ISOLATION, CHARACTERIZATION AND SCREENING OF *Bacillus* spp. FROM RHIZOSPHERE SOILS OF BLACK GRAM (*Vigna mungo* L.)

ABSTRACT

The present study entitled "Isolation, characterization and screening of *Bacillus* spp. from rhizosphere soil 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 rhizosphere soil samples were collected from black gram fields in Raichur and Kalaburagi. Twenty-five isolates of *Bacillus* were purified and subjected to morphological and biochemical characterization. Morphological analysis revealed that the isolates grown on Nutrient Agar were Gram-positive, motile, endospore-forming rods. Biochemical characterization showed that all isolates were positive for methyl red and catalase tests, 14 were positive for starch hydrolysis, and 11 for urease test, while all were negative for indole test. All 25 *Bacillus* isolates were further screened for their plant growth-promoting potential. IAA production ranged from 5.06 to 8.44 µg/ml, with isolate BR-15 showing the highest level. Phosphate solubilization zones varied from 13.11 to 17.30 mm, where BR-15 again exhibited the maximum activity. In ammonia production, one isolate was strongly positive (+++), four were moderate (++), and three were negative. Eight isolates exhibited antifungal activity against *Fusarium* sp., with BR-15 recording the highest inhibition (62.50%). Overall, isolate BR-15 demonstrated superior PGP and biocontrol potential, highlighting its promise as an effective bioinoculant for sustainable black gram production.

Keywords: *Bacillus*, black gram, IAA production, phosphate solubilization, biocontrol activity, bioinoculant.

1. INTRODUCTION

Black gram (*Vigna mungo* L.) belongs to the family leguminaceae and regarded as the third most important pulse crop in India. India remains the largest producer and consumer, contributing over 70% of global production, with an output of 2.78 million tonnes from 4.63 million hectares and an average productivity of 600 kg/ha (Ministry of Agriculture and Farmers Welfare, 2021-2022). Major growing states include Madhya Pradesh, Uttar Pradesh, Maharashtra and Karnataka, where it is cultivated mainly during the *kharif* season.

Black gram is a short-duration, warm-season crop that matures within 90-120 days. It is valued for its nutritional richness, containing 24-26% protein, 60% carbohydrates, and

Commented [AA1]: The purpose of this research is not yet clear about the benefits of this isolation

Commented [AA2]: The aim and problem of this research is whether it is only to find pure isolates or is there another aim in applying the results of the isolates?

Commented [AA3]: Has research ever been conducted on the isolation of Bacillus sp microbes from other types of plants?

minerals such as potassium, phosphorus, and calcium. Apart from being an important source of dietary protein, it serves as green manure, cover crop, forage, silage, and hay.

Among PGPR microbes, *Bacillus* is one of the most potential genera due to their spore forming ability, *Bacillus* sp. are ubiquitous in nature and are often found in higher concentrations in plant-associated soils thereby increasing the adaptation of *Bacillus* strain to commercial formulation and field application (Liu and Sinclair, 1993). *Bacillus* is frequently isolated from rhizosphere, these species are also common plant endophytes. The Gram positive bacterium *Bacillus subtilis* is known to positively influence plant growth, vitality and the ability of the plant to cope with pathogens often resulting in higher yield. *Bacillus mucilaginous* has been reported for its capability of solubilizing potassium (Wu *et al.,* 2005) and phosphate (Idriss *et al.,* 2002).

2. MATERIALS AND METHOD

2.1 Survey and collection of rhizosphere soils of black gram

Commented [AA4]: The reason for taking 25 soil samples located in Raichur and Kalaburagi is not yet clear.

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

2.2 Isolation and biochemical characterization of *Bacillus* spp. from black gram

2.2.1 Isolation and purification of *Bacillus* spp. from rhizosphere soil

Bacillus spp. was isolated from collected rhizosphere soil samples by serial dilution plating on Nutrient agar medium. Sterile water blanks (9 ml) were prepared in test tubes by autoclaving. Then 1 g of collected soil sample was weighed and transferred to the 9 ml sterile water blank which gives 10^{-1} dilution. Same procedure was repeated up to 10^{-6} and 10^{-7} dilution. Then 0.1 ml of suspension from appropriate dilution (10^{-6} and 10^{-7}) was transferred to the Petri plate containing nutrient agar medium. Three replications were maintained for each dilution. these Petri plates were incubated in an inverted position at 30^{o} C for 48 hours (Pankaj Kumar *et al.*, 2012).

