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Isolation and Biochemical Characterization of Rhizobia from Rice Rhizosphere and Their Effect on Rice Growth Promotion

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ABSTRACT

Biofertilizer is a relatively safer, environmental friendly and cost-effective approach as an alternative to reduce chemical fertilizer usage. The selection of bacterial strains with multiple beneficial characteristics are important to maximize the effectiveness on the host plant. Rhizobium, a nitrogen fixing bacteria is found both as free living in soil or in associative form within the root nodules of host legumes. Nine different colonies of Rhizobium spp. were isolated from which two isolates isolate 7 and 9 were finally selected on the basis of their better growth on yeast extract mannitol agar for their biochemical characterization and study its efficacy on rice growth promotion. The results has successfully demonstrated the effectiveness of isolated rhizobial strains from rice field with multiple beneficial characteristics on vigor of rice seedlings under controlled condition. The result proved to be a vital information in the development of a liquid biofertilizer for rice.

Key words: *Rhizobium, Rice, Isolates, YEMA*

INTRODUCTION

Nitrogen is an essential nutrient for plant growth and development. Plants cannot directly use dinitrogen gas, which makes up about 80 % of the atmosphere. Plants absorbs only available nitrogen in the form of ammonium and nitrates from the soil. The limited availability of nitrogen and increase in requirement of this element by the crop for growth has increased the use of chemical fertilizers[1,2]. Most of the nitrogen applied in form of chemical fertilizer is lost in the environment through gaseous emissions, runoff, erosion and leaching which leads to greenhouse effects, nitrate pollution of surface and ground water causing eutrophication and compromising agricultural sustainability. However, due to the increased prices, availability and the environmental concerns of chemical fertilizers, there is an urgent need to find alternative strategies that can ensure competitive crop yields, provide environmental safety and protection by maintaining ecological balance in agro-ecosystem.

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In this context, now a days the use of the nitrogen fixing bacteria in agricultural practices has gained importance. Only some prokaryotes are capable to utilize atmospheric nitrogen through a process known as biological nitrogen fixation (BNF), which is the conversion of atmospheric N_2 to NH_3[3,4].

Among the soil bacteria, unique group called Rhizobia has a beneficial effect on the growth of plants. Although rhizobia naturally infect legumes as host plants, some *Rhizobium* strains can form symbiotic relationships with non-legume species[5]. Several researches demonstrated the ability of *Rhizobium* to colonize the roots of non-legumes showing various PGP traits like phytohormone production[6] , phosphate solubilizer[7], nitrogen fixers[8], vitamins[9] and siderophores[10] and enhancement in stress resistance[11].

Rice is the major world food crop and it is the most important staple diet for nearly 3 billion people, approximately half of the world population[12]. If the consumption trend continues, there is need of increase in the production of rice. To increase the production of rice, huge amount of chemical fertilizer is applied by the farmers which can be hazardous to environment. The increase of production must be achieved through improvement in agricultural productivity. Microbes are considered as beneficial having key factor in maintaining soil quality and rice production through biological nitrogen fixation[13], increased root growth [14] with enhanced nutrient uptake[15], phytohormone production[16], plant growth enhancement stimulation by other beneficial bacteria and fungi[17] and biocontrol of plant diseases[18]. Peng *et al*. (2002), reported the rhizobial inoculation known for their symbiotic relationship with legumes, could also increase rice grain yield.

Thus, it is essential to isolate native rhizobia from rice grown fields for multiple beneficial effects on rice crop and for potential biofertilizer development. In the present study, isolation from rhizospheric soil of rice and screening of rhizobia with multiple plant growth-promoting abilities were conducted and also assessed their effectiveness on root and shoot development and biomass production of rice *cv*. Gurjari in crates under net house conditions.

