



Isolation and Identification of Microorganisms Growing on the Root of Leguminous Plants (Groundnut, Soya bean and Pea)

Bukola Catherine Akin-Osanaiye^{1*}, Oluwatobi Olaife Arowolo¹ and Ifeyomi Wilfred Olobayotan¹

¹Department of Microbiology, Faculty of Science, University of Abuja, Federal Capital Territory, Abuja, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. The work is a research project of an undergraduate student of author OOA under the supervision of author BCAO with the assistance of author IWO. All the authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJBGMB/2019/v2i330061

Editor(s):

(1) Dr. Arulselvan Palanisamy, Adjunct Associate Professor, Muthayammal Centre for Advanced Research (MCAR), Muthayammal College of Arts and Science, Tamil Nadu, India.

Reviewers:

(1) Yemisi Jeff-Agboola, University of Medical Sciences, Nigeria.
(2) Aimé Christian Kayath, Université Marien Ngouabi, Republic of the Congo.
(3) Noubissi Paul Aimé, University of Buea, Cameroon.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/51463>

Original Research Article

Received 24 July 2019
Accepted 26 September 2019
Published 03 October 2019

ABSTRACT

Study on the isolation and identifications of bacteria associated with the root of legumes were conducted using Spread Plate Technique. The frequencies of occurrences of the bacteria isolate showed that a total of sixteen (16) bacteria belonging to three genera and four species were isolated from the leguminous plants. Maximum number recovered from sample collected from the root of groundnut was seven (7) followed by Soya bean with five (5) while Pea recorded the least number of four (4). Role of *Bacillus subtilis* in the soil around the leguminous plant was the highest, which covered about 37.50% of the total isolates. Other bacteria that were also isolated from the soil around the legumes root include *Bacillus cereus* and *Staphylococcus aureus* which covered about four (4) each representing 25.0% of the total isolates while *Pseudomonas aeruginosa* recorded the least value of 12.50%. The bacteria isolated from the root of the legumes were not significantly different ($P < 0.05$). The bacteria have Nitrogen-fixing potential, having isolated from three leguminous plants which include Soya bean, Groundnut and Pea.

*Corresponding author: E-mail: tou_femi@yahoo.com;

Keywords: Isolation; identification; leguminous; nitrogen-fixing.

1. INTRODUCTION

The family Leguminosae comprises about 750 genera and more than 18,000 species, from which only 8 % are cultivated plants. Little information is available on wild legumes and their root-associated bacteria. These interactions are particularly important when they result in tolerance to extreme environmental conditions such as severe drought, elevated temperature and salinity [1]. The rhizosphere is a microecological zone in direct proximity of plant roots. It is often operationally defined as the soil that clings to roots after being gently shaken in water. The actual extent of the rhizosphere is dependent on the zone of influence of the plant roots and associated microorganisms. This area of soil is considered to be the most biodiverse and dynamic habitat on Earth [2]. It is widely accepted that rhizosphere and rhizoplane microorganisms can influence plant growth and development. The term plant growth-promoting rhizobacteria (PGPR) was coined for the bacterial biocontrol agents of the rhizosphere. Some years later, the term plant growth-promoting bacteria (PGPB) was proposed to designate rhizobacteria that enhance plant growth by other ways [3]. Plant growth-promoting (PGP) activities have been reported for a series of bacterial species including *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* [4,5,6]. Although the mechanisms by which PGPB promote plant growth are not fully understood [5,7] these bacteria can improve plant growth by Nitrogen fixation, phytohormone and siderophore production, phosphorus solubilization, and disease control [3,1]. Moreover, the root-surrounding environment, the rhizosphere, is a dynamic soil site of nutrient turnover. Bacteria around plant roots perform a wide range of metabolic activities and are able to make use of a wide range of low molecular mass organic compounds and of some more complex compounds as carbon and energy sources [8]. These microorganisms play major roles in nutrient transformation and element cycling and influence the availability of these nutrients for plant uptake [9]. Accordingly, studies suggest that proteobacteria, actinobacteria, non-sporulating rods, pseudomonads, and actinomycetes are among the most dominating populations of bacteria within the rhizosphere [10]. Due to their small mass, they only account

