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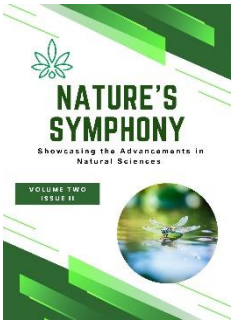

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Research Article

Auxin Production and Bio-control Potential of *Bacillus megaterium* and *Rhizobium* Enhanced the Growth of *Zea mays* (L.)

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Plant growth promoting rhizobacteria (PGPR), Phytohormones, Indole acetic acid (IAA), Rhizosphere, L-tryptophan

ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) can potentially protect plants from phytopathogens. They can also enhance the vegetative growth parameters of plants. This research aimed to screen *Bacillus* and *Rhizobium* strains for their auxin production ability and to assess their bio-control activity against phytopathogens. Strains were tested for auxin production using colorimetric analysis and their bio-control activity was checked using dual culture assay. Pot trials assessed the influence of bacterial strains on vegetative growth parameters of maize. The data of the current study was analyzed by one-way ANOVA test using SPSS 20 software. The results showed that the bacterial strains produced a statistically significant amount of auxin at different concentrations of L-tryptophan. Strains Z-28 (9 µg/ml), Z-56 (10 µg/ml), and Z-63 (8 µg/ml) produced maximum amount of auxin at 0 µg/ml, 500 µg/ml and 1000 µg/ml L-tryptophan, respectively. For bio-control activity, Z-06, Z-63, Z-19, and Z-18 exhibited antifungal activity against *Macrophomina phaseolina* and *Fusarium oxysporum*. In pot trials, it was observed that Z-63 recorded a 7.6% improvement in shoot length and a 47% improvement in root length compared to control. Similarly, this strain (Z-63) also showed a 41% enhancement in fresh weight of plants. For dry weight, Z-19 (73%) showed statistically significant results as compared to the control. Among consortiums, M1 (72%) and M3 (51%) produced a maximum enhancement in root length and fresh weight over control. Similarly, M1 (66%) produced significant improvement in dry weight in comparison with control. Thus, it was proved that *Bacillus* and *Rhizobium* strains have plant growth-promoting abilities and bio-control potential against plant pathogens. Future aspects of our study provide an eco-friendly and economically efficient approach for the rhizo-engineering of soil with PGPR which would help to overcome the prevailing issue of global food insecurity.

1. INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are good colonizers of plant surfaces and natural soil inhabitants of rhizosphere (Sati *et al.*, 2023). They promote plant growth by different mechanisms such as nitrogen fixation, phosphate solubilization, and phytohormones production. The capability of PGPRs to produce auxin is the most frequently used mechanism for explaining PGPRs' beneficial impacts on plant growth. It is secreted as a

secondary metabolite by 80% of rhizospheric microbes (Mukherjee *et al.*, 2024). The most important and naturally found auxin is indole-3-acetic acid (IAA). Other molecules that are involved in anabolism of IAA include indole-3-acetamide, indole-3-pyruvate, and indole-3-acetaldehyde. PGPR modifies auxin and helps plants to increase root and shoot length and number of roots (Navid *et al.*, 2023). IAA is generated from tryptophan with the help of several bacterial populations, primarily PGPR, and hence plays a significant role in plant growth stimulation. It has been found that IAA-producing strains of *Bacillus* and *Pseudomonas* enhanced the growth parameters of chickpeas (Lata *et al.*, 2024).

In addition to auxin, cytokinins are also an important class of phytohormones that regulate vascular development, shoot growth, and cell division start. Cytokinins also perform the development of flower and fruit, pathogen interaction, and root and shoot extension (Timofeeva *et al*., 2024). Gibberellin (GAs), an important phytohormone, is required for seed germination and plant health. Similarly, many plant growth-promoting microorganisms create identical secondary metabolites that work as plant growth stimulants (Ali *et al.*, 2024a).

Nitrogen deficiency has many effects on plants. Soil microorganisms are responsible for approximately 90% of natural nitrogen fixation on our planet (Hasan *et al.*, 2024). The second most important macronutrient in growth and development of plants is phosphorus. Phosphate-solubilizing bacteria are microorganisms that solubilize phosphorus. *Bacillus*, *Rhizobium*, and *Pseudomonas* are highly significant phosphate solubilizers. Organic acids are produced and released by phosphate solubilizing bacteria, causing acidity in the cells of microbes and their atmosphere, as a result, phosphate ion release (Das Mohapatra *et al.*, 2024).

