g l-arabinose and 1 g sodium gluconate. It was further supplemented with 88 µg $CoCl_2$, 83 µg $ZnCl_2$ and 1 µg brilliant green (BG) which served as the selecting agent for *Bradyrhizobium* species (Tong and Sadowsky, 1994). The pH of the medium was adjusted to 6.8.

Molecular characterization and identification

Healthy seeds of a soybean variant (TGX1448-2E) which was of similar size and color were selected for pre-germination. Seeds were surface sterilized by immersing 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. Afterwards, the seeds were again rinsed twice with sterile distilled water and were 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 radicals were 0.5 to 1.0 cm long (Woomer et al*.,* 2011).

The presumptive Bradyrhizobial colonies were subcultured in 50 mL of freshly prepared yeast-mannitol broth in sterile erlenmeyer flasks (250 mL) and incubated at room temperature on a rotary shaker for 3 days (Woomer et al*.,* 2011).

Planting was done in the screen house (dimension 10.97x5.18 m) at the Department of Microbiology, Ahmadu Bello University, Zaria. Plastic cups (dimension 10.7x6.2 cm) with at least three holes on the bottom were used for planting. 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 centimeter below the surface. Pre-germinated seeds were picked with sterile forceps and one seed in each hole was 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. Two weeks after planting, the plants were thinned to two uniform plants per cup and were inoculated with the culture yeast-mannitol broth using a fresh pipette for each isolate (Woomer et al*.,* 2011).

The experiment was set up as a Randomized Complete Block Design (RCBD) with three replications. The treatments were assigned as follows: isolates from the harvested plants, a commercial inoculant (BIOFIX) which was supplied by the Department of Soil Science, Institute of Agricultural Research (IAR), Zaria Kaduna State and two controls; non-inoculated control with nitrogen (positive control) and without nitrogen (negative control). For the positive control, nitrogen was applied as 5 mL of 0.05% KNO_3 (w/v) solution once every week (Howienson and Dilworth, 2016).

At 7 weeks after planting, harvesting was done. The soil was washed of the plants using a gentle stream of water, the shoots were cut and their fresh weight was determined. The nodules were detached from the root, counted and sliced open with a sterile razor blade to check for pink interior. The percentage effective nodules were calculated for each isolate by counting the number of nodules with pink interior, dividing it by the number of nodules formed and multiplying it by 100 (Machido, 2010). After that, the fresh weight of the nodules was determined and both the shoots and nodules were placed in an aluminum foil for drying to constant weight at 70°C for 2 days. The dry weight of nodules and shoots were recorded (Woomer et al*.,* 2011). The dry nodules, shoots and roots of plants were ground together, passed through 2 mm sieve and the nitrogen content was determined by the micro-Kjeldhal method (Machido, 2010).

DNA extraction: The best performing isolate from each site of sampling based on nodule number, effective nodules, nodule dry weight and plant nitrogen content was selected for molecular analysis. The isolates were grown on YEMA for 5 days and DNA was isolated from the bacterial colonies by using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany), following the manufacturer's instructions.

PCR-amplification: The primers fD1 (27F) (AGAGTTTGATCMTGGCTCAG) and rP3 (1492R) (TACG-GYTACCTTGTTA CGACTT) were used for the amplification of 16S rDNA gene fragments. The polymerase chain reaction (PCR) was done in 25-µl aliquots using Applied Biosystems 9700 thermal cycler. The reaction mix contained 12.5 µl of qiagen toptaq Taq master mix, 0.5 µl of 10 µM Reverse primer, 0.5 µl of 10 µM Forward primer, 2.5 µl of coral load, 4 µl of nuclease free water and finally 5 µl of Template.The PCR reactions were performed with an initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, 55°C primer annealing for 1 min and 72°C extension for 1 min, followed by a final extension step at 72°C for 10min. Amplicons were electrophoresed in 1.5% agarose gel (Invitrogen) with ethidium bromide. Purification and sequencing of the PCR-amplified DNA fragments were done in INQABA Biotec, South Africa. Sequences were analysed using MEGA X and BLAST (Basic Local Alignment Search Tool) and compared to sequences available in the GenBank Database at the National Center for Biotechnology Information (NCBI) for identification of the closely related species with the isolates (Marinković et al., 2017).