The bacterial colonies exhibiting the creamy white colonies were selected, purified, sub cultured and stored on the slants of nutrient agar for further morphological and biochemical studies.

2.3 Morphological and biochemical characterization of *Bacillus* spp.

All the selected isolates were checked for their purity and then studied for the colony morphology and pigmentation. The cell shape and Gram reaction were recorded as the standard procedure given by (Barthalomew and mittewar, 1950).

2.3.1 Colony character

Colony characters of all isolates were studied on solid surface of Nutrient Agar medium in Petri dishes. The observation on colour, shape and size of the colonies were recorded.

2.3.2 Gram's staining

A loopful of inoculum from 24 h old culture was taken and mixed with a drop of sterile distilled water placed in the centre of the slide. The suspension was spread out on slide using the tip of inoculation needle to make a thin suspension. The smear was dried in air and fixed through mild heating by passing the lower side of the slide three to four times over the flame. Then each smear was covered with crystal violet for 30 seconds followed by washing each slide with distilled water for few seconds. Then each slide was covered with gram's iodine solution for 60 seconds, then washed with 95% ethyl alcohol followed with distilled water and drained. Safranin was then applied for 30 seconds and washed with distilled water and dried. The bacteria that appeared purple was referred to as Gram-positive and those that will be pink as Gram-negative (Aneja, 2003).

2.3.3 Endospore staining

Smears of organisms to be tested for endospores were prepared and heat fixed. Smear was covered with a piece of absorbent paper cut to fit the slide and will be placed on a wire gauze on a ring stand. Paper was saturated with malachite green and slide was heated until steam was seen rising from the surface. Reheating was done as needed to keep the slide steaming for about three minutes. As the paper began to dry malachite green was added drop by drop to keep it moist, so that the temperature is not appreciably reduced. Later the paper was removed with tweezers and the slide thoroughly rinsed with tap water. Further the slide was drained and counterstain with 0.5% safranin for 45 seconds and washed with tap water,

blot dried and examined. The vegetative cells appeared red and the spores appeared green (Aneja, 2003; Harley and Prescott, 2002).

2.3.4 Indole test

Test cultures were inoculated into pre-sterilized SIM agar tubes and incubated at 28 ± 2 °C for 48 hours. Following incubation, 10 drops of Kovac's reagent were added to each tube. The appearance of a red coloration was considered positive for indole production.

2.3.5 Methyl red test

The test bacteria were aseptically inoculated into MR-VP broth using a sterilized inoculating needle. After incubation, a few drops of alcoholic methyl red solution were added to each tube. The development of a red colour indicated the conversion of glucose to stable acids, confirming mixed acid fermentation and a positive methyl red test.

2.3.6 Starch hydrolysis

The ability of the isolates to hydrolyse starch were examined. Starch agar plates were inoculated with the test culture and incubated at 30°C for three days. After incubation the plates were flooded with Lugol's iodine solution, allowed to stand for 15-30 minutes and observed for clear zone around the colony which indicates hydrolyses of starch (Eckford, 1972).

2.3.7 Urease test

Overnight grown culture was inoculated with sterilized urea broth and incubated for 48 hrs at 37°C. Change in colour of the broth from orange to pink was taken as a positive for the test. (James and Sherman, 1992).

2.3.8 Catalase test

The isolates were flooded with 1ml of 3% hydrogen peroxide and observed for production of gas bubbles. The occurrence of gas bubbles was recorded positive for catalase activity (Blazevic and Ederer, 1975).

2.4 Screening of *Bacillus* sp. isolates for its beneficial traits

Commented [AA5]: Why not do PCR and SDS page tests so that the phylogenetic tree can really be found?

2.4.1 Indole acetic acid (IAA) production

IAA production in 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 30 ml Nutrient broth supplemented with DL- tryptophan at the rate of 100 µg/ml and incubated at 30°C for eight days under stationary conditions of growth. After centrifugation 2 ml of Salkowaski reagent was added to 2 ml of culture supernatant, mixed and allowed to stand for 30 min for the development of pink colour and colour intensity was estimated at 500 nm using spectrophotometer against a reagent blank.