for a small amount of the biomass within the soil. Metabolites released from the root hairs of the plants act as chemical signals for bacteria to actively move towards the root surface where they can obtain nutrients [11]. Additionally, bacteria known as plant-growth-promoting-rhizobacteria (PGPR) colonize roots very efficiently. They perform important functions to satisfy plant health and growth in various ways. Furthermore, symbiotic relationships with plants lead to benefits for both the plant and the PGPR, such as better nutrient uptake and stimulation of root growth [12]. A better knowledge of these bacteria and their implications on plant physiology could change traditional crop management practices regarding plant nutrition and defense mechanisms. For a maximum exploitation of the plant-bacteria association, effective bacteria must be selected in plant studies that take specific ecological conditions into consideration, e.g., crop management, soil, temperature, etc. The aim of this study was to isolate and identify microorganisms growing on the root of leguminous plants [13].

2. MATERIALS AND METHODS

2.1 Collection of Soil Sample

A total of fifteen (15) soil sample were collected randomly from the roots of three (3) different leguminous plants in Gwagwalada, FCT-Abuja, Nigeria.

2.2 Methods

The sterilization of glass wares such as conical flasks, beaker and test tubes after washing with detergent was carried out in hot air oven at 160°C for 2 hours. A total of fifteen (15) soil samples were obtained randomly from the roots of three (3) different leguminous plants in Gwagwalada FCT-Abuja. Five samples each were gotten from each from the melon, groundnut and mucunna plants. At each location, about 10 g of soil were weighed from the region of root nodules of the legumes. Soil samples were collected in the sterile urine sample bottles and brought to the Microbiology laboratory of the Department of Biological Sciences, University of Abuja, for the isolation and identification of microorganisms associated with the roots of legumes.

2.3 Preparation and Sterilization of Media

Nutrient agar was used in this study and prepared according to the manufacturer instruction thus; 28 grams of nutrient agar was weighed and suspended in 1000 mL of distilled water. It was shaken carefully so as to dissolve completely; cotton wool was wrapped in aluminum foil and was used to cover the mouth of the conical flask which was transfer to the autoclave for sterilization. It was autoclaved for 15 minutes at 121°C. After autoclaving the agar was placed on the work bench and allowed to cool to 47°C before dispensing about 20 mL was poured into sterile petri dishes and allowed to solidify.

2.4 Isolation of Bacteria Associated with Root of Legumes

The bacteria were isolated from the soil around the root of the legumes using the spread plate technique. One gram (1 g) of the soil samples were dissolved in 10 mL sterilized distilled water. The soil suspensions were diluted up to 10^5 . The samples were inoculated on already prepared nutrient agar plates. The inoculated plates were then incubated at 37°C for 24 hours. Colony developments were observed after incubation period. The colony count was done using colony counter. The counted colonies were expressed as colony-forming units per gram of sample [14]. All the experiments were done in replicates.

2.5 Preparation of Pure Isolates

Each distinct colony was be sub-cultured on another freshly prepared nutrient agar plates to obtain pure isolates. Sub-cultured plates were incubated at 37°C for 24 hours. The pure colonies were subjected to biochemical characterization according to Cowan and Stell (1993) and [15].

2.6 Identification of Bacteria Isolates

Isolates obtained were identified on the basis of biochemical tests, Gram staining reactions which include the microscopic features and morphological assessment through macroscopic features. Among the characteristics used were: colonial characteristics such as size, surface appearance, texture and colour of the colonies [15].

2.7 Cultural Characteristics

Colonies were observed for size, texture, colour, shape, colony surface and edges/margin.

