Research Article

Isolation, Characterization and Growth of Rhizobium Strains under Optimum Conditions for Effective Biofertilizer Production

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ABSTRACT

Rhizobium is a soil habitat, gram negative bacterium which is associated symbiotically with the roots of leguminous plants. Screening and selecting the most effective strain is important for biological nitrogen fixation. The present work was undertaken to shed some light on different characteristics, growth and Phytohormone (Indole Acetic Acid) secretion of Rhizobial strains (Rhizobium trifolii, Rhizobium phaseoli, Rhizobium leguminosarum and Bradyrhizobium japonicum). The study revealed that by a series of morphological and biochemical test conducted, all the four strains were gram negative, rod shaped and mucous producing. All the strains grew well at pH 6 and 7, temperature 34 °C and at salt concentration 4%. Bradyrhizobium japonicum was found to be negative to bromothymol blue test, while all other three strains were positive depicting the former to be slow growing and later to be fast growing. All the strains were resistant to Penicillin and sensitive to Tetracycline. Rhizobium phaseoli produced starch hydrolysis while Rhizobium trifolii was positive in Caesinase test. B. japonicum, R. trifolii and R. phaseoli showed sufficient growth in Urea test and Lysine decarboxylase test. All four strains hydrolyzed lipase and Catalase enzyme. Rhizobium trifolii and Rhizobium phaseoli utilized citrate and in utilization of carbon sources, fast growing strains were able to utilize carbon in comparison to slow growing one. Experiments for Indole Acetic Acid production under aerobic and anaerobic conditions showed maximum production in aerobic condition i.e., 0.4 µg ml⁻¹ by Rhizobium trifolii and 0.6 µg ml⁻¹ by Rhizobium phaseoli. Rhizobium was further applied as a biofertilizer for significant improvement in plant growth and yield.

Keywords: Isolation, Characterization, Biofertilizers, Indole Acetic Acid, Plant growth.

INTRODUCTION

Legumes can establish an agronomically and ecologically important symbiosis that leads to the development of new plant organ (legume nodule) in response to nitrogen fixing bacteria1. Symbiosis is not only important for legume crops but also in Nitrogen cycle2. Biological Nitrogen fixation is a process by which atmospheric nitrogen (N₂) is converted into ammonia and subsequently available for plants. Legumes such as beans, clover, soybean and pea help to feed the meat producing animals as well as humans. Crop yield is improved by nodulating plants. In agriculture, perhaps 80% of the biologically fixed nitrogen comes from symbiosis involving leguminous plants and bacteria of family Rhizobiaceae. The family Rhizobiaceae currently involves six genera: Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium and Bradyrhizobium, which are collectively referred to as Rhizobia3. It has been estimated that 1g of soil may contain a community of 10⁹ microorganisms with Rhizobia representing around 0.1% of soil microbes or 10⁶ g⁻¹ soil⁻¹.

In the soil the bacteria are free living and motile, feeding on the remains of dead organisms. Free living Rhizobia cannot fix nitrogen and possess a different shape from bacteria found in nodules. They are regular in structure, appearing as straight rods while the nitrogen fixing forms exists as irregular cells called bacteroids. Rhizobia are generally found in large number in the regions which are close to the plant roots known as Rhizosphere. Beneficial free-living soil bacteria are usually referred to as Plant Growth Promoting Rhizobacteria (PGPR). Independent of the mechanisms of vegetal growth promotion, PGPRs colonalizes the rhizosphere, the rhizoplane or the roots itself. Soil temperature, acidity and rainfall are important conditions required by Rhizobia for its growth and development. To identify efficient isolates of Rhizobia, one has to understand the growth, development and characteristics (pH, nutrient availability of Rhizobia). It has been reported that Phaseoli can live on simple synthetic media4. The classical phenotypic characterization of Rhizobia has been the first method employed when classifying unknown strains of Rhizobia5. Rhizobial cells have large irregularly shaped nuclear region in center surrounded by narrow region of denser protoplasm. Resistance of nodule forming bacteria refers to the intrinsic resistance to antibiotics in terms of normal growth. Applying antibiotics, typified Rhizobium isolates according to their nodulation efficiency6. Phenotypic methodologies play a significant role in identification and characterization of Rhizobia. Rhizobium strains secrete growth hormones like IAA which shows positive influence on plant growth and play an important role in formation and development of root nodules7. Biofertilizers are known as microbial inoculants, which are artificially multiplied cultures of certain soil microbes that can improve soil fertility and crop productivity. They are...
organic products containing living cells of different types of microorganisms, which have the ability to convert nutritionally important elements from unavailable to available form through biological process. Keeping in view the importance of Rhizobium in legume plants, the present study was undertaken to characterize and study different strains of Rhizobium and select the effective strains for optimum biofertilizer production.

MATERIALS AND METHODS

Isolation of Rhizobium species

The Rhizobium isolates were obtained from the root nodules of Trifolium (clover), Vigna radiata (mung bean), Glycine max (soybean) and Lens culinaris (lentil) plants. Nodules located on the roots were spherical (2-4 mm) and pink in color. Root nodules were sterilized in 95% (v/v) ethanol for 10 seconds and then washed 7 times with sterile distilled water. Individual nodules were crushed with sterile glass rods and streaked on Yeast Extract Mannitol agar containing 0.0025% (w/v) Congo red. After incubation for 2-3 days at 30 °C, single colonies were selected and restreaked on YEM agar for purity.

