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Evaluation of *Rhizobacteria* for growth promotion in black gram (*Vigna mungo*)

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Contribution | **Nosha, O.** conducted the experimental work, collected the data, performed the statistical analysis and prepared the first draft of the manuscript. **B. Ali** designed the experiment and checked the final draft of the study.

Black gram (*Vigna mungo* L.) is a highly nutritious crop in Asia. The present study was aimed to evaluate the effect of the *Bacillus* strains on shoot length, fresh weight, dry weight, and number of pods of the black gram. Biochemical characterization was carried out for reliable identification. A seed germination assay was conducted with mixed cultures (Z-06+Z-43, Z-06+Z-07, Z-02+Z-43, Z-02+Z-07) of bacterial strains. Monocultures showed a germination percentage in the range of 14% to 50% while the mixed cultures showed a germination percentage in the range of 33% to 100%. Auxin bioassay was carried out in which strains were inoculated in the medium containing tryptophan and also without tryptophan. Z-07 and Z-43 demonstrated significantly more promising results, outperforming Z-02 and Z-06 by 83.3% and 62.5%, respectively. Analysis of auxin-producing strains was performed by Fourier Transformed Infrared spectroscopy (FTIR). A pot trial was carried out of the auxin-producing strains in monocultures and mixed cultures. Two harvestings were performed after 1 month and 2 months, respectively. In both harvestings, plant parameters (shoot length, fresh weight, dry weight, and number of pods) were improved after treating the seeds with the bacterial cultures as compared to control. The strains Z-07 and Z-43 showed enhanced results in the auxin bioassay, leading to notable increases in plant growth parameters.

Keywords: Plant growth-promoting *Rhizobacteria* (PGPR), bacterial auxin production, FTIR spectroscopy, seed germination assay.

INTRODUCTION: Black gram (*Vigna mungo* L.) is a very nutritious grain legume crop. The majority of black gram farms are located in South and Southeast Asia (Kaewwongwal *et al.*, 2015). In Pakistan, black gram was cultivated on 17,000 hectares of land during 2016-2017, yielding 7,000 tons of total production. Punjab is the primary province that produces black gram, although being grown throughout the nation. In comparison to other grain legumes, the productivity of black gram in the nation has remained relatively low (Qayyum *et al.*, 2019). It is regarded as a top-notch, simple-to-digest source of nutrient-dense, high-grade protein. The majority of Asians who eat vegetarian diets benefit greatly from its substantial lysine content (Goyal *et al.*, 2021). In South Asian vegetarian diets, it is a mainstay due to its high nutritional value. About 24%-26% protein, 60% carbohydrates, 1.3% lipids, phosphorous, potassium, iron, and calcium are found in mature dried black gram seeds. In addition, various important amino acids, vitamins, and minerals, such as vitamin B3, vitamin A, vitamin B1, and vitamin B2, are present (Nair *et al.*, 2024).

The rhizosphere of the plant contains a lot of microbes performing numerous functions and roles that are considered essential for plant growth. In this way, the health of the rhizosphere determines the health of the plants that reside in it (Hakim *et al.*, 2021). Microorganisms in soil are thought to be the soil's active compartment as they are involved in the decomposition and regulation cycles (Chodak *et al.*, 2013). The diversity of microorganisms in soil changes with the increase in depth of the soil (Brussaard *et al.*, 2007). Because of this, soil microbial diversity tells us about the health of the soil (Nielsen *et al.*, 2002).

Conventional agricultural practices, like the use of chemicals pesticides, herbicides, fungicides, and fertilizers, protect crop plants against diseases and improve output. Agricultural chemicals contain chemical compounds that pollute the land, atmosphere, and water, all of which are hazardous for the environment (Al-Ani, 2018). These substances threaten the diversity of soil microorganisms (Bikrol *et al.*, 2005; Santos *et al.*, 2020) and fungal communities (Streletskii *et al.*, 2022). They are also the cause of the extinction of fish (Weltje *et al.*, 2013), bees (Mukherjee *et al.*, 2022), and plants (Matarczyk *et al.*, 2002; Bruni *et al.*, 2013).