2.4.2 Phosphate solubilization

All the isolates were screened for phosphate solubilization. Modified Pikovskaya's agar with insoluble Dicalcium phosphate, a loopful of each culture placed on 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).

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

2.4.3 Ammonia production

Freshly grown bacterial cultures were inoculated into 10 ml of peptone water in each tube and incubated at 28 ± 2 °C for 48–72 hours. Following incubation, 0.5 ml of Nessler's reagent was added to each tube, and the development of a yellow coloration was observed (Cappuccino and Sherman, 1992).

2.4.4 Biocontrol efficiency of bacterial isolates

Biocontrol efficiency was screened by a dual culture method in which both bacterial isolate and test fungi was 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 *Bacillus* 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.

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

3. RESULTS AND DISCUSSION

3.1 Source and details of microbial cultures used in the experiment

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

3.2 Isolation and purification of *Bacillus* spp. isolates

All 25 isolates of *Rhizobium* spp. (RR-1 to RR-25) and *Bacillus* spp. (BR-1 to BR-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.

3.3 Morphological characterization of *Bacillus* spp. isolates

Based on a thorough microscopic and morphological analysis that included evaluations of colony appearance, cell shape, motility and Gram staining, the isolates of *Bacillus* species were characterized. Morphological characteristics of each isolate are presented in (Table 2).

3.3.1 Colony morphology

The isolates colony morphology varied greatly, their colours ranged from creamy to white, their sizes ranged from small to medium and their forms were either irregular or round. This pattern has also been described by Sandeep *et al.* (2011), who found that all of the

standard strains and isolated cultures formed completely white, round, smooth and shiny colonies when seen morphologically.

3.3.2 Gram staining

All 25 isolates showed crystal violet colour on the Gram reaction, indicating that they were Gram positive. These findings concurred with those of Chari *et al.* (2018), who found that on microscopic examination, all forty-four isolates tested positive for the Gram reaction and endospore development.

3.3.3 Endospore staining

Endospore staining results for all *Bacillus* isolate tested were positive. These results are in line with Baumann's (2004) description of *Bacillus* species as endospore-forming, Gram-positive bacteria that are frequently found in soils.

3.3.4 Motility

All *Bacillus* spp. isolates were found to be motile. The outcomes are consistent with those of Schwenk *et al.* (2022), who also noted that motility was a prevalent feature of isolates of *Bacillus* species.

3.4 Biochemical characterization of *Bacillus* spp. isolates

The biochemical studies pertaining to chemical reactions were performed on all 25 isolates. All biochemical test findings were presented in (Table 3).

3.4.1 Indole test

All 25 isolates of *Bacillus* spp. had negative indole test results. The findings are comparable to those of a study conducted by Lakkannavar (2020), who isolated and described *Bacillus* sp. from cereal rhizosphere soil. All forty isolates had negative Indole test results.

Fig 1. Pure culture of *Bacillus*

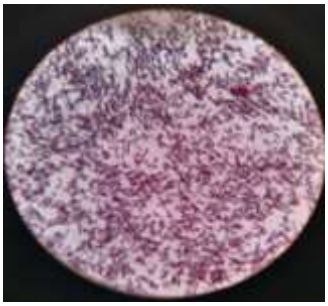

fig.2* Gram staining of *Bacillus

3.4.2 Methyl red test

All 25 *Bacillus* isolates were positive for methyl red test. Alif (2016) discovered that five of the eight *Bacillus* isolates examined were methyl red positive, which is consistent with similar findings.

3.4.3 Starch hydrolysis

Of the 25 isolates of *Bacillus* spp., 14 demonstrated successful starch hydrolysis. This indicates their ability to secrete extracellular amylase enzymes that break down starch into simple sugars. Similar findings were reported by (Baumann, 2004).

3.4.4 Urease test

Eleven of the 25 isolates had positive test results, while the other 14 had negative results. In a similar study (Lakkannavar, 2020) found that out of 40 isolates, 22 had positive test results and the remaining 18 had negative results.

3.4.5 Catalase test

All twenty-five isolates shown positive result for the catalase test. Awais *et al.* (2007) reported similar outcomes, with the isolates demonstrating catalase positive.

