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Phenotypic and biochemical characterization of rhizobia nodulating bean *(Vigna Unguigulata)* and groundnut *(Arachis hypogaea)* from the soil of ATBU, Yalwa Campus Bauchi State, Nigeria

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Abstract
Plant rhizosphere is a very complex niche in which soil, plants, and microbe interaction occur, and plant roots harbor an assembly of different beneficial and deleterious microorganisms. The nitrogen-fixing attribute of legumes with rhizobia plays a key role in maintaining soil fertility for legumes and subsequently non-leguminous plants. The identification and characterization of indigenous *rhizobium* strains is a prerequisite for sustainable crop production. This research was carried out to isolate and characterize morphologically and using biochemical methods the bacteria from the root nodules of groundnut and bean. Thirteen bacteria were isolated from the root of groundnut (38%) and beans (62%). Based on the colony characteristics, cell morphology, and the ability to absorb Congo red dye seven (G1, G1 (2), G2, G2 (2), B2.3 (2), G2 (2), and B2.3 (1)) out of the thirteen tested isolates were suspected to be rhizobia. The result of the study also shows a variation in diversity and the type of bacteria nodulating the root of groundnut and beans.
However, further phenotypic and molecular characterization is required to confirm the isolates given that, are rod shape which is a characteristic of rhizobia. This study could an innovative way of improving plant nutrient availability or it could be a starting point of molecular studies.

Keywords: characterization, biochemical traits, root nodulating, rhizobia, groundnut and beans

Introduction
Agriculture is one of the most important factors contributing to the economic growth of Nigeria in the 70s. Nigeria has 75 percent of its land suitable for agriculture, but only 40% of this landmass is cultivated (Ayodele *et al.*, 2013) [2] In order to reap a better harvest, farmers inoculate the soil with fertilizers. Fertilizers come in two types, are either chemical or biofertilizers. Increasingly high inputs of chemical fertilizers during the last 150 years have not only left soils degraded, polluted, and less productive but have also posed severe health and environmental hazards. Organic farming methods such as the use of biofertilizers would solve these problems and make the ecosystem healthier. Approximately 22 million hectares of land are now cultivated organically. Organic farming represents less than 1 percent of the world's conventional agricultural production and about 9 percent of the total agricultural area,

Biofertilizer is a type of manure with a specific content of large beneficial microorganism population with the ability to enhance productivity by fixing nitrogenous, solubilizing soil phosphorus, and synthesis of growth promoting substances such as vitamin and hormones. (Glick, 1995) [13] These bioinoculants enhance plant growth and yield. Biofertilizers are products containing living cells of different types of microorganisms that have the ability to mobilize nutritionally important elements from non-usable forms through biological stress. It can be defined as substances that contain living microorganisms that colonize the rhizosphere, or the interior of the plant. These promote growth by increasing the availability of primary nutrients and/or growth stimulus to the target crop when applied to seed, plant surfaces, or soil (Muraleedharan *et al*, 2010) [21]. Several research have demonstrated that a higher population of beneficial microorganisms in soil could increase nutrient retention. This led to germination of up to 20 percent, Increase in the availability and uptake of nitrogenous and phosphorus in plants. Improve the status of soil fertility and Promote soil good health and crop productivity (Archulate, 2009). Beneficial microorganisms in biofertilizers improves and increases plant growth and protect plants from disease and pests (El-yazeid *et al*., 2007).

Biofertilizers have demonstrated great potential as an alternative, renewable, and environmental friendly (ecofriendly) sources of plant nutrients and are an important component of Integrated Nutrient Management (INM) and Integrated Plant Nutrition System (IPNS) (Raghuwanshi, 2012). Application of Biofertilizers promises the saving of half nitrogen and phosphorus fertilizers, improve soil structure/ fertility for sustainable agriculture, reduce soil-borne diseases, reduce environmental dangers of chemical fertilizer and insecticides. Biofertilizer promote plant growth by increasing the availability of primary nutrients or growth stimulus to the target crop when applied to seed, plant surfaces, or soil (Muraleedharan *et al*, 2010) [21]. It is therefore important to develop an effective means for sustainable agriculture in Nigeria. The study aims to isolate and determine the potency of root nodulating bacteria as biofertilizers for sustainable agriculture in Bauchi, Nigeria. To isolate root nodulating bacteria in some leguminous plants. To characterize

the root nodulating bacteria base on morphology and biochemical test.

