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Improving Yield and Quality through Salt-tolerant PGPR Inoculation: A Focus on Vigor Index and Growth Promotion of Wheat (*Triticum aestivum*)

Tulasa Khatik[1], Jainish Panchal[1], Yesha Master[2] and Smita Parekh[1]*
[1]*Department of Microbiology, The Mandvi Education Society, Veer Narmad South Gujarat University, District: Surat, Gujarat, India*
[2]*Deparment of Life Science, GSFC University, District: Vadodara, Gujarat, India*
*For correspondence: smitaparekh92@yahoo.com
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Abstract

One of the most detrimental abiotic factors affecting crop development and output, particularly in coastal regions, is salinity. The use of Plant Growth Promoting Rhizobacteria (PGPR) as a treatment can be a valuable tool for improving crop resilience and productivity in saline soils, which are a major constraint to agriculture worldwide. In the current study, bacterial isolates were obtained from soil samples that were collected from different coastal regions of Gujarat state, including Surat district (Dumas, Hajira, and Ubharat), Dwarka district, and Porbandar district (Madhavapur). Isolates were examined for their Plant Growth Promoting (PGP) activities to enhance wheat growth under salt stress. The production of ammonia, hydrogen cyanide (HCN), indole acetic acid (IAA) and gibberellic acid were among the PGPR activities that were used to screen the potential PGPR isolate. For salt stress tolerance, M3 isolate (*Acinatobacter lowffi*) grew well in 7.5% NaCl followed by 12.5% NaCl. Validation of nitrogen fixing isolates was performed using nifH gene amplification. Using 16S rRNA sequencing, significant isolate with salt tolerance and PGPR activity were molecularly characterised. Vigor index was measured for a plant's overall health and growth potential. Simultaneously, growth factors like seedling emergence, root development and leaf growth were taken into consideration. The results showed that five out of fifteen isolates obtained on nitrogen free Ashby's agar showed high salt tolerance potential along with nitrogen fixation capacity as per PCR studies. However, the application of M3 was proven to be better halotolerant PGPR candidate under 7.5% and 12.5% salt stress compared to the control group that did not receive inoculum treatment and NPK half dose (T12 and T14). Seeds inoculated with M3 showed higher vigor index than the uninoculated seeds. The findings indicated that M3 demonstrated good potential as PGPR for salinity mitigation approach for coastal wheat production. Therefore, further studies on various wheat varieties and under field conditions are recommended.

Keywords: Nitrogen fixing bacteria; Halotolerant bacteria, PGPR Rhizobacteria, Biofertilizer, Seed germination, Vigor index

Introduction

In light of the expected population growth, agricultural output will play a major role in ensuring future food security. The current agricultural production rise is not sufficient to meet the food requirement of 10 billion people by 2050, according to the Global Agricultural Productivity (GAP) Index (Etesami and Beattie 2018; Sabagh *et al.* 2021; Sharma *et al.* 2021; John *et al.* 2023) for food security. The most important staple crops are wheat, rice and maize are the world's most important crops, accounting for a significant amount of daily energy and protein intake. For both people and cattle, wheat is a vital flour crop. Since it is an excellent source of carbs, it has nutritive and physiological benefit. Among these key cereals, wheat (*Triticum aestivum* L.) is ranked top because of its domestication and significant primary staple crop in the world (Sabagh *et al.* 2021; Yaghoubian *et al.* 2022). A variety of stresses are experienced by plants, including insect attacks, phytopathogens, extremes of temperature, salt, drought, flooding and inadequate nutrients. A significant issue contributing to the decline in crop growth and productivity worldwide is the excessive concentration of salts in the soil, among other environmental stressors. Over 1,000 m ha of land has been affected by salinity worldwide and 10% of agricultural lands are at risk of becoming salinized due to continued fertilizer use and suboptimal irrigation water. The percentage of dissolved salt in saline soil is high. Since it might be metabolized into NaCl, Na_2CO_3 and Na_2SO_4 in the soil, Na^+ constitutes one of the most widespread salt-dissolved constituents (Sharma *et al*. 2016; Etesami and Beattie 2018; Khumairah *et al*. 2022; John *et al*. 2023).

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In an attempt to mitigate these impacts, farmers are irrigating their fields and applying fertilizers to restore soil mineral nutrients; yet chemical inputs lead to contamination of the soil and water (Yaghoubian *et al*. 2022). Plant halobionts, known as Halotolerant plant growth-promoting rhizobacteria (H-PGPR), enhance plant development in saline soil. Plant roots are home to H-PGPR isolates, which can coexist and interact to form a rhizosphere bacterium (Khumairah *et al.* 2022). Salt-tolerant rhizobacteria have evolved a variety of approaches for surviving in highly saline conditions. One essential approach is the ability to accumulate suitable osmolytes to maintain intracellular osmotic balance where they form the nitrogenase enzyme, which has a potential involvement in biological nitrogen fixation activity (Etesami and Beattie 2018; Abbas *et al*. 2019). The enzyme is made up of the molybdenum iron dinitrogenase (MoFe protein), encoded by the nifDk genes, and the iron (Fe) containing dinitrogenase reductase (Fe protein), transcribed by the nifH gene. Nitrogen fixer has an adaption mechanism that protects it from the concentration of oxygen molecules because nitrogenase is an oxygen-sensitive enzyme (Nikul and Goswami 2021).

