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PJMLS

Print ISSN: 2707-4471. Online ISSN: 2707-448X

Research Article	Pak-Euro Journal of Medical and Life Sciences
DOI: 10.31580/pjmls.v5i1.2433	Copyright © All rights are reserved by Corresponding Author
Vol. 5 No. 1, 2022: pp. 101-112	
www.readersinsight.net/pjmls	
Submission: February 16, 2022	**Revised:** March 30, 2022 **Accepted:** March 31, 2022

EVALUATION OF *BACILLUS* AND *RHIZOBIUM* STRAINS TO ENHANCE THE GROWTH OF *VIGNA RADIATA* (L.) UNDER DROUGHT STRESS

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Abstract

In the present study, strains of Bacillus and Rhizobium were evaluated for their efficacy to produce auxin and to enhance the growth of Vigna radiata (L.) under drought stress. Colorimetric analysis indicated the auxin production in bacterial culture supernatants at various concentrations of 0, 200, and 500 µg ml^{-1} L-tryptophan. At 500 µg ml^{-1} of L-tryptophan, B. subtilis Z-24 and B. simplex Z-38 produced significant levels of 41.4 and 24.8 µg ml^{-1} auxin, respectively. High Performance Liquid Chromatography (HPLC) also detected the auxin production by bacterial strains. In vitro rooting assay also recorded a maximum increase in the number of lateral roots with B. simplex Z-02, over control. For pot trials, seeds treated with single and mixed bacterial cultures were grown under 28%, 20% and 10% field capacities (FC) of loamy soils. At 28% FC (normal water level), a maximum increase of 28% dry weight was observed for R. rosettiformans Z-30; as compared to the respective control with P≤0.05. At 10% FC (highest water stress), significant increases of 87% and 70% in fresh weight and root length were recorded by B. cereus Z-59 and B. simplex Z-37, respectively; (P≤0.05). In the case of mixed culture, C-3 (Z-38, Z-20 and Z- 30) and C-2 (Z-59, Z-20 and Z-23) were the most effective to enhance up to 68% dry weight and 13% shoot length of plants under drought stress (10% FC); (P≤0.05). Results showed that Bacillus and Rhizobium strains have the potential to produce auxin and improved the growth of mung beans under drought-stressed conditions.

Keywords: *Bacterial auxin production, Biofertilizers, Biotization, Plant growth-promoting rhizobacteria, Plant-microbial interaction, Plant trials*

INTRODUCTION

Drought stress is considered one of the major limiting factors for the yield, growth and productivity of plants. In arid or semi-arid areas, it creates an impediment to the yield of crops (1, 2). It causes great damage to plants by badly disturbing the plant water relation from cellular to organismic level. It performs a vital part in the generation of reactive oxygen species (ROS) (3).

Plants present a variety of strategies to mitigate the adverse impacts of water stress. Some physiological alterations occur that help in the growth of plants during water stress conditions. One of the brisk responses is stomatal closure leading to a decrease in the rate of transpiration. This mechanism is linked to the accumulation of abscisic acid which may in turn trigger signaling genes (4). Plants also alleviate the drought stress effects by increasing the root to shoot ratio. This leads to a decrease in evaporation rate and an increase in water use efficiency (5).

It has been reported in the literature that microbial activity performs a crucial part in the stability and integrity of the ecosystem by increasing soil fertility (6). Plant growth-promoting rhizobacteria (PGPR) may induce a variety of physiological adaptations in plants leading to increased tolerance of abiotic stress. The relationship of plant development may be witnessed by analyzing the association of PGPRs and roots of plants and the effect of auxins on shoots and roots (7).

Among the PGPR, *Bacillus* due to their spore-forming capability is of great importance to mitigate plant water stress (8). Moreover, they produce biologically active compounds such as siderophores, indole-3-acetic acid (IAA), antifungal metabolites and a variety of defense enzymes like peroxidase and chitinase

(9-11). Biotization is also an important area of research in this regard for using microorganisms as co-culture to improve the yield of crops (12).

Plants have adopted certain biochemical strategies to mitigate drought stress. Auxin helps plants by regulating the development of flowers, seed dormancy, growth tropism, root patterning etc. It has been observed that auxin homeostasis is necessary for the stress environment as it also influences abscisic acid synthesis which contributes a lot to managing stress response (13). L- tryptophan acts as a source for the biosynthesis of auxins in plants and bacteria (14). Microbial IAA affects plant growth, development and helps them to persevere in stress conditions. Evaluation and detection of IAA levels is a crucial part of the study of the activity of PGPRs (15).

Mung bean (*Vigna radiata* L.) is an important agronomic crop that has been used extensively to study positive plant-microbe interactions. It has been reported that abiotic stress may cause a hindrance in the growth, development, and nodulation of mung beans (16). Co-inoculation of rhizobia with PGPRs in this scenario may improve the growth of leguminous plants (17).

Therefore, the objective of this study was to screen *Bacillus* strains based on their ability to biosynthesize *in vitro* auxin and to evaluate their role along with *Rhizobium* to enhance the growth of mung beans under normal and water-stressed conditions.

MATERIALS AND METHODS

BACTERIAL STRAINS

Bacillus and *Rhizobium* strains, isolated from drought prone areas of Pakistan, were selected from the culture collection of the Institute of Microbiology and Molecular Genetics (MMG), University of the Punjab, Pakistan. The list of the selected strains with accession numbers is given in Table I. Strains were refreshed and routinely maintained on Luria-Bertani agar (L-agar). Ten *Bacillus* strains were selected for morphological characterization which were subjected to Gram and endospore staining by following the protocol of Cappucino and Sherman (18). Strains were carefully evaluated for their color, morphology and arrangement of cells under the microscope.