Growth of Rhizobial strains under aerobic and anaerobic conditions

For determination of growth of Rhizobial strains, serial dilutions (10 X) were made in 9 ml distilled water and an aliquot (100 μl) from decimal dilutions was used to inoculate YEM agar (Vincent, 1970) media for the growth of Rhizobium. For providing aerobic conditions, plates were incubated at 30 °C for 3-5 days in BOD incubator and for anaerobic conditions; plates were incubated at rotary shaker (150 rpm) for 24-48 hours. To measure the growth, colonies of Rhizobium from 10^-7 dilutions were counted using formulae adopted by 12, 13.

\[
\text{Viable Cell Count (CFU/ml) = \frac{\text{No of colonies}}{\text{Volume of inoculum} \times \text{Dilution Factor}}}
\]

Colony and Morphological Characteristics

Colony Morphology of isolates was examined on YEM agar plates. Log phase culture (0.1 ml quantity of strain) was spread on YEM agar. After incubation for 2-3 days at 30 °C, individual colony was characterized on the basis of colony- shape, size, color, texture and Gram stain reaction.

pH Variation Assay

The ability of Rhizobial isolates to grow at different pH was tested in YEM broth by adjusting the pH to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 with NaOH and HCl. Growth at different pH was determined by measuring O.D. at 610 nm. Each experiment was performed in triplicates.

Temperature Tolerance

Temperature Tolerance was investigated by incubating bacterial cultures in YEM agar at 29 °C, 34 °C and 37 °C. Control plates were incubated at 24 °C for 3-5 days.

Salt Variation Assay

The ability of Rhizobial cultures to grow in different salt concentrations was tested by streaking them on YEM medium containing 0.5%, 1%, 2%, 3%, 4% (w/v) NaCl. Intrinsic Antibiotic Resistance

The isolates were tested for antibiotic sensitivity by Kirby Bauer disc diffusion on YEM agar. Six antibiotics-Penicillin (10 µg), Chloramphenicol (30 µg), Kanamycin (30 µg), Tetracycline (30 µg), Nalidixic Acid (30 µg) and Streptomycin (10 µg) were used for examining the antibiotic resistance of Rhizobium strains. 200 µl of mother culture of each strain was inoculated over the entire surface of YEM agar media. Agar was allowed to dry for 3-5 minutes. Antibiotic disks were placed equidistantly at the center of the 60 mm plates and were incubated at 30 °C for 5-7 days. Resistance to antibiotic was detected by inhibition zone formed around the disks.

Effect of Metal Salts

All the isolates were tested for their sensitivity to metals by agar dilution method. Freshly prepared YEM agar plates were amended with metal salts i.e., HgCl2, CuSO4, Al2(SO4)3 and ZnSO4 at 1 and 0.01 % (w/v) concentrations. Effect of metal salts was determined by Rhizobial growth after incubating the plates at 30 °C for 2 days.

Bromothymol Blue Test

The YEM was enriched with 1% (w/v) Bromothymol blue to selectively identify fast and slow growing Rhizobia. All samples were subjected to grow on BTB added YEM media. Positive sample showed yellow color to acid production after incubation for 48 hours at 28 °C.

Starch Hydrolysis

This test was performed to determine the capability of Rhizobium to use starch as a carbon source. Starch Agar Medium was inoculated with Rhizobium and analyzed for starch utilization. Iodine Test was used to determine the capability of microbes to use starch. A drop of iodine (0.1N) was spread on 24 hour old culture and clear zone of inhibition were formed.

Caesinase Test

Rhizobium strains were inoculated on Skimmed Milk Agar medium (caesin 5 g, yeast extract 2.5 g, glucose 1 g and agar 15 g). Components were mixed with distilled water in a conical flask and were boiled and sterilized. Alternatively, skimmed milk powder was prepared by dissolving 1 g of skimmed milk in 100 ml distilled water and was autoclaved. Sterilized skimmed milk was mixed into the first flask and was poured over the plates. Inoculated plates were incubated at 34 °C for 3 days and color change was examined.

Urease Test

Urease Test was performed by inoculating the isolates on Urease medium (peptone 1 g, dextrose 1 g, NaCl 5g,
KH₂PO₄ 2 g, Phenol red 0.012 g). The plates with modified media were inoculated with *Rhizobial* isolates and incubated for 5 days at 30°C.

**Lysine Decarboxylase Test**

*Rhizobium* strains were streaked on Bromoresol Purple Falkow medium (peptone 5 g, yeast extract 3 g, glucose 1 g, Bromoresol purple 0.02 g, distilled water 1 liter). *Rhizobial* strains were inoculated on the media and were left for incubation at 34°C for 1 day.

**Catalase Test**

This test was performed to study the presence of enzyme Catalase which hydrolyzes H₂O₂ into H₂O and O₂ in bacterial strains²⁰. *Rhizobial* colonies (2-3 days old) were taken on glass slide and one drop of H₂O₂ (30%) was added. Appearance of gas bubble indicated Catalase enzyme presence.

**Lipase Test**

Lipase presence around bacterial colonies was detected by supplementing YEM with 1% (w/v) Tween 80²¹.

**Citrate Test**

Citrate utilization as a carbon source was examined by replacing mannitol from YEM agar with equal amount of sodium citrate and Bromothymol blue (25 mg/l). Plates with modified media were inoculated and incubated for 24 - 48 hours²².