The aim of the present study was to isolate Nitrogen fixing *Azotobacter* species often with salt tolerant potential. To accomplish this goal, soil samples were collected from various coastal regions of Gujarat state and appropriate media was used. Further validation of nitrogen fixing property was done through PCR to check presence of nifH gene. Nitrogen fixers were tested for their salt tolerant potential using different NaCl concentrations accompanied with other growth promoting tests. Organisms showing good PGP activity were further subjected to *in vivo* pot assay. Pot assay assisted in identifying a better halotolerant nitrogen fixing organism. Finding of this study will be helpful to explore salt tolerant PGPR with growth promoting traits fostering soil fertility improvement, the growth of coastal plants and support the general resilience and health of coastal ecosystems.

Materials and Methods

Collection of soil samples

Soil samples were obtained from various places affected by salinity. Five soil samples were taken from the rhizosphere of weeds from different locations including Dwarka, Madhavpur, Dumas, Hajira and Ubharat (Table 1). The soil from salinity affected coastal regions were carefully collected in sterile plastic bags and brought to the lab for microbiological examination. Soil samples were stored at 4°C prior to microbial analyses.

Isolation of nitrogen fixing Bacteria

Isolation of nitrogen fixing bacteria was done according to method described by Sharma *et al.* (2021). Serially diluted soil samples were initially inoculated on Jensen's media to obtain nitrogen fixing organisms. Subsequently isolates obtained on Jensen's media were further inoculated on selective media such as Ashby's Mannitol Agar for the isolation of *Azotobacter* sp. Incubation was done at room temperature for 8 days. Isolates having nitrogen fixation potential will give blue colour pigmented growth on Jensen's media and dew drop colonies on Ashby's mannitol agar media.

Morphological characterization

Bacterial isolates were characterized by colony characteristics using light microscopy (100x). The cell shape, size, elevation, margin, surface, consistency, pigmentation and opacity were examined.

Capsule staining

Maneval's method for capsule staining as given by Anthony (1931) was performed for the conformation of *Azotobacter* spp. Microbes were examined under 100x oil immersion microscope.

Screening of isolates at varying concentration of NaCl

The isolates' capacity to withstand varying salt concentrations was examined according to method outlined by Sharma *et al*. (2021). The bacterial isolates were streaked on different NaCl concentration containing nutrient media (5, 7.5, 10 and 12.5%) followed by incubation at 25 ± 2°C for 5 days to observe growth.

In vitro screening of isolates for plant growth-promoting (PGP) activities

Quantitative estimation of ammonia production: With few modifications in methods described by Kerbab *et al.* (2021) and Lee *et al.* (2021) were followed for quantitative estimation of Ammonia. The ammonia production ability of isolates was examined using 10 mL Asbhy's mannitol broth and cultured for 4 days at 28 ± 2°C temperature. After incubation, 0.5 mL of Nessler's reagent was added and colour change from yellow to brown confirmed ammonia production.

HCN production: Hydrogen cyanide, one of the secondary metabolites generated by the PGPR, is crucial to the biological control of pathogens. HCN production was detected using the procedure outlined by Sharma *et al.* (2021). Bacterial isolates that could tolerate salt were streaked over a nutritional agar with 4.4 g/L glycine concentration. Filter paper discs were dipped in 0.5% picric acid produced in 2% sodium carbonate and placed on a medium consisting of glycine followed by 4 days of incubation at 28°C. If color change of the filter paper was noticed from deep yellow to orange to brown indicates HCN production by bacterial isolates.

Table 1: Soil samples collection from various coastal regions of Gujarat

District	Collection areas	Coordination	Elevation (masl)	pH
Dwarka	Dwarka (D)	22°14'21"N 68°58'04"E	15 m	8
Porbandar	Madhavpur (M)	17°45'82"N 78°30'14"E	-	8.4
Surat	Dumas (DU)	21°04'5"N 72°42'26"E	20.37 m	7.5
	Hajira (H)	21°09'15"N 72°44'27"E	29.51 m	7.2
	Ubharat (U)	21°00'36"N 72°43'58"E	29 m	8.4

masl = meters above sea level

Estimation of growth hormone production

Indole acetic acid (IAA): Indole acetic acid production by bacterial isolates was estimated by the method given by Kerbab *et al.* (2021). Freshly grown cultures were inoculated into 10 mL of nutrient broth supplemented with 1% L-tryptophan and kept in an incubator shaker at 37 ± 2°C (120 rpm) for 48 h. After incubation, cultures were centrifuged at 10,000 rpm at room temperature for 15 min. A 2 mL of supernatant was used and 2% Salkowski reagent (0.5 *M* $FeCl_3$ in 35% $HClO_4$) was added followed by two drops of orthophosphoric acid. The resulting mixture was allowed to react in dark for development of colour. Subsequently, the colour density was measured at 530 nm after two h. The IAA concentrations were calculated from the standard graph of IAA.

Gibberellic acid: Gibberellic acid production by bacterial isolates was estimated by the method given by Patel and Minocheherhomji (2020) with few modifications. A loopful of bacterial culture was added to 30 mL of nutrient broth and allowed to incubate at 37°C for seven days. After incubation, bacterial culture was centrifuged at 9000 rpm for 10 min. A 15 mL of supernatant was taken to which 2 mL of zinc acetate was added and kept for 2 min. To this, 2 mL of K_3FeCN_6 solution was added and centrifuged at 9000 rpm for 10 min. Subsequently, 5 mL of supernatant was taken and 5 mL of 30% hydrochloric acid was added followed by incubation at 25°C for 75 min. The resulting reaction mixture was used to measure absorbance at 780 nm with a UV-VIS spectrophotometer against a blank (5% hydrochloric acid).