MATERIALS AND METHODS

Sampling of rhizospheric soil of Rice for bacterial isolation

Soil samples were collected from rhizosphere of three varieties of rice field Gurjari, Narmada and IR-64. Soil samples from rhizosphere of rice plants were collected carefully by uprooting the root system and placing in a sterile polythene bag for transport and stored at 4°C.

Isolation of Rhizobium from soil samples

For isolation 10 gram of rhizospheric soil was suspended in 90 mL of sterile DDW in sterile flask and was mixed properly. 100 µL of 10^5 diluted suspension was kept on solid YEMA plate and spreading was done. It was incubated for 24-72 hours at 30°C in incubator. Morphologically different Rhizobial strains were isolated and preserved at 4 °C for further characterization. The isolates were subcultured in fresh YEMA slants once in a month and maintained at 4 °C.

Biochemical characterization

The Gram's reaction was performed as per standard protocol. Physiological and biochemical characterizations of the bacterial isolates such as urease, nitrate reduction, IMViC and carbon source utilization were examined according to the standard methods. The isolates were identified accordance with the Bergey's manual of determinative bacteriology.

2.4 Nitrogen Fixation Ability

The plant growth promoting effect showed by rhizospheric isolates is directly attributed to its capacity to fix atmospheric nitrogen into the forms utilized by plants. Isolates were inoculated into the nitrogen free broth containing glucose as carbon source and cultures were grown at 30 ± 2°C for seven days and nitrogen fixation was measured by Micro-Kjehldahl method[20]. Sugar utilization was estimated by DNS method. The rate of nitrogen fixation was expressed as mg nitrogen fixed per gram of glucose consumed.

Phosphate Solubilizing ability on solid and liquid medium

The phosphate solubilizing activities of the isolates were assayed by spotting the cultures on Sperber media. The plates were incubated at 30°C for one week and the phosphate solubilization efficiency was measured. The isolated strains were evaluated for their phosphate-solubilizing activity in broth culture. Erlenmeyer flasks (250 ml) containing 100 ml of the liquid Sperber's broth were inoculated with 100 µl of bacterial suspension (approx. 10^7cfu/ml). Each treatment was replicated three times. Flasks were incubated at 30+ 2°C on rotary shaker (150 rpm). Measurement of pH and liberated P by Vanado-molybdate method was carried out after 2, 4 and 6 days[21]. The graph of OD versus concentration of phosphate in µg was plotted for the standard and samples were compared to calculate P concentration.

Indole Acetic Acid (IAA) production

The isolates were inoculated in Glucose Phosphate Broth (GPB) with the addition of tryptophan (1µg/ml) and incubated at 28°C for 48 hours. The culture was centrifuged at 7000 rpm for 7 minutes and 1.0 mL of the supernatant was mixed with 2 mL of Salkowsky's reagent and incubated for 30 min. The IAA concentration was measured by spectrophotometer at 535 mm in three replicates, the levels of IAA production were estimated referring IAA standard graph[22].

Potassium Solubilization Ability

The isolates were screened for their ability to solubilize potash. The isolates were spot inoculated on Alendreskov's media containing mica as a raw insoluble potash substrate. Plates were incubated at 30+2°C and examined for the zone of solubilization by colonies to check their potash solubilization activity[23].

Plant Inoculation Test in in vitro and net house conditions

In vitro experiment evaluated the effect of the rhizospheric isolates inoculation on rice seed germination. Seeds were surface sterilized with 0.1% $HgCl_2$ solution for 5 min and rinsed thoroughly with distilled water 3-4 times. Sterilized seeds were inoculated with 5 mL of rhizospheric isolates cultured in YM broth for 30 min and were kept on previously sterilized germination paper and incubated at 28°C for 12 days. Seed germination was observed after 24 h through a 4-days period. Root and shoot length were measured after the twelfth day. The experiment was planned as a completely randomized design with 5 replications for each isolate.

