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Bacillus cereus: an effective bio-inoculant for promoting salt stress tolerance of rice seedlings under saline soil conditions

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are a powerful tool to maintain sustainable agriculture and promote plant resistance to biotic and abiotic types of stress. Salinity, a major abiotic stress hampers plant growth, development, and yield. Salt-tolerant PGPR are effective agents for ameliorating salinity effects on rice plants. The present study endeavoured to isolate, determine halotolerant ability, characterize Plant Growth Promoting (PGP) traits, and finally observe the effect of PGPR strain on plant growth promotion of rice plants under saline and non-saline conditions. Based on the 16S rRNA gene sequencing technique, the rhizobacterial strain DB2 was identified as *Bacillus cereus* ATCC 14579(T) from NCMR, NCCS Pune. To check the growth-promoting ability, the strain was inoculated with two rice genotypes named Chinsurah Nona I (salt tolerant-non aromatic) and Badshabhog (aromatic) under polyhouse conditions. Results showed a significant increment in relative water content (RWC), total chlorophyll content (TCC), root length (RL), and shoot length (SL) in both rice genotypes inoculated with DB2 under both saline and non-saline conditions. Under non-saline conditions enhancement of RWC, TCC, RL, and SL was better in DB2 inoculated Chinsurah Nona I than in Badshabhog inoculated with DB2. Whereas, DB2-inoculated Badshabhog showed more recovery of RWC, TCC, RL, and SL than DB2-inoculated Chinsurah Nona I under saline conditions. Under salt stress conditions, inoculation with the rhizobacterial strain showed a significant reduction in electrolytic leakage (EL) in rhizobacteria inoculated with both rice genotypes. Moreover, DB2 inoculation showed a significant reduction in Na^+ content in the roots of Chinsurah Nona I (44.6%) and Badshabhog (24.5%) rice genotypes. The present study has indicated that the application of salt-tolerant PGPR may be an effective and sustainable solution for rice cultivation under salt-stress conditions.

Keywords

Bacillus cereus; Na^+ content; PGPR; Rice; Salinity; Sustainability

Introduction

Abiotic and biotic types of stress are major constraints to plant growth and productivity worldwide (1). Salinity, a type of abiotic stress, also impairs plant growth and yield, especially in rice plants. Being a glycophyte by nature, rice is inherently not able to tolerate saline conditions (2). Soil with electrical conductivity (EC) 3.5 dsm^{-1} and 7.2 dsm^{-1} showed detrimental effects on rice plant growth and productivity, with 10% and 50% yield reduction, respectively (3). Chronic exposure of plants to salt stress leads to some primary effects, such as cellular dehydration, water potential

reduction, and ion toxicity, and also causes secondary effects including stomatal closure, protein destabilization, reduced enzyme activity, retired cellular metabolic activity, decreased cell and leaf expansion, increased ion concentration and membrane destabilization (4-6). Rice (*Oryza sativa* L.) belonging to the Poaceae family of monocotyledonous flowering plants is one of the most important food crops globally. Fifty percent of the world's population relies on rice to meet 50%-80% of their caloric needs (7). The demand for rice production is rising day by day to nourish the growing human population. It has been reported that more than 50% of all arable land will be consumed by salinity by 2050 (8). In the present-day context, using saline soil for farming is a big challenge. Several approaches have been implemented to reduce salt stress but they are not ideal for maintaining a sustainable environment and agriculture (2). Therefore, strategies that can overcome plant growth, development, and productivity in response to abiotic stress in an environmentally friendly manner need to be developed.

Several adaptation mechanisms in plants to overcome the deleterious effects of salt stress include stomatal regulation, osmotic adjustment, nutritional balance, ionic homeostasis, Reactive oxygen species (ROS) scavenging, and stress signaling (4). Genetic technology has been applied to develop salt-tolerant rice varieties (2). Pokkali, the first salt-tolerant rice variety was introduced by Sri Lanka formerly called Ceylon in the year 1939 (9). The application of PGPR could be an effective environmentally friendly agent to improve vegetative growth and yield of rice under environmental stress conditions. Various published works have provided evidence for the commanding roles of PGPR in alleviating the effects of salt on plants under salt-induced conditions (2, 10-12). Moreover, it has been proven that PGPR is a good alternative to chemical fertilizers and maintains soil fertility. Some bacterial genera including *Rhizobium*, *Thiobacillus*, *Bacillus*, *Streptomyces*, *Serratia*, *Azospirillum*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Klebsiella*, *Acinetobacter,* and *Alcaligenes* have been shown to play fundamental roles in promoting plant growth under saline conditions (13, 14).

PGPR can show plant growth-promoting traits such as nitrogen fixation (NF), phosphate solubilization (PS), Indole acetic acid (IAA) production, exopolysaccharides (EPS) production, hydrogen cyanide (HCN) production and ammonia (NH_3) production. IAA is an important plant growth regulator that promotes root length and branches that improve water uptake from the soil and protect plant cells from desiccation (14). Salt tolerant - IAA-producing PGPR has been reported to ameliorate salt-induced detrimental effects on plants (15). EPS is too efficient in protecting plants from environmental stresses like heavy metal pollution, salinity drought, etc. (16). EPS exerts protection for microbes and promotes bacterial colonization on plant root surfaces by possessing an essential role in bacterial biofilm formation (17). Under saline conditions, EPS-producing microbes play dual roles *viz.* down regulation of sodium ion (Na^+) absorption from soil by plant root and reaching the ions to stem by binding the Na^+ in the soil and up regulation of nutrient uptake from soil (18).

Plant-PGPR interactions are well established. Research is going on to gather information concerning the effects of PGPR on plant growth and development under environmental stress conditions. Recent studies demonstrated that halotolerant PGPR is an effective agent for improving shoot length, root length, yield, chlorophyll content, etc. under saline conditions (19). A study proved that co-inoculation of *Brevibacterium frigoritolerans* W19 (rhizospheric bacteria) and *Bacillus safeness* BTL5 (endophytic bacteria) enhanced the production of phenylalanine ammonia-lyase and antioxidant enzymes such as catalase, superoxide dismutase, and polyphenol oxidase in rice seedlings under high salt concentration (20). Thus, the present study was an attempt to isolate, characterize, and identify salt-tolerant PGPR from the paddy field of Gosaba, a coastal salt-affected area in West Bengal, India, and to check their effects on the morphological, physiological, and biochemical parameters of the salt tolerant rice genotype Chinsurah Nona I and one aromatic rice genotype Badshabhog under saline and non-saline conditions.