One of the most promising methods is the introduction of microbes that promote plant development; yet, our understanding of the molecular pathways underlying plant-microbe interactions is still lacking (Antoszewski *et al.*, 2022). Plants and related microbes interact in a variety of ways during agriculture. The oldest occupation in human history is agriculture, which has been carried out for generations (Gupta *et al.*, 2022).

Research on soil-beneficial microorganisms, and plant growth-promoting rhizobacteria (PGPR), has increased due to the elevation in the demand for sustainable agriculture (Hussain *et al.*, 2023). To achieve this goal of improving the plant's growth and development and consequently, its productivity, PGPRs are regarded as the most important organisms that affect the growth of the host (Zahoor *et al.*, 2017). A key factor in controlling the growth of plants is phytohormone production. Several phytohormones have been studied and are important for plant growth, including ethylene, salicylic acid, gibberellins, cytokine, auxin, and brassinosteroids. Moreover, salicylic acid and brassino steroids also contribute to the phytohormone biocontrol role (Orozco-Mosqueda *et al.*, 2023).

The structure of the root system has a crucial role in determining the uptake of resources below ground and, in turn, the overall fitness of the plant. Plant hormone auxin is necessary for almost all stages of root development, from cellular to systemic (Roychoudhry and Kepinski, 2022). The auxin coordinates numerous essential activities in plant growth, development, and environmental adaptability. Certain biosynthesis, homeostasis, transport, and signal transduction pathways are linked to auxin actions (Zhang *et al.*, 2022). The importance of auxin in the growth, development, and stress response of the model plant *Arabidopsis thaliana* and wheat, maize and rice (Gill *et al.*, 2021).

OBJECTIVES: In the present study, bacterial strains of the genus *Bacillus* were used, and biochemical characterization was done. The role of bacterial auxin on the plant growth parameters was determined using plant *V. mungo.* Auxin-producing strains were then inoculated to *V. mungo* in a pot trial. After the PGPR-*Vigna* interaction, the stimulatory effects of bacterial auxin were demonstrated on the plant's growth.

MATERIALS AND METHODS: **Bacterial strains:** The bacterial strains used in this study were *B. simplex* Z-02 (KT027590), *B. megaterium* Z-06 (KT027594), *B. pumilus* Z-07 (KT027595) and *Exiguobacterium acetylicum* Z-43 (KT027616). These strains were collected and purified from the culture collection of Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore.

Biochemical characterization: Gram staining and spore staining were carried out. After that, some basic biochemical testing was done including oxidase and catalase tests to determine if the strains can produce cytochrome c oxidase enzyme and catalase enzyme. Starch hydrolysis (to determine of the strains had ability to hydrolyze starch using enzymes), MR-VP (to determine if the bacterial strains were capable of fermenting several forms of glucose), SIM (to determine the motility and production of sulfide and indole by strains), urease (to identify if the strains had ability to hydrolyze urea into ammonia and carbon-dioxide), phosphate solubilization (to identify if the strains were capable of converting insoluble phosphorous to its soluble form), and citrate (to identify if strains could utilize citrate as the sole source of carbon and energy) tests were also performed. To evaluate the fermentation of sugars and hydrogen sulfide production, Triple sugar-iron (TSI) was performed. To assess the mineralization process carried out by the rhizobacteria, nitrate reduction test was performed to determine if the bacterial strains have ability to convert nitrates into nitrites. All apparatus and required media were autoclaved prior to use and biochemical testing was carried out under sterilized conditions. Protocols for each test were taken from the manual of James Cappucino and Sherman Natalie.

***In Vitro* auxin production by Rhizobacteria:** In vitro, auxin production of the plant growth-promoting rhizobacteria (PGPR)

was determined by using the L-tryptophan as the amino acid precursor. This bioassay was performed in the form of five replicates for each bacterial strain. The inoculum was given in L-broth supplemented with L-tryptophan. The incubation was done at 37°C for 72 h. After incubation, the 1.5 mL broth was transferred to an eppendorf and centrifuged at 10,000 rpm for 10 min. and 1 mL supernatant was transferred to a clean test tube comparing 2 mL Salkowski's reagent was added and incubated at dark for 30 min. and then analysed using a spectrophotometer at 535 nm.

