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SCREENING *BACILLUS* STRAINS FOR AUXIN PRODUCTION AND THEIR POTENTIAL TO STIMULATE THE GROWTH OF *VIGNA RADIATA* (L.)

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Abstract

Auxin producing rhizobacteria are known to enhance plant growth and play a major role in the development of agronomical growth parameters. The main objective of this study was to evaluate the potential of Bacillus strains on the growth of Vigna Radiata (L.) under natural environmental conditions. The Microscopic analysis showed that strains were gram-positive and endospore former. Biochemical analysis revealed positive results for catalase and citrate test while negative for oxidase, urease, nitrate, TSI, and starch hydrolysis. The strains except Z-16 were tested negative for phosphate solubilization. Colorimetric analysis of strains at 0µg/ml of L-tryptophan recorded very low levels of auxin production. However, at 1000 µg/ml L-tryptophan, maximum auxin was produced by Bacillus subtilis Z-16 which was 85% over control. For pot trials surface sterilized seeds were treated with bacteria suspension and were sown into pots under natural environmental conditions. These trials resulted in maximum shoot length by strain of Bacillus subtilis Z-16 (85%), after 8 weeks of germination. Similarly, for fresh weight, B. subtilis produced recorded a 34% increase, over control. Analysis of dry weight proved that B. aerius Z-54 was giving maximum weight after 8 weeks of germination (12%). A mixture of Z-03 and Z-54 produced 27% improvement in shoot length while a 1-fold improvement in fresh weight. The analysis of all these vegetative growth characteristics proved that these strains can be used as effective bio fertilizers to enhance crop productivity.

Keywords: *Bio fertilizer, Phosphate solubilizing microorganism, Plant Growth-promoting rhizobacteria, Vigna radiata (L.)*

INTRODUCTION

The loose material that covers the majority of land is called soil. Many distinct types of soil are created by processes like leaching, weathering, and microbiological activity (1). The term plant growth promoting rhizobacteria (PGPR) also known as plant health promoting bacteria (PHPB) refers to bacteria colonizing roots of plant. The way PGPR aids plants involves several diverse processes. Auxins (IAA), cytokines, and gibberellins (GA), abscisic acid (ABA) are formed by some PGPR and encourage the growth of roots and shoots (2). Phosphorus and other mineral-based nutrients are soluble, which makes them more accessible to plants (3). Improved plant health and growth can result from enhanced interactions between microorganisms and plants due to the presence of phytohormones in the rhizosphere (4). They also help plants absorb nutrients and synthesize specific molecules, or they can shield plants (5).

Microorganisms can produce auxin through two different pathways: the tryptophan-dependent pathway, which gradually converts tryptophan to IAA, and the tryptophan-independent pathway, which uses alternative intermediates (6). Auxin affects many aspects of plant growth (7). It helps to maintain xylem differentiation and reduces dropping of fresh leaves and fruits; thereby promoting detachment of old leaves. It regulates the regulation of different essential genes in rhizobia which are involved in attachment of rhizobia to roots and plants signal regulators. This has also been used by gardeners for weed management and is produced at apex of shoot. However changes in auxin are the requirement for nodule organogenesis (8). Auxin also regulates the development of flowers, seed dormancy and growth tropism (9). *Bacillus aryabhattai* strain SRB02, isolated from the soybean rhizosphere, was found to produce significant amounts of gibberellins, IAA, and jasmonic acid (JA), which are known to boost soybean growth and lower oxidative stress (10).

There are various techniques for analyzing auxin production, including spectrophotometry, FTIR, HPLC, GC-MS, and LC-MS. Among these FTIR spectroscopy can quantitatively and qualitatively analyze the chemical components of cells, such as lipids, proteins, carbohydrates, biopolymers, and phyto-hormones like auxin, making it highly informative and repeatable (11).

Many *Bacillus* species, including *B. megaterium, B. circulans, B. coagulans, B. subtilis, B. azotofixans, B. macerans, B. velezensis,* and others, have been identified as PGPR. Various direct and indirect processes of *Bacillus* spp.-induced plant growth promotion include nitrogen fixation (BNF), phosphorus and other nutrient solubilization and mineralization and synthesis of phyto-hormones (12).