**Utilization of Carbon Source**

All the *Rhizobium* strains were tested for the utilization of different carbon sources by replacing mannitol in YEM agar with four different carbon sources and two organic acids. Growth was measured as colony diameter after 72 hours of incubation at 30 ± 1°C.

**Effect of UV radiations on the antibiotic sensitivity of *Rhizobium***

This investigation was done to analyze the effect of UV radiation on the antibiotic sensitivity of *Rhizobial strains* i.e., (*Rhizobium trifoli* and *Rhizobium phaseoli*). Freshly cultivated colony containing YEM agar plates were exposed under UV radiations (λ = 400 nm, intensity = 0.2-1 µW/cm²) in laminar air flow cabinet for 1 to 3 minutes respectively. UV exposed colonies were added into freshly prepared YEM broth which was incubated at rotary shaker (150 rpm) for 2 days. After 2 days, serial dilutions (10⁻³) of these broths were made and the culture was evenly spread over YEM agar plates. To determine the increase or decrease in the sensitivity of *Rhizobial* strains towards antibiotics, antibiotic disks were placed equidistantly at the center of 60 mm petri plates and zones of inhibition around antibiotic disks were formed.

**Indole Acetic Acid Production (IAA)**

All four *Rhizobial* strains were further screened for IAA production by inoculating them into 100 ml conical flasks containing YEM broth supplemented with L-tryptophan. The flasks were incubated at 30 ± 1°C on rotary shaker (150 rpm) for 72 hours. After incubation medium was centrifuged at 5000 x g for 20 minutes and cell free supernatant was used for IAA extraction.²³

To 10 ml of supernatant, 2 ml Salkowski reagent was added and incubated for 30 minutes under darkness. Amount of IAA produced was determined calorimetrically at 540 nm²⁴.

**Solid Biofertilizer Production**

**Determination of Water Holding Capacity**

In 10 g carrier material, 10 ml distilled water was added and the solution was filtered using Whatmann filter paper 1. The amount of filtered water was measured to determine the amount of water held by the carrier.

**Preparation of the solid (carrier) biofertilizer**

The pure culture of *Rhizobium* strains were established in 250 ml of growth media under sterile conditions. The carrier material was crushed, screened through 100-200 mesh sieves and weighed about 50 g. The cells of respective strains were immobilized on carrier depending on the water holding capacity i.e. upto 50% of it. After proper mixing it was left for 2-10 days during which the *Rhizobial* cells multiplied by a process called Curing. "*Rhizobial* Inoculants" were packaged and stored.

**Quantitative Analysis of Solid Biofertilizer**

30 g of inoculant was dispensed in 250 ml distilled water and was kept on rotary shaker (150 rpm) for 10 minutes. Serial dilutions upto 10⁻⁸ were made by suspending 10 ml aliquot of dilution in 90 ml distilled water. 10⁻⁵ to 10⁻⁹ dilutions were spread over YEM agar plates and plates were incubated at 28 ± 2°C for 3-5 days. Colony number was counted and calculated figures were measured in terms of per gram of carrier.

**Qualitative Analysis of Solid Biofertilizers**

10 g of each solid biofertilizer was weighed in petri plate and then sterilized for 3 hrs at 170°C. Weighed sample was mixed with distilled water and was filtered using Whatmann filter paper 1 to obtain filtrate.

**pH measurement**

Filtrate was maintained upto 25 ml in measuring flask. 2-3 drops of pH detector was added and color change of the sample was matched with the sample using pH strip.

**Estimation of Moisture**

5 g of prepared sample of biofertilizer was weighed in clean, dry petri dish and heated in oven for 5 hrs at 65 ± 1°C. Cooled in desiccator and weighed. Per cent loss in weight was reported as moisture content.

\[
\text{Moisture \% by weight} = \frac{100 (B - C)}{(B - A)}
\]

A= Weight of petri dish

B= Weight of petri dish plus sample

C= Weight of petri dish plus moisture content
Estimation of Electrolytic Conductivity

Fresh sample of biofertilizer was passed through 2-4 mm sieve. Filterate was prepared with 20 g sample and 100 ml distilled water in a ratio 1:5. Electrical Conductivity was measured using Conductivity meter calibrated using 0.01 M KCl.

Estimation of Carbon

10 g of sample was dried in oven at 105 °C for 6 hrs in a crucible and was ignited in muffle furnace at 600-700 °C for 6-8 hrs. Sample was cooled at room temperature and was kept in desiccator.

\[
Total \text{ Organic matter} \% = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Weight of Sample taken}} \times 100
\]

\[
Total \text{ C} \% = \frac{\text{Total Organic matter} \%}{1.724}
\]

Estimation of Nitrogen

1 g of sample was taken in 100 ml of kjeldahl flask. 5 mg of salt (K2PO4, Zn, CuSO4) were added with 3 ml H2SO4. After digestion, 10 ml distilled water was added. The distillate was collected in a conical flask containing 10 ml of standard acid solution and 4 drops of methyl red indicator. This solution was titrated against standard standard NaOH solution. Nitrogen content was determined by using following formula:

\[
\text{Nitrogen} \% = \frac{(\text{Sample titre} - \text{Blank sample} \times \text{NaCl} \times 14 \times 100)}{\text{Sample weight} \times 100}
\]

Liquid Biofertilizer Production

The mother culture of Rhizobium strains in Yeast Extract Mannitol (YEM) medium was established in 250 ml of growth media under sterile conditions. Inoculated broths were incubated in BOD for 1 week at 28±2 °C. After 1 week, the viable cell count should be about 1 x 10^7 cells/ml. Once the required cell count was achieved, the liquid biofertilizers were used directly, packed and stored.

