Dear Editor,

I am pleased to submit a research article titled “**Symbiotic bacteria with plant growth promoting traits inherent in ground nut (*Arachis hypogaea*) tissues**” for consideration for publication in your reputable journal: Journal of Experimental Research.

The investigation is in line with the journal’s aim of describing work related to microbiology and biotechnology.

This manuscript has not been published and is not under consideration for publication elsewhere and the authors have no conflicts of interest to disclose.

Thank you for your consideration.

Yours faithfully,

Dr Nicholas Ozede Igiehon

Symbiotic bacteria with plant growth promoting traits inherent in ground nut (*Arachis hypogaea*) tissues

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ABSTRACT

Plant growth-promoting bacteria (PGPB) play a crucial role in enhancing and promoting overall plant development. This study investigated the presence and potential benefits of plant growth-promoting bacteria (PGPB) within the root nodules of groundnut (*Arachis hypogaea*) plants, harvested from rose farm at Opkowvin town, Udu local government area, Delta state, Nigeria. Three (3) mature and healthy groundnut plants (sample A, B and C) were uprooted, the samples were aseptically packaged and transported to the laboratory. Symbiotic endophytic bacterial counts were determined using spread plate methods. Bacterial isolates were identified using cultural and morphological characteristics. Bacterial isolates were further subjected to biochemical identification. Plant growth promotion tests were conducted with the production of indole acetic acid, ammonia and hydrogen cyanide. Sample (A) revealed $4.85\text{x}10^3$ cfu/ml, (B) 3.51×10^4 cfu/ml while (C) 2.68×10^4 cfu/ml. Sample A had the highest bacterial colony count of $4.85\text{x}10^3$ cfu/ml while sample C had the least count of 2.68×10^4 cfu/ml. Four bacterial isolates displayed had growth promotion potentials namely *Psedomonas putida*, *Azotobacter*, *Enisifer melitoti* and *Bradyrhizobium japonicum*. These bacteria exhibit distinct mechanisms for enhancing plant growth including biocontrol potential, promotion of nutrient uptake and may reduce the reliance on synthetic fertilizers and enhance crop yields.

Keywords: crop yield, eco-friendly, nutrient uptake, phenotypic characterization, root nodule

INTRODUCTION

Plant growth-promoting bacteria (PGPB) play a crucial role in enhancing the growth and health of plants by facilitating nutrient uptake, suppressing pathogens, and promoting overall plant development (Pandya *et al*., 2017). In the case of groundnut (*Arachis hypogaea*), a fascinating symbiotic relationship exists between the plant and specific bacteria residing in its root nodules. This relationship primarily involves nitrogen-fixing bacteria, such as *Bradyrhizobium spp*. and other rhizobia (Peix *et al.,* 2015).

Rhizobia are soil bacteria capable of establishing a symbiotic association with legume plants where they can reside inside root or stem nodules and fix atmospheric nitrogen (Cardoso *et al.,* 2018). Symbiosis between legume plants and rhizobia in soil is widely studied and is highly significant in agriculture (Peix *et al.,* 2015). Rhizobia usage in agriculture enhanced crop productivity and thereby reduced the cost of inorganic fertilizers (Rajendran *et at.*, 2012). Nodules, especially those collected from the field are not always occupied by rhizobia or by a single microorganism. There is a huge diversity among the endophytic bacteria in different legume nodules (Kumar *et al*., 2017; Dekaka *et al*., 2020). Nodules of pea have been described to contain both the nitrogen-fixing symbiont and associative organism such as *Micromonospora*. Molecular diversity of bacterial endophytes varies from genera to genera and species to species of different plants and even in different tissues of a single plant (Ferchichi *et al.,* 2019).

