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Auxin Production by *Bacillus simplex* Enhanced the Growth of *Zea mays* (L.) under *In-Vitro* and *In-Vivo* Conditions

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ABSTRACT: *In the current study Bacillus simplex was evaluated for its role in improving the growth of maize (Zea mays (L.)) under lab and natural environmental conditions. Bacterial strains were tested for several biochemical traits and auxin biosynthesis. Maize seeds were surface sterilized and treated with bacterial strains as single and mixed cultures to evaluate the enhancement of maize vegetative growth parameters. All bacterial strains exhibited positive results for catalase, citrate and nitrate while negative for urease, indole, triple sugar iron and oxidase test. Colorimetric analysis revealed significant production of auxin by Z-13 (6.7µg/ml), Z-09 (5.8µg/ml), Z-37 (5.7 µg/ml), Z-38 (5.7µg/ml), Z-02 (5 µg/ml), Z-37 (4.5 µg/ml) and Z-04 (3.5 µg /ml) at 1000µg/ml concentration of L-tryptophan. At 0 µg/ml conc of L-tryptophan, Z-37, Z-13, Z-04, Z-09 and Z-38 also manifested significant levels of auxin biosynthesis. In-vitro rooting assay revealed that Z-09, Z-35 and Z-37 showed significant increase in root length i.e., 1.2, 1.18 and 1.11-fold, respectively, as compared to control. Pot trials under natural conditions manifested significant improvements in shoot length by Z-38 (67.1%), Z-13 (51%), and Z-09 (43%), over control. Most significant results for fresh weight (120%) and dry weight (100%) in maize were shown by strain Z-38. Among mixed cultures C-1 (Z-02, Z-04, Z-09) improved shoot length (42%) and fresh weight (45%) with respect to control. Improvement in vegetative growth parameters of maize suggests that B. simplex can be used as an effective biofertilizer.*

Keyword: Auxin, PGPR, Plant Growth, Biochemical Test, Maize, Rooting assay

INTRODUCTION

The first goal of agriculture was to secure survival by providing the necessity of feeding. Conventional agricultural approaches that provide fruitful yield are highly dependent on the use of chemical fertilizers. The widespread use of chemical fertilizers raises environmental concerns and a question on the consumer's health. In order to cope with all these problems an environmentally friendly approach is highly required in farming practices (Vocciate et al., 2022).

The term "plant growth promoting rhizobacteria" (PGPR) also known as plant health promoting bacteria (PHPB) or nodule promoting bacteria (NPB) refers to bacteria colonizing roots of plant. A variety of bacteria strains like *Pseudomonas*, *Bacillus*, *Psychrobacter* and *Brevibacter* are known to enhance plant growth (Basu et al., 2021). The use of these *Rhizobacterium* reduces the need of chemical fertilizers to decrease cost of production and environmental hazards (Vocciante et al., 2022). The role of PGPR has been demonstrated in promoting the growth of wheat (Iqbal et al., 2023), maize and lettuce (Gonzalez et al., 2023).

Bacillus spp. due to its spore forming capability are recognized as exceptional

PGPRs for enduring stress conditions. They help in plants' growth by sequestering heavy metals in soil, induced systemic resistance (ISR), production of hormones like indole acetic acid, cytokinin, gibberellic acid etc. There is several *Bacillus* spp. that has been documented as PGPRs including *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, *B. thuringiensis*, *B. cereus*, *B. megaterium*, *B. simplex* etc. Several studies demonstrated *B. simplex* as PGPR due to its involvement in enhancement of growth of Kiwi, Arabidopsis and tomato (Schwartz et al., 2013).