Quantitative Analysis of Liquid Biofertilizer

Quantitative Test was performed using viable or Plate Count Method18.

Qualitative Analysis of Liquid Biofertilizer

In qualitative assessment of liquid biofertilizers, pH and visible contaminants were checked. Gram staining was done by method of Vincent18. Also, a loopful of broth was streaked on Glucose-Peptone agar at 28 ± 2 °C for 2 hours and results were examined.

Pot Cultivation Test

Two most efficient strains (Rhizobium trifolii and Rhizobium phaseoli) were evaluated for their potential to enhance the growth and yield of Trifolium (clover) and Vigna radiata (mung bean) plants grown in pots. Black sand was procured from nearby under construction area of National Center of Organic Farming (NCOF), Ghaziabad. Black sand having pH 7.8, E.C. 2.3 dSm⁻¹, organic matter 0.96% and total nitrogen 0.06% was autoclaved using an autoclavable bag at 121 °C for 20-30 minutes and cooled to room temperature. Seeds of Trifolium (clover) and Vigna radiata (mung bean) were surface sterilized before sowing using 0.1% Mercuric chloride (HgCl₂) for 2 minutes in petri plates. Seeds were soaked in their respective biofertilizers for 1 hour before sowing under laminar air flow chamber. Pots with sterile black sand were filled upto one third of the pot height.10 ml of nutrient solution was added to each pot everyday for growth. The pots were arranged randomly at ambient light and temperature according to completely randomized design and each treatment was replicated thrice. Plant growth was examined periodically.

Statistical Analysis

All experiments were performed in triplicate and the standard error of mean values was calculated. The means were tested according to Students t-test for significant differences among the samples. A statistical significance was accepted at P<0.05 using SPSS software.

RESULTS AND DISCUSSION

Isolation of Rhizobium strains

Four strains viz., Rhizobium trifolii, Rhizobium phaseoli, Bradyrhizobium japonicum and Rhizobium leguminosarum were recovered from root nodules of Trifolium (clover), Vigna radiata (mung bean), Glycine max (soybean) and Lens culinaris (lentil) plants and were screened for characteristic studies.

Growth of Rhizobium strains under aerobic and anaerobic conditions

Greater viable colonies (10⁷ CFU/ml) were found in Rhizobium trifolii in aerobic conditions as compared to anaerobic conditions (10⁵ CFU/ml). While amongst the four test isolates, maximum survival efficiency was seen in aerobic condition. The difference in survival efficiency under aerobic and anaerobic conditions may be attributed to relative tolerance to experimental and cultural conditions. Rank of isolates on the basis of growth - Rhizobium trifolii>Rhizobium phaseoli>Rhizobium leguminosarum>Bradyrhizobium japonicum (Figure 1).

Figure 1: Colony counts of Rhizobial strains. STR1 (Rhizobium phaseoli), STR2 (Rhizobium trifolii), STR3

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(Bradyrhizobium japonicum), STR4 (Rhizobium leguminosarum). Maximum viable colonies were observed in Rhizobium trifolii (10⁹ CFU/ml) and least number of colonies were seen in Bradyrhizobium japonicum (10³ CFU/ml). Maximum survival efficiency was found in aerobic condition by all the strains in comparison to anaerobic condition.

Colony and Morphological Characteristics

All the isolates were gram negative, non-spore forming and motile rods. The colonies of all isolates except Bradyrhizobium japonicum were found to be circular, 2-4 mm, milky white, transparent (Table 1).

It was found that all the three strains i.e., Rhizobium trifolii, Rhizobium phaseoli and Rhizobium leguminosarum were fast growers as they showed convex elevation in Yeast Extract Mannitol medium, described by Jordan in his work on Rhizobium phaseoli. Also, Bradyrhizobium japonicum was found to be a slow grower as the colonies formed by this isolate were 3.1 mm, translucent, whitish pink and glistening.

<table>
<thead>
<tr>
<th>Strains Characters</th>
<th>Rhizobium phaseoli</th>
<th>Rhizobium trifolii</th>
<th>Bradyrhizobium japonicum</th>
<th>Rhizobium leguminosarum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td>circular</td>
<td>circular</td>
<td>circular</td>
<td>circular</td>
</tr>
<tr>
<td>Size</td>
<td>2-4 mm</td>
<td>2-4 mm</td>
<td>3.1 mm</td>
<td>2.5 mm</td>
</tr>
<tr>
<td>Color</td>
<td>Milky white</td>
<td>Milky white</td>
<td>Whitish pink</td>
<td>white</td>
</tr>
<tr>
<td>Opacity</td>
<td>transparent</td>
<td>transparent</td>
<td>translucent</td>
<td>transparent</td>
</tr>
<tr>
<td>Motility</td>
<td>motile</td>
<td>motile</td>
<td>Motile</td>
<td>motile</td>
</tr>
<tr>
<td>Bacterium Shape</td>
<td>Rod shaped</td>
<td>Rod shaped</td>
<td>Rod shaped</td>
<td>Rod shaped</td>
</tr>
<tr>
<td>Grams nature</td>
<td>Gram Negative</td>
<td>Gram Negative</td>
<td>Gram Negative</td>
<td>Gram Negative</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strains</th>
<th>NaCl (w/v)</th>
<th>Temp (°C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Rhizobium phaseoli</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Rhizobium trifolii</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bradyrhizobium japonicum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rhizobium leguminosarum</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(**+**): High growth; (+): Moderate; (-): No growth.

