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ISOLATION, CHARACTERIZATION AND SCREENING OF *RHIZOBIUM* SP. FROM ROOT NODULES OF BLACK GRAM (*VIGNA MUNGO* L.)

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ABSTRACT

The present investigation entitled "Isolation and characterization of *Rhizobium* sp. from root nodules of black gram (*Vigna mungo* L.)" was carried out in the Department of Agricultural Microbiology, College of Agriculture, UAS Raichur during 2024-25. A total of 25 root nodule samples were collected from black gram fields in Raichur and Kalaburagi. Twenty-five isolates of *Rhizobium* were purified and subjected to morphological and biochemical characterization. Morphological characterization revealed that *Rhizobium* isolates isolated on YEMA media were Gram-negative, motile, rod shaped. Biochemical tests indicated that out of 25 *Rhizobium* isolates, 15 were positive for indole, 14 were positive for methyl red, 16 were positive for starch hydrolysis, 12 were positive for bromothymol blue test and all were positive for catalase test. All 25 *Rhizobium* sp. isolates were screened *in vitro* for their plant growth promoting (PGP) traits. Nitrogen fixation ranged from 6.60 to 20.25 mg/g, with isolate RR-5 showing the highest activity. All isolates produced indole-3-acetic acid (IAA) between 5.38 µg/ml and 20.28 µg/ml, and RR-5 recorded the maximum. Phosphate solubilization zones varied from 11.02 mm to 15.02 mm, while ammonia and hydrogen cyanide (HCN) production were positive in most isolates, indicating PGP potential and biocontrol ability. 10 isolates exhibited biocontrol efficiency ranging from 14.44% to 41.74% against *Fusarium* sp. demonstrating their potential as effective bioinoculants for sustainable crop production.

Keywords **:** *Rhizobium*, Black gram, Nitrogen fixation, IAA, Phosphate solubilization.

Introduction

Black gram (*Vigna mungo* L.) belongs to family leguminaceae and regarded as the third most important pulse crop in India. It is a short-duration warm-season crop that matures within 90-120 days and is valued for its high nutritive profile, soil fertility restoration and wide adaptability (Karamany, 2006). The crop is highly valued due to its excellent digestibility, making it an important component of vegetarian diets. In addition to its use as human food, also utilized as green manure, cover crop, forage, silage, hay and chicken pasture thereby serving multiple agricultural roles (Nair *et al*., 2024). Although its primary use is for seed and food production, black gram is also suited for double cropping systems especially when cultivated after cereals like barley, wheat and oat as practiced in regions such as Serbia. Furthermore, it offering both early-season forage and later seed production, thereby enhancing its utility in diverse farming systems. Black gram seeds are highly nutritious containing high amounts of protein (24-26%), carbohydrates (60%), fat (1.3%) and also reported to be rich in potassium, phosphorous and calcium with good amount of sodium. Black gram can obtain nitrogen (N) from atmosphere by fixation in their root nodules in symbiosis with soil rhizobia and thus have the potential to perform well in N deficit soils.

Rhizobium is well known species of a group of bacteria that acts as primary symbiotic nitrogen fixer. Rhizobia are Gram-negative, rod-shaped, motile bacteria that inhabit the soil and colonize legume roots and fixes the atmospheric nitrogen symbiotically (Tilak *et al*., 2005). The activities like solubilization of insoluble phosphates, production of plant growth

regulators, hormones, siderophore and production of antagonistic substances play an important role in plant growth. The legume *Rhizobium* interaction is the result of specific recognition of host legume by *Rhizobium*.

Material and Methods

Survey and collection of root nodules of black gram

A survey was conducted to collect the black gram samples from the fields. A total of 25 root nodule samples of black gram were collected from the fields located in Raichur and Kalaburagi, isolates were designated as RR-1 to RR-25.

Isolation and purification of *Rhizobium* isolates

The selected black gram root nodules were surface sterilized by using 0.1% mercuric chloride for two to three minutes and then nodules were washed in sterile distilled water for three to four times to make them free from excessive chemicals. Then the nodules were transferred to test tubes containing sterile distilled water. Using the blunt end of a surface sterilized glass rod, nodules were crushed to form a suspension. In the meantime, several Petri plates containing yeast extract mannitol agar (YEMA) with congored were prepared. When the agar solidified, with the help of an inoculation needle, one loopful of nodule suspension was streaked in a zig-zag manner on the agar medium and the plates were incubated at 28 ± 2 °C for 3-5 days (Sushma *et al.,* 2017).

After careful selection of single colony which is white, round and did not absorb congored were further purified by streak plate method on fresh YEMA plates. These purified cultures of root nodulating bacteria were maintained on agar slants of YEMA medium for further studies.

