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Screening growth promoting potential of *Halotolerant bacillus* strains in maize under saline stress

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Contribution | **Bano, I.,** conducted the experimental, collected the data; **S. Navid** prepared the draft; **B. Ali** conceived this study

This current study was conducted to screen halotolerant bacterial strains for their ability to enhance the productivity of *Zea mays* (L,) under saline stress. The soil samples were collected from the rhizosphere of *Acacia arabica* (L.) tree growing in saline areas. Halotolerant strains were isolated by using salt amended growth media. The final taxonomic status of strains was confirmed by 16S RNA sequence analysis. The strains were analysed for auxin production, phosphate solubilisation, antibiotic sensitivity, and hydrogen cyanide production. The effect of isolated strains on the growth of maize was checked under both *in vitro* and *in vivo* conditions. The sequence analysis confirmed local strains belonged to the genus *Bacillus*. For auxin production, strains B7and B4 were recorded to produce a 1-fold and 96% improvement, in the presence of L-tryptophan. Rooting assay recorded up to a 70% increase with B3 strain, over control. *In vivo* pot trials at 100 mM NaCl, B1 (82%) recorded statistically significant improvement in shoot length, over control. While for dry weight B5, and B3 were giving 2-fold and 1-fold improvement in growth, respectively. For fresh weight, B5 strain recorded a maximum growth response of 1.5-fold at 200mM NaCl, over the control in vivo. The results of current study confirmed that salt-tolerant strains can minimize the effects of salinity on the growth of maize plants.

Keywords: Salinity, rhizobacteria, growth promotion, *Zea mays*, plants, stress

INTRODUCTION: According to Trade Development Authority of Pakistan, Pakistan produces 10 million tons of maize annually utilizing an area of 17 million hectares. Soil salinity is a major ecological stress that can influence crop growth and productivity. Around 20% of cultivated land is affected by salinity, and this percentage is rising. It reduces areas of cultivated land, and the quality of crops. Salinity influences the crop productivity as well as soil structure, soil properties, and the balance of the soil profile (Singh, 2022; Singh, 2022) Saline soils cause disturbs physiological and biochemical processes of plant growth, like seed germination, photosynthesis, and uptake of nutrients. Also it causes osmotic and oxidative stress, ion toxicity, and limitation in uptake of water (Muhammad *et al.*, 2024). Salty soils contain toxic ions like sodium, chlorine, and boron, which affect seed germination and lead to ion toxicity. Sometimes, phosphorus uptake is decreased in saline soils due to the involvement of phosphate ions with calcium cations (Xie *et al.*, 2022). Somatic growth of plants is affected in salty soils due to the disrupted process of photosynthesis. It may decrease leaf area, efficiency of photosystems, and chlorophyll content. Salinity disturbs photosynthesis by limiting the presence of CO_2. It causes a reduction in mesophyll and stomatal content in spinach (Umarov *et al.*, 2024). Identification of salt-tolerant microorganisms can encourage salt tolerance in crops and also reduce the burden on arable lands. The ability of Plant Growth Promoting Rhizobacteria (PGPR) to cause resistance to different abiotic stresses is supported by ecological conditions, which may include the microclimate, weather, and soil structure and properties of soil (Gwa and Ekefan, 2024). Phytohormones produced by PGPRs are involved in different biochemical and physiological responses (Tariq *et al.*, 2024). Phytohormones help in growth promotion, the seedling process, and the uptake of nutrients. Five main phytohormones are auxin, gibberellin, cytokinin, ethylene, and abscisic acid. Similarly, the ability of PGPR to solubilize phosphate and break HCN increases its ability to induce salt tolerance in plants (Ranjan *et al.*, 2024). Indole-3-acetic acid (IAA) is also a growth-promoting phytohormone and it is included in the category of auxins. Auxin is involved in variety of mechanisms for growth in plants like enlargement of leaves, increase in area of leaves, and number of leaves, flower and fruit development and cell elongation, apical dominance, responses toward different factors, primary and lateral root growth and leaf morphogenesis in plants (Kumari *et al.*, 2024). The salt tolerant PGPR on inoculation improve the growth of crops in salt stress environment. They provide help to crops by enhancing their mechanisms which include; activation of antioxidation defense by activating enzymes like SOD (superoxide dismutase), catalase and peroxidase, soil aggregation by producing EPS which can reduce salt stress because of its composition which binds sodium ions, alteration in morphology of root to access water, increase in stomatal conductance to improve photosynthetic activity and regulation of tolerant genes in response to stress (AbuQamar *et al.*, 2024). *Bacillus* is a commonly found genus of rhizobacteria. They release many metabolites to form strong contact with higher plants. Bacterial inoculation of seeds improves plant health and survival rates. Different *Bacillus* strains have beneficial effects on different plants like raspberries and wheat. They play an important role in agricultural development, with wide applications and have become an integral part of the sustainable agricultural system. In the U.S, 65% to 75% of crops such as cotton, corn, and grains are treated with commercial *Bacillus* products, which combat soil-borne pathogens like *Fusarium spp.* and *Rhizoctonia spp.* (Wu *et al.*, 2025).