<table>
<thead>
<tr>
<th>Strains Antibiotics</th>
<th>Inhibition zone diameter in cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rhizobium phaseoli</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>0.9 ± 0.01</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>0.7 ± 0.01</td>
</tr>
<tr>
<td>Penicillin</td>
<td>ND</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1.2 ± 0.02</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not detectable

<table>
<thead>
<tr>
<th>Metal Salts Strains</th>
<th>HgCl₂</th>
<th>ZnSO₄</th>
<th>CuSO₄</th>
<th>Al₂SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1%</td>
<td>0.01%</td>
<td>1%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Rhizobium phaseoli</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rhizobium trifolii</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bradyrhizobium japonicum</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rhizobium leguminosarum</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

“+” represents resistance of Rhizobial strains to metal salts and “–” represents sensitivity of Rhizobial strains to metal salts.
Table 5: Effect of different carbon sources on growth and acid production by *Rhizobium* strains.

<table>
<thead>
<tr>
<th>Strains Parameter</th>
<th><em>Rhizobium phaseoli</em></th>
<th><em>Rhizobium trifolii</em></th>
<th><em>Bradyrhizobium japonicum</em></th>
<th><em>Rhizobium leguminosarum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.0 ± 0.01</td>
<td>4.5 ± 0.05</td>
<td>3.0 ± 0.03</td>
<td>5.0 ± 0.02</td>
</tr>
<tr>
<td>Glucose</td>
<td>12.0 ± 0.04</td>
<td>10.5 ± 0.03</td>
<td>9.0 ± 0.02</td>
<td>11.0 ± 0.04</td>
</tr>
<tr>
<td>Sucrose</td>
<td>9.0 ± 0.03</td>
<td>8.0 ± 0.01</td>
<td>7.0 ± 0.01</td>
<td>8.0 ± 0.02</td>
</tr>
<tr>
<td>Lactose</td>
<td>6.5 ± 0.03</td>
<td>7.0 ± 0.02</td>
<td>5.0 ± 0.01</td>
<td>6.3 ± 0.04</td>
</tr>
<tr>
<td>Oxalate</td>
<td>3.5 ± 0.02</td>
<td>2.0 ± 0.04</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>5.0 ± 0.03</td>
<td>4.0 ± 0.03</td>
<td>3.0 ± 0.03</td>
<td>5.0 ± 0.05</td>
</tr>
</tbody>
</table>

ND=Not detectable

Table 6: Summary of the Biochemical tests for different strains of *Rhizobium*.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Strains</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram Reaction</td>
<td>Bromothymol Blue</td>
<td>Starch hydrolysis</td>
<td>Caseinase</td>
</tr>
<tr>
<td>SRP</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>STR</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>SBJ</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>SRL</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Growth is signified by "+" and poor growth is signified by "−". SRP – *Rhizobium phaseoli*, STR – *Rhizobium trifolii*, SBJ – *Bradyrhizobium japonicum* and SRL – *Rhizobium leguminosarum*

Table 7: Diameter of inhibition zones (in cm) formed around antibiotic discs inoculated with *Rhizobial* strains after exposure to UV-rays.

<table>
<thead>
<tr>
<th>Strains Antibiotics</th>
<th>Inhibition zone diameter in cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min.</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>ND</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>0.4 ± 0.04</td>
</tr>
</tbody>
</table>

*: minutes, ND= Not detectable

Table 8: Characterization of produced Liquid & Solid Biofertilizers.

<table>
<thead>
<tr>
<th>Forms</th>
<th>Liquid Biofertilizer</th>
<th>Solid Biofertilizer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Rhizobium trifolii</em></td>
<td><em>Rhizobium phaseoli</em></td>
</tr>
<tr>
<td>Appearance</td>
<td>Without odor</td>
<td>Without odor</td>
</tr>
<tr>
<td>Variable Cell Counts</td>
<td>1×10⁸ CFU ml⁻¹</td>
<td>0.5×10⁸ CFU ml⁻¹</td>
</tr>
<tr>
<td>Particle size in carrier (mm)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pH</td>
<td>7.1</td>
<td>6.4</td>
</tr>
<tr>
<td>Electrolytic conductivity (dsm⁻¹)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Carbon content (%)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Nitrogen content (%)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Non target bacterial contaminants</td>
<td>05</td>
<td>05</td>
</tr>
<tr>
<td>Gram stain</td>
<td>Gram negative</td>
<td>Gram negative</td>
</tr>
<tr>
<td>Glucose -Peptone</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

ND= Not detectable
The bacterial isolates were grown in Yeast Extract Mannitol (YEM) agar media and colony observation was done after 2-3 days of incubation at 30 ± 2 °C. Experiments were performed in triplicate. Table-1 represents the morphological and cultural characteristics of all the strains indicating *Rhizobium phaseoli*, *Rhizobium trifolii* and *Rhizobium leguminosarum* to be fast growing, and *Bradyrhizobium japonicum* to be slow growing.

