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Evaluation of plant growth promoting traits of Soybean (*Glycine max* L.) root nodulating *Rhizobium* and it's characterization

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ABSTRACT

Present study conducted with an aim of isolating and identifying rhizobial strains associated with soybean root nodules and assessing their plant growth-promoting properties. Total 131 isolates were screened for their biotechnological potential for promoting plant growth. From which, nine isolates were chosen for further analysis based on their in vitro plant growth-promoting activities including indole-3-acetic acid production, mineral solubilization, nitrogen fixation, hydrogen cyanide (HCN) production. All the screened isolates showed positive test for N_2-fixation. Among the isolates tested for mineral solubilization, strain BJ1 and BJ8 efficiently solubilized phosphate as they showed maximum solubilization index (6 mm). BJ5 was found efficient for potassium solubilization and BJ4 and BJ5 for Zinc solubilization. Highest IAA production was exhibited by strain BJ7 (110.9 µg/ml) and BJ9 (90 µg/ml) and lowest by BJ1 (10 µg/ml).

Key words: *Soybean, Glycine max, Rhizobium, bioinoculants, PGP Traits.*

Introduction

The increasing global population necessitates increased agricultural productivity, and the future agricultural system must be intensive and sustainable to meet the increasing population's needs. But the soil deficiencies hinder plant growth, leading to increased reliance on chemical fertilizers for higher crop yields (Glick, 2012; Etesami *et al.*, 2017). Indiscriminate use of chemical fertilizers, particularly nitrogenous and phosphorus, has led to substantial pollution of soil, air and water (*Gupta et al.*, 2015). Over-exploitation of fertilizers resulting in ever-less crop productivity per unit area, decline in fertility and erosion that leads to the irreversible deleterious environmental consequences that may be impossible to reverse. In such circumstances, crop productivity may not be significantly improved even by heavy applications of synthetic fertilizers (Masciarelli *et al.*, 2014). Beneficial soil bacteria that competitively colonized plant roots and stimulated plant growth and thereby reducing the circumstantiality of plant diseases termed as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1981;).

PGP bacteria can fix atmospheric nitrogen, providing plant hosts with necessary nitrogen (Chanway and Woll, 1991); they produce siderophores, which sequester iron from soil (Schippers, 1988); synthesize phytohormones such as auxins (Zeffa *et al.*, 2020); affect plant growth promotion and root nodulation (Glick, 2012); cytokinins and gibberellins which can enhance or regulate various stages of plant growth (Zeffa *et al.*, 2020); may have mechanisms for the solubilization of minerals, conversion of insoluble form of phosphates into soluble forms (Linu *et al.*, 2009) and solubilization of K-bearing minerals (Etesami *et al.,* 2017).

Soybean (*Glycine max* L.) is most cultivated legumes worldwide (Ciampitti and Salvagiotti, 2018). Being legume, it has a ability to form agronomically and ecologically important symbiotic association with nitrogen fixing rhizobia (micro-symbiont) (Datta *et al.,* 2015). Genus *Bradyrhizobium* (Jordan, 1982) is considered the main rhizobial genus that establishes a symbiotic association with soybean (Sugiyama *et al.*, 2015).

Symbiotic nitrogen fixation (SNF) is a mutual beneficial interaction, root nodules (macro-symbiont) provide microaerobic condition where rhizobia can fix atmospheric nitrogen into ammonia and that exchange for plant photosynthates, which provides about 60-70% of total nitrogen requirement of the plant (Jaiswal *et al.*, 2016; Sawale and Phirke, 2022). The amount of N_2-fixed (kg ha^{-1}) by soybean have been reported up to 450 kg N_2 ha^{-1} (Unkovich and Pate, 2000), reduces the dependence of farmers on inorganic nitrogenous fertilizers in crop cultivation (Collino *et al.,* 2015).

The objective of this study was to isolate and characterised indigenous rhizobial strains which are already adapted to local conditions and that holds potential to be included in inoculant formulations for locally accepted verities of soybean.

Materials and Methods

Isolation of rhizobia from soybean root nodules

Soybean plants were collected for nodule samples. The plants were uprooted and preserved at 4-7°C. Nodules were separated, cleaned, disinfected, and crushed into saline solution to release rhizobial bacteroids. Purified bacterial isolates were obtained on Yeast extract mannitol agar (YEMA) with Congo red [composition in % (w/v): Yeast extract 0.1, Mannitol 1, K_2HPO_4 0.05, $MgSO_4$ 0.02, NaCl 0.01, Congo red 0.0025, Agar 1.5, pH 6.8 ± 0.2] incubated at 28 ± 2°C for 2-7 days. (Maruekarajtinpleng *et al.,* 2012). Growth sub-cultured and preserved for longer time by covering slant surfaces by sterile glycerol (Pervin *et al.,* 2017).

