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Effect of Indole Acetic Acid (IAA) Produced *Rhizobium* Species on Groundnut and Black Gram Seed Germination

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Abstract

This study was aimed at isolating and identifying *Rhizobium* species from the soil of ground nut and black gram plant root. It is to explore and maximize their contributions to soil nitrogen fertilization in place of synthetic fertilizers. Totally five different *Rhizobium* species were isolated and identified using standard microbiological and biochemical techniques. This study revealed that the *Rhizobium sp*. have increased indole acetic acid (IAA) by was observed during its growth in modify YEMA. Production, plant growth promotion activity and root colonization of *Rhizobium sp.* studies showed enhanced IAA synthesis, more seed germination and overall improvement in plant growth. Control was also maintained where the groundnut and black gram seeds were sown without bacterial inoculums.

Keywords: *Rhizobium*, Groundnut, Indole acetic acid, Black gram.

INTRODUCTION

Rhizobia are legume root-nodule bacteria. They are soil bacteria that induce the formation of special structures (nodules) on the roots of the leguminous plants. Inside these nodules, the *Rhizobia* fix nitrogen. This means that they convert de-nitrogen into ammonia. Ammonia is toxic, so it is rapidly assimilated in to organic compounds, most of which the bacteria pass

to the plant to fulfill its nutritional need for the nitrogen. Nitrogen fixation energy-intensive processes and the bacteria take carbon compounds from the plant to fuel it.

Many physiological activities of plants are regulated by a variety of plant growth regulators and phytohormones. A large amount of information regarding phytohormones, nitrogen fixation, nodules (development, morphogenesis and physiology) and hormone production by microorganisms had enriched the literature from time to time. Among which IAA is play a critical role in nodule and nodule bacteria for the nodule development and symbiotic relationship. IAA has been implicated in virtually all aspects of plant growth and development [1].

Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L- tryptophan metabolism produced by several microorganisms including Plant Growth-Promoting Rhizobacteria (PGPR) [2]. Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as PGPR. PGPR can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (like auxin, gibberellin, and ethylene), siderophores, HCN and antibiotics [3].

The first objective of this study was to isolate and screen indigenous Indole acetic acid producing bacteria. The second was to purify the IAA and screen their abilities of plant growth promoting rhizobacteria attributes in groundnut and blackgram seed. Besides, optimization study intended for high IAA production was carried out with physicochemical parameters such as carbon and nitrogen source, with and without supplement of tryptophan, pH and temperature.

MATERIALS AND METHODS

Isolation of *Rhizobium* from root nodules of the Ground nut plants

Ground nuts plants were carefully uprooted and the root system was washed under running water to remove the adhesive soil particles. The color of the nodules varied from brown to pink depending on the state of pigment present in them. For experiment healthy unbroken pink nodules were selected.

Surface Sterilization of root nodules

The sterilizing agent, 0.1 % mercuric chloride and 3-5% hydrogen peroxides were used. The nodules were immersed in sterilizing agents for 4-5 mints and then washed repeatedly

with sterile distilled water. Then they are washed in 70% ethyl-alcohol followed by washing with sterile distilled water again.

Isolation of *Rhizobium* by serial dilution method

Nodules were washed in a small aliquot of sterile distilled water with the help of a glass rod. Yeast Extract Mannitol Agar (YEMA) plates (Himedia) were prepared and sterilized by autoclaving. Ten fold serial dilution of nodular extract was prepared by taking 1g of nodular extract into 10 ml of sterile distilled water and mixed well to get nodular extract suspension. One ml of nodular extract suspension was diluted with 9 ml of sterile distilled water making the dilution to 10^{-2}. Similarly dilution up to 10^{-8} was made separately for each nodular extract. Suspension (0.1 ml) of nodular extract was prepared from 10^{-3} to 10^{-8} dilutions and inoculated into sterile YEMA plates. The sample was spreaded throughout the YEMA plates and inoculated Petri plates were incubated for 4-7 days in an incubator at 37^{o}C.

Identification of *Rhizobium* sp.

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. Flagellation test was carried out to test for motility using (Loffler's mordant).

Determination of Biochemical Nature of IAA

The isolation of the microorganisms was done as follows. 10g of *Rhizosphere* soil in 250mL flask was taken and 90 ml sterile distilled water was added. It was incubated on rotary shaker at 120 rpm for 10 min. 1ml sample was serially diluted up to 10^{-7}. 0.1 ml of diluted sample was plate on sterile Luria Bertani (LB) agar (Himedium). Single colonies were picked up and streaked on sterile LB media, India) and incubated for 3 days at 28°C. Agar plates to get pure culture. Well isolated colonies were observed for morphological characterization. Total 10 isolates were obtained from different Rhizospheric soil. The isolates were further checked for IAA production.