COLORIMETRIC ANALYSIS FOR BACTERIAL AUXIN PRODUCTION

For auxin quantification, 0, 200 and 500 µg ml^{-1} concentrations of L-tryptophan was supplemented in L-broth medium (15 ml). *Bacillus* and *Rhizobium* strains were inoculated as single culture in the L-broth medium. Culture flasks were then incubated at 120 rev/ min on a shaker at 37°C for 72 h. After that, centrifugation of culture media was accomplished at 5000 rpm for 10 min to remove the bacterial cells. 2 ml Salkowski reagent was added in the test tubes having 1 ml of bacterial supernatant. For 30 min, tubes were kept in the dark for the establishment of pink to red color. Afterward, the optical density of bacterial suspensions was noted at 535 nm. To calculate bacterial auxin production, different concentrations of standard IAA were used to plot the standard curve.

HPLC ANALYSIS FOR BACTERIAL AUXIN

For HPLC analysis, L-broth was inoculated with bacterial strains as mentioned above. After centrifugation, 10 ml supernatant was transferred to separate tubes and pH was adjusted at 2.5-3.0. Then the supernatant was mixed with 10 ml ethyl acetate to make a fraction of pure auxin. After the addition of ethyl acetate tubes were shaken vigorously and left for overnight. Ethyl acetate fraction was separated in beakers and dried in the water bath at 60°C. Dried extracts were then dissolved in 2 ml methanol. Extracted auxin was examined by using an HPLC model (integrated with hypersil-keyston ODS C-18 column) having dimensions of 5 µm; 4.6 x 250 nm. Methanol, water, acetic acid at a ratio of 36: 64: 1 with a flow rate of 1 ml min^{-1} was used as mobile phase. At 220 nm sample elutes were detected. Auxin was detected by integrating the areas under the peaks. For comparison, as a standard authentic IAA (Sigma) was used.

ROOTING ASSAY

This experiment was executed to demonstrate the bacterial auxin effect on the development of primary or lateral roots in mung beans. For seed sterilization, the certified seeds of mung bean were soaked for 2-3 min in 0.1% $HgCl_2$ followed by washing of seeds with autoclaved distilled water 3 times. For seed inoculation, Petri plates were lined with double filter paper and autoclaved. Filter papers were later soaked in 10 ml of autoclaved distilled water. Afterward, 10 ml L-broth was prepared in flasks and inoculated with respective bacterial strains and incubated on a shaker as mentioned above. Next, in each Petri plate, 3 ml of specified bacterial strain inoculum was dispensed. As a control, seeds treated with water were used. In each Petri plate, around 8 seeds were placed at equal distances and incubated in dark at room temperature for 7-8 days. To maintain moisture on filter paper, water was routinely checked and supplemented. After incubation, rooting was observed in each plate and root length and number of lateral roots were reported.

POT TRIALS UNDER NORMAL CONDITIONS

For pot trial under normal conditions, 10 *Bacillus* (Z-06, Z-10, Z-15, Z-17, Z-24, Z-35, Z-37, Z-38, Z-59, Z-66) and 4 *Rhizobium* strains (Z-18, Z-20, Z-23, Z-30) (Table 1) were selected. For inoculum preparation, strains were cultivated on L-Agar plates and incubated for 24 h at 37°C. Then bacterial growth was collected and dissolved in 10 ml autoclaved distilled water in falcon tubes to adjust culture density equal to 2 McFarland Standard. For mixed culture, an equal volume of bacterial suspension (Bacillus and Rhizobium) was added in culture tubes. Seed sterilization was achiFeved as mentioned earlier. Afterward, bacterization of seeds was carried out by soaking seeds for 15 min in bacterial suspensions Afterward, pots of dimensions 7.5×7.7 cm were filled with autoclaved sand and soil (1:1). In total, 5 seeds were sown in triplicate in each pot. Overall, 30 pots for *Bacillus* and 12 for rhizobia were placed. Similarly, 18 pots were also placed for mixed cultures. For mixed cultures, C-1 (Z-66, Z-10, Z-20), C-2 (Z-37, Z-59, Z-30), C-3 (Z-59, Z-24, Z-23), C-4 (Z-35, Z-17, Z-20), C-5 (Z-15, Z-06, Z-30) and C-6 (Z37, Z-38, Z-18) combinations (C) were selected. For control, water-treated seeds were kept for comparison. Then, pots were incubated at 25°C for 7 days. The watering of pots was carried out at regular intervals. After 7 days, harvesting was accomplished to record vegetative growth parameters (root and shoot length).

EXPERIMENTS UNDER WATER STRESS CONDITIONS

Pot experiments were also executed to gauge the efficacy of *Bacillus* and *Rhizobium* to enhance the growth of mung bean plants under water stressed conditions. For pot trials, 3 distinct *Bacillus* and *Rhizobium* strains were selected. For seed bacterization, microbial inoculum (single or mixed cultures) was prepared as mentioned above. Then seed sterilization and bacterization were also accomplished as mentioned earlier. Afterward, soil and sand were autoclaved and mixed in 1:1 in the pots. For inoculation 18 pots for *Bacillus* and 18 for rhizobia were placed. Similarly, 18 pots were also placed for mixed cultures. For mixed culture, C-1 (Z-37, Z-23, Z-30), C-2 (Z-38, Z-20, Z-30) and C-3 (Z-59, Z-20, Z-23) combinations were evaluated. For control, seeds treated with water were used for comparison. All the pots were placed in a wirehouse under natural environmental conditions. According to 3 different field capacities (FC) applied i.e., 28%, 20% and 10%, pots were categorized. Soil moisture meter was used to monitor and maintain the soil moisture content. After interval of 7 days plants were harvested and the roots and shoots length were recorded. The dry and fresh weight of plants was also recorded as per protocol.

STATISTICAL ANALYSIS

Data recorded for bacterial auxin synthesis and vegetative growth parameters were analyzed through Analysis of Variance (ANOVA) by using SPSS 20 software. By applying DMRT (Duncan's multiple range test) with $P \leq 0.05$, means of different values were separated.