Mung bean *Vigna radiata* (L.) is one of the most significant pulse crops, farmed in tropical and subtropical climates worldwide. Mung beans can thrive in both dry and irrigated environments and have great potential for use in crop rotation systems and dry farming areas. They are important for agriculture (growing green manure crops to increase soil fertility), food, fuel, and the economy (13). The primary goal of this study was to determine the ability of *Bacillus* strains to produce auxin and to evaluate their potential to enhance the growth parameters of *Vigna radiata* (L.) under natural environmental conditions.

MATERIALS AND METHODS

BACTERIAL STRAINS

Different Bacillus strains i.e *Bacillus aryabhattai* (Z-03), *Bacilllus oceansediminis* (Z-14), *Bacillus subtilis* (Z-16), and *Bacillus aerius* (Z-54) were selected in present study. These strains were selected for their association with the rhizosphere and their plant growth promoting attributes. These *Bacillus* strains were revived from the microbial bank at the Institute of Microbiology and Molecular Genetics and refreshed on L-agar plates.

MORPHOLOGICAL CHARACTERIZATION OF BACTERIAL STRAINS

Gram staining was performed to analyze differential staining properties of bacterial cell wall. Seven glass slides were taken and were degreased. Thin bacterial smears of fresh cultures were flooded with crystal violet, iodine and safranin sequentially while washing at each step. The morphology, color and arrangement of cells were evaluated by observing slides under a 100X oil immersion microscope. For spore staining smear of 72-hour-old culture were air dried and heat fixed. Then, flooded the smear with 1% Malachite green with steam, and then after washing safranin was added as a counter stain. All slides were placed in a slanting position for drying purposes and were observed under a microscope under 100X oil immersion following manual protocols of Cappuccino and Sherman 2002.

BIOCHEMICAL CHARACTERIZATION

For biochemical testing catalase, oxidase, citrate, TSI, nitrate, urease test and starch hydrolysis tests were performed using procedures of Cappuccino and Sherman 2002 (14). The fresh culture was inoculated in the respective growth media and results were noted after 24-hour incubation.

PHOSPHATE SOLUBILIZING TEST

This test was performed to identify the ability of bacteria which solubilize phosphate in the medium. Sterile plates having Pikovskaya's agar media were streaked and incubated for 2 days for 37°C. After incubation, clear zone formation was noted for respective strains.

COLORIMETRIC ANALYSIS FOR IAA PRODUCTION BY BACTERIA

For auxin quantitative analysis two concentrations 0 and 1000µg/ml of L-tryptophan were used in duplicate. For one strain 4 flask having 15ml L- broth was prepared and autoclaved. A stock solution of L-tryptophan was made by adding 0.2 g L-tryptophan in 20 ml autoclaved distilled water. For 1000µg/ml concentration 1.5ml L-tryptophan from stock was added to L- broth. All the flasks were inoculated with respective Bacillus strains. Culture flasks were incubated at 130rpm on a shaker incubator at 37°C for 72 hours. After incubation, there were harvesting of bacterial cultures. Centrifugation of culture was done at

5000 rpm for 10 minutes to obtain supernatant. 1ml bacterial supernatant was dispensed in a test tube then 2 ml of Salkowski reagent was added in it. Test tubes were incubated in the dark in the absence of sunlight for 30 minutes. After incubation the pink color development was observed and optical densities were measured at 535nm.

FTIR ANALYSIS

This analysis was done to identify organic and inorganic components of bacterial extracts. The analysis is based on the method in which Infrared light changes the dipole moment of molecules having specific vibrational patterns. Strains were inoculated in L-broth with L-tryptophan added in it. After 48 hours of incubation cultures were centrifuged at 5000 rpm for 10 minutes and the supernatant was separated. The supernatants were subjected to FTIR analysis using transmission mode and wave number 400-4000cm^{-1}. Samples were simply placed in the path of an Infrared light wave. As the light passes through the sample the transmitted energy is measured and a peak was generated. The temperature of the room was maintained at 25ºC. The spectra were first preprocessed to avoid the variations due to spectral acquisition and to get accurate results.

POT TRAILS ASSAY

Four bacterial strains along with four mixed cultures were used in pot trials. Bacterial cultures were prepared in 10ml of autoclaved distilled water. For mixed cultures equal volumes of each culture were taken and mixed. The mixed cultures were used (Z-03, Z-54), (Z-16, Z-54), (Z-14, Z16), (Z-03, Z-14). The turbidity of bacterial suspension was adjusted to maintain a density equal to McFarland 2. Mung seeds *Vigna radiata* (L.) were sterilized by using 0.1% HgCl2 and then treated with bacterial suspensions. Seeds that were soaked in autoclaved distilled water were used as control. The sterile seeds were treated with bacterial suspension of 6.0×10^8 cells/ml of density. The dimensions of the pots were 25×27cm poured with soil and placed in the natural environment (wire house) at the Institute of Microbiology and Molecular Genetics. 10 seeds that were treated with the bacterial culture suspension were sown carefully by using sterile forceps at a distance of 10cm. Slight water was given to pots and allowed to germinate. Pots were placed under direct sunlight with average day temperature of 21°C.