Morphological and biochemical characterization of the *Rhizobium* isolates

Colony character

Colony characters of all isolates were studied on solid surface of yeast extract mannitol agar medium in Petri plates. Colony colour, shape and size were recorded (Rai *et al.,* 2013).

Gram staining and microscopic examination

A thin smear of root nodulating bacteria was made and heat fixed. Smear was stained with crystal violet for one minute, then washed with water and flooded with iodine. Decolorize with 95 per cent alcohol for 30 seconds. Then it was counter stained with safranin for one minute, air dried and it was observed under microscope. The stained microscope slides were examined through a bright field microscope under oil immersion for gram reaction and cell morphology. Typical Gram-negative, rod-shaped cells were inferred as *Rhizobium* sp. (Buchanan and Gibbson, 1974).

Congo red test

Prepared CRYEMA plates (YEMA medium supplemented with an aliquot of 2.5 ml of a 1% solution of congored dye to a litre of YEMA), pipette out 1 ml of the culture suspension from 10^{-4} dilution to CRYMA plates, plates were incubated in inverted position at 28-30°C for 5 days, observed the plates for the appearance of *Rhizobium* (Somasegaran and Hoben, 1994).

Growth on Bromothymol blue media

Freshly prepared YEMA with 0.5 per cent BTB (in alcoholic solution) was dispensed aseptically in sterile Petri plates. Each isolate was streaked on yeast extract mannitol agar plates containing bromothymol blue. The fast/acid production isolates were turn the medium to yellow colour while the slow/alkali production isolates will turn the medium from green to blue colour (Somasegaran and Hoben, 1994).

Indole production

To the pre-sterilized SIM agar tubes, the test cultures were inoculated. The tubes were incubated at 28 ± 2°C for 48 hours. After incubation, each tube was added with 10 drops of Kovac's reagent. The development of red colour was taken as positive for the indole production (Sneka *et al*., 2022).

Methyl red test

The test bacteria were inoculated aseptically into the MR-VP broth with the help of sterilised inoculating needle. Alcoholic solution of methyl-red is dropped into each test tube and observed that the bacteria has converted glucose to a stable acid, as indicated by conversion of methyl red from yellow to red colour indicates mixed acid fermentation and methyl red test is positive.

Catalase test

Isolates of 48 hours old flooded with hydrogen peroxide and observe liberation of effervescence of oxygen around the bacterial colonies (Graham and Parker, 1964).

Starch hydrolysis

The starch hydrolysis test was performed to determine the ability of microorganisms to use starch as a carbon source (De Oliverira, 2007). Starch agar media were inoculated with isolates and analysed for starch utilization. Iodine test was used to determine the capability of microorganisms to use starch. Drops of iodine solution (0.1 N) were spread on 24 hours old

cultures grown in Petri plates. Formation of blue colour indicated non utilization of starch and *vice versa*.

Screening of *Rhizobium* sp. for its plant growth promoting potential

Estimation of *in vitro* nitrogen fixation

Nitrogen fixation by each *Rhizobium* isolate was studied according to the method described by (Humphries, 1956). The YEM broth supplemented with L-glutamic acid at 100 mg/L was used in this study. To a 250ml conical flask, 100 ml of the above medium was dispensed and autoclaved at 15 lbs pressure for 15 minutes. The *Rhizobium* isolates were grown separately for 24 hours in YEM broth and inoculated at 2 ml/100 ml of the medium. Duplicate samples were kept for each isolate. The flasks were incubated at 28±2 °C for 7 days.

After seven days of incubation, the culture was homogenized. Five ml of the homogenized culture was withdrawn and digested with 5 ml concentrated H_2SO_4 and 200 mg catalytic mixture (K_2SO_4: $CuSO_4$, Selenium) (100:10:1 ratio) until the contents become clear. After cooling, the volume was made up to 25 ml with distilled water. Then, 5 ml of aliquot was transferred to micro-kjeldhal distillation unit. An aliquot of 10 ml of 40 % sodium hydroxide was added and steam distilled. Ammonia evolved was collected over 2 per cent boric acid (20 ml) containing 2 drops of double indicator (83.3 mg bromocresol green + 16.6 mg methyl red indicator dissolved in 10 ml of 95% ethanol) and back titrated against 0.005 N H_2SO_4 (1 ml of 0.005 N of H_2SO_4 0.0007g of N) and substituting the titre value in the formula per cent N was calculated.

$$N(\%) = \frac{\text{Titer value} \times 0.014 \times \text{N. of } H_2SO_4 \times \text{Vol. made}}{\text{Vol. of sample made}} \times 100$$

Indole Acetic Acid (IAA) production

IAA production by different bacterial isolates was detected according to Gordon and Weber (1951). Bacterial isolates were tested for their ability to produce indole acetic acid (IAA). Cultures were inoculated in 30ml Nutrient broth supplemented with DL- tryptophan at the rate of 100µg/ml and incubated at 30 ºC for eight days under stationary conditions of growth. After centrifugation 2ml of salkowaski reagent was added to 2ml of culture supernatant, mixed and allowed to stand for 30 min for the development of pink colour and colour intensity was measured at 500nm using spectrophotometer against a reagent blank.