Phylogenetic analysis: Sequence alignment was

conducted using Clustal W. The phylogenetic tree was constructed using the 16S rRNA sequences of the isolate and those of the related strains obtained from Genbank. The partial 16S rRNA gene sequences of the isolates were aligned with those of the related Bradyrhizobium species and the evolutionary history was inferred using the Neighbor-Joining method with 1000 bootstrap support (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 13 positions in the final dataset. The tree was drawn to scale, with branch lengths (5.04331166) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Statistical analysis

Data collected on nodulation, shoot and nitrogen content were subjected to analysis of variance (ANOVA) and where the F-ratios were found to be significant, Duncan multiple range test was used to separate the means. Correlation analysis was established between the parameters assessed. These statistical analyses were carried out using Statistical Analysis System (SAS) v9.4 software (SAS Institute, 2013).

Results

Isolate characterization

A total of 18 strains presumptively identified as *Bradyrhizobium* species based on their cultural properties were isolated. Of these, 12 isolates were recovered from the harvested plants from three sites (A, D and E) while 6 isolates were recovered from the plants harvested from the other two sites (B and D). Colonies were small, whitish in colour and grew within 10-13 days of incubation on BJSM.

Relative performance of the isolates and BIOFIX based on nodulation

The performance of the isolates based on nodulation varied with different isolates as presented in Table 1. It was observed that isolate A3 produced significantly higher number of nodules than all other isolates while isolates C2 and the commercial inoculant (BIOFIX) formed the least number of nodules. There was no nodule formation in the positive and negative controls.

Table 1. Relative performance of indigenous species of *Bradyrhizobium* isolated from five sites on Ahmadu Bello University Farm

Treatments	Nodulation parameters assessed per plant, n=3			
	Number of nodules per plant*	% Effective nodules per plant*	Fresh weight of nodules, mg*	Dry weight of nodules, mg*
A1	13.33^{abcdef}	83.33^{a}	246.67^{ab}	55.33^{abc}
A2	18.33^{abcde}	96.97^{a}	266.67^{ab}	61.00^{ab}
A3	29.33^{a}	76.00^{a}	156.67^{bcd}	35.33^{abcd}
A4	25.67^{abc}	73.10^{a}	373.33^{a}	78.00^{a}
B1	22.33^{abcd}	85.87^{a}	256.67^{ab}	59.67^{ab}
B2	23.67^{abc}	64.93^{a}	196.67^{abcd}	40.67^{abcd}
B3	5.33^{def}	61.67^{a}	96.67^{bcd}	21.00^{bcd}
C1	24.00^{abc}	79.83^{a}	126.67^{bcd}	30.33^{abcd}
C2	2.33^{ef}	66.67^{a}	20.00^{cd}	5.67^{cd}
C3	10.33^{bcdef}	84.23^{a}	106.67^{bcd}	27.67^{abcd}
D1	21.00^{abcd}	87.83^{a}	143.33^{bcd}	32.33^{abcd}
D2	16.00^{abcdef}	69.43^{a}	166.67^{abcd}	40.67^{abcd}
D3	27.33^{ab}	96.77^{a}	230.00^{abc}	54.00^{abc}
D4	10.33^{bcdef}	55.83^{a}	90.00^{bcd}	24.67^{bcd}
E1	17.33^{abcdef}	60.83^{a}	123.33^{bcd}	31.67^{abcd}
E2	7.67^{cdef}	39.43^{ab}	70.00^{bcd}	18.00^{bcd}
E3	23.33^{abc}	86.43^{a}	193.33^{abcd}	42.33^{abcd}
E4	8.00^{cdef}	92.60^{a}	96.67^{bcd}	23.67^{bcd}
BIOFIX	2.33^{ef}	66.67^{a}	26.67^{cd}	6.00^{cd}
PC	0.00^{f}	0.00^{b}	0.00^{d}	0.00^{d}
NC	0.00^{f}	0.00^{b}	0.00^{d}	0.00^{d}