Vigour index =germination (%) × total plant length

In nethouse experiment was performed with plastic crates containing soil for rice growth. Seedlings were treated with rhizobial isolate cultured in YM broth for 15 mins and were sowed in plastic crates along with sterile YM broth in uninoculated control. 75% of Ammonium sulphate of recommended dose was given in the crates having treatment of isolates and 100% in crates with uninoculated control. At 30, 60 and 90 days after transplanting (DATP) shoot length was measured and at harvest shoot and root length was measured, number of tillers per plants was counted, the fresh and dry weight was measured.

RESULTS AND DISCUSSION

Isolation and characterization of Rhizobium

Rhizobium was isolated from rhizosphere of rice plant grown in rice field from Main Rice Research Station, Nawagam, AAU, Anand. Two isolates from the total of seven isolates have been selected on basis of morphological and biochemical studies and were then subjected to PGP traits such as quantification of N_2 fixation, phosphate and potassium solubilization rates and qualitative observation of siderophore.

The colonies of the two isolates (isolate 7 and 9) were large convex, opaque, smooth and circular mucilaginous colony giving light pink colour on YEMA medium (Plate 1). The cells of both the isolates were G -ve, rods which is characteristic feature of genus *Rhizobium*.

pH tolerance

Both the isolates have wide pH tolerance range (5.0-8.0) (Table 1). Best growth of both the

isolates was observed in the pH range of 5.0-7.0.

Antibiotics resistance profile

In the present study (Table 2) it was found that, both the isolates were highly resistant to tested antibiotics except isolate 9 showing high sensitivity to polymyxin-B (300 µg/disc).

Biochemical characterization

The results of tests for specific breakdown products are presented in Table 3. The data revealed that both the isolates were ornithine utilization, nitrate reductase, esculine hydrolysis positive and isolate 7 was found positive for ONPG, lysine utilization, urease and citrate utilization and both were found negative for phenyl alanine deamination, H_2S production, Vogesproskauer's, methyl red, Indole and Malonate utilization.

The present study also indicates that both the isolate were able to utilize large group of carbohydrate (Table 4). Both the isolates can utilize xylose, maltose, dextrose, fructose, galactose, L-arabinose, glycerol and cellobiose.

Evaluation of PGPR traits of both the isolates in in vitro

Both the isolates were characterized for plant growth promoting attributes nitrogen fixation, P and K solubilization, production of Indole-3-Acetic Acid (IAA).

N_2 fixing capacity

In vitro nitrogen fixation efficiency of isolates was assessed before seed inoculation and efficacy testing through pot trials. Both the isolates were confirmed to have ability of fixing atmospheric nitrogen. The nitrogen fixing potentiality of isolate 7 was 31.5 and isolate 9 was 26.76 mg N g^{-1} of glucose consumed.

Phosphate solubilization capacity

Phosphate solubilization on solid media

Both the isolates were tested for their phosphate solubilization capacity on Sperber's media and showed clear zone (Plate 2) around colony which shows ablility to solubilize tri-calcium phosphate. The ability of the isolates to solubilize insoluble organic phosphate in form of Ca-Phytate was also tested on solid MS media containing Ca- Phytate as sole source of phosphate. isolate 7 and 9 both were found positive for phytase enzyme production.

Phosphate solubilization efficiency in liquid medium

Phosphate solubilization assay in liquid medium was performed with TCP in Sperber's broth. Observations were recorded on 2^{nd}, 4^{th} and 6^{th} day after inoculation and release of P was estimated by Vanedo-molybdate method.

Data regarding phosphate solubilization activity of isolates are presented in Table 5. Estimation of P in the medium revealed that both the strains released P from tri calcium phosphate (TCP). At 4 DAI, isolate 7 recorded maximum P release (655.2 µg ml^{-1}).