RESULTS

BACTERIAL STRAINS

Different *Bacillus* and *Rhizobium* strains (Table I) isolated from drought prone areas of Pakistan were maintained and refreshed on L-Agar plates. The color and morphology of *Bacillus* strains were carefully recorded. All *Bacillus* cells were gram positive rods with cells arrangements as *diplobacillus* and *streptobacillus* and *endospore* formers.

Table I. Drought tolerant *Bacillus* or *Rhizobium* strains used in the present study

S. No.	Strains	Identified as	Accessions
1	Z-02	*Bacillus simplex* Z-02	KT027590
2	Z-04	*B. simplex* Z-04	KT027592
3	Z-06	*B. megaterium* Z-06	KT027594
4	Z-07	*B. pumilus* Z-07	KT027595
5	Z-08	*B. pumilus* Z-08	KT027596
6	Z-10	*Lysinibacillus xylaniliticus* Z-10	KT027598
7	Z-15	*B. cereus* Z-15	KT027603
8	Z-16	*B. subtilis* Z-16	KT027604
9	Z-17	*B. cereus* Z-17	KT027605
10	Z-24	*B. subtilis* Z-24	KT027606
11	Z-28	*B. megaterium* Z-28	KT027647
12	Z-35	*B. simplex* Z-35	KT027608
13	Z-37	*B. simplex* Z-37	KT027610
14	Z-38	*B. simplex* Z-38	KT027611
15	Z-39	*B. pumilus* Z-39	KT027612
16	Z-44	*B. subtilis* Z-44	KT027617
17	Z-45	*B. aryabhattai* Z-45	KT027618
18	Z-56	*B. megaterium* Z-56	KT027627
19	Z-59	*B. cereus* Z-59	KT027630
20	Z-66	*B. subtilis* Z-66	KT02736
21	Z-18	*Rhizobium daejeonense* Z-18	KT027637
22	Z-20	*R. herbae* Z-20	KT027639
23	Z-23	*R. nepotum* Z-23	KT027646
24	Z-30	*R. rosettiformans* Z-30	KT027644

COLORIMETRIC ANALYSIS FOR BACTERIAL AUXIN DETECTION

Colorimetric analysis indicated that bacterial strains produced significant auxin levels in the presence or absence of the precursor L-tryptophan (Fig. 1). At 0 µg L-tryptophan, *B. subtilis* (Z-24), *L. xylaniliticus* (Z-10) and *B. aryabhattai* (Z-45) produced 30.4 µg ml^{-1}, 24 µg ml^{-1} and 19.9 µg ml^{-1} of auxin, respectively. At 200 µg ml^{-1} L-tryptophan concentration, significant level of auxin was produced by *L. xylaniliticus* (Z-10) **(**33 µg ml^{-1}**),** *B. subtilis* **(**Z-24) (32 µg ml^{-1}) and *B. simplex* **(**Z-37) (21 µg ml^{-1}). At the highest L-tryptophan concentration (500 µg ml^{-1}), the maximal auxin levels were reported by *B. subtilis* **(**Z-24) (41.4 µg ml^{-1}), *B. megaterium* **(**Z-06) (36 µg ml^{-1}) and *L. xylaniliticus* (Z-10) (30.4 µg ml^{-1}).

Analysis of extracted auxin was achieved using an HPLC (model integrated with hypersil-keyston ODS C-18 column). Auxin standard peak was observed at 2.87 which was comparable to the peaks obtained from the extracts of *B. pumilus* (Z-08), *B. subtilis* (Z-66) and *B. cereus* (Z-15) in Fig. 2.

ROOTING ASSAY

Primary root length along with the number of lateral roots was recorded after 7-8 days under *in vitro* conditions (Fig. 3). Statistical analysis showed significant improvements for root length and number of roots per plant in comparison with water treated seedlings. For instance, *B. simplex* Z-02 (266%), *B. pumilus* (Z-08) (233%) and *B. pumilus* (Z-07) (18%) were proved to be effective in terms of increase in root length and number of lateral roots as compared to controls. Fig. 4 showed the impact of *Bacillus* strains on the root growth of *V. radiata*.

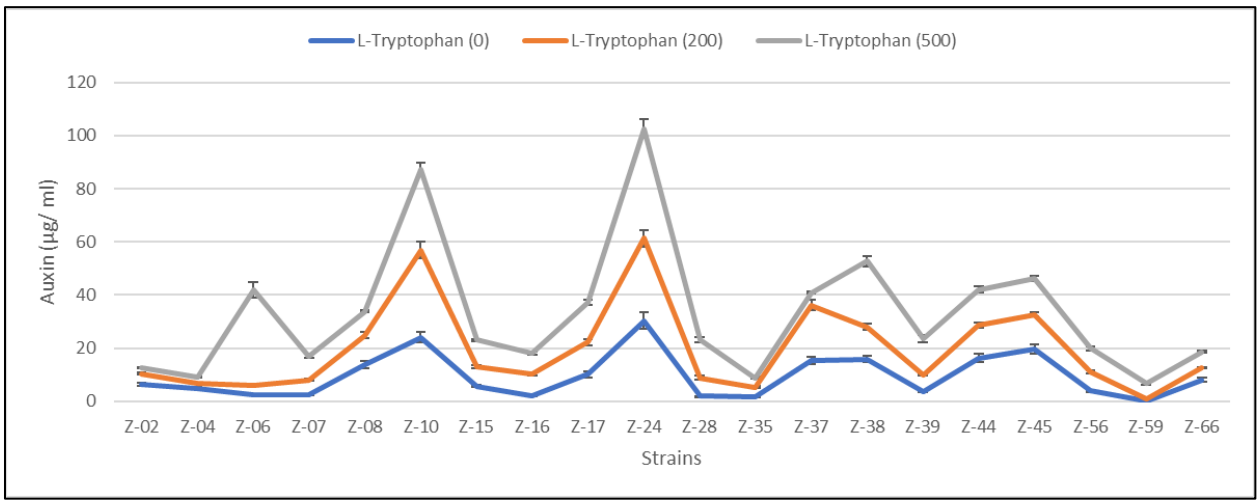

Fig. 1. Effect of different L-tryptophan concentrations on bacterial auxin production. Mean ± S.E. 2 replicates