Key: *Means (3 replicates) in the same column with the same letter(s) are not significantly different at ($p \geq 0.05$).
A1-A4: Isolate from site 1; B1-B3: Isolate from site 2; C1-C3: Isolate from site 3;
D1-D4: Isolate from site 4; E1-E4: Isolate from site 5; BIOFIX: Commercial isolate;
PC: Plant fertilized with KNO_3; NC: Plant without inoculant nor fertilized with KNO_3.

The nodules produced by the isolates showed relatively high percentage effectiveness with isolate E2 having the least ability. Isolate A4 exhibited the highest nodule fresh and dry weight while isolate C2 and the commercial inoculant (BIOFIX) had the least.

Relative performance of the isolates and BIOFIX based on shoot weight and plant nitrogen content

Table 2 shows the relative performance of the tested isolates based on shoot weight and nitrogen content. It was observed that isolate A4 also had the highest shoot fresh and dry weight; however, there was no significant difference in the values obtained for all the isolates, commercial inoculants and controls. Isolates A2, A4 and D1 were found to have significantly higher nitrogen content than the rest of the test inoculants. Most of the parameters assessed were found to have a significant positive correlation with one another ($p<0.05$, $p<0.01$, $p<0.001$) (Table 3). There was a non-significant positive correlation ($p\geq0.05$) between the effective nodules formed and dry weight of shoots.

Table 2. Relative performance of indigenous species of *Bradyrhizobium* isolated from five sites on Ahmadu Bello University Farm based on shoot weight and plant nitrogen content

Treatments	Fresh weight of shoots, mg	Dry weight of shoot, mg	Nitrogen content of plants, %*
A1	2276.7^{a}	573.3^{a}	1.88^{ab}
A2	1996.7^{a}	518.3^{a}	2.30^{a}
A3	1800.0^{a}	408.7^{a}	1.99^{ab}
A4	2563.3^{a}	640.7^{a}	2.21^{a}
B1	1793.3^{a}	444.0^{a}	1.88^{ab}
B2	2246.7^{a}	578.7^{a}	1.97^{ab}
B3	1660.0^{a}	416.0^{a}	1.96^{ab}
C1	1430.0^{a}	339.7^{a}	1.81^{ab}
C2	1446.7^{a}	369.0^{a}	1.68^{ab}
C3	1436.7^{a}	344.3^{a}	1.52^{ab}
D1	1290.0^{a}	289.0^{a}	2.25^{a}
D2	1846.7^{a}	436.3^{a}	1.74^{ab}
D3	1833.3^{a}	443.7^{a}	2.13^{ab}
D4	1596.7^{a}	413.3^{a}	1.92^{ab}
E1	1723.3^{a}	430.0^{a}	1.96^{ab}
E2	1163.3^{a}	266.7^{a}	1.77^{ab}
E3	1356.7^{a}	307.7^{a}	1.86^{ab}
E4	1376.7^{a}	347.7^{a}	1.57^{ab}
BIOFIX	1333.3^{a}	351.0^{a}	1.64^{ab}
PC	1606.7^{a}	375.0^{a}	1.85^{ab}
NC	1400.0^{a}	343.0^{a}	1.36^{b}

Key: *Means (3 replicates) in the same column with the same letter(s) are not significantly different at $p\geq0.05$.
A1-A4: Isolate from site 1; B1-B3: Isolate from site 2; C1-C3: Isolate from site 3;
D1-D4: Isolate from site 4; E1-E4: Isolate from site 5; BIOFIX: Commercial isolate;
PC: Plant fertilized with KNO_3; NC: Plant without inoculant nor fertilized with KNO_3.