FTIR analysis: For FTIR analysis, bacterial strains were inoculated to autoclaved L-broth. Supernatant (0.01 mL) was combined with 10 mL of ethyl acetate. Tubes were shaken vigorously and left overnight after adding ethyl acetate. The ethyl acetate fraction was separated and dried at 60°C in a water bath and methanol (2 mL) was used to dissolve the dried extracts. An FTIR analysis was performed to look at the extracted auxin.

Seed germination assay: For this purpose, the bacterial strains were inoculated in L-broth and incubated at 37°C for 24 h. The sterile double filter paper was placed in sterile petri plates moist with sterile distilled water. For sterilization seeds were soaked in $HgCl_2$ (0.1%) for 2 min. and washed with sterile distilled water at least 3 times. Each bacterial suspension (5 mL) was combined with 10 seeds per plate. This assay was also carried out with mixed cultures (Z-02 + Z-07, Z-06 + Z-43, Z-02 + Z-43, Z-06 + Z-07). Plates were incubated in dark for 5 days and number of germinated seeds were counted.

In-vitro analysis: L-agar was prepared and autoclaved before being poured into sterilized petri plates. Bacterial cultures were swabbed and subsequently transferred to test tubes containing autoclaved distilled water to create bacterial suspensions. The density of these suspensions was standardized to McFarland Standard 2, corresponding to approximately 600 million cells per milliliter. Each bacterial suspension (5 mL) was combined with 10 seeds per plate, which were then allowed to stand for 25 min. Following this incubation period, the bacterial culture treated seeds were sown in pots (25 x 27 cm) with three replicates of each bacterial strain, including a control group. The pots were watered regularly to maintain optimal growing conditions. The first harvest was conducted approximately one month after sowing, with a second and final harvest occurring two months post-sowing. During each harvesting phase, the following parameters were measured: shoot length (cm), fresh weight (g), and dry weight (g) of the plants. Additionally, the number and length of pods were recorded at the final harvest.

Statistical analysis: Data from auxin production and plant growth parameters was subjected to ANOVA by using SPSS software. The mean values were separated and compared by using Duncan's multiple range test ($P \leq 0.05$).

RESULTS AND DISCUSSION: Biochemical identification: For gram staining, all strains except Z-06 showed positive results (figure 5). All strains showed the formation of endospores. For the SIM test, Z-02 and Z-07 showed H2S production, none of the strains showed indole positive results, while, all strains tested positive for motility. All strains were capable of reducing nitrates into nitrites. For TSI, all strains except Z-43 showed blackening that indicates the presence of H2, while Z-43 showed the absence of carbohydrate fermentation results with red slant/ red butt (table 1).

Sr. No.	Biochemical Test	Z-02	Z-06	Z-07	Z-43
1	Catalase	Positive	Positive	Positive	Positive
2	Oxidase	Negative	Negative	Negative	Negative
3	Starch hydrolysis	Positive	Negative	Positive	Negative
4	Phosphate solubilization	Negative	Negative	Negative	Negative
5	Citrate	Positive	Positive	Positive	Positive
6	Urease	Negative	Negative	Negative	Negative
7	Nitrate	Positive	Positive	Positive	Positive
8	MR	Positive	Weakly Positive	Negative	Positive
9	VP	Negative	Negative	Negative	Negative

Table 1: Results of biochemical tests of all four strains.

***In Vitro* auxin production:** Without tryptophan, *B. simplex*, *B. megaterium*, *B. pumilus* and *Exi. acetylicum* produced 1.20 µg/mL, 1.20 µg/mL, 2.40 µg/mL and 0.80 µg/mL auxin, respectively. With tryptophan *B. simplex, B. megaterium, B. pumilus* and *Exi. acetylicum* produced 1.20 µg/mL, 1.20 µg/mL, 7.20 µg/mL and 3.20 µg/mL auxin, respectively (table 2).