Fusarium infection in agricultural land causes large yield losses and is very difficult to control (Joncy, 2024). Plant-associated bacteria are used to control *Fusarium*-induced pathogenesis because fungicides have many harmful effects on plant and human health. It has been determined that *Bacillus* species are effective antagonists of *Macrophomina phaseolina*. Microbial species are used in bio-control procedures to provide fungal pathogens with more environmentally friendly solutions. Different rhizobia species enhance plant growth and stop the growth of numerous pathogens in soil, including *M. phaseolina, R. solani*, and *Fusarium sp.*, in both leguminous and non-leguminous plants (Ghoniem *et al.*, 2024). *Pseudomonas* and species of *Bacillus* make up the vast majority of bacteria examined for bio-control action because *Bacillus* can produce endospores (Hof and Schrecker, 2024). *B. megaterium* and *Rhizobium spp.* can also act as bio-control agents because they have activity against fungal pathogens like *Fusarium oxysporum*. It can help to reduce food waste worldwide and also reduce the use of fungicide sprays which are harmful to both plants and other species (De Oliveira-Paiva *et al.*, 2024).

Bacillus species are among the best-studied experimental systems in bacteriology. Multiple species of *Bacillus* occur in agricultural fields that can promote crop health in different ways. Some of these species directly stimulate plant growth either through enhancement in the acquisition of nutrients or through stimulation of host plant's defense mechanisms before infection. *B. megaterium* and *Rhizobium spp.* act as bio-fertilizer and enhance the growth of plants. They can replace use of chemical fertilizer which is harmful not only to plants but also to human health (Gao *et al.*, 2024). *Rhizobium* is a bacterial genus that forms root nodules in plants. These bacteria exist with legumes in a symbiotic relationship. They take nitrogen from surroundings and plants use it, to

grow in soil that has low nitrogen (Wulandari *et al.*, 2024).

Zea mays (L.), generally known as corn or maize, is a cereal plant in *Poaceae* (grass) family that produces an edible grain. It is a staple crop and the most consumed crop worldwide. It ranks in second position in the world productivity index with production of 6.3 tons per hectare (Ríos-Ruiz *et al.*, 2024).

The primary objective of our study was to check the bio-control potential of *Bacillus megaterium* and *Rhizobium* strains against fungal pathogens. Moreover, bacterial ability to promote the vegetative growth parameters of *Zea mays* (L.) under natural environmental conditions was also evaluated.

2. MATERIAL AND METHODS

2.1 Microorganisms (Bacteria and Fungi)

The samples of bacterial strains *Bacillus megaterium* Z-63, Z-56, Z-06, Z-28 and *Rhizobium* spp. Z-18, Z-19, and Z-30 were taken from the culture collection of the Institute of Microbiology and Molecular Genetics, University of the Punjab. The fungal cultures *Macrophomina phaseolina* and *Fusarium oxysporum* were obtained from the Faculty of Agricultural Sciences, University of the Punjab, Lahore.

2.2 Morphological and Biochemical Characterization

The streak plate method was used to isolate colonies of bacterial strains and determine their morphological characteristics. Gram and spore staining were performed for morphological characterization of strains. Biochemical attributes of strains were evaluated by catalase, MR, VP test, nitrate, and urease test (N, 2002).

2.3 Auxin Quantification

Auxin analysis was done by colorimetric method to check the ability of bacterial strains to produce auxin *in vitro* in the absence or presence of L-tryptophan. For this purpose, bacterial strains were cultivated in 20 ml of L-broth supplemented with 0µg ml^{-1}, 500µg ml^{-1} and 1000 µg ml^{-1} of L-tryptophan in three replicates. Flasks were incubated for 72 hours on shaker at 120 rev/min at 37ºC. After 3 days of incubation, bacterial culture was taken in Eppendorf and centrifuged at 8000 rpm for 10 minutes. 1 ml supernatant was used to check biosynthesis of auxin by adding 2 ml Salkowski reagent. Salkowski's reagent was made by mixing 0.5M $FeCl_3$ solution and 35% perchloric acid. After the addition of Salkowski's reagent, samples were incubated in dark for 30 minutes for formation of color. Optical density (O.D) was recorded at 535 nm. 2 ml of Salkowski reagent was taken as blank.