Ammonia production

Freshly grown bacterial cultures were inoculated in 10ml peptone water in each tube and incubated for 48-72 h at 28 ± 2 °C. Nessler's reagent (0.5ml) was added in each tube and the development of the yellow colour indicated ammonia production (Cappuccino and Sherman, 1992).

Phosphate solubilization

All the isolates were screened for phosphate solubilization. Modified Pikovskaya's agar containing insoluble dicalcium phosphate was used, a loopful of each culture placed at the centre of Petri plates and incubated at 37°C for 48 to 72 h. Appearance of hollow zone around the colonies infers positive phosphate solubilizing ability. The solubilization zone was determined by subtracting the diameter of bacterial colony from the diameter of total zone. Phosphate solubilizing index was calculated by using formula (Pikovskaya, 1948).

$$\text{Solubilization index (SI)} = \frac{\text{Colony diameter (mm)} + \text{Halozone diameter (mm)}}{\text{Colony diameter}}$$

HCN production

Cultures were streaked on nutrient agar amended with 4.4g/L glycine. A Whatman filter paper no. 1 dipped in 0.5% picric acid solution (in 2% sodium carbonate) placed inside the lid of the plates. Plates were sealed with parafilm and incubated at 37°C for 7 days. If paper turned yellow to brown in colour, it showed HCN production by the isolates.

Biocontrol efficiency of bacterial isolates

Biocontrol efficiency was screened by a dual culture method in which both bacterial isolate and test fungi were inoculated in a single Potato Dextrose Agar (PDA) media plate. The test fungi (5 mm diameter disc) was inoculated at the centre of the potato dextrose agar plate and 24 h old culture of *Rhizobium* isolate was spot inoculated at the corner of the plate and incubated for four to eight days at 27°C. Antifungal activity was indicative as mycelia growth of test fungus inhibited in the direction of active bacteria, the level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist colony from the bacterial growth radius. The width of the inhibition zone between the pathogen and bacteria was evaluated as the inhibition zone (Dennis and Webster, 1971) using below mentioned formula.

$$\% \text{ inhibition} = \frac{\text{Pathogen growth in control (mm)} - \text{Pathogen growth in treatment}}{\text{Growth of pathogen in control (mm)}} \times 100$$

Results and Discussion

Source and details of microbial cultures used in the experiment

Twenty-five root nodule samples were collected from the black gram plants cultivated in Raichur and Kalaburagi (Table 1).

Isolation and purification of *Rhizobium* sp. isolates

All 25 isolates of *Rhizobium* sp. (RR-1 to RR-25) were sub cultured and agar slants were made with three duplicates of each isolate to preserve the isolates, which were then routinely utilized for a variety of tests.

Morphological characterization of *Rhizobium* sp. isolates

The findings of the analysis of the colony colour, shape, size, motility and Gram staining of all 25 *Rhizobium* isolates are shown in (Table 2).

Colony morphology

Rhizobium sp. isolates on YEMA medium showed a range of colony features, including round to irregular shapes, small to medium sizes and white to pale white coloration. According to Meti *et al*. (2015), 40 rhizobial colonies usually displayed white, creamy, circular colonies on YEMA following 72 hours of incubation, which supports these findings.

Gram staining

Each of the 25 isolates of *Rhizobium* sp. was Gram-negative. Similar findings were obtained by Sushma *et al*. (2017) reported that all 40 isolates were Gram negative and short rods characteristic of *Rhizobium* sp. (Table 3).

