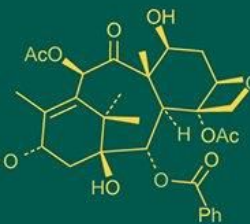
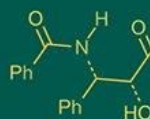


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Isolation and functional characterization of rhizobia from dggv-2 variety of green gram

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Abstract

Green gram (*Vigna radiata* L.) is a vital pulse crop contributing to nutritional security and soil fertility through biological nitrogen fixation. However, yield potential remains underutilized due to the use of outdated rhizobial inoculants and suboptimal strain selection. This study aimed to isolate, biochemically characterize and functionally evaluate native rhizobial strains associated with the DGGV-2 variety of green gram. A total of 32 bacterial isolates were recovered from 31 samples collected from Dharwad district and screened using morphological and biochemical tests for confirmation of *Rhizobium* spp. five isolates exhibited characteristic rhizobial biochemical profiles (oxidase and catalase positive, citrate utilization positive, negative Congo red absorption and absence of lactic acid production). Functional characterization revealed significant variability in plant growth-promoting traits, including IAA production (2.11-9.43 µg/ml), ammonia production, siderophore formation, HCN production, antifungal activity, and *in vitro* nitrogen fixation capacity (0.27-1.96%). Among these, isolates DR-114 and DR-107 exhibited superior performance across multiple parameters. These findings underline the importance of biochemical and functional screening for identifying efficient native rhizobia with potential application as bioinoculants to enhance growth, yield, and soil fertility in green gram cultivation.

Keywords: Biochemical characterisation, green gram, isolation, morphological characterisation, PGPR traits, *rhizobium*

1. Introduction

Green gram (*Vigna radiata*) also known as mung bean is valued for its nutritional benefits, including high protein content, essential amino acids, vitamins and minerals and plays a pivotal role in the agricultural economy and food security. It is widely cultivated legume crop accounting 10% of production and 16% of area for all pulses. The average productivity of mung bean is 721 kg ha⁻¹ with an area of 7.3 million hectares globally with a production of 5.3 million tonnes grains. Seventy percent of production produced globally is split between India and Myanmar and China (iipr.icar.gov.in/mungbean).

In India 5.5 million hectares are under cultivation of green gram with production of 3.17 million tonnes at a productivity of 570 kg ha⁻¹. The dominant contributors to mung bean cultivation in terms of area and production are Rajasthan (46% and 45% respectively) with Madhya Pradesh, Maharashtra, Karnataka, Odisha, Bihar, Tamil Nadu, Gujarat, Andhra Pradesh and Telangana also playing significant roles, as stated in the Annual Report (2022-23) by AICRP on Kharif pulses.

Green gram is also known for its ability to fix nitrogen in the soil, making it an important crop for sustainable agricultural practices. In recent years, the global production of green gram has been experiencing fluctuations due to several challenges affecting both yield and productivity. However, the productivity levels of green gram in many regions still fall short of their potential with factors such as soil degradation, climate change and inadequate agronomic practices limiting yield potential. The global demand for green gram has been steadily increasing, owing to its high nutritional value and the rising demand for plant-based protein sources. In order to reach its potential yield, one promising approach to improve both the productivity and sustainability of green gram cultivation is the inoculation with efficient *Rhizobium* species (Bhabai *et al.*, 2019)^[4].

The key challenges in cultivation of green gram includes inadequate water management, limited availability of high-quality inoculants, poor soil health and vulnerability of the crop

to various pests and diseases along with lack of awareness and technical knowledge among farmers a regarding the proper application of microbial inoculants. Environmental stresses such as drought, heat, and soil salinity further exacerbate the difficulties in achieving optimal productivity. These constraints necessitate further research into the development of resilient and efficient *Rhizobium* strains, better inoculation techniques, and more effective management practices that can enhance both crop productivity and sustainability in green gram cultivation (Mir *et al.*, 2024) [13]