Indole acetic acid (IAA) production

Both the isolates were grown in Glucose Phosphate Broth supplemented with 0.5 µg ml^{-1} and 1.0 µg ml^{-1} of tryptophan for IAA production (Table 6). For 0.5 µg ml^{-1} supplementation of tryptophan, variable response was seen in isolates for IAA production. On 4 DAI and 2 DAI isolate 9 showed highest IAA production (12.4 µg ml^{-1}) and (11.1 µg ml^{-1}).

For 1.0 µg ml^{-1} supplementation of tryptophan, variable response was shown by isolates in terms of IAA production. At 2 DAI and 4 DAI isolate 9 showed higher IAA production (24.4 µg ml^{-1}) and (17.9 µg ml^{-1}).

Potash solubilization efficiency

Both the isolates were tested for their potash solubilizing efficiency on Alendreskov's media containing mica as natural 'K' substrates. Both the isolates showed zone of potash solubilization on Alendreskov's mica media (Table 7).

Plant growth promoting effects of proven isolates on rice cv. Gurjari

As both the isolates were found to possess one or more PGP traits, their plant growth promoting activity was tested in laboratory on rice *cv.* Gurjari.

In vitro effect of isolates on rice cv. Gurjari

Seed inoculation of both the isolates had significant effect on growth and development of germinating rice *cv.* Gurjari in laboratory. Both the isolates showed increase in seed

germination compared to non-inoculated seeds (Table 8).

Both the isolates showed 100% germination of Gurjari seeds indicating no adverse effect, however in control only 85% germination was recorded. Seedling vigor index (SVI) was higher in both isolate 9 (1504) and isolate 7 (1482) as compared to control (1037). Shoot length, root length, fresh weight and dry weight was higher in both isolates as compared to control.

Effect of isolates on shoot, root length, number of tillers, plant fresh and dry weight of rice cv. Gurjari in plastic crates under net house conditions

Treatment of isolate 7 along with 75% RDF (recommended dose of fertilizer) was statistically superior to all the treatments for shoot (70.23) and root length (31.15 cm) at harvest (Table 9).

Table 10 shows effect of diazotroph inoculation on number of tillers, root and shoot fresh and dry weight (g). Isolate 7 along with 75% RDF (N:P) showed highest number of tillers (5.05 numbers) which was found close to 100% RDF (5.03).

Table 1: pH tolerance test of the isolates

Isolates	pH range tested								
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Isolate 7	-	-	-	++	++	++	++	-	-
Isolate 9	-	-	-	++	++	++	+	-	-

Note: - = No growth, + = Moderate growth, ++ = Good growth, +++= Excellent growth

Table 2: Antibiotic resistance profile of the isolates

Isolates	Antibiotic tested										
	A (10)	**Cb (100)**	**P (300)**	**S (10)**	**R (5)**	**T (30)**	**K (30)**	**V (30)**	**Se (100)**	**G (10)**	**C (10)**
Isolate 7	HR	HR	HR	HR	HR	HR	HR	HR	HR	HR	HR
Isolate 9	HR	HR	HS	HR	HR	HR	HR	HR	HR	HR	HR

G- Gentamicin	**A**- Ampicillin	**T**- Tetracycline	**P**-Polymyxin-B
C- Chloramphenicol	**S**- Streptomycin	**K**- Kanamycin	**R**- Rifampicin
Cb- Carbenicillin	**Se**- Spectinomycin	**V**-Vancomycin	

Symbols	Radius of inhibition zone (mm)
R	5- 10mm
HR	< 5mm
S	11-20mm
HS	> 20mm

Table 3: Biochemical reactions of the isolates

Sr. No.	Biochemical tests	Isolate 7	Isolate 9
1.	ONPG	+	-
2.	Lysine utilization	+	-
3.	Ornithine utilization	+	+
4.	Urease	+	-
5.	Phenyl alanine deamination	-	-
6.	Nitrate reductase	+	+
7.	H_2S production	-	-
8.	Citrate utilization	+	-
9.	Vogesproskauer's	-	-
10.	Methyl red	-	-
11.	Indole	-	-
12.	Malonate utilization	-	-
13.	Esculine hydrolysis	+	+