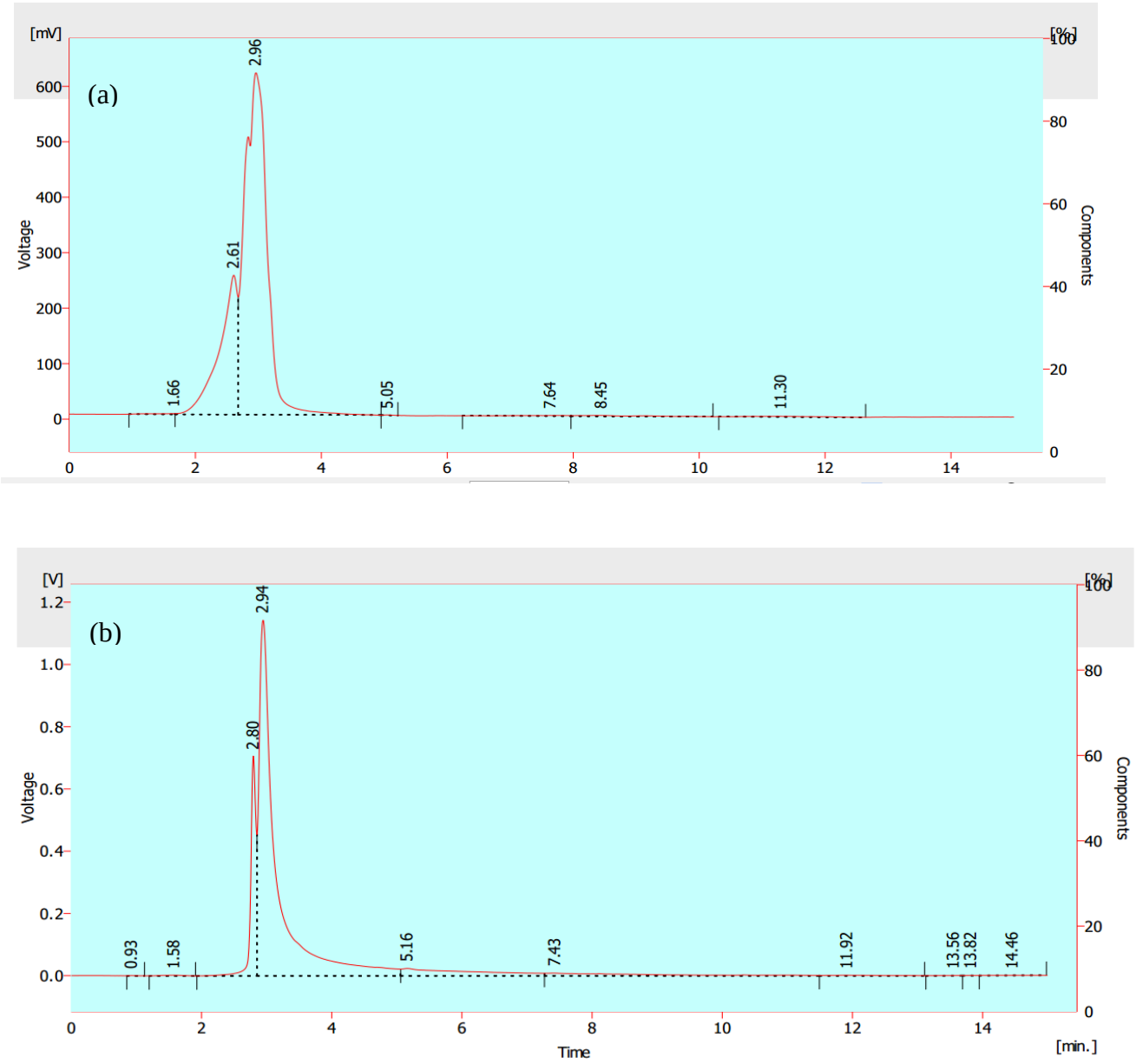

Fig. 2. Chromatograms obtained from HPLC analysis showing the auxin production. Peak obtained from(a) auxin standard at 2.8 retention time; (b) *B. pumilus* (Z-08)

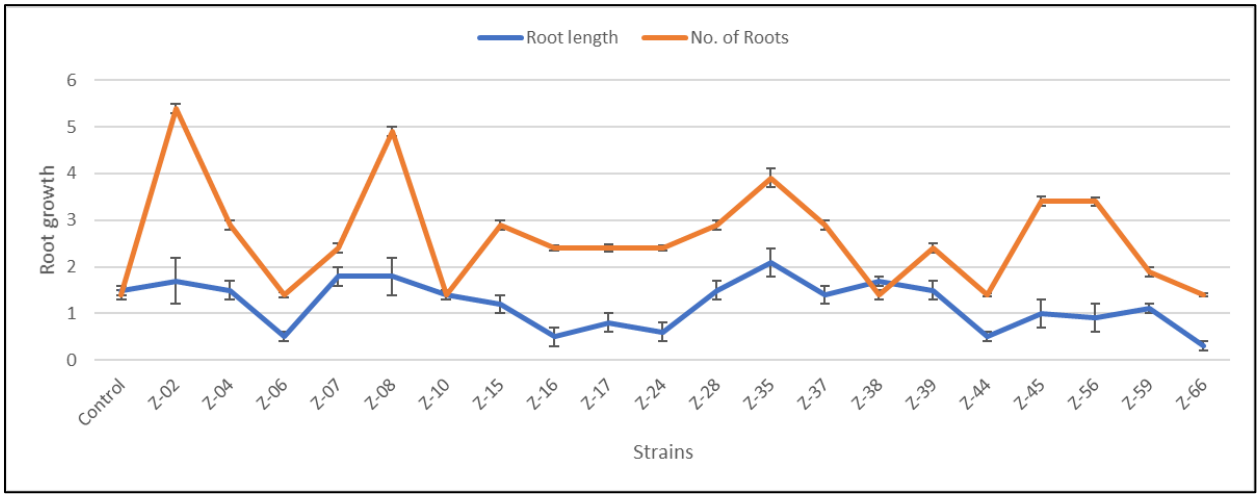

Fig. 3. Effect of different *Bacillus* strains on root length and number of roots per plant in Petri plate bioassay. Mean ± S.E. 4 replicates