Rhizobium, a genus of bacteria that forms a symbiotic relationship with leguminous plants. *Rhizobium* bacteria are known to fix atmospheric nitrogen, enriching the soil with this essential nutrient, thereby reducing the need for synthetic fertilizers. This natural nitrogen fixation not only benefits plant growth but also contributes to improving soil fertility over time. The inoculation of green gram with specific *Rhizobium* strains has been shown to enhance plant growth, increase nitrogen availability, and promote better root development, leading to higher yields. Identification of rhizobia strains for effective inoculation of legumes with plant growth promoting traits is key for enhancing yield (Monika *et al.*, 2017) [14]. The standard strain NC-92 which is presently used under Agro climatic zone 3, 8 and 11 was isolated in early 1980's. So, the attempt is made for isolation of rhizobial strains from the fields under UAS, Dharwad and screening for functional traits that have potential for improving plant growth. Through this study, we hope to contribute to the development of sustainable agricultural practices by recommending the microbial strains as bio fromulants for plant growth and yield promotion.

2. Material and Methods

2.1 Collection of rhizospheric soil and root nodules of green gram samples and isolation of bacteria from samples.

The samples were collected from 45 days old green gram plants from the fields of college of agriculture, UAS-Dharwad, Dharwad district, Karnataka. Healthy plants were selected and carefully uprooted. The sample collection was performed gentle shaking in order to collect the rhizospheric soil. Healthy roots with big nodules and rhizospheric soil samples and were placed in a polythene bag separately, labelled and then brought to the laboratory. The nodules were detached from the roots under aseptic conditions and surface sterilized using 2.5% sodium hypochlorite for 3 min and then rinsed thoroughly with sterilized distilled water for six to eight times to remove the chemicals residues. The nodules then crushed aseptically with a sterile glass rod in a test tube that contained 1 mL sterile water. One micro litre of the nodule suspension was spread on Petri-dishes that contained YEMA media. The rhizospheric soil was serially diluted and plated on YEMA at 10^{-6} concentration. These Plates were incubated for 3 days at 30°C Single colonies were selected at the end of the incubation period and further purified on fresh YEMA Plates and cultures stored at 4°C in refrigerator for further characterization (Somasegaran and Hoben, 1994) [20].

2.2 Characterization of bacterial isolates based on morphological and biochemical features

Isolates were streaked on YEMA medium and incubated at 28 ± 2 °C for 2 days. After incubation, isolated colonies were

characterized for morphological traits *viz.*, colony size, shape, elevation, margin and colour (Vincent, 1970) [24]. Gram staining was performed as per Graham and Parker (1964) [8]. A loopful of pure culture was smeared on a glass slide, air dried, and heat-fixed. The smear was stained with crystal violet for 1 min, rinsed, treated with iodine for 1 min then decolorized with 95% ethanol for 30 sec, washed, blot dried and counterstained with safranin. After final rinse and air drying, slides were observed under bright-field microscope using oil immersion for Gram reaction and cell morphology.

The biochemical tests performed included Oxidase test, Catalase test, Citrate utilization, Ketolactose test, growth on YEMA + Congo red medium, YEMA + BTB medium, Hofer's alkaline test, and growth on glucose peptone agar. The isolated were screened for oxidase presence by using 5-6 drops of 1% Wuster's reagent were added to a filter paper strip, and a loopful of overnight YEMA culture was smeared. Isolates turning blue/violet were oxidase positive (Cappuccino and Sherman, 1996) [5]. Catalase activity of isolates was detected using 100 µl of 3% hydrogen peroxide. The reagent was placed on a slide and mixed with overnight YEMA culture. Oxygen effervescence indicated a positive result (Graham and Parker, 1964) [8]. Simmon's citrate agar was prepared, autoclaved, streaked with overnight YEMA isolates, and incubated at 28 ± 2 °C for 24 hrs. Colour change from green to blue indicated citrate utilization (Simmons, 1926) [18]. Two confirmatory test were performed for bacterial isolates for differentiating them from *Agrobacterium* sps. The first was performed according to protocol of Vincent (1970) [24] using CRYEMA. The sterile media was added to plated and isolates were streaked with overnight YEMA isolates and incubated at 28 ± 2 °C for 2-3 days. Colony appearance and characteristics were recorded periodically and little or no Congo red absorption indicated rhizobia. The second one was performed according to protocol given by Bernearts and Deley (1963) [2]. Sterile ketolactose agar plates were streaked with isolates and incubated at 28 ± 2 °C for 36 hrs. Plates were flooded with Benedict's solution. Isolates failing to change blue to yellow were considered *Rhizobium* sp. For differentiating *Rhizobium* and *Bradyrhizobium* isolates (Vincent, 1970) [24]. YEMA+BTB plates (pH 7.0) were streaked with isolates. Acid producers turned the medium yellow (fast-growing rhizobial), while alkali producers turned it blue (slow-growing bradyrhizobial). Conducted glucose peptone agar test according to Vincent, 1970. The isolated were incubated at 28 °C for 2-3 days on glucose peptone agar with bromocresol purple with overnight bacterial isolates and plates were observed for change in colour after incubation.