Materials and Methods

Isolation and characterization of plant growth-promoting traits performed by selected rhizobacterial isolates

Rhizobacterial isolation was done from the rice rhizosphere soil of Gosaba (Latitude- 22.1652 °N, Longitude- 88.8079 °E), a coastal salt-affected area in South 24 Parganas, West Bengal, India. Isolation of rhizobacterial isolates was carried out by a simple dilution plating method. Rhizobacterial isolates were primarily selected based on their maximum salt-tolerant capacity. After that, the selected isolates were allowed to characterize some plant growth-promoting (PGP) traits like, IAA production, EPS production, PS, NF, NH_3 production, HCN production, catalase test, protease test, and antifungal property. With some modification of the method (21), nutrient agar media (composition per L: peptone- 5 g, NaCl- 5 g, beef extract- 1.5 g, yeast extract- 1.5 g and agar- 15, final pH 7.4±0.2 at 25 °C) supplemented with different concentrations of sodium chloride (NaCl) (0.34 M, 0.68 M, 1.02 M, 1.36 M, 1.70 M and 2.04 M NaCl) were used to determine the halotolerance ability of the rhizobacterial isolates. According to standard protocols, characterization of some plant growth-promoting (PGP) traits like, IAA (22), PS (23, 24), NF ability (25), NH_3 production (26), EPS production (27), HCN production (28), antifungal property (29), catalase test (30) and protease test (31) of selected rhizobacterial isolates was done. Furthermore, the effect of NaCl (0.34 M, 0.68 M, 1.02 M, and 1.36 M) on IAA production, EPS production, NH_3 production, and NF by best-performing rhizobacterial isolate was determined. Growth of the best-performing rhizobacterial isolate was recorded for 28 h at 2 h intervals to prepare the growth curve of the isolate. For this purpose, the isolate was inoculated separately in 15

conical flasks containing nutrient broth (NB) and incubated in a BOD incubator at 28 ± 2 °C.

Identification of the best-performing rhizobacterial isolate using 16S rRNA gene sequencing and MALDI-TOF MS-based biotyping and phylogenetic analysis of the strain

Based on salt tolerant ability and PGP characterization, the best-performing rhizobacterial isolate was selected and identification of the isolate was carried out by 16S rRNA gene sequencing and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) based bio typing from National centre for Microbial Resource- National Centre for Cell Sciences (NCMR-NCCS), Pune. Standard phenol/chloroform extraction method (32) was followed for genomic DNA extraction which was followed by PCR amplification using the forward primer 27F (5'AGAGTTTGATCMTGGCTCAG3') and reverse primer 1492R (5'CGGTTACCTTGTTACGACTT3') (33). The strain was deposited to NCMR-NCCS, Pune. For determining the Evolutionary position, the Phylogenetic tree was constructed using MEGA X software (34) following the Bootstrap method (with 1000 Bootstrap replications) - LogDet (Jukes and Cantor correction) model (35).

Polyhouse experiment

Rice seeds collection

In the present study, salt tolerant non-aromatic rice seeds of Chinsurah Nona I (CN I) were collected from Chinsurah Rice Research Station (CRRS), Chinsurah (22° 53' 44" N, 88° 24' 8" E). Seeds of aromatic Badshabhog (BB) rice were collected from Central Rice and Seed Multiplication Farm (CRSMF) of The University of Burdwan (23° 15' 0.3636" N, 87° 50' 55.8132″ E), Burdwan. Both stations are situated in West Bengal, India.

Experimental setup preparation

For each experiment, a total of 4 experimental sets were prepared including Uninoculated-without salt treated or control (C), Inoculated-without salt treated (T1), Uninoculated-Salt treated (T2), and Inoculated-salt treated (T3) for both plants.

Bacterial inoculum preparation and application on Rice seeds and seedlings

A loop of bacterial culture was inoculated in NB and kept in a BOD shaker incubator at 28 ± 2 °C for 24 h. After applying trial experiments, around 10^7-10^8 CFU mL^{-1} of freshly prepared bacterial suspension was considered as optimal for plant inoculation treatment. In this present study, the bacterial inoculum was applied following a method as demonstrated in Fig. 1. For an uninoculated set sterile distilled water was used.

Soil preparation and salt treatment

Initially in soil, nutrient content like nitrogen (N), phosphorous (P), and potassium (K) was 4.9 g kg^{-1}, 0.18 mg kg^{-1}, and 0.11 mg kg^{-1} respectively. Then the soil was dried properly and allowed for sterilization. The sterile soil was transferred to non-leaky plastic pots (3 kg in each pot). Inorganic fertilizer was applied as per the recommendation of CRRS, Chinsurah (Urea 0.01%, Triple superphosphate 0.0025%, and Muriate of Potash 0.008%). For salt-treated experimental sets, salinization of soil was done by using Sodium chloride (NaCl) solution to maintain soil EC as 6 dsm^{-1}. Sterile distilled water was applied to the experimental set without salt treatment.

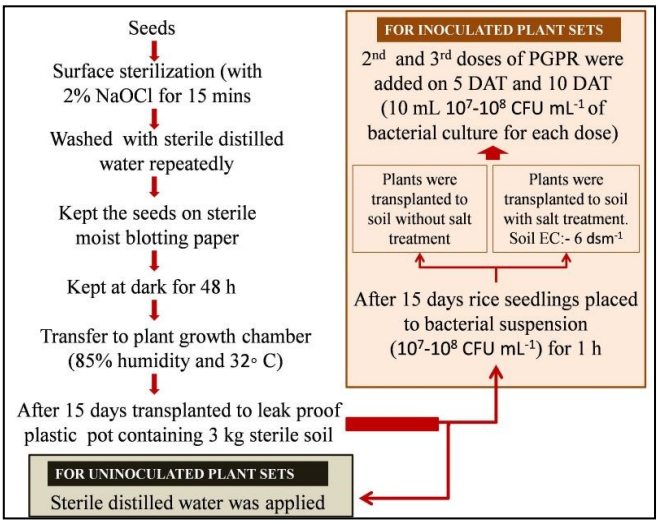

Fig. 1. Method for applying bacterial inoculation to rice plants.

Measurement of root length (RL) and shoot length (SL)

Root length and shoot length of rice seedlings were measured on the 15th day after transfer (DAT).

Relative water content (RWC)

At 15 DAT RWC percentage of rice plants was measured following the procedure (36). For this purpose, the fresh weight of the leaves of rice plants was taken. Then the leaves were dipped in distilled water and kept in the refrigerator for 24 hours. After blotting, the full turgid weight of the leaves was taken. Then the leaves were dried by keeping them in a hot air oven at 72 °C for 24 h and dry weight was taken. RWC (%) was recorded using the formula:

RWC (%) = [(fresh weight - dry weight) / (fully turgid weight - dry weight)] × 100

Electrolytic Leakage (EL)

Leaf discs of similar diameter and similar number for every experimental set were submerged separately into 25 mL double distilled water. The step was followed by another three sequential steps including gentle shaking for 10s, keeping overnight at 4 °C, and autoclave at 121 °C for 15 min. EC values were taken after each step using electrical conductivity (EC) meter and % of Electrolyte leakage (EL) was taken following the formula (37)

EL (%) = $[(EC_1 - EC_0) / (EC_2 - EC_0)] \times 100$

Where, EC_0- initial EC after 10s of shaking; EC_1- EC after keeping overnight at 4 °C; EC_2- EC after autoclave.

Total chlorophyll content (TC) and carotenoid content

Approximately 500 mg of fresh leaves of 30 days rice plants were homogenized using 80% acetone and then the spectrophotometric reading of the leaf extract was taken at 645, 663, and 470 nm and TC and carotenoid content was measured according to the formulas (38).

Chlorophyll a (Chl a): 12.7(A_{663}) - 2.69(A_{645})

Chlorophyll b (Chl a): 22.9(A_{645}) - 4.68(A_{663})

Total Chlorophyll: 20.2(A_{645}) + 8.02(A_{663})

Carotenoid Content: [(1000A_{470}) -(1.82 Chl a) -(85.02 Chl b)]/198

Observation of salt content in root– uninoculated and inoculated with bacterial isolate

Energy-dispersive X-ray analysis (EDAX) study was done by *USIC, The University of Burdwan* to observe salt accumulation in roots. For this study, root samples (15 DAT) were collected from salt-treated-PGPR uninoculated and salt-treated-PGPR inoculated experimental sets of CN I and BB rice plants. It was already established EDAX study was the confirmatory method for mineral content analysis of salt-tolerant rice (39).