Table 4: Carbohydrates utilization by isolates after 48 hours of inoculation

Sr. No.	Carbon source	Isolate 7	Isolate 9	Sr. No.	Carbon source	Isolate 7	Isolate 9
1.	Lactose	+	-	18.	Inositol	-	-
2.	Xylose	+	+	19.	Sorbitol	-	-
3.	Maltose	+	+	20.	Mannitol	-	-
4.	Fructose	+	+	21.	Adonitol	+	-
5.	Dextrose	+	+	22.	Arabitol	-	-
6.	Galactose	+	+	23.	Erythritol	-	-
7.	Raffinose	+	-	24.	α- methyl-D-glucoside	-	-
8.	Trehalose	+	-	25.	Rhamnose	+	-
9.	Melibiose	+	-	26.	Cellobiose	+	+
10.	Sucrose	+	-	27.	Melezitose	-	+
11.	L-Arabinose	+	+	28.	α- methyl-D-Mannoside	-	-
12.	Mannose	+	-	29.	Xylitol	-	-
13.	Inulin	+	-	30.	D-Arabinose	-	-
14.	Sodium gluconate	-	-	31.	Glucose	-	-
15.	Glycerol	+	+	32.	Sorbose	-	-
16.	Salicin	-	-	33.	Control	-	-
17.	Dulcitol	-	-				

Table 5: *In vitro* phosphate solubilization efficiency of isolates in liquid medium

Isolates	Available P concentration (µg ml^{-1} or ppm)		
	2 DAI	4 DAI	6 DAI
Initial	-	-	-
Isolate 7	118.9	655.2	169.2
Isolate 9	123.7	364.4	364.3

Table 6: *In vitro* IAA production efficiency of isolates supplemented with tryptophan 0.5 and 1 µg ml^{-1}

Isolate	IAA concentration (0.5 µg ml^{-1})		IAA concentration (1µg ml^{-1})	
	2 DAI	4 DAI	2 DAI	4 DAI
Isolate 7	10.07	9.14	23.44	15.07
Isolate 9	11.1	12.4	24.4	17.9

Table 7: *In vitro* Potash solubilization activity of isolates

Isolates	Potash solubilization efficiency Zone (SI < 2mm)
Isolate 7	+
Isolate 9	+

Table 8: *In vitro* effect of isolates on rice *cv.* Gurjari

Treatments	Germination %	Shoot length (cm)	Root length (cm)	Fresh weight (mg)	Dry weight (mg)	Seed vigour index
Control	85	6.34±0.270	5.86±0.320	58.2±1.4	22.0±0.65	1037
Isolate 7	100	7.4±0.627	7.42±0.259	89.0±4.08	38.8±1.7	1482
Isolate 9	100	7.68±0.062	7.46±0.397	103.6±2.84	39.0±2.5	1504
S. Em.±		0.310	0.278	2.983	1.48	
CD at 5 %		0.956	0.858	9.193	4.585	
CV %		9.72	9.79	8.73	10.53	

Table 9: Effect of isolates and RDF on shoot and root length (cm) of rice plant at harvest

Treatment	Shoot length at 30 DATP (cm)	Shoot length at 60 DATP (cm)	Shoot length at 90 DATP (cm)	At harvest	
				Shoot length (cm)	Root length (cm)
Control	34.68±0.79	49.53±0.44	62.23±1.38	64.55±1.18	23.80±1.17
100% RDF	35.50±0.48	52.90±0.83	66.45±1.26	69.93±0.99	26.00±0.70
75% RDF + isolate 7	40.00±1.96	54.23±.096	66.45±0.52	70.23±1.77	31.15±1.70
75%RDF + isolate 9	41.87±0.57	53.13±1.20	67.55±0.44	66.80±0.41	26.10±1.59
S.Em.±	1.120	0.900	0.995	1.191	1.350
CD at 5 %	3.451	2.773	3.067	3.669	4.161
CV %	5.89	3.43	3.03	3.51	10.09