OBJECTIVES: The primary objective of this study was to analyse the ability of halotolerant *Bacillus spp.* from the rhizosphere of *Acacia arabica* (L.) to promote the growth of *Zea mays* (L.) under salt stress. The strains were screened for auxin production and then used as bioinoculants to evaluate the potential to enhance plant growth in pot trials.

MATERIALS AND METHODS: **Sample collection and isolation**: Rhizospheric soil samples were collected from the plant *Acacia arabica* (L.) growing in the vicinity of Khewra salt mine, Punjab, Pakistan. The concentrations (0M, 0.25M and 0.5M) of common salt were added to the L-agar for isolation of salt tolerant bacteria strains. The sample dilutions were spreader and then streaked on L-agar plates with respective salt concentration. Bacterial strains were characterized morphologically by recording morphology, size, margin and elevation of colony. Pure cultures of bacteria were subjected to Gram's staining to evaluate their morphological characteristics. Spore staining was also accomplished to detect the endospore formation by bacterial strains.

The 16S rRNA gene sequencing: DNA was isolated using AquaPure genomic DNA isolation kit (BIO-RAD) in accordance with the instructions of manufacturer. Gel electrophoresis of isolated DNA samples was done to find DNA. Forward primer 27F (5'AGA GTT TGA TCC TGG CTC AG 3') and reverse primer 1522r (5' AAG GAG GTG ATC CA(AG) CCG CA 3') were used for amplification. PCR master mix of Dream Taq™ was used, and reaction mixture was incubated in thermocycler. For every step of PCR, temperature and time was specified in thermocycler. For denaturation step 94°C for 5 min., for annealing step 55°C for 1 min. and for extension step 72°C for 5 min. After PCR, products were kept at -20°C. For gel extraction, protocol of the Thermoscientific Genejet DNA Purification Kit was followed. Results of sequencing were studied by Chromas software. To check the homology between sequences Basic Local Alignment Search Tool (BLAST) was used.

Auxin production by colorimetric method: Potential of bacterial isolates to produce auxin under *in vitro* conditions were estimated in presence or absence of L-tryptophan. Strains were cultured in 20 ml L-broth having 0 and 0.5M NaClin 6 replicates. Three different concentrations of L-tryptophan (0, 200, and 400 µg/ml) were used for auxin quantification. Incubation was done at 37°C at 120 rev/min for 72 hours. After incubation, 1ml culture was centrifuged for 1 minute at 5000 rpm. The supernatant was then used to check the biosynthesis of auxin by adding Salkowski's reagent. Samples were incubated in dark for 20 minutes. After incubation, color change was observed and optical density (O.D) was taken at 535 nano meter and recorded. The standard curve constructed by using standard auxin was used to quantify bacterial auxin production.

Rooting assay with bacterial strains: For rooting assay, sand was taken in culture tubes, and autoclaved. Bacterial isolates were cultured on L-agar plates and were used to prepare bacterial suspensions adjusted to 10^7 CFUs/ ml. Seeds of corns (*Zea mays*)

were treated with $HgCl_2$ solution for 1-2 min. Seeds were washed repeatedly with autoclaved distilled water. Bacterization of seeds was done by soaking them in bacterial suspension for 25-30 min. For control, seeds were dipped only in water. After seed treatment, seeds were sowed to 1 cm depth in culture tubes with the help of sterile forceps. Sand was slightly moistened with distilled water. Culture tubes were incubated at room temperature for seed germination. After seed germination, root length (RL), shoot length (SL), number of roots and leaves, fresh weight (FW) and dry weight (DW) were noted after 2 weeks.