Scanning Electron Microscopy (SEM) study of the rhizobacterial strain and its colonization in root

For the SEM study of the strain, sample preparation of freshly prepared bacterial culture was done following the method (40). Root samples of inoculated seedlings were collected at 15 DAT and washed carefully with sterile distilled water. The root samples were cut into 1 cm and prepared for SEM study following the method (41). The SEM study was done using Zeiss *Gemini SEM 300 from USIC, The University of Burdwan.*

Statistical analysis

In every experimental data, a mean of 3 replicates was used. For the quantitative estimation of IAA and EPS production by rhizobacterial isolates (Fig. 2, 3) and quantitative estimation of IAA, and EPS production by rhizobacterial isolate DB2 under NaCl treatment (Fig. 4A, 4B), statistical analysis was done using one-way ANOVA. Duncan's multiple range test (DMRT) in SPSS 29.0.1.0 was used for significant tests of SL, RL, RWC, EL, TCC, and carotenoid content for each rice genotype. Above the bars, different letters were used to indicate statistically significant differences between groups. The significance level of all experimental data was at $p<0.05$.

Results

Halotolerance and Plant Growth Promoting (PGP) traits of the rhizobacterial isolates

Except for rhizobacterial isolate DB4 other isolates including, DB1, DB2, DB3, DB5, and DB6 showed halotolerant ability upto 1.36 M NaCl concentrations (Table 1).

Based on maximum salt tolerance capacity all the isolates except DB4 were selected for further experiments. All isolates showed positive responses to the qualitative tests, like N_2 fixation and NH_3 production (Table 2). DB2, DB3, and DB6 showed HCN production capacity (Table 2).

The isolates DB2 and DB6 also showed positive results to other qualitative assay like catalase and protease (Table 2). Only DB2 performed phosphate solubilizing capacity positively showing a clear halo zone surrounding the bacterial colony and Phosphate Solubilizing Index (PSI) was 1.28 (Table 3).

Table 1. Influence of salt on rhizobacterial growth ('+' indicates positive result where ++++>+++>++>+ and '-' indicates negative result. Control represents no salt treatment)

Strains	Control	Salt (NaCl) Concentration [M]					
		0.34	0.68	1.02	1.36	1.70	2.04
DB1	++++	+++	++	+	+	-	-
DB2	++++	+++	++	+	+	-	-
DB3	++++	+++	+++	++	+	-	-
DB4	++++	+++	+	+	-	-	-
DB5	++++	+++	++	+	+	-	-
DB6	++++	+++	++	+	+	-	-

Table 2. Plant Growth Promoting traits performed by the selected rhizobacterial isolates ('P' and 'N' indicate positive and negative results respectively)

PGP traits	DB1	DB2	DB3	DB5	DB6
IAA Production	P	P	P	P	P
N_2 Fixation	P	P	P	P	P
Ammonia Production	P	P	P	P	P
EPS Production	P	P	P	P	P
Phosphate Solubilization	N	P	N	N	N
HCN Production	N	P	P	N	P
Antifungal property	N	P	N	N	N
Catalase	N	P	N	N	P
Protease	N	P	N	N	P

Table 3. Phosphate solubilization index (PSI) by rhizobacterial isolate DB2

Isolate	Bacterial colony diameter halozone diameter / Colony diameter	Phosphate Solubilizing Index (PSI)
DB2		(0.9/0.7)=1.28

DB2 also showed antifungal activity (61.29%) against the fungal strain *Alternaria alternata* (Table 4).

Table 4. Antifungal properties of rhizobacterial isolate DB2

Isolate	Fungal strains	Antifungal activity (%)	Inhibition (%)
DB2	*Alternaria alternata*	{(R-r) / RX100} R= fungal colony away from the bacterial colony r = fungal colony towards the bacterial colony	61.29

The strain DB2 continued its N_2 fixation and NH_3 production up to 1.36 M NaCl concentrations (Table 5).

Table 5. Effect of salt stress on plant Growth Promoting traits performed by the selected rhizobacterial strain DB2 (+++++>++++>+++>++>+)

Strain	PGP traits	Control	Salt (NaCl) Concentration (M)			
			0.34	0.68	1.02	1.36
DB2	NH_3 production	+++++	++++	+++	++	+
	NF	+++++	++++	+++	++	+

DB2 showed the highest number of EPS and Indole Acetic Acid (IAA) production which were 0.85 mg mL^{-1} and 11.61 µg mL^{-1} respectively (Fig. 2, 3).

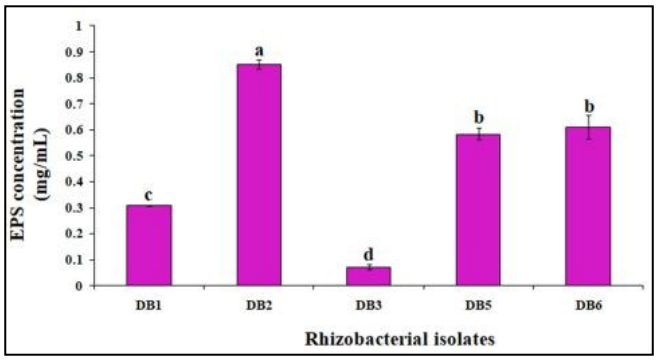

Fig. 2. Quantitative estimation of EPS production by rhizobacterial isolates.

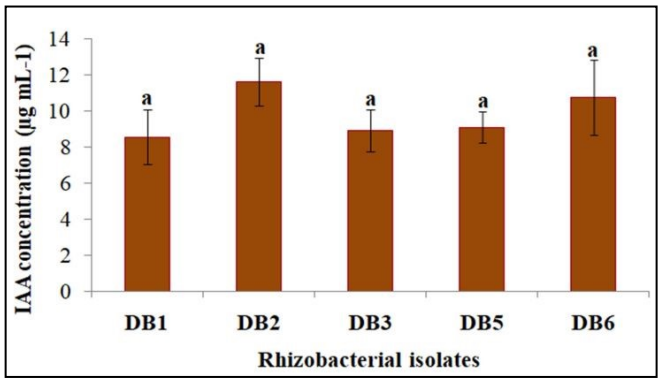

Fig. 3. Quantitative estimation of IAA production by rhizobacterial isolates.

Under salt treatment, the growth of DB2 gradually decreased with increasing salt concentration however, EPS production was maximal in 0.34 M NaCl concentration (1.85 mg mL^{-1}) which was followed by 1.02 M (1.50 mg mL^{-1}), control (1.11 mg mL^{-1}), 0.68 M (1.06 mg mL^{-1}) and 1.36 M (0.72 mg mL^{-1}) respectively (Fig. 4A). DB2 showed IAA production up to 1.36 M NaCl concentration where IAA production showed to decrease as the salt concentration was increased (Fig. 4B).