**Biochemical Characterization**

All isolates were grown in YEM medium with pH 4-9. In the present study optimum pH for *Rhizobial* growth was found to be 6-7. Minimal growth of isolates was exhibited at pH 4 and pH 9. Deora and Singhal\(^2\) noted that growth started after 34 °C temperature tolerance started after 34 °C in which 50% of the strain i.e. *Rhizobium trifolii* and *Rhizobium phaseoli* survived while other strains had no growth at this temperature.

Some *Rhizobium* strains have been shown to grow under high salt concentration\(^2\). In the investigation, it was found that all the strains showed growth at 0.5% (w/v) NaCl and continued growth upto 4% (w/v) NaCl concentration. Also, it was found that *Rhizobium phaseoli* showed maximum growth which is similar to the findings of\(^2\) who stated that *Rhizobium phaseoli* is a halo tolerant *Rhizobium* (Table 2). Maximum growth was seen at pH 6 and pH 7. Temperature 34 °C and salt concentrations 0.5 and 4% (w/v) (NaCl). All the experiments were repeated 3 times.

Test isolates were assessed against six antibiotics namely Penicillin (10 µg), Chloramphenicol (30 µg), Kanamycin (30 µg), Tetracycline (30 µg), Nalidixic Acid (30 µg) and Streptomycin (10 µg). The evaluation of intrinsic resistance to antibiotics showed that all the test isolates exhibited high resistance to Penicillin (Table 3). *Rhizobium phaseoli*, *Rhizobium trifolii* and *Rhizobium leguminosarum* exhibited sensitivity to Tetracycline. *Bradyrhizobium japonicum* showed sensitivity to Nalidixic acid and Chloramphenicol. Antibiotic resistance pattern observed from the above table was Penicillin > Streptomycin > Kanamycin > Chloramphenicol > Nalidixic acid > Tetracycline. The results displayed in the table 3 show the average values of Tetracycline. The results displayed in the table 3 show the average values of 3 experimental set up. Standard deviations are calculates as deviations of the measurements at each data point.

Hartman and Amarger\(^28\) emphasized that intrinsic antibiotic resistance of different *Rhizobial* strains belonging to same species have a significant phenotypic characteristics.

The results obtained in the research agrees to the report of other author Yosey who found a diversity of reaction by *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *viciae* regarding resistance to antibiotics. Also, the present study is in accordance to Hungria and Vargas\(^27\) that this character of bacteria is due to bacterial permeability to antibiotics hydrophobicity, electrical charge and amount of antibiotic.

Many bacteria have developed controlled system to differentiate and cope up with harmful metal ions. Assessing the effect of metal salts on *Rhizobium* strains showed that all the strains were sensitive to mercuric chloride (HgCl\(_2\)). On the other hand, ZnSO\(_4\) and CuSO\(_4\) had least antibacterial effect at 0.1% (w/v) and 1% (w/v) concentration. Most metal ions have to enter bacterial cells in order to produce physiological and toxic effect. Table 4 represents overall pattern of sensitivity of *Rhizobial* strains towards metal salts observed through the data: HgCl\(_2\) > Al\(_2\)SO\(_4\) > CuSO\(_4\) = ZnSO\(_4\). All the strains were tested for sensitivity and resistance to metal salts at 1% and 0.01% (w/v) concentrations. Experiments were repeated 3 times. Maximum sensitivity was observed in all the strains due to mercuric chloride (HgCl\(_2\)).

All isolates were streaked on Bromothymol blue added YEM selective media for further confirmation. All four strains showed growth in 2 days and turned YEM media from blue to yellow confirming their nature of being fast growers and acid producers.

However, *Bradyrhizobium japonicum* was negative to Bromothymol blue test, as there was no color change in the medium confirming a slow growing, alkali producing nature. Javed and Asghari\(^27\) also characterized the

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**Table 9: Effects of Bioinoculants on seed germination and morphological parameters of *Trifolium* (clover) and *Vigna radiata* (mung bean).**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parameters</th>
<th>C1 Rhizobium trifolii</th>
<th>C2 Rhizobium phaseoli</th>
<th>L1 Rhizobium trifolii</th>
<th>L2 Rhizobium phaseoli</th>
<th>S1 Rhizobium trifolii</th>
<th>S2 Rhizobium phaseoli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Germination (days)</td>
<td></td>
<td>7.5 ± 0.01</td>
<td>4.5 ± 0.01</td>
<td>10.5 ± 0.05</td>
<td>15.5 ± 0.05</td>
<td>11 ± 0.02</td>
<td>14.2 ± 0.08</td>
</tr>
<tr>
<td>Plant height (cm)</td>
<td></td>
<td>1.2 ± 0.04</td>
<td>2.4 ± 0.01</td>
<td>2.5 ± 0.04</td>
<td>2.6 ± 0.01</td>
<td>2.5 ± 0.02</td>
<td>2.8 ± 0.02</td>
</tr>
<tr>
<td>Fresh weight (g/plant)</td>
<td></td>
<td>0.6 ± 0.04</td>
<td>0.2 ± 0.04</td>
<td>0.9 ± 0.01</td>
<td>0.4 ± 0.03</td>
<td>0.8 ± 0.02</td>
<td>0.5 ± 0.02</td>
</tr>
<tr>
<td>Dry weight (g/plant)</td>
<td></td>
<td>0.3 ± 0.01</td>
<td>0.4 ± 0.01</td>
<td>4.3 ± 1.01</td>
<td>4.5 ± 0.05</td>
<td>3.7 ± 0.03</td>
<td>4.8 ± 0.02</td>
</tr>
<tr>
<td>Fresh biomass (g/plant)</td>
<td></td>
<td>3.2 ± 0.01</td>
<td>4.1 ± 0.02</td>
<td>5.8 ± 0.02</td>
<td>6.0 ± 0.02</td>
<td>6.3 ± 0.02</td>
<td>7.1 ± 0.02</td>
</tr>
<tr>
<td>Number of pods per plant</td>
<td></td>
<td>3.2 ± 0.01</td>
<td>4.1 ± 0.02</td>
<td>5.8 ± 0.02</td>
<td>6.0 ± 0.02</td>
<td>6.3 ± 0.02</td>
<td>7.1 ± 0.02</td>
</tr>
</tbody>
</table>