Plant growth promoting rhizobacteria are well characterized based on their biochemical attributes like production of phytohormones (Haque et al., 2020). These hormones include abscisic acid (ABA), gibberellins (GA), ethylene, auxin and cytokines (Karnwal et al., 2023). Auxin is the master controller of plant growth. It is the major plant growth regulator and aids in signaling (Sati et al., 2023). Microorganisms are capable of producing auxin *in vitro* and in rhizosphere. Exogenous supplementation of microbial IAA increases growth in inoculated plants (Pantoja et al., 2023). Auxin production in rhizosphere triggers the physiological

response in host. Indole-3-acetic acid (IAA) is the most abundant auxin quantitatively (Karnwal et al., 2023). More than 85% of rhizospheric bacteria are IAA producers. It plays a significant role in cell proliferation, fruit development and leaves senescence. IAA enhances the growth of many parts of plants i.e., roots, stems, leaves and flowers. In monocots and dicots IAA enhances the length of primary and lateral roots respectively. Studies also show that water and nutrient availability to plants were improved by microbial IAA synthesis in Canola (de Aquino et al., 2023).

The L-tryptophan is the main precursor for synthesis of IAA and supplementation of cultural media with this amino acid results in high auxin production. Different genera of bacterial strains are known to produce auxin in the presence of L-tryptophan. It is the major root exudate released in rhizospheric areas of soil. Inoculation of PGPR in the presence of precursor helps in increasing root and shoot length of plant (Iqbal et al., 2023). The ability of PGPR in soil and culture was increased several folds by application of L-tryptophan (Onrit et al., 2023). Other essential traits associated with PGPR include biological nitrogen fixation, phosphate solubilization and hydrogen

cyanide (HCN) production (Chamkhi et al., 2023).

B. simplex is an elongated shaped, gram-positive motile bacterium capable of forming central, para central or sub terminal spores. These are the most abundant strains in rhizosphere and can explore biofertilizers. Co-inoculation of *Bacillus* with *rhizobium* strains enhances the root length and shoot length, making them important phyto-stimulators.

Maize (*Zea mays* L.) is the second most rated crop widely used in food stuff or fodder. Cultivation of maize is easy as it needs less fertile soil and fewer chemicals (Erenstein et al., 2022). The primary goal of this study was to screen *Bacillus simplex* for their ability to produce auxin in vitro and to check the effect of their inoculation on the growth of *Zea mays* (L.).

MATERIALS AND METHODS

Bacterial strains

Distinct *B. simplex* strains (Z-02, Z-04, Z-09, Z-13, Z-35, Z-37 and Z-38) were revived from the culture collection of the Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan. Strains of *B. simplex* were subjected to gram staining, endospore staining and biochemical tests including catalase test, oxidase test,

citrate test, triple sugar iron test, nitrate test, indole test and urease test following the protocols of Cappuccino and Sherman (2002).

Colorimetric analysis for IAA production by bacteria

For auxin quantitative analysis two concentrations 0 and 1000 μ g/ml of L-tryptophan was used in duplicate. For one strain 4 flasks containing 15 ml L-broth were prepared and autoclaved. Stock solution of L-tryptophan was made by adding 0.2 g L-tryptophan in 20 ml autoclaved distilled water. For 1000 μ g/ml concentration 1.5ml L-tryptophan from stock was added to L-broth. All the flasks were inoculated with respective *Bacillus* strain. Culture flasks were incubated at 37°C for 3 days on a rotatory shaker at 120 rpm. After incubation, centrifugation of cultures was done at 5000 rpm for 10 min to remove the bacterial cells. About 2ml Salkowski reagent was dispensed in falcon tubes with 1 ml bacterial supernatant. These falcons were incubated for 30 min in the absence of light. The falcon tubes were observed for the formation of pink color and optical densities were measured at 535nm. For calculating bacterial auxin production 14 different concentrations 0, 5, 10, 20, 30 up to 120 μ g/ml of

standard auxin were prepared to make the standard curve (Ali et al., 2009).