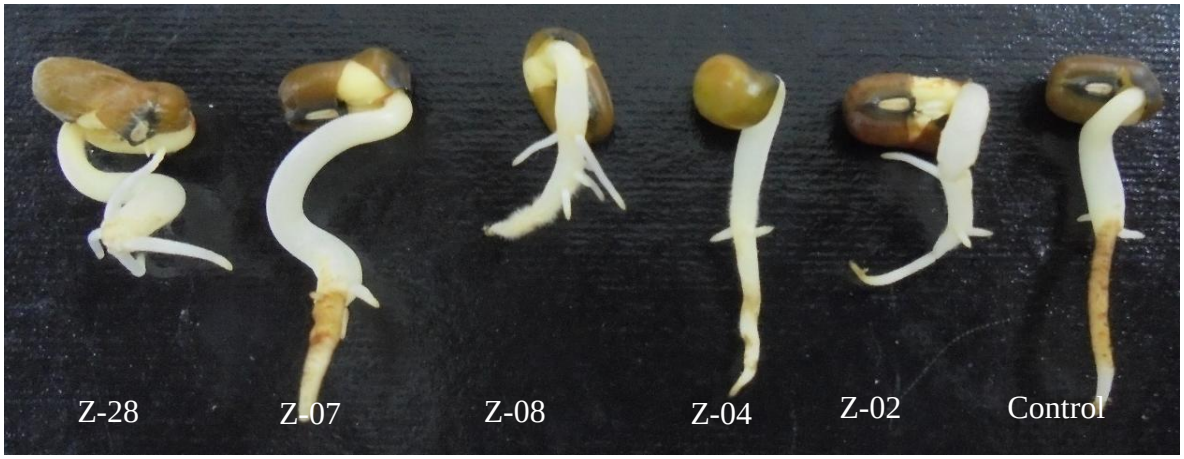

Fig. 4. Root growth response of mung bean with *B. megaterium* (Z-28), *B. pumilus* (Z-07), *B. pumilus* (Z-08), *B. simplex* (Z-04), *B. simplex* (Z-02) and Control.

POT TRIALS UNDER NORMAL CONDITIONS

Statistical analysis showed a significant difference in results for shoot length and root length in pot trials under normal conditions (Fig. 5). Plants treated with *B. simplex* (Z-37) (8%), *B. megaterium* (Z-06) (7%), *B. simplex* (Z-38) (7%), *B. cereus* (Z-59) (7%) and mixed cultures C-1 (12%), C-3 (12%), C-2 (9%) showed improvements in shoot length over control. While in case of root length *B. cereus* (Z-17) (4%), *B. simplex* (Z-38) (4%), *R. rosettiformans* (Z-30) (5%) and mixed cultures combinations C1 (10%), C2 (3%), C4 (2%) showed improvements as compared to control.

POT TRIALS UNDER WATER-STRESSED CONDITIONS

Pot trial under water stress showed the potential of bacterial strains in mitigating the water scarcity. Plants having bacterial inoculum showed significant improvement in growth as compared to plants without bacterial treatment. Statistical analysis showed that at 10% FC, *B. simplex* (Z-37), C-1, and C-3 showed 70%, 170% and 85%; enhancement in root length as compared to respective control. At 20% FC, significant increase for root length was recorded for *R. rosettiformans* (Z-30), *B. cereus* (Z-59) and C-3 showed 81%, 85% and 43%, respectively compared to respective control (Fig. 6). For fresh weight (10% FC), statistically significant results were obtained with *B. cereus* (Z-59), C-2 (mixed culture), C-3 (mixed culture) (Fig. 7).

At 28% FC, around 40% increase was recorded with *R. nepotum* (Z-23) for dry weight of plant. Similarly, at 20% FC, Z-30 and C-2 (mixed culture) recorded significant increases. Whereas at highest water stress (10% FC), Z-38, Z-59 and C-3 (mixed culture) showed significant increases in dry weight (Fig. 8). For shoot length statistically comparable results were recorded at different water regimes. However, mixed cultures (C-2, C-3) gave promising results at 10% FC in comparison with the respective control (Fig. 9).