Preliminary identification of halotolerant bacteria

The isolates were characterized by their morphological, cultural, and biochemical properties. Various biochemical tests were performed. Bergey's manual of systematic bacteriology, second edition, was utilized to identify bacterial isolates (Marakana *et al*. 2018).

Molecular characterization of N_2 fixers using PCR technique

PCR technique requires template DNA and it is extracted according to method of Ali *et al*. (2015). Selected isolates were first grown for 12 h in Luria-Bertani broth. After removing 1.5 mL of the culture, it was centrifuged for 5 min at 13,000 × g. Cell pellets were utilized, rinsed and resuspended in 200 μL of TE buffer (10 m*M* Tris–HCl, 1 m*M* EDTA; pH 8). After keeping for 5 min in boiling water with 1% sodium dodecyl sulphate, the cells were lysed. After that, the resultant lysate was extracted with phenol : chloroform (1:1) followed by two extractions using 24:1 ratio of chloroform to isoamyl alcohol. The aqueous fraction was further used and 0.1 volume of 3 *M* sodium acetate (pH 5.2) and 0.5 volume of isopropanol were added and incubated for 30 min at −20°C. After centrifuging the precipitated nucleic acids at 13,000 × g for 20 min, the resultant pellet was washed with 70% v/v ethanol. The pellet of nucleic acid was dissolved in appropriate volume of TE buffer and utilized as a template for the nifH gene PCR amplification. The PCR amplification was done using the primers F 5'CTTAATGTTTGCACTGAGCA3', and R 5'GAATTACAAACGTGACTCGT3'. Each PCR reaction mixture (50 μL) contained 0.5 μL Taq DNA polymerase, 5 μL Taq buffer, 5 μL triphosphates (dNTPs), 5 μL (100 ng μL^{-1}) of each primer (Lamizadeh *et al.* 2016). PCR condition includes initial denaturation (94°C) for 1 min, denaturation (94°C) for 30 s, annealing (58°C) for 1 min, extension (72°C) for 1 min (for 35 cycles) and then final extension (72°C) for 5 min.

***In vivo* assay**

A pot experiment was performed to assess the effect of selected halotolerant bacterial isolates (DU2, DU3, DU6, M3 and H5) on wheat production using a half dose of Nitrogen, Phosphorus and Potassium (NPK) fertilizer. The soils were autoclaved and utilized in a pot experiment. The cultures of the five selected isolates were transferred to 50 mL nutrient broth and grown aerobically in a flask on a shaker (150 rpm) at 30°C for 48 h. Surface sterilized seeds were immersed in bacterial suspensions with cell densities of 10^8 CFU mL^{-1} for 24 h. Sterile distilled water was used to immerse uninoculated seeds (controls). Each pot held around 2 kg of soil and the five treated seeds were planted in it at a depth of 1 cm below the soil's surface. A total of 14 treatments were given as randomized block design (RBD): T1: DU2+ 7.5% NaCl conc. + half dose of NPK, T2: DU3+ 7.5% NaCl conc. + half dose of NPK, T3: DUG + 7.5% NaCl + half dose of NPK, T4: M3+ 7.5% NaCl Conc. + half dose of NPK, T5: H5 + 7.5% NaCl conc. + half dose of NPK, T6: DU2 + 12.5% NaCl Conc. + half dose of NPK, T7: DU3 + 12.5% NaCl Conc. + half dose of NPK, T8: DU6 + 12.5% NaCl Conc. + half dose of NPK, T9: H5 + 12.5% NaCl Conc. + half dose of NPK, T10: M3+12.5% NaCl Conc. + half dose of NPK, T11: 7.5% NaCl + half dose of NPK, T12: 12.5% NaCl + half dose of NPK, T13: 7.5% NaCl and T14: 12.5% NaCl. The experiment was conducted in a pot with a photoperiod of 16 h of light and average day/night temperatures were 26 ± 2°C and 16 ± 2°C, respectively. Soils were sprinkled with 7.5 and 12.5% NaCl water to see

how salt affected plant growth. The pots were watered on a regular basis depending on the soil moisture level. After 15 days, the growth was measured in terms of shoot height, root height, fresh and dried shoot weight, root weight, and seedling leaf count. The germination percentage and vigor index were calculated using the formulas provided by Lee *et al.* (2021).

Germination percentage (GP) = $\left(\frac{\text{Number of germinated seeds}}{\text{Total number of seeds}}\right) X\,100$.

Vigor index (I) = Germination percentage × seedling length (shoot length + root length).

16S rRNA sequencing

Universal primers were employed to amplify the 16S rRNA sequences of the salt-tolerant NPKGP isolates. The PCR amplified product was purified and sequenced from Techno Enterprise, Pune, India. Using aligner software, the forward and reverse sequence data were combined to produce the consensus sequence for the 16S rRNA gene. BLAST searches were conducted using the 16S rRNA gene sequence against the NCBI GenBank's 'nr' database. A multiple alignment program called Clustal W was used to align the first ten sequences, which were selected based on their highest identity score. The distance matrix and phylogenetic tree were constructed using MEGA 10.