Phosphate solubilisation test: Pikovskaya's medium was used to screen phosphate solubilizing ability of the bacterial strains. After streaking, incubation was done at 28°C for 7 days. Plates were observed for a clear zone, and results were recorded (Navid *et al*., 2024)

Antibiotic susceptibility test: Heavy inoculum was given to plates having Mulller Hinton agar. Antibiotic discs (Bioanalysis®) of ampicillin (10µg), gentamycin (10µg), neomycin (30µg), chloramphenicol (30µg), erythromycin (15µg), and tetracycline (30µg) were used for analysis. These discs were shifted to the surface of agar. Incubation was done at 37°C for 24 h. After incubation, the zone of inhibition was measured for each disc. The results were noted and compared with the standard chart for antibiotics obtained from CLSI (Clinical Laboratory Standard Institute).

Hydrogen cyanide production test: Bacterial colonies were swabbed on L-agar plates. Filter papers dipped in a 2% solution of sodium carbonate in 0.5% picric acid were placed on agar plates. Incubation was done at 30°C for 5 days. A color change from orange to brownish-red was observed and recorded for HCN production.

Pot trials with salt stress: Pot trial was performed to estimate the effect of isolates on growth promotion of *Z. mays* in salt stress. For pot trials, pots filled with soil and arranged in the wire house of the Institute of Microbiology and Molecular. Seeds were treated with bacterial suspension as mentioned above. After that, seeds were shifted to pots 1 cm deep with sterile forceps. For each bacterial strain, 8 pots were arranged. Those 8 pots include replicates of 0 mM, 50 mM, 100 mM and 200 mM salt stress (NaCl). Each pot contained 6 seeds. Seed germination started in 12 days. After seed germination, water with different salt concentrations was added in soil. For control, 8 pots were arranged in which seeds treated with water were planted. Moreover, salt stress of 0 mM, 50 mM, 100 mM and 200 mM NaCl was given to control. Parameters of growth like RL (root length), SL (shoot length), germination rates, (FW) fresh weight, and DW (dry weight) were noted after 3 weeks of germination (Navid *et al*., 2024).

Statistical analysis: Data from auxin analysis, rooting assay and pot trials was subjected to ANOVA (analysis of variance), and Duncan's test ($P \leq 0.05$) to separate means of different treatments.

RESULT AND DISCUSSION: Morphological characterization of bacteria: Morphology of bacterial isolates was confirmed by Gram's staining. Microscopic studies showed that all strains were gram positive rods. Moreover, all strains were able to produce resistant bodies called endospores.

Analysis of 16s rRNA gene sequences: Sequence analysis showed that all strains belong to genus *Bacillus*. B3, B4, B5, B7, S3 and S4 showed 100% similarity with different species of *Bacillus subtilis* (table 1). Whereas, S5 was similar to *Aneurinibacillus sp.* Sequences were submitted to gene bank. Accession numbers were given to each sequence by NCBI. By using these accession numbers, a phylogenetic tree was made by Molecular Evolutionary Genetics Analysis version 4 (MEGA4) (figure 1A).