2.8 Morphological Characteristics

Morphological characterization through Gram staining was observed. The procedure is as follows: A moderately thin smear of the cultures (to be identified) will be made on clean grease free slides. The slides will be air dried and the reversed sides will be quickly passed three times over a flame that is, heat fixed to avoid wash-off during subsequent process of flooding. The slides will be stained with crystal violet for 60 seconds and washed with water. The slides would then be flooded with Grams iodine for 60 seconds, washed with water and the slide decolorized with acetone for 5 seconds. They will be further flushed with water and then counter stained with safranin for 30 seconds. The slides will be finally washed with water and allowed to air dry before they are examined under oil immersion objective lens of the microscope.

2.9 Sugar Fermentation Test

One tube each labelled accordingly with the appropriate 10% aqueous solution of the test sugar (lactose, sucrose and glucose) as well as an inverted Durham tube (free from air bubbles) that has been fully filled with already sterilized broth. About 2 drops of bromocresol blue was added as the indicator for gas production. Each test tube was aseptically inoculated with 1mL of the suspension of the bacterial isolates. Un-inoculated sugar solution in test tubes served as the control. The preparations were incubated for 24 hours at 37°C. Presence of bubbles in the Durham tube indicates positive Gas production and change in colour of the bromocresol blue to yellow confirms positive fermentation.

2.10 Starch Hydrolysis

Ten percent (10%) solution of soluble starch was prepared in sterile water and steam for 1 hour. Twenty milliliter (20 mL) of this solution was added to 100 mL already prepared nutrient agar plates. Inoculated starch agar was incubated for 5 days, and then flooded with dilute iodine solution. Hydrolysis was indicated with clear zones around the growth.

2.11 Biochemical Tests

The biochemical characteristics used include; oxidase test, coagulase test and IMViC test (citrate utilization test, indole test, methyl red and voges-proskauer test).

2.12 Citrate Utilization Test

This test was carried out by inoculating the slope and stabbing the butt of a 5 mL Simmon's citrate agar with the test organism. An uninoculated control was also setup in each case. These were incubated at 37°C for 48 hours, growth indicated that the organism is able to use citrate as a sole carbon source and is usually accompanied by the medium turning from green to bright blue [15].

2.13 Catalase Test

Three (3) mL of hydrogen peroxide solution was dispensed in a sterile test tube and several colonies of the test organism was picked and immersed in the hydrogen peroxide solution using an inoculating loop. It was observed for immediate bubbling which indicates positive result [15].

2.14 Coagulase Test

A drop of distilled water was placed on a clean grease free slide and a colony of the test

organism was picked and emulsified on the drop of water to make a thick suspension. A loopful of plasma was also added to the suspension and mixed gently to observe for clumping of the organism within 10 seconds [15].

2.15 Determination of Frequency of Occurrence of Bacteria Isolates

The frequency of occurrence of the isolated bacterial associated with the leguminous plants was determined by taking the sum of all the numbers of the organisms in each sample and the percentage was calculated as:

$$\frac{\text{Number of each Isolates}}{\text{Total number of Isolates}} \times 100$$

3. RESULTS

3.1 Colony Counts of the Bacterial Isolates

The results of the colony counts of the bacteria isolated from root of leguminous plant are shown in Table 1.

3.2 Identification of Bacterial Isolates

The results of the morphological and biochemical characteristics are presented in Tables 2 and 3 respectively.

Table 1. Colony count of bacterial isolates

Samples	Density (CFU/mL)		
	Soya Bean	Groundnut	Pea
1	$4.90 \times 10^{10} \pm 0.30$	$5.20 \times 10^{10} \pm 1.10$	$5.70 \times 10^{10} \pm 0.40$
2	$5.15 \times 10^{10} \pm 0.95$	$4.20 \times 10^{10} \pm 0.40$	$5.20 \times 10^{10} \pm 0.30$
3	$4.80 \times 10^{10} \pm 0.80$	$4.50 \times 10^{10} \pm 0.10$	$7.80 \times 10^{10} \pm 0.40$
4	$5.90 \times 10^{10} \pm 0.20$	$5.10 \times 10^{10} \pm 0.10$	$6.75 \times 10^{10} \pm 0.45$
5	$5.40 \times 10^{10} \pm 0.20$	$5.00 \times 10^{10} \pm 0.30$	$4.50 \times 10^{10} \pm 0.40$

