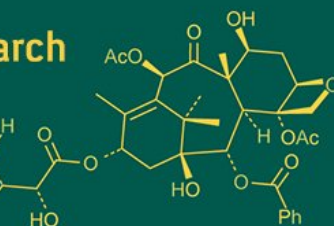
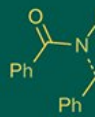


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Isolation, Characterization and Plant Growth-Promoting Potential of *Rhizobium* Isolates from Green Gram

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Abstract

Green gram is an important short-duration pulse crop in which beneficial rhizobacteria contribute to nutrient acquisition and plant growth. The present study aimed to isolate and characterize *Rhizobium* from green gram root nodules and evaluate their plant growth-promoting characteristics. Twenty root nodule samples were collected from green gram-growing fields in Raichur, Yadgiri and Kalaburagi districts of Karnataka. The isolates were obtained on yeast extract mannitol agar supplemented with Congo red and characterized based on colony morphology, Gram reaction, motility, Congo red absorption, bromothymol blue reaction, indole production, methyl red and catalase activity. Plant growth-promoting characteristics were evaluated based on indole acetic acid production, ammonia production, phosphate solubilization and nitrogen content. 20 isolates coded GRL-1 to GRL-20 were obtained. All isolates were Gram-negative, rod-shaped, motile and catalase positive and did not absorb Congo red. 11 isolates showed an acid reaction and nine showed an alkaline reaction on bromothymol blue medium, while 14 and 13 isolates were positive for indole and methyl red tests, respectively. All isolates produced indole acetic acid, ranging from 7.04 to 46.17 µg/mL. Phosphate-solubilization zones ranged from 10.83 to 15.10 mm, while nitrogen content ranged from 7.34 to 20.85 mg/g. All isolates produced ammonia. GRL-11 recorded the highest indole acetic acid production, phosphate-solubilization zone and nitrogen content. The findings indicated that the isolates possessed multiple plant growth-promoting characteristics, with GRL-11 showing the greatest potential among the isolates evaluated.

Keywords: Green gram; *Rhizobium*; indole acetic acid; phosphate solubilization; nitrogen content; plant growth promotion

Introduction

Green gram (*Vigna radiata* L.) is an important short-duration pulse crop widely cultivated in India. It is valued for its nutritional quality, adaptability to diverse agro-climatic conditions and suitability for multiple and sequential cropping systems. The crop generally matures within 60–80 days, making it suitable for intensive cropping systems. Green gram seeds contain approximately 20–25% protein, 53–67% carbohydrates and 0.7–1.9% lipids, along with vitamins, essential amino acids and minerals ^[1]. Green gram contributes to soil fertility through its symbiotic association with rhizobia, which form root nodules and contribute to biological nitrogen fixation. In addition to their symbiotic role, rhizobia may contribute to plant growth through plant growth-promoting characteristics such as phosphate solubilization and production of phytohormones, particularly indole-3-acetic acid ^[2]. The performance of rhizobial isolates may vary according to their physiological and biochemical characteristics. Native isolates associated with locally cultivated green gram may therefore provide useful candidates for plant growth promotion. Screening of isolates for multiple functional traits is important for identifying efficient strains with potential application as microbial inoculants. Therefore, the present study was undertaken to isolate and characterize *Rhizobium* from green gram root nodules and evaluate their plant growth-promoting characteristics, with emphasis on indole acetic acid production, ammonia production, phosphate solubilization and nitrogen content.

Materials and Methods

Sample collection

A total of 20 root nodule samples of green gram were collected from fields located in Raichur, Yadgiri and Kalaburagi districts of Karnataka. The isolates obtained from the root nodules were coded GRL-1 to GRL-20.

Isolation and purification of *Rhizobium* isolates

Selected green gram root nodules were surface sterilized with 0.1% mercuric chloride for 2–3 min and washed three to four times with sterile distilled water. The nodules were crushed in sterile distilled water using a surface-sterilized glass rod to prepare a suspension. Yeast extract mannitol agar supplemented with Congo red was prepared in Petri plates, and one loopful of the suspension was streaked in a zig-zag manner. The plates were incubated at 28 ± 2 °C for 3–5 days. Single white, round colonies that did not absorb Congo red were selected and purified by repeated streaking on fresh medium [3].