Strains	Without Tryptophan (0µg/mL)	With Tryptophan (500µg/mL)
Z-02	1.20±0.20 (a)	1.20±0.20 (a)
Z-06	1.20±0.49 (a)	1.20±0.20 (a)
Z-07	2.40±0.40 (b)	7.20±0.97 (c)
Z-43	0.80±0.20 (a)	3.20±0.66 (b)

Table 2: Production of auxin by bacterial strains with or without tryptophan in vitro.

The capability of auxin-producing bacterial strains to promote soybean sprout plant growth was identified in the study. Twelve of the ninety isolates that were tested showed a significant increase in seedling development (Wahyudi *et al.*, 2011). In the present study, all the strains showed some extent (14%-50%) of seedling growth while the mixed cultures of *B. megaterium* + *Exi. acetylicum* and *B. megaterium* + *B. pumilus* showed 100% and 96% increase in growth. IAA, often known as native auxin, is a phytohormone. It is a signaling molecule that promotes the development of plant roots and boosts water absorption (Martínez-Viveros *et al.*, 2010). IAA causes the plant to produce more lateral and adventitious roots, loosens the cell wall, and release exudates that feed bacteria present in the rhizosphere (Ahemad and Kibret, 2014). Tryptophan was used as an amino acid precursor for *Bacillus* species to demonstrate synthesis of indole-3-acetic acid, and after adding Salkowski's reagent bacteria was capable of turning the color of the medium from yellow to pink.

The concentration of auxin produced by the bacterial strains was 6.57µg/ml (Rayavarapu and Padmavathi, 2016). Similar results were observed in a study conducted by Joseph (Joseph *et al.*, 2007), all strains of the *Bacillus* showed a significant increase in the synthesis of auxin by the strains. In the present study, similar results were observed. Two out of four (*B. pumilus* and *Exiguobacterium acetylicum*) bacterial strains under observation showed increased synthesis of auxin when supplemented with the precursor tryptophan in the medium while *B. simplex* and *B. megaterium* showed moderate auxin synthesis. A study conducted by Wahyudi *et al.* (2011) demonstrated that auxin produced by the *Bacillus* species was analyzed after adding L-tryptophan to the growth media with a spectrophotometric technique. The strains were observed to produce auxin in a medium containing tryptophan in it. The range of the auxin produced by the bacterial samples was 0.81-86.82 mg/ml. In the present study, the auxin concentration was also measured with a spectrophotometer and the range of auxin produced by bacterial strains was 0.8-7.2 µg/mL.

FTIR analysis: The peaks observed in the present study were at ~3300 and ~1020 indicating =C-H and C-OH stretch, respectively as shown in figure 1.

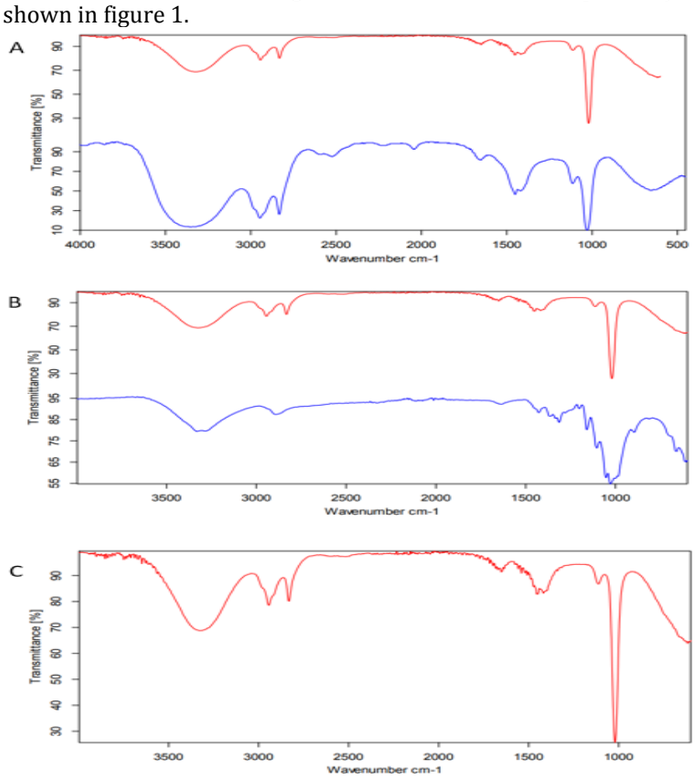

Figure 1: (A) Library spectra and Query spectrum (red), (B) Reference spectra and Query spectra (red), (C) Spectrum of B. simplex obtained by FTIR analysis.