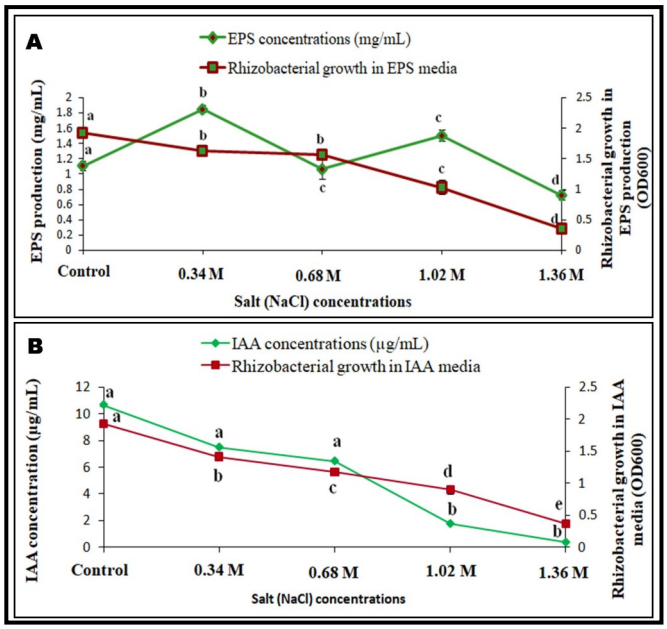

Fig. 4. Quantitative estimation of PGP traits by rhizobacterial isolate DB2 under salt stress conditions–(A) EPS production, (B) IAA production. For EPS production, the control set contains 0.085M NaCl. For IAA production, no NaCl was added to the control set.

Rhizobacterial isolate DB2 showed positive responses to all PGP traits (Fig. 5, Table 2).

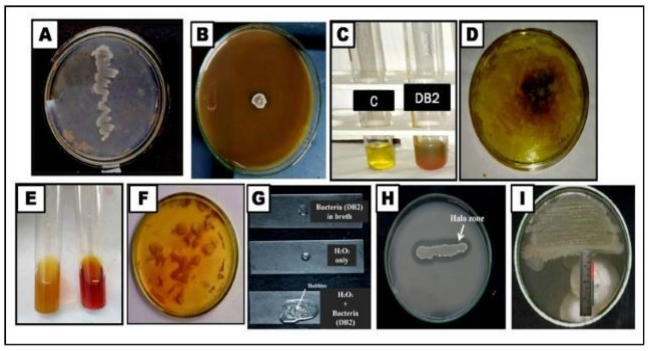

Fig. 5. Qualitative assay of PGP traits by rhizobacterial isolate DB2 – (A) Nitrogen-fixing ability, (B) Phosphate solubilization ability, (C) Ammonia production, (D) HCN production, (E) EPS production, (F) IAA production, (G) Catalase production, (H) Protease production, (I) Antifungal property.

The growth curve study indicated that the maximum growth rate of the rhizobacterial isolate DB2 was from 4 h to 6 h and maximum growth was at 24 h (Fig. 6).

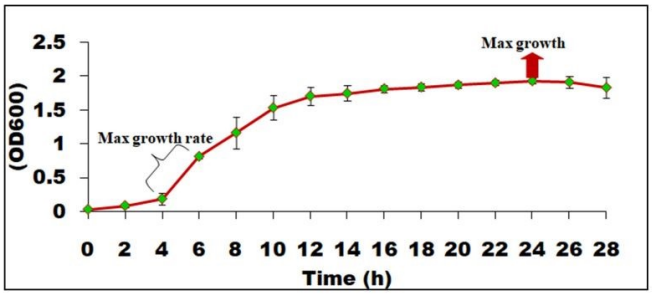

Fig. 6. Growth curve of the rhizobacterial strain DB2.

Molecular Identification of the best-performing PGPR isolates

The rhizobacterial isolate DB2 was selected for molecular identification. For 16S rRNA gene sequencing, ~1200 bp for the isolate was sequenced and revealed 100% similarity with *Bacillus cereus* ATCC 14579(T) (NCMR accession number: MCC 4881) (Table 6).

Using the Neighbor-joining bootstrap method (Jukes and Cantor correction) evolutionary relation of the strain with an additional 17 members of the genus *Bacillus* was inferred (Fig. 7A).

Depending upon MALDI-TOF-based bio typing, NCMR stated that the bacterial strain DB2 showed the best match with *Bacillus cereus* 47Y2 (Score value 2.175) which was followed by *Bacillus cereus* 4080 (Score value 2.153) (Table 6, Fig. 7B).

Table 6. Molecular identification of rhizobacterial isolate DB2 from NCMR-NCCS, Pune

Molecular identification of rhizobacterial strain DB2			
I. Molecular identification by 16Sr DNA sequencing			
Closest Neighbour	**Similarity (%)**	**Accession no.**	
Bacillus cereus ATCC 14579 (T)	100	MCC 4881	
II. Molecular identification by MALDI TOF-based bio typing			
Best match	**MALDI Score**	**Second Match**	**MALDI Score**
Bacillus cereus 47Y2	2.175	*Bacillus cereus* 4080	2.153