C1, C2: Control; L1, L2: Liquid Biofertilizer; S1, S2: Solid Biofertilizer.
Rhizobium from soil root nodule with the same biochemical test.

Starch Hydrolysis Assay was examined to determine the production of reducing sugar from starch in bacteria. Clear zone around colonies were observed in Rhizobium phaseoli and Bradyrhizobium japonicum after addition of iodine.

Similar results were observed by De Olivera, who found Rhizobium can utilize starch from different carbon sources. Rhizobium trifolii showed positive Caseinase test and all other strains were found to be negative. Urease test was performed on urease medium incubated for 5 days. All the test isolates showed utilization of urease enzyme but Rhizobium leguminosarum had no growth. Bubble formation around bacterial colonies of all four strains showed positive Catalase test. These findings are in contradiction to the results of Elsheikh30.

Lysine decarboxylase test was performed using Bromocresol purple falkow media. Results showed change in the color of medium inoculated with Rhizobium phaseoli, Rhizobium trifolii and Bradyrhizobium japonicum but no such color change was found in the medium inoculated with Rhizobium leguminosarum. Lipase Test was found to be positive for all Rhizobial strains.30 found that Bradyrhizobium japonicum also showed a positive test to Lysine.

Utilization of Citrate as a carbon source was found to be positive in Rhizobium phaseoli and Rhizobium trifolii (the fast growing Rhizobia). However, slow growing Bradyrhizobium japonicum and Rhizobium leguminosarum showed no color change, exhibiting negative Citrate test.

Fast growing Rhizobia utilizes a wide range of carbon sources as compared to slow growing Rhizobia24. In the present study, all the strains showed good growth on Glucose, Sucrose and Starch (Table 5).

However poor growth was observed in Bradyrhizobium japonicum (slow growing) as compared to all the three strains. Similar observations were reported in Rhizobium isolates from Sesbania sesban32. Poor growth was seen in utilization of organic acids in all the strains of Rhizobia. Results presented above Table 5 are the mean of 3 experimental set up.

The bacterial isolates were grown in the media specific for the different tests and different characteristics were observed. Experiments were repeated 3 times.

After a series of cultural, morphological and biochemical test (Table 6), it was seen that Rhizobium trifolii and Rhizobium phaseoli produced optimum results under different test conditions.

These findings are in correlation with the literature survey that suggests both these strains fix the atmospheric nitrogen at a significant level (130 and 80-110 kg ha\(^{-1}\))29.

Effects of UV- Radiations on Antibiotic Sensitivity of Rhizobium

Antibiotics i.e. (Tetracycline and Kanamycin) which were found to be toxic to the Rhizobium strains were tested for observing the growth patterns of Rhizobial strains (Rhizobium trifolii and Rhizobium phaseoli) after their exposure to UV – rays (λ = 400 nm, intensity = 0.2-1 µW/cm\(^2\)) in laminar air flow at different time intervals. Rhizobium phaseoli showed resistance to Tetracycline at all time intervals (Table 7). But, Rhizobium trifolii was still sensitive to the effect of tetracycline at all time intervals. However, sensitivity in Rhizobium trifolii decreased at a marginal rate after exposure to UV-light. Results presented are the mean of 3 individual experimental set up.

Table 7 infers that sensitivity towards antibiotics, somehow, decreased after the strains were exposed to UV radiations under laminar air flow and the strains showed reasonable resistance. Also the strains may become resistant if they are exposed for a longer period of time, thereby, causing genotypic and phenotypic changes in them. This effect also shows that a mutagen may increase the resistance of Rhizobial strains towards antibiotics.

Indole Acetic Acid Production

The plant hormone production in nodules by symbiont and its transport to host by nodular symbiont was first reported by24. Rhizobium is known to produce IAA in culture supernatant and carbon sources present influences its production25.

In the present research, Rhizobium strains were checked to study their ability for IAA production under in vitro condition. When strains were grown in tryptophan supplemented YEM, production of IAA appeared to be maximum. Although inducing the concentration of L-tryptophan enhanced IAA production in Rhizobium, but it appears from analysis that IAA production increased 5-10 folds when the bacterium were provided with aerobic condition as compared to anaerobic conditions. This shows that along with L-tryptophan, oxygen is also an essential element required for Indole Acetic Acid Production. Maximum IAA produced by both strains under aerobic conditions is Rhizobium trifolii = 0.4 µg ml\(^{-1}\) and Rhizobium phaseoli = 0.6 µg ml\(^{-1}\).