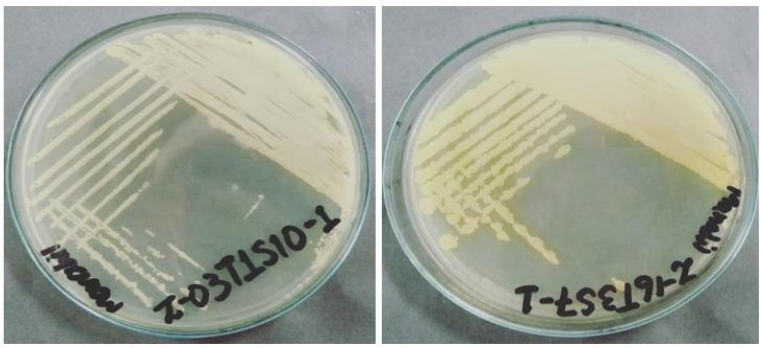
STATISTICAL ANALYSIS

Data from colorimetric analysis of auxin production and pot trials was subjected to analysis of variance (ANOVA) by using SPSS 20 software. Mean values from treatment were compared by using Duncan's Multiple Range Test (DMRT) ($p\leq0.05$) (a descriptive test) to find out significant differences among means of different groups.

RESULTS

BACTERIAL STRAINS

Different strains of *Bacillus* (Table I) were purified by repeated quadrant streaking method on L-agar as shown in Fig. 1.



(A) (B)

Fig. 1. Shows the growth of Z-14 and Z-54 by quadrant streaking method

Table I. List of *Bacillus* strains used in the current study with their GenBank accession numbers

Sr. No.	Strains	Identified as	Accessions
1.	Z-03	*Bacillus aryabhattai*	KT027591
2.	Z-14	*B. oceansediminis*	KT027602
3.	Z-16	*B. subtilis*	KT027604
4.	Z-54	*B. aerius*	KT027625

MORPHOLOGICAL CHARACTERISTICS OF BACTERIAL STRAINS:

All the strains were tested as gram-positive rod-shaped bacteria. Results of spore staining showed that all strains can form green endospores as indicated by the green color under the microscope. Results of biochemical analysis showed that all strains were positive for catalase and citrate test. The strains were tested to be negative for oxidase and urease. For the nitrate test Z-03 showed negative results and the rest showed positive activity. Results of the Triple sugar iron test showed Z-54 was giving positive activity with H_2S gas production while all others were tested to be negative. Strain Z-16 showed a positive result for starch hydrolysis while the rest were tested to be negative. All results were compiled in Table II.

Table II. Biochemical characterization of different bacterial strains

Sr. No.	Bacterial strains	Catalase test	Oxidase test	Citrate test	TSI test	Urease test	Nitrate test	Phosphate solubilizing test	Starch test
1.	Z-03	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
2.	Z-14	+ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve
3.	Z-16	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve
4.	Z-54	+ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve

PHOSPHATE SOLUBILIZING TEST

A strain of Z-16 showed phosphate solubilization as exhibited by a clear zone. All the other test strains were phosphate solubilizing negative. There was no clear zone around strains as shown in Fig. 2.