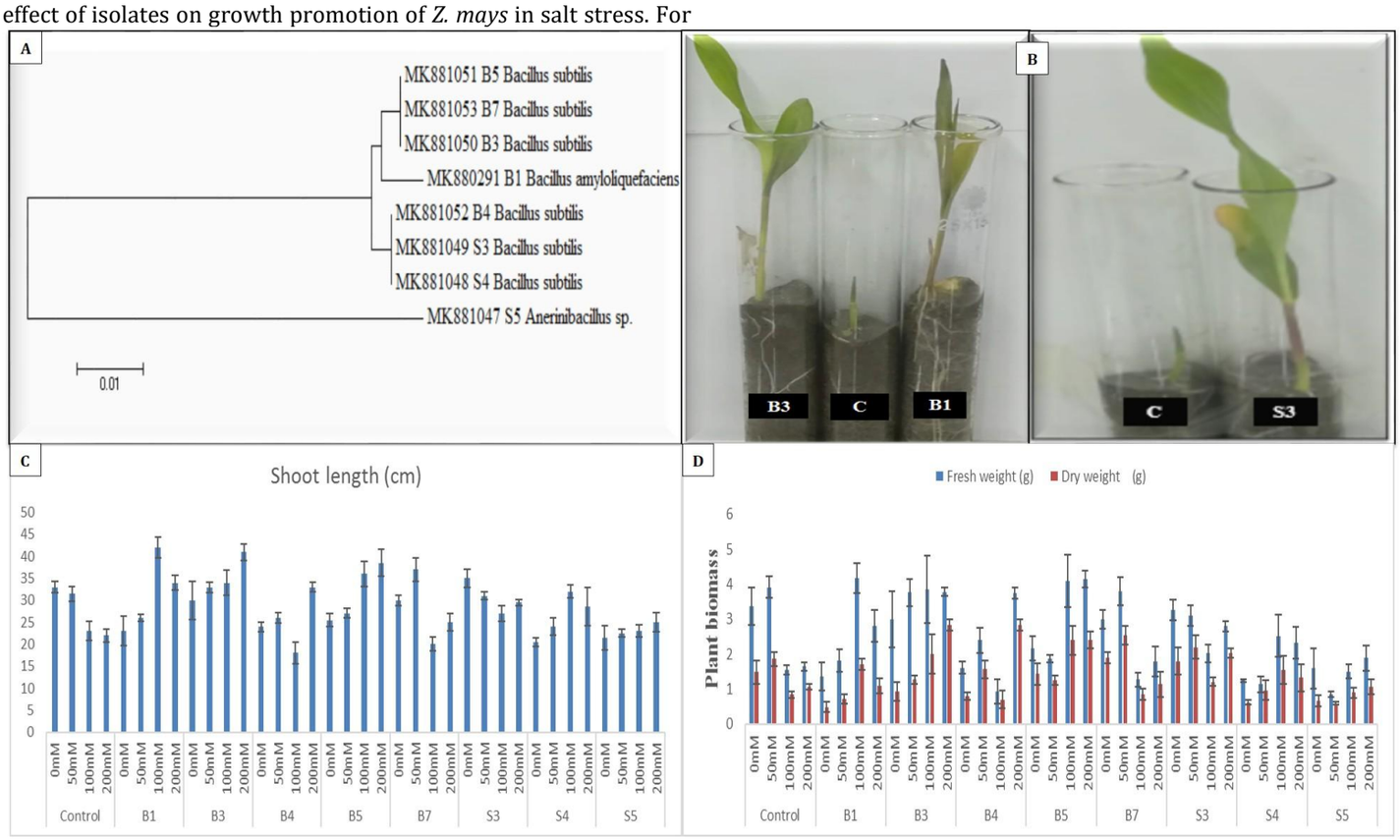

Figure 1: Phylogenetic tree, comparison of halotolerant bacteria isolated from the rhizosphere of *Acacia arabica* L. (A), results of in-vitro rooting assay under normal environmental conditions (B), effect of bacterial inoculations on shoot length of maize under salt stress in pot trials (C) and effect of bacterial treatments on plant biomass in pot trials under salt stress (D).

Sr no.	Strains	GenBank
1	*Bacillus amyloliquefaciens* B1	MK880291
2	*Bacillus subtilis* B3	MK881051
3	*Bacillus subtilis* B4	MK881053
4	*Bacillus subtilis* B5	MK881050
5	*Bacillus subtilis* B7	MK881052
6	*Bacillus subtilis* S3	MK881049
7	*Bacillus subtilis* S4	MK881048
8	*Aerinibacillis species* S5	MK881047

Table 1: Bacterial strains used in research.

