Research Article

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

Abhinav Datta[1], Ravi Kant Singh*[2], Shahina Tabassum[3]
[1]Department of Biotechnology, IMS Engineering College, Ghaziabad, Uttar Pradesh, India.
[2]Department of Biotechnology, Noida Institute of Engineering & Technology, Gr. Noida, Uttar Pradesh, India.
[3]Scientific Officer, National Centre of Organic Farming (NCOF), Ghaziabad, Uttar Pradesh, India.
***Corresponding author's E-mail:** rksingh.iitr@hotmail.com

Accepted on: 23-03-2015; **Finalized on:** 30-04-2015.

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 ^{o}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 Urease 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 bacteria[1]. Symbiosis is not only important for legume crops but also in Nitrogen cycle[2]. Biological Nitrogen fixation is a process by which atmospheric nitrogen (N_2) 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 *Rhizobia*[3]. It has been estimated that 1g of soil may contain a community of 10^9 microorganisms with *Rhizobia* representing around 0.1% of soil microbes or 10^6 g^{-1} soil[4].

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 nods while the nitrogen fixing forms exists as irregular cells called bacteriods. *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[5]. 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 media[6]. The classical phenotypic characterization of *Rhizobia* has been the first method employed when classifying unknown strains of *Rhizobia*[7,8]. *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 efficiency[9]. 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 nodules[10].

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[11].

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[12].

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[13]:

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

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[12].

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[14].

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[15].

Intrinsic Antibiotic Resistance

The isolates were tested for antibiotic sensitivity by Kirby Bauer disc diffusion on YEM agar[16]. 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[17]. Freshly prepared YEM agar plates were amended with metal salts i.e., $HgCl_2$, $CuSO_4$, Al_2SO_4 and $ZnSO_4$ 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[18].

Starch Hydrolysis

This test was performed to determine the capability of *Rhizobium* to use starch as a carbon source[19].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,

KH_2PO_4 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 Bromocresol Purple Falkow medium (peptone 5 g, yeast extract 3 g, glucose 1 g, Bromocresol 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 H_2O_2 into H_2O and O_2 in bacterial strains[20]. *Rhizobial* colonies (2-3 days old) were taken on glass slide and one drop of H_2O_2 (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 80[21].

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 hours[22].

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 trifolii* and *Rhizobium phaseoli*). Freshly cultivated colony containing YEM agar plates were exposed under UV radiations (λ = 400 nm, intensity = 0.2-1 μW/cm^2) 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 flaks 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 nm[24].

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^{-9} 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 filterate.

pH measurement

Filterate 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.

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

A= Weight of petri dish

B= Weight of petri dish + material before drying (heating)

C= Weight of petri dish + material after drying (heating)

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 ^{o}C for 6 hrs in a crucible and was ignited in muffle furnace at 600-700 ^{o}C for 6-8 hrs. Sample was cooled at room temperature and was kept in desiccator.

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

$$Total\ C\% = \frac{Total\ Organic\ matter\ \%}{1.724}$$

Estimation of Nitrogen

1 g of sample was taken in 100 ml of kjeldahl flask. 5 mg of salt (K_2PO_4, Zn, $CuSO_4$) were added with 3 ml H_2SO_4. 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:

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

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 ^{o} C. After 1 week, the viable cell count should be about 1 x 10^9 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 Method[18].

Qualitative Analysis of Liquid Biofertilizer

In qualitative assessment of liquid biofertilizers, pH and visible contaminants were checked. Gram staining was done by method of **Vincent**[18]. 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 ^{o}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 ($HgCl_2$) 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 (10^9 CFU/ml) were found in *Rhizobium trifolii* in aerobic conditions as compared to anaerobic conditions (10^2 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).