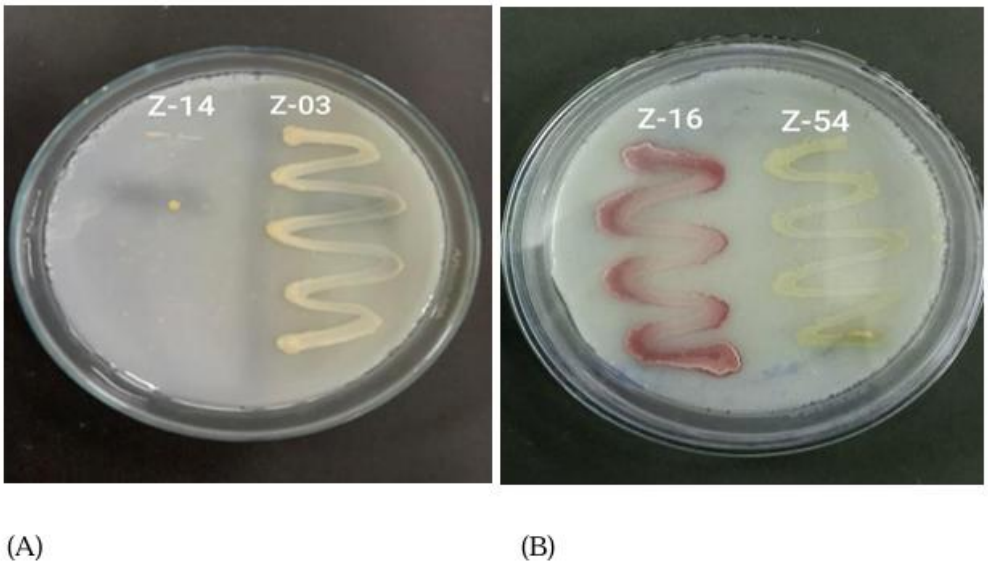


Fig. 2. Shows the results of phosphate solubilizing test. All strains are negative. There is no clear zone observed. While Z-16 strain showed positive activity

COLORIMETRIC ANALYSIS FOR IAA PRODUCTION BY BACTERIA

Colorimetric analysis showed that bacterial strains produced significant levels of auxin in the presence or absence of L-tryptophan in vitro. At 0µg/ml L-tryptophan strains Z-03, Z-14, Z-16, and Z-54 produced 0.6 µg/ml, 2.4 µg/ml, 1.0 µg/ml and 1.0 µg/ml auxin while at 1000 µg/ml L-tryptophan, significant levels of auxin were produced by Z-16 which was 85% over control. Similarly, Z-03 (82%), Z-54 (66%), and Z-14 (36%) also produce high levels of auxin as compared to control respectively as shown in Fig. 3.

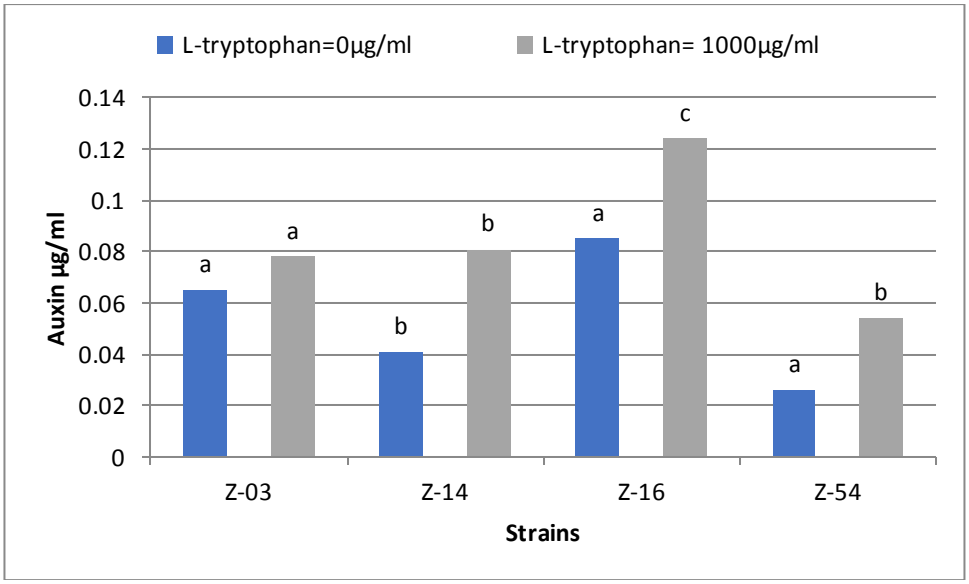


Fig. 3. Bacterial auxin production at two different concentrations of L-tryptophan 0µg/ml and 1000µg/ml. The bars represent the mean of five replicates. Different alphabets on bars show significant differences between treatments by Duncan's Multiple Range Test (DMRT) p≤0.05

FTIR ANALYSIS OF STRAIN Z-03 AND Z-16

The analysis was done to check auxin production by bacteria and peaks were obtained which confirmed the presence of auxin produced by these strains (Fig. 4). For strain Z-16 and Z-03, a peak of OH group was obtained at 3400cm^{-1}. An aromatic peak occurred at wave number 1450 cm^{-1} which manifested a double bond. Also a peak for carboxyl group appeared at wave number 2700 cm^{-1}. The peak at 1012 cm^{-1} shows a carbon nitrogen stretch. So comparison of these sample peaks with that of standard peaks confirmed the presence of indole acetic acid in our samples.