Statistical analysis

Every experiment was conducted in triplicate to determine the significance of many microbial strains under different growth conditions in comparison to the control sample. Means were calculated and compared to SD. Duncan's test was carried out after the one-way ANOVA. The mean value of different treatments was compared using multiple comparisons when significant differences were found within each row. All statistical tests were performed with a significance level of 5% ($P < 0.05$). SPSS 20.0 was used to perform all kind of statistics.

Results

The present study investigated the existence of halo-tolerant N_2 fixing bacteria in the rhizospheric zone from five soil samples derived from coastal saline area of various districts of the Gujarat state including Dwarka, Porbandar and Surat (Fig. 1). The pH of soil samples was also assessed (Table 1). Jensen's medium was used as selective media to isolate nitrogen fixing microorganisms. Total 31 nitrogen fixing isolates were obtained from five different soil samples on Jensen's agar medium. Flat, translucent, sticky colonies with pigments in shades of blue, light green and brown were seen. Here, Ashby's Mannitol Agar used as the selective medium to grow especially the *Azotobacter* spp. On Ashby's mannitol agar plate, a total of 15 colonies were observed (DU1, DU2, DU3, DU4, DU6, H1, H2, H5, H8, H12, M3, M8, U14, U18 and U20) as depicted in Table 2. All the 15 isolates were showing positive Capsule's staining.

Investigating the salt tolerance and PGP activity of isolated bacteria

All of these 15 isolates were tested for their salt tolerant capacity at varying salt dosages. Out of 15 isolates, only five isolates such as DU2, DU3, DU6, M3 and H5 were grow at the maximum NaCl (12.5%) concentration (Fig. 2). The ability of salt-tolerant bacteria for plant growth-promoting characteristics was investigated, including ammonia synthesis, phosphate solubilization, HCN and IAA. As shown in Table 3, all of these five salt-tolerant bacteria demonstrated significant PGP activity. The highest IAA and gibberellic production was obtained in M3, which was about 8.231μg/mL and 916.5 μg/mL, respectively. Therefore, all these 5 isolates were further selected for the nifH gene presence through PCR amplification technique.

Biochemical characterization

Biochemical testing was used to characterise a nitrogen-fixing bacterium. Results of various biochemical tests, including Methyl-red, Voges-Proskauer, citrate utilisation, H_2S production, starch and urea hydrolysis and Tripple Sugar Iron (TSI), were seen following their corresponding incubation periods (Table 4).

Molecular characterization of nitrogen fixing bacteria

Specific primer was used for nifH amplification (F 5'CTTAATGTTTGCACTGAGCA3', R 5'GAATTACAAACGTGACTCGT3'). Amplified product was observed at ~259 bp (Fig. 3-4) with some non-specific bands. Surprisingly, all five isolates showed nifH amplification. All these isolates are tolerant to high salt concentration. This suggests that isolated organisms having high tolerance to saline condition were having nitrogenase enzyme. Presence of nitrogenase gene suggests its nitrogen fixation potential. All these five isolates were further subjected to pot study.

Pot experiment

For pot experiments, isolates with the highest tolerance to salinity as well as other growth promoting characteristics that promote plant development, such as nitrogen fixation, growth hormone production, and HCN production were chosen. Beneficial PGPR inoculation of seeds greatly improved wheat seed germination and seedling vigor index. With PGPR isolate, the pace of enhancement differed, though. As illustrated in Table 5 and Fig. 5, the outcomes of the pot study demonstrated that inoculating wheat seed with inoculum M3 had a beneficial influence on all evaluated growth parameters when compared to the control group that did not receive inoculum treatment and NPK half dose (T12 and T14). M3 has shown 2898 and 2240 vigor

Table 2: Bacterial isolates isolated on different media from soil samples of coastal regions

District	Collection areas	Nutrient agar (N)	Jensen's agar (J)	Ashby's agar (A)
Dwarka	Dwarka (D)	D1 to D10	Undetected	Undetected
Porbandar	Madhavpur (M)	M1 to M8	M1, M2, M3, M7, M8	M3, M8
Surat	Dumas (DU)	DU1 to DU6	DU1 to DU6	DU1, DU2, DU3, DU4, DU6
	Hajira (H)	H1 to H12	H1 to H12	H1, H2, H5, H8, H12
	Ubharat (U)	U1 to U20	U1, U4, U7, U8, U12, U14, U18, U20	U14, U18, U20
Total no. of isolates		56	31	15

Table 3: Plant growth promoting activities of salt-tolerant bacterial isolates

Isolates	Growth on 12.5% NaCl conc.	HCN production activity	Phosphate solubilization activity	Ammonia production	IAA production (μg/mL)	GA production (μg/mL)
DU2	++	+++	Undetected	+++	Undetected	590.9
DU3	++	++	Undetected	++	Undetected	535.5
DU6	++	++	Undetected	+++	Undetected	487.9
M3	+++	++	Undetected	+++	8.231	916.5
H5	++	++	Undetected	+++	6.722	583.1

Where, +: low; ++: medium; +++: high; HCN: Hydrogen Cyanide: IAA: Indole Acetic Acid: GA: Gibberellic Acid