2.3 Detection of PGP traits of rhizobial isolates under *in vitro* conditions.

Estimation of Indole Acetic Acid (IAA): IAA produced by *Rhizobium* isolates was quantified using yeast extract mannitol broth supplemented with 0.01% tryptophan. The isolates incubated at 28 ± 2 °C for 3 days under shaking. After centrifugation, the supernatant was mixed with orthophosphoric acid and Salkowski's reagent. Development of pink colour read at 540 nm indicated IAA production. Uninoculated broth served as control and IAA concentration was determined from a standard curve (Gordon and Weber, 1951) [7]. For checking ammonia production, rhizobial isolates were inoculated into peptone

water and incubated for 48 hrs at 28 ± 2 °C. Addition of Nessler's reagent causing brown to yellow colour change indicated positive ammonia production (Manasa *et al.*, 2017) [12]. HCN production was assessed by streaking isolates on YEMA + glycine plates. Filter paper pads soaked in picric acid solution were placed in lids and plates were sealed and incubated at 28 ± 2 °C for 7-8 days. Colour intensity (no change to orange) indicated HCN production strength (Wei *et al.*, 1991) [25].

Siderophore production was screened using Chrome Azurol S (CAS) assay (Schwyn and Neilands, 1987) [16]. Rhizobial isolates were spotted on CAS agar plates and incubated at 28 ± 2 °C for 2-3 days. Orange halo around colonies indicated positive production and zone diameter was recorded. Estimation of *in vitro* nitrogen fixation potential by rhizobial isolates was done under symbiotic conditions. Green gram seeds (DGGV-2) were surface sterilized, treated with overnight rhizobial isolates and sown in sterilized Leonard jar assemblies containing vermiculite and N-free nutrient solution. After 45 DAS, plants were dried and nitrogen content in shoots and roots was estimated using micro-Kjeldahl method (Jackson, 1967) [10]. Antifungal activity (*In vitro*) of rhizobial isolates was against *Agroathelia rolfii* and *Colletotrichum lindemuthianum* using dual culture technique on nutrient agar. Radial growth of fungi was measured and percentage inhibition calculated by using formula:

$$\text{Inhibition (\%)} = \{(R-r)/R\} \times 100$$

Where r = fungal radius opposite bacterial colony and R = fungal radius without bacteria (Kumari and Khanna, 2014) [11].

2.4 Identification of the rhizobial isolates by 16S rRNA gene sequence analysis

Pure cultures of efficient rhizobial strains cultured in YEM broth until log phase for molecular identification. The BLAST program was used to compare the sequences obtained from Eurofins Genomics India Pvt. Ltd to those from the NCBI and the Clustal W software was used to align the sequences and the MEGA7 software was used to build phylogenetic trees. The neighbour-joining approach was used to infer the dendrogram (Mohammad Imran Mir *et al.*, 2021) [26].

2.5 Statistical analysis

The data were analysed statistically by analysis of variance (ANOVA; Genstat 20. version) in a completely randomized design (CRD) for greenhouse to evaluate the efficiency of diazotrophic bacteria. Significance of differences between the treatment means was tested at $P = 0.01$ (Mir *et al.*, 2021) [26].

3. Results

3.1 Collection of samples and isolation of rhizobial spp.

A total of 15 rhizospheric soil samples and 17 root nodules samples were collected from college of agriculture, UAS-Dharwad of Dharwad district in Karnataka. The geographical details were collected using GPS system and presented in Table 1. The samples were processed and plated to isolate rhizobial spp. using standard protocol. A total of 32 bacterial isolates were selected from YEMA Plates. The isolates were coded as DR-94 to DR-125 on

basis of region from which they collected Dharwad. All 32 were picked and purified (four-way streaking method) and stored at 4 °C for further study for characterization.