2.4 Anti-Fungal Assay

Autoclaved potato dextrose medium was poured into sterile petri plates aseptically. A 2 mm fungal plug was developed from master culture using a sterile cork-borer and was placed 2 cm from edge of PDA agar plate. Then bacteria isolates were streaked using an aseptic technique with use of a sterile cotton swab on the other side of plate leaving a margin of 2cm from edges. Petri plates were kept for incubation at 28˚C for 7 days and zone of inhibition of fungal growth around bacteria was observed.

2.5 In Vivo Pot Trials

For pot trails healthy maize seeds were obtained from a certified plant nursery. The seeds were surface sterilized using 0.1% mercuric chloride solution and allowed to stand for 3 minutes. Seeds were then washed thoroughly with autoclaved distilled water. For seed inoculation, bacterial suspensions were prepared by using a fresh bacterial sample that was grown on L-agar media. The cell pellet was dissolved in 10 ml autoclaved distilled to adjust 6×10^8 cells. Then seeds were soaked in bacterial suspension for almost 30 minutes for

priming purposes. Thirty-three medium-sized pots were filled with 110 grams of soil and placed in a wire-house. At least 4-5 seeds inoculated by respective strains were sowed in pots in triplicates. After one-week seeds were germinated. Pots were watered daily. After a month, plants were harvested and various parameters of growth, for example shoot length, root length, leaf number, root number, dry and fresh weight of plants were measured.

2.6 Statistical Analysis

The data collected from these experiments was analyzed by using One-way ANOVA and Duncan's multiple range test ($p<0.05$) on SPSS 20 software.

3. RESULTS

3.1 Morphological and Biochemical Characterization

All strains were positive in gram staining except Z-63 strain and all strains were spore formers (Figure 1). Z-63, Z-56, Z-18, Z-19, Z-30 showed fermentation of dextrose, Z-28 had fermentation of dextrose and sucrose, and Z-06 indicated not any carbohydrate fermentation. No strain showed any hydrogen sulfide production (Figure 2). All strains were positive for catalase, methyl red and Voges Proskauer test but Z-28 were negative for Voges Proskauer test. All strains were negative for urease and nitrate reduction test (Table 1).

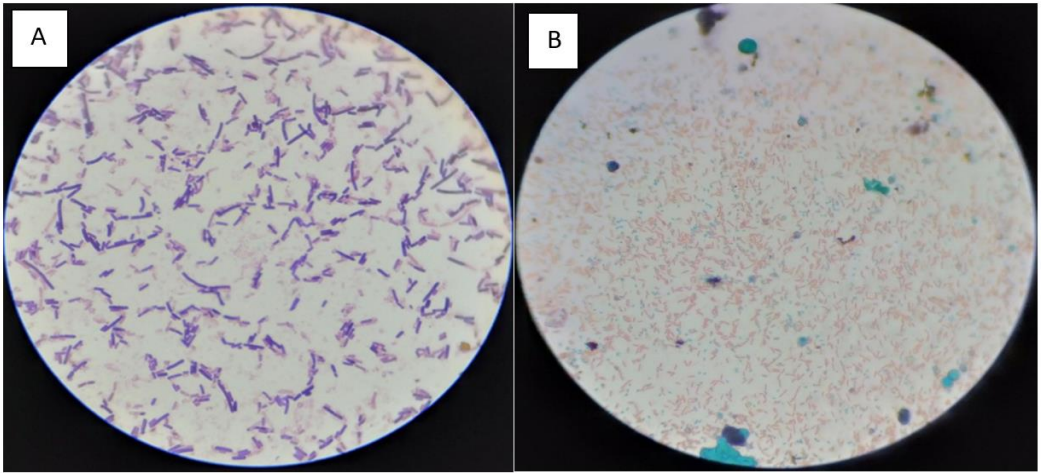

Figure 1. A= Gram staining of the *Bacillus megaterium* (Z-06), B= Spore staining of *B. megaterium* (Z-56)

Table 1. Results of MR/VP/Catalase/Nitrate reduction and Urease test

Sr.No	Strains	MR	VP	Catalase	Nitrate	Urease
1.	Z-63	+	+	+	-	-
2.	Z-56	+	+	+	-	-
3.	Z-06	+	+	+	-	-
4.	Z-28	+	-	+	-	-
5.	Z-18	+	+	+	-	-
6.	Z-19	+	+	+	-	-
7.	Z-30	+	+	+	-	-