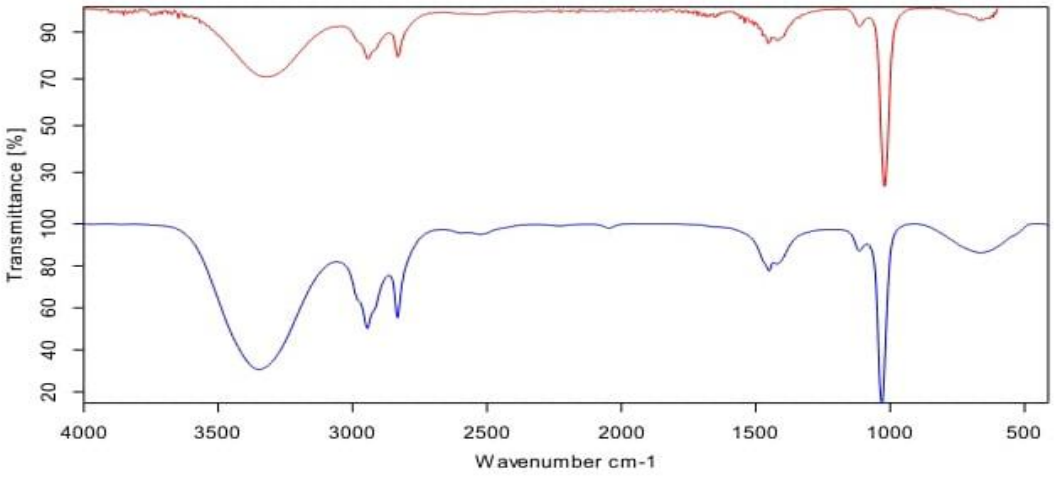


Fig. 4. FTIR analysis of Z-16 (blue peaks) in comparison with standard

POT TRIALS ASSAY

Vegetative parameters were measured after four weeks and eight weeks of seed germination and results were compiled. The results of single or mixed bacterial culture after 4 weeks of germination are shown in Figure 5 and 6. Maximum shoot length and fresh weight was observed by Z-16 and Z-54. For dry weight Z-03 and Z-54 gave maximum values after 4 weeks of germination (Fig. 7). After 8 weeks of germination Z-16 promoted shoot length that was 27% over control while Z-54 and Z-14 produced 18% and 9% improvement in shoot length. For fresh weight Z-16 promoted growth to its maximum that was 34% as compared to control. While Z-14 and Z-54 showed 8% and 3% improvement in fresh weight over control. Among the combinations C4 (Z-03, Z-14) promotes shoot length by 27% while, C3 (Z-14, Z-16) produced 18%, C1 (Z-03, Z-54) produced 18% and C2 (Z-16, Z-54) produced 9% improvement in shoot length. For fresh weight strain Z-16, Z-14 and Z-54 produced 34%, 8% and 3% improvement over control. While for

mixed cultures C1 (Z-03, Z-54) and C2 (Z-16, Z-54) produced 1.05 folds and 0.2 folds improvement in fresh weight as compared to control. For dry weight Z-54 gave maximum growth promotion that was 12% over control. While among mixtures C1 (Z-03, Z-54) and C3 (Z-14, Z-16) produced 27% and 25% improvement of dry weight as compared to control. Results were compiled and arranged in Table III.

Table III. Effects of single and mixed cultures of *Bacillus* strains on the growth of Vigna radiata (L.) after 8 weeks of seed germination under axenic conditions

Sr. No.	Strains	Fresh weight	Dry weight	Shoot length
1.	Control	0.57±0.11(ab)	0.47±0.08(a)	10.60±0.60(a)
2.	Z-03	0.52±0.66(a)	0.35±0.72(a)	10.75±0.94(a)
3.	Z-14	0.62±0.11(ab)	0.37±0.51(a)	11.55±0.83(a)
4.	Z-16	0.87±0.17(ab)	0.43±0.48(a)	13.45±0.77(a)
5.	Z-54	0.59±0.12(ab)	0.54±0.99(a)	12.57±0.71(a)
6.	Z-03,Z-54	1.17±0.41(b)	0.65±0.14(a)	12.40±0.67(a)
7.	Z-16,Z-54	0.72±0.21(ab)	0.40±0.09(a)	11.88±1.31(a)
8.	Z-14,Z-16	0.56±0.11(ab)	0.63±0.12(a)	12.66±1.08(a)
9.	Z-03,Z-14	0.53±0.10(ab)	0.40±0.56(a)	13.25±1.03(a)