Groundnut (*Arachis hypogaea*) is an important oilseed crop suitable for cultivation in tropical areas of the world (Ibañez *et al*., 2014). There are three phenotypes in groundnut such as bunching, spreading and semi-spreading (Tajima *et al.,* 2007). To the best of knowledge no attempts have been made to reveal the R and PE bacterial diversity from groundnut nodules and their role in nodulation and plant growth promotion (Mohite, 2013). With the introduction of several new cultivars in recent times, rhizobia inoculants with highly competitive nodulation efficiency are necessary for effective nodulation and nitrogen fixation. Rhizobium might have interaction with other PE (Pande *et al.,* 2017), however, this work suggest that a tripartite interaction is established between R, PE and groundnut plant (Martínez-Hidalgo and Hirsch, 2017), hence, this study aims to reveal the cultivable R and PE in bunch and semi-spreading groundnut phenotypes and to explore the plant growth promotion ability of these endophytes in different groundnut phenotypes.

MATERIALS AND METHODS

Sample collection

Fresh root nodules of groundnut plants (*A. hypogaea*) were harvested from Rose farm at Opkowvin town, Udu local Government Area, Delta state. Three (3) mature peanut plants were uprooted from the ground, and root nodules were collected from the uprooted plants, and were aseptically packaged in sterile ziploc bags and transported to the laboratory for bacteriological assessment.

Identification of plant

The plant *A. hypogaea* was identified in its vernacular names by the farmers and confirmed to be the same as those previously authenticated by the herbarium at the University of Benin, Plant Biology and Biotechnology Department. The specimen was lodged at the herbarium and assigned voucher number UBH-A352.

Surface disinfection of root nodules

The root nodules were washed thoroughly under running tap water to remove loose soil and debris. They were then transferred to a container filled with 70% ethanol and allowed to soak for 60 seconds to kill surface contaminants. After the ethanol treatment, the root nodules were rinsed in sterile water to remove the residual ethanol. The surface-sterilized nodules were macerated in a sterile mortar and pestle.

Serial dilution

Serial dilution of the macerated solution was made by using a sterile pipette to transfer 1 ml from the macerated sample to 9 ml of sterile water (stock solution). Then, 1 ml was taken from the stock solution to a tube containing 9 ml of sterilized distilled water (labelled 10^{-1}). An aliquot of 1 ml from the 10^{-1} test tube was also transferred to another 9 ml of sterilized distilled water (labelled 10^{-2}). One ml (1 ml) each of the serially diluted samples was dispensed with a micropipette and transferred into the corresponding labeled Petri dishes containing nutrient agar. The cultures were incubated at 28±2 °C in an incubator for 24 hrs. Microbial load of the nodule samples was determined visibly by counting the colonies after 24 hours of incubation. The microbial load/ml was then determined by the formula below:

$$cfu/ml = \frac{\text{number of colonies}}{\text{volume plated x dilution factor}}$$

Cultural characterization of the symbiotic endophytic bacteria was also done.

Sub-culturing of symbiotic endophytic bacterial isolates

A single isolated colony of the bacteria from the mixed culture was teased with sterilized wire loop and was streaked on freshly prepared nutrient agar medium. The inoculated nutrient agar plates were incubated at 28±2 ℃ in an incubator for 24 hrs.

Gram staining and Biochemical test

Following sub-culturing of these bacterial isolates, Gram staining and a series of biochemical tests were conducted, such as catalase, oxidase, indole, and citrate tests

PLANT GROWTH PROMOTION TEST

Assay for indole acetic acid (IAA) production

IAA production is a property of symbiotic endophytic and rhizosphere bacteria that stimulate and facilitate plant growth. Bacterial cultures were grown on their respective media at 28 + 2 °C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCla solution). Development of pink color indicated IAA production.

NH_3 production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48-72 hrs at 28 ± 2 °C. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was a positive test for ammonia production.

Hydrogen cyanide production

Bacterial isolates were screened for the production of HCN. Briefly, nutrient agar was amended with 4.4 g glycine/I and bacteria were streaked on modified agar plate. A Whatman filter paper soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28+2°C for 4 days. Development of orange to red color indicated HCN production.