Determination of cultural, morphological and biochemical traits of isolates

Cultural characteristics like size, shape, diameter, colour, transparency, consistency, mucous production, were recorded and compared with standards of Bergey's Manual of Determinative Bacteriology (Halt *et al.,* 1994). Morphological traits of strains were observed by micrometry, simple, Gram's, spore, capsular, flagellar staining and motility techniques. Rhizobial strains were subjected for different biochemical characterization. All the biochemical tests were performed in triplicate by using following standard methods and results were recorded. Thus, catalase test (Graham and Parker, 1964), oxidase test (Kovaks, 1956), citrate utilization (Koser, 1923), gelatine liquefaction (Sadowsky *et al.,* 1983), H_2S test (Zobell and Feltham 1934), Ammonia production (Phirke, 2014), urea hydrolysis (Christensen, 1946), Methyl red and Voges-Proskauer test (PohIman, 1931), Potassium hydroxide solubility (Suslow *et al.*, 1982), and starch hydrolysis (Oliveira *et al.,* 2007).

Determination of PGP characteristics of isolated *Rhizobia*

Plant growth-promoting activities of rhizobial strains were assessed qualitatively (plate-based assay) and quantitatively (liquid assay).

Nitrogen fixation: Rhizobial strain's ability to fix atmospheric nitrogen was assessed by inoculating them on nitrogen-free broth medium (NFB), without addition of nitrogen source. At 30°C, the plates were incubated for 24-48 h and observed for bacterial growth as qualitative evidence of the atmospheric nitrogen fixation (Mozumder *et al.,* 2022).

P-Solubilization: A single well isolated colony of *Rhizobium* was spotted on Pikovskaya's medium and incubated at 28 ± 2 °C and observed until formation of transparent "halos" around each colony up to for 7 days (Wang *et al.,* 2017).

Potassium solubilization: A loopful of fresh growth of the rhizobial strains was spotted on Aleksandrov medium and kept at a temperature of 28 ± 2 °C until the formation of translucent "halos" around each colonies, which is due to the solubilization of K source (Parmar and Sindhu, 2013).

Zinc solubilization: Zinc solubilization examined by spot inoculation on Bunt and Rovira medium [composition (g/L): glucose (10), ammonium sulphate (1), potassium chloride (0.2), di-potassium hydrogen phosphate (0.1), magnesium sulphate (0.2), agar (15), Zn source (Zinc oxide) (0.1%) and pH (7.0 ± 0.2), incubated at 28 ± 2°C] (Javed *et al.,* 2018).

Detection and quantification of indole-3-acetic (IAA) producing activity: IAA production detected by the method of Gordon and Weber (1951). Rhizobial strains were grown in YEM medium supplemented with L-tryptophan (0.1% w/v) and without L-tryptophan, incubated at 30°C for 96 h. After incubation, centrifuged at 3000 rpm for 20 minutes, 2 ml of cell-free supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski reagent (2 ml 0.5M $FeCl_3$ solution mixed in 50 ml of 35% (v/v) perchloric acid). For quantification, Spectra was taken at 540 nm and the level of IAA produced was estimated using a standard graph. The experiment was duplicated for each isolate.

Hydrogen cyanide (HCN) production test: Rhizobial strains were streaked on YEM agar supplemented with glycine (4.4 g L^{-1}). An autoclaved filter paper (Whatmann no. 1) was soaked with picric acid (0.5%) and Na_2CO_3 (2%) solution and placed in the top of the petri dishes. The dishes were sealed with parafilm, then packed with black paper and incubated for 28 ± 2°C for 4 days (Lorck, 1948).

Results and Discussion

Determination of cultural, morphological and biochemical traits of isolates

In this study, soybean root nodulating rhizobia which bears multiple beneficial traits for plants were attempted to isolate from soybean root nodules and rhizospheric soil of Amravati region, Maharashtra. Hundreds of isolates, isolated on Congo red YEMA plates were rigorously studied to get standard characteristics of *Rhizobium* in accordance with Bergey's Manual. Selected isolates showed cultural and morphological characteristics similar to genus *Rhizobium* which indicated that the isolated strains belonged to genus *Rhizobium* (Table 1), these were in accordance with the earlier findings of Gachande and Khansole (2011) and Graham and Parker (1964).