Table 3: Correlation analysis between the parameters assessed

Parameters	Number of nodules	Effective nodules formed	Nodule fresh weight	Dry weight of nodules	Shoot fresh weight	Dry weight of shoots	Nitrogen content of plants
Number of nodules		0.528***	0.699***	0.708***	0.504***	0.466***	0.491***
Effective nodules formed			0.558***	0.579***	0.246*	0.238^{ns}	0.373**
Nodule fresh weight				0.985***	0.722***	0.704***	0.636***
Dry weight of nodules					0.752***	0.740***	0.641***
Shoot fresh weight						0.987***	0.623***
Dry weight of shoots							0.616***
Nitrogen content of plants							

The correlation is significant at *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

Sequence analysis of the 16S rRNA Gene of the isolates

Amplification of 16S rRNA Gene of the selected isolates (A4, B2, C1, D3 and E3) produced a single PCR product with the expected amplicon size of 1484 bp (Figure 2). The result of the blast analysis using the NCBI Data base showed similarity of the isolates with reference strains belonging to the genus *Bradyrhizobium* (Table 4). A phylogenetic tree built with the sequences obtained grouped the isolates without any close reference strain. However, isolate B2 showed closeness with *Bradyrhizobium guangdongense* with 95% sequence identity (Figure 3). The scale bar indicates the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The accession numbers of the associated strains are followed by their names.

Table 4. Similarities of 16srRNA gene sequences of the isolates with those available in Genbank

Isolate number	Closest sequence match in Genbank	Maximum identification, %	Accession number	E- value
A4	*Bradyrhizobium japonicum* RV9	78	KY940048.1	1e-155
B2	*Bradyrhizobium guangdongense* CCBAU 51649	95	CP030051.1	4e-40
C1	*Bradyrhizobium sp.* UFLA05-149	91	MH651765.1	0.0
D3	*Bradyrhizobium sp.* B918	78	MH688810.1	9e-163
E3	*Bradyrhizobium sp.* UFLA05-149	92	MH651765.1	0.0

Key: A4, B2, C1, D3 and E3- Selected isolates

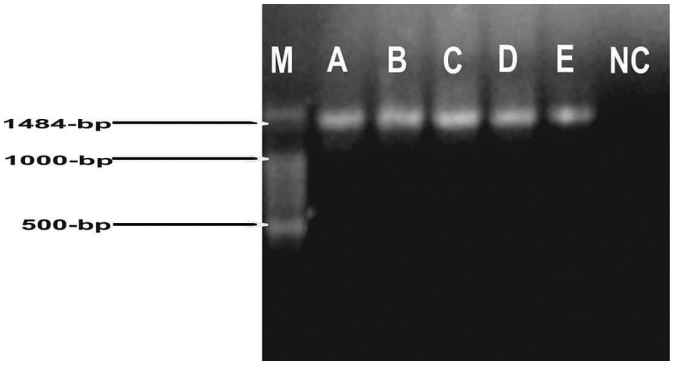

Figure 2 Amplicon of 16S rRNA gene of *Bradyrhizobium* spp. (1484bp) isolated from soybean plants

(Key: M: Molecular Marker; Lane A: Isolate A4 - isolate from site 1; Lane B: Isolate B2 - isolate from site 2; Lane C: Isolate C1 - isolate from site 3; Lane D: Isolate D3 - isolate from site 4; Lane E: Isolate E3- isolate from site 5; Lane NC: Negative control - Premix with sterile water).

