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Isolation and Characterization of Nitrogen Fixing Bacteria from Babchi (*Psorolea corylifolia* L.) and Testing them for Plant Growth Promotion Traits *in vitro*

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A B S T R A C T

Two isolates of *Rhizobium* were isolated from Babchi (*Psorolea corylifolia*) root nodules, which showed positive tests for catalase test, oxidase test, nitrate reduction test and starch hydrolysis while negative test for methyl red test, Voges-Proskauer test, urease test, gelatin lignification test, methylene blue test, urease test, gelatin liquification test, methylene blue test, lactose test, citrate utilization test and hydrogen sulphide production test. The isolate PCRI-1 and PCRI-2 exhibited a great variation in N fixation by of 24.35 mg/g and 20.70 mg/g of sucrose consumed, respectively. The amount of IAA produced by these isolates was 37.73 µg/ml and 32.40 µg/ml respectively. These isolates showed variation in PO_4 solubilization efficiency in the range of 19.1 to 17.8 per cent, with solubilization zone of 6.2 mm and 4.2 mm, respectively. Its characterization and testing of plant growth promotion traits is the first attempt from the state of Maharashtra, from root nodules of Babchi.

Introduction

Psoralea corylifolia (Bakuchi), which belongs to the Leguminosae family, is a medicinal plant of immense biological importance due to its magical effects against several skin disease such as psoriasis, leukoderma, and leprosy. This plant is also pharmacologically studied for its chemoprotective, antioxidant, antimicrobial, and anti-inflammatory properties (Khushboo *et al.*, 2010). The seed powder and paste are used in indigenous medicine as laxative, aphrodisiac, anthelmintic, diuretic stomachic, stimulant and diaphoretic in febrile conditions (Ambreen *et al.*, 2013). The root nodulating bacteria, also known as plant growth promoting rhizobacteria, are in symbiotic association with leguminous plants. These symbiotic bacteria along with the legume give maximum contribution of global nitrogen fixation. Rhizobiaceae family contains six genera viz., *Rhizobium*, *Sinorbizobium*, *MesoRhizobium*, *AlloRhizobium*, *AzoRhizobium* and *BradyRhizobium* (Okazaki *et al.*, 2004). Rhizobiacteria effectively colonize plant root and increases plant growth by production of various plant growth hormones, B-solubilizing

activity, N_2 fixation and biological control activity (Deshwal *et al.*, 2011). The *Rhizobium* legume symbiosis in superior to other nitrogen fixing systems due to its high potential (Sanaa and Fawziah, 2005). Dispersion of host plants to new geographical locations might serve as a major source for these new rhizobia species. Only 57 % of 650 genera of leguminous plants have been studied for nodulation. Exploration of large number of legume species can potentially lead to the identification of many more rhizobial species. Therefore, identification and exploration of such potential rhizobia with plant growth promoting properties will be useful for sustainable agriculture.

Materials and Methods

Collection of root nodules and isolation of *Rhizobium*

For the isolation of nitrogen fixing *Rhizobium* the root-nodules of Babchi plant was collected from AICRP on Medicinal, Aromatic Plants and Betelvine Project, M.P.K.V., Rahuri. The collected nodules were washed under tap water to remove adhering soil particles. The healthy, fresh, plump and pinkish root nodules were selected and surface sterilized with 95 per cent ethanol for 5-10 sec., followed by 3 % (v/v) sodium hypochloride solution for 2-4 min and washed with sterile distilled water for 3-4 times. The selected nodules were crushed with the help of sterilized glass rod to obtain a milky suspension of bacteriods. these were streaked on yeast extract mannitol agar (YEMA pH 7.0) media plantes and incubated at 3°C (Aneja, 2003). After two days of incubation, colonies of nitrogen fixing bacteria were obtained.

Confirmation of nitrogen fixing bacteria as *Rhizobium*

All the six bacterial isolates obtained from the rot nodules of Babchi were tested for their growth on comes red yeast extract manitol agar (RYEMA), Lactose agar (LA) for their further an formation as *Rhizobium* isolates. As *Rhizobium* colonies did not absorb Congo red colour while common contaminants absorb Congo red colour in this way *Rhizobium* was differentiated. On the lactose agar medium presence of yellow colour confirms the presence of common contaminant *Agrobacterium* sp. white absorbance of yellow colour indicates presence of *Rhizobium* sp. as these *Rhizobium* does not convert lactose to 3 leaf lactose. This is one of ways to differentiate *Rhizobium* and other common contaminants.