Morphologically, both the fast and slow-growing soybean rhizobia were Gram negative, short rods, motile, and non-spore forming. Biochemically, oxidase, catalase positive and Indole, methyl red, Voges Proskauer negative were considered as putative rhizobial isolates. Our results (Table 1) were in accordance with previous findings Graham and Parker (1964) and Deshmukh *et al.* (2019). A total of 131 putative rhizobial isolates were obtained. Out of those, only nine showed positive reaction for most of PGP traits, that were selected.

Carbohydrate fermentation performed using Six different carbon sources, i.e. fructose, sucrose, mannose, galactose, mannitol and lactose. Results of carbohydrate utilization tests (Table 2) showed that the fast growers utilized a greater assortment of carbohydrates than the slow growers (Sadowsky *et al.*, 1983).

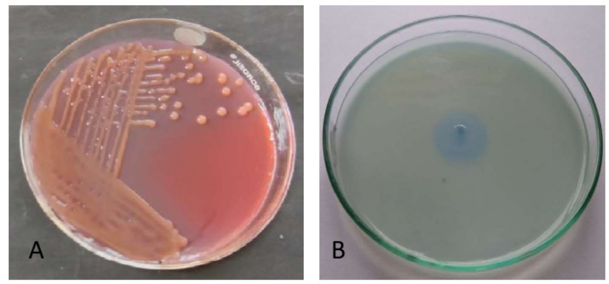

Fig. 1. (A) Colonies of *Rhizobium* on CR-YEMA media, (B) Growth of *Rhizobium* on NFB medium.

Screening of bacteria for plant growth- promoting properties

Plant growth-promoting activities of rhizobial strains were assessed qualitatively (plate-based assay) and quantitatively (liquid assay) depicted in Table 3.

Ability of rhizobial strains to fix atmospheric nitrogen (N_2) was determined by inoculating rhizobial strains on NFb medium. All the nine isolate tested showed positive reaction for N_2-fixation. As they gave blue colour reaction on NFB medium (Figure 1). Our findings were in support to previous findings of Mozumder *et al.* (2022).

Detection and quantification of IAA production: Salkowski reagent was used to determine IAA production. After the addition of reagent, colour development was started within minutes in a tube containing highest IAA concentration and continued to increase in intensity for a period of 30 min. In the quantitative measurement, the maximum value of IAA was obtained by bacterial strain BJ4 as it produced 70.1µg/ml and BJ7 produced 110.1µg/ml. Lowest was produced by BJ1 that is 10.3 µg/ml, moderate IAA production was by bacterial strain BJ4- 70.1µg/ml, BJ6- 50.8µg/ml, BJ8- 30.1µg/ml, The results showed that L-tryptophan stimulated IAA production and strain BJ7 was found to be most efficient IAA producing soybean root nodulating

Table 1. Cultural, morphological and Biochemical characteristics of *Rhizobium*

Isolates No.	Colony characters on CR-YEMA	Morphological characters				Biochemical characters								
		Gram's character	Motility	Spore formation	Capsule formation	1	2	3	4	5	6	7	8	9
BJ-1	Circular, 2-3 mm, whitish pink, slimy, translucent	Gram negative, short rod	Motile	Non-sporulating	Capsulated	-	+	-	+	+	+	+	+	+
BJ-2	Small, Circular, whitish pink, slimy, opaque, convex	Gram negative, thick rod	Motile	Non-sporulating	Capsulated	-	+	-	+	+	+	+	+	+
BJ-3	Circular, size 2-3 mm, whitish pink, slimy, translucent, convex	Gram negative, thick rod	Motile	Non-sporulating	Capsulated	-	+	-	+	+	+	+	-	+
BJ-4	Circular, size 2-3 mm, whitish pink, slimy, translucent, convex	Gram negative, short rod	Motile	Non-sporulating	Capsulated	-	+	-	+	+	+	+	+	+
BJ-5	Circular, size 2-3 mm, whitish pink, slimy, translucent, convex	Gram negative, short rod	Motile	Non-sporulating	Capsulated	-	+	-	+	-	+	+	+	+
BJ-6	Circular, size 2-3 mm, whitish pink, slimy, translucent	Gram negative, short rod	Motile	Non-sporulating	Capsulated	-	+	-	+	+	+	+	-	+
BJ-7	Circular, size 2-4 mm, pink, slimy, translucent, convex	Gram negative, short rod	Motile	Non-sporulating	Capsulated	-	+	-	+	-	+	+	-	+
BJ-8	Circular, size 2-4 mm, whitish pink, slimy, translucent	Gram negative, short rod	Motile	Non-sporulating	Capsulated	-	+	-	+	+	+	+	+	+
BJ-9	Circular, size 2-3 mm, whitish pink, slimy, translucent, convex	Gram negative, short rod	Motile	Non-sporulating	Capsulated	-	+	-	+	+	+	+	-	+