Rooting assay under laboratory conditions

The procedure was conducted under lab conditions (*in-vitro*) according to Tanveer and Ali, (2022). Seeds were sterilized by soaking them in 0.1% mercuric chloride solution for 60 sec. Seeds were then rinsed with autoclaved distilled water three times. Bacterial cultures were prepared by swabbing on poured plates of L-agar and incubated overnight at 37°C. Bacterial suspensions from these plates were prepared in autoclaved distilled water and density was adjusted equal to 6.0×10^8 CFU/ml using McFarland 2. For mixed cultures C1 (Z-02, Z-04, Z-09), C2 (Z-09, Z-13, Z-35), C3 (Z-35, Z-37, Z-38) were selected. Petri plates were lined with filter paper of size 9 mm and autoclaved. Then surface sterile seeds were transferred to 10 autoclaved petri plates aseptically. Seeds were then treated with pure as well as mixed cultures by soaking them in bacterial cultures for 25 min. For control, seeds soaked in autoclaved distilled water were used. Treated seeds were then arranged on petri plates having filter paper (5 seeds per plate). For maintaining moisture 8ml autoclaved distilled water was dispensed in each

petri plate. The petri plates were then incubated under dark conditions at room temperature for a week. After 7 days rooting was detected in each plate and vegetative parameters were noted.

Pot trials under natural conditions

Pot trials under natural conditions (*in-vivo*) were conducted using 7 bacterial strains along with 3 mixed cultures as mentioned above. Bacterial cultures were prepared in autoclaved distilled water and density was adjusted equal to 6.0×10^8 CFU/ml. Corn seeds were sterilized and treated with bacterial suspensions as mentioned above. Seeds treated with autoclaved distilled water were used as control. Then pots of dimension 19×20 cm were filled with soil and placed in a wire house with natural environmental conditions at Institute of microbiology and molecular genetics. 10 seeds treated with bacterial suspensions were then sown using sterile forceps in respective pots at a distance of 10 cm. All pots were lightly watered and allowed to germinate and sprout out (Raheem *et al.*, 2018).

Statistical analysis

Data from pot trials, *in-vitro* assay and auxin production was analyzed through “Analysis of Variance” (ANOVA) and

means were compared with “Duncan’s multiple range test ($p \leq 0.05$).

RESULTS

Bacterial strains

Different strains of *Bacillus simplex* i.e., Z-02, Z-04, Z-09, Z-13, Z-35, Z-37 and Z-38 with Accession numbers KT027589, KT027592, KT027597, KT027601, KT027608, KT027610, and KT027611, obtained from the culture collection of Institute of Microbiology and Molecular Genetics, were retrieved by repeated quadrant streaking on L-agar plates. All the strains were gram positive and endospore formers. All bacterial strains were recorded positive for catalase, nitrate and citrate while negative for indole, urease, TSI and oxidase test.

Colorimetric analysis for IAA production by bacteria

Colorimetric estimation showed that bacterial strains biosynthesized significant levels of auxin irrespective of addition of L-tryptophan. The supernatant of 72 h old cultures was mixed with Salkowski reagent and placed in dark for 30 min. Tubes were observed for presence of pink color and optical densities were observed. It was observed that bacterial cultures showed significant production of auxin like Z-35

(6.8 µg/ml), Z-13 (6.7 µg/ml), Z-09 (5.8 µg/ml), Z-37 (5.7 µg/ml), Z-02 (5 µg/ml), Z-04 (3.5 µg/ml), Z-37 (4.5 µg/ml). Strains Z-02 and Z-35 showed significant increase in auxin levels (85% and 83%) respectively over control.

Comparison with control also showed that Z-37 (66%), Z-13 (42%), Z-04 (40%), Z-09 (18%) and Z-38 (9.6%) showed promising increase in auxin levels (Fig. 1).