Table 10: Effect of isolates and RDF on number of tillers, plant shoot and root weight (g) of rice *cv.* Gurjari

Treatment	Number of tillers	Plant fresh weight(g)	Plant dry weight(g)
Control	4.10±0.13	37.43±2.30	14.41±0.35
100% RDF	5.03±0.23	42.65±0.46	16.00±1.60
75% RDF+ isolate 7	5.05±0.15	43.61±0.81	19.51±1.65
75%RDF+ isolate 9	4.23±0.26	39.17±1.32	18.47±0.93
S.Em.±	0.200	1.407	0.830
CD at 5 %	0.617	4.335	2.556
CV %	8.71	6.71	10.00

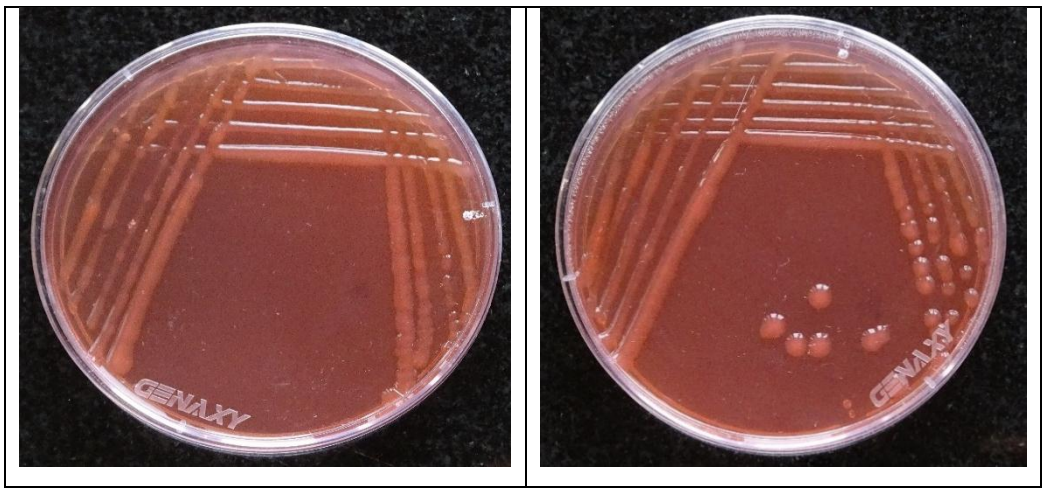

Plate 1: Colonies of isolates on YEMA medium

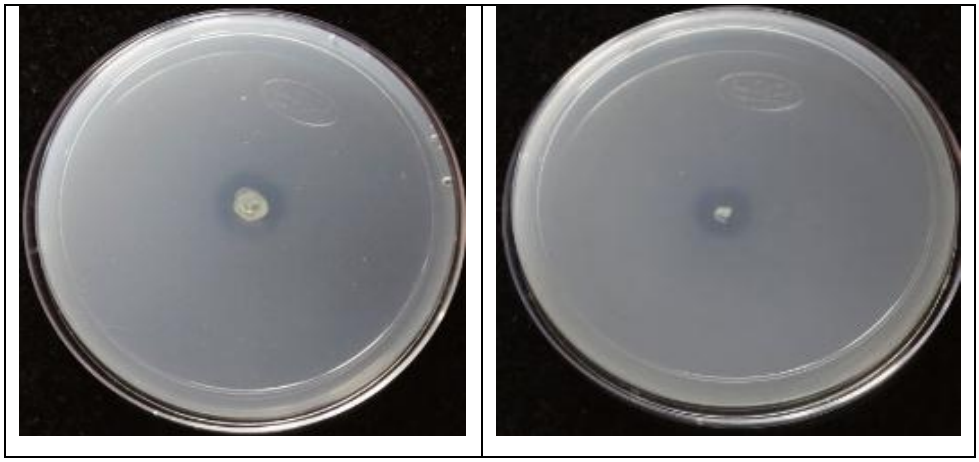

Plate 2: Phosphate solubilization by isolate7 and 9 showing zone on solid medium

DISCUSSION

Rhizobium is generally found mainly in association with the leguminous plants but here free living *Rhizobium* was isolated from the rhizosphere of rice and it showed significant increase in growth parameters of rice[14].