Auxin estimation was done using the HPLC (High-performance liquid chromatography) technique and also FTIR-analysis. Both techniques showed the maximum peak of auxin produced by bacterial samples, nearly equal to the standard peak (Yadav *et al.*, 2022). Another study carried out the quantification of auxin using Thin Layer Chromatography (TLC), Gas Chromatography, and FTIR analysis. All the techniques showed the presence of auxin produced by the bacterial strains (Lakshmanan *et al.*, 2022). In the present study, the quantification of auxin was carried out by FTIR analysis and a large peak was noticed.

Seed germination: For monocultures, *B. simplex* treated seeds showed 50% germination, *B. megaterium* treated seeds showed 14% germination, *B pumilus* treated seeds showed 14% germination and *Exi. acetylicum* showed 46% germination. For mixed cultures, *B. megaterium* + *Exi. acetylicum* showed 100% seed germination, *B. megaterium* + *B. pumilus* showed 76% seed germination, *B. simplex* + *Exi. acetylicum* showed 33% seed germination and *B. simplex* + *B. pumilus* showed 95% seed germination. As a whole, seed germination increased when seeds were treated with monocultures and mixed cultures. Seed germination is shown in figure 2.

Pot trials with black gram: Different parameters were measured after harvesting including fresh weight, dry weight and length of the plant. Length of plants was measured with the help of a ruler while fresh weight and dry weight were measured by using weighing balance. Also, the number of pods were evaluated and their length was measured with the help of a ruler. The length of pods in comparison to the control is shown in figure 4. On first harvesting, strains and their mixed cultures showed only marginal improvements in shoot length and fresh weight, however, Z-07 showed statistically significant results in the dry weight of the plant. On second harvesting, strains and mixed cultures showed no improvement in shoot length; Z-02 and Z-43 showed significant improvement in fresh weight; Z-02, Z-43, Z-06+Z-07 showed significant results in dry weight of the plant. On the other hand, for pod length, all strains and their mixed cultures showed a marginal increase (table 3).

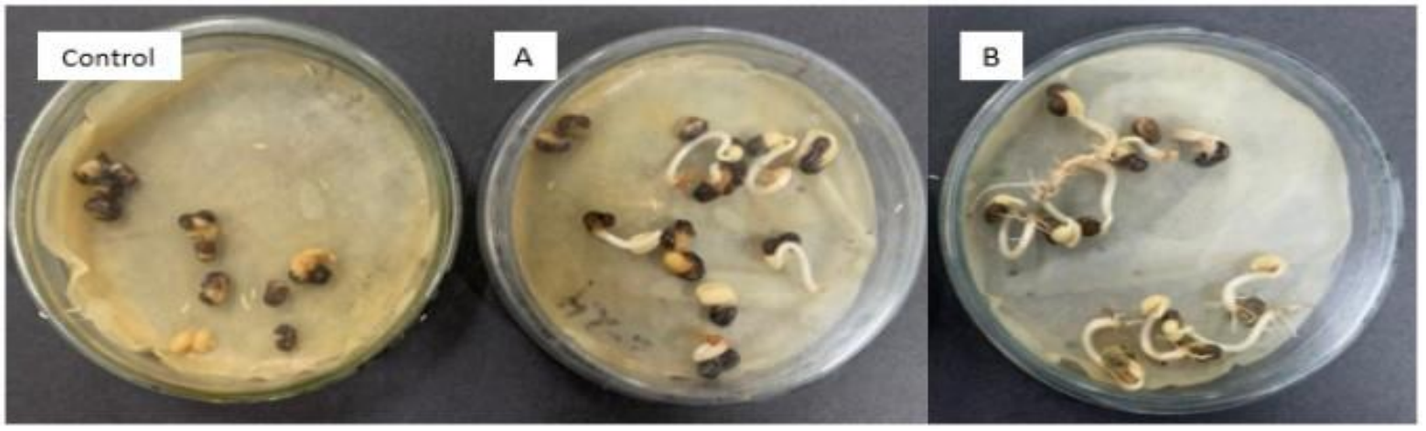

Figure 2: Germination of seeds when treated with *B. simplex* (A) and the mixed cultures of *B. megaterium* and *B. pumilus* (B).

