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Isolation and Identification of *Rhizobium* Species from Root Nodules of *Arachis hypogaea* L. and *Vigna mungo* (L.) Hepper in Tamil Nadu, India

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Abstract: Soil bacteria called rhizobia are very important and have been used in agricultural practices for decades. Rhizobium forms a symbiotic relationship with plant legumes to fix atmospheric nitrogen. Due to this symbiosis, root nodules are formed where in rhizobia fixes atmospheric nitrogen to ammonia. This paper reviews the ability for nitrogen fixation in *Arachis hypogaea* and *Vigna mungo* and their genetic and edaphic factors were studied. Genetic analysis was studied using Random Amplified Polymorphic DNA (RAPD)-PCR of the isolates. The nodulation ability of rhizobium was studied and the plants were grown at green house temperature.

Keywords: Rhizobium, *Arachis hypogaea*, *Vigna mungo*, (RAPD)-PCR, etc

1. Introduction

Nitrogen fixation using biological agents is mostly associated with legumes and contributes to the sustainable agricultural development [1]. This is a very good example of a symbiotic relationship accounting to the beneficial plant-microbe interaction [2]. Biological nitrogen fixation (BNF) is the cheapest and environment friendly procedure in which nitrogen fixing micro-organisms, interacting with leguminous plants, fix aerobic nitrogen into soil [3]. Atmospheric nitrogen fixation is carried out by microorganisms to fixed forms of nitrogen, such as ammonia and nitrate to be used by the plants. Nitrogen (N) is a constituent of proteins, enzymes, chlorophyll, and growth regulators to plants and its deficiency causes reduced growth, leaf yellowing, reduced branching and small trifoliate leaves in legumes [4]. Legumes have been studied extensively since they harbour nitrogen-fixing bacteria that fix nitrogen in their root nodules. Nitrogen fixation is carried out by nitrogen fixing bacteria called rhizobia belonging to the family Rhizobiaceae.

They maintain a symbiotic relationship with legumes of the family Fabaceae to fix atmospheric nitrogen as they are unable to do so without a plant host. Legumes secrete flavonoids which are recognised by the bacteria and induce them to synthesis lipochitooligosaccharides (LCOs), also known as nod factors, which are responsible for the nodulation process [5]. To, establish an effective symbiosis, two main classes of bacterial symbiosis genes are needed: nodulation and nitrogen fixing genes. The enzymes encoded by the nodulation genes such as *nodABC* are responsible for the synthesis of nod factors or LCOs that interact with the plant flavonoids [1]. *nodD* is one of the most important regulatory nodulation gene, that is involved in the transcription of other nod genes in the presence of plant signal inducers such as flavonoids, thus initiating the nodulation process [5]. Nitrogenase enzyme (*nifHDK*) which is responsible for the fixation of atmospheric nitrogen is encoded by the nitrogen fixation genes (*nif* and *fix)* [1].

The genus *Bradyrhizobium* which is slow-growing rhizobia generally form nodules in Arachis *hypogaea.*

However, fast-growing species of Rhizobium has also been seen to nodulate this plant [6]. *Vigna mungo,* on the other hand is nodulated by the genus *Bradyrhizobium, Rhizobium* and *Sinorhizobium* [7]. In India, groundnut yields 550 to 1100 kg ha^{-1} in different years and consequently the total production of the country also varied from 4.3 to 9.6 million tons. The groundnut (Arachis *hypogaea L.)* is a highly nutritious food; are rich in protein, minerals and vitamins. The groundnut contain fat 34-54% and very important in crop rotation system as they help in biological nitrogen fixation. Black gram (*Vigna mungo* L.), is one of the important pulses crop, grown throughout the country. The crop is resistant to adverse climatic conditions and improves the soil fertility by fixing atmospheric nitrogen in the soil. It has been reported that the crop produces equivalent to 22.10 kg of N/ha., which has been estimated to be supplement of 59 thousand tonnes of urea annually. The pulse 'Black gram' plays an important role in Indian diet, as it contains vegetable protein and supplement to cereal based diet.

Therefore, this study was carried out to isolate and identify the nitrogen-fixing bacterium (*Rhizobium* spp.) from *Arachis hypogaea* L. and Vigna *mungo L*. so as to explore their environmental friendly contributions to soil fertilization.

2. Materials and Methods

2.1. Sample Collection [8]

Plants were collected from 31 agricultural field sites in different districts of Tamil Nadu, 16 sites for *Arachis hypogaea* and 15 sites for *Vigna mungo* sample by using Tamil Nadu Agricultural Department Survey.

The legume plants were uprooted; the roots along with mature nodules were thoroughly washed in running water until the removal of adhering soil particles. The collected nodules were kept in sterile polythene bags and for further investigation.

2.2. Isolation and Purification of *Rhizobium* from Root Nodules [8]

The collected nodules were washed several times with sterile distilled water and surface sterilized using 0.1% mercuric chloride solution for 1 min, 70% ethanol for 4-5 min and washed in distilled water; it was transferred to 70% ethanol for 2 min and finally washed in distilled water to remove all the traces of sterilants.