DISCUSSION

The present research proved that pure and mixed cultures of *Bacillus* have ability to up regulate the growth and development of *Vigna radiata* (L.). Strains were tested biochemically and it was observed that all strains were gram positive, spore formers which showed positive results for catalase and citrate test while negative for oxidase, urease test. For nitrate test Z-03 showed negative results and the rest were showing positive activity. Results of Triple sugar iron test showed Z-54 was giving positive activity with H_2S gas production while all other were tested to be negative. Strain Z-16 showed a positive result for starch hydrolysis while the rest were tested to be negative. The strains were tested negative for phosphate solubilization. *B. megaterium* is the largest known species of *Bacillus*. Chains of *Bacillus* are common. The spore forming bacteria form endospore which is heat resistance and provide protection in harsh environmental conditions (15).

Auxin biosynthesis was examined in the isolates. In the absence of L-tryptophan the auxin production is low. After increase in the concentration of L-tryptophan from 0µg/ml to 1000µg/ml, the auxin production increases and was maximum for *Bacillus aerius* (7µg/ml) that was 85% as compared to control while for Z-03 it 82% over control as represented by Figure 3. It has been reported that the quantity and accessibility of the tryptophan, the precursor, played a key role in the tryptophan-dependent processes that produce Indole acetic acid (IAA) (16). Research shows that there is an increase in auxin production as tryptophan concentration increased using different concentrations of L tryptophan (0, 200, 400, 600, 800, 1000, 1200µg/ml) (17). Significantly higher amounts of IAA were produced by three isolates that were molecularly recognized as *B. aryabhattai, B. megaterium,* and *B. cereus* (18). Bacterial strains produced significant amount of auxin in the supernatant concentration 500µg/ml in presence of L-tryptophan as compared to control (19).

In the pot trials Z-54 and Z-16 produced maximum value of shoot length and fresh weight (Fig. 5 and Fig. 6). In first harvesting, plants grown by inoculation of strains Z-16, Z-54 have commendable increase in fresh weight than others. It was observed that after 8 weeks of germination Z-16 promoted shoot length and fresh weight by 27% and 34% respectively over control. While the mixture of Z-03 and Z-54 produced 1.05-fold improvement in fresh weight as compared to control. Similarly, for dry weight there was 12% improvement in growth exhibited by Z-54 while the mixture of Z-03 and Z-54 produced 27% improvement in dry weight as compared to control. It has been reported that strain *Bacillus simplex* improved the growth of *Zea mays* (L.) under *in-vitro* and *in-vivo* conditions (20). In second harvesting, the plants grown by inoculation of mixed culture Z-03, Z-54 have admirable increase in fresh weight and shoot length. While studying the effect of bacterial strains on mung bean researchers found a statistically significant increase in shoot dry weight (21). When *A. thaliana* plants were inoculated with bacteria, there were noticeable increase in plant fresh weight (22). *Bacillus* strains have a significant effect on the growth of *V. radiata* (L.). Highest shoot length of 4.25% was found for *B. subtilis* Ra1S6, *while B. zhangzhouensis* R7S6 showed a significant

increase in the number of tillers (23). Studies report *Pseudomonas geniculata* strain can act as plant growth promoter and thus can help in the remediation of heavy metal (Cr, Cd, and Pb) contaminated soil (24). Researchers have reported that *Bacillus* species have ability to enhance endogenous auxin production in *Triticum aestivum* var. Inqalab-91 (25). Azeem *et al.* (2022) reported an improvement in fresh weight and dry weight (56% and 103%) in maize by *Bacillus* treatments. Also the mixture of compost, NPK fertilizers and PGPR improved the sunflower yield in in vitro trials and pot trials (26). Improved seed germination rates of corn and tomato has been exhibited by using microbial supernanents with varying pH levels (27). Besides *Bacillus* and *Azospirillum*, other species of *Paenibacillus, Burkholderia* and *Pseudomonas* genera play a vital role in better growth and yield of the crop (28). A significant effect of bio fertilizers application in mung bean was observed on the yields, nutrient uptake and soil health. Co-inoculation of Rhizobium and PGPR increased the nodule number, nodule dry weight, plant dry weight, grain yield, straw yield, in mung bean and improved soil health over the no inoculation (29). The plants grown with single bio fertilizer of *Rhizobium sp.* did not show significant results in the morphological and physical parameters. On the other hand, mixed bio fertilizers i.e., *Rhizobium* sp., *P. putida* and *F. aurantia* showed observable yield of Mung bean (30).