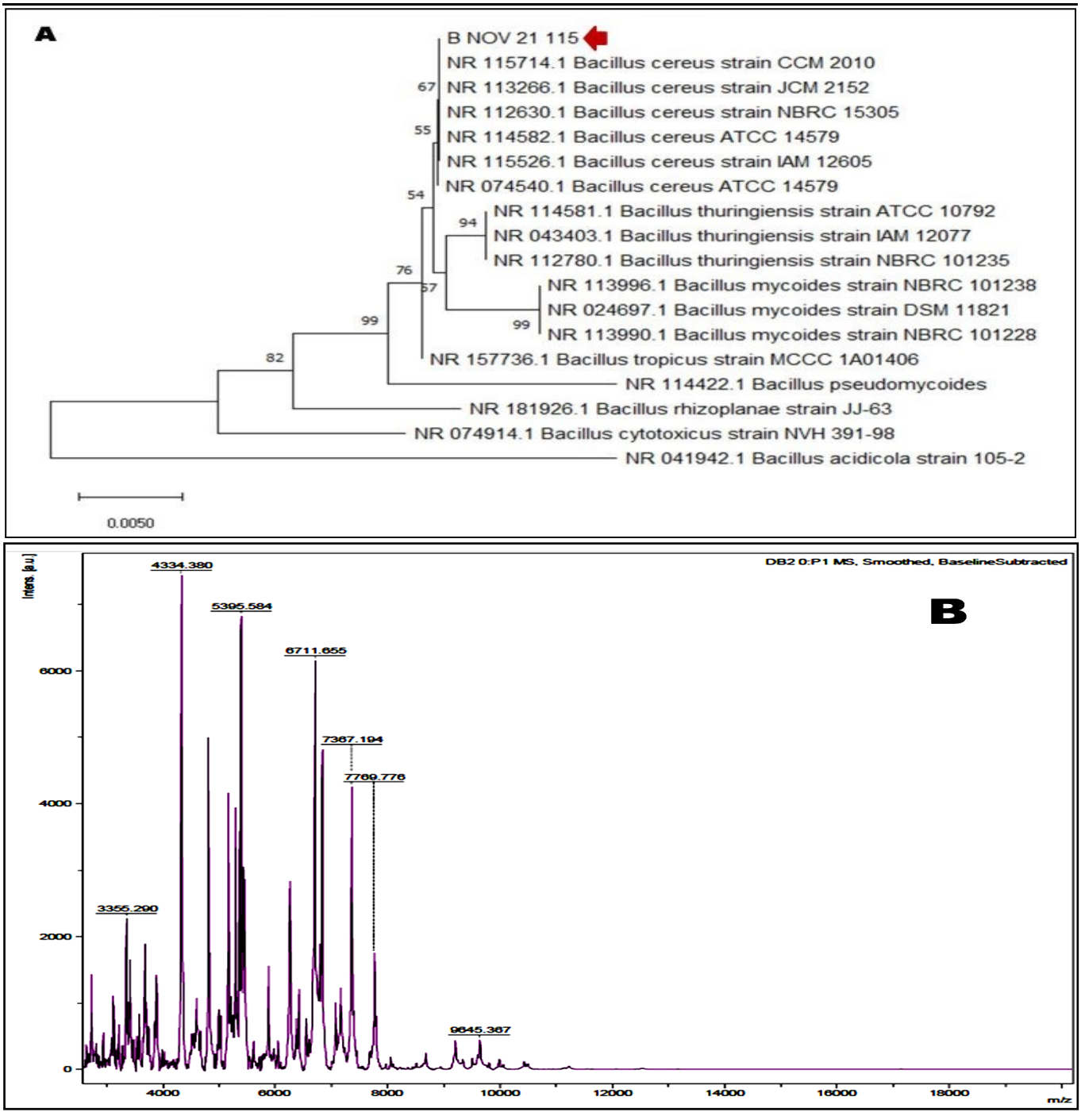

Fig. 7. Molecular Identification of the rhizobacterial strain DB2 – (A) Maximum Likelihood Neighbour joining phylogenetic tree using MEGA11 software with Jukes and Cantor correction (1969). (B) MALDI-TOF spectra analysis of ribosomal protein

Effect of Bacterial Inoculation on shoot length and root length of rice genotypes

Saline condition hampered the shoot length of both rice genotypes CN I (31.9%) and BB (57.9%). Under normal growing conditions, inoculation with DB2 was shown to enhance shoot length in CN I (28%) and BB (26.3%). Under salt treated condition, compared to uninoculated plant sets significant enhancement of shoot length (95%) was recorded for BB and CN I it was 36.5% (Fig. 8A). Under saline conditions, root length reduction was 21.1% for CN I and 42.8% for BB. Inoculation with DB2 under both saline and non-saline conditions was found to increase root length for both rice varieties (Fig. 8B).

Effect of Bacterial Inoculation on the Relative Water Content and Electrolytic leakage of rice genotypes

Salt stress was shown to hamper the RWC of BB more than CN I. The rhizobacterial isolate DB2 showed its ability to promote the percentage of RWC under both normal growing and salt-treated conditions. Under normal growing conditions, bacterial inoculation enhanced RWC of CN I (9%) and BB (4.7%). The inoculation of DB2 improved the RWC of CN I and BB by 19% and 42% respectively under saline conditions (Fig. 9A).

Under non-saline conditions, the very least enhancement of EL by application of DB2 was found in both rice genotypes which were 1.4% for CN I and 0.6% for BB. Under salt stress conditions, a significant reduction of EL was found in inoculated CN I (43.4%) and BB (70.6%) compared to uninoculated sets (Fig. 9B).

Effect of Bacterial Inoculation on Total chlorophyll content (TC) and carotenoid content of rice genotypes

Salinity reduced TC both in CN I (20.9%) and BB (47%) in comparison to the control plant set. Bacterial inoculation improved TC under both saline and non-saline conditions. Under non-saline conditions TC of DB2 inoculated CN I enhanced by 26.6% while BB treated with DB2 increased by 17.5%. Compared to uninoculated plant sets, under saline conditions, inoculation with DB2 enhanced 26.4% TC in CN I and 32.5% in BB (Fig. 10A). Besides these, inoculation with DB2 showed to increase carotenoid content in CN I (23.5%) and BB (9.7%) compared to uninoculated plants under normal growing condition. Under salt treatment increment of carotenoid content was observed for inoculated- CN I (17.3%) and inoculated- BB (6.3%) in comparison to salt-treated uninoculated plant sets (Fig. 10B).

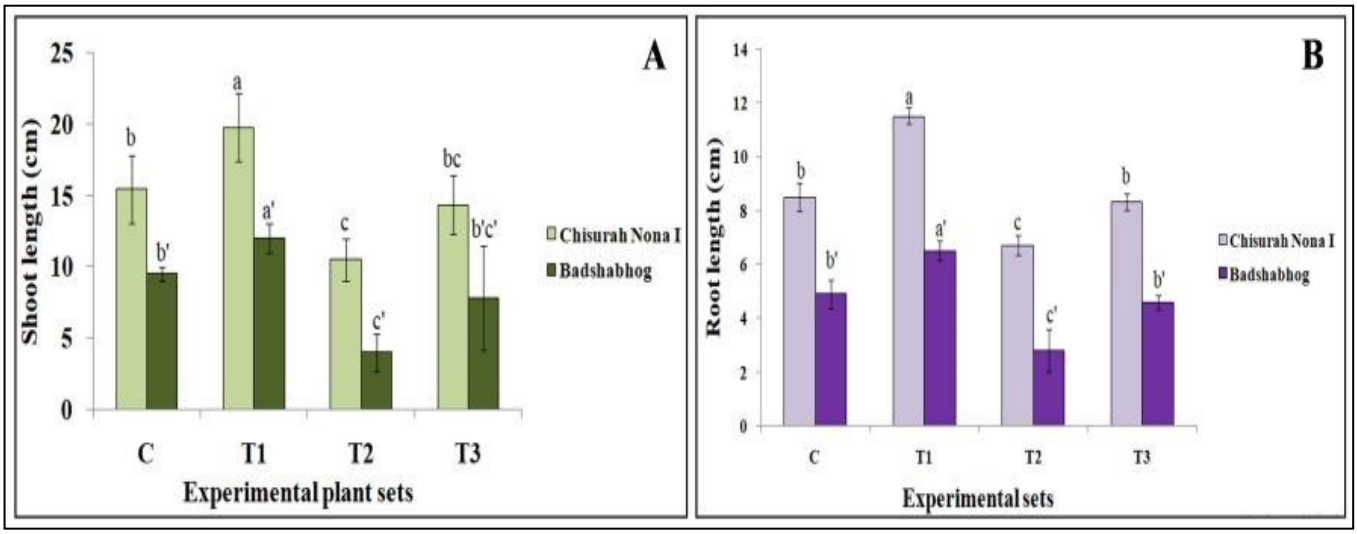

Fig. 8. Effect of rhizobacterial strain DB2 on morphological traits of two rice genotypes Chinsurah Nona I and Badshabhog– (A) Shoot length, (B) Root length (where, C-Control, T1-Inoculated with DB2, T2- Salt treated, T3-Salt treated + inoculated with DB2). For each rice genotype, above the bars, different letters indicate statistically significant differences between groups (Mean±SD, n=3, DMRT in SPSS 29.0.1.0., p<0.05).