1-MRVP test, 2- Citrate utilization test, 3-Indole test, 4- Oxidase test, 5- Starch hydrolysis test, 6-KOH solubility test, 7- Catalase test, 8- Gelatine hydrolysis test, 9- Nitrate reduction test. (+): Positive test, (-): Negative test.

Rhizobia (Table 4).

As the L-tryptophan is a precursor for indole-3-acetic acid, YEM media with L-tryptophan was more suitable for IAA production as compared with YEM without L-tryptophan. Our findings were in support to previous findings of Mohite (2013).

Determination of mineral solubilization activity: The solubilization of insoluble phosphate was examined by spot inoculation. A clear zone around each spot during 1 week at 28 ± 2°C was considered positive evidence of phosphate solubilization (Figure 2 A). Among the nine rhizobial strains tested, BJ1 (6.00 mm) and BJ6 (6.00 mm) showed maximum solubilization index by utilizing insoluble phosphate sources effectively in the selective medium. While strains BJ2 and BJ9 were unable to solubilise phosphate from the medium, thus did not form clear zone around colonies. A least phosphate solubilization zone was noticed with other tested strains (Table 5). In addition to phosphate, potassium and zinc were also essential nutrients required for optimum growth of plants.

Similarly, the isolates were screened to get potassium solubilization activity. Results were presented in Figure 2 C. The maximum solubilization of K was observed by bacterial isolates BJ5 and BJ8 as they showed maximum solubilization index for K-solubilization that was measured about 4.66mm and 4.00mm respectively in diameter were shown in (Table 5).

Zinc solubilization was also examined. Among the rhizobial strains tested, all the isolates showed maximum zone by utilizing insoluble zinc source effectively in the selective medium (Figure 2B). Only the strains BJ3 was unable to utilize insoluble zinc source from medium (Table 5).

Degree of mineral solubilization varied in varying levels with the incubation period and the organisms involved. When these rhizobial strain inoculated in liquid medium, in both the case of P and K solubilization, there were reduction in pH of the media, due to the production of weak organic acids by rhizobial strain that decreased the pH of medium. Similar type of results were reported earlier by Bagyalakshmi *et al.* (2017). The solubilization index calculated as the ratio of the total diameter (colony + halo zone) to the colony (Patel *et al.*, 2023). The solubilization index ranged between 4.25 to 6.00, 3.40 to 4.66, and 3.71 to 4.28 for phosphate, potash and zinc-solubilizing bacteria, respectively (Table 5).

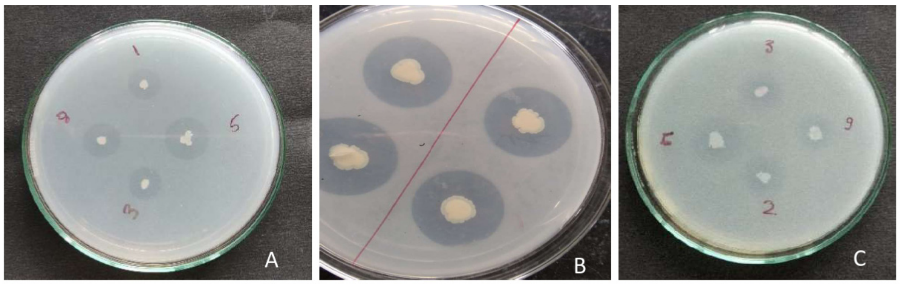

Fig. 2. Mineral solubilization (A) Phosphate solubilization by *Rhizobial* isolates on Pikovskaya's agar, (B) Zinc solubilization by rhizobial isolates, and (C) Potash solubilization by rhizobial isolates on Aleksandrow agar.