RESULTS

Sample (A) revealed 4.85×10^3 cfu/ml, (B) 3.51×10^4 cfu/ml while (C) 2.68×10^4 cfu/ml. Sample A had the highest bacterial colony count of 4.85×10^3 cfu/ml while sample C had the least count of 2.68×10^4 cfu/ml. The cultural characteristics of these bacterial isolates showed distinctive features. They exhibited a variety of shapes, including circular, filamentous, and irregular forms. The majority of these isolates appeared flat, raised and convex shapes when viewed on a slide. Additionally, the edges or margins of most isolates displayed a smooth and slightly wavy pattern. Majority of these isolates were creamy, while a few leaned towards a whitish appearance. They displayed both chain-like and single-cell arrangements, with the predominant cell shape being rod-like in structure. Four bacterial isolates displayed had growth promotion potentials namely *Psedomonas putida*, *Azotobacter*, *Enisifer melitoti* and *Bradyrhizobium japonicum.*

Table 1: Microbial Count of Symbiotic Endophytic Bacteria Collected from Root Nodule Obtained from Groundnut Plant Collected from Farm

Farm	Plant sample	Bacterial count (cfu/ml)
Rose	A	4.83×10^3
Rose	B	3.51×10^4
Rose	C	2.68×10^4

Key: cfu/ml=Colony forming unit per millimeter

Table 2: Cultural, Morphological and Biochemical Characteristics of Symbiotic Endophytic Bacterial Isolates from Groundnut Root Nodules.

Cultural Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Form	Filament	Circular	Irregular	Circular	Irregular	Irregular	Irregular	Circular	Circular
Elevation	Raised	Raised	Convex	Raised	Convex	Umbonate	Flat	Convex	Flat
Margin	Filiform	Curled	Entire	Entire	Curled	Lobate	Lobate	Entire	Entire
Colour	Cream	Cream	Yellowish	Whitish	Cream	Cream	Cream	Cream	Whitish
Morphological Characteristics									
Cell type	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Cell arrangement	Single	Single	Chain	Single	Single	Chain	Chain	Chain	Chain
Biochemical characteristics									
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	-	+	-	+	+	+	-	+
Indoles	-	-	-	+	-	-	-	+	-
Citrate	+	+	+	+	+	+	+	+	+
Suspected Isolates	*Azotobacter*	*Rhizobium leguminosarum*	*Azotobacter*	*Psedomonas putida*	*Enisifer melitoti*	*Azotobacter*	*Azotobacter*	*Bradyrhizobium japonicum*	*Verticuliae*

Key: + = Positive, - = Negative

Table 3: Gram Staining of Symbiotic Endophytic Bacteria Isolated from Groundnut Plant Root Nodules

Bacterial Isolates	**Gram Staining**
Azotobacter	+
Rhizobium leguminosarum	-
Azotobacter	+
Psedomonas putida	+
Enisifer melitoti	-
Azotobacter	+
Azotobacter	+
Bradyrhizobium japonicum	+
Verticuliae	+

Key: + = Gram positive, - = Gram negative.

Table 4: Plant Growth Promoting Test of Symbiotic Endophytic Bacterial Isolates

Bacterial Isolates	Plant Growth Promotion Test		
	Indole Acetic Test	**Ammonia**	**Hydrogen Cyanide**
Azotobacter	-	-	-
Psedomonas putida	-	+	+
Enisifer melitoti	+	+	-
Bradyrhizobium japonicum	-	-	+

Key: + = Positive, - = Negative

DISCUSSION

Symbiotic endophytic bacterial isolated from ground nut root tissues were characterized phenotypically and isolates were screened for their plant growth promoting traits such as ammonia production, hydrogen cyanide production and indole acetic acid production test, The result indicated that *P. putida* and *E. melitoti* tested positive to ammonia production, *P. putida* and *B. japonicum* tested positive to hydrogen cyanide production while *Azotobacter* tested negative for all.