The sterilized root nodules were crushed by adding small aliquots of sterile water, which was 10^{-1} dilution and was serially diluted up to 10^{-7}. The diluted suspensions 10^{-5}-10^{-7} were selected and 0.1 ml of suspension was inoculated in plates containing sterile Yeast Extract Mannitol Agar medium [YEMA] with Congo red. The inoculated plates were incubated at 30 ± 2°C for three days.

At the end of the incubation period the Rhizobial colonies appeared white, translucent and elevated. They were inoculated aseptically on YEMA medium. The isolates were purified, sub cultured and stored for further investigation.

2.3. Identification of *Rhizobium* [8]

The isolates were subjected to gram staining and checked for motile characteristics (motility test). The isolates were identified via growth in YEMA medium with Congo red, litmus milk agar and lactose agar, and the tests include polyhydroxy butyrate (PHB) test and Hoffer's alkaline broth test.

The isolates were then subjected to biochemical analysis which includes Indole Test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Hydrogen sulfide production test, Nitrate reduction test, Urease test, Catalase test, Oxidase test, Starch hydrolysis, Gelatin hydrolysis test, Triple Sugar Iron Agar Test, Mac-Conkey Agar Test

2.4. RAPD-PCR Analysis of Genomic DNA

2.4.1. DNA Extraction [9]

YEM broth culture (1.5 ml) containing *Rhizobium* isolates was centrifuged at 12,000 rpm for 10 min at 4°C. The pellets were resuspended and lysed in 200 µl lysis buffer (40 mM Tris-acetate pH 7.8; 20 mM sodium-acetate; 1 mM EDTA and 1% SDS). After mixing 66 µl of 5 M NaCl was added to remove cell debris and proteins. The viscous mixture was then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was transferred into a fresh tube to which an equal volume of chloroform was added and gently inverted until a milky solution was formed. The solution was centrifuged at 12,000 rpm for 3 min after which the extracted supernatant was transferred into another tube and DNA was precipitated with 100% ethanol, dried and re-dissolved in 50 µl of pure water. The concentration and purity of DNA was estimated spectrophotometrically at 260-280 nm.

2.4.2. RAPD-PCR Amplification [10]

For RAPD-PCR 10 oligonucleotide OPI-06 primer (AAGGCGGCAG) was used. The PCR reaction mixture (25 µl) contained 1 µl of 50 µg genomic DNA, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1.5 µl of Taq DNA polymerase, 1 µM primer and rest of the volume was adjusted with distilled water. The amplification reaction was performed in thermocycler. The amplified PCR products were separated by gel electrophoresis on 1.5% (w/v) agarose gel and visualized under UV-transilluminator. 1 kb DNA ladder was used as marker.

2.4.3. Construction of Dendrogram Based on Statistical Analysis [11]

The amplified bands were scored as 1 and 0 based on band presence and absence, respectively. Size of amplified bands were estimated using gel pro analyser software. The binary data set was used to calculate the pairwise similarity index and to assemble the corresponding similarity matrix. The matrix obtained was used to generate a dendrogram using the UPGMA method (Unweight pair group method Arithmetical means). The distances in the dendrogram were compared with the genetic distances between genotypes pairs to

calculate the cophenetic correlation. All the analyses were performed with the aid of the 2006 version of Non-linear Dynamics – PC computer program.

2.5. Testing Nodulation Ability of *Rhizobium* [8]

2.5.1. Test Tube Method

Nitrogen-free agar medium (e.g. Jensen's medium) was used to prepare agar slants in boiling test tubes. Seeds were surface sterilized with 30% H_2O_2 for 10 min and transferred aseptically. On germination, they were inoculated with 1 ml rhizobial suspension. At regular intervals, the moisture content of the tube was checked and the nutrient solution replenished when necessary. The tubes were incubated for 3-4 weeks in growth cabinets, the plants were examined and nodulation data was obtained. This method restricts the growth of seedlings in closed test tubes.

2.5.2. Plastic Cup Method

By using a plastic cup, seedlings were grown on soil. Plastic cups were filled with 500 g of sterilized soil. After surface sterilization, the seeds were sown in the pot and inoculated with 10 ml of rhizobial suspension. Plants were harvested and shoot length, root length and nodule number was observed.

2.5.3. New Nodulation Ability Test

The Leonard Jar method was modified to check the nodulation ability of the plants. This method has been used for the first time in our work. This was done using 1 litre water bottle. The bottle was cut into 2 portions. The upper portion of the bottle was inverted and placed into the lower portion (reservoir). The mouth of the bottle should be present 2-3 cm above the base of the reservoir. The growth medium (soil) in the bottle is irrigated by a cotton wick running the length of the bottle and extending out of the mouth and into the reservoir containing the nutrient solution. In this work, a cotton wool was used as a wick since it is adequate and easy to obtain. A small amount of cotton stuffed into the neck of the bottle will aid in securing the position of the wick and prevent the soil from setting in the reservoir. Wick material should be boiled in water and dried prior to use. This removes air trapped in the wick and improves water conductivity. While holding the wick in a central position, the bottle is filled with sterilized soil. The soil was packed to minimize air spaces. The soil in the bottle was moistened by adding 150-200 ml of N-free nutrient solution. The nutrient solution was allowed to saturate the soil. The reservoir was filled with 400 ml of nutrient solution.