3.2 Morphological and biochemical characterization of bacterial isolates

The rhizobial isolates were morphologically characterized based on YEMA media for colony shape, colour, surface margin, elevation, cell shape and Gram reaction. Details of morphological studies are presented in Table 2. All the isolates on YEMA Plates exhibited colony shape as circular, translucent white in colour due to exo-polysaccharides production with entire margin (Plate 1). Further convex elevation was observed in all CFU's. All 32 rhizobial isolates exhibited milky white to creamy white colony colour. The cell shape of isolates was varied as cocci, rod, vibrio and isolates were gram positive and gram negative observed under microscope with a magnification of 40X. Based on morphological characterization a total of 12 bacterial isolates, which were gram negative and rod shape cells exhibiting colony shape as circular and convex elevation with entire margin and colour varying from creamy white to milky white along with translucent exopolysaccharides production were taken further for biochemical characterization.

The bacterial isolates exhibiting oxidase and catalase activity and capable of using citrate as carbon source, no red colour absorption on YEMA+CR media, changing media colour of YEMA+BTB from green to yellow, no lactic acid production on ketolactose media and no growth on glucose peptone agar media can be tentatively confirmed as rhizobial isolates. Out of 12 isolates tested only 5 isolates exhibited typical biochemical characteristics (Plate 2) that were listed in Bergey's manual of determinative bacteriology.

3.3 Functional characterization (Plant growth promoting traits) of rhizobial isolates

The tests on functional characterization of all 30 rhizobial isolates revealed that isolates exhibit varying levels of PGPR traits Table 3 and Plate 3. IAA production by isolates was in range of 0.2116 to 0.9432 µg/ml. The highest amount of IAA was produced by the isolate DR-114 (0.9432 µg/ml) followed by DR-29 (0.7511 µg/ml) and least by DR-116 (0.2116 µg/ml). All rhizobial isolates were positive to ammonia production by changing media colour from brown to yellow after adding Nessler's reagent. All isolate except DR-100 were positive HCN production and sub characterized based on intensity of brown colour developed in media i.e., medium brown (moderate, ++), light brown (weak, +) and non-producer (-). All isolates exhibit DR-116 were positive for siderophore production. Antifungal activity against *Agroathelia rolfii* and *Colletotrichum lindemuthianum* exhibited by rhizobial isolates was in range of 12.23 to 35.60 percent for *Agroathelia rolfii* and 11.23 to 45.67 percent for *Colletotrichum lindemuthianum*. The highest level of inhibition was exhibited by DR-107 (35.60 and 45.67 percent) for both *Agroathelia rolfii* and *Colletotrichum lindemuthianum* respectively. When *in-vitro* nitrogen fixation capacity was tested by using Leonard jar assembly, at 45 DAS rhizobial isolates were in range of 1.96-0.27 percent, where DR-114 (1.96%) followed by DR-100 (0.27%) exhibited highest range and DR-100 exhibited lowest level plant nitrogen.

4. Discussion

In the present study a total of 31 samples (15 rhizospheric soil samples and 17 root nodule samples) were collected for isolation of rhizobial bacteria. The systematic approach of morphological, biochemical and functional characterization followed for accurate identification of rhizobial isolates with plant growth-promoting potential. From the samples collected a total of 32 bacterial isolates which are exhibiting different colony characters were isolated and purified using selective (YEMA) media. The morphological assessment revealed that 12 isolates displayed typical *Rhizobium* like traits, including creamy white colonies, convex elevation, production of exopolysaccharides and gram-negative rod-shaped cell morphology. Exopolysaccharide production important as it facilitates successful root hair infection and protects bacteria against desiccation and environmental stresses, which are common challenges in pulse-growing regions. The tested traits are consistent with the descriptions reported in Bergey's Manual of Determinative Bacteriology and supported by previous studies on rhizobia from mung bean and cowpea nodules (Somasegaran and Hoben, 1994; Swapna *et al.*, 2023) [20, 23]. Studies reported by Stella *et al.* (2020) [22] and Elhoshi *et al.* (2023) [6] where they isolated 103 and 25 bacterial isolates respectively using similar methodology from cowpea and root nodules of faba bean plants respectively.