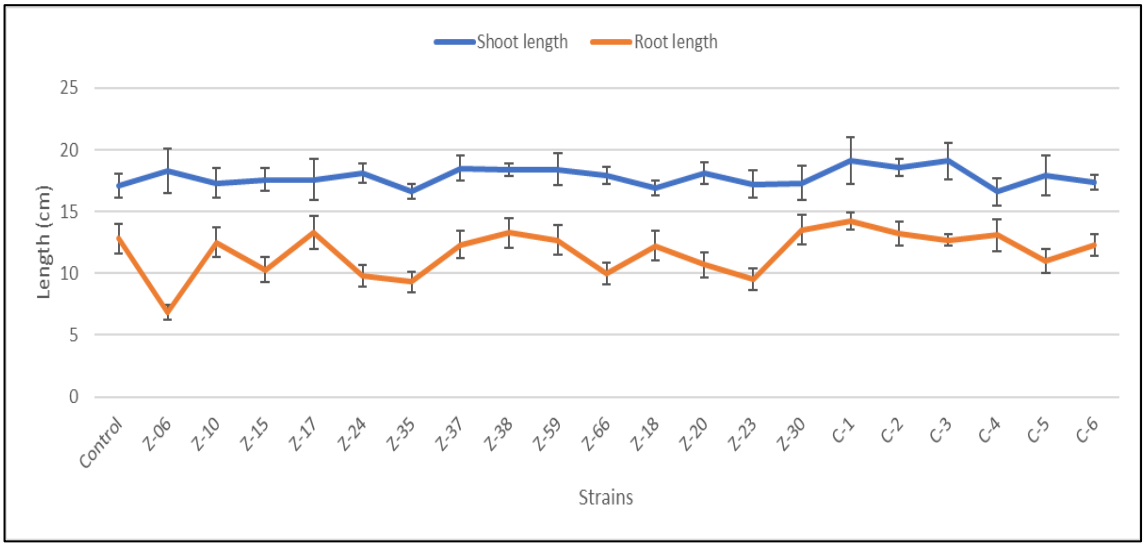

Fig. 5. Effect of different *Bacillus* and *Rhizobium* strains on shoot length and root length in pot trials under normal conditions. Mean ± S.E. of 4 replicates

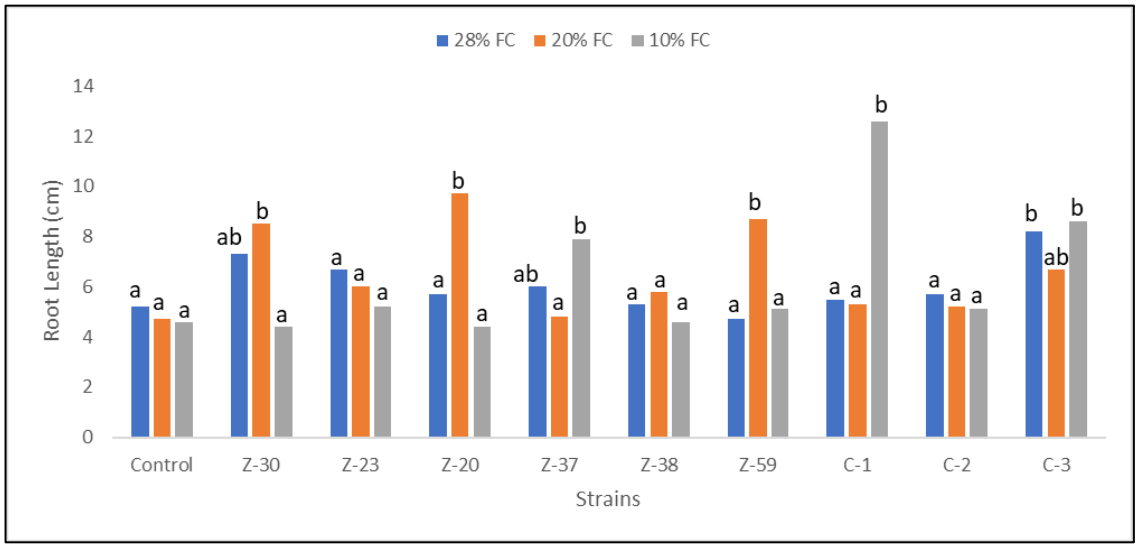

Fig. 6. Effect of *Bacillus* and *Rhizobium* strains on root length of plants at different soil field capacities. The bar represents the Mean ± S.E. of 5 replicates. Different alphabets represent a significant difference in mean values by using DMRT (Duncan's Multiple Range Test); (P≤0.05).

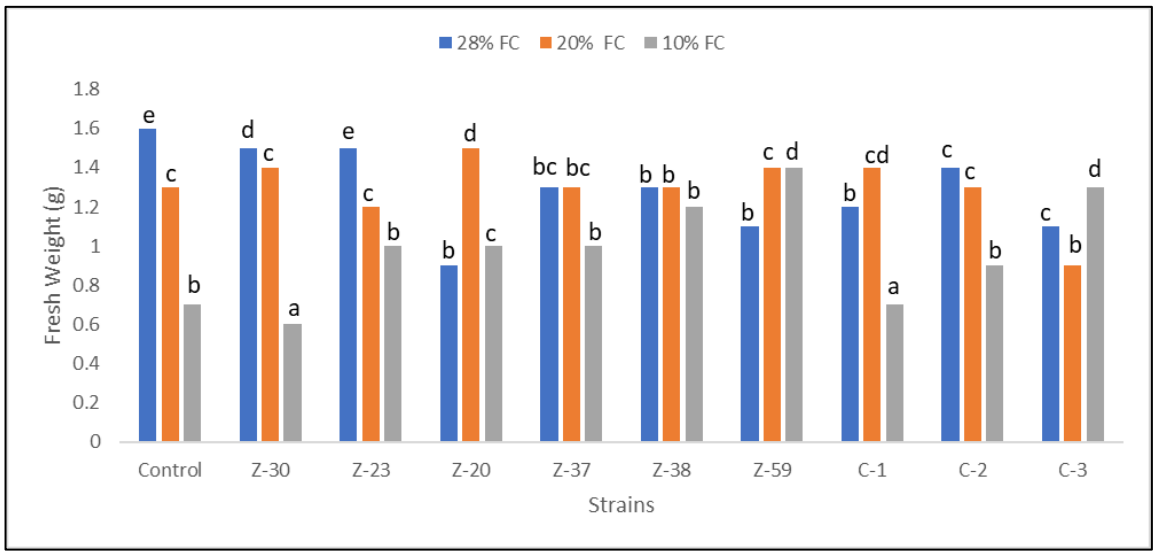

Fig. 7. Effect of *Bacillus* and *Rhizobium* strains on fresh weight of plants at different soil field capacities. The bar represents the Mean ± S.E. of 5 replicates. Different alphabets represent a significant difference in mean values by using DMRT (Duncan's Multiple Range Test); P≤0.05.