Vigna mungo and *Arachis hypogaea* seeds were germinated and sown; the jars were inoculated with 10 ml of rhizobial suspension. Control plans served as control. The plants were harvested after 6 weeks and the shoot length, root length and number of nodules was observed.

2.5.4. Green House Experiment

The green house pot trials were conducted to test the nodulation ability of *Rhizobium*. Before planting, the seeds were surface sterilized by 30% H_2O_2 for 5 min. Pot were surface sterilized by alcohol and filled with 2 kg of sterilized soil. Seeds were sown and inoculated with 20 ml of rhizobia suspension. Green house temperatures varied between 29°C (day) and 21°C (night). Pots were irrigated at regular intervals. After 45 days, plants were harvested and shoot length, root length and nodule number was observed.

3. Results

3.1. Morphological Characteristics of *Rhizobium*

Thirty-one strains of rhizobia were isolated from nodulating *Arachis hypogaea* and *Vigna mungo* in different districts agricultural sites in Tamil Nadu. All the rhizobial isolates appeared to be dominant growing on YEMA medium and 31 isolates were designated as Rh01 to Rh31. Phenotypically most of the isolates were fast growers and failed to absorb Congo red in the medium. Mostly the growth of isolates was obtained on third day of incubation. Some of the isolates were slow-growers and growth was obtained after 3-5 days.

The morphology of *Rhizobium* colony on YEMA medium was mostly circular, mucoid, white and translucent. *Rhizobium* Rh07 and Rh22 colony showed less mucoid whereas the colonies of all isolates were mucoid and highly mucoid after three days of incubation at 28°C. Under the microscope, all the isolates were non-motile, gram negative and rod shaped bacteria (Table 1).

Table 1. *Morphological characteristics of Rhizobium isolates.*

S. No.	Rhizobium isolates	Colony character	Cell shape	Gram's staining	Cell motility	Growth obtained (days)
1.	Rh01	highly mucoid	Rod	-ve	-ve	3 days
2.	Rh02	highly mucoid	Rod	-ve	-ve	3 days
3.	Rh03	Mucoid	Rod	-ve	-ve	5 days
4.	Rh04	Mucoid	Rod	-ve	-ve	5 days
5.	Rh05	Mucoid	Rod	-ve	-ve	3-5 days
6.	Rh06	Mucoid	Rod	-ve	-ve	3-5 days
7.	Rh07	less mucoid	Rod	-ve	-ve	5 days
8.	Rh08	Mucoid	Rod	-ve	-ve	3 days
9.	Rh09	mucoid white	Rod	-ve	-ve	5 days
10.	Rh10	Mucoid	Rod	-ve	-ve	3 days
11.	Rh11	highly mucoid	Rod	-ve	-ve	3 days

S. No.	Rhizobium isolates	Colony character	Cell shape	Gram's staining	Cell motility	Growth obtained (days)
12.	Rh12	highly mucoid	Rod	-ve	-ve	3 days
13.	Rh13	Mucoid	Rod	-ve	-ve	3 days
14.	Rh14	Mucoid	Rod	-ve	-ve	3 days
15.	Rh15	Mucoid	Rod	-ve	-ve	3 days
16.	Rh16	Mucoid	Rod	-ve	-ve	3 days
17.	Rh17	Mucoid	Rod	-ve	-ve	3 days
18.	Rh18	highly mucoid	Rod	-ve	-ve	3 days
19.	Rh19	Mucoid	Rod	-ve	-ve	3 days
20.	Rh20	Mucoid	Rod	-ve	-ve	3 days
21.	Rh21	highly mucoid	Rod	-ve	-ve	3 days
22.	Rh22	less mucoid	Rod	-ve	-ve	5 days
23.	Rh23	highly mucoid	Rod	-ve	-ve	3 days
24.	Rh24	mucoid white	Rod	-ve	-ve	3 (or) 5 days
25.	Rh25	Mucoid	Rod	-ve	-ve	3 (or) 5 days
26.	Rh26	mucoid white	Rod	-ve	-ve	5 days
27.	Rh27	Mucoid	Rod	-ve	-ve	3-4 days
28.	Rh28	highly mucoid	Rod	-ve	-ve	3 days
29.	Rh29	highly mucoid	Rod	-ve	-ve	3 days
30.	Rh30	highly mucoid	Rod	-ve	-ve	3 days
31.	Rh31	highly mucoid	Rod	-ve	-ve	3 days