However, morphological features alone are insufficient for accurate identification because many soil bacteria can produce similar colony morphologies. Biochemical characterization was therefore critical for confirming true rhizobial identity and excluding look-alike genera such as *Agrobacterium*. All selected isolates were oxidase- and catalase-positive, indicating an active cytochrome oxidase system and the ability to neutralize reactive oxygen species, which is critical for survival in the rhizosphere where oxidative stress is common. Citrate utilization indicated that all the isolates could metabolize organic acids as sole carbon sources, a useful adaptation in nutrient-limited soils. The negative results for ketolactose tests and lack of Congo red absorption on YEMA + CR medium helped differentiate rhizobia from *Agrobacterium* spp., thus minimizing false positives. When isolates grown on YEMA+BTB for differentiating slow growing alkaline producers with fast growing acid producers, 5 isolated changed color from green to yellow indicating fast growing acid producers and the rest ten bacterial isolates changed colour to blue indicating alkaline producers. By looking into Bergey's manual of determinative bacteriology, the isolates showing positive for oxidase activity, catalase activity, positive for citrate utilization, negative to ketolactonic acid production and no congo red absorption on YEMA media along with changing YEMA +BTB media into blue color were tentatively identified as rhizobial isolates. In present study a total of 5 bacterial isolates out of 15 tested were tentatively identified as rhizobial isolates.

The above findings are in line with reports of Sneka *et al.* (2022) [19] where bacteria isolates showing *Rhizobium* traits were selected based on negative results on congo red absorption, lactose production, growth on glucose peptone agar and Holfer's alkaline test. Similar results reported by Ozdogan *et al.* (2023) [15] by studying 10 bacteria isolated from root nodules of sugar beans in Turkey where 2 isolates were negative for congo red absorption, 8 isolates turned YEMA + BTB to yellow and negative for growth on glucose peptone media. Molecular identification of 10 bacteria isolates revealed that eight of the isolates were identified as

Agrobacterium tumefaciens, while two isolates (F4DC and F6DC) were identified as *Rhizobium gallicum*.

All the five rhizobial isolates were tested for their functional characters for overall plant growth promotion. All the isolates were tested positive for indole-3-acetic acid (IAA) production showing their potential to increase root growth, cell division, differentiation and development of vascular bundles. Ammonia production by all isolates confirms their potential role in supplying readily available nitrogen to plants, in addition to their symbiotic nitrogen fixation ability. Siderophore production was recorded in all isolates except DR-116, demonstrating their ability to sequester Fe³⁺ and enhance micronutrient availability to plants, which is critical for chlorophyll synthesis and plant metabolic functions, particularly in calcareous soils where iron is often limiting.

In addition to nutrient mobilization, several isolates showed biocontrol potential through hydrogen cyanide (HCN) production and antifungal activity. The strong inhibition of *Agroathelia rolfii* and *Colletotrichum lindemuthianum* by isolates such as DR-107 and DR-114 suggests that these rhizobia could suppress soil-borne and seed-borne pathogens, thereby contributing to sustainable plant protection. These dual benefits (nutrient mobilization and pathogen suppression) make such isolates attractive candidates for use as bioinoculants. The ability of the isolates to fix atmospheric nitrogen under *in vitro* conditions was confirmed through Leonard jar assemblies, where DR-114 exhibited the highest nitrogen accumulation (1.96%). This highlights its potential as a superior strain for enhancing nitrogen availability and reducing dependence on synthetic fertilizers.

The similar results were reported by Soni *et al.* (2023) [21] and Sharma *et al.* (2024) [17]. Nineteen bacterial isolates were examined by Soni *et al.* (2023) [21] for PGP activities where IAA production all isolates shown positive results with the range of 76.25-12.00 µg/ml and 7 isolates, 12 isolates are positive for siderophore production 5 isolates are negative for ammonia production and all isolates except 2 shown positive for HCN production. Sharma *et al.* (2024) [17] studied 61 bacterial isolates where 19 isolates shown better PGPR traits with highest of IAA production (76.97±1.68 mg/l), Ammonia production (38.59±0.19 mg/l), Siderophore production (49.21±1.83 percent), Phosphate solubilization (4897.73±25.53 mg/l) confirmed through quantitative analysis.