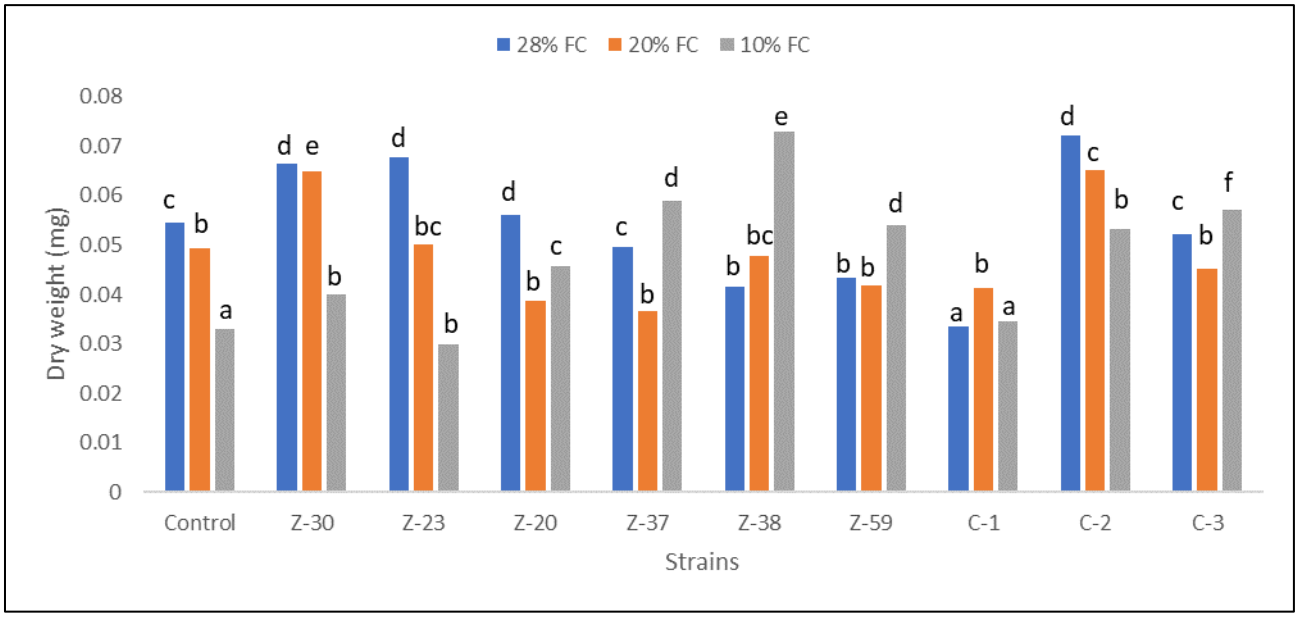

Fig. 8. Effect of *Bacillus* and *Rhizobium* strains on the dry weight of plants at different soil field capacities. The bar represents the Mean ± S.E. of 5 replicates. Different alphabets represent a significant difference in mean values by using DMRT (Duncan's Multiple Range Test); P≤0.05.

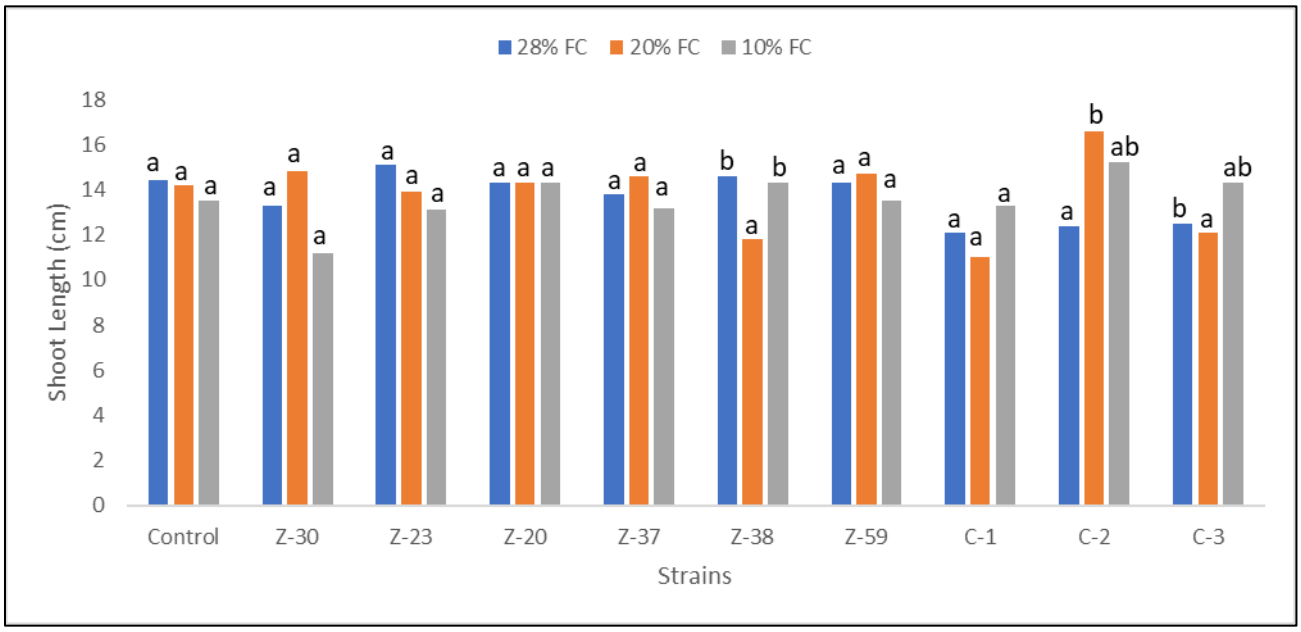

Fig. 9. Effect of *Bacillus* and *Rhizobium* strains on shoot length of plants at different soil field capacities. The bar represents the Mean ± S.E. of 5 replicates. Different alphabets represent a significant difference in mean values by using DMRT (Duncan's Multiple Range Test); P≤0.05.