Materials and Methods

The study was carried out in Abubakar Tafawa Balewa University (ATBU) Bauchi Yelwa campus, in the Laboratory and botanical garden of the Department of Biological Sciences, Faculty of Science. The samples collection was taken in April 2017. Root nodules of Beans and Groundnut were collected from the Botanical Garden

Media Preparation

The isolate was obtained from Beans (*Vigna unguigulata*) and Groundnut (*Arachis hypogaea*). Congo Red Yeast Extract Mannitol Agar Media was used for the isolation of the root nodulating bacteria. All material listed below were properly measured with a weighing balance in order to have an accurately prepared media.

Materials Require for the media preparation include: Yeast extract (1.0g), Mannitol (10.0g), Dipotassium phosphate (0.5g) Magnesium sulphate (0.2g), Sodium chloride (0.1g), Congo red (0.025), Agar (20g). Final pH (at 25°C) 6.8±0.210 ml of Congo red stock solution was (dissolved in 250 mg of Congo red in 100ml water) to 1 liter after the PH was adjusted to 6.8 before adding agar. And Yeast extract mannitol agar (YEMA) was used for the cultivation of the Rhizobium species to obtain a pure isolate. Materials required for the preparation of the YEMA are the same as the above except for Congo red.

Isolation of root nodulating bacteria

Root nodules of Beans (*Vigna unguigulata*) and Groundnut (*Arachis hypogaea*) were used to isolate root nodulating bacterial strains collected from the Botanical Garden of the Faculty of Science. The collected roots were washed in tap water, the nodules erewere separated and surface sterilized in 0.01% HgCl for 5 minutes followed by washing in sterilized distilled water, the nodules were then dipped in 70% ethyl alcohol for 3minutes, and after it was washed in sterilized distilled water, and crushed with a sterilized glass rod in a small aliquot of sterile water. A small amount of the suspension was placed on Congo red combined with the yeast extract mannitol (YAM) agar medium to obtain sparse and distinct colonies. The colonies were cultivated on the Yeast Extract Material (YEM) medium enriched with Bromothymol Blue (BTB) which was used in detecting the production of an acid growth reaction at the end of incubation 2-3 days at 28°C.

Biochemical Test

The Identification of root nodulating bacteria was conducted first using morphological observations. The most important differential stain used in bacteriology is the Gram stain. A thin smear of the bacterial isolate was prepared on the glass slide, air-dried, and heat-fixed. It was stained in the following sequential order: covered with crystal violet for 30 seconds, washed with distilled water, covered with Lugo's iodine solution for 60 seconds, washed with distilled water, covered with 95 % ethyl alcohol and washed immediately, washed with distilled water, counterstained with safranin for 30 seconds and finally washed with distilled water. The stained and air-dried slides were examined under a microscope using the oil-immersion objective technique. Gram-positive bacteria retain the color of crystal violet and stain in purple color, while the Gram-negative take the color of counterstain safranin and appear pink in color. All biochemical tests conducted was done according to Bergey's Manual of Determinative Bacteriology (Miwa *et al.,* 2009), which includes the following tests Gram staining, (shape, morphology, arrangement), Urease, Oxidase, catalase, citrate, and gram staining was conducted for all the pure isolates obtained.

Results

Table 1 Morphological characterization of Beans (*Vigna unguigulata*) and Groundnut (*Arachis hypogaea*)

Table 1: Below summarized the colour and texture of beans and groundnut seeds.

Character	Beans	Groundnut
Color	Yellow	Cream White
Surface	Smooth/Wet	Rough/Discrete
Texture	Slime/Mucous	Not slime

Colony characteristics of the isolates

The colony of the tested isolates varied in colour with the plant type on BTB agar. All the colonies from groundnut were creamy white and that from beans were Yellow. Similarly, all colonies from groundnut were slow growers compared to isolates from beans (Table 2)

Table 2: Colony characterization of the isolates

Isolates	Color produced on BTB agar	Fast/Slow grower
GT1	Cream White	Slow
GT1.2	Cream White	Slow
GT2	Cream White	Slow
GT2.2	Cream White	Slow
G3	Cream White	Slow
B1	Yellow	Fast
B1.(2)	Yellow	Fast
B2. (1)	Yellow	Fast
B2.(2)	Yellow	Fast
B2.(31)	Yellow	Fast
B2.3(2)	Yellow	Fast
B3.3(1)	Yellow	Fast
B3.3(2)	Yellow	Fast

Morphological characteristics of the isolates

Morphological characterization of the tested isolates (Table 3) shows that all the isolates groundnut were rod in shape except G1 which is cocci. Inversely, all the isolates from the bean were cocci except B2.3 (2) which is rode shape. The colony arrangement of all the isolates in both groundnut and bean was single except in B2. (2) Which has a cluster arrangement