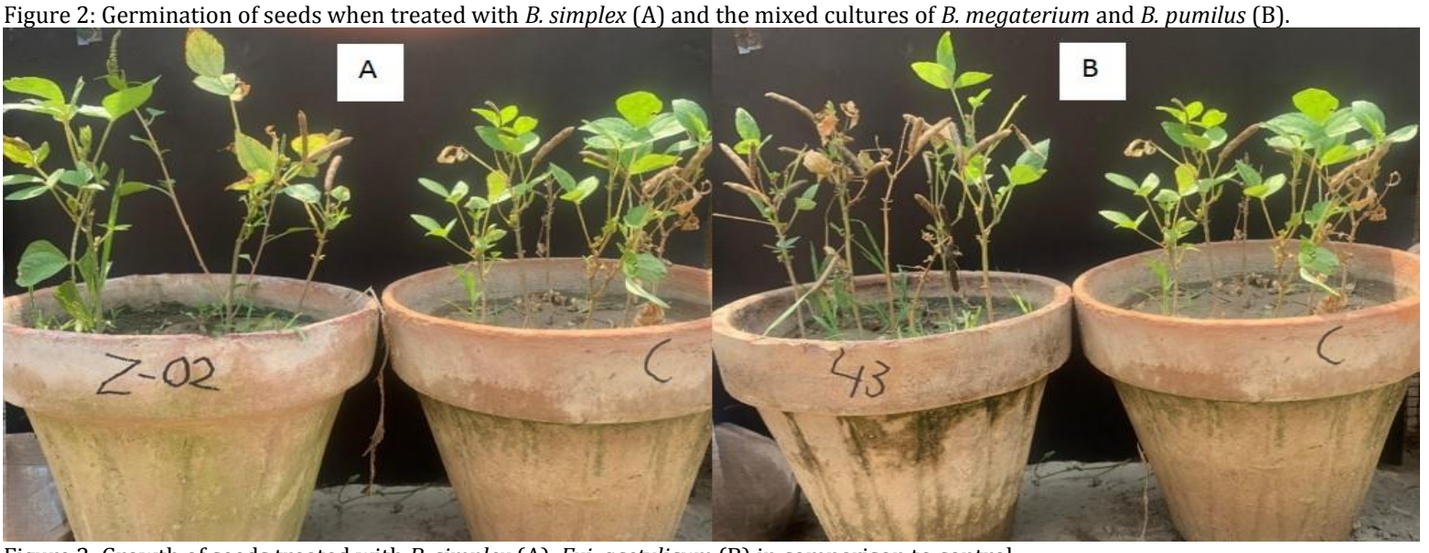

Figure 3: Growth of seeds treated with *B. simplex* (A), *Exi. acetylicum* (B) in comparison to control.

Figure 4: Length of pods grown by seeds treated with *Exi. acetylicum* (A) and *B. megaterium* + *B. pumilus* (B) in comparison to control