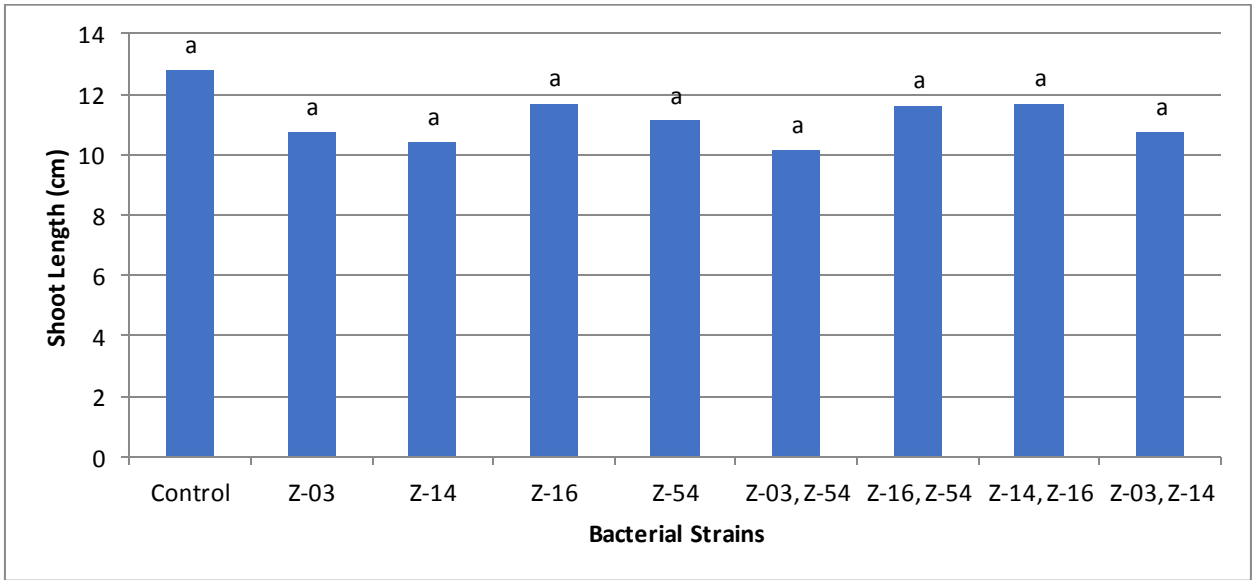


Fig. 5. Effect of bacterial inoculum on shoot length of *V. radiata* (L.), 4 weeks after seed germination. The bars represent the mean of three replicates. Different alphabets on bars show significant differences between treatments by Duncan's Multiple Range Test (DMRT) p≤0.05