Morphological and biochemical characterization

Colony colour, shape and size were recorded on yeast extract mannitol agar. For Gram staining, a thin bacterial smear was prepared and heat fixed. The smear was stained with crystal violet for 1 min, treated with iodine, decolorized with 95% alcohol for 30 s and counterstained with safranin for 1 min. The preparation was examined under a bright-field microscope using the oil-immersion objective. Gram reaction, cell shape and motility were recorded [4].

Congo red test

Congo red-yeast extract mannitol agar plates were prepared by supplementing the medium with 2.5 mL of 1% Congo red solution per litre. One millilitre of culture suspension from the 10^{-4} dilution was inoculated onto the plates. The plates were incubated in an inverted position at 28–30 °C for 5 days and observed for colony colour and Congo red absorption [3].

Growth on bromothymol blue medium

Yeast extract mannitol agar containing 0.5% bromothymol blue in alcoholic solution was prepared and dispensed aseptically into sterile Petri plates. Each isolate was streaked onto the medium and incubated. Acid-producing isolates changed the medium to yellow, whereas alkali-producing isolates changed the medium from green to blue [3].

Indole production

Test cultures were inoculated into pre-sterilized SIM agar tubes and incubated at 28 ± 2 °C for 48 h. After incubation, 10 drops of Kovac's reagent were added. Development of a red colour was recorded as a positive reaction for indole production [5].

Methyl red test

The test bacteria were inoculated aseptically into methyl red–Voges-Proskauer broth using a sterilized inoculating needle. After incubation, methyl red reagent was added. Development of a red colour was recorded as a positive methyl red reaction, indicating mixed-acid fermentation [6].

Catalase test

24-hour old cultures were tested with hydrogen peroxide. Immediate effervescence due to liberation of oxygen was recorded as a positive reaction [7].

Screening for plant growth-promoting characteristics

Indole acetic acid production

Indole acetic acid production was detected according to Gordon and Weber [8]. Bacterial isolates were inoculated into 30 mL nutrient broth supplemented with DL-tryptophan (100 µg/mL) and incubated at 30 °C for 8 days under stationary conditions. After incubation, cultures were centrifuged. Two millilitres of supernatant were mixed with 2 mL Salkowski reagent and allowed to stand for 30 min for pink colour development. Colour intensity was measured at 500 nm using a spectrophotometer against a reagent blank. Indole acetic acid concentration was determined using a standard curve prepared with known concentrations.

Ammonia production

Freshly grown bacterial cultures were inoculated into 10 mL peptone water and incubated for 48–72 h at 28 ± 2 °C. Nessler's reagent was added to the tubes. Yellow colour development indicated ammonia production [6]. Ammonia production was recorded qualitatively as low (+), moderate (++) or strong (+++) according to colour intensity.

Phosphate solubilization

All isolates were screened for phosphate solubilization using modified Pikovskaya's agar containing insoluble dicalcium phosphate. A loopful of each culture was placed at the centre of the Petri plate and incubated at 37 °C for 48–72 h. A clear halo around the colony indicated phosphate-solubilizing ability. The solubilization zone was measured as the clear zone surrounding the colony [9].

Estimation of nitrogen content

Nitrogen content of each isolate was estimated according to Humphries [10]. Yeast extract mannitol broth supplemented with L-glutamic acid (100 mg/L) was used. One hundred millilitres of medium was dispensed into a 250-mL conical flask and autoclaved at 15 lb pressure for 15 min. The isolates were grown separately for 24 h in yeast extract mannitol broth and inoculated at 2 mL per 100 mL of medium. Duplicate samples were maintained for each isolate and incubated at 28 ± 2 °C for 7 days. After incubation, the culture was homogenized. Five millilitres of homogenized culture were transferred to a digestion flask and digested with 5 mL concentrated H₂SO₄ and 200 mg catalytic mixture (K₂SO₄: CuSO₄: selenium, 100:10:1) until the contents became clear. After cooling, the volume was made up to 25 mL with distilled water. An aliquot of 5 mL was transferred to a micro-Kjeldahl distillation unit, followed by addition of 10 mL of 40% sodium hydroxide. The mixture was steam distilled and the evolved ammonia was collected over 20 mL of 2% boric acid containing two drops of double indicator. The distillate was back-titrated against 0.005 N H₂SO₄, and nitrogen content was calculated from the titre value.