Rhizobium was successfully isolated on YEMA medium similar culture growth was reported on YEMA medium[24]. Wide pH range was tolerated by both the isolates similar results of pH tolerance were obtained by both native and exotic strains of Rhizobium in the laboratory conditions where no growth was observed below pH 5 among pH levels 4 t0 7[25].

For distinguishing the inoculated rhizobial strains from indigenous rhizobia present in soil survival, persistence and competitiveness are the major factors determining their successful use as inoculants[26]. To determine these properties, use of intrinsic antibiotics resistance is the simplest and most commonly used method for strain identification[27] further isolates having multiple antibiotic resistance have greater advantage in establishing themselves as inocula in natural soil conditions as well as in any ecological niche.

Biochemical characterization of rhizospheric bacterial isolates 7 and 9 were matching to that of genus Rhizobium according to[28] which was supported by[24] who had obtained positive results for Vogesproskauer's, citrate, maltose, galactose and arabinose utilization. Nitrogen fixation ability of the isolates was detected similarly it was detected by[29] ranging from 5.45 to 9.26 mg N g^{-1} substrate from 5 days old *Azotobacter* culture.

The present findings established the phosphate solubilization as an additional benefit of diazotrophic nitrogen fixer bacterial isolates and thereby, they can also improve the availability of phosphorous in rice rhizosphere. Similar findings were reported by[30] where in the maximum phosphate solubilizing rate (61.87 µg mL^{-1}) at 12 days was obtained by rhizobial isolate UPMR3l.

The present results showed that inoculation of native diazotrophic isolates can improve plant growth by increasing nutrient uptake by root system. Accumulation of IAA enhance morphological development in plant including cell elongation as well as prevention of senescence which ultimately results in root growth promotion and thereby increase nutrient and water uptake capacity of plant[31].

In presence of tryptophan, the microbes release greater quantities of IAA and related compounds[32]. Tryptophan may induced the synthesis of enzymes as functional substrate of IAA. Similarly, Joshi and Shekhawat, (2015) obtained maximum production of IAA at 3 mg l^{-1} of tryptophan concentration by two isolates identified as *Rhizobium* and *Azotobacter* isolated from rice rhizosphere soil. The bacteria might produce acids, alkalis or chelants which enhance the release of elements from potassium bearing minerals such as mica[30].

Plant fresh and dry weight was found higher than that of control it may be due to the formation and development of numerous root branching; root hairs and better primary and secondary lateral roots which may have increased the nutrient uptake capacity of roots[34].

The results of [35] supports our results where they found that inoculation of strains of *R. phaseoli* and *R. leguminosarum* in rice cultivar "Super Basmati" have increased the growth parameters i.e. number of tiller (46%); paddy yield (43%), plant biomass (18%), straw dry weight (45%) and 1000-grain (25%) and plant height (28%).

CONCLUSION

Rhizobium bacteria can significantly promote seed emergence and seedling attributes which benefits the early seedling establishment influence rice plant growth with reduction of 25 % chemical fertilizers of rice cultivar Gurjari. Moreover, inoculation of *Rhizobium* isolates *via* seedling dip makes its application suitable for farmers growing transplanted paddy.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors contribution:

A. Patel designed and conducted the experiment and prepared the manuscript with the assistance of M. Mankad; R. V. Vyas and Subhash N., mentor the whole experiment, checked and corrected the manuscript.

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