Table 4: Biochemical characterization of selected isolates

Test	DU2	DU3	DU6	M3	H5
M-R test	+	+	+	+	+
V-P test	-	-	-	-	-
Simmons citrate	+	+	+	+	+
H_2S production	-	+	-	+	+
Starch hydrolysis	+	+	+	-	+
Urea hydrolysis	-	-	-	-	-
TSI test	Slant (AL)	(AL and g)	(AL and g)	(AL and g)	(AL and g)
	Butt (A and g)	A	A	A	A

Where, + (positive), - (negative), AL (alkaline), A (acidic), g (gas production); M-R: Methyl Red test: V-P: Voges-Proskauer test: TSI: Tripple Sugar Iron test

Fig. 1: Sample collection sites: (**A**) Dwarka (**B**) Madhavpur (**C**) Hazira (**D**) Dumas (**E**) Ubharat

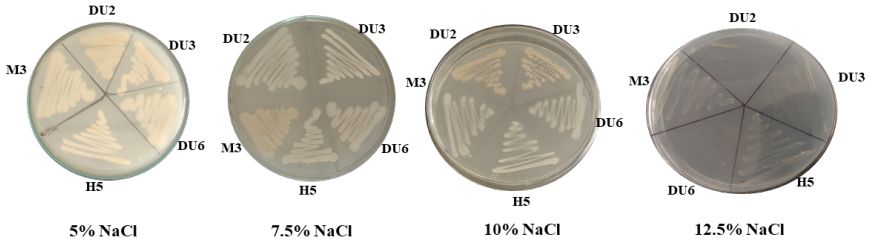

Fig. 2: Isolates obtained on Ashby's selective media for the isolation of nitrogen fixing organisms further screened for salt tolerance using different salt containing agar media. Five isolates (DU2, DU3, DU6, H5, M3) out of fifteen is showing good growth on different NaCl concentrations (5%, 7.5%, 10% and 12.5%) containing media

index at 7.5 and 12.5% NaCl treatments, respectively which is quite impressive compared to other PGPR and control. From Table 5, it can be inferred that Isolate M3 has shown 21.53 ± 1.616 cm shoot length, 9.03 ± 0.251 cm root length, 0.285 ± 0.005 g fresh and 0.026 ± 0.004 g dry seedling weight at 12.5% NaCl treatment (Fig. 4). In comparison with control treatments where seedlings are treated with half dose of NPK without bio-inoculum (T12) and completely devoid of NPK dose and bio-inoculum (T14). Therefore, M3 (*Acinetobacter lwoffii*) has exhibited a significant effect on shoot and root length as well as fresh and dry weight of seedlings. Moreover, seeds treated with lower (7.5%) and

higher concentration of NaCl (12.5%) were not much affected in terms of growth parameters with increasing saline conditions while other isolates have shown drastic fall in growth parameters with increasing salinity. Results of seedling vigor index and growth parameters suggested the remarkable role of M3 followed by DU2, DU6, DU3 and H5, respectively. It can be deduced from Fig. 4 that *A. lwoffii* (M3) is a most substantial halotolerant PGPR for wheat crop growth and development. To support this claim, statistical tests were performed. To identify the significant difference between different PGPR isolates, one way ANOVA was performed. The results of One-Way ANOVA have shown that *P*-value was less than 0.05 for all the treatments. Hence, means of each isolates' treatments were different compared to control. Further, post hoc Duncan's test was performed to identify and prove the outstanding growth promoting effect of M3 on seedling growth promoting parameters compared to other isolates. The F value for M3 was 5.406 and *P*-value was 0.038 suggested the superior difference of means of seedling growth parameters (Table S1). This statistical claim supported *A. lwoffii* (M3) as a potent PGPR for wheat seedling growth.

16s rRNA gene sequencing for identification of halo-tolerant bacterial isolates

The PGPR isolates M3 were chosen for molecular identification due to their promising salt tolerance and plant growth-promoting capabilities (Fig. 6-7). The isolate M3 with *A. lwoffii* strain JCM 6840 (NCBI accession number: NR_113346.1) showed 100% similarity to the sequences that were examined in the NCBI nucleotide database using BLAST-N.

Discussion

One of the most frequently received environmental threats to crop quality and yield is an increase the salt content in the soil (Tyerman *et al*. 2019). Salinity affects about 20% of the world's farmed land and about half of its irrigated land. Rhizobacteria that promote plant growth have been employed in agriculture because they can promote plant growth in a variety of ways, including by fixing nitrogen and generating plant growth regulators (Hayes *et al*. 2019). The elimination of salt as a result of the formation of conductive pores linked to advantageous soil aggregation may be the cause of the decrease in salinity stress (Sharma *et al.* 2021).