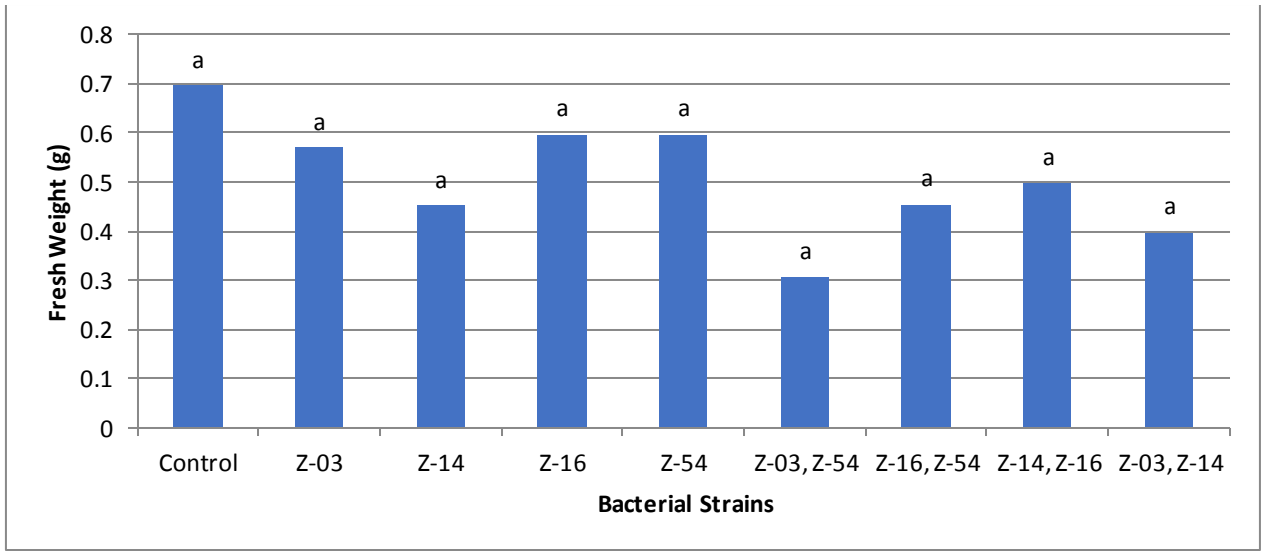


Fig. 6. Effect of bacterial strains on fresh weight of *V. radiata* (L.), 4 weeks after seed germination. The bars represents mean of three replicates. Different alphabets on bars show significant differences between treatments by Duncan's Multiple Range Test (DMRT) p≤0.05

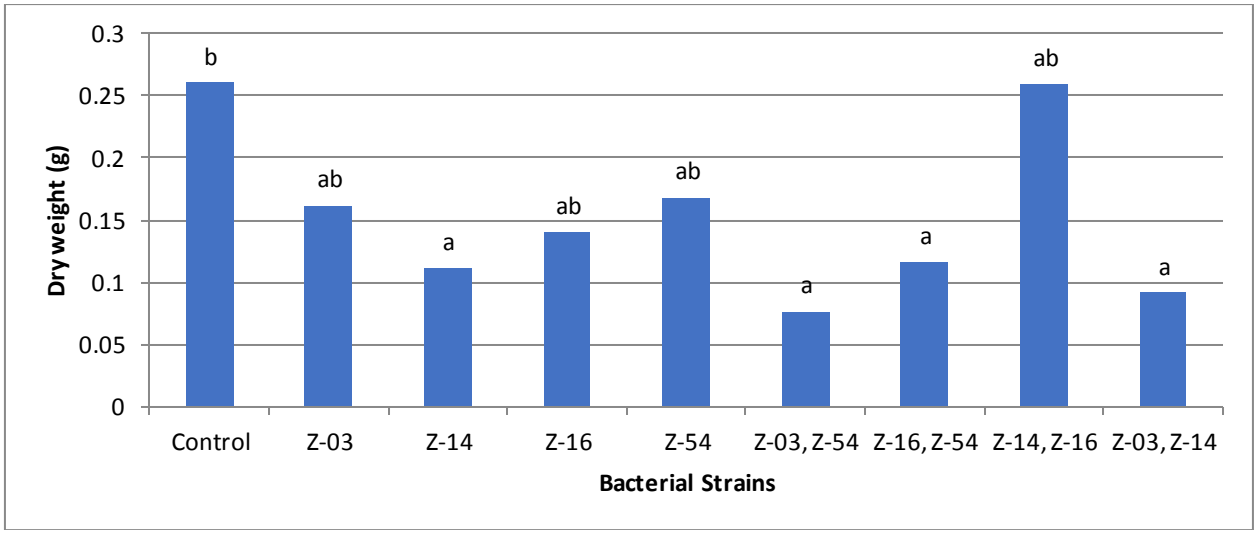


Fig. 7. Effect of bacterial treatment on dry weight of *V. radiata* (L.), 4 weeks after seed germination. The bars represent the mean of three replicates. Different alphabets on bars show significant differences between treatments by Duncan's Multiple Range Test (DMRT) p≤0.05

CONCLUSION

This study concluded that auxin production by *Bacillus* strains is directly related to the presence of precursor L-tryptophan and promotes the vegetative growth parameters of *Vigna Radiata* (L.). The results of pot trials proved that these strains are an excellent replacement of chemical fertilizers. Improvement in shoot length, fresh weight, and dry weight show that these strains have the potential for plant growth promotion, especially the strain *Bacillus subtilis,* which maximizes growth in pot trials. Future research in producing high-yield and low-cost PGPR formulations with less phyto-toxicity will be valuable and will help to improve the yield of wheat, maize, barley, sunflower and many other agronomical significant crops both under normal as well as stressed environments.

Conflict of Interest:

Authors have no conflict of interest.

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