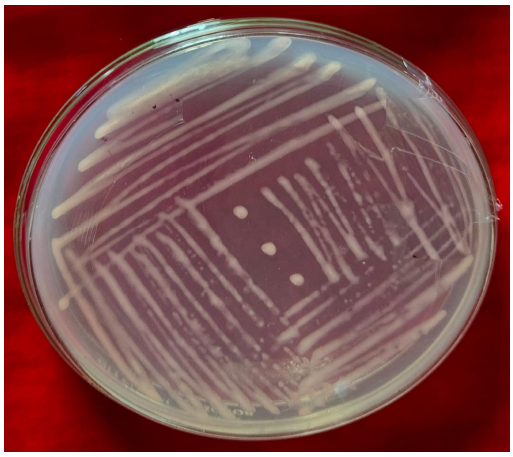

Plate 1: Pure culture of *Rhizobium*

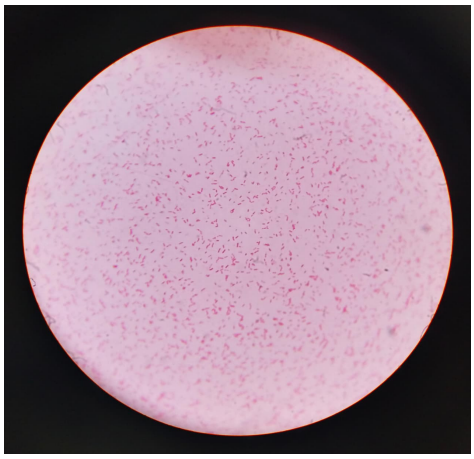

Plate 2: Gram staining of *Rhizobium*

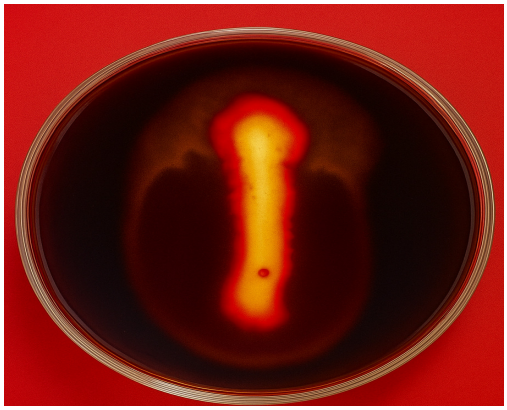

Plate 3: Starch hydrolysis

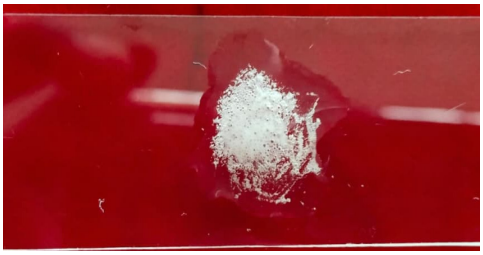

Plate 4: Catalase test

Motility

All *Rhizobium* sp. isolates were motile (Table 3). Motility is an important trait that helps rhizobia move toward root exudates, initiate infection threads and establish symbiosis with legumes. Our findings are consistent with what Fathy *et al*. (2021) found in the root nodules of *Vicia faba.*

Biochemical characterization of *Rhizobium* sp. isolates

For further identification the isolates were subjected to various biochemical tests and the results are presented in the (Table 3).

Congo red test

All 25 *Rhizobium* sp. isolates in the congored test did not absorb congored dye and remained white in colour. The results were consistent with those of Nagalingam *et al*. (2020), who reported that none of the isolates absorbed the congored color when streaked over CR-YEMA.

Indole test

Out of the 25 isolates, 15 *Rhizobium* sp. isolates were found to produce cherry ring, which validated the indole test's positive results. Similar findings were reported by Ustad *et al*. (2023), who found that four of the ten isolates from pigeon pea root nodules tested positive for indole test.

Methyl red test

Fourteen of the twenty-five isolates of *Rhizobium* sp. tested positive for the methyl red test. The results are in agreement with the 34 isolates of green gram that Sneka *et al*. (2022) identified, of which half tested positive for methyl red. According to Rasool *et al.* (2015), the test organism is MR positive since the MR medium stayed red after adding methyl red, a pH indicator showing that the organism produces acid.

Bromothymol blue test

In YEMA medium enriched with bromothymol blue, 12 of the 25 isolates of *Rhizobium* generated acid and became yellow after 48 hours of incubation.

Mondal *et al*. (2016) also found that all of the isolates DMP1, DMP2, DMP3, KNP4, KNP5, KNP6, KKP7, KKP8 and KKP9 developed a yellow colour, indicating that they are acidic in nature. Similar results were reported by Altai *et al*. (2022), who found that most rhizobial isolates coloured the YEMA-BTB medium yellow, indicating the formation of acid. Others however caused it to turn blue, indicating the creation of alkaline compounds.

Starch hydrolysis

Sixteen out of the twenty-five isolates tested positive for starch hydrolysis. The outcomes corroborated the findings of Editha *et al*. (2017), who found that five out of the 55 isolates screened had the capacity to hydrolyse starch.

Catalase test

All 25 *Rhizobium* sp. isolates were showed positive results for catalase test. As stated by Meti *et al*. (2015), all 40 isolates showed catalase positive and oxygen effervescence around the bacterial colonies when exposed to hydrogen peroxide.

***In vitro* screening of *Rhizobium* sp. isolates for their plant growth promoting potential**

All 25 *Rhizobium* sp. isolates went through various *in-vitro* screening tests and the results of each investigation were presented below (Table 4).