In this investigation, all soil samples were taken from the coastal regions of Gujarat. The alkaline composition of the soil may have an impact on nutrient availability for plant growth. These pH variations may have caused the sample collecting sites' poor soil structure and infertility. Total fifteen isolates were found to be salt tolerant at different salt concentrations in the current study. The isolates DU2, DU3, DU6, H5 and M3 have demonstrated the highest salt

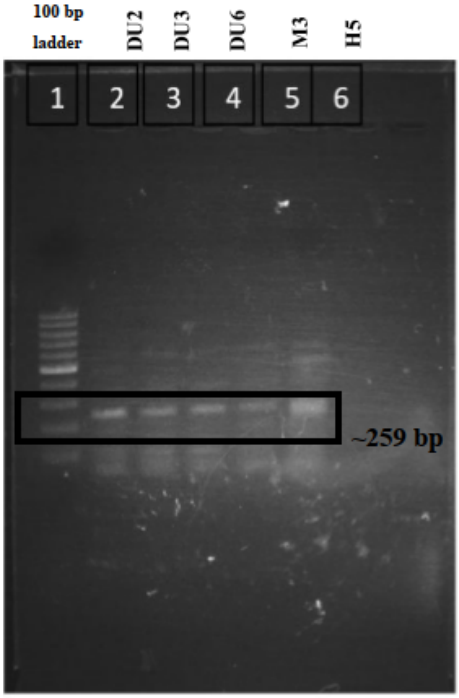

Fig. 3: Amplification of nifH gene from genomic DNA of the selected isolates

tolerance at 12.5%. In a comparable study, Sharma *et al.* (2021) identified 13 isolates that demonstrated salt tolerance up to 7.5% salt concentration, with only two isolates able to thrive at 10% concentration. Ma *et al.* (2019) conducted a similar investigation and found that *Pseudomonas libanensis* TR1 treated *Helianthus annuus* and *P. libanensis* showed strong resistance to saline stress at 8%. One more study in line with this was conducted by Mahmood *et al*. (2019) where they have isolated 80 isolates out of 150 demonstrates the tolerance towards the soil salinity. They identified *Streptomyces* and *Bacillus* strains as an efficient strain against soil salinity (1250 m*M*). According to this review of literatures, the M3 has more salt tolerant capacity (12.5%) when compared to the previous studies. Moreover, all the five isolates showed the expression for nifH gene *via* PCR studies suggests their potential nitrogenase activity in saline environment (Fig. 3).

The isolates M3 exhibiting notable salt tolerance and PGPR ability were identified as *A. lwoffii via* 16S rRNA gene sequencing. *Acinetobacter* sp. is a gram-negative bacterium found in the rhizosphere of many plants. It is known to produce IAA, gibberellin, ammonia, solubilizes phosphate, potassium and zinc, hence, gained a greater importance in agriculture worldwide (Mujumdar *et al.* 2023). Recently, another species from *Acinetobacter* genus documented for its ability to resist salt stress in dry seedling (Sun *et al*. 2024). Moreover, in contrast to our study, Awlachew *et al*. (2024) reported a species of *Acinobacter* genus that was showing good germination at 150 m*M* (0.87%) and 300 m*M* (1.75%) salt

Table 5: Effect of Plant Growth Promoting Rhizobacteria (PGPR) on plant growth promoting parameters (15 DAS)

Treatment (Inoculum) + NaCl conc.	Germination Percentage (%)	Shoot length (cm)	Root length (cm)	Fresh seedling weight (g)	Dry seedling weight (g)	Vigor index (I)
T1 (DU2) + 7.5%	70%	16.53 ± 0.152	6.20 ± 0.4	0.245 ± 0.017	0.025 ± 0.003	1591.1
T2 (DU3) + 7.5%	60%	11.51 ± 0.225	5.06 ± 0.450	0.157 ± 0.003	0.014 ± 0.001	990
T3 (DU6) + 7.5%	70%	15.56 ± 0.929	4.53 ± 0.117	0.151 ± 0.010	0.023 ± 0.001	1330
T4 (H5) + 7.5%	70%	14.00 ± 1.00	6.00 ± 0.2	0.233 ± 0.002	0.012 ± 0.003	1400
T5 (M3) + 7.5%	90%	26.83 ± 1.527	7.00 ± 0.458	0.334 ± 0.017	0.031 ± 0.003	2898
T6 (DU2) + 12.5%	60%	8.03 ± 0.321	6.53 ± 0.907	0.152 ± 0.014	0.013 ± 0.002	1020
T7 (DU3) + 12.5%	50%	11.50 ± 0.1	3.55 ± 0.350	0.144 ± 0.005	0.018 ± 0.002	750
T8 (DU6) + 12.5%	40%	14.53 ± 0.152	6.50 ± 0.781	0.142 ± 0.011	0.021 ± 0.002	880
T9 (H5) + 12.5%	40%	5.06 ± 0.251	1.03 ± 0.251	0.225 ± 0.015	0.011 ± 0.004	240
T10 (M3) + 12.5%	80%	21.53 ± 1.616	9.03 ± 0.251	0.285 ± 0.005	0.026 ± 0.004	2240
T12 (1/2NPK) +7.5%	60%	8.00 ± 0.2	3.06 ± 0.404	0.156 ± 0.012	0.013 ± 0.002	663.6
T14 +12.5%	50%	9.06 ± 0.208	5.08 ± 0.381	0.149 ± 0.004	0.011 ± 0.003	707

*DAS: Days after sowing. Values are Mean ± SD of three replicates (n=3). DAS: Days after sowing; NPK: Nitrogen, Phosphorus, and Potassium