Characterization of *Rhizobium* isolates

The *Rhizobium* isolates were characterized morphologically, physiologically and biochemically, morphological characterization includes colony colour shape, diameter, margin, elevation and consistency as well as shape of bacteria, mortility and gram reaction as pe standard procedure of Aneja (2003). Physiological characterization includes growth at different range of temperature and pH value, biochemical characterization includes methyl red-Voges-Proskauer test, catalase test, oxidase test, urease test, nitrate reduction test, starch hydrolysis test, gelatin liquification test, methylene blue test, lactose test, citrate utilization test and hydrogen sulphide production test.

Efficacy of *Rhizobium* isolates for plant growth promotion traits

The plant growth promotion traits viz., nitrogen fixation, phosphate solubilization and indole acetic acid production were also tested for the *Rhizobium* isolates.

Nitrogen fixation: The test *Rhizobium* isolates were screened for their nitrogen fixation ability on the basis of per mg of sucrose consumed and the amount of nitrogen fixed

was estimated by Micro-Kjeldhal's method (Jackson, 1976).

Phosphate solubilization: The plates were prepared with Pikovaskaya's medium. The test *Rhizobium* isolates was streaked on the media of plates and incubated in an incubator at 28°C for 7-8 days. The presence of clearing zone around the test *Rhizobium* isolates was used as indicator for positive phosphate solubilization.

Indole acetic acid (IAA) production: The test *Rhizobium* isolates were grown in 25 ml of nutrient broth amended with 50 µ/ml of treyptophan in 100 ml flank for 24 hrs at 28°C on rotary shaker. After completion of incubation period test cultures were harvested by centrifuged at 10,000 rpm for 25 min at 4°C. Two-three drops of arthophosphoric acid was added to 2 ml of cell free supernatant and the development of colour was observed. The presence of a pink colour indicates positive reaction for middle acetic acid production.

Results and Discussion

The six bacteria isolates were tested for the growth on Congo red yeast extract, mannitol agar and lactose agar mediums. Out of six isolates, isolate-1 (PCRI-1) and isolate-2 (PCRI-2) did not absorb the Congo red colour and maintain their original milky white colour when grown on CRYEMA medium plates (Table 1). While, isolate-3 (PCRI-3), isolates (PCRI-4), isolate-5 (PCRI-5) and isolate-6 (PCRI-6) absorbed red colour of Congo when grown on CRYEMA medium plates. When these six isolates were grown on lactose agar, isolate-1 (PCRI-1) and isolate-2 (PCRI-2) did not shown any yellow colour of colonies while isolate-3 (PCRI-3), isolate-4 (PCRI-4), isolate-5 (PCRI-5) and isolate-6 (PCRI-6) shown yellow colour and around the colonies of these isolates. It clearly initiated that isolate-1 (PCRI-1) and isolate-2 (PCRI-2) were of *Rhizobium* sp. while rest of the isolates no 3 (PCRI-2), 4 (PCRI-4), 5 (PCRI-5) and 6 (PCRI-6) were contaminant and may be *Agrobacterium* sp. Thus, finally two isolates were confirmed to be of *Rhizobium* genus.

Morphological characterization

The *Rhizobium* isolates PCRI-1 and PCRI-2 were characterized morphologically on YEMA media plates (Table 2).

The *Rhizobium* isolate PCRI-1 was white translucent in colour, circular in shape and have colony diameter of 2-3 mm with entire margin, raised (convex) and sticky mucold in consistency; whereas, isolate PCRI-2 was milky while translucent, circular in shape with 2-4 mm in colony diameter, locate margin and convex in elevation with sticky mucold consistency. Both the isolates were rod shaped, motile and gram negative in reactions.