3.5 Screening of *Bacillus* spp. isolates for their plant growth promoting potential

All 25 *Bacillus* spp. isolates were subjected to various *in-vitro* screening tests and the results of each investigation were presented below (Table 4).

3.5.1 Indole acetic acid production by *Bacillus* spp. isolates

Bacillus spp. isolates were cultivated in a tryptophan-containing culture medium to determine their capacity to produce IAA. A pink colour appeared upon the use of Salkowski's reagent, signifying the production of IAA. A spectrophotometer was used to measure the quantity of IAA. IAA production varied between 5.06 µg/ml and 8.44 µg/ml across the 25 isolates. The greatest amount was produced by isolate BR-15 (8.44 µg/ml), which was followed by BR-25 (8.26 µg/ml). BR-2 produced the least amount of IAA (5.06 µg/ml).

A greenhouse experiment by Akinrinlola *et al*. (2018) showed that seven out of the twelve tested strains, including all strains of *Bacillus safensis* and *Bacillus megaterium*, produced indole-3-acetic acid (IAA). Elsoud *et al*. (2023) isolated seven isolates Far1, Far13, Far20, Far21, C1, R8 and S5. These isolates were screened on nutrient broth for IAA production among them, isolates Far1, R8 and S5 were the highest IAA producers.

3.5.2 Phosphate solubilization by *Bacillus* spp. isolates

Phosphate solubilization by *Bacillus* spp. isolates showed variation, with solubilization zones ranging from 13.11 mm to 17.30 mm. Isolate BR-15 recorded the highest solubilization zone (17.30 mm), followed by BR-25 (16.73 mm). The smallest zone was observed in BR-6, measuring 13.11 mm. The clear halo zones formed around the colonies indicate phosphate solubilization efficiency, showing a direct relationship between zone size and solubilizing ability.

According to Shika devi *et al.* (2023), seven isolates (SR3, SR2, SR11, SR13, SR20, SR56, and SR18) exhibited phosphorus-solubilizing ability. Among these, isolate SR2 recorded the largest solubilization zone on Pikovskaya’s agar with a phosphate solubilization index of 11 mm, surpassing the other bacterial isolates.

3.5.3 Ammonia production

Among the 25 *Bacillus* isolates tested for ammonia production, one isolate showed strong (+++), 4 isolates showed moderate (++), the majority showed low production (+) and 3 isolates were negative. Development of a yellow colour indicated a positive reaction.

Similar findings were reported by Ahmad *et al.* (2008), who observed that all rhizobacterial isolates tested produced ammonia, thereby enhancing nutrient availability for host plants.

3.5.4 Biocontrol efficiency of *Bacillus* spp. bacterial isolates

Out of the 25 *Bacillus* spp. isolates tested, 8 isolates, namely BR-1 (25.25%), BR-3 (47.50%), BR-5 (40.25%), BR-9 (57.25%), BR-10 (50.50%), BR-15 (62.50%), BR-23 (15.00%) and BR-25 (42.25%) exhibited varied percentage of inhibition against the pathogen *Fusarium* sp., indicating their potential biocontrol efficiency.

Similar observations were reported by Shikadevi *et al.* (2023) screened four isolates (SR13, SR2, SR18, and SR56) for antagonistic activity against *Fusarium oxysporum* and *Rhizoctonia solani*. Among these, isolates SR2 and SR18 exhibited significant inhibition zones against *F. oxysporum*. The maximum growth inhibition recorded by SR2 and SR18 against *F. oxysporum* was 67% and 48% respectively in contrast to the control.

Table 1. Details of samples collection from rhizosphere soils of black gram crop from different location