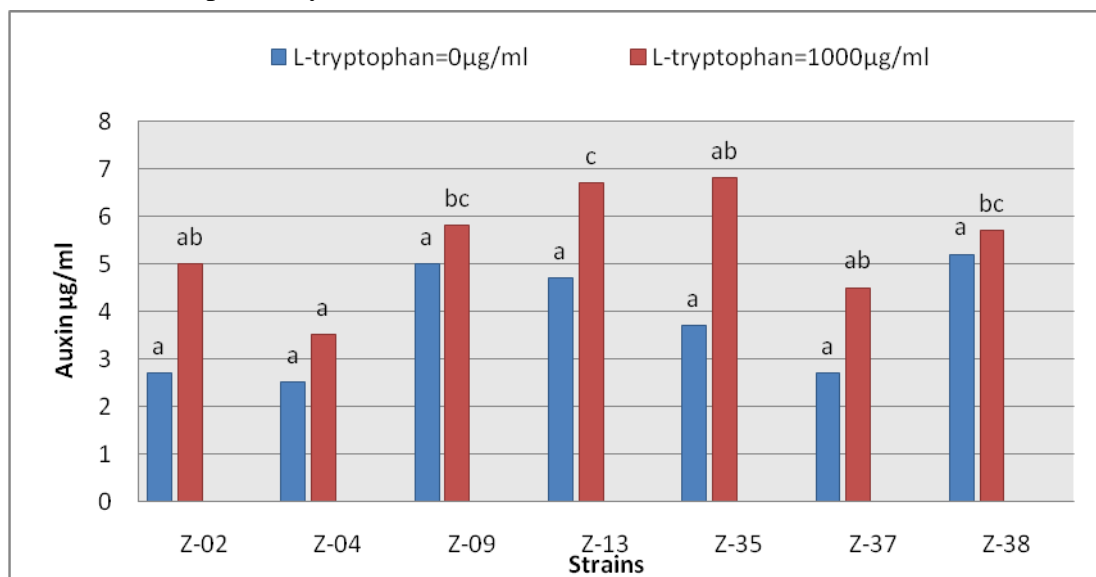


Fig. 1. Bacterial auxin production at two different concentrations of L-tryptophan 0 µg/ml and 1000 µg/ml. The bars represent Mean of 5 replicates. Different alphabets show significant differences between treatments by Duncan’s Multiple Range Test (DMRT) $p \leq 0.05$.

Rooting assay under laboratory conditions

Surface sterilized seeds were treated with bacterial suspension of McFarland 2 and allowed to germinate in dark under *in vitro* conditions. The water supply was monitored regularly. Length of radical roots and shoots were measured *in-vitro* after a period of 7 days. Bacterial treated seeds should significantly increase in root and shoot length over control (Fig. 3). In rooting

assay, strains of Z-09, Z-35 and Z-37 showed statistically significant increase (1.2 fold, 1.18 fold and 1.11 fold) respectively in root length over control. While for other strains Z-13, Z-38, Z-02 and Z-04, percentage increase of 98%, 76%, 63% and 38% was observed respectively over control. In case of mixed cultures C-3 (Z-35, Z-37, Z-38) exhibited a maximum (1.18 fold) increase while other mixtures C-1 (Z-02, Z-04, Z-09) and C-2 (Z-09, Z-13, Z-35) also showed positive effects on root

length 63% and 76% respectively over control. For shoot length a maximum fold increase was noted in Z-35 treated plants (1.18 folds). A significant increase in shoot length was observed by Z-38 (96%), Z-37 (88%), Z-13 (85%), while promising promotion was revealed by Z-02 (29%), Z-04 (33%)

and Z-09 (48%) over control. For mixed cultures treated plants again C-3 (Z-35, Z-37, Z-38) showed 81% increase in shoot length while C-2 showed 25% increase in shoot length over control. C-1 exhibited 3% decrease in shoot length over control (Fig. 2).