The investigation into the presence and characteristics of plant growth-promoting bacteria (PGPB) within the root nodules of groundnut plants has unveiled valuable insights into the symbiotic relationships that underpin sustainable agriculture. This study employed a comprehensive approach, encompassing morphological characteristics, biochemical tests, and plant growth-promoting assays, to identify and elucidate the potential roles of *Pseudomonas putida*, *Azobacter*, *Bradyrhizobium japonicum*, and *Ensifer meliloti* in promoting plant growth and overall crop productivity. It was reported by Kloepper *et al.,* (1980) that specific strains of the *Pseudomonas* *fluorescens-putida* group have recently been used as seed inoculants on crop plants to promote growth and increase yields. A similar observation was obtained by Farah *et al*. (2006) which stated that PGPB may indirectly influence the growth of plants by producing siderophores. Siderophores bind to the available form of iron Fe^{3+} in the rhizosphere, thus making it unavailable to phytopathogens thereby protecting plants from pathogenic attack.

One of the primary steps in this research involved the observation and analysis of the morphological characteristics of the isolated bacteria. The distinct features of these microbes, such as colony morphology, cell shape, and staining characteristics, provided initial clues for their identification. The presence of key morphological traits, consistent with known PGPB species, confirmed their identity. To be specific the IAA experiment revealed the production of IAA by symbiotic endophytic bacterial species (Table 4). The findings from this study indicated that *Enisifer melitoti* may possess the genetic properties required for the synthesis of IAA, which indicates that they can promote the formation of lateral roots and root hairs. This can improve the plant's ability to absorb water and nutrients from the soil, leading to better overall plant health and productivity, which is partly in line with the study of Daniela *et al*. (2018) who indicated that *Bradyrhizobium sp*. has the ability to produce IAA and this capacity has been demonstrated *in vitro* with soybean seed germination.

The study of Preyanga *et al*. (2021) revealed that groundnut nodule endophytic bacterial diversity is vast and not influenced by plant phenotypes. When two different bacterial species were co-inoculated into groundnut plants, they significantly improved the plant growth than single inoculation. Their study suggest new and beneficial bio-fertilizer formulation that will enhance and promote plant growth and reduce the application of chemical fertilizer. Findings from this study revealed significant potential PGPB that could have eco-friendly agricultural applications. Harnessing the potential of these beneficial microbes could lead to sustainable agricultural practices. Utilizing PGPB as biofertilizers or biopesticides may reduce the reliance on chemical inputs, mitigating the environmental impact of agriculture.

Comparative analysis of our results with previous studies on PGPB in legume-rhizobia interactions highlights both commonalities and differences. While certain mechanisms of plant growth promotion may be conserved across these interactions, the specific species involved and their impact on crop health can vary. Understanding these effects is crucial for tailoring microbial applications to specific crop-legume systems. This study pave way for future research direction.

Investigating the potential of these isolated PGPB strains as bio-inoculants in field trials could provide valuable insights into their practical applications in agriculture. Furthermore, exploring the genetic and molecular aspects of these microbes may unveil novel strategies for enhancing their effectiveness as plant growth promoters.

CONCLUSION

The use of morphological characteristics, biochemical tests, and plant growth-promoting assays have revealed the presence and significance of *Pseudomonas putida*, *Azobacter*, *Bradyrhizobium japonicum*, and *Ensifer meliloti* in the root nodules of groundnut plants. The multifaceted mechanisms employed by these microbes offer promising prospects for sustainable and eco-friendly agricultural practices. Furthermore, future study will expand on the complexities of plant-microbe interactions, enhancing crop productivity while minimizing the environmental footprint of agriculture.

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CONFLICTS OF INTEREST

All authors have no conflicts of interest to disclose.

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