3.2. Identification of *Rhizobium*

The Rhizobia isolates did not show growth in Hofer's alkaline broth (pH 11), since rhizobia are unable to grow at high pH as they are non ketolactose producers [12]. All rhizobial isolates showed positive results in PHB test. In Litmus milk reaction, *Rhizobium* fermented the lactose and produced alkaline reaction in the litmus milk. However, in YEMA medium it was found that Agrobacteria absorbed the Congo red, whereas the rhizobia colonies stand out as white, mucoid and translucent.. Pure *Rhizobium* isolates are unable to grow on lactose [13]. Strains were routinely maintained on yeast extract mannitol (YEM) agar slants [8] and kept at 48°C. Purity was assured by routine plating on YEM agar supplemented with Congo red and selecting uniform colonies [14] (Table 2).

Table 2. *Identification test for Rhizobium.*

S. No	Test	Results
1.	Polyhydroxy butyrate stain	Positive
2.	Congo red	Negative
3.	Hofer's alkaline broth	Negative
4.	Lactose agar	Negative
5.	Litmus milk reaction	Alkaline reaction

3.3. Biochemical Characteristics of *Rhizobium* Isolates

The results shown indicated that all the isolates of Rhizobia were positive to the Indole, nitrate reduction, urease, catalase, Oxidase and Mac-Conkey agar test. This is in comparison to the biochemical tests which were performed on *Arachis hypogaea* showed that most were positive for catalase, Oxidase, Voges – Proskauer and indole tests. None of the isolates produced H_2S in hydrogen sulfide production test and utilized citrate as sole source of carbon.

Starch hydrolysis and gelatin hydrolysis test were positive for all isolates except Rh07 and Rh22 isolates. These isolates were slow-growers and unable to utilize starch and gelatin components. Rhizobial cells produce gelatinase enzyme and negative gelatinase activity also feature of *Rhizobium* [15]. In methyl red test, acid produced from the glucose, the isolates Rh02, Rh03, Rh08, Rh19, Rh22 and Rh29 were positive, whereas remaining *Rhizobium* isolates were negative.

In Voges-Proskauer test, acetoin was produced from glucose; the isolates Rh01, Rh05, Rh06, Rh07, Rh09, Rh13, Rh17, Rh24 and Rh30 were positive whereas other isolates were negative. In triple sugar Iron agar test, all the rhizobial isolates fermented glucose and sucrose and also produced gas in the test tubes. However the isolates did not ferment lactose. Pure cultures of the isolates were made and then subjected to Gram reaction. The Gram negative isolates were further subjected to biochemical tests including catalase, oxidase, Voges-Proskauer and indole tests for confirmation (Table 3).

Table 3. *Biochemical characteristics of Rhizobium isolates.*

S. No	Rhozobium isolates	IND	MR	VP	CIT	H_2S	NR	URE	CAT	OXID	STA	GEL	TSI	MAC
1.	Rh01	+	-	+	-	-	+	+	+	+	+	+	A/K, G	+
2.	Rh02	+	+	-	-	-	+	+	+	+	+	+	A/K, G	+
3.	Rh03	+	+	-	-	-	+	+	+	+	+	+	A/K, G	+
4.	Rh04	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
5.	Rh05	+	-	+	-	-	+	+	+	+	+	+	A/K, G	+
6.	Rh06	+	-	+	-	-	+	+	+	+	+	+	A/K, G	+
7.	Rh07	+	-	+	-	-	+	+	+	+	-	-	A/K, G	+

S. No	Rhozobium isolates	IND	MR	VP	CIT	H_2S	NR	URE	CAT	OXID	STA	GEL	TSI	MAC
8.	Rh08	+	+	-	-	-	+	+	+	+	+	+	A/K, G	+
9.	Rh09	+	-	+	-	-	+	+	+	+	+	+	A/K, G	+
10.	Rh10	+	+	-	-	-	+	+	+	+	+	+	A/K, G	+
11.	Rh11	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
12.	Rh12	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
13.	Rh13	+	-	+	-	-	+	+	+	+	+	+	A/K, G	+
14.	Rh14	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
15.	Rh15	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
16.	Rh16	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
17.	Rh17	+	-	+	-	-	+	+	+	+	+	+	A/K, G	+
18.	Rh18	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
19.	Rh19	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
20.	Rh20	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
21.	Rh21	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
22.	Rh22	+	+	-	-	-	+	+	+	+	-	-	A/K, G	+
23.	Rh23	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
24.	Rh24	+	-	+	-	-	+	+	+	+	+	+	A/K, G	+
25.	Rh25	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
26.	Rh26	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
27.	Rh27	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
28.	Rh28	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+
29.	Rh29	+	+	-	-	-	+	+	+	+	+	+	A/K, G	+
30.	Rh30	+	-	+	-	-	+	+	+	+	+	+	A/K, G	+
31.	Rh31	+	-	-	-	-	+	+	+	+	+	+	A/K, G	+