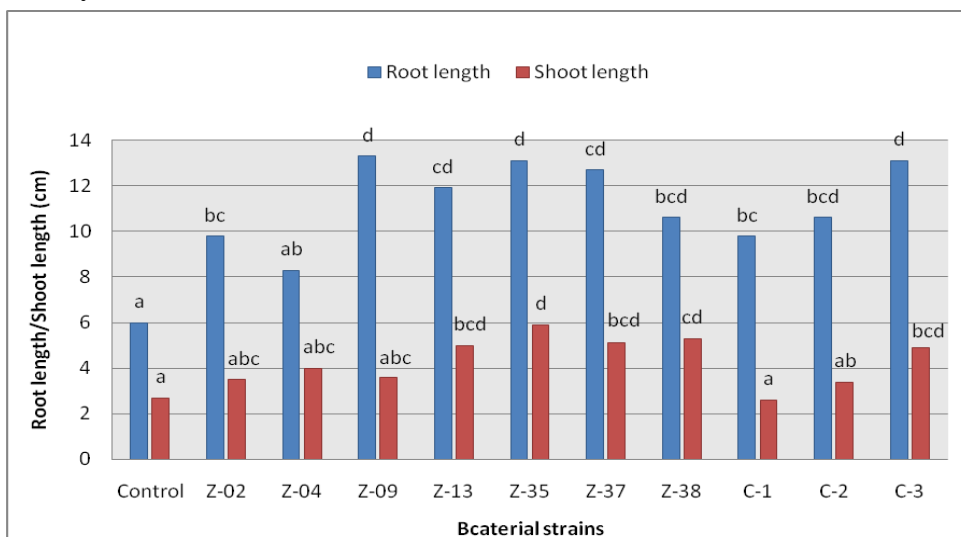


Fig. 2. Effect of bacterial treatments on root and shoot length of *Z. mays* (L.) in *in vitro* assay after 7 days of incubation. The bars show Mean of 5 replicates. Different alphabets show significant differences between treatments by “Duncan’s Multiple Range Test” (DMRT); $p \leq 0.05$.

Pot trials under Natural conditions

There was a significant improvement in values of bacterial treated seeds. Statistically significant results of shoot length were shown by Z-38 (67.1%), Z-13 (51%), Z-09 (43%) over control. While Z-04 (28%), Z-35 (23%), Z-37 (21%) and Z-02 (18%) also showed promising results. For mixed bacterial cultures C-1 (Z-02, Z-04, Z-09) showed maximum shoot length promotion

(28%) while C-2 and C-3 showed 26% and 23% promotion in shoot length over control. Plants treated with Z-38 and Z-09 showed 1.2 fold and 1 fold increase in fresh weight over control which was significant statistically. Z-35 and Z-04 treated plants showed half fold increase in fresh weight over control. Other treatments like Z-13 (86%), Z-02 (45%), Z-37 (22%) while mixed cultures C-1 (45%), C-2 (40%) and C-3 (36%) exhibited marginal increase in fresh

weight as compared to control (Fig. 3). One fold increase in dry weight was observed by treatment Z-38 which shows statistically significance while Z-02, Z-04, Z-13, Z-37 along with all mixed cultures showed 33% increase in dry weight as compared to control. No % increase in dry weight was observed by Z-35 treatment (Fig. 4).

Vegetative parameters recorded after 8 weeks of seed germination also showed significant improvements. Plants treated with Z-13 (42%) and Z-09 (39%) showed statistically significant increase in shoot length when compared to control. Promising results were shown by other culture treatments Z-38 (24%), Z-35 (9.7%), Z-37 (7.8%), Z-02 (6%),

C-1 (8%), C-2 (2.8%) and C-3 (4.17%). Results of fresh weight revealed that Z-37 (69%), Z-09 (61%), Z-04 (45%), Z-13 (42%) exhibited statistically significant results over control while Z-35 (16%) and Z-02 (15%) showed promising results. A percentage decrease in fresh weight was observed by Z-38 (11%) and mixed cultures C-1 (3%), C-2 (34%) and C-3 (32%) (Fig 5). In the second harvest bacterial treatments showed an enhancement in dry weight of plants i.e., Z-02 (5 fold), Z-04 (4 fold), Z-09 (3 fold), Z-13 (3 fold) and Z-35 (2.8 fold) while among mixed cultures C-1 (136%), C-3 (9%). However, C-2 showed no increase in dry weight over control (Fig. 6).