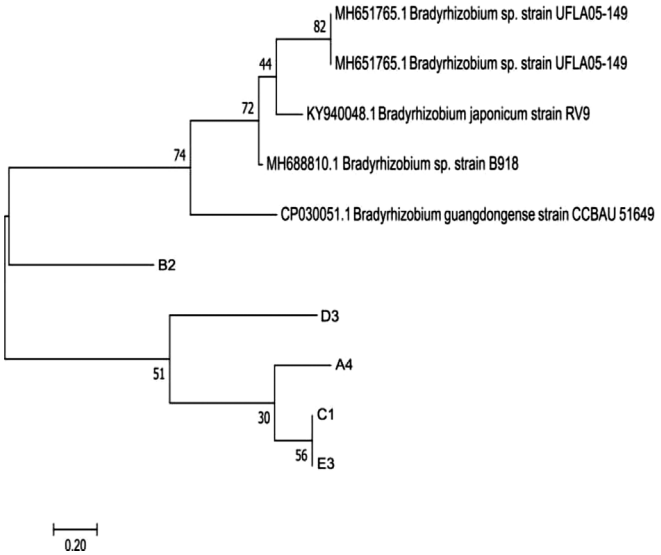

Figure 3. Neighbor Joining Tree showing the evolutionary relationships of the selected *Bradyrhizobium* strains (A4, B2, C1, D3 and E3) isolated from soybean plant and the associated strains obtained from Genbank

Discussion

Biotechnological applications in agriculture offer a sustainable solution to soil fertility, plant diseases, food security and many other issues. The use of seed inoculants developed from local strains of Rhizobia has great potential in improving plant yield, especially in nutrient-poor soils. In this study, the species of *Bradyrhizobium* grew after 10 days, which is typical of slow growing rhizobia on Yeast mannitol medium (Rodriguez-Navarro et al., 2021). Nodulation was the highest in Isolate A3; however, the number of nodules produced did not reflect its effectiveness as it had lower nodule fresh and dry weight than some isolates which have lower number of nodules. These could be because isolates which induce bigger nodules generally produced fewer nodule numbers, while those which induce smaller nodules produce greater nodule numbers (Gyogluu et al., 2018). It has been indicated by Abd El-Maksoud and Keyser (2010), that great number of nodules can be formed by a strain fixing little or no nitrogen. Some isolates could also produce high number of nodules and as well high biomass; this can be seen in the case of isolate A4 which had the highest nodule dry weight. The performance of the standard inoculant (BIOFIX) used in this study based on nodulation was very low; this contradicts the work of Imrana (2017) who reported a significant amount of effective nodules produced by BIOFIX inoculant. This low performance could be due to low temperature of the weather as at the time the experiment was conducted (December 2018 - February 2019); since it is not native to Nigerian soils, it was not able to adapt well to the extreme weather condition. Koskey et al. (2017) reported the superiority of native

rhizobia over introduced inoculants in four different agroecological zones in Kenya with no history of previous inoculation. It was further indicated that the average performance of BIOFIX in nitrogen fixation could be due to the soil properties and unfavorable agroecological conditions in Eastern Kenya, which affects the rhizobia-legume interaction within the rhizosphere. There is an indication that poorly compatible associations can result in root nodule formation with minimal (sub-optimal) or no (ineffective) N_2-fixation (Melino et al., 2012), this can be seen in the case of isolate C2 which produced the lowest nodule number and also had the least biomass. There is also a suggestion that rhizobia which fix little or no nitrogen could exhibit parasitic behavior (Ouma et al., 2016). The nodules produced by the isolates showed high percentage effectiveness with isolate E2 having the least significant value. This is in accordance with the work of Abubakar and Ado (2016) who reported that soils of the Northern Guinea Savanna contain indigenous rhizobia with high relative efficiency. The effectiveness of these isolates resulted in the higher shoot biomass and nitrogen content as demonstrated by the positive and highly significant correlation between the nodule dry weight, shoot dry weight and plant nitrogen content (r=0.740, 0.641, 0.616, respectively). This supports the indication that shoot weight is usually highly correlated with total nitrogen content; as such they are routinely used as an indicator of relative strain effectiveness (Woomer et al., 2011). This result also concurs with the assertion made by Delić et al. (2010), that there is a direct relationship between nodule formation and nitrogen accumulation in legumes. Howienson and Dilworth (2016) also indicated in their study that the important parameter to quantify in the assessment of the symbiotic effectiveness of rhizobia with legume is the extent of effective nodulation and the yield of biomass by the legume. This is because measuring the amount of nitrogen fixed by single plants does not add much insight as opposed to the information provided by nodulation and yield data.