Physiological characterization

The *Rhizobium* isolates PCRI-1 and PCRI-2 were tested against different temperature ranging from 15°C to 45°C (Table 3). The isolate PCRI-1 required optimum temperature of 35-40°C for its growth, while the isolate PCRI-2 required optimum temperature of 30-35°C and both were merophilic in nature. Both the isolates were also tested against different YEMA media with variant pH values ranging from 4.5 to 8.5 pH. The optimum pH ranges in between 6.5 to 7.5, which indicates that they were neutral phallic in nature.

Biochemical characterization

Biochemical characterization of the *Rhizobium* isolates were carried out for Indole test, Methyl red and Voges-Proskauer test (MRVP test), Catalase test, Oxidase test, Nitrate reduction test, Urease test, Starch hydrolysis, Gelatin liquification, Methylene blue test, Lactose assay, Citrate utilization test and H_2S production test for differentiation of the isolated strains of *Rhizobium* (Table 4).

Table.1 Confirmation tests for bacterial isolates

Bacterial isolates	Growth on differential media		Absorption of red colour on CRYRMA media	Absorption of yellow colour on lactose agar media	Possible organisms
	CRYRMA	LA			
PCRI-1	++	--	-ve	-ve	*Rhizobium* sp.
PCRI-2	++	--	-ve	-ve	*Rhizobium* sp.
PCRI-3	++	++	+ve	+ve	*Agrobacterium* sp.
PCRI-4	++	++	+ve	+ve	*Agrobacterium* sp.
PCRI-5	++	++	+ve	+ve	*Agrobacterium* sp.
PCRI-6	++	++	+ve	+ve	*Agrobacterium* sp.

++ = Full growth = No growth
Presence of red colour = Positive test (+ve)
Absence of red colour = Negative test (-ve)
Presence of yellow colour = Positive test (+ve)
Absence of yellow colour = Negative test (-ve)

Table.2 Morphological characterization of *Rhizobium* isolates

Sr. No.	*Rhizobium* isolate	Colony morphology						Bacterial morphology		
		Colour	Shape	Diameter (mm)	Margin	Elevation	Consistency	Shape	Motility	Gram reaction
1.	PCRI-1	White and translucent	Circular	2-3	Entire, Smooth	Convex	Sticky and Mucoid	Rod	Motile	Negative
2.	PCRI-2	Milky white and translucent	Circular	2-4	Lobate smooth spreading type	Convex	Sticky and Mucoid	Rod	Motile	Negative

Table.3 Effect of different temperature on the growth of *Rhizobium* isolates

Sr. No.	Isolate no.	Temperature (°C)							Optimum temperature requirement	Classification
		15	20	25	30	35	40	45		
1	PCRI-1	+	+	+	+	++	++	+	35-40°C	Mesophile
2	PCRI-2	+	+	+	++	++	+	--	30-35°C	Mesophile

++: Full growth, +: Scanty growth, --: No growth

Table.4 Biochemical characterization of *Rhizobium* isolates PCRI-1

Sr. No.	Biochemical test	PCRI-1	PCRI-2
1.	Indole test	-	-
2.	Methyl red test	-	-
3.	Voges-Proskauer test	-	-
4.	Catalase test	+	+
5.	Oxidase test	+	+
6.	Nitrate reduction test	+	+
7.	Urease test	-	-
8.	Starch hydrolysis test	+	+
9.	Gelatin liquification test	-	-
10.	Methylene blue test	-	-
11.	Lactose assay	-	-
12.	Citrate utilization test	-	-
13.	H_2S production test	-	-

+: Positive, -: Negative

The *Rhizobium* isolate PCRI-1 and PCRI-2 showed positive reaction against catalase, oxidase, nitrate reduction and starch hydrolysis test while negative reactions for rest of the tests conducted. On the basis of these characterization these isolates tentatively belongs to *Rhizobium leguminosarum* and which further needs to be further confirmed by molecular techniques.

Plant growth promotion traits of *Rhizobium* isolates

The *Rhizobium* isolates PCRI-1 and PCRI-2 were also tested for their plant growth promotion traits viz., nitrogen fixation, phosphate solubilization, indole acetic production *in vitro*.

Nitrogen fixation

The amount of nitrogen fixed by *Rhizobium* isolates during incubation in broth culture indicated that the *Rhizobium* isolate PCRI-1 fixed nitrogen 24.35 mg/g of sucrose consumed whereas isolate PCRI-2 fixed nitrogen 20.70 mg/g of sucrose consumed. It clearly indicates that both isolates possessed nitrogen fixation capacity with little variation among them.