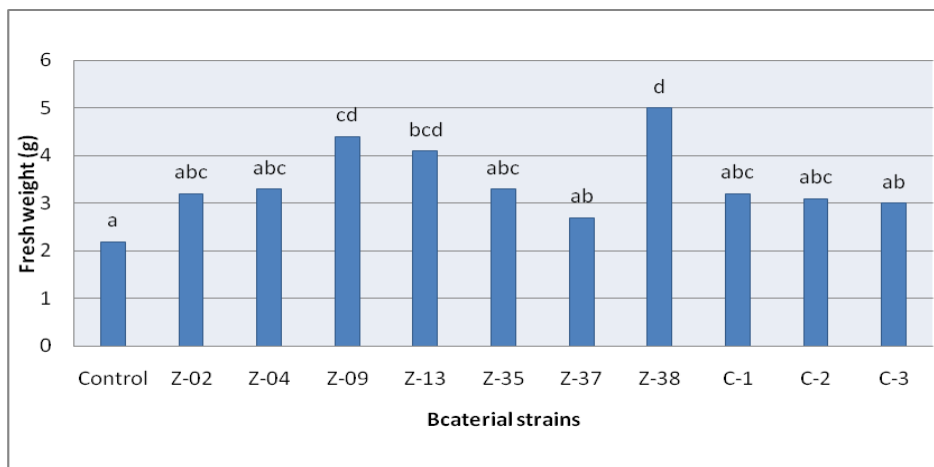


Fig. 3. Effect of bacterial inoculation on fresh weight of *Z. mays* (L.), 4 weeks after seed germination. The bars show Mean of 5 replicates. Different alphabets show significant differences between treatments by “Duncan’s Multiple Range Test” (DMRT) $p \leq 0.05$.

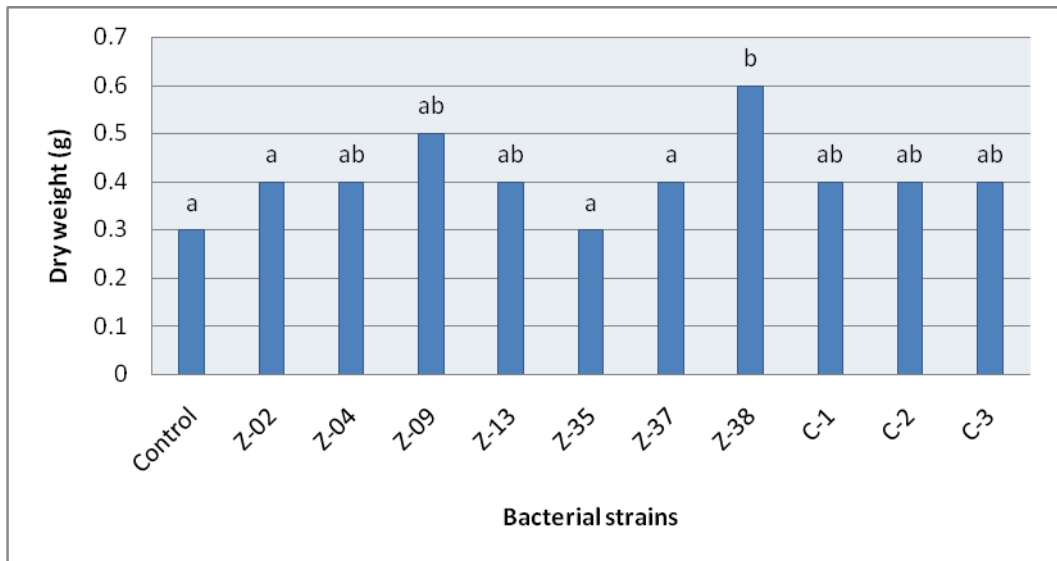


Fig. 4. Effect of bacterial inoculation on dry weight of *Z. mays* (L.), 4 weeks after seed germination. The bars show Mean of 5 replicates. Different alphabets show significant differences between treatments by “Duncan’s Multiple Range Test” (DMRT) $p \leq 0.05$.