Bacterial auxin production: Bacterial strains exhibited increase in auxin production in the presence of L-tryptophan. Strains were showing promising results in the presence of 200 µg/mL and 400 µg/mL L-tryptophan. It was observed that at 200µg/Ml L–tryptophan, B7 and B4 recorded around 1-fold increase, over control. Similarly, at 400 µg/ml L-tryptophan, B4 was producing 2-fold auxin, whereas, strains B5 and B1 recorded 1-fold increase as compared to control (table 2). Auxin analysis revealed that at 200 µg/ml L–tryptophan, B7 was producing maximum amount of auxin that was 1-fold over control. Researches have reported that in presence of 1000 µg/mL L-tryptophan, maximum auxin was produced by *Bacillus subtilis* Z-16 which was 85% over control (Saboor *et al.*, 2024). Similarly, other studies revealed significant

production of auxin by some *Bacillus* strains, i.e. Z-13 (6.7µg/mL) and Z-09 (5.8µg/mL), at 1000µg/mL concentration of L-tryptophan (Navid *et al.*, 2023).

Sr no.	Strains	L-tryptophan 0µg/mL	L-tryptophan 200 µg/Ml	L-tryptophan 400 µg/mL
1	**B1**	0.374 (b)	0.65 (c)	1.053 (b)
2	**B3**	0.375 (b)	0.673 (c)	0.955 (b)
3	**B4**	0.386 (b)	0.759 (cd)	1.096 (b)
4	**B5**	0.478 (c)	0.803 (d)	1.076 (b)
5	**B7**	0.293 (b)	0.676 (c)	0.946 (b)
6	**S3**	0.158 (a)	0.441 (b)	0.701 (ab)
7	**S4**	0.071 (a)	0.403 (b)	0.721 (ab)
8	**S5**	0.142 (a)	0.112 (a)	0.267 (a)

Table 2: Auxin production by bacterial strains in the presence and absence of L-tryptophan.
Mean of triplicates. Different alphabets in same column indicate significant difference among treatments using Duncan's multiple range test ($p \leq 0.05$).

Sr no.	Strains	Root length RL (cm)	Shoot length SL (cm)	Fresh weight FW(g)	Dry weight DW (g)
1	**Control**	14 (b)	10 (a)	0.54 (a)	0.17 (a)
2	**B1**	11 (a)	16 (c)	0.75 (e)	0.24 (c)
3	**B3**	20 (c)	17 (d)	0.70 (d)	0.24 (c)
4	**B4**	17 (c)	10 (a)	0.67 (c)	0.21 (b)
5	**B5**	14 (b)	12 (ab)	0.90 (f)	0.25 (c)
6	**B7**	14 (b)	15 (c)	0.61 (b)	0.20 (b)

Table 3: Effect of bacterial treatment on the growth of *Zea mays* (L.) under in vitro conditions.
Mean of duplicates. Different letters in same column indicate significant difference among treatments using Duncan's multiple range test ($P \leq 0.05$).

Sr no.	Strains	Diameter of zone of inhibition in mm					
1	**B1**	20(R)	27(S)	22(I)	30(S)	23(S)	18(I)
2	**B3**	14(R)	25(S)	20(I)	25(S)	23(S)	22(S)
3	**B4**	20(R)	20(S)	20(I)	21(S)	18(I)	25(S)
4	**B5**	15(R)	20(S)	18(I)	22(S)	22(I)	23(S)
5	**B7**	15(R)	20(S)	18(I)	25(S)	25(S)	25(S)
6	**S3**	20(R)	20(S)	28(I)	25(S)	21(I)	20(S)
7	**S4**	25(R)	23(S)	20(I)	23(S)	20(I)	30(S)
8	**S5**	25(R)	20(S)	18(I)	14(I)	15(I)	26(S)

Table 4: Antibiotic sensitivity pattern of halotolerant bacterial strains against different antibiotics.

Rooting assay: In comparison to control, all strains showed plant growth promotion (figure 1B). Bacterial isolates B3, B4 and S4 showed 40%, 25% and 11% increase in root length, respectively, over control. For shoot length, strains B1, B3 and B7 showed 60%, 70% and 50% increases in shoot length, respectively, over control (table 3).

Antibiotic susceptibility test: Antibiotic discs of six antibiotics ampicillin, gentamycin, neomycin, chloramphenicol, erythromycin and tetracycline were shifted to agar surface in sterile conditions. All the isolated strains formed zone of inhibitions in the range of 14 – 30 mm. All strains were resistant to ampicillin, but sensitive to other antibiotics. Strain B4, B5, S3 and S4 were showing intermediate activity with neomycin and erythromycin (table 4).