SI. No.	Place	Crop	Isolate code	Source of sample
1	New area, UASR	Black gram	BR-1	Rhizosphere soil
2	New area, UASR	Black gram	BR-2	Rhizosphere soil
3	New area, UASR	Black gram	BR-3	Rhizosphere soil
4	CoA Raichur	Black gram	BR-4	Rhizosphere soil
5	CoA Raichur	Black gram	BR-5	Rhizosphere soil
6	CoA Raichur	Black gram	BR-6	Rhizosphere soil
7	Seed unit, UASR	Black gram	BR-7	Rhizosphere soil
8	Seed unit, UASR	Black gram	BR-8	Rhizosphere soil
9	Seed unit, UASR	Black gram	BR-9	Rhizosphere soil
10	Kalmala	Black gram	BR-10	Rhizosphere soil
11	Kalmala	Black gram	BR-11	Rhizosphere soil
12	Kalmala	Black gram	BR-12	Rhizosphere soil
13	Kallur	Black gram	BR-13	Rhizosphere soil
14	Kallur	Black gram	BR-14	Rhizosphere soil
15	Kallur	Black gram	BR-15	Rhizosphere soil
16	Gabbur	Black gram	BR-16	Rhizosphere soil
17	Gabbur	Black gram	BR-17	Rhizosphere soil
18	Gabbur	Black gram	BR-18	Rhizosphere soil
19	ZARS, Kalaburagi	Black gram	BR-19	Rhizosphere soil
20	ZARS, Kalaburagi	Black gram	BR-20	Rhizosphere soil
21	ZARS, Kalaburagi	Black gram	BR-21	Rhizosphere soil
22	ZARS, Kalaburagi	Black gram	BR-22	Rhizosphere soil
23	ZARS, Kalaburagi	Black gram	BR-23	Rhizosphere soil
24	ZARS, Kalaburagi	Black gram	BR-24	Rhizosphere soil
25	ZARS, Kalaburagi	Black gram	BR-25	Rhizosphere soil

Table 2. Morphological characteristics of *Bacillus* spp. isolates isolated from rhizosphere soils of black gram.

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

Table 3. Biochemical characteristics of *Bacillus* spp. isolates isolated from rhizosphere soils of black gram

SI. No.	Isolate code	Indole test	Methyl red test	Starch hydrolysis	Urease test	Catalase test
1	BR-1	-ve	+ve	+ve	-ve	+ve
2	BR-2	-ve	+ve	-ve	-ve	+ve
3	BR-3	-ve	+ve	+ve	+ve	+ve
4	BR-4	-ve	+ve	-ve	-ve	+ve
5	BR-5	-ve	+ve	+ve	-ve	+ve
6	BR-6	-ve	+ve	-ve	-ve	+ve
7	BR-7	-ve	+ve	+ve	+ve	-ve
8	BR-8	-ve	+ve	-ve	-ve	+ve
9	BR-9	-ve	+ve	-ve	+ve	+ve
10	BR-10	-ve	+ve	+ve	-ve	+ve
11	BR-11	-ve	+ve	-ve	-ve	+ve
12	BR-12	-ve	+ve	+ve	-ve	+ve
13	BR-13	-ve	+ve	-ve	+ve	+ve
14	BR-14	-ve	+ve	+ve	+ve	+ve
15	BR-15	-ve	+ve	+ve	+ve	+ve
16	BR-16	-ve	+ve	+ve	-ve	+ve
17	BR-17	-ve	+ve	-ve	+ve	+ve
18	BR-18	-ve	+ve	+ve	+ve	+ve
19	BR-19	-ve	+ve	+ve	-ve	+ve
20	BR-20	-ve	+ve	+ve	-ve	+ve
21	BR-21	-ve	+ve	-ve	-ve	+ve
22	BR-22	-ve	+ve	-ve	+ve	+ve
23	BR-23	-ve	+ve	+ve	+ve	+ve
24	BR-24	-ve	+ve	+ve	-ve	+ve
25	BR-25	-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 *Bacillus* spp.