***In vitro* Nitrogen fixing ability of *Rhizobium* sp. isolates**

The ability of the *Rhizobium* sp. isolates to fix nitrogen *in vitro* was assessed, the findings are shown in (Table 4). The isolates nitrogen fixation ranged from 6.60 mg/g to 20.25 mg/g of mannitol used. Isolate RR-5 has the maximum nitrogen fixation (20.25 mg/g), followed by the reference strain (19.40 mg/g). On the other hand, isolates RR-6 (6.98 mg/g) and RR-24 (6.60 mg/g) had the lowest nitrogen fixation.

Related research was conducted by Seidu *et al.* (2025), Nitrogen fixation and plant growth promotion (PGP) tests revealed that, all ten *Bradyrhizobium* isolates had nitrogen fixing abilities. Specifically, isolate P4A18 demonstrated a greater ability to fix nitrogen by exhibiting maximum growth. Tu *et al*. (2021) revealed that the isolate Bra6 showed the highest nitrogen-fixing activity *in vitro*. Co-inoculation of Bra6 with *Pseudomonas* sp. Pse2 in sterile flask experiments significantly enhanced nodulation, biomass, and nutrient uptake compared to controls.

***In vitro* IAA production**

Amount of Indole Acetic Acid (IAA) produced by each isolate of *Rhizobium* sp., All 25 isolates were found to be capable of producing IAA with quantities varying between 5.38 µg/ml and 20.28 µg/ml of the medium. Out of them, isolate RR-5 produced the most IAA (20.28 µg/ml).

Rhizobium sp. isolated from the root nodules of a leguminous shrub that yields pulses, *Cajanus cajan*,

were shown to produce substantial levels of indole acetic acid during growth on basal medium supplemented with L-tryptophan, according to similar findings published by (Datta and Basu, 2000).

According to parallel research by Wanjofu *et al*. (2022), every *Mesorhizobium* strain tested positive for the synthesis of IAA. LMG17149 produced the least amount of IAA, whereas the commercial strain IC59 produced the greatest.

Out of the ten isolates examined, only eight were able to produce Indole Acetic Acid (IAA), according to Verma and Pal (2020) findings. Additionally, PGP-2 produced the most IAA out of the eight isolates, followed by PGP-9, PGP-5 and PGP-10.

Phosphate solubilization

Phosphate solubilization by *Rhizobium* sp. isolates showed variation, with solubilization zones ranging from 11.02 mm to 15.02 mm. Isolate RR-5 recorded the highest solubilization zone (15.02 mm). The colonies were formed clear halo zones indicate phosphate solubilization efficiency, showing a direct relationship between zone size and solubilizing ability.

Corresponding results were obtained by (Sridevi and Mallaiah, 2009) *Rhizobium* isolates tested on Pikovskaya's agar exhibited varying phosphate-solubilizing capacities. The isolate from *Cassia absus* showed the maximum solubilization, while the strain from *Sesbania sesban* recorded the lowest. And related research was conducted by Alikhani *et al*. (2006) Rhizobial isolates showed considerable ability to mobilize inorganic P, with 44% solubilizing TCP. Among them, *Rhizobium leguminosarum* bv. *viciae* exhibited the highest P solubilization. Satyanandam *et al*. (2013) reported, among the 19 strains tested, only eight strains (VM-1, VM-2, VM-3, VM-8, VM-9, VM-11, VM-15 and VM-17) formed clear zone around the colonies was noted after 24 h of incubation on Pikovskaya's agar medium, with the zone size increasing up to 72 h.

Ammonia production (NH3)

Among 25 *Rhizobium* isolates tested for ammonia production, one isolate exhibited strong production (+++), 6 isolates demonstrated moderate production (++), the remaining isolates displayed low production (+) of ammonia and 1 isolate tested negative for ammonia production. Development of the yellow colour was observed, indicating a positive result for the test.

Verma and Pal (2020) found that eight of the ten rhizobacterial isolates examined produced a large amount of ammonia, whereas the other two, PGP-3 and PGP-6 were shown to produce ammonia rather weakly. Similar findings were reported by Ahmad *et al.* (2008), who found that all isolates produced ammonia.

Hydrogen cyanide production (HCN)

Plant growth-promoting rhizobacteria (PGPR) are known to produce hydrogen cyanide as one of their primary biocontrol mechanisms. Six of the 25 isolates of *Rhizobium* that were examined for the ability to produce hydrogen cyanide (HCN) scored positive. Two isolates out of them showed moderate (++) HCN production and four isolates showed low production of HCN.