When compared with the standard strain NC-92, which has been widely used since the 1980s as a commercial inoculant under agro-climatic zones 3, 8 and 11, the native isolates demonstrated comparable or superior plant growth-promoting attributes. Notably, isolates DR-114 and DR-107 exhibited higher IAA production and greater antifungal activity against *Colletotrichum lindemuthianum* and *Agroathelia rolfii* than NC-92, suggesting a stronger ability to enhance root development and suppress soil-borne pathogens. Although NC-92 recorded slightly higher nitrogen fixation (2.54%) under *in vitro* conditions, the combined functional traits of DR-114 (1.96% N fixation, strong siderophore production and dual biocontrol activity) and DR-107 (high IAA production and 45.67% fungal inhibition) indicate a broader spectrum of benefits for plant growth and health. This suggests that the selected native isolates could perform equally well or better under field conditions, especially when used in consortia, potentially replacing or complementing NC-92 as a more effective and locally adapted inoculant.

Table 1: Details of root nodules and soil samples collected from Dharwad district

Sl. No.	Location	Isolate code	Sample type	Crop	GPS Coordinates		
					Latitude (N)	Longitude (E)	Elevation (Above msl)
1	Dharwad	DR-94	Rhizosphere soil	Green gram	15°49'37"	74°98'71"	701
2	Dharwad	DR-95	Rhizosphere soil	Green gram	15°49'58"	74°98'46"	697
3	Dharwad	DR-96	Rhizosphere soil	Green gram	15°49'07"	74°98'83"	70-1
4	Dharwad	DR-97	Rhizosphere soil	Green gram	15°49'09"	74°98'96"	696
5	Dharwad	DR-98	Rhizosphere soil	Green gram	15°49'54"	74°98'69"	694
6	Dharwad	DR-99	Rhizosphere soil	Green gram	15°49'38"	74°98'81"	697
7	Dharwad	DR-100	Rhizosphere soil	Green gram	15°49'42"	74°98'76"	695
8	Dharwad	DR-101	Rhizosphere soil	Green gram	15°49'42"	74°98'76"	696
9	Dharwad	DR-102	Rhizosphere soil	Green gram	15°49'55"	74°98'90"	690
10	Dharwad	DR-103	Rhizosphere soil	Green gram	15°49'63"	74°98'98"	712
11	Dharwad	DR-104	Rhizosphere soil	Green gram	15°49'70"	74°98'05"	696
12	Dharwad	DR-105	Rhizosphere soil	Green gram	15°49'78"	74°98'12"	695
13	Dharwad	DR-106	Rhizosphere soil	Green gram	15°49'86"	74°98'20"	690
14	Dharwad	DR-107	Rhizosphere soil	Green gram	15°49'42"	74°98'76"	678
15	Dharwad	DR-108	Rhizosphere soil	Green gram	15°49'99"	74°98'34"	692
16	Dharwad	DR-109	Root nodules	Green gram	15°49'07"	74°98'42"	695
17	Dharwad	DR-110	Root nodules	Green gram	15°49'14"	74°98'49"	691
18	Dharwad	DR-111	Root nodules	Green gram	15°49'54"	74°98'69"	702
19	Dharwad	DR-112	Root nodules	Green gram	15°49'29"	74°98'63"	680
20	Dharwad	DR-113	Root nodules	Green gram	15°49'41"	74°98'74"	692
21	Dharwad	DR-114	Root nodules	Green gram	15°49'48"	74°98'81"	706
22	Dharwad	DR-115	Root nodules	Green gram	15°49'56"	74°98'88"	700
23	Dharwad	DR-116	Root nodules	Green gram	15°49'64"	74°98'95"	705
24	Dharwad	DR-117	Root nodules	Green gram	15°49'71"	74°98'02"	896
25	Dharwad	DR-118	Root nodules	Green gram	15°49'79"	74°98'09"	708
26	Dharwad	DR-119	Root nodules	Green gram	15°49'87"	74°98'16"	709
27	Dharwad	DR-120	Root nodules	Green gram	15°49'95"	74°98'23"	710
28	Dharwad	DR-121	Root nodules	Green gram	15°49'41"	74°98'74"	689
29	Dharwad	DR-122	Root nodules	Green gram	15°50'10"	74°98'37"	684
30	Dharwad	DR-123	Root nodules	Green gram	15°49'48"	74°98'81"	678
31	Dharwad	DR-124	Root nodules	Green gram	15°49'26"	74°98'51"	698
32	Dharwad	DR-125	Root nodules	Green gram	15°49'42"	74°98'76"	674