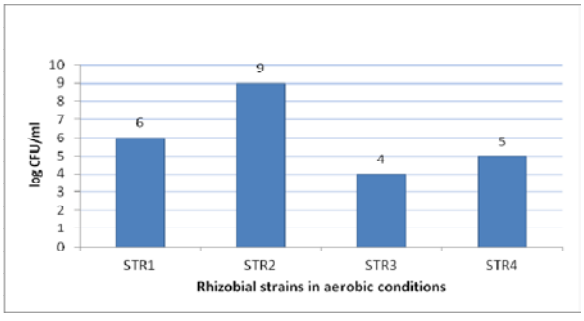

Figure 1: Colony counts of *Rhizobial* strains. STR1 (*Rhizobium phaseoli*), STR2 (*Rhizobium trifolii*), STR3

(*Bradyrhizobium japonicum*), STR4 (*Rhizobium leguminosarum*). Maximum viable colonies were observed in *Rhizobium trifolii* (10^9 CFU/ml) and least number of colonies were seen in *Bradyrhizobium japonicum* (10^4 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**[12] 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 glistering.

Table 1: Morphological Study of *Rhizobial* Strains

Strains Characters	*Rhizobium phaseoli*	*Rhizobium trifolii*	*Bradyrhizobium japonicum*	*Rhizobium leguminosarum*
Shape	circular	circular	circular	circular
Size	2-4 mm	2-4 mm	3.1 mm	2.5 mm
Color	Milky white	Milky white	Whitish pink	white
Opacity	transparent	transparent	translucent	transparent
Motility	motile	motile	Motile	motile
Bacterium Shape	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Grams nature	Gram Negative	Gram Negative	Gram Negative	Gram Negative

Table 2: Tolerance of *Rhizobium* strains to pH, Temperature and NaCl concentrations

Strains	NaCl (w/v)					Temp (°C)			pH					
	0.5	1	2	3	4	29	34	37	4	5	6	7	8	9
Rhizobium phaseoli	++	++	++	++	++	+	++	+	–	+	++	++	++	–
Rhizobium trifolii	++	+	+	+	++	+	++	+	+	+	++	++	+	–
Bradyrhizobium japonicum	+	+	+	+	+	+	++	–	–	+	++	++	+	+
Rhizobium leguminosarum	++	+	+	+	++	+	++	–	–	+	++	++	++	+

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

Table 3: Inhibition zones (in cm) formed around the disks of antibiotics inoculated with *Rhizobial* strains.

Strains Antibiotics	Inhibition zone diameter in cm			
	Rhizobium phaseoli	*Rhizobium trifolii*	*Bradyrhizobium japonicum*	*Rhizobium leguminosarum*
Nalidixic Acid	0.9 ± 0.01	1.6 ± 0.02	3.5 ± 0.04	1.7 ± 0.01
Kanamycin	0.7 ± 0.01	1.5 ± 0.02	2.9 ± 0.03	2.0 ± 0.02
Penicillin	ND	ND	0.1 ± 0.01	ND
Streptomycin	0.3 ± 0.01	1.6 ± 0.02	2.0 ± 0.02	1.8 ± 0.02
Tetracycline	1.2 ± 0.02	3.7 ± 0.03	3.0 ± 0.02	4.8 ± 0.04
Chloramphenicol	ND	2.3 ± 0.02	3.2 ± 0.02	1.9 ± 0.02

ND = Not detectable

Table 4: Effect of metal salts on the growth of *Rhizobium strains.*

Metal Salts Strains	$HgCl_2$		$ZnSO_4$		$CuSO_4$		Al_2SO_4	
	1%	0.01%	1%	0.01%	1%	0.01%	1%	0.01%
Rhizobium phaseoli	–	–	+	+	+	+	+	+
Rhizobium trifolii	–	–	+	+	+	+	–	–
Bradyrhizobium japonicum	–	–	+	+	+	+	+	+
Rhizobium leguminosarum	–	–	+	+	+	+	–	–

"**+**" 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.