(+) Positive; (-) No Reaction; IND – Indole; MR – Methyl red; VP – Voges-Proskauer; CIT – Citrate utilization; H_2S – Hydrogen Sulfide Production; NR – Nitrate reduction; URE – Urease; CAT – Catalase; OXID – Oxidase; STA – Starch hydrolysis; GEL – Gelatin hydrolysis; TSI – Triple Iron agar; Mac – Mac-Conkey agar

3.4. RAPD-PCR Analysis

Genomic DNA was isolated from all Rhizobia isolates and separated by agarose gel electrophoresis. Total genomic DNA from 31 Rhizobia isolates were used as templates in amplification reactions with primer in PCR-RAPD analysis. The PCR products from the different isolates were separated by agarose gel electrophoresis. The primers yield multiple DNA products ranging in size from 250-1000 bps.

The dendrogram obtained by statistical analysis of gel data, fell in to three clusters. This first cluster consisted of 11 Rhizobia isolates, i.e., Rh1, Rh13, Rh19, Rh7, Rh20, Rh8, Rh16, Rh2, Rh27, Rh3 and Rh6. The second cluster consisted of two groups. The second major cluster comprised most of *Rhizobium* isolates. The first group of second cluster consisted of Rh14, Rh22, Rh4, Rh21, Rh10, Rh26, Rh5, Rh9, Rh11 and Rh23 whereas Rh17 and Rh18 isolates fell in to second group. Only eight Rhizobial isolates were grouped with in the third major cluster, viz., Rh15, Rh12, Rh24, Rh28, Rh31, Rh25, Rh30 and Rh29 isolates.

The similarity between Rhizobial isolates varied from 0.2 – 0.8. There was no 100% similarity between any two strains. The highest similarity coefficient (0.8) was computed between many strains whereas the lowest similarity coefficient (0.2) was computed between some of the Rhizobial strains (Figure 1). PCR-RAPD is a useful tool to conduct persistence and competitiveness studies in rhizobia stains when inoculated in soils as inoculants [16].

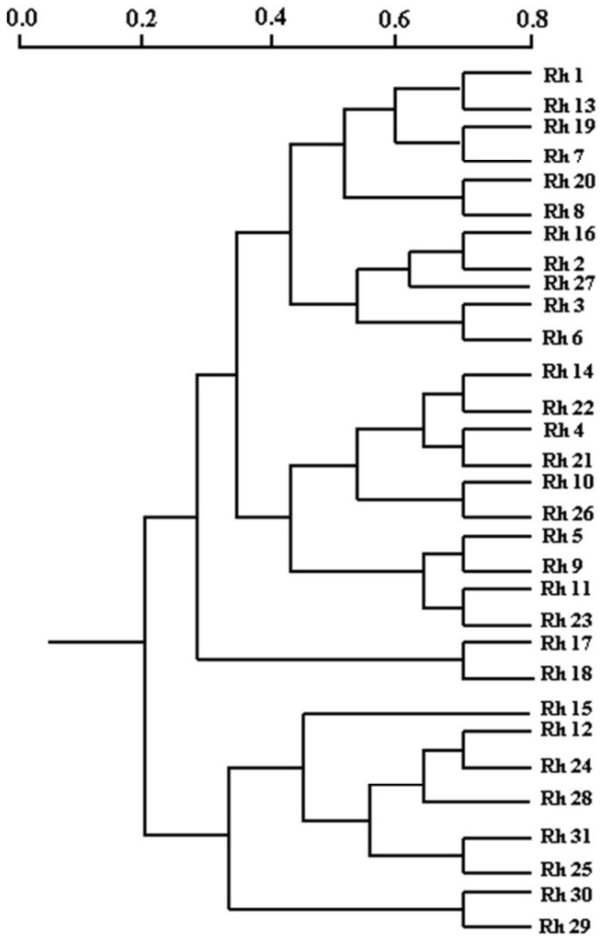

Figure 1. *Dendrogram of the 31 rhizobia strains based on Random amplified polymorphic DNA (RAPD-PCR) using phylogenetic analysis.*

3.5. Nodulation Ability by *Rhizobium*

Out of 31 Rhizobia isolates, 2 isolates were selected for better growth behaviour and coded namely VRS 1 and ARS 2 (VRS- *Vigna mungo* species and ARS- *Arachis hypogaea* species) respectively. Nodulation ability test was carried out using VRS 1 rhizobia strain used for *Vigna mungo* and ARS 2 rhizobial strain used for *Arachis hypogaea* for all the nodulation ability tests. Results obtained also showed that the nodules formed by *Rhizobium* strains were healthy and pink in colour.

3.5.1. Test Tube Method

VRS1 and ARS2 rhizobia isolates produced abundant nodulation on *Vigna mungo* and *Arachis hypogaea* legumes. In *Vigna mungo* nodule fresh weight (26mg), plant dry weight (317 mg) and *Arachis hypogaea* nodule fresh weight (32 mg), plant dry weight (330 mg), also increased with inoculation of *Rhizobium* strains, whereas number of nodules, nodule fresh weight and plant dry weights were reduced in control plants (Table 4).