+ positive, - negative

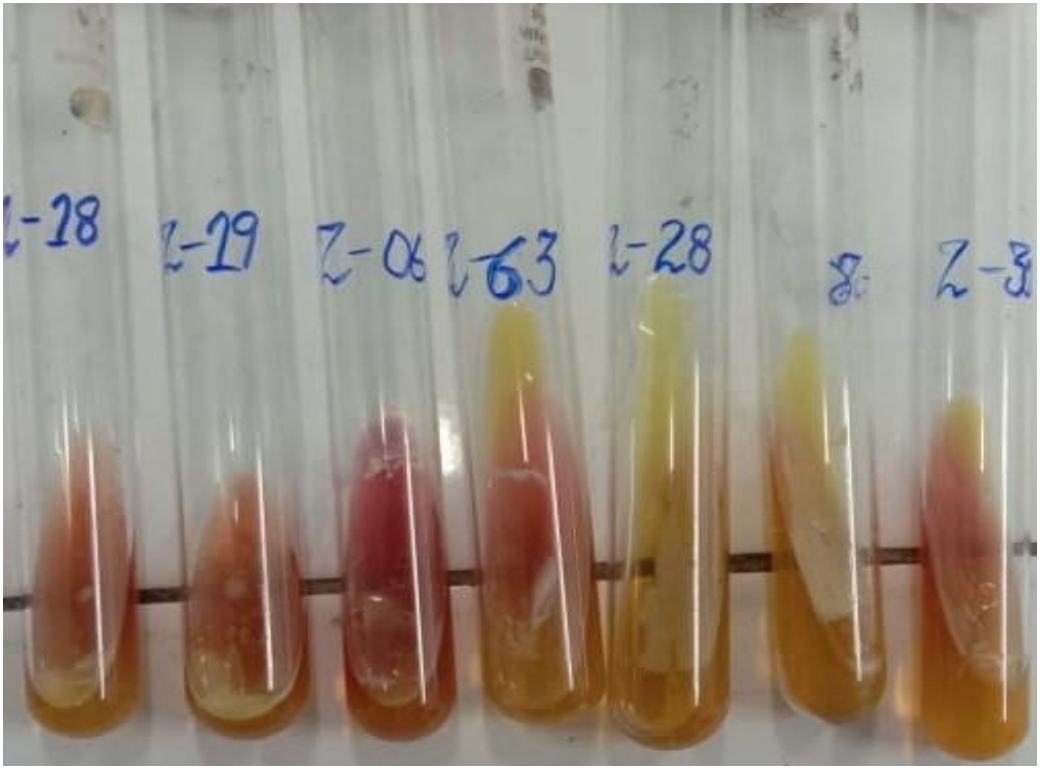

Figure 2. TSI agar test of bacterial strains *Bacillus megaterium* Z-63, Z-28, Z-56, Z-06 and *Rhizobium* Z-30, Z-18, Z-19

3.2 Auxin Quantification

By using a colorimetric assay, it was possible to determine auxin production by bacteria *in vitro* in the presence or absence of L-tryptophan. The amounts of L-tryptophan used in L-broth were 0 µg, 500 µg and 1000 µg. All strains produced different amounts of auxin at different concentrations of L-tryptophan. At 0µg/ml Z-28 and Z-30 produced a maximum auxin concentration that was 9 µg/ml. At 500 µg/ml concentration of L-tryptophan Z-56 produced maximum auxin that was 66% over control. Also, Z-30, Z-19 and Z-06 produced a significant amount of auxin that was 9 µg/ml, 8 µg/ml and 8 µg/ml respectively. At 1000 µg/ml L-tryptophan concentration maximum auxin was produced by Z-56 was 66% over control while Z-63 and Z-19 produced 12% more auxin than control (Table 2).

3.3 Bio-control Activity

In this experiment, antifungal assay was performed against plant fungal pathogen *Macrophomina phaseolina* and *Fusarium oxysporum*. The zone of inhibition of fungal growth around bacteria on potato dextrose agar plate confirmed antifungal activity of that bacteria. Z-06 and Z-63 strains of *B. megaterium* and Z-19, Z-18 strains of *Rhizobium* have antifungal activity against *M.phaseolina* and *F. oxysporum* (Figure 3).