Strains	Shoot Length (cm)	Fresh Weight (g)	Dry Weight (g)
		One month	
Control	17.92±0.78 (a)	1.13±0.19 (a)	0.28±0.04 (ab)
Z-02	19.50±0.63 (a)	1.35±0.34 (a)	0.31±0.06 (ab)
Z-06	18.42±0.69 (a)	1.36±0.30 (a)	0.30±0.07 (ab)
Z-07	19.50±0.71 (a)	1.68±0.20 (a)	0.44±0.06 (b)
Z-43	19.33±1.41 (a)	1.60±0.28 (a)	0.30±0.04 (ab)
Z-02+Z-07	20.17±1.45 (a)	1.54±0.22 (a)	0.32±0.07 (ab)
Z-06+Z-43	17.00±1.47 (a)	0.97±0.38 (a)	0.36±0.08 (ab)
Z-02+Z-43	17.40±1.08 (a)	0.10±0.17 (a)	0.21±0.03 (a)
Z-06+Z-07	19.00±0.70 (a)	1.56±0.17 (a)	0.37±0.04 (ab)
		Two months	
Control	18.43±0.99 (b)	1.13±0.21 (ab)	0.53±0.06 (abc)
Z-02	17.00±1.24 (ab)	1.62±0.36 (b)	0.64±0.09 (bc)
Z-06	16.67±1.09 (ab)	1.36±0.36 (ab)	0.50±0.13 (abc)
Z-07	16.94±0.53 (ab)	1.13±0.15 (ab)	0.49±0.04 (abc)
Z-43	16.29±1.16 (ab)	1.42±0.31 (b)	0.80±0.15 (c)
Z-02+Z-07	16.91±0.75 (ab)	0.10±0.21 (ab)	0.42±0.07 (ab)
Z-06+Z-43	14.20±0.93 (a)	0.53±0.08 (a)	0.29±0.07 (a)
Z-02+Z-43	15.67±1.33 (ab)	0.81±0.22 (ab)	0.60±0.12 (abc)
Z-06+Z-07	17.36±0.72 (ab)	0.86±0.11 (ab)	0.68±0.10 (bc)
	Pod Length (cm)		
Control	2.85±0.15 (a)		
Z-02	3.04±0.12 (a)		
Z-06	3.30±0.38 (a)		
Z-07	2.99±0.13 (a)		
Z-43	3.28±0.16 (a)		
Z-02+Z-07	3.23±0.12 (a)		
Z-06+Z-43	3.14±0.17 (a)		
Z02+Z-43	3.05±0.10 (a)		
Z-06+Z-07	3.08±0.13 (a)		

Table 3: Effect of *Bacillus* strains on vegetative parameters of *V. mungo* (L.)