Table 2: Morphological characterization of bacterial isolates

Sl.no	Isolate code	Colony shape	Colour on YEMA	EPS production	Surface margin	Elevation	Cell shape	Gram reaction
1	DR-94	Circular	Milky white	+	Entire	Convex	Rod	G-ve
2	DR-95	Circular	Creamy white	+	Entire	Convex	Rod	G+ve
3	DR-96	Circular	Milky white	+	Entire	Convex	Rod	G-ve
4	DR-97	Circular	Creamy white	+	Entire	Convex	Cocci	G+ve
5	DR-98	Circular	Milky white	+	Entire	Convex	Rod	G-ve
6	DR-99	Circular	Milky white	+	Entire	Convex	Vibrio	G+ve
7	DR-100	Circular	Milky white	+	Entire	Convex	Rod	G-ve
8	DR-101	Circular	Creamy white	+	Entire	Convex	Cocci	G-ve
9	DR-102	Circular	Milky white	+	Entire	Convex	Cocci	G+ve
10	DR-103	Circular	Milky white	+	Entire	Convex	Rod	G-ve
11	DR-104	Circular	Creamy white	+	Entire	Convex	Rod	G+ve
12	DR-105	Circular	Milky white	+	Entire	Convex	Vibrio	G-ve
13	DR-106	Circular	Creamy white	+	Entire	Convex	Rod	G+ve
14	DR-107	Circular	Milky white	+	Entire	Convex	Rod	G-ve
15	DR-108	Circular	Milky white	+	Entire	Convex	Vibrio	G-ve
16	DR-109	Circular	Milky white	+	Entire	Convex	Rod	G+ve
17	DR-110	Circular	Creamy white	+	Entire	Convex	Rod	G-ve
18	DR-111	Circular	Milky white	+	Entire	Convex	Rod	G-ve
19	DR-112	Circular	Milky white	+	Entire	Convex	Cocci	G+ve
20	DR-113	Circular	Creamy white	+	Entire	Convex	Rod	G-ve
21	DR-114	Circular	Milky white	+	Entire	Convex	Rod	G-ve
22	DR-115	Circular	Milky white	+	Entire	Convex	Rod	G-ve
23	DR-116	Circular	Milky white	+	Entire	Convex	Rod	G-ve
24	DR-117	Circular	Creamy white	+	Entire	Convex	Vibrio	G-ve
25	DR-118	Circular	Milky white	+	Entire	Convex	Rod	G+ve
26	DR-119	Circular	Milky white	+	Entire	Convex	Vibrio	G-ve
27	DR-120	Circular	Creamy white	+	Entire	Convex	Cocci	G+ve
28	DR-121	Circular	Milky white	+	Entire	Convex	Vibrio	G-ve
29	DR-122	Circular	Creamy white	+	Entire	Convex	Rod	G+ve
30	DR-123	Circular	Milky white	+	Entire	Convex	Rod	G+ve
31	DR-124	Circular	Milky white	+	Entire	Convex	Rod	G+ve
32	DR-125	Circular	Milky white	+	Entire	Convex	Vibrio	G-ve

+: Positive for exopolysaccharide production; G-ve: Gram negative; G+ve: Gram positive

Table 3: Biochemical characterization of bacterial isolates

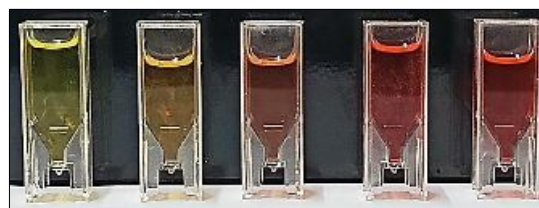
Sl. No.	Isolate code	Oxidase test	Catalase test	Citrate utilization	YEMA+CR test	YEMA+BTB test	Ketolactose test	Growth on GPA	Tentative identification
1	DR-94	+	+	+	-	Blue	-	-	<i>Bradyrhizobium</i> spp.
2	DR-96	+	+	+	-	Yellow	-	-	<i>Rhizobium</i> spp.
3	DR-98	+	+	+	-	Blue	-	-	<i>Bradyrhizobium</i> spp.
4	DR-100	+	+	+	-	Yellow	-	-	<i>Rhizobium</i> spp.
5	DR-103	+	+	+	-	Blue	-	-	<i>Bradyrhizobium</i> spp.
6	DR-107	+	+	+	-	Yellow	-	-	<i>Rhizobium</i> spp.
7	DR-110	+	+	+	-	Blue	-	-	<i>Bradyrhizobium</i> spp.
8	DR-111	+	+	+	-	Blue	-	-	<i>Bradyrhizobium</i> spp.
9	DR-113	+	+	+	-	Yellow	-	-	<i>Rhizobium</i> spp.
10	DR-114	+	+	+	-	Yellow	-	-	<i>Rhizobium</i> spp.
11	DR-115	+	+	+	-	Blue	-	-	<i>Bradyrhizobium</i> spp.
12	DR-116	+	+	+	-	Yellow	-	-	<i>Rhizobium</i> spp.