Characterization of IAA Production

To determine the amounts of IAA produced by each isolate, colorimetric techniques performed with Van Salkowski reagent using the Salkowski's method [4]. The isolates was grown in yeast malt dextrose broth (YMD broth) (Himedia, India) and incubated at 28 °C for 4 days. The broth was centrifuged after incubation. Supernatant was reserved and 1ml was mixed with 2ml of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) and kept in the

dark. The optical density (OD) was recorded at 530 nm after 30 min. and 120 min. IAA production was compared in YMD and LB media. YMD medium was compared with and without tryptophan.

Extraction and Purification of IAA

Isolates was cultivated in YMD broth and it was centrifuged by 1000 rpm 15 min. The supernatant was collected and mixed with ethyl acetate (1:2). After vigorous shaking it was allowed to stand for 10 min. IAA was extracted within solvent layer. The procedure was repeated 3 to 4 times.

Plant Growth Promotion Activity of IAA Producing *Rhizobium* sp.

Plant growth promoting potential of *Rhizobium* sp was checked at pot assay and pot assay level. Seed groundnut and black gram surface sterilized with 0.1% $HgCl_2$ bacterized with IAA rich YEMA containing *Rhizobium sp.* Its bio-efficacious potential was measured by simultaneous bacterization of groundnut and with the standard culture of *Rhizobium sp.*

Pot Assay

To study the effect of IAA producing Rhizospheric isolates on plant growth, pot assay was performed. Local black gram and groundnut (Lokvan) seeds were used for seed coating. The black gram seeds were surface sterilized by immersing in 95% ethanol for 30Sec and mercury chloride (0.2%) for 3 min. Then further to remove traces of mercury chloride, the disinfected seeds were washed 5 times by sterile distilled water. 0.1ml overnight grown culture (0.5 OD) was applied on seed surface for seed coating.

Seeds were dried and sowed into sterile soil as carrier. Six seeds were sown in each pot used per pot at equal distance and experiment was performed in triplicates for each isolates. The uncoated seeds were used as control. Pots were irrigated with sterile distilled water every day and kept in sunlight. At the interval of every 5th day, plant was up rooted and seedlings were measured for shoot and root length and chlorophyll content up to 15^{th} day.

RESULT AND DISCUSSION

Collection of Soil Sample

The rhizospheric soil samples were collected from fields growing ground nut from Mulluvadi Village, Arcot Taulk, Vellore district, Tamil Nadu. All bacterial strains were isolated on their respective media; *Rhizobium* was isolated on yeast extract mannitol agar.

Isolation of Rhizobium

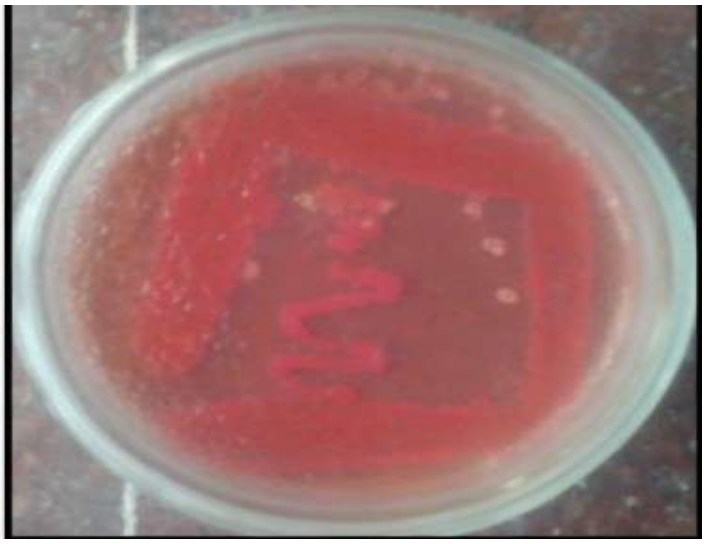
The *Rhizobium sp*. was isolated from fresh and healthy mature soil sample of groundnut plant and checked purity. By means of serial dilution and formation of mucoid colonies, growth on selective YEMA medium.

Cultural Characteristics

Macro and Micro Morphology

Rhizobium colony pink color, mucoid, softness in growth on selective YEMA media. (Figure 1). There organisms was identified by their specific test like Gram staining, short, Gram negative rod pink color is observed. The organism was identified rod shaped smooth morphology.

Figure 1. Selective YEMA Media in Isolation of *Rhizobium* sp.



Biochemical Test

The biochemical tests performed on the isolates showed that most were positive for catalase, Oxidase, Voges – Proskauer and indole tests. Only one *Rhizobium* isolate from Grount nut was negative to oxidase test. These findings are in close agreement with previously characterized the *Rhizobium* from soil and root nodules of groundnut with same positive biochemical tests [5]. Similarly studies reported that the nodulation pattern in legume plants by screening through the same tests [6]. Singh *et al.,* [7] characterized *Rhizobium* strain from the roots of groundnut bacterial species (Table 1). These findings corroborate with the results of Singh *et al.,* (2008) also reported these sugar tests positive during isolation and characterization of *Rhizobium meliloti* on most of leguminous plant roots.