Colony diameter in mm after 72 hour incubation				
Strains Parameter	***Rhizobium phaseoli***	***Rhizobium trifolii***	***Bradyrhizobium japonicum***	***Rhizobium leguminosarum***
Control	4.0 ± 0.01	4.5 ± 0.05	3.0 ± 0.03	5.0 ± 0.02
Glucose	12.0 ± 0.04	10.5 ± 0.03	9.0 ± 0.02	11.0 ± 0.04
Sucrose	9.0 ± 0.03	8.0 ± 0.01	7.0 ± 0.01	8.0 ± 0.02
Lactose	6.5 ± 0.03	7.0 ± 0.02	5.0 ± 0.01	6.3 ± 0.04
Oxalate	3.5 ± 0.02	2.0 ± 0.04	ND	ND
Pyurvate	5.0 ± 0.03	4.0 ± 0.03	3.0 ± 0.03	5.0 ± 0.05

ND=Not detectable

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

Characteristics									
Strains	**Gram Reaction**	**Bromothymol Blue**	**Starch Hydrolysis**	**Caesinase**	**Urease**	**Catalase**	**Lysine De Carboxylase**	**Lipase**	**Citrate**
SRP	–	+	+	–	+	+	+	+	+
STR	–	+	–	+	+	+	+	+	+
SBJ	–	–	+	–	+	+	+	+	–
SRL	–	+	–	–	–	+	–	+	–

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.

Inhibition zone diameter in cm						
Strains Antibiotics	***Rhizobium phaseoli***			***Rhizobium trifolii***		
	1 min.	2 min.	3 min.	1 min	2 min.	3 min. [a]
Tetracycline	ND	ND	ND	0.7 ± 0.01	0.5 ± 0.04	0.4 ± 0.02
Kanamycin	0.4 ± 0.04	0.2 ± 0.03	0.1 ± 0.01	0.4 ± 0.02	0.3 ± 0.05	0.1 ± 0.03

[a]: minutes, ND= Not detectable

Table 8: Characterization of produced Liquid & Solid Biofertilizers.

Forms	**Liquid Biofertilizer**		**Solid Biofertilizer**	
	Rhizobium trifolii	***Rhizobium phaseoli***	***Rhizobium trifolii***	***Rhizobium phaseoli***
Appearance	Without odor	Without odor	Black	Black
Variable Cell Counts	1×10^{9} CFU ml^{-1}	0.5×10^{8} CFU ml^{-1}	3×10^{6} Cell g^{-1}	5×10^{7} Cell g^{-1}
Particle size in carrier (mm)	ND	ND	0.20 ± 0.05	0.18 ± 0.02
pH	7.1	6.4	7.2	6.5
Electrolytic conductivity(dsm^{-1})	ND	ND	1.42 ± 0.01	0.98 ± 0.01
Moisture (%)	ND	ND	22 ± 0.02	27±0.02
Carbon content (%)	ND	ND	12.74 ± 0.05	14.36 ± 0.05
Nitrogen content (%)	ND	ND	0.81 ± 0.01	0.67 ± 0.01
C/N ratio	ND	ND	15.72	21.43
Non target bacterial contaminants	05	05	ND	ND
Gram stain	Gram negative	Gram negative	ND	ND
Glucose -Peptone	Negative	Negative	ND	ND

ND= Not detectable

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

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

C1, C2: Control; L1, L2: Liquid Biofertilizer; S1, S2: Solid Biofertilizer.

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**[25] has proposed that slight variation in the pH of medium might have an enormous effect on the growth of *Rhizobium*.

The maximum temperature at which all the strains grew was 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[26]. 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[27] 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 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: $HgCl_2 > Al_2SO_4 > CuSO_4 = ZnSO_4$. 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**[29] also characterized the

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 Caesinase 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 **Elsheikh**[30].

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 *Rhizobia*[31]. 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 sesban*[32]. 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})[33].

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 $\mu W/cm^2$) 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 by[34]. *Rhizobium* is known to produce IAA in culture supernatant and carbon sources present influences its production[35].