Each value represents Mean ± Standard deviation of two independent experiments

Table 2. Morphological characteristics of bacterial isolates

Macroscopic characteristics						
Isolates	Shape	Colour	Edge	Elevation	Surface	Optical
A	Rod	White	Entire	Flat	Rough	Translucent
B	Rod	White	Entire	Flat	Rough	Opaque
C	Rod	Green	Entire	Flat	Smooth	Opaque
D	Cocci	Cream	Entire	Raised	Smooth	Opaque

Keys: A= Isolate one, B= Isolate two, C= Isolate three, D= Isolate four

Table 3. Biochemical characteristics of isolates

Isolates	Biochemical tests						Probable organisms
	GR	S	CA	CO	CI	L	
A	+	+	+	-	+	+	<i>Bacillus subtilis</i>
B	+	-	+	-	-	-	<i>Bacillus cereus</i>
C	+	-	+	-	-	-	<i>Pseudomonas aeruginosa</i>
D	+	-	+	+	-	-	<i>Staphylococcus aureus</i>

Keys: + = Positive, - = Negative, CA = Catalase test, CO = Coagulase test, GR = Gram reaction, S = Starch hydrolysis, L = Lactose and CI = Citrate utilization test

3.3 Occurrence of Bacteria Isolates

The frequencies of occurrences of the bacteria isolates are represented in Table 4 while Table 5 shows the role of the bacteria isolates in the soil with leguminous plant.

4. DISCUSSION

In the present study, it appears that *Bacillus* species, *Staphylococcus* species and *Pseudomonas* species are associated with the roots of some leguminous plants. These bacteria represent many species associated with the leguminous plants. Sixteen (16) bacteria belonging to three genera and four species were isolated from the leguminous plants. Maximum number recovered from sample collected from the root of groundnut was seven (7) followed by melon with five (5) while mucunna recorded the least number of four (4). This is in agreement

with [16] who reported 114 bacterial isolates from 15 root nodules, of which nearly 60% were rhizobia while the remainder was identified as belonging to several other genera of which eight species were exclusively found only in root nodules. Probably all the organisms whose presence has a beneficial relation might get associated with the plant nodules. There are several reports depicting bacteria isolated from roots as plant growth promoting [17]. In this study, the role of *Bacillus subtilis* in the soil with leguminous plant was the highest, which covered about 37.50% of the total isolates. This may be due to the spore-forming capability of many bacilli they are readily adaptable to field applications. *Bacillus* species also have been shown to have positive growth effect on rhizobial-plant interaction. Other bacteria that were also isolated in this study from the legumes root include *Bacillus cereus* and *Staphylococcus aureus* which covered about four (4) each

Table 4. Frequencies of occurrence of bacterial isolates

Locations	Number of sample	Isolates	Frequency
Soya Bean	5	<i>Staphylococcus aureus</i>	2
		<i>Bacillus subtilis</i>	2
		<i>Bacillus cereus</i>	1
		<i>Bacillus cereus</i>	1
Groundnut	5	<i>Bacillus subtilis</i>	3
		<i>Pseudomonas aeruginosa</i>	1
		<i>Staphylococcus aureus</i>	1
Pea	5	<i>Bacillus subtilis</i>	1
		<i>Bacillus cereus</i>	2
		<i>Pseudomonas aeruginosa</i>	1
Total	15		15

Table 5. Role of Microorganisms in soil with leguminous plants

Bacteria isolates	Frequencies of occurrence	Percentage (%)
<i>Bacillus subtilis</i>	5	38.00
<i>Bacillus cereus</i>	4	25.00
<i>Staphylococcus aureus</i>	4	25.00
<i>Pseudomonas aeruginosa</i>	2	13.00
Total	15	100

representing 25.0% of the total isolates while *Pseudomonas aeruginosa* recorded the least value of 12.50%. This agrees with [18] who reported that *Bacillus* species were isolated from the root nodules of soyabean.