3.4 *In Vivo* Pot Trial

The results of pot trial were compared to control, and it was observed that *B. megaterium*

Z-63 (7.6%), Z-56 (4.8%), Z-06 (13.3%), and Z-28 while Rhizobium strains, Z-18 (3.6%), Z-19 (0.1%), Z-30 (6.5%), show improvement in shoot length. When compared to control *B. megaterium* strain Z-63 (47.1%), Z-56 (86%), Z-06 (87.9%), Z-28 (59.2%) and Z-18 (65.5%), Z-(19 99%), Z-30 (98%) while among consortiums Mix 1 (72.6%), Mix 2 (35%) and Mix 3 (72%) showed significant increase in root length. For fresh weight, *B. megaterium* Z-63 strain showed 41.1%, Z-56 52.2%, Z-06 48.7%, Z-28 3.5% and Z-18 27.5%, Z-19 56.1%, while Rhizobium Z-30 showed 81.1% improvement in growth. While for mixtures, Mix 1 (39.7%), and Mix 3 (51.7%) showed improved fresh weight of plants. Compared to control *B. megaterium* Z-63 strain showed 1.69 folds, Z-56 1.86 folds, Z-06 1.86 folds, and Z-18 1.49 folds, improvement in dry weight while among mixtures, Mix 3 resulted in 1.22 folds improvement in dry weight (Figure 4).

Table 2. L-tryptophan dependent auxin production of bacterial strains

Sr. No	Strains	L tryptophan (µg/ml)		
		0µg	500µg	1000µg
		Auxin production (µg/ml)		
1.	Z-63	7±0.5(ab)	6±0.6(a)	8±1.2(ab)
2.	Z-56	6±0.0(a)	10±2.3(b)	10±2.1(b)
3.	Z-06	8±0.0(bc)	8±0.3(ab)	7±0.3(ab)
4.	Z-28	9±0.0(c)	7±0.6(ab)	9±0.3(ab)
5.	Z-18	7±0.6(bc)	6±0.3(a)	7±0.0(a)
6.	Z-19	7±0.3(bc)	8±1.0(ab)	8±0.3(ab)
7.	Z-30	9±0.5(c)	9±0.3(ab)	8±0.6(ab)

"Mean and ± S.E of 3 replicates, different letter indicates significance difference among mean value using Duncan's multiple range test ($p \leq 0.05$)"

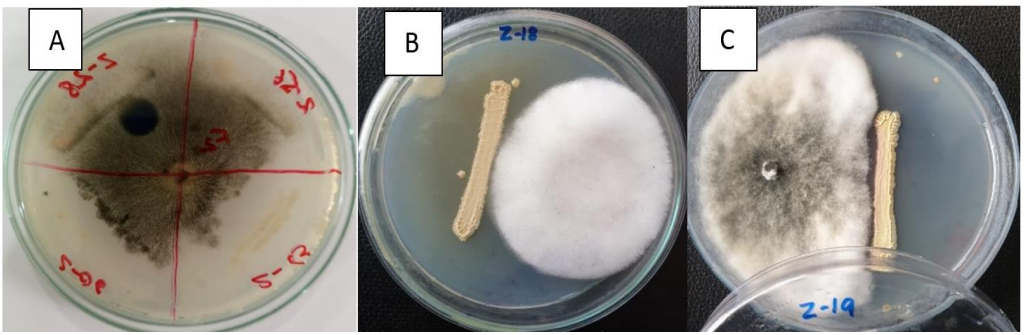

Figure 3. Antifungal Assay of bacteria A= *Bacillus megaterium* strains activity against *Macrophomina phaseolina*, B= Antifungal activity of *Rhizobium* Z-18 against *Fusarium oxysporum*, C= Antifungal activity of *Rhizobium* Z-19 against *M. phaseolina*

Figure 4. Plant microbe interaction A= *Bacillus megaterium* Z-63, B= *Rhizobium* Z-19

4. DISCUSSION

The current study aimed to evaluate antifungal activity and plant growth-promoting ability of bacterial strains to stimulate the growth of *Zea mays* under natural environmental conditions. For this purpose, different biochemical tests were performed to check different characteristics of strains. In this study, it was determined that a strain's capacity to promote growth depends on its ability to produce auxin *in vitro*. Maize seeds treated with various bacterial strains were used in pot trials to test their ability for growth promotion. Most *Bacillus* strains had higher auxin production than *Rhizobium*. *Pseudomonas* and *Bacillus* are most frequently isolated genera of PGPR, according to recent studies (Lyng *et al.*, 2024). Strains Z-06, Z-63 of *Bacillus megaterium* and Z-19, Z-18 of *Rhizobium* had antifungal activity against *Macrphomina phaseolina* and *Fusarium oxysporum*. A study revealed bio-control activity of PGPR against a variety of fungal phytopathogens. Also, these specific metabolites interfered with growth and metabolic process of phytopathogens even at low concentrations.