Similar findings were made by Kumar *et al*. (2012) found that two isolates, FBJ6 and FBS4 produced HCN. According to Manasa *et al.* (2017), eight of the fifteen *Rhizobium* isolates produced HCN. Furthermore, GNR-1 received a moderate (++) score for HCN production, while RR-1 demonstrated strong (+++) HCN production out of the eight isolates. However, it was shown that the remaining six isolates MR-2, BGR-1, GNR-2, GGR-2, SFR-2, and SYR-1 were poor (+) in their synthesis of HCN.

Biocontrol efficiency of *Rhizobium* sp. bacterial isolates

Out of the 25 *Rhizobium* sp. isolates tested, 10 isolates, namely RR-2 (30.62%), RR-5 (41.74%), RR-7 (30.18%), RR-8 (19.51%), RR-12 (27.92%), RR-15 (16.81%), RR-18 (24.22%), RR-19 (14.44%), RR-21 (33.36%) and RR-25 (36.18%) exhibited varying degrees of inhibition against the pathogen *Fusarium* sp., indicating their potential biocontrol efficiency.

Parallel studies conducted by Nagalingam *et al*. (2020) isolated 8 *Rhizobium* isolates, screened for antagonist activity against fungal growth in dual culture assay. Out of these, 3 isolates showed inhibition potential *viz*, ISSA-2 (55.40%), ISSA-3 (38.80%) and ISSA-4 (50.70%).

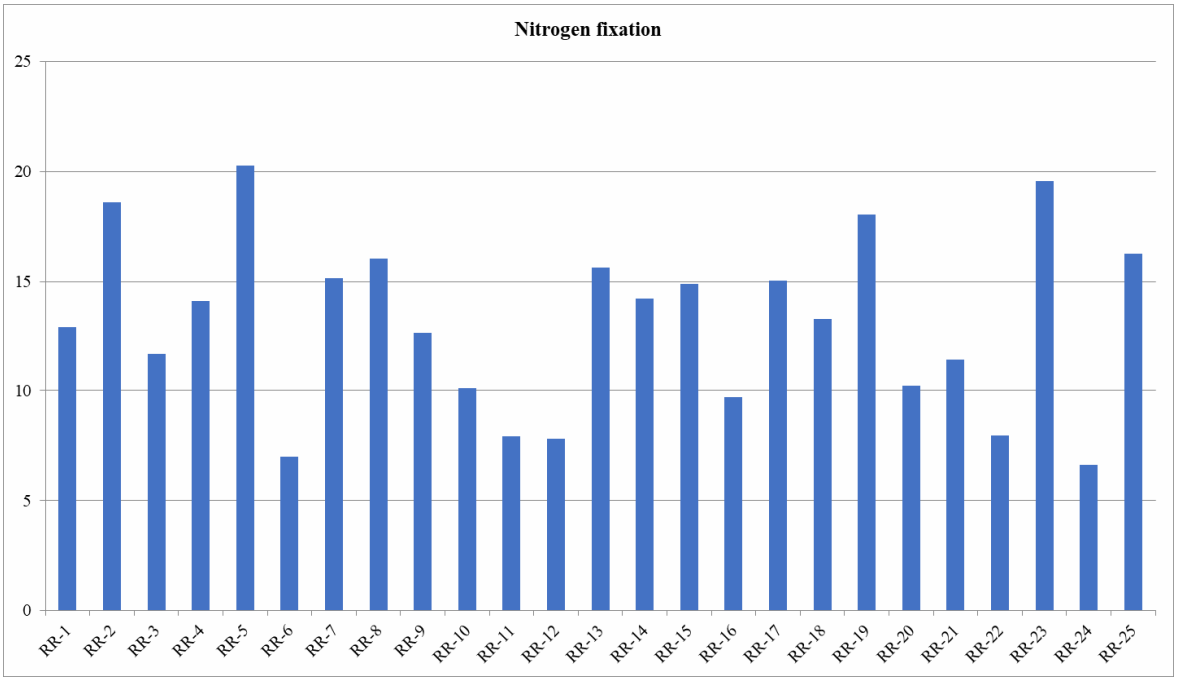

Fig. 1 : *In vitro* Nitrogen fixation for plant growth promoting characteristics of *Rhizobium* spp. isolates

Conclusion

The study successfully isolated, characterized and screened twenty-five *Rhizobium* isolates from the root nodules of black gram (*Vigna mungo* L.), confirming their identity as Gram-negative, motile, rod-shaped bacteria with distinct biochemical traits. In vitro screening revealed considerable variability among isolates in plant growth-promoting attributes such as nitrogen fixation, IAA production, phosphate solubilization, ammonia, and HCN production, as well as biocontrol activity against *Fusarium* sp. Among all isolates, RR-5 consistently exhibited superior performance across multiple traits, indicating its strong potential as an efficient bioinoculant. The findings highlight the significance of selecting native, high-performing *Rhizobium* strains to enhance black gram productivity and contribute to sustainable pulse cultivation through eco-friendly biofertilization.