DISCUSSION

In the current study, we have evaluated the impact of drought resistant *Bacillus* and *Rhizobium* strains on *Vigna radiata* (L.) growth improvement, under normal and water stressed conditions. The scarcity of water and minerals is one of the lethal environmental stress factors for the growth of plants in arid and semi-arid areas. Nevertheless, the inoculation of plant growth promoting rhizobacteria (PGPR) improves the growth of plants under abiotic stress conditions. PGPR produces useful substances like antibiotics, siderophores and phytohormones such as auxins to enhance plants growth (19). Therefore, in the present study strains of genus *Bacillus* and *Rhizobium* were screened for auxin production to evaluate their positive impact on plant growth. Strains were screened colorimetrically for auxin production in the presence of L-tryptophan. Auxin was also detected in bacterial culture supernatants by using High-Performance Liquid Chromatography (HPLC). The maximum level of *in vitro* auxin production was recorded in *B. subtilis* Z-24 in L-tryptophan supplemented media. *In vitro* studies have shown the production of different types of

auxins (indolic compounds) in L-tryptophan supplemented medium (20). Auxin truly affects the rooting system of plant and help plant in mineral uptake; hence, increasing plants growth (21, 22). Our results demonstrated that each bacterial strain has affected the rooting to a variable degree. These variable results in the rooting assay are well justified by varying degrees of production of auxin. For instance, *B. subtilis* Z-24 produced the highest levels of auxin and also showed a significant increase in the number of lateral roots. *In vitro* experiments with wheat seedlings showed a significant effect on rooting by different PGPRs by Raheem et al. (20). Another study also revealed the effect of PGPRs on *Arabidopsis thaliana* shoots and roots development by influencing division and differentiation of cells in primary and lateral roots development which consequently leads to shoot development (23). It has been reported that there is not only auxin that may stimulate the growth of plants but some other substances like nitric oxide, carotenoids as in the case of *Azospirillum brasilense* that contribute to better growth of plants (24). Stimulation of plants by *Bacillus* is well reported by Ahmad et al. (25). Besides *Bacillus* and *Azospirillum,* there are also some species of *Paenibacillus*, *Burkholderia* and *Pseudomonas* genera that play a vital role in better growth and yield of the crop (26). There are some other factors viz. 3-hydroxy-2- butanone (acetoine) and 2, 3-butanediol by *Bacillus spp.* that promotes plant growth (27).

Pot trials performed with stress and without stress conditions manifested the effect of bacterial auxin on the growth of mung beans. Bacterial strains under normal water conditions exhibited statistically non-significant results. Nonetheless, an increase in root length was observed with *B. simplex* Z-38 and *B. cereus* Z-17 while *B. simplex* Z-37 showed improvement in shoot length. Pot trials conducted under wirehouse conditions also manifested an increase in grain weight, the number of tillers and root elongation of wheat (20).

Drought stress is lethal for plant growth as it may inhibit the growth of plants due to high levels of ethylene production. The application of PGPRs may defend them from stress condition impacts and make them to sustain their growth (28). In our single strain treatment, there was an insignificant increase in shoot length of the plant over water treated control. While in the case of dry weight, fresh weight and root length showed a significant increase in growth with *B. simplex* (Z-37) and *B. cereus* **(**Z-59). While in the case of mixed culture under water stress, M-2 and M-3 showed remarkable results when juxtaposed with respective control. Thus, our results are in accordance with the results obtained from the inoculation of *Rhizobium* and *Pseudomonas* strains on mung beans (29). Effect of PGPRs on the enhancement of shoot and roots' fresh and dry weight in sunflower pot trials under drought stress are also reported by Siddique et al. (30). Co-inoculation of *Rhizobium galegae and Pseudomonas spp.* in *Galega officinalis* also exhibit remarkable results by increasing its fresh weight, shoot and root length as compared to a single inoculum of *R. galegae* (31). The effect of different field capacities applied on *Cymbopogon citratus* is also elaborated by Mirzaei et al. (32). Our results are also in accordance with the study conducted on PGPRs and Rhizobium with mung bean plants that showed significant growth promotion under stress conditions (33). Drought tolerant PGPRs have also increased maize biomass and roots establishment under water stress (34). Another study for evaluation of the effect of rhizobacteria on wheat plant spike length and seed weight provided with different water regimes also confirmed our results (20). Co-inoculation of rhizobia and PGPRs may act as excellent biofertilizers due to the synergistic effect of different strains to enhance plant growth and cope with abiotic stress. Co-inoculation of soybean with *Bradyrhizobium japonicum* and PGPR *Pseudomonas putida* has shown enhance drought tolerance, plant growth, nodulation and nutrient uptake under water-stressed conditions (35). As this was *in-vitro* study, there is a great opportunity for us to expand the study for field trials.

CONCLUSION

Our study concludes that PGPRs produce varying levels of *in vitro* auxin owing to difference in concentrations of supplemented L-tryptophan. They facilitate the growth of plants under normal and drought conditions. Improvement in growth of roots by *B. simplex* Z-02, *B. pumilus* Z-08, M-1 mixed culture inoculation showed that PGPRs may assist in plants growth by elevating minerals and water absorption. Especially, *R. nepotum* (Z-23), *B. simplex* (Z-37) and *B. cereus* (Z-59) exhibited significant results under water-

stressed conditions. Overall, *Bacillus* and *Rhizobium* in both single and combinations showed good potential to promote the growth of mung bean plants under drought stress conditions. Finally, strains showed good potential to be used in field trials to enhance the growth of agronomic crops.

Contribution of authors:

Sana Tanveer conducted the experimental work and collected the data and prepared the first draft of the manuscript. Basharat Ali conceived this study, performed the statistical analysis and checked the final draft of this study.

Conflicts of interest:

There are no conflicts of interest among the authors.

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