The discovery of new genera, species and strains of soybean Bradyrhizobia for inoculant production necessitates the understanding of its genetic diversity (Gyogluu et al., 2018). As such, phylogenetic analysis was carried out on the best performing isolates from each site of sampling. The BLAST analysis on the NCBI Database showed similarity of the isolates with reference strains belonging to the genus *Bradyrhizobium.* A study conducted by Chibeba et al. (2017) indicated that 75% of the 87 indigenous isolates trapped by promiscuous soybean cultivar (TGX) from soil of Mozambique were *Bradyrhizobium* while the rest 25% were *Rhizobium* genera. Imrana (2017) also reported that the isolates obtained from soil of Nigeria which were effective in nitrogen fixation belong to the genera *Bradyrhizobium.*

A phylogenetic tree of the 16SrRNA sequences from the selected isolates showed only 78-95% similarity to the reference strains from Genbank. However, isolate B2 showed close affiliation with *Bradyrhizobium guangdongense* with 95% sequence identity. *Bradyrhizobium guangdongense* is a novel species of *Bradyrhizobium* which was isolated from peanut (Li et al., 2015). In addition, the reference strains *Bradyrhizobium japonicum* RV9 and *Bradyrhizobium sp.* UFLA05-149 were isolated from green gram and forage peanut, respectively (Shahid and Khan, 2018; Sá et al., 2019). This result supports the fact that the soybean cultivar Tropical Glycine Cross (TGX) used in this study was developed to nodulate freely with indigenous rhizobia, presumably strains of *Bradyrhizobium* spp. which nodulate legumes in the “cowpea cross-inoculation” or “cowpea miscellany” group (Abdullahi et al., 2013). The rest of the isolates which stood alone without any close reference strain could imply that they belong to new species of *Bradyrhizobium* as it is well established that two organisms that have less than 97% homology of 16S rRNA sequence belong to two different species (Berrada and Fikri-Benbrahim, 2014), although further molecular investigation is required. However, it has been shown that the resolving power of this technique is limited in the studies of strains or closely related species whose divergence is very recent (Berrada and Fikri-Benbrahim, 2014).

Conclusion

The nodules produced by the eighteen strains of *Bradyrhizobium* which were evaluated for nodulating potential showed high percentage effectiveness with isolate A4- *Bradyrhizobium japonicum* RV9 having significantly higher nodule dry weight (78.00 mg) than the rest of the test isolates (5.67-61 mg). The commercially available soybean inoculant, BIOFIX, used performed poorly compared to the isolates in this study, having a nodule dry weight of 6.0 mg. There was positive correlation between the nodule dry weight, shoot dry weight and plant nitrogen content (r=0.740, 0.641, 0.616, respectively) at $p<0.001$. The most efficient strains of *Bradyrhizobium* isolated from each sampling site based on nodule number, effective nodules, nodule dry weight and plant nitrogen content were A4, B2, C1, D3 and E3. Sequence analysis of these high performing isolates indicated that they showed similarity with reference strains belonging to the genus *Bradyrhizobium* (A4-

Bradyrhizobium japonicum RV9, B2- *Bradyrhizobium guangdongense* CCBAU 51649, C1- *Bradyrhizobium sp.*UFLA05-149, D3- *Bradyrhizobium sp.* B918 and E3- *Bradyrhizobium sp.*UFLA05-149). A phylogenetic tree of the 16SrRNA sequences from the selected isolates showed only 78-95% similarity to the reference strains from Genbank. Isolate B2 showed closeness with *Bradyrhizobium guangdongense* with 95% sequence identity. These five strains of *Bradyrhizobium* can be suggested for use as potential candidates for inoculant production based on their effectiveness.

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Conflict of Interest

No potential conflict of interest to be declared.

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