Table 1 : Details of samples collection from nodules of black gram crop from different location

SI. No.	Place	Crop	Isolate code	Source of sample
1	New area, UASR	Black gram	RR-1	Root nodule
2	New area, UASR	Black gram	RR-2	Root nodule
3	New area, UASR	Black gram	RR-3	Root nodule
4	CoA Raichur	Black gram	RR-4	Root nodule
5	CoA Raichur	Black gram	RR-5	Root nodule
6	CoA Raichur	Black gram	RR-6	Root nodule
7	Seed unit, UASR	Black gram	RR-7	Root nodule
8	Seed unit, UASR	Black gram	RR-8	Root nodule
9	Seed unit, UASR	Black gram	RR-9	Root nodule
10	Kalmala	Black gram	RR-10	Root nodule
11	Kalmala	Black gram	RR-11	Root nodule
12	Kalmala	Black gram	RR-12	Root nodule
13	Kallur	Black gram	RR-13	Root nodule
14	Kallur	Black gram	RR-14	Root nodule
15	Kallur	Black gram	RR-15	Root nodule
16	Gabbur	Black gram	RR-16	Root nodule
17	Gabbur	Black gram	RR-17	Root nodule
18	Gabbur	Black gram	RR-18	Root nodule
19	ZARS, Kalaburagi	Black gram	RR-19	Root nodule
20	ZARS, Kalaburagi	Black gram	RR-20	Root nodule

21	ZARS, Kalaburagi	Black gram	RR-21	Root nodule
22	ZARS, Kalaburagi	Black gram	RR-22	Root nodule
23	ZARS, Kalaburagi	Black gram	RR-23	Root nodule
24	ZARS, Kalaburagi	Black gram	RR-24	Root nodule
25	ZARS, Kalaburagi	Black gram	RR-25	Root nodule

Table 2 : Morphological characteristics of *Rhizobium* spp. isolates isolated from root nodule of Black gram

Sl. No.	Isolate Code	Colony morphology			Microscopic characterization		
		Colony colour	Shape	Size	Gram staining	Cell shape	Motility
1	RR-1	White	Circular	Small	Negative	Rod	Motile
2	RR-2	Pale white	Irregular	Medium	Negative	Rod	Motile
3	RR-3	White	Irregular	Small	Negative	Rod	Motile
4	RR-4	White	Circular	Small	Negative	Rod	Motile
5	RR-5	Pale white	Circular	Medium	Negative	Rod	Motile
6	RR-6	Pale white	Circular	Small	Negative	Rod	Motile
7	RR-7	Pale white	Irregular	Small	Negative	Rod	Motile
8	RR-8	White	Circular	Medium	Negative	Rod	Motile
9	RR-9	White	Irregular	Small	Negative	Rod	Motile
10	RR-10	White	Irregular	Small	Negative	Rod	Motile
11	RR-11	Pale white	Irregular	Small	Negative	Rod	Motile
12	RR-12	White	Circular	Medium	Negative	Rod	Motile
13	RR-13	Pale white	Circular	Medium	Negative	Rod	Motile
14	RR-14	Pale white	Irregular	Small	Negative	Rod	Motile
15	RR-15	Pale white	Circular	Small	Negative	Rod	Motile
16	RR-16	White	Irregular	Small	Negative	Rod	Motile
17	RR-17	White	Irregular	Small	Negative	Rod	Motile
18	RR-18	White	Circular	Small	Negative	Rod	Motile
19	RR-19	Pale white	Circular	Medium	Negative	Rod	Motile
20	RR-20	White	Irregular	Small	Negative	Rod	Motile
21	RR-21	Pale white	Irregular	Medium	Negative	Rod	Motile
22	RR-22	Pale white	Irregular	Medium	Negative	Rod	Motile
23	RR-23	White	Circular	Small	Negative	Rod	Motile
24	RR-24	Pale white	Circular	Small	Negative	Rod	Motile
25	RR-25	Pale white	Irregular	Small	Negative	Rod	Motile

Table 3 : Biochemical characteristics of *Rhizobium* spp. isolates isolated from root nodules of black gram