The HCN production*:* Bacterial isolates were screened for antifungal metabolite i.e., HCN production. Two strains *Bacillus amyloliquefaciens* (B1) and *Aneurinibacillus sp.* (S5) showed positive results as evident by brown colour. The other six strains (B3, B4, B5, B7, S3 and S4) showed no colour change and were recorded negative for HCN.

Phosphate solubilisation test: Bacterial isolates were assessed for phosphate solubilisation ability in Pikovskaya's medium. After incubation, clearing zones were not shown by any strains, thus, recorded as non-phosphate solubilizes.

Pot trials: Seeds were germinated for 15 days and parameters of plant growth like shoot length (SL), number of leaves (NL), fresh weight (FW) and dry weight (DW) were recorded. It was observed at 50 mM NaCl, strain B7 (19%) and B3 (6%) improved shoot length over control. At 100 mM, B1 (82%), B5 (56%), B3 (47%) and S4 (39%) strains recorded improvements in shoot length as compared to control. While, fresh weight, B5 (1.6-fold), B1 (1.5-fold), B3 (1.4-fold), and S4 (66%) showed statistically significant results, over control. While for dry weight, B5, and B3 recorded, respectively, 2-ford and 1-fold increases, over control. At 200 mM salt stress, B3 (86%), B5 (72%), B1 (54%), B4 (50%) and S3 (31%) improved shoot length, over control. For fresh weight, B5, and B3 recorded 1.5-fold and 1-fold increases, respectively, as compared to control. Similar, for dry weight, B3 and B5 showed 1.6-fold and 1.2-fold increases, over control (figure 1D). Rooting assay revealed that bacterial isolate B3, showed 42% increase in root length as compared to control. Also, previous studies revealed that *Bacillus megaterium* recorded a 7.6% improvement in shoot length and a 47% improvement in root length of *Zea mays* (L.) compared to control (Shoaib *et al.*, 2024). Our study reported that at 50mM NaCl strain B7 (19%) was improving shoot length over control. At 100mM, B1 (82%) and B5 (56%) were showing improvement in shoot length over control (figure 3). Likewise, at 200 mM salt stress B3 (86%), B5 (72%), B1 (54%), B4 (50%) and S3 (31%) were improving shoot length over control. Studies show that halotolerant microbes solubilize phosphate by 54% and help in enhancing mustard seed growth under saline stress (14). Similar inoculation of maize plant with some rhizobacteria isolated from wild halophytes improved the biochemical responses of plant to saline stress (Sharma *et al.*, 2026). In our study, B5 (1.6-fold), B1 (1.5-fold), B3 (1.4-fold), and S4 (66%) showed statistically significant improvement in fresh weight, over control at 200mM salt stress (figure 4). Recent studies report that Halophillic *Pseudomonas* was reported to alleviate salt stress and improved the growth of peanut under salt stress (Atici *et al.*, 2025). Moreover, *Bacillus spezizinni* has ability to induce salt tolerance and enhance the growth of cherry tomato in hydroponic cultivation system (Trung and Tri, 2025). Synthetic consortium of PGPR improved the metabolic profile and productivity of Lettuce in suboptimal regime (Masmoudi *et al.*, 2025). It is inferred that Glomus in synergism with Bacillus cereus help in the amelioration of adverse effects of salt stress in wheat plant (Sabah *et al.*, 2025). Also, studies have reported that iron-silicon nanoparticles (NPs) and plant growth-promoting rhizobacteria can reduce abiotic stress and enhance crop yield (Aghaei *et al.*, 2025).

CONCLUSION: The current study confirmed that auxin producing, halotolerant bacterial strains isolated from salt stressed soil have the ability to enhance the growth of *Zea mays* (L.). *B. amyloliquefaciens* B1, *B. subtilis* B3 and *B. subtilis* B5 showed statistically significant enhancement in growth parameters and were involved in reducing deleterious effects of salinity on plant growth. Also, the improvement in growth by these strains was directly related to its ability of auxin production. Future prospects

of our research include modern applications of PGPR in soil by coupling them with some fluorescent proteins. This will help to better understand the distribution of strains in soil along with a detailed analysis of their mechanism in plant growth promotion.

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