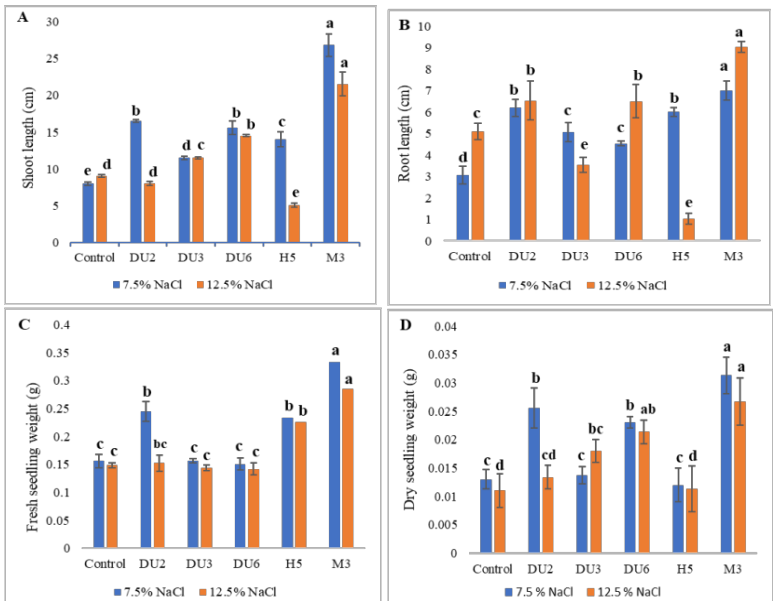

Fig. 4: Effect of selected isolates on (**A**) Shoot length, (**B**) Root length, (**C**) Fresh seedling weight and (**D**) Dry seedling weight under 7.5% and 12.5% NaCl concentration. Values are Mean ± SD of three replicates (n = 3). SPSS 20.0 was used for statistical analysis. One-way ANOVA followed by post hoc Duncan's test was used to indicate significant difference compared to control at $P \leq 0.05$. Different letters on bars indicate significant difference compared to control

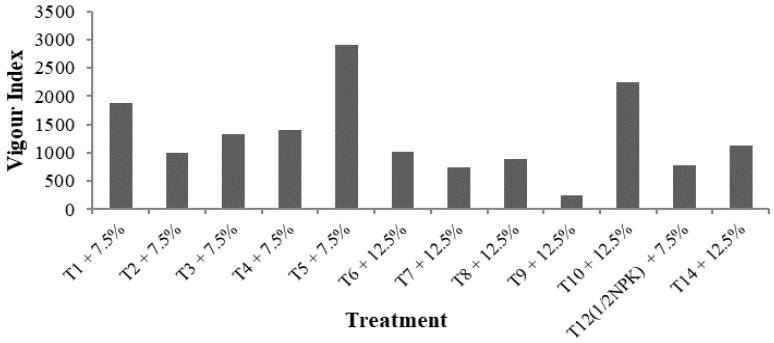

Fig. 5: Effect of different nitrogen fixing salt tolerant bacterial isolates and salinity on vigor index of *Triticum aestivum* L. seeds after 15 days of germination compared to control groups without bio-inoculants

concentrations. On the other hand, *A. lwoffii* was showing very effective seed germination at 12.5% salt concentration. Therefore, M3 (*A. lwoffii*) is an excellent PGPR boosting plant growth under salt stress, compared to previous investigations. Other PGPR such as *Bacillus* strains were showing good salt tolerance up to 18% NaCl concentration and possible mechanism was modulation of phytohormones in wheat under salt stress (Ayaz *et al.* 2022). *P. fluorescens*

is another promising PGPR showing good potential under saline soil (Al-Bardini *et al*. 2024). Another study using *Rhizobium leguminosarum* and *Paenibacillus polymyxa* as potent PGPR for wheat crop improvement under saline stress were taken into consideration where salt concentration up to 1.5 M (8.7%) were taken and got positive results with respect to *in vivo* studies. Compared to present study *A. lwoffii* is still better as PGPR in combating highly saline condition (12.5% Salinity) (El-Sayed and Hagab 2020). In a study of challenging salt stress, a consortium of PGPR *Bacillus* sp., *Azospirillum brasilense*, *A. lipoferum* and *P. stutzeri* were used for wheat crop under 150 m*M* (0.87%) NaCl stress showing improved antioxidant enzymes and proline synthesis; however, *A. lwoffii* (M3) is still better alone, a promising candidate challenging highly saline condition (12.5%) (Ilyas *et al*. 2020). The possible mechanisms behind better wheat crop yield and mitigating saline stress are reduction of osmotic, oxidative, and ethylene stress. They may be the method to mitigate salt stress in wheat crop. Nitrogen fixation, the production of phytohormones including indole acetic acid (IAA) and gibberellic acid (GA), the solubilization of potassium and phosphate, and the synthesis of enzymes that can control ethylene levels all directly promote the wheat plant growth (Kerbab *et al*. 2021). The expression analysis of selected genes by qPCR revealed the role of Salt overly sensitive genes (SOS 1 and SOS 4) present in PGPR suggests the potential role in wheat plant under saline stress (Haroon *et al*. 2021).

The effect of PGPR treatments on seedling growth was determined on the basis of germination percentage and vigor index. One qualitative feature of the germination process that is generally translated into a quantitative attribute, most commonly a percentage, is a single seed's ability to germinate, which is based on a binary respond (germinated/non-germinated). The percentage of seeds in a sample that arise up to the level of peak germination is termed as germination energy, where the maximum amount of germination on a given day is known as peak germination (Bahru *et al.* 2012). The vigor index, which indicates the health of the seedlings produced, considers both the germination percentage and the radical length. A greater vigor index suggests that the seedlings are in better health. In our study, isolate M3 (*A. lwoffii*) primed seeds have shown maximum vigor index suggested its potential role on seedlings health. Saraf and Jha (2011) for *Jatropha curcas* and Kanchana *et al.* (2014) for *Capsicum annuum*, Nawaz *et al.* (2020) for *Triticum aestivum* used appropriate PGPR to study seed germination and seedling vigor.