Table 3: Morphological characteristic of the isolates

Isolate	G1	G1(2)	G2	G2(2)	G3	B1	B1.(2)	B2.(1)	B2.(2)	B2.3(1)	B2.3(2)	B3.3(1)	B3.3(2)
Shape	Cocci	Rod	Rod	Rod	Rod	Cocci	Cocci	Cocci	Cocci	Cocci	Rod	Cocci	Cocci
Arrangement	Single	Single	Single	Single	Single	Single	Single	Single	Cluster	Chain	Single	Single	Single

Key: G, Groundnut; B, Beans

Biochemical characterization of the isolates

The biochemical characterization of the thirteen isolates isolated from the root nodule of bean and groundnut revealed that three (23%) of the isolates were catalase positive and 77% were catalase negative. These include groundnut G1 (2), and G3 while among the bean isolates only B2.3 (2). All the isolates tested positive to citrate (85%) except G2 and B1. (2) From groundnut and beans accounting for (15%) respectively. For the urease test, all the isolates tested positive. Three of the thirteen isolates were oxidase positive (23%) and the rest (77%) were found to be oxidase negative as shown in Table 3. Below. The presumptive test also indicates that 77% of the isolates were to be gram-positive and only 23% gram-negative. These include G2 (2) from groundnut and B2.3 (1) and B2.3 (2) from beans (Table 3)

Table 4: Biochemical characterization of the isolates

Isolate	G1	G1(2)	G2	G2(2)	G3	B1	B1.(2)	B2.(1)	B2.(2)	B2.3(1)	B2.3(2)	B3.3(1)	B3.3(2)
Catalase	-	+	-	-	+	-	-	-	-	-	+	-	-
Citrate	+	+	-	+	+	+	-	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	+	-	-	-	-	-	-	-	+	+	-	-
Gram stain	+	+	+	-	+	+	+	+	+	-	-	+	+

Key: G, Groundnut; B, Beans

Discussion

This study reports on the phenotypic and biochemical characterization of root nodulating bacteria associated with roots of groundnut and beans. Thirteen bacteria were isolated from the root nodules of Beans (*Vigna unguigulata*) and Groundnut (*Arachis hypogaea*). Base on the colony characteristics, cell morphology, and the ability to absorbed Congo red dye, ten of the isolates were suspected to be rhizobia (Table 3). These isolates were all rod shape and the isolate cultured on YEMA media containing Congo red dye produced colonies that were whitish and yellow indicating that the isolates did not absorb the dye. The inability of the isolate to absorbed Congo red dye is a unique character of rhizobia (Khanafari, *et al.,* 2012; Datta, *et al*., 2015; Naz *et al* 2009).

The isolates obtained (Table 1) from beans were yellow in color and almost wet, mucilaginous, and transparent, while that of groundnut are cream white in color, almost dry, and non-mucilaginous. This is in agreement with the results of Fred *et al.,* (1932) [12] who observed similar strains as in the present study. According to the growth rate on YEMA medium, rhizobia are categorized into two groups, fast -and -slow-growing rhizobia. In this study, all the two groups of rhizobia were observed. All the isolated rhizobia from groundnut when grown on YEMA plates containing BTB, produced creamy whitish color colonies were considered as slow-growing rhizobia (Table 2). However, all rhizobia isolated from beans produced yellow colonies owing to acid production on the medium with a high amount of mucus after 2 days of incubation were considered as fast growers (Turk and Keyser, 1992; Zhang *et al*., 1991) [32, 39].

The result of the study showed a variation in the type of rhizobia nodulating the roots of groundnut and beans. Slow-growing rhizobia nodulate the root of groundnut while fast-growing rhizobia nodulate the root of beans. Saeki *et al.*, (2005), Vietnam, and Sharma *et al.,* (2010), use of YMA-BTB medium for classifying indigenous beans root nodules of fast and slow-growing groundnut rhizobia based on acid or alkali production. Both the fast and slow-growing rhizobia (Table 3) were found to be positive for the urease test, 77% positive for gram staining, 85% citrate utilization, and 23% for oxidase and catalase activity. From the above result, only isolate B2.3(2) was confirmed to be *Rhizobium* species as it tested negative to gram stain, tested positive to citrate, urease, oxidase and catalase test as opposed to other isolates. However, further phenotypic and molecular studies should be conducted on the rest of the isolated especially GT1. (2), GT2, GT2 (2), G3 to confirm the isolates since the isolates were rod shape which is a characteristic of rhizobia. This study could be the starting point of molecular study.

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