Results

Isolation and morphological characterization of *Rhizobium* isolates

A total of 20 root nodule samples were collected from green gram plants grown in Raichur, Yadgiri and Kalaburagi districts of Karnataka. Twenty isolates coded GRL-1 to GRL-20 were obtained and maintained on agar slants. The isolates produced white to pale-white, round to irregular colonies of small to medium size on yeast extract mannitol agar. All isolates were Gram-negative, rod-shaped and motile (Table 1).



Fig 1: Pure culture of *Rhizobium* isolates

Biochemical characterization of *Rhizobium* isolates

All 20 isolates were negative for Congo red absorption. On yeast extract mannitol agar containing bromothymol blue, 11 isolates showed an acid reaction and nine showed an alkaline reaction. 14 isolates were positive for indole production, while 13 were positive for the methyl red test. All 20 isolates showed positive catalase activity (Table 2).



Fig 2: Indole production and methyl red test

In vitro plant growth-promoting characteristics

All 20 isolates produced indole acetic acid, with values ranging from 7.04 to 46.17 $\mu\text{g/mL}$. GRL-11 recorded the highest value (46.17 $\mu\text{g/mL}$), followed by GRL-8 (41.74 $\mu\text{g/mL}$), whereas GRL-5 recorded the lowest value (7.04 $\mu\text{g/mL}$). All 20 isolates produced ammonia. GRL-11 showed strong production (+++). GRL-1, GRL-5, GRL-6, GRL-9, GRL-12, GRL-14, GRL-17, GRL-18 and GRL-20 showed moderate production (++), while the remaining isolates showed weak production (+). Phosphate-solubilization zones ranged from 10.83 to 15.10 mm. GRL-11 recorded the highest zone (15.10 mm), whereas GRL-15 recorded the lowest zone (10.83 mm). Nitrogen content ranged from 7.34 to 20.85 mg/g. GRL-11 recorded the highest nitrogen content (20.85 mg/g), whereas GRL-4

recorded the lowest value (7.34 mg/g). The complete screening results are presented in Table 3.



Fig 3: Indole acetic acid production and phosphate solubilization

Discussion

The white to pale-white, round to irregular colonies and Gram-negative, rod-shaped and motile characteristics observed in the present study were consistent with previously reported rhizobial isolates. Meti reported white to creamy circular colonies on yeast extract mannitol agar [11], while Nguyen *et al.* reported Gram-negative, rod-shaped rhizobial isolates [12]. Fathy *et al.* also reported motile rhizobial isolates [13]. All isolates showed negative Congo red absorption, which is a characteristic commonly used during preliminary characterization of rhizobial isolates. Nagalingam *et al.* reported comparable observations among rhizobia isolated from pigeonpea root nodules [14]. The acid and alkaline reactions observed on bromothymol blue medium indicated variation among the isolates in their reaction on the medium. Acid-producing reactions among rhizobial and related bacterial isolates have also been reported previously [15, 16]. 14 isolates produced a positive indole reaction, indicating variation in indole-producing ability among the isolates. Shanmitha *et al.* reported indole production among green gram rhizobial isolates [17]. The positive catalase reaction recorded for all isolates was also in agreement with the characterization of rhizobia reported by Rajpoot and Panwar [18]. All 20 isolates produced indole acetic acid, but the amount varied considerably among isolates. GRL-11 recorded the highest production of 46.17 $\mu\text{g/mL}$. Abbasi *et al.* reported IAA production ranging from 5.5 to 31 $\mu\text{g/mL}$ among rhizobial isolates [19]. Wanjofo *et al.* also reported plant growth-promoting activity among rhizobial strains isolated from diverse sources [20]. The variation in IAA production among isolates may be associated with differences in their metabolic ability to synthesize IAA under the assay conditions. All isolates produced ammonia, although the intensity differed among isolates. GRL-11 showed strong production, while nine isolates showed moderate and the remaining isolates showed weak production. Verma and Pal reported ammonia production among rhizobacterial isolates with variation in