SI. No.	Isolate code	IAA production (µg/ml)	P Solubilization (mm)	Ammonia production	*Fusarium* sp. (% inhibition)
1	BR-1	6.52^{ij}	14.01^{fgh}	+	25.25
2	BR-2	5.06^{o}	13.20^{j}	-	0.00
3	BR-3	5.93^{klm}	13.45^{ij}	+	47.50
4	BR-4	7.14^{efgh}	15.74^{bc}	+	0.00
5	BR-5	6.89^{hi}	14.22^{efg}	+++	40.25
6	BR-6	6.24^{jkl}	13.11^{j}	+	0.00
7	BR-7	5.83^{lm}	13.76^{hi}	+	0.00
8	BR-8	7.52^{de}	14.58^{de}	++	0.00
9	BR-9	7.77^{cd}	15.38^{c}	+	57.25
10	BR-10	5.64^{mn}	13.88^{j}	+	50.50
11	BR-11	7.38^{defg}	16.04^{b}	-	0.00
12	BR-12	6.93^{ghu}	14.32^{def}	+	0.00
13	BR-13	5.37^{no}	14.69^{d}	+	0.00
14	BR-14	6.24^{jkl}	13.80^{ghi}	+	0.00
15	BR-15	8.44^{a}	17.30^{a}	++	62.50
16	BR-16	6.98^{fghi}	15.39^{c}	+	0.00
17	BR-17	7.45^{def}	13.74^{hi}	+	0.00
18	BR-18	7.27^{efgh}	13.83^{ghi}	+	0.00
19	BR-19	6.06^{jklm}	16.12^{b}	++	0.00
20	BR-20	5.12^{o}	13.55^{hij}	+	0.00
21	BR-21	7.85^{bcd}	13.86^{ghi}	+	0.00
22	BR-22	6.33^{jk}	15.44^{c}	-	0.00
23	BR-23	6.12^{jklm}	14.54^{de}	+	15.00
24	BR-24	5.90^{klm}	15.98^{b}	+	0.00
25	BR-25	8.26^{ab}	16.73^{a}	++	42.25
26	Ref.strain	8.057^{abc}	17.70^{a}	+	60.25

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

4. CONCLUSION

Commented [AA6]: The main goal is to support the growth of black bean plants. This can be included in the introduction.

Twenty-five *Bacillus* isolates were obtained from the rhizosphere soils of black gram and characterized as Gram-positive, motile, endospore-forming rods showing diverse biochemical traits. Most isolates were positive for catalase and methyl red tests, while several showed starch hydrolysis and urease activity. *In vitro* screening revealed variation in PGP traits, with BR-15 showing the highest IAA production (8.44 µg/ml), phosphate solubilization (17.30 mm), and maximum inhibition of *Fusarium* sp. (62.50%). Overall, BR-15 emerged as the most efficient isolate with strong plant growth-promoting and biocontrol potential, indicating its suitability as a promising bioinoculant for sustainable black gram cultivation.

5. REFERENCES

Ahamad, F., Ahmad, I., & Khan, M. S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research, 163(2), 173-181. https://doi.org/10.1016/j.micres.2006.04.001

Akinrinlola, R. J. (2018). *Evaluation of Bacillus strains for plant growth-promotion potentials on corn (Zea mays), wheat (Triticum aestivum), and soybean (Glycine max)* (Master's thesis, University of Nebraska). https://digitalcommons.unl.edu/agronhortdiss/137/ .

Alif, F. A., 2016, Isolation and identification of orange M2R and green GS dye degrading bacteria from textile sludge (soil) samples and determination of optimum growth conditions. *Ph. D. Thesis*, BRAC Univ., Dhaka (Bangladesh).

Aneja, K. R. (2003). Experiments in microbiology, plant pathology and biotechnology. New Age International. https://books.google.com/books/about/Experiments_In_Microbiology_Plant_Pathol.html?id=2_1xAAAAMAAJ .

Directorate of Economics and Statistics, Ministry of Agriculture and Farmers Welfare, Government of India. (2022). *Crop production statistics information system*. https://upag.gov.in/ .

Awais, M., Shah, A. A., Hameed, A., & Hasan, F. (2007). Isolation, identification and optimization of bacitracin produced by Bacillus sp. *Pak. J. Bot.*, *39*(4), 1303-1312. https://www.pakbs.org/pjbot/papers/148_PJB-2007-148.pdf .

Mittwer, T., Bartholomew, J. W., & Kallman, B. J. (1950). The mechanism of the Gram reaction. II. The function of iodine in the Gram stain. Stain Technology, 25(4), 169-179. https://doi.org/10.3109/10520295009110986 .

Bauman, R. W., 2004, Microbiology, Pearson Education Inc., San Francisco, CA

Blazevic, D. J., & Ederer, G. M. (1975). Principles of biochemical tests in diagnostic microbiology. Wiley. https://library.nust.na/cgi-bin/koha/opac-detail.pl?biblionumber=112183 .

Cappuccino, J. C. and Sherman N., 1992, Microbiology: A Laboratory Manual, New York, 1: 125-79.