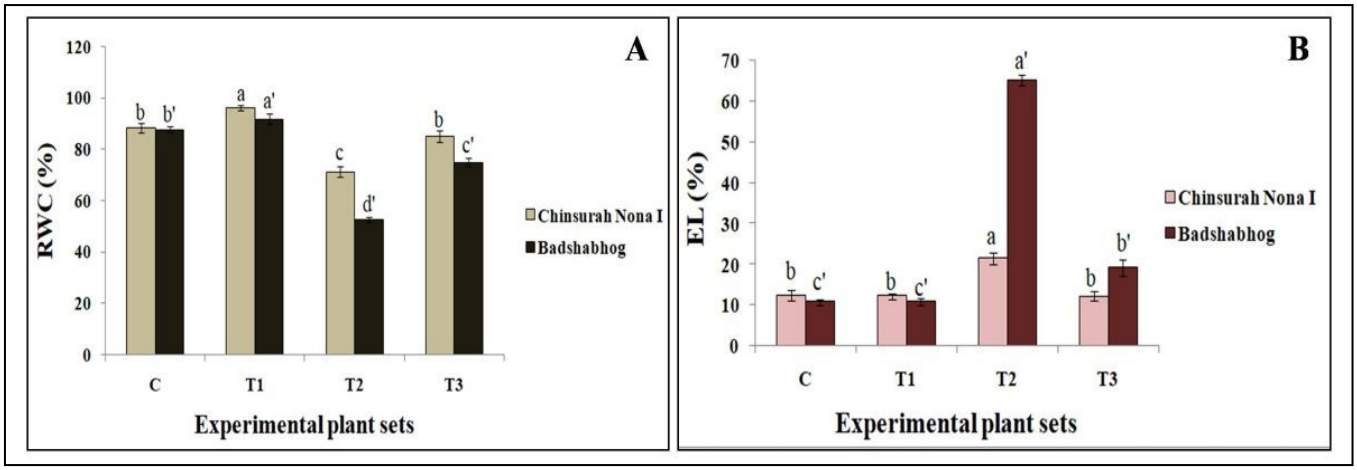

Fig. 9. Effect of rhizobacterial strain DB2 on physiological traits of two rice genotypes Chinsurah Nona I and Badshabhog– (A) Relative Water Content (RWC), (B) Electrolytic Leakage (EL) (where, C-Control, T1-Inoculated with DB2, T2- Salt treated, T3-Salt treated + inoculated with DB2). For each rice genotype, above the bars, different letters indicate statistically significant differences between groups (Mean±SD, n=3, DMRT in SPSS 29.0.1.0., p<0.05).

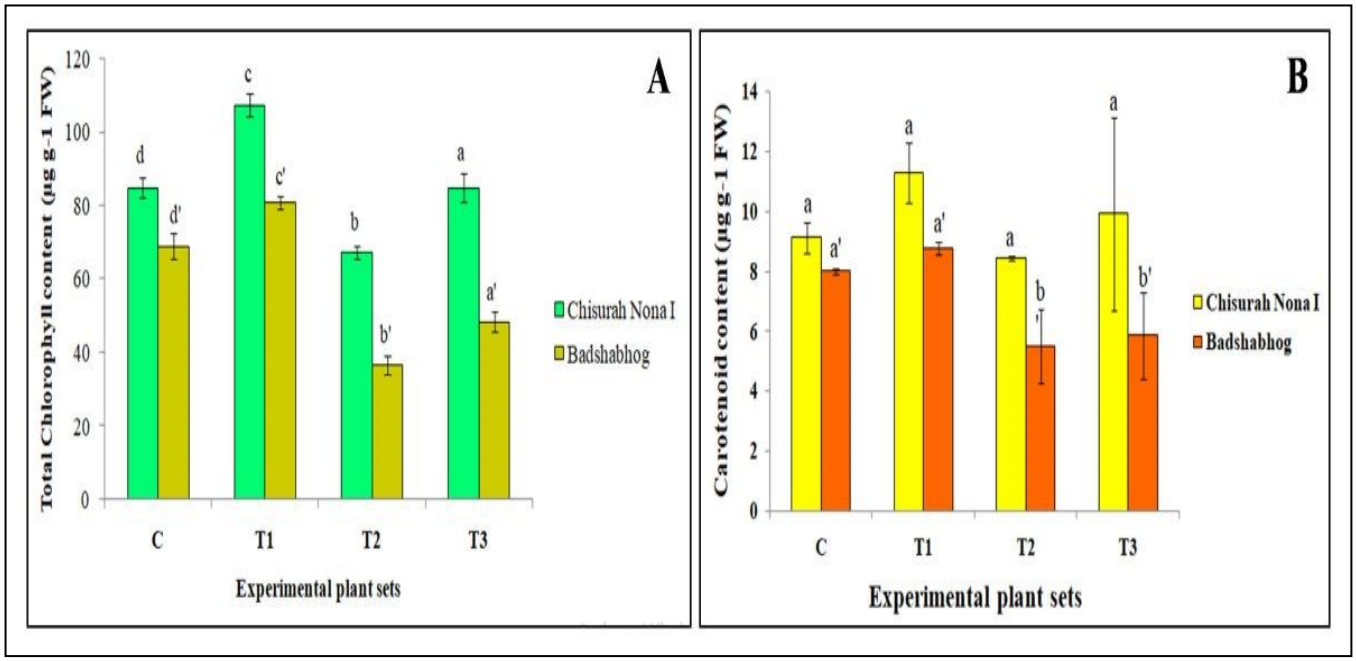

Fig. 10. Effect of rhizobacterial strain DB2 on biochemical traits of two rice genotypes Chinsurah Nona I and Badshabhog– (A) Total Chlorophyll Content (TCC), (B) Carotenoid Content (where, C-Control, T1-Inoculated with DB2, T2- Salt treated, T3-Salt treated + inoculated with DB2). For each rice genotype, above the bars, different letters indicate statistically significant differences between groups (Mean±SD, n=3, DMRT in SPSS 29.0.1.0., $p<0.05$).

Effect of bacterial inoculation on root Na^+ content of rice cultivars

EDAX study recorded a decrease in Na^+ content in roots of CN I (44.6%) (Fig. 11A, 11B) and BB (24.5%) by application of DB2 (Fig. 11C, 11D).

Identification of Bacterial Root Colonization by SEM study

SEM study revealed the surface morphology of the rhizobacterial isolate DB2 (Fig. 12A) and the successful colonization of DB2 in the root of the CN I rice variety (Fig. 12B).