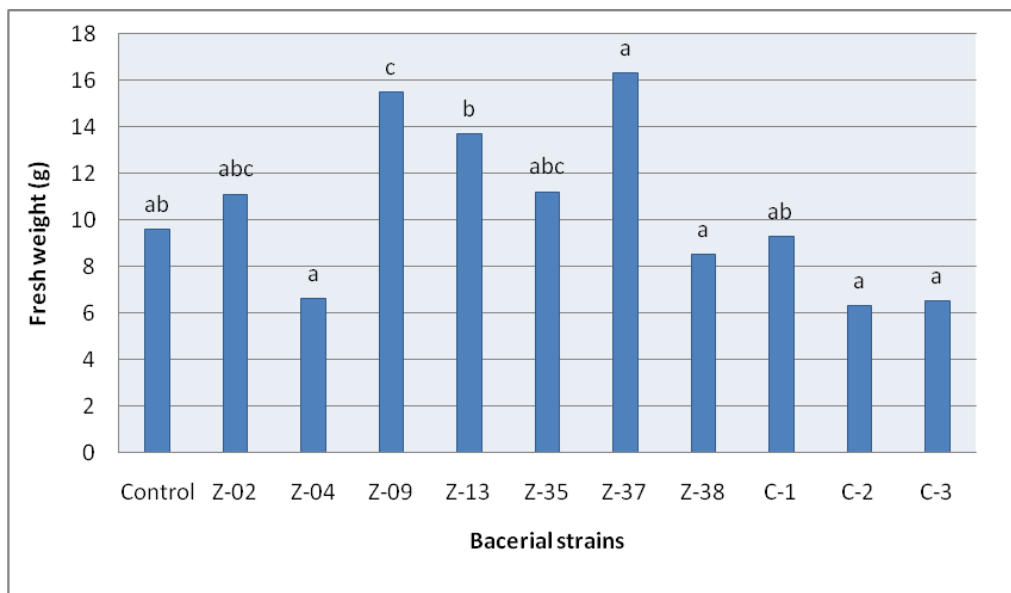


Fig. 5. Effect of bacterial inoculation on fresh weight of *Z. mays* (L.), 8 weeks after seed germination. The bars show Mean of 5 replicates. Different alphabets show significant differences between treatments by “Duncan’s Multiple Range Test” (DMRT) $p \leq 0.05$.

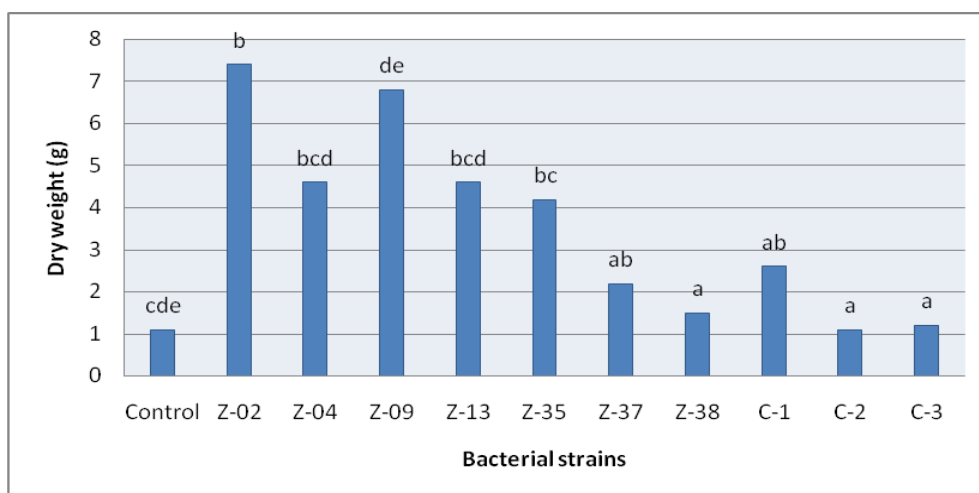


Fig. 6. Effect of bacterial inoculation on dry weight of *Z. mays* (L.), 8 weeks after seed germination. The bars show Mean of 5 replicates. Different alphabets show significant differences between treatments by “Duncan’s Multiple Range Test” (DMRT) $p \leq 0.05$.

DISCUSSION

This research was conducted to estimate the potential of pure and mixed varieties of *Bacillus simplex* to up regulate the growth and development of maize (*Zea mays* L.). Strains of *B. simplex* were subjected to seven biochemical tests. They were recorded positive for catalase, nitrate and citrate while negative for indole, urease, TSI and oxidase test. We tested *B. simplex* strains for the quantitative production of auxin. Auxin is an important phytohormone which is a primary criteria to choose the best plant growth promoting rhizobacteria. The effect PGPR on growth regulation depends on host plant, rhizobacteria, inocula concentration and environmental conditions (Shah et al., 2017).