Chaitra, L., 2020, Interaction effect of *Bacillus* spp. and *Glomus intraradices* on growth and yield of ragi. *M.Sc. Thesis*, Univ. Agric. Sci. Raichur (India).

Chari, K. D., Reddy, R. S., Triveni, S., Trimurtulu, N., Rani, C. V. D., & Sreedhar, M. (2018). Isolation and characterization of abiotic stress tolerant plant growth promoting Bacillus spp. from different rhizospheric soils of Telangana. *Biosciences Biotechnology Research Asia*, *15*(2), 485-494. https://doi.org/10.13005/bbra/2653 .

Dennis, C., & Webster, J. (1971). Antagonistic properties of species-groups of Trichoderma. I. Production of non-volatile antibiotics. Transactions of the British Mycological Society, 57(1), 25-39. https://doi.org/10.1016/S0007-1536(71)80077-3 .

Devi, S., Sharma, S., Tiwari, A., Bhatt, A. K., Singh, N. K., Singh, M., Kaushalendra, & Kumar, A. (2023). Screening for multifarious plant growth promoting and biocontrol attributes in Bacillus strains isolated from indo gangetic soil for enhancing growth of rice crops. Microorganisms, 11(4), 1085. https://doi.org/10.3390/microorganisms11041085 .

Eckford, M. O. (1927). Thermophilic bacteria in milk. American Journal of Hygiene, 7(3), 201–221. https://doi.org/10.1093/aje/7.3.201 .

Elsoud, M. M. A., Hasan, S. F., & Elhateir, M. M. (2023). Optimization of Indole-3-acetic acid production by Bacillus velezensis isolated from Pyrus rhizosphere and its effect on plant growth. Biocatalysis and Agricultural Biotechnology, 50, 102714. https://doi.org/10.1016/j.bcab.2023.102714 .

Gordon, S. A., & Weber, R. P. (1951). Colorimetric Estimation of Indoleacetic Acid. Plant Physiology, 26(1), 192-195. https://doi.org/10.1104/pp.26.1.192 .

Idriss, E. E., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R., Bochow, H., Richter, T., & Borriss, R. (2002). Extracellular phytase activity of Bacillus amyloliquefaciens FZB45 contributes to its plant-growth-promoting effect. Microbiology, 148(7), 2097-2109. https://doi.org/10.1099/00221287-148-7-2097 .

James, C. R. and Sherman, N., 1992, Microbiology and laboratory manual, Rockland Community college, Suffern, New York, 3rd ed. The Benjamin/Cummings Publishing *Co. Inc*., Redwood city, California.

Kumar, P., Dubey, R.C., & Maheshwari, D.K. (2012). Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. Microbiological Research, 167(8), 493-499. https://doi.org/10.1016/j.micres.2012.05.002 .

Liu, Z. L., & Sinclair, J. B. (1993). Colonization of soybean roots by Bacillus megaterium B153-2-2. Soil Biology and Biochemistry, 25(7), 849-855. https://doi.org/10.1016/0038-0717(93)90087-R .

Pikovskaya, R. I., 1948, Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Microbiol*., 17: 362-370.

Prescott, L. M. and Harley, J. P., Laboratory Exercises in Microbiology, 2002, 5th Edition, McGraw-Hill Inc., New York.

Sandeep, C., Raman, R. V., Radhika, M., Thejas, M. S., Patra, S., Gowda, T., Suresh, C. K., & Mulla, S. R. (2011). Effect of inoculation of Bacillus megaterium isolates on growth, biomass and nutrient content of Peppermint. Journal of Phytology, 3(11), 19-24. https://updatepublishing.com/journal/index.php/jp/article/view/2731 .

Schwenk, V., Dietrich, R., Klingl, A., Märtlbauer, E., & Jessberger, N. (2022). Characterization of strain-specific Bacillus cereus swimming motility and flagella by means of specific antibodies. PLoS ONE. https://doi.org/10.1371/journal.pone.0265425 .

Wu, S., Jia, S., Sun, D., Chen, M., Chen, X., Zhong, J., & Huan, L. (2005). Purification and characterization of two novel antimicrobial peptides Subpeptin JM4-A and Subpeptin JM4-B produced by Bacillus subtilis JM4. Current Microbiology, 51(5), 292–296. https://doi.org/10.1007/s00284-005-0004-3 .