production levels ^[21]. Ammonia production by beneficial rhizobacteria may contribute to plant nutrient availability and therefore represents a useful trait during preliminary screening. Phosphate-solubilization ability also varied among the isolates. GRL-11 recorded the highest solubilization zone of 15.10 mm, whereas GRL-15 recorded the lowest zone of 10.83 mm. Alikhani *et al.* reported variation in phosphate-solubilizing ability among rhizobial isolates ^[22]. Kumar and Ram reported phosphate-solubilization zones up to 18 mm among positive rhizobial strains ^[23]. Such variation may be associated with differences among isolates in their ability to release organic acids and other compounds involved in phosphate mobilization. Nitrogen content varied from 7.34 to 20.85

mg/g, with GRL-11 recording the highest value. Related nitrogen-associated characteristics have been reported among rhizobial and *Bradyrhizobium* isolates ^[24, 25]. Because the present assay estimated nitrogen content by digestion, distillation and titration, the observed values are interpreted as nitrogen content rather than direct evidence of biological nitrogen fixation. Overall, GRL-11 showed the strongest combined performance among the isolates, with the highest IAA production, phosphate-solubilization zone and nitrogen content and strong ammonia production. The occurrence of several plant growth-promoting characteristics in a single isolate makes GRL-11 a promising candidate for further evaluation.

Table 1: Morphological characteristics of *Rhizobium* spp. isolates isolated from root nodules of green gram

Sl. No.	Isolate Code	Colony morphology			Microscopic characterization		
		Colony colour	Shape	Size	Gram staining	Cell shape	Motility
1	GRL-1	Pale White	Irregular	Medium	Negative	Rod	Motile
2	GRL-2	White	Irregular	Medium	Negative	Rod	Motile
3	GRL-3	White	Circular	Small	Negative	Rod	Motile
4	GRL-4	Pale white	Circular	Small	Negative	Rod	Motile
5	GRL-5	White	Circular	Small	Negative	Rod	Motile
6	GRL-6	White	Irregular	Medium	Negative	Rod	Motile
7	GRL-7	Pale white	Irregular	Medium	Negative	Rod	Motile
8	GRL-8	Pale white	Circular	Small	Negative	Rod	Motile
9	GRL-9	White	Irregular	Small	Negative	Rod	Motile
10	GRL-10	Pale white	Circular	Medium	Negative	Rod	Motile
11	GRL-11	White	Irregular	Medium	Negative	Rod	Motile
12	GRL-12	Pale white	Circular	Small	Negative	Rod	Motile
13	GRL-13	Pale white	Circular	Medium	Negative	Rod	Motile
14	GRL-14	White	Irregular	Small	Negative	Rod	Motile
15	GRL-15	White	Irregular	Medium	Negative	Rod	Motile
16	GRL-16	Pale white	Circular	Small	Negative	Rod	Motile
17	GRL-17	White	Irregular	Small	Negative	Rod	Motile
18	GRL-18	White	Circular	Small	Negative	Rod	Motile
19	GRL-19	Pale white	Irregular	Medium	Negative	Rod	Motile
20	GRL-20	White	Circular	Medium	Negative	Rod	Motile

Table 2: Biochemical characteristics of *Rhizobium* spp. isolates isolated from root nodules of green gram