Table 2. Sugar utilization by fast and slow-growing soybean rhizobia

Isolates	Carbohydrates												Colour produced on BTB-YEMA
	Sucrose		Galactose		Mannose		Lactose		Fructose		Mannitol		
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	
BJ-1	+	+	+	-	+	+	-	-	+	+	+	+	Yellow
BJ-2	+	-	+	-	+	-	-	-	+	-	-	-	Blue
BJ-3	-	-	-	-	-	-	-	-	-	-	+	+	Blue
BJ-4	-	-	+	+	+	+	-	-	+	+	+	-	Blue
BJ-5	-	-	+	-	+	-	+	-	+	+	+	+	Yellow
BJ-6	+	+	+	+	+	+	+	-	-	-	+	-	Yellow
BJ-7	+	-	-	-	-	-	-	-	+	+	+	+	Yellow
BJ-8	-	-	+	-	+	+	+	-	-	-	+	-	Blue
BJ-9	+	+	+	+	+	+	-	-	+	-	+	+	Yellow

(+): Positive test, (-): Negative test.

Table 3. Determination of PGP traits of selected bacterial isolates

Isolates	N_2-fixing activity	P-solubilization activity	K-solubilization activity	Zinc solubilisation	IAA production	HCN production	NH_3 production	Total PGPR Traits
BJ-1	+	+++	-	+++	+	-	+	5
BJ-2	+	-	+	+	+	+	+	6
BJ-3	+	++	+	-	+	+	++	6
BJ-4	+	+	++	++	+++	+	+	7
BJ-5	+	++	+++	++	+	-	+	6
BJ-6	+	+	+	+	++	+	+	7
BJ-7	+	++	-	+	+++	+	+	6
BJ-8	+	+++	+	+	+	+	+	7
BJ-9	+	-	+	+	+++	-	++	5

+ or - sign shows the positive or negative reactions and number of + sign denoted the intensity of PGP traits.

Table 4. Production of IAA by bacterial isolates

Isolates	BJ-1	BJ-2	BJ-3	BJ-4	BJ-5	BJ-6	BJ-7	BJ-8	BJ-9
IAA (µg/ml) (W/O Tryptophan)	10.1	10.3	9.9	40.1	20.2	40.8	20.6	20.2	10.6
IAA (µg/ml) (W Tryptophan)	10.3	20.9	20.2	70.1	20.2	50.8	110.9	30.1	90.1

Table 5. Mineral solubilization by rhizobial isolates

	Phosphate solubilization by isolates			Potassium solubilization by isolates			Zinc solubilization by isolates		
Isolate	Colony diameter (mm)	Zone diameter (mm)	Solubilization index (SI)	Colony diameter (mm)	Zone diameter (mm)	Solubilization index (SI)	Colony diameter (mm)	Zone diameter (mm)	Solubilization index (SI)
BJ1	2	10	6.00	-	-	-	7	23	4.28
BJ2	-	-	-	4	11	3.75	9	22	3.44
BJ3	3	12	5.00	4	11	3.75	-	-	-
BJ4	4	12	4.00	6	15	3.50	8	24	4.00
BJ5	4	15	4.75	3	11	4.66	7	21	4.00
BJ6	3	9	4.00	4	10	3.50	9	25	3.77
BJ7	4	13	4.25	-	-	-	9	26	3.88
BJ8	3	15	6.00	3	9	4.00	8	23	3.87
BJ9	-	-	-	5	12	3.40	-	-	-

(-): absence of solubilisation activity

Determination of hydrogen cyanide (HCN) production: Microbial cyanides plays a role in suppression of many plant diseases. Although its role in plant disease suppression firmly established, but it may be involved in the induction of intrinsic plant resistance (Devi and Thakur, 2018). In the present study, nine soybean root nodulating rhizobial strains were screened qualitatively to produce HCN on nutrient agar plates supplemented with 0.44% glycine. Rhizobial strains BJ2, BJ3, BJ4, BJ6, BJ7 and BJ8 showed positive reaction for HCN production (Table 3). As they turned the colour of filter paper from yellow to red-brown, rest of three were did not show HCN production.

Conclusion

From this study, we have isolated nine efficient soybean root nodulating native rhizobial strains from various locations of Amravati, Maharashtra. Cultural, morphological and biochemical testing of the isolates showed that all isolates characterized belonged to genus *Rhizobium*, included in the fast and slow-growing groups, were able to fix atmospheric nitrogen, produced IAA and had the ability to solubilize minerals like phosphate, potash and zinc. The results of our isolates consistently showed high N_2-fixation efficiency, highlight their importance as PGP *Rhizobium* and recommend these strains as biofertilizers. Further studies of isolates would be performed to evaluate their impact on locally accepted varieties of soybean under field conditions.

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Conflict of Interest- None

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