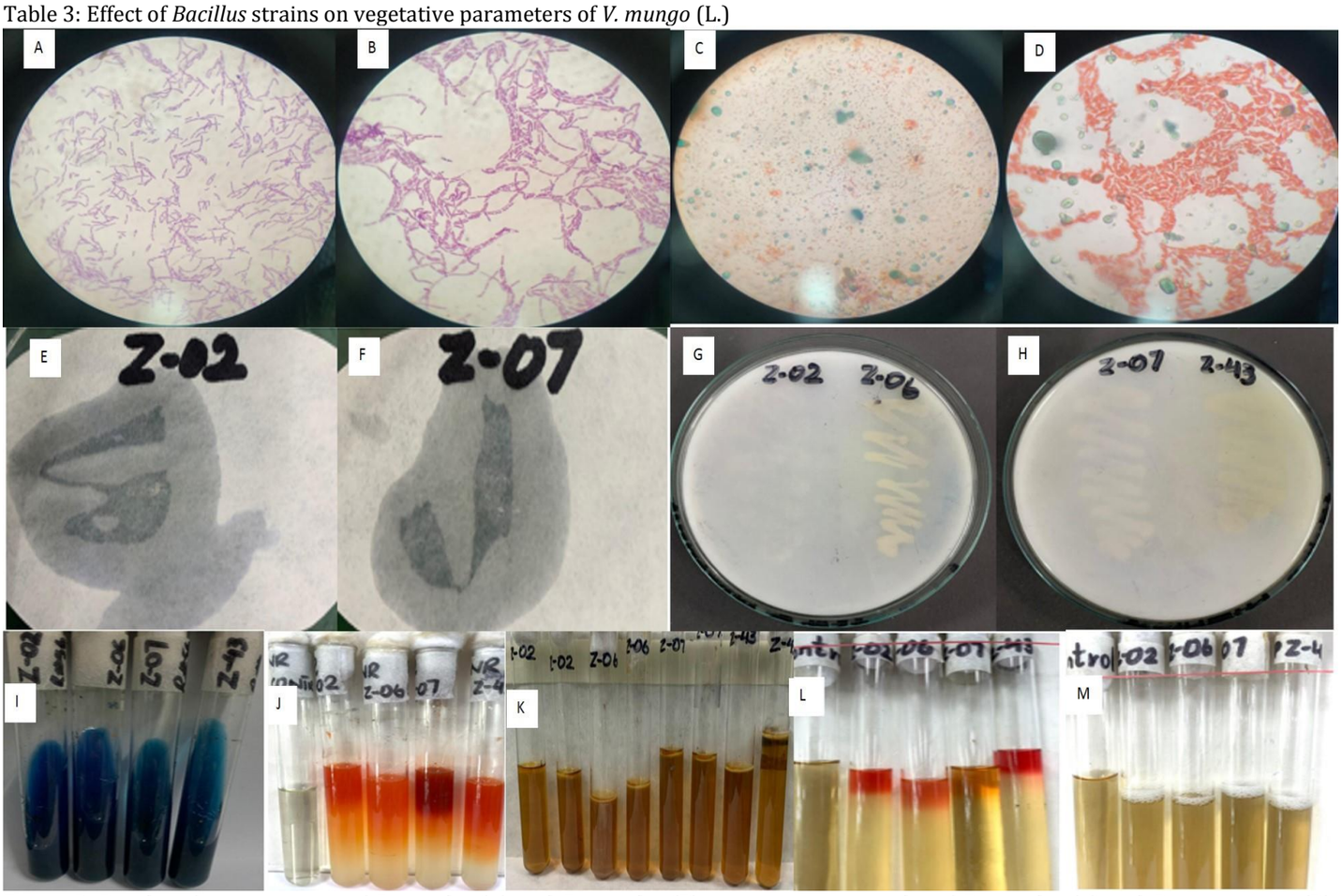

Figure 5: Results of gram staining of *B. simplex* (A) and *B. pumilus* (B), spore staining results of *B. pumilus* (C) and *B. megaterium* (D), Negative results of oxidase test *B. simplex* (E) and *B. pumilus* (F), negative results for phosphate solubilization test, positive results for citrate test (G), positive test results for nitrate reduction test (H), results of SIM (sulfur, indole, motility) test (J), positive and Negative test results for (K) MR, (L) VP.

A pot trial was carried out on *Vigna radiata* (L.) seeds treated with different *Bacillus* and *Rhizobium* strains. A significant difference was observed in plant growth parameters including root length and shoot length. Bacterial strains showed improvement in plants as compared to the plants treated with no bacteria i.e. control (Tanveer and Ali, 2022). In another study, PGPR plants in the absence of cadmium in soil showed significant results in plant growth parameters. However, in the presence of cadmium, PGPR-treated plants showed metal toxicity (Rabiya Ikram and Basharat Ali, 2018). Similar results were found in the study conducted by Swain (Swain *et al.*, 2007), who observed that IAA generated by *B. subtilis* has a beneficial effect on the edible tubercle *Dioscorea rotundata* L., increasing the stem and root's length and fresh weight. In the present study four *Bacillus* (Z-02, Z-06, Z-07, Z-43) and their mixed

cultures (Z-02+Z-07, Z-06+Z-43, Z-02+Z-43, Z-06+Z-07) treated seeds of *V. mungo* (L.) plant were observed for any change in plant growth parameters in pot trial. All the *Bacillus* strains showed a slight increase in the shoot length. Z-02 showed a significant improvement in fresh weight, while, Z-43 showed a significant increase in dry weight of the plant.

CONCLUSION: It is concluded that *Bacillus* strains have the ability to release good amounts of IAA under *in vitro* conditions. In pot trials, strains showed promising potential to enhance the vegetative and yield parameters of *V. mungo*. The bacterial strains (Z-02, Z-06, Z-07, and Z-43) have the potential to synthesize IAA and to promote plant growth in pot trials.

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LIFE SCIENCE REPORTING: In current research article no life science threat was reported

ETHICAL RESPONSIBILITY: This is original research, and it is not submitted in whole or in parts to another journal for publication purpose.

INFORMED CONSENT: The author(s) have reviewed the entire manuscript and approved the final version before submission.

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