Maximum amount of auxin was produced by Z-38 (6.8 $\mu\text{g/ml}$). Comparison showed that Z-02 strain produced 85% more auxin in the presence of 1000 $\mu\text{g/ml}$ L-tryptophan than control. Auxin production is related to L- tryptophan concentration. Studies have been conducted on quantitative detection of bacterial auxin and it has been reported that *B. pumilis* produced maximum auxin (103 $\mu\text{g/ml}$) at 0.5 mg L- tryptophan which is much higher than 22 $\mu\text{g/ml}$ auxin in the absence of L-tryptophan (Dashti et al., 2021). Roots play very critical role in plant development as they help in uptake of water and minerals. *Bacillus* species are known to improve average length and area of roots (Yu et al., 2023). Rooting assay *in vitro* evaluated that the test strains were able to directly influence

the vegetative growth parameters of *Z. mays* L. and there was a significant difference in values over control. Our study reported the best rooting response of maize by strains of Z-09 and Z-35 which showed 120% and 118% increase in root length over control as evident by auxin production while in mixed cultures the combination of C-3 (Z-35, Z-37, Z-38) (118%) proved to be the best growth promoter in maize. Screening of PGPR report that *B. subtilis* increases root length of *Sinapis alba* (L.) by 28% under sterile soil conditions (Minut *et al.*, 2023). Microbial consortia of two or more PGPR have positive impact on organic maize under varying climate conditions (Hett *et al.*, 2023).

In *in vivo* experiments (pot trials) response of different plant parameters was recorded statistically. Bacterial species showed better responses as compared to control. Plant biomass was related to shoot length which was related to time of harvest. In our research the maximum increase in shoot length of maize was produced by Z-38 (67.1%), while in combinations C-1 (Z-02, Z-04, Z-09) produced maximum enhancement in shoot length (28%). Results after 8 weeks of germination showed that Z-13 treated plants resulted in maximum increase in shoot length

(42%) over control and among mixed cultures C-1 (Z-02, Z-04, Z-09) was considered good driver of fast plant growth. In our study the statistically significant result for fresh weight and dry weight of maize was recorded by strain Z-38 (120% and 100%). While in mixed cultures C-1 (Z-02, Z-04, Z-09) produced 45% and 42% increase in plant fresh weight and dry weight than control. These values were recorded 4 weeks after seed germination. Vegetative parameters after 8 weeks revealed Z-09 (61%) and C-2 (34%) were increasing fresh weight to maximum. For dry weight Z-02 produced 5 fold increase while mixed culture C-1 (136%) produced significant results. Tanveer and Ali (2022) has demonstrated the comparable results with Z-02 *B. simplex* on enhancement of growth of *Vigna radiata* (L.) under drought stress. Plants treated with *B. simplex* (Z-37) showed an increase of 8% under normal conditions. Azeem *et al.* (2022) reported an increase in fresh weight and dry weight (56% and 103%) in maize by *Bacillus* treatments. Other studies have reported that *B. albus* and *B. proteolyticus* increase weight of peanut plant (1.5-1.9 times) as compared to control (Trinh *et al.*, 2022).

CONCLUSION

It was concluded that bacterial auxin production was directly related to concentration of L- tryptophan in culture media. Enhancement in vegetative characteristics of maize by *Bacillus simplex* shows that PGPR are an excellent environmentally friendly replacement of chemical fertilizers. Improvement in root growth of maize by different strains showed that these strains can enhance plant growth by improving water and mineral uptake. Especially the strains of Z-02, Z-09, and Z-37 best improved the growth. In plant-microbe experiments both single and mixed cultures of *B. simplex* showed great potential of improving plant growth of maize both *in vitro* experiments and field trials. Future research in producing higher yield and less expensive PGPR formulations with reduced phytotoxicity and increased self-life will be helpful.

Contribution of Authors

Shafaq Navid conducted the experimental work and collected the data. Sana Tanveer prepared the 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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