Sl. No.	Isolate code	Congo red test	Bromothymol blue test	Indole test	Methyl red test	Catalase test
1	GRL-1	-	+	+	+	+
2	GRL-2	-	-	-	+	+
3	GRL-3	-	-	+	-	+
4	GRL-4	-	+	+	-	+
5	GRL-5	-	+	-	+	+
6	GRL-6	-	-	+	+	+
7	GRL-7	-	+	-	-	+
8	GRL-8	-	-	-	+	+
9	GRL-9	-	-	+	-	+
10	GRL-10	-	+	+	+	+
11	GRL-11	-	+	+	+	+
12	GRL-12	-	+	+	-	+
13	GRL-13	-	-	-	+	+
14	GRL-14	-	-	+	-	+
15	GRL-15	-	+	+	+	+
16	GRL-16	-	-	-	+	+
17	GRL-17	-	+	+	-	+
18	GRL-18	-	+	+	+	+
19	GRL-19	-	-	+	+	+
20	GRL-20	-	+	+	+	+

Table 3: *In vitro* screening of *Rhizobium* isolates for plant growth-promoting characteristics

SI. No.	Isolate code	IAA ($\mu\text{g/ml}$)	Ammonia production	Phosphate Solubilization (mm)	Nitrogen fixation (mg/g)
1	GRL-1	34.57 ^c	++	12.11 ^g	15.34 ^f
2	GRL-2	22.17 ^h	+	11.24 ⁱ	13.82 ⁱ
3	GRL-3	16.83 ^k	+	14.56 ^b	8.37 ⁿ
4	GRL-4	25.17 ^g	+	13.32 ^e	7.34 ^p
5	GRL-5	7.04 ^s	++	14.10 ^c	16.60 ^e
6	GRL-6	12.83 ⁿ	++	12.88 ^f	10.4 ^l
7	GRL-7	9.42 ^q	+	11.46 ^{hi}	15.37 ^f
8	GRL-8	41.74 ^b	+	14.70 ^b	19.25 ^b
9	GRL-9	10.34 ^p	++	11.46 ^{hi}	11.68 ^k
10	GRL-10	7.68 ^r	+	12.25 ^g	14.11 ^{gh}
11	GRL-11	46.17 ^a	+++	15.10 ^a	20.85 ^a
12	GRL-12	25.65 ^f	++	14.24 ^c	12.80 ^j
13	GRL-13	13.18 ^{mn}	+	13.22 ^e	15.48 ^f
14	GRL-14	32.35 ^d	++	12.76 ^f	7.94 ^o
15	GRL-15	18.16 ^j	+	10.83 ^j	14.30 ^g
16	GRL-16	13.60 ^m	+	11.34 ⁱ	9.43 ^m
17	GRL-17	11.45 ^o	++	12.06 ^g	14.06 ^{hi}
18	GRL-18	16.07 ^l	++	14.32 ^c	13.86 ⁱ
19	GRL-19	21.48 ⁱ	+	11.60 ^h	18.38 ^c
20	GRL-20	28.34 ^e	++	13.58 ^d	16.93 ^d
21	Ref. strain	21.84 ^{hi}	+	14.6 ^b	19.40 ^b

Mean values followed by the same letter are not significantly different based on Duncan's multiple range test ($P < 0.05$), a > b > c.

+++; Strong ++; Moderate +; Low

Conclusion

20 *Rhizobium* isolates were obtained from green gram root nodules collected from Raichur, Yadgiri and Kalaburagi districts of Karnataka. The isolates showed characteristic morphological and biochemical properties, including Gram-negative, rod-shaped and motile cells, absence of Congo red absorption and positive catalase activity. 11 isolates showed an acid reaction and nine showed an alkaline reaction on bromothymol blue medium, while 14 and 13 isolates were positive for indole and methyl red tests, respectively. All isolates exhibited multiple plant growth-promoting characteristics. Indole acetic acid production ranged from 7.04 to 46.17 $\mu\text{g/mL}$, phosphate-solubilization zones ranged from 10.83 to 15.10 mm and nitrogen content ranged from 7.34 to 20.85 mg/g. All isolates produced ammonia. GRL-11 recorded the highest indole acetic acid production, phosphate-solubilization zone and nitrogen content and showed strong ammonia production. The findings indicate that the isolates possessed multiple plant growth-promoting characteristics, with GRL-11 showing the greatest potential among the isolates evaluated.

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Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

All authors contributed to the planning and execution of the study. The first author conducted the laboratory work, compiled the results. The co-authors contributed to

supervision, interpretation of results, critical review and final approval.

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