SI. No.	Isolate code	Congored test	Indole test	Methyl red test	Bromothymol blue test	Starch hydrolysis	Catalase test
1	RR-1	-ve	-ve	+ve	-ve	+ve	+ve
2	RR-2	-ve	+ve	-ve	+ve	-ve	+ve
3	RR-3	-ve	-ve	+ve	-ve	-ve	+ve
4	RR-4	-ve	-ve	+ve	-ve	+ve	+ve
5	RR-5	-ve	+ve	+ve	+ve	+ve	+ve
6	RR-6	-ve	+ve	-ve	+ve	-ve	+ve
7	RR-7	-ve	+ve	-ve	+ve	+ve	+ve
8	RR-8	-ve	+ve	-ve	-ve	+ve	+ve
9	RR-9	-ve	+ve	+ve	+ve	-ve	+ve
10	RR-10	-ve	-ve	+ve	-ve	+ve	+ve
11	RR-11	-ve	-ve	+ve	+ve	+ve	+ve
12	RR-12	-ve	+ve	-ve	-ve	-ve	+ve
13	RR-13	-ve	-ve	+ve	-ve	-ve	+ve
14	RR-14	-ve	-ve	+ve	-ve	+ve	+ve
15	RR-15	-ve	+ve	+ve	+ve	+ve	+ve
16	RR-16	-ve	+ve	-ve	+ve	+ve	+ve
17	RR-17	-ve	-ve	-ve	-ve	-ve	+ve
18	RR-18	-ve	+ve	+ve	-ve	+ve	+ve
19	RR-19	-ve	+ve	-ve	+ve	-ve	+ve

20	RR-20	-ve	-ve	+ve	-ve	+ve	+ve
21	RR-21	-ve	+ve	-ve	+ve	+ve	+ve
22	RR-22	-ve	-ve	+ve	-ve	+ve	+ve
23	RR-23	-ve	+ve	-ve	+ve	-ve	+ve
24	RR-24	-ve	+ve	+ve	+ve	+ve	+ve
25	RR-25	-ve	+ve	-ve	-ve	+ve	+ve

+ve: Positive for the test, -ve: Negative for the test

Table 4 : *In vitro* screening for plant growth promoting characteristics of *Rhizobium* spp. isolates

SI. No.	Isolate code	Nitrogen fixation (mg/g)	IAA (µg/ml)	Ammonia production	HCN Production	Phosphate Solubilization (mm)	*Fusarium* sp. (% inhibition)
1	RR-1	12.92^{j}	11.86^{hi}	+	-	13.98^{cde}	0.00
2	RR-2	18.60^{c}	11.20^{j}	+	+	11.9^{ijkl}	30.62
3	RR-3	11.65^{k}	12.35^{h}	++	-	11.77^{kl}	0.00
4	RR-4	14.11^{h}	15.11^{f}	+	-	13.36^{f}	0.00
5	RR-5	20.25^{a}	20.28^{a}	+++	++	15.02^{a}	41.74
6	RR-6	6.98^{o}	13.65^{g}	+	-	11.2^{mn}	0.00
7	RR-7	15.15^{g}	15.23^{f}	+	-	12.88^{g}	30.18
8	RR-8	16.05^{e}	8.88^{l}	+	-	14.07^{cd}	19.51
9	RR-9	12.65^{j}	16.03^{d}	++	-	13.6^{ef}	0.00
10	RR-10	10.11^{l}	7.88^{m}	+	-	11.55^{lm}	0.00
11	RR-11	7.92^{n}	5.38^{o}	+	-	11.02^{n}	0.00
12	RR-12	7.80^{n}	6.83^{n}	+	+	12.23^{ij}	27.92
13	RR-13	15.64^{f}	10.54^{k}	+	-	14.22^{bc}	0.00
14	RR-14	14.23^{h}	14.94^{f}	+	-	11.7^{kl}	0.00
15	RR-15	14.90^{g}	15.46^{ef}	++	-	11.83^{jkl}	16.81
16	RR-16	9.70^{m}	12.16^{h}	+	+	13.39^{f}	0.00
17	RR-17	15.05^{g}	15.77^{de}	++	-	12.32^{hi}	0.00
18	RR-18	13.30^{i}	11.44^{ij}	-	+	12.69^{gh}	24.22
19	RR-19	18.03^{d}	7.60^{m}	+	-	13.4^{f}	14.44
20	RR-20	10.20^{l}	13.23^{g}	+	-	11.11^{mn}	0.00
21	RR-21	11.40^{k}	17.50^{c}	++	-	12.28^{hij}	33.36
22	RR-22	7.95^{n}	18.06^{b}	+	-	13.75^{def}	0.00
23	RR-23	19.55^{b}	19.86^{a}	++	++	11.48^{lmn}	0.00
24	RR-24	6.60^{o}	8.48^{l}	+	-	11.12^{mn}	0.00
25	RR-25	16.25^{e}	12.27^{h}	+	-	12.02^{ijk}	36.18
26	Ref.strain	19.40^{b}	19.80^{a}	+	+	14.6^{b}	40.20

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

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