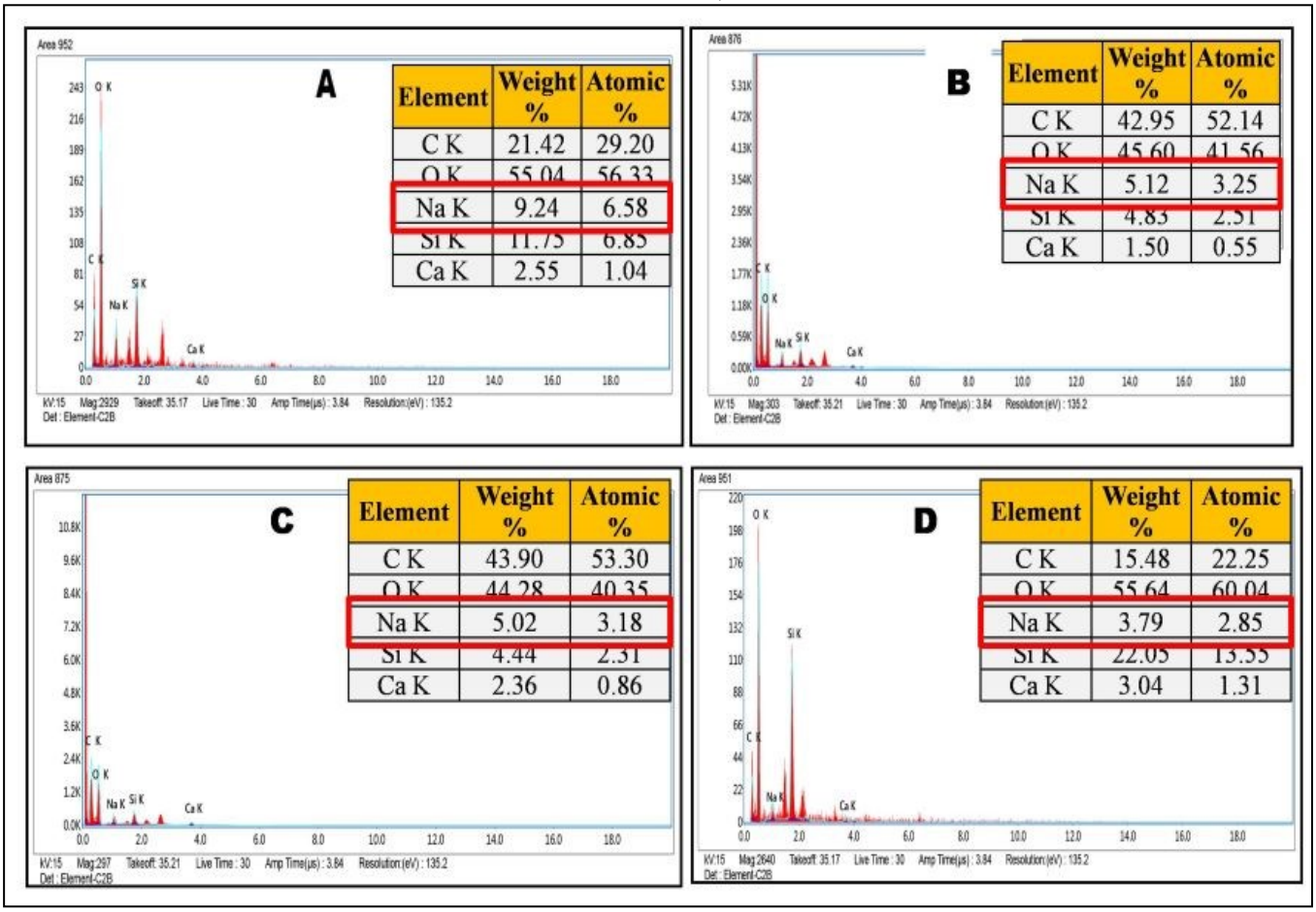

Fig. 11. Effect of rhizobacterial strain DB2 on NaCl uptake by roots of two rice genotypes under saline conditions. A and B for Chinsurah Nona I (CN I), C and D for Badshabhog (BB). (A) NaCl content in CN I root before DB2 inoculation, (B) NaCl content in CN I root after DB2 inoculation, (C) NaCl content in BB root before DB2 inoculation, (D) NaCl content in BB root after DB2 inoculation.

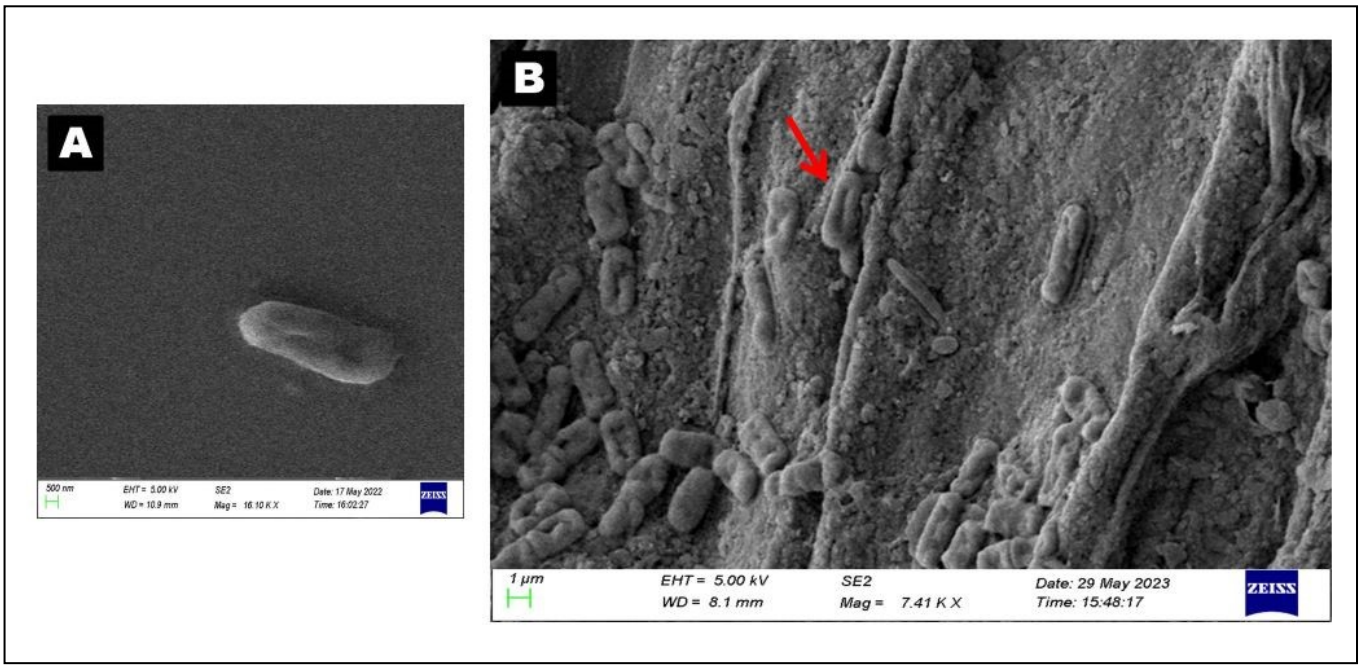

Fig. 12. SEM micrograph showing – (A) Rhizobacterial strain DB2 and (B) Colonization of DB2 on the root of a CN I rice plant.

Discussion

Among different types of environmental constraints, soil salinization has tremendous impacts on the shrinkage of agricultural production and food security. According to a study, around the world, salt stress causes 20-50% yield loss for important food crops, like rice, maize, and wheat (42). Thus, new eco-friendly, sustainable methods are urgently needed to overcome the salinity problem. Salt-tolerant PGPR is the most effective and environmentally sound for managing adverse conditions (2, 43). Some important bacterial genera including *Bacillus*, *Pseudomonas*, *Exiguobacterium*, *Klebsiella,* and *Steptomyces* have already been reported as important bio-inoculants to overcome the salinity problem (2, 43, 44).