Table 4. *Nodulation ability by test tube method in Arachis hypogeae and Vigna mungo.*

S. No	Rhozobial strains	Host plant	Nodule number/plant	Nodule fresh weigh/plant (g)	Plant dry weight/plant (g)
1.	Control	*Vigna mungo*	4	8	256
2.	VRS 1	*Vigna mungo*	10	26	317
3.	Control	*Arachis hypogaea*	10	18	305
4.	ARS 2	*Arachis hypogaea*	17	18	330

3.5.2. Plastic Cup Method

The VRS1 and ARS2 rhizobia strains successfully formed nodules on *Vigna mungo* (30) and *Arachis hypogaea* (110) plants respectively. Nodule weights of *Vigna mungo* plants nodulated by VRS1 were 0.5 g and plant dry weight 10g/p. Maximum nodule number was scored after inoculating with ARS2 on *Arachis hypogaea* than control plants. Nodule fresh weights 1.2 g/p and plant dry weight 14g with ARS2 strain whereas 0.9 g/p (N.f.w) and 9 g/p (p.d.w) were observed an control plants. The *Rhizobium* inoculation significantly increased the nodule number, nodule weight and plant weight (Table 5).

Table 5. *Nodulation ability by plastic cup method in Arachis hypogeae and Vigna mungo.*

S. No	Rhozobial strains	Host plant	Nodule number/ plant	Nodule fresh weigh/ plant (g)	Plant dry weight/plant (g)
1.	Control	*Vigna mungo*	18	0.25	7
2.	VRS 1	*Vigna mungo*	30	0.5	10
3.	Control	*Arachis hypogaea*	82	0.9	9
4.	ARS 2	*Arachis hypogaea*	110	1.2	14

3.5.3. New Nodulation Ability Test

An assay with Leonard jars was performed by modifying Leonardo Jar method. VRS1 isolates formed more number of nodules (22), nodule fresh weight (0.4 g/plant) and plant dry weight (3.6 g/p) than the control. ARS2 symbiotically effects *Arachis hypogaea* thus increasing the number of nodules (35), nodule fresh weight (0.6 g/p) and plant dry weight (4.5 g/p) whereas control plants show decreased nodule number (16), nodule fresh weight (0.24 g/p) and plant dry weight (2.1 g/p). The results suggest that VRS1 and ARS2 *Rhizobium* strains nodulated effectively with leguminous plants and also contributed significantly to the nitrogen economy of the plant (Table 6).

Table 6. *New nodulation ability test in Arachis hypogeae and Vigna mungo.*

S. No	Rhozobial strains	Host plant	Nodule number/plant	Nodule fresh weight/plant (g)	Plant dry weight/plant (g)
1.	Control	*Vigna mungo*	10	0.18	2.5
2.	VRS 1	*Vigna mungo*	22	0.4	3.6
3.	Control	*Arachis hypogaea*	16	0.24	2.1
4.	ARS 2	*Arachis hypogaea*	35	0.6	4.5

3.5.4. Green House Experiment

Control plants in greenhouse experiment had no effect on growth characteristics and nodule numbers of the *Vigna mungo* and *Arachis hypogaea* as compared to *Rhizobium* inoculated plants. *Rhizobium* strains increased the nodule number, nodule fresh weight and plant dry weights of both leguminous plants. Nodule weights of *Vigna mungo* and *Arachis hypogaea* plants were 0.6 g and 1g per plant respectively, whereas 0.24g and 0.7g per plant were observed in control plants of *Vigna mungo* and *Arachis hypogaea* (Table 7).

Table 7. *Nodulation ability by green house (pot) experiment method in Arachis hypogeae and Vigna mungo.*

S. No	Rhozobial strains	Host plant	Nodule number/ plant	Nodule fresh weigh/plant (g)	Plant dry weight/ plant (g)
1.	Control	*Vigna mungo*	15	0.24	7.1
2.	VRS 1	*Vigna mungo*	32	0.6	9.2
3.	Control	*Arachis hypogaea*	60	0.7	8.1
4.	ARS 2	*Arachis hypogaea*	106	1	11.4

4. Discussion

4.1. Morphological Characteristics of *Rhizobium*

All the isolates were found to be gram negative, rod shaped and non-motile. After three days of incubation, high production of mucus was identified in 35% of the isolates, 13% were white and 52% isolates were mucoid, opaque colonies. Vincent [8] reported that the *Rhizobium* grown on YEMA medium produced small to medium sized colonies. This result was confirmed by using Bergey's Manual of determinative bacteriology [17].

4.2. Identification of *Rhizobium*

Instead of Hofer's alkaline medium, YEMA medium was used for the growth of Rhizobia isolates. The pH of the medium during the growth of isolates was changed from 7 to 6, thus showing the production of acid which is a characterstic *Rhizobium* to produce acid during growth. Similar findings were made by Kumari *et. al*. [18] for the characterization of *Rhizobium* isolates from *Indigofora* species and confirmed that those organisms which have the above said characterstics were used to identify the Rhizobial isolates.