Biofertilizer Production

Rhizobium biofertilizers are recommended for grain legumes to improve the productivity and to augment soil nitrogen status. A good strain of Rhizobium should be used in biofertilizer production which is capable of forming effective nitrogen fixing nodules on legumes. Rhizobium cells were immobilized on carriers, which is an inert material used for mixing with broth so that inoculants can easily be handled, packed, stored, transported and used. The broth containing Rhizobial cells were mixed with carrier. The moisture content was...
maintained at 35-40%. During this period, *Rhizobium* cells multiplied, by a process called curing. Thereafter, *Rhizobium* inoculants were used, packed and stored. *Rhizobium* inoculation is a well known agronomic practice to ensure adequate nitrogen fixation of legumes instead of N-fertilizers. Co inoculation benefits the plant growth\textsuperscript{26}. Quantitative analysis revealed that sufficient amount of growth on the basis of viable cell count was recorded in both the forms of *Rhizobium* biofertilizer (Table 8). However, *Rhizobium trifoli* as an inoculant exhibited higher growth in liquid (1×10\textsuperscript{7} CFU/ml) form while *Rhizobium phaseoli* as an inoculant exhibited higher growth in carrier (5×10\textsuperscript{7} cell/g) form.

Quality of biofertilizer is one of the most important factors resulting in their success or failure and acceptance or rejection by end-user, the farmers. Basically, quality means the number of selected microorganism in the active form per gram or milliliter biofertilizer. Quality standards are available only for *Rhizobium* in different countries. Quality has to be controlled at various stage of production (during mother culture stage, carrier selection, broth culture stage, mixing of broth and culture, packing and storage). When solid biofertilizers were assessed for quality check; both the inoculants exhibited improved properties (Table 8).

Sufficient amount of nitrogen and carbon content was found in both the strains. Neutral pH, sufficient moisture and moderate electrical conductivity revealed that both the inoculants were effective to be used in pot culture test. Good cultural characters without contamination (Table 8) were seen in liquid biofertilizers prepared for both the strains. All the experiments were repeated thrice and standard deviations were calculated.

**Pot Cultivation Test**

The growth of *Trifolium* (clover) and *Vigna radiata* (mung bean) plants with different forms of *Rhizobium trifoli* and *Rhizobium phaseoli* biofertilizers were compared to the control after 8-10 days of sowing under normal growth conditions. The growth and yield promoters such as germination days, plant height, fresh and dry weight were significantly increased by solid and liquid biofertilizer applications in comparison to the control (Table 9). Significant improvement in the fresh biomass was recorded upon inoculation with two isolates in comparison to uninoculated control.

**Naserirad**\textsuperscript{27} indicated that inoculation with biofertilizers containing *Azotobacter* and *Azospirillum* increased plant height, leaf number, fruit mean weight and yield in comparison to control which is similar to the our work but with different microbial biofertilizer. The stimulatory effects of biofertilizers used in the presence research are in accordance with the results obtained by **Chauhan**\textsuperscript{38} who found that inoculation of *Azospirillum* as a biofertilizer-markedly increased pods number and seed yield of *Brassica napus* L. plants over the non inoculated plants. In addition, **Buragohain**\textsuperscript{39} found that sugarcane yield was significantly higher in the cultivated crops with *Azotobacter* that uninoculated crops.

However one of the most commonly reported plant growth promotion mechanism by bacteria is the changing of morphological and physiological changes in root system\textsuperscript{40}. An increase in the number of lateral roots and root hairs cause addition of root surface available for nutrients and water uptake. Higher water and nutrient uptake by inoculated roots caused an improved water status of plant, which in turn could be the main factor enhancing plant growth\textsuperscript{41}.

Results have revealed that biofertilization has performed significant improvement in plant productivity and yield. Results presented are mean of three individual experimental set up. Maximum growth was found in *Vigna radiata* (mung bean) plants inoculated with *Rhizobium phaseoli* liquid biofertilizer (plant height 15.5 cm) and solid biofertilizers (plant height 14.2 cm). Significant increase in all the growth and yield parameters was observed i.e. germination days, plant height, fresh and dry weight, fresh biomass and pod number after inoculating the plants with biofertilizers (Table 9).

**CONCLUSION**

The present investigation seems to be promising approach to consider the optimum production and utilization of agricultural byproducts through conversion. It is suggested that two fast growing, acid producing strains (*Rhizobium trifoli* and *Rhizobium phaseoli*) act as a potential candidate to be used in nitrogen fixation and lab based experiments. The second part of the research points out that resistance to antibiotic of *Rhizobium* strains increased after UV exposure (\(\lambda = 400\) nm, intensity = 0.2-1 \(\mu W/cm^2\)). The optimum Indole Acetic Acid (IAA) production was found in presence of L-tryptophan induced Yeast Extract Mannitol (YEM) medium under aerobic conditions. Inoculation of solid and liquid biofertilizers resulted into enhanced plant growth by providing balanced nutrient supply. Optimum growth was observed in *Vigna radiata* (mung bean) plant inoculated with *Rhizobium phaseoli* liquid biofertilizer (plant height 15.5 cm) and solid biofertilizer (plant height 14.2 cm). These findings allow us a new scope for extensive research in Agricultural Biotechnology.

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