The Auxin production test was performed *in vitro* using different concentrations of L-tryptophan (0 µg, 500 µg and 100 µg) and then optical density was taken on 535nm. The graph showed that Z-56 strain of *B. megaterium* exhibited excellent results and had the highest auxin production rate than other strains. The inoculation of PGPR that produced auxin in plants, resulted in a diverse range of reactions, ranging from effective growth promotion to growth inhibition (Pantoja-Guerra *et al.*, 2023).

In the *in-vivo* experiment, seeds were grown with an equal mixture of sand and soil. Every pot held five plants. Root and shoot length,

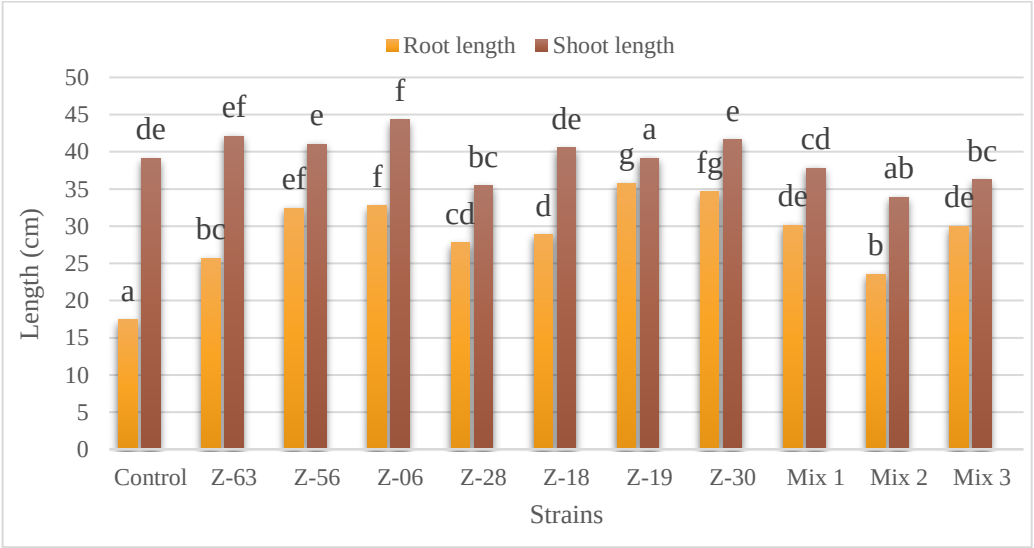

Figure 5. Effect of bacteria on root and shoot length of maize plant

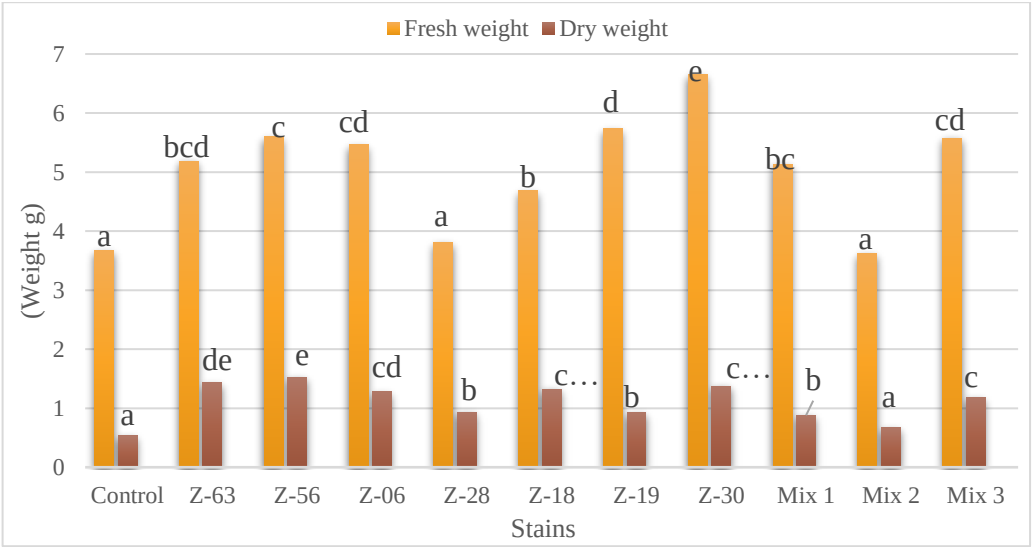

Figure 6. Effect of bacteria on fresh and dry weight of maize plant

fresh and dry weight of maize plant were noted. Pot trials showed that *B. megaterium* Z-06 showed excellent results with an increase in shoot length of about 13.3%, over control (Figure 5). Strains of *Rhizobium* Z-30 and Z-18 also showed good results with 6.5% growth improvement. According to one study, *B. subtilis* exhibited the ability to increase the shoot length and growth yield of sour cherry, and also protected plants against disease caused by a fungus *Alternaria* (Choub *et al.*, 2024). PGPR strains also showed tremendous results in increasing root length of maize plants in pot trails. Strain Z-19 of *Rhizobium* showed

excellent results with an increase in root length of plant by about 99%. Mixture 1 and Mixture 3 of bacterial strains which contain *B. megaterium* and *Rhizobium* strains also caused an increase in root length of maize plant as compared to control. According to recent studies, mixing two or more different PGPR strains resulted in greater growth promotion and stress management in barley (Ferioun *et al.*, 2024). When compared with control, Z-30 strain of *Rhizobium* showed promising results with 81% increase in fresh weight of maize plant. Similarly, *B. megaterium* Z-56 showed promising results in enhancing dry weight of maize plants (Figure 6). Studies revealed that use of PGPR helped to improve the stress management genes against cucumber mosaic virus in ridge gold (Karthikeyan *et al.