4.3. Biochemical Characteristics of *Rhizobium* Isolates

All the isolates were shown to be positive to Indole, Nitrate reduction, Urease, Catalase, Oxidase and Mac-Conkey agar tests. All 31 isolates showed no production of H_2S and utilized citrate as a sole source of carbon. These findings are in close agreement with Javed and Asghari who have previously characterized the *Rhizobium* from soil and root nodules of groundnut with same positive biochemical tests [19]. Similarly, Oblisami [20] studied the nodulation pattern in legume plants by screening through the same tests and reported similar results. Singh *et al*. characterized *Rhizobium* strain from the roots of groundnut bacterial species. These findings corroborate with the results of Singh *et al.* [21], and Erum and Bano [22] who also reported these sugar tests positive during isolation and characterization of *Rhizobium meliloti* on most of leguminous plant roots.. De Oliveira *et al* [23] also observed that the *Rhizobium* strains utilize the starch obtained from the different sources. Biochemical tests are performed by various workers for the identification of rhizobial isolates [24]. Similar work has been done with the strains of root nodulating bacteria were isolated from the root nodules of *Arachis hypogaea* L. and *Telfairia occidentalis* plants growing in south-east, Nigeria [25].

4.4. RAPD-PCR Analysis

The RAPD technique was used to detect the polymorphisms among the 31 strains of Rhizobia used in the study. The number of amplified products ranged from 4-8 per isolate. Amplification by PCR method has been done by many workers [26]. El-Fiki [27] reported that RAPD finger printing were used for strain identification the assessment of genetic diversity with in a field population of *Rhizobium*.

4.5. Nodulation Ability by *Rhizobium*

VRS1 and ARS2 Rhizobial isolates were selected and used in nodulation ability tests. VRS1 and ARS2 strains were found to form nodules in *Vigna mungo* and *Arachis hypogaea* respectively. Both these isolates showed abundant nodulation, whereas nodules were decreased in uninoculated control plants. The number of nodules, nodule fresh weight and plant dry weight were increased by VRS1 and ARS2 Rhizobial isolates in all nodulation ability tests such as test tube method, new nodulation ability test, plastic cup method and green house experiment. Mahmood and Athar [28] have stated that the highest number of nodules were produced by Rhizobial isolates in *Vigna mungo*. Several reports describe in successful experiments where wild rhizobia are more effective in nitrogen fixation than their compatible host [29].

5. Conclusion

Rhizobium is important for its nitrogen-fixing ability in the environment via symbiotic relationship with the root nodules of plants. The nodulation ability is governed by various nodulating genes present in its plasmids, making it transferable to other species. This study confirmed that due to its nodulation ability, Rhizobium is able to fix nitrogen in *A. hypogeae* and *V. mungo*. This organism is believed to increase better agricultural practices when inoculated in plant legumes. Thus, Rhizobium reduces the stress of using synthetic nitrogen fertilizers in the environment.

References

[1] Marta L., Ana A. and Solange O. 2014. Legume growth-promoting rhizobia: An overview on the Mesorhizobium genus. *Microbiological Research.* 169: 2-17.

[2] Francisco X. N., Clarisse B., Bernard R. G., and Márcio J. R. 2016.. *Review Article* The Role of Rhizobial ACC Deaminase in the Nodulation Process of Leguminous Plants.

[3] Md. Jakaria A. M., Md. Mehedi H., Md. Mizanur R., and Mamun O. R. 2013. Isolation and characterization of *Rhizobium* spp. and determination of their potency for growth factor production. *International Research Journal of Biotechnology* (ISSN: 2141-5153) Vol. 4 (7): 117-123.

[4] Zephania S., Kelvin M., Amare G. and Patrick A. N. 2014. Isolation and Characterization of Nitrogen Fixing Rhizobia from Cultivated and Uncultivated Soils of Northern Tanzania. *American Journal of Plant Sciences*. 5: 4050-4067.

[5] Oblisami G. 1995. *in vitro* growth of five species of ectomycorrhizal fungi, *Euro J for Path*, Vol. 1-7: 204-210.

[6] Sanjay K. J., Levini A. M. and Felix D. Dakoraa. 2017. Phylogenetically diverse group of native bacterial symbionts isolated from root nodules of groundnut (*Arachis hypogaea L.*) in South Africa. *Systematic and Applied Microbiology,* 40: 215-226.

[7] Dušica D., Olivera S., Nataša R., Djordje K., Dragana J. and Bogić M. 2010. Nodulation and N2 Fixation Effectiveness of *Bradyrhizobium* Strains in Symbiosis with Adzuki Bean, *Vigna angularis.* Vol. 53: 293-299.