Phosphate solubilization

The per cent PO_4 solubilization by *Rhizobium* isolates PCRI-1 and PCRI-2 during incubation in both cultures was 19.1 and 17.8 per cent, respectively with solubilization of

6.2 mm and 4.2 mm, respectively. It clearly indicates that both the isolates exhibited phosphate solubilization ability.

Indole acetic acid production

The *Rhizobium* isolates PCRI-1 and PCRI-2 when tested for their indole acetic production test indicated that both the isolates have ability of indole acetic acid production. The isolate PCRI-1 production 37.73 µg/ml of IAA whereas isolate PCRI-2 produced 32.40 µg/ml of IAA. These results clearly indicated that both the isolates possessed plant growth promotion activity to namely, nitrogen fixation, phosphose solubilization and indole acetic acid production.

This is the first attempt from the state of Maharashtra to isolate *Rhizobium* from root nodules of Babchi its characterization and testing the plant growth promotion traits.

Out of the six isolates, from the root nodules of Babchi, PCRI-1 and PCRI-2 were unable to grow on Lactose agar media and rest of isolates viz. isolate PCRI-3, PCRI-4, PCRI-5 and PCRI-6 showed full growth. Results also showed that on CRYEMA media isolate1 and 2 did not absorb the Congo red colour which clearly indicated that these isolates were of *Rhizobium*. Similar results were earlier reported by Deshwal and Chaubey (2014) and Shetta *et al.,* (2011). Both bacterial isolate viz., PCRI- 1 and PCRI- 2 were motile, Gram negative and rod shaped. The morphological characterization indicated that isolates PCRI-1 was dull white in colour, entire in margin, convex in elevation and circular in form. The isolate PCRI-2 was whitish in colour, lobate in margin, elevated in elevation and irregular and spreading type of form. The isolate PCRI-1 and PCRI-2 showed pin head type small sized colonies on CRYEMA and secreted high mucilaginous compound around the colonies as just mention elsewhere (Arora *et al.,* 2001; Deshwal *et al.,* 2014). The *Rhizobium* isolates showed optimum temperature requirement of 35-40°C and the optimum pH of 6.5-7.5. Similar observation was made by Baoling *et al.,* (2007), Deora *et al.,* (2010) and Singh *et al.,* (2011). Both isolates showed positive results for catalase test, oxidase test, nitrate reduction test and starch hydrolysis test while negative results for indole test, MRVP test, urease test, gelatin liquification test, methylene blue test, lactose test, citrate utilization and H2S production test. Similar results were obtained by many researchers Deora and Singhal (2010), Bhattacharya *et al.,* (2013), Rajpoot and Panwar (2013), Patil *et al.,* (2014). On the basis of cultural, morphological, physiological and biochemical characterization shown by the isolate PCRI-1 and PCRI-2, they were tentatively identified as *Rhizobium leguminosarum*. However for their exact identification, molecular characterization and cross inoculation studies are necessary. However, Prabha *et al.,* (2013) made attempt to isolate *Rhizobium* from *Psorolea corylifolia* L. and isolated two species viz. *Rhizobium leguminosarum* and *Ensifer meliloti*. In our study we have isolated only one species i.e. *Rhizobium leguminosarum.* As far as the plant growth promotion traits are concerned the *Rhizobium* isolate PCRI-1 was found to be superior in terms of indole acetic acid production, nitrogen fixation and phosphate solubiization. Satyanandam *et al.,* (2014) reported that some *Rhizobium* species were found to be involved in phosphate solubilization and this ability of phosphate solubilization by the *Rhizobium* strains could be exploited as PGPR. Gupta *et al.,* (2014) studied the isolation of rhizobactor and selection of plant growth promoting bacteria via their biochemical screening like N_2 fixation, phosphate solubilization and indole acetic acid production. On the basis of in vitro and in vivo experiment, both the isolate seems to be promising to have

beneficial effect on the plant growth promoting parameters of Babchi and the co-inoculation of these two *Rhizobium* isolates has most significant effect over control.

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