*, 2024). It has been reported that PGPR played an important role in rhizo-remediation and growth enhancement of chickpeas in hydrocarbon-contaminated soil (Ali *et al.*, 2024b). Real-time PCR conducted to improve and optimize the effectiveness of PGPR showed that biochar was very helpful for optimization of PGPR in soil (Iosa *et al.*, 2024). Also, consortium of *Bacillus* species acted as a bio-based remedy to protect pea plant from wilt disease (Raza *et al.*, 2024). Research experiments showed that multiple strains of *Pseudomonas* shared a positive interaction with a minimum inhibitory concentration (MIC) ranging from 0.8mM and 0.6mM values for cadmium. Also, *P. fluorescens* had better anti-phytopathogenic activity against *Alterneria brassicae, Alterneria brassicola, Verticillium longisporum, Fusarium oxysporum* than *P. putida (Bhardwaj et al., 2024)*. Out of 150 isolated strains, 15 showed enhancement in growth of *Vigna Radiata* (L.) by suppressing genes causing charcoal rot disease (Khan *et al.*, 2024). Also reshaping of rhizospheric microbiome with PGPR increases yield and extracts of medicinal plant *Pseudostellaria heterophylla* (Ng *et al.*, 2024). Also, *Bacillus subtilis*, *Bacillus aerius* and *Bacillus aryabhattai* significantly improved agronomical growth parameters of *Vigna Radiata* (L.) (Saboor *et al.*, 2024).

5. CONCLUSION

Our findings concluded that *B. megaterium* Z-56 has the highest auxin production ability in the presence of precursor L- tryptophan. Strains Z-06, Z-56 of *B. megaterium* and Z-30, Z-18, and Z-19 of *Rhizobium* increased root and shoot length of plant by 13.3% in pot trials. Similarly, strains also increased fresh and dry weight of maize plants in pot trials. Moreover, strains *B. megaterium* Z-63, Z-06 and *Rhizobium* Z-19, Z-18 also have the best bio-control potential against *M. phaseolina* and *F. oxysporum*. These PGPRs may be helpful for our agricultural land on a commercial scale and can be used to improve the yield of edible crops.

Conflicts of Interest

The authors have no conflicts of interest.

Contribution of Authors

Fiza Shoaib conducted experimental work and collected the data. Shafaq Navid prepared the draft of the manuscript. Basharat Ali conceived this study and checked the final draft of the study.

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