[8] Vincent, J. M., 1970. *A Manual for the Practical Study of Root- Nodule Bacteria.* IBP Hand Book, Oxford; Blackwell Scientific Publication, Vol. 15.

[9] Sambrook, J., and Russell, D. 2001. *Molecular Cloning; A Laboratory Manual.* 3rd Cold Spring Laboratory, Cold Spring Horbour, New York.

[10] Treaumroog, N. and Boonkerd, N., 1998. The Detection of *Bradyrhizobium* spp. and *B. japonicum* by primer- based technology and direct DNA extraction. *Pl.* Soil, 204: 127-134.

[11] Rohif, F. J., 1990. NTSYS-PC numerical taxonomy and multivariance analysis system, Version 1.60, Exeter Software.

[12] Gaur Y. D. and Sen A. N. 1981. Cultural and biochemical characterstics of root nodule bacteria of chickpea (*Cicer arietinum* L*.). Zbl. Bakt. II Abst*., 136: 307-3126.

[13] Kucuk C., Kivanc M., and Kinaci E. 2006. Characterization of *Rhizobium* spp. isolated from Bean. *Turk. J. Biol*., 30: 127-132.

[14] Stella C., Marcela P., Marta V and Adriana F. 1999. Nodulation in peanut (*Arachis hypogaea L.*) roots in the presence of native and inoculated rhizobia strains. *Applied Soil Ecology* 13: 39-44.

[15] Hunter W. J., Kuykendall L. D., and Manter D. L. 2007. Rhizobium *Selenireducens* spp. A selenite-reducing α-Proteobacteria isolated from a bioreactor. *Curr. Microbiol*., 55: 455-460.

[16] Pinto P. P., Paiva E., Purcino H., Passos R. V. M., and Sa N. M. H. 2004. Characterization of rhizobia that nodulate *Arachis pintoi* by RAPD analysis*. Braz. J. Microbiol.,* 35 (3): 1517-1520.

[17] Holt J. G., Kreig N. R., Sneath P. H. A., Staley J. T., and Williams S. T. 1994. *Bergey's Manual of Determinative Bacteriology*, 9th edn. Baltimore; Published by Williams and Wilkins, A. Wavely Company, 787.

[18] Kumari B. S., Ram M. R., and Mallaiah K. V., 2010. Studies on nodulation, biochemical and analysis and protein profiles of rhizobium isolated from *Indigofora* sp. *Mal. J. Microbiol*., 6 (2); 133-139.

[19] Javed K. and Asghari B. 2008. "Potential allelopathic effects of sunflowers on microorganisms," *Afri. J. biotech*. Vol. 7: 4208-4211.

[20] Oblisami G. 1995. "an *in vitro* growth of five species of ectomycorrhizal fungi," *Euro J for Path*, Vol. 1-7: 204-210.

[21] Singh, R. K. and Singh K. 2008. "Characterization of Rhizobium strain isolated from Trigonella foenumgraecum (Fenugreek)," *Africa. J. Biotech.*, Vol. 7: 3671-3676.

[22] Erum S. and. Bano A. 2008. "Variation in phytohormone production in Rhizobium strains at different altitudes of Northern areas of Pakistan" *Int. J. Agric. Biol.*, Vol. 10: 536-540.

[23] De O, A. N., De Oliveira, L. A. Andrade, J. S., and Chargas, J. A. F. 2007. Rhizobia amylase production using various starchy substances as carbon substrates*. Braz. J. Microbio.,* 208-216.

[24] Vishal K and Abhishek C. 2014. Isolation and Characterization of *Rhizobium leguminosarum* from Root nodule of *Pisum sativum* L*. Journal of Academia and Industrial Research (JAIR).* Vol 2.

[25] Agah, M. V., Orji J. O., Nnachi A. U., Chukwu O. S., Udu-Ibiam O. E., Nwachi A. C., Olaosebikan O. O. 2016. Isolation and Identification of *Rhizobium* species from Root Nodules of *Arachis hypogaea* L. and *Telfairia occidentalis* in South-East, Nigeria. *International Journal of Science and Research (IJSR)*, Vol. 5: 2319-7064.

[26] Pinto P. P., Paiva E., Purcino H., Passos R. V. M., and Sa N. M. H. 2004. Characterization of rhizobia that nodulate *Arachis pintoi* by RAPD analysis*. Braz. J. Microbiol.,* 35 (3): 1517-1520.

[27]] El-Fiki, 2006. Genetic diversity in Rhizobium determined by Random Amplified Polymorphic DNA analysis*. J. Agric. Soc. Sci*., 2 (1): 1-4.

[28] Mahmood A. and Athar M. 2008. Cross inoculation studies; Response of *Vigna mungo* to inoculation with rhizobia from tree legumes growing under arid environment*. Int. J. Environ. Sci. Tech.,* 5 (1): 135-139.

[29] Zahran H. H. 2001. Rhizobia from wild legumes; diversity, taxonomy, ecology, nitrogen and biotechnology. *J. Biotech*., 91: 143-153.