Table 1. Biochemical test

S. No	Name of the test	Result
1.	Indole	+
2.	Methyl Red	-
3.	Voges – Proskauer	+
4.	Citrate utilization	-
5.	Catalase	+
6.	Oxidase	+

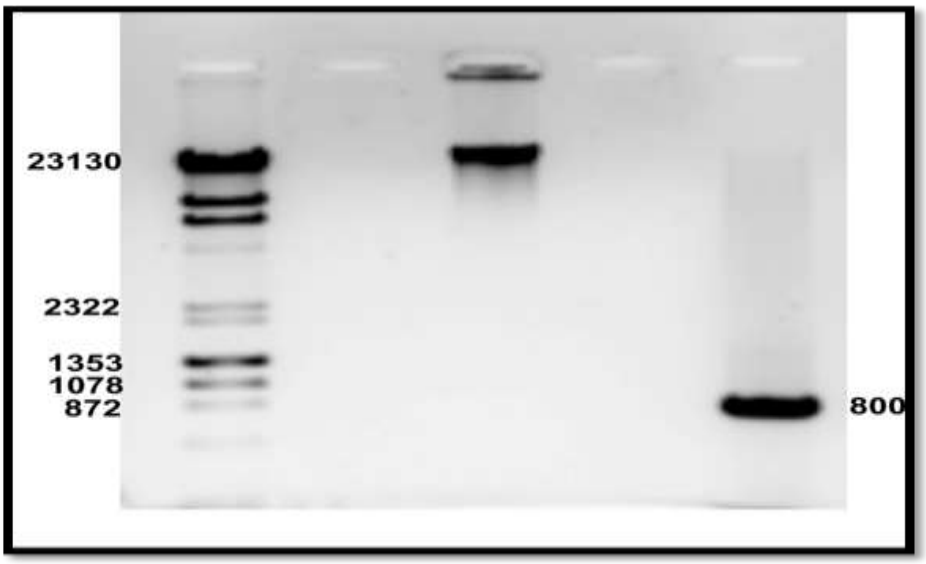
\+ Positive - Negative

Molecular Techniques

DNA Isolation

The genomic DNA was isolated from the potent isolate *Rhizobium* sp. and separated by agarose gel electrophoresis. A Sharp band was observed using the doc imaging system (UVP).

Figure 2. Agarose gel Electrophoresis



Screening Potential of Seed

Pot Culture

Rhizobium sp. YEMA broth inoculated in 37^0C, for 24 hrs. After the centrifuge in culture for supernatant collected and soaked for seed in black gram and groundnut in 1 hrs, inoculated in soil at 15^{in} days after observed for shoot length, color and leaf length, daily observed in seed germination respectively (Figure 3 &Table 2).

Figure 3. Pot Assay test.

Ground nut

Black gram plant

Table 2. Influence of Producing *Rhizobium* on Seed Germination both groundnut and Black gram seed.

S. No	Characters	Ground nut		Black gram	
		Control	Test	Control	Test
1.	Leaf color	Dark green	Light green	Dark green	Light green
2.	Root length	1.5 cm	2.2 cm	0.8 cm	1.4 cm
3.	Shoot length	3.5 cm	4.2 cm	4 cm	7 cm
4.	Root Branches	Few	Higher	-	-
5.	Leaf numbers	22 Nos	28 Nos	2 Nos	5 Nos

Extraction of IAA

Isolation was cultivated in YEMA broth and it was centrifuged by 15 min. The Supernatant was reserved and 1ml was mixed with 2ml of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) and kept in the dark. Spectrophotometric the optical density (OD) was recorded at 530 nm after 30 min and 120 min. OD was reaction Spectrophotometric of IAA production was compared in YMD and LB media.

Isolate from different sources showed IAA production [9, 10]. In the present study IAA production in *Azotobacter* isolates are in agreement with earlier reports. The ability of bacteria to produce IAA in the *rhizosphere* depends on the availability of precursors and uptake of microbial IAA by plant. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the *rhizosphere* and other PGP activities [11].

IAA, a member of the group of phytoharmones, is generally considered to be the most important native auxin. All ten isolates are positive for IAA production but among those five isolates br1, br2, br3, mr2 and wr2 were selected as potential IAA producers. Most or studies from the earlier work showed that IAA producing organisms are Gram negative [12,13]. Few Gram positive strains belong to Bacillus strain known to produce IAA [14]. Present study showed that five IAA positive strains were Gram positive.

CONCLUSION

Rhizobium is an important microorganism for the environment because of its nitrogen-fixing ability when in symbiotic relationship with plants. This study definite that the root nodules of black gram and groundnut plants harbour the nitrogen-fixing bacterium-*Rhizobium.* It also showed that these plants when inoculated with *Rhizobium* isolates perform better. This organism will greatly enhance agricultural production, if they are often used to inoculate legume plants, thereby reducing the environmental threat of synthetic nitrogen fertilizers.

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