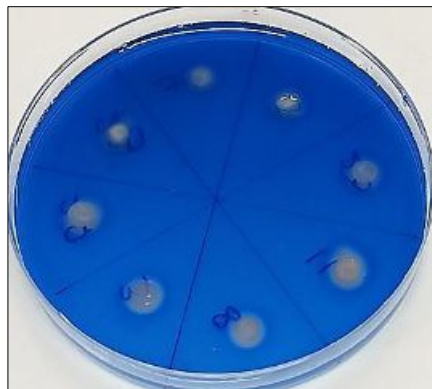
+: Positive results - : Negative results

Table 4: Functional characterization of rhizobial isolates

Isolate code	IAA ($\mu\text{g/ml}$)	Ammonia production	HCN production	Siderophore production	Antifungal activity (%)		<i>In-vitro</i> plant N ₂ fixation (%)
					<i>Agroathelia rolfii</i>	<i>Colletotrichum lindemuthianum</i>	
DR-100	05.458	+	-	+	19.87	13.20	0.27
DR-107	09.432	+	+	-	35.60	45.67	1.70
DR-113	07.418	+	+	+	19.19	20.19	1.75
DR-114	07.511	+	++	+	25.09	21.09	1.96
DR-116	02.116	+	++	-	12.23	11.23	1.30
NC-92	05.183	+	-	-	15.82	16.85	2.54
0.715					01.10	00.80	00.06
2.028					03.50	01.56	00.12

Ammonia production: + indicates positive results and - indicates negative; HCN production: + weak HCN producer, ++ strong HCN producer; Siderophore production: + indicates the growth of isolates on CAS agar, ++ indicates zone of solubilization by isolate on CAS agar and - indicates no growth on CAS agar

**Plate 1:** Pure culture of DR-114 isolate**C.** Growth on ketolactose media**Plate 2:** Biochemical characterization of bacterial isolates**A.** Growth on CRYEMA media**A.** Invitro nitrogen estimation**B.** Ammonia production test**B.** IAA production by rhizobial isolates



C. Siderophore production by rhizobial isolates



D. HCN production of rhizobial isolates

Plate 3: Functional characterization of rhizobial isolates

5. Conclusion

The present study demonstrated that systematic isolation, biochemical and functional characterization of rhizobial isolates are indispensable steps for identifying efficient strains with high potential for enhancing green gram productivity. Biochemical profiling proved crucial for differentiating true rhizobia from non-target bacteria and for confirming key metabolic features such as oxidase, catalase and citrate utilization that are essential for efficient symbiosis. The functional characterization further highlighted the diverse plant growth promoting traits exhibited by the isolates, including IAA production, ammonia release, siderophore formation, HCN production, antifungal activity and nitrogen fixation potential. These attributes collectively contribute to improved root architecture, enhanced nutrient uptake, suppression of soil-borne pathogens and overall plant vigour. Importantly, the integration of both biochemical and functional characterization ensured the selection of isolates with proven symbiotic efficiency and multiple plant growth-promoting abilities, rather than relying solely on morphological traits. This holistic approach strengthens the reliability of recommending specific strains, such as DR-114 and DR-107, as potential bioinoculants for the DGGV-2 variety of green gram. Future studies should focus on multilocal field trials and genomic characterization to validate the consistency of these isolates under variable agro-climatic conditions and to explore their potential for inclusion in commercial bioformulations aimed at sustainable pulse production.

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