The present study represents the salt stress tolerance and PGP traits of the selected PGPR isolates and the effect of the isolate DB2 on two rice genotypes under salt treatment. The isolate showed halotolerance ability to 1.36 M NaCl concentration and significantly responded to PGP traits. The highest accumulation of EPS by DB2 was found in 0.34M NaCl supplemented media which was followed by 1.02 M NaCl amended media. It was proven that EPS-producing microbes are capable of reducing sodium ion transport into the stem and inducing salt stress tolerance in plants (18). Bacillus tequilensis (UPMRB9) was found to produce the most EPS in a 1.5 M NaCl-containing medium (2). Bacteria that make EPS around plant roots can reduce the harmful effects of salt and toxic soil by storing Na+ through EPS secretion (2, 45). In our study, rhizobacterial isolate DB2 showed the highest IAA production and showed its capability for IAA production under salt stress conditions of upto 1.36 M of NaCl. Previously, it has been well established that IAA-producing bacterial species belonging to the genus *Bacillus* can protect plants from salt stress (2, 46). IAA is an important PGP trait that may act as an important signaling molecule in plant development regulation. Research supports the idea that IAA improves abiotic stress tolerance and modulates plant growth by increasing other phytohormones, such as Abscisic acid (ABA) (16). It was identified that *Bacillus aryabhattai* (UPMRE6) and *Bacillus tequilensis* (UPMRB9) as efficient P solubilizers (2). *Bacillus aryabhattai* (AB211) has been reported as an efficient phosphate solubilizer under saline conditions (47). In the current study, only the strain DB2 showed a clear halo zone (PSI: - 1.28) on the plate containing Pikovskaya's agar media. Moreover, in the present study, all the selected isolates DB1, DB2, DB3, DB5, and DB6 showed NF and NH_3 production capacity whereas DB2 showed the capacity to fix nitrogen and NH_3 production under saline conditions upto 1.36 M of NaCl. Three bacterial isolates in this study, DB2, DB3, and DB6 were able to produce HCN whereas catalase and protease production was done by DB2 and DB6. It was reported that *Bacillus cereus* B8W8 is a proficient producer of HCN and protease (48). Moreover, *Bacillus cereus* B8W8 showed antifungal properties against *Penicillium digitatum* and *Monilinia laxa* (47). This study indicated antifungal activity of the bacterial isolate DB2 (61.29%) against *Alternaria alternata*.

Total chlorophyll content, carotenoid content, RWC, and EL are important indicators of salt stress tolerance in plant tissues. Without the salt stress condition, Chinsurah Nona I inoculated with DB2 showed the highest enhancement of chlorophyll content, which is 26.6% over the uninoculated plant set. This inoculation with DB2 increased the chlorophyll content of Badshabhog by 32.5% and Chinsurah Nona I by 26.4% under salinity conditions. It was reported that an enhancement in Total chlorophyll content over uninoculated sets of rice varieties namely BRRI dhan67, Putra-1, and MR297 was achieved by the application of species of *Bacillus* under normal growing and salt stress conditions (2). We found that saline condition hampered carotenoid content more in Badshabhog than in Chinsurah Nona I. Compared to uninoculated sets, inoculation of DB2 improved carotenoid content in Chinsurah Nona I (17.3%) and

Badshabhog (6.3%) under salt-treated conditions. Earlier it was investigated on maize crops and proved the ability of *Bacillus* sp. PM_{31} to enhance carotenoid content under saline conditions (49). Relative water content (RWC), an important marker of water status in a plant body helps determine the tolerance level of the plant to water deficit or salinity (50, 51). Our study showed that salt stress inhibited the RWC of Badshabhog more (>2 times) than Chinsurah Nona I. Application of DB2 showed improvement of RWC in both rice varieties under normal growing conditions. Moreover, under saline conditions, an increase of 42% for Badshabhog and 19% for Chinsurah Nona I of RWC by application of DB2 was recorded in our study. Generally, salt stress hampers RWC in the plants. Previous studies have shown improvement of RWC in salinity in important food crops such as rice and maize by application of *Bacillus* sp. (2, 49). EL is one of the key indicators to determine plant responses to different types of biotic and abiotic stresses including drought, salinity, heavy metals, and pathogenic attack (52). It was found that the level of EL was more pronounced in salt-purposed eggplant leaves than in intolerant ones under salt stress (53). In a study, it was observed that a decrease in EL in rice plants inoculated with *Bacillus* compared to uninoculated plants under saline conditions (2). Particularly EL measures the percentage of electrolyte extruded due to loosening of cell membrane integrity (54). In the present study, EL increased more in Badshabhog than in Chinsurah Nona I under the salt stress conditions. Inoculation with DB2 showed a significant reduction in the EL in Chinsurah Nona I (43.4%) and Badshabhog (70.6%) grown on saline soils. Moreover, inoculation of DB2 was shown to promote the shoot and root length of both rice genotypes under normal growing and saline conditions. Improvement of root and shoot length of maize by the application of *Bacillus* sp. PM_{31} was proved in a previous study (49). A study already demonstrated the ability of *Bacillus* to reduce Na^+ translocation to shoot and root in BRRI dhan67 (2). We observed a decrease in Na^+ accumulation in the roots of Chinsurah Nona I (44.6%) and Badshabhog (24.5%) by application of DB2 which may prove the ability of DB2 to interfere with Na^+ uptake by plant roots. In our study, DB2 showed a significant response to PGP traits and acted as an important bioinoculant to improve salt stress tolerance in rice genotypes. This conclusion was backed up by SEM studies that clearly showed how plants and microbes interact by showing DB2 colonization around Chinsura Nona I roots. Previous studies (2, 55) have also demonstrated the colonization of PGPR in plant roots.

Conclusion

In the present study, we intend to investigate the effect of bacterial inoculation on the salt stress tolerance of rice plants under polyhouse conditions. In our study, among the two rice genotypes, Badshabhog was more affected by salinity than Chinsurah Nona I. Inoculation with DB2 (*Bacillus cereus* ATCC 14579(T) improved shoot length, root length, TC, carotenoids, and RWC in both rice genotypes under saline and non-saline conditions. Under normal conditions, enhancement of RWC, TCC, RL, and SL was better in DB2-inoculated Chinsurah Nona I than in Badshabhog inoculated with the same strain. However, the inoculation under saline conditions and treatment with DB2 exhibited better results for these parameters in Badshabhog than in Chinsurah Nona I. Under saline soil conditions, salt-tolerant rhizobacterial strain DB2 improved salt stress tolerance in both rice genotypes by improving shoot length, root length, TC, carotenoids, and RWC and also by decreasing EL and root Na^+ content. In addition to this, strain DB2 showed a positive response of the PGP properties such as EPS production, IAA production, N_2 fixation, NH_3 production, HCN production, antifungal property, catalase, and protease activity. Overall, this work revealed the ability of DB2 to enhance salt stress tolerance in rice plants which may be due to the presence of PGP trait and halotolerance ability of the rhizobacterial strain DB2. Rice is a staple food crop worldwide and aromatic rice varieties are popular having a valuable position in the global market. The results indicated that the rhizobacterial strain DB2 can reduce the effect of salt on the aromatic rice genotype Badshabhog under saline conditions which may be helpful to increase the yield of Badshabhog under salinity. DB2 makes plants better at fighting off salt stress, which suggests that the strain could be a cheap and environmentally friendly way to improve plant growth while avoiding salt problems in areas that get a lot of salt.

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Authors' contributions

The main concept of the work was provided by SD. Literature study and lab work were done by DC. DC prepared the manuscript. CM helped DC in lab work. ShD, CM, and SyD helped with manuscript formatting. Finally, all authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest: The authors do not have any conflict of interest to declare.

Ethical issues: None.

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