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[36]. 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 trifolii* as an inoculant exhibited higher growth in liquid (1×10^9 CFU/ml) form while *Rhizobium phaseoli* as an inoculant exhibited higher growth in carrier (5×10^7 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 trifolii* 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[37] 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**[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**[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[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[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 trifolii* 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 (λ = 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.

REFERENCES

1. Schultze M, Kondrosi A. Regulation of Symbiotic root nodule development. Annual review of genetics. 32, 1998, 33-37.
2. Arujo ASF, Figuerido MBV, Monteiro RTR. Potential of biological nitrogen fixation as indicator of Soil pollution. Nova Science Publisher, New York, 2008.
3. Vance CP. Legume Symbiotic nitrogen fixation: Agronomical aspects. Kluver Academic Publishers, Dorderecht, 1998, 509-530.
4. Thies J, Singelton P, Bohlool B. Influence of size of indigenous *Rhizobial* population on establishment and symbiotic performance of *Rhizobia* in field legumes, Applied and Symbiotic Micro, 57, 1991, 19-28.

5. Datta A, Singh RK, Kumar S. An Effective and Beneficial Plant Growth Promoting Soil Bacterium "Rhizobium": A Review. Annals of Plant Sciences. 4(01), 2015, 933-942.
6. Subba RNS. Soil Microbiology, Soil Microorganism and plant growth. Oxford and IBH publishing Co. Pvt. Ltd, New Delhi, India, 1999.
7. Zhang X, Harper R, Karsisto M, Lindstrom K. Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. International J. Systematic Bacteriol, 41, 1991, 104-113.
8. Wolde-meskel E, Berg T, Peters NK, Frostegard A. Nodulation status of native woody legumes and phenotypic characteristic of associated *Rhizobia* in soils of southern Ethiopia. Biology and Fertility of Soils, 40, 2004, 55-66.
9. Kremer RF, Paterson IA. Nodulation Efficiency of legume inoculation as determined by intrinsic antibiotic resistance. Appli. Environ. Microbiol., 43, 1982, 636-642.
10. Nutman PS. Study framework for symbiotic Nitrogen Fixation. Edited by Nutman WJR, Postgate and Rodriguez Barrueco (Acad. Press, London), 1977, 443.
11. Vessey JK. Plant growth promoting Rhizobacteria as biofertilizers, Plant Soil., 255, 2003, 571-586.
12. Jordan DC. Family III Rhizobiaceae In: Kreig NR, Holt JG (Eds.), Bergey's Manual Of Systematic Bacteriology, Williams and Wilker, Battinore., 1984, 234-242.
13. James GC. Native Sherman Rockland Community College, State University of New York., The Benjamin/Coming Publishing Company.Inc., 1978, 75-80.
14. Lindstorm K. Metabolic properties maximum growth temperature and phage sensitivity of *Rhizobium* sp. (*Galega*) compared with fast growing *Rhizobia*. FEMS Microbial letters, 50, 1988, 277-287.
15. Graham P, Sadowsky MJ, Keyser HH. Proposed minimal standards for the description of new genera of roots and stem nodulating bacteria.Syst.bacteriol. 41, 1991, 582-587.
16. Bauer AW, Kirby WMM., Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol, 45, 1966, 493-496.
17. Cervates C, Chavez J, Cardova NA, Denamora P. Resistance to metal by *Pseudomonas acrugenosa*, Clinical Isolates Microbiol. 48, 1986, 159-163.
18. Vincent JM. A manual of practical study of Root nodule Bacteria., Blackwell Scientific publication, Oxford, London, 1970.