5. CONCLUSION

In conclusion, this study shows that *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* have the potential to fix-Nitrogen, having isolated from three leguminous plants which include Melon, Groundnut and Mucunna. The beneficial effects of legumes in agriculture have been recognized even before the principles of crop rotation were established. Symbiotic nitrogen fixation in legumes in association with rhizobia is important in the development of sustainable agriculture. Using symbiotically fixed Nitrogen instead of chemical fertilizers decreases the need for application of chemical nitrogen fertilizers to crops.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Barea JM, Pozo MJ, Azcon R, Azcon-Aguilar C. Microbial co-operation in the rhizosphere. *Journal of Experimental Botany*. 2005;56:1761-1778.
2. Hinsinger P, Bengough AG, Vetterlein D, Young IM. Rhizosphere: Biophysics, biogeochemistry and ecological relevance. *Plant Soil Plant and Soil*. 2009;321(1-2): 117-152.
3. Bashan Y, Holguin G. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: Biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. *Soil Biology and Biochemistry*. 1998;30:1225-1228.
4. Versalovic J, Schneider M, De Bruijn FJ, Lupski JR. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Cell Biology*. 1994;5:25-40.
5. Glick BR. The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology*. 1995;41:109-114.
6. Han J, Sun L, Dong X, Cai Z, Sun X, Yang H, Wang Y, Song W. Characterization of a novel plant growth-promoting bacteria strain, *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. *Systemic and Applied Microbiology*. 2005;28:66-76.
7. Ahmad F, Ahmad I, Khan MS. Screening of free-living rhizospheric bacteria for their multiple plant growth-promoting activities. *Microbiology Research*, 2008;163:173-181.
8. Misko AL, Germida JJ. Taxonomic and functional diversity of pseudomonads isolated from the roots of field grown canola. *Microbial Ecology*. 2002;42:399-407.
9. Dunfield KE, Germida JJ. Diversity of bacterial communities in the rhizosphere and root interior of fieldgrown genetically modified *Brassica napus*. *Microbiology Ecology*. 2001;38:1-9.
10. Sylvia D, Fuhrmann J, Hartel P, Zuberer, D. Principles and applications of soil microbiology. Pearson education Inc. New Jersey. 2005;23-25.
11. Nihorimbere V, Ogena M, Thonart P. Beneficial effect of the rhizosphere microbial community for plant growth and health. *Science of The Total Environment*. 2011;15: 5-11.
12. Kent AD, Triplett EW. Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annual Review of Microbiology*, 2002;56:211-236.
13. Giongo, Adriana, Anelise Beneduzi, Adriana Ambrosini, Luciano Kayser Vargas, Marcos Roberto Stroschein, Flávio Luiz Eltz, Maria Helena Bodanese-Zanettini, Luciane Maria Pereira Passaglia. Isolation and characterization of two plant growth-promoting bacteria from the rhizoplane of a legume (*Lupinus albus*) in sandy soil. *Revista Brasileira de Ciência do Solo*. 2010;34(2):361-369.
14. Geetha R, Maheshwari HP, Sanket JJ. Isolation and characterization of nodule-associated *Exiguobacterium* sp. from the root nodules of Fenugreek (*Trigonella foenum-graecum*) and their possible role in plant growth promotion. *International Journal of Microbiology*, 2012;55:1-8.
15. Chesebrough M. Medical laboratory manual for tropical countries. Second edition, University Press, Cambridge, Great Britain, 2006;11:377.
16. Sturz AV, Christie BR, Matheson BG, Nowak J. Biodiversity of endophytic bacteria which colonize red clover nodules,

- roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils*. 1997;25(1):13–19.
17. Mishra J, Singh R, Arora NK. Plant growth-promoting microbes: diverse roles in agriculture and environmental sustainability. Krumar V, Kumar M, Sharma S, Prasad R. (Eds.). *Probiotics and Plant Health*, Springer. 2017;71–111.
18. Bai Y, D'Aoust F, Smith DL, Driscoll BT. Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. *The Canadian Journal of Microbiology*. 2002;48(3):230–238.

© 2019 Akin-Osanaiye et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
<http://www.sdiarticle4.com/review-history/51463>