In order to boost crop yields, salt-tolerant bacteria with PGPR activity may be helpful in managing agricultural fields affected by salt. Alternatively, agricultural plants can be genetically modified to exhibit salt tolerance, or halophilic bacteria and their genes can be mined for salt-tolerant PGPR activity.

Conclusion

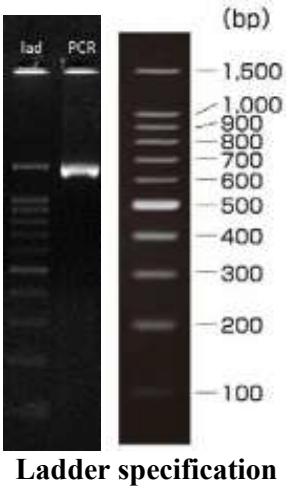

Fig. 6: 16s rRNA gene amplification by PCR
(Lane 1:100 bp marker; lane 2: 16 sRNA amplicon of Isolate M3)

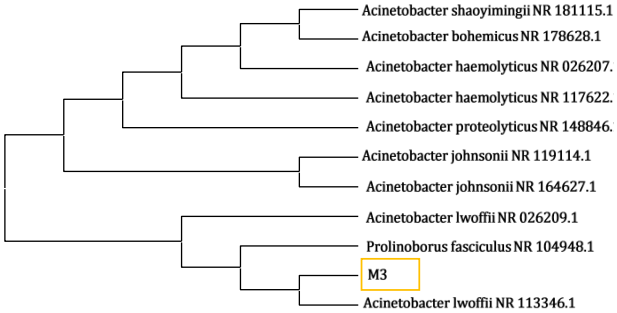

Fig. 7: The 16S rRNA gene sequences of salt-tolerant PGPR bacterial isolate (M3) and related sequences from the NCBI were analysed to construct phylogenetic tree using the maximum likelihood method. Scale bar: 0.005 substitutions per nucleotide position. M3: *Acinetobacter lwoffii*

The present work identified the halotolerant PGPR with its plant growth-promoting characteristics in wheat cultivated in salinized soil. Five (DU2, DU3, DU6, M3 and H5) out of 15 bacterial isolates were found to be salt tolerant at high (12.5%) salt concentrations were exhibiting good PGP activities that help wheat crop to grow under salt affected regions especially coastal regions. Moreover, every salt-tolerant bacterium exhibited notable PGP activity. The isolate M3 has showed the better PGP activities was identified as *A. lwoffii.* This isolate can be utilized as effective bioinoculants to improve wheat growth in saline soil. Further field trials to demonstrate the effectiveness of *A. lwoffii* under various condition ensures scalability and reliability. Scaling up of *A. lwoffii* as biofertilizer can reduces the dependency on chemical fertilizers and pesticides. Use of such a promising salt tolerant bio-inoculants at saline coastal lands not only mitigate salt stress problem but decreases fertilizer mediated pollution, promotes root growth, improves soil structure and boosting

farmer incomes which can ultimately contribute the sustainable agriculture practices.

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Author Contributions

TK and SP: Planned the experiments and Methodology, TK, JP: Investigation, Writing and editing, YM: Statistically analyzed the data.

Conflict of Interest

The authors report there are no competing interests to declare.

Data Availability

Data will be made accessible upon reasonable request from the corresponding author.

Ethics Approval

Not applicable to this paper.

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Table S1: The results of One-Way ANOVA followed by Post-hoc Duncan's test

ANOVA followed by Duncan's test

				Sum of Squares	df	Mean Square	F	Sig.
DU2	Between Groups	(Combined)		0.000	5	0.000	0.000	1.000
		Linear Term	Contrast	0.000	1	0.000	0.000	1.000
			Deviation	0.000	4	0.000	0.000	1.000
	Within Groups			12.000	12	1.000		
	Total			12.000	17			
DU6	Between Groups	(Combined)		507.903	5	101.581	213.355	.000
		Linear Term	Contrast	136.811	1	136.811	287.350	.000
			Deviation	371.092	4	92.773	194.856	.000
	Within Groups			5.713	12	.476		
	Total			513.616	17			
M3	Between Groups	(Combined)		114.636	5	22.927	75.240	.000
		Linear Term	Contrast	1.647	1	1.647	5.406	.038
			Deviation	112.989	4	28.247	92.698	.000
	Within Groups			3.657	12	.305		
	Total			118.293	17			
DU3	Between Groups	(Combined)		.053	5	.011	100.899	.000
		Linear Term	Contrast	.035	1	.035	332.270	.000
			Deviation	.018	4	.004	43.057	.000
	Within Groups			.001	12	.000		
	Total			.054	17			
H5	Between Groups	(Combined)		.001	5	.000	12.671	.000
		Linear Term	Contrast	.000	1	.000	26.607	.000
			Deviation	.000	4	.000	9.187	.001
	Within Groups			.000	12	.000		
	Total			.001	17			