19. De Oliveria AN, De Oliveria LA, Andrade A, Chagas JAF. *Rhizobia* amylase production using various starchy substances as carbon substrates, Braz. J. Microbiol. 38, 2007, 208-218.
20. Faddin M. Biochemical test for identification of Medical bacteria. Williams and Walkins, Baltimore, USA., 1980, 151-154.
21. Kumari BS, Ram MR, Mallaih KV. Studies on nodulation, biochemical analysis and protein profiles of *Rhizobium* isolates from *Indigofera* species, Malaysian J. Microbiol, 2009, 133-139.
22. Koser SA. Utilization of salts of organic acids by colon aerogenes group. J. Bacteriol. 8, 1923, 493-520.
23. Sinha BK, Basu PS. Indole-3-acetic acid metabolism in root nodules of *Pongamia pinnata* (L) Pierre. Biochem Physiol., 176, 1981, 218-227.
24. Gordan SA, Weber RP. Colorometric estimation of IAA., Plant Physiol., 26, 1951, 192-195.
25. Deora GS, Singal K. Isolation, biochemical characterization and preparation of biofertilizers using *Rhizobium* strain for commercial use, Biosci. Biotech. Res. Comm. 3(2), 2010, 132-136.
26. Kucuk C, Kivan M, Kinaci E. Characterization of Rhizobium sp. Isolated from Bean, Turk. J. Biol. 30, 2006, 127-132.
27. Hungria M, Vargas MAT. Environmental factors offering nitrogen fixation in grain legumes in tropics with an emphasis on Brazil Field Crops., Res. 65, 2000, 151-164.
28. Hartman A, Amarger N. Genotypic diversity of an indegenious *Rhizobium meliloti* field population assessed by plasmid profiles DNA fingerprinting and inertion sequence typing., Can. J. Microbiol., 37, 1991, 600-608.
29. Javed K, Asghari B. Potential allelopathic Effects of sunflowers on microorganisms. Afri. J. Biotech., 7(22), 2008, 4208-4211.
30. Elsheikh EAE, Wood M. Soil Biology Biochem, 21, 1989, 883-887.
31. Graham PH, Parker CA. Diagnostic features in characterization of root nodule bacteria of legumes., Plant Soil., 20, 1964, 383-396.
32. Helmish FA, El-Mokadem MT, Zekry SHA. Nutritional requirement and invertase activity of *Rhizobium* nodulating Sesbania seban roots Fur Microbiol., 148, 1993, 582-587.
33. Gupta A, Saxena AK, Gopal M, Tilak KVBR. Effect of plant growth promoting rhizobacteria on competitive ability of introduced *Bradyrhizobium* sp. (*Vigna*) for nodulation. Microbiological Research, 153, 1998, 113–117.
34. Hunter WJ. Indole-3-acetic acid production by bacteriods from soybean root nodules. Plant physiol., 76, 1989, 31-36.
35. Ghosh A, Basu PS. Production and Metabolism of Indole Acetic acid in root nodules of *Phaseolus mungo*. Microbiol Res. 161, 2006, 362-366.
36. Sarig S, Okon Y, Blum A. Effect of *Azospirillum brasilence* inoculation on growth dynamics and hydraulic conductivity of *Sorghum bicolor* roots. J. Plant Nutr., 15, 1922, 805-819.
37. Naserirad H, Sokymanifar A, Naseri R. Effect of integrated application of Biofertilizer on Grain Yield, Yield components and Associated Traits of Maize cultivars. American Eurasian J. Agric. 10(2), 2011, 271-277.
38. Chauhan DR, Paroda S, Katria OP, Singh KP. Response of Indian mustard (*Brassica juncea*) to biofertilizeers and nitrogen. Ind. J. Agron., 40, 1995, 86-90.
39. Buragohain SK. Efficacy of biofertilizers with varying levels of N on growth, yield and quality of sugarcane (*Saccharum officinarum* L.) Ind. Sugar, 49, 2000, 847-850.
40. Dalla Santa OR, Hernándes Alvarez GLM, Junior PR, Soccol CR. *Azospirillum* sp. Inoculation in wheat, barley and oats seeds green house experiments. Brazillian Archives of Biology and Technology, 47(6), 2004, 843-850.
41. Bai Y, Zhou X, Smith DL. Crop ecology, management and quality: Enhanced Soybean plant growth resulting from co-inoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. Crop Science, 43, 2003, 1774-1781.

Source of Support: Nil, **Conflict of Interest:** None.