

Article

Effect of Salt-Tolerant Bacterial Inoculations on Rice Seedlings Differing in Salt-Tolerance under Saline Soil Conditions

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Abstract: Salt-tolerant plant growth-promoting rhizobacteria (PGPR) could be an alternative to alleviate salinity problems in rice plants grown in the coastal areas. This study was conducted to isolate and characterize salt-tolerant PGPR and observe their effects on the physiological and biochemical properties of rice plants grown under non-saline and saline glasshouse conditions. Three strains were selected based on their salt-tolerance and plant growth-promoting properties under in vitro saline conditions. These strains were identified as *Bacillus tequilensis* (UPMRB9), *Bacillus aryabhatai* (UPMRE6), and *Providencia stuartii* (UPMRG1) using a 16S rRNA technique. The selected strains were inoculated to three different rice varieties, namely BRRI dhan67 (salt-tolerant), Putra-1 (moderate salt-tolerant), and MR297 (salt-susceptible) under glasshouse conditions. Results showed that the MR297 rice variety inoculated with UPMRB9 produced the highest total chlorophyll content, with an increment of 28%, and lowest electrolyte leakage of 92%. The Putra-1 rice variety also showed a 156% total dry matter increase with the inoculation of this bacterial strain. The highest increase of relative water content and reduction of Na/K ratio were found upon inoculation of UPMRE6 and UPMRB9, respectively. The biggest significant effects of these bacterial inoculations were on relative water content, electrolyte leakage, and the Na/K ratio of the BRRI dhan67 rice variety under saline conditions, suggesting a synergistic effect on the mechanisms of plant salt-tolerance. This study has shown that the application of locally-isolated salt-tolerant PGPR strains could be an effective long-term and sustainable solution for rice cultivation in the coastal areas, which are affected by global climate change.

Keywords: PGPR; salt-tolerant; rice; salinity; dry matter

1. Introduction

Globally, plant growth and productivity are being hampered by various biotic and abiotic stresses [1]. Among them, salinity is regarded as one of the important agricultural problems, as it affects more than 20% of the total and irrigated land [2]. Rice (*Oryza sativa* L.) is one of the staple food crops across the globe and the demand is rising to feed the ever-increasing human population. Rice is a glycophyte plant by nature; it is inherently prone to salinity and will show a wide and vivid detrimental response to an increased salt level in the soil [3]. Moreover, the utilization of saline soil

for agriculture has become necessary due to the lack of arable land. Several physical and chemical strategies for salinity mitigation have been tested but these methods are not feasible, causing adverse impacts on the ecosystem, thereby creating other problems. Therefore, identifying and developing an eco-friendly strategy that can ameliorate plant growth in response to abiotic stresses is vital in the current agricultural systems, which have to cope with the effect of climate change [4].

Having this in mind, the genetic technology to develop salt-tolerant rice variety and the utilization of PGPR could be the answer to improve rice cultivation on saline soils [5]. Several reports have been published on the effectiveness of PGPR on improving the growth of crops under environmental stress conditions [6,7]. In addition to the direct beneficial effect of PGPR, it has also been reported to conserve soil fertility, thus ensuring a favorable alternative to inorganic fertilizers and pesticides for sustainable agriculture systems [8]. Several bacterial genera, such as *Alcaligenes*, *Azospirillum*, *Bacillus*, *Clostridium*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, *Thiobacillus*, *Serratia*, and *Streptomyces*, were used and tested as plant growth-promoters (PGP) under saline conditions [9]. Various researches have shown the commanding role of PGPRs in mitigating salt stress in different crop plants [10–14].

Plant growth-promoting rhizobacteria can play an important role during the seedling growth stage through several mechanisms, including biological N fixation, P solubilization, boosting of photosynthetic activities, chelation of iron through siderophores production, and synthesis of plant growth regulators such as indole-3-acetic acid (IAA) [15–20]. These positive traits of PGPR during the establishment of the crop seedling will significantly enhance plant growth during the later growth stage. Salt-tolerant PGPR has been reported to possess various salt-tolerant mechanisms, namely through the production of exopolysaccharide (EPS) and the formation of a biofilm, which have been proven to restrict Na^+ uptake under saline soil conditions [21]. Bacterial flocculation or aggregate formation as a response to salinity, which are closely associated with the production of bacterial exopolysaccharides, help bacteria to survive in stressed environments and assist the host plant in tolerating various stresses [22]. Exopolysaccharides play a fundamental role in the formation of a bacterial biofilm, which enhances bacterial colonization on plant root surfaces [23]. Gholami et al. [24] showed enhanced germination of seed and vigorous growth of maize seedling by PGPR inoculation during the early plant growth stage, consequently resulting in an increased yield. According to Yoshida [25], the accumulation of essential nutrients for plant growth, such as N, P, K, and S, at the early stage of rice seedling growth is very crucial for the subsequent vegetative and reproductive stages.

The relationship between PGPR and plants has been widely known; however, there is a dearth of information regarding the effect of salt-tolerant PGPR on the growth of high yielding salt-responsive rice varieties. This is of utmost importance considering the perspective of salinity mitigation in coastal salt-affected rice-growing areas, in which seawater intrusion has become an urgent threat in recent years due to global climate change. Thus, the present study was undertaken as a starting point to characterize and identify salt-tolerant PGPR isolated from Malaysian coastal saline rice cultivation areas and to observe their effect on physiological and biochemical characteristics of three rice varieties differing in salinity tolerance under non-saline and saline glasshouse conditions.

2. Materials and Methods

2.1. Determination of Plant Growth-Promoting Properties of Selected Bacterial Isolates

The bacterial strains were isolated from the coastal salt-affected rice-growing areas in the northern part of Peninsular Malaysia. The bacterial strains were primarily selected based on their profuse growth on 1.5 M of a NaCl-amended tryptic soy agar (TSA) media plate. Thereafter, the selected isolates were screened for several salt-tolerance properties, namely exopolysaccharide production, floc yield production, biofilm formation; and for uptake of Na and plant growth-promoting properties, namely IAA production, P and K solubilizations, and N_2 fixation under a NaCl-amended tryptic soy broth (TSB) medium. The salt-tolerance properties were done following the protocol of Hong et al. [26] with some modifications. The IAA was determined following the colorimetric procedure as described by

Gordon and Weber [27] with some modifications. The P and K solubilizing activities were determined following the protocol by Tan et al. [28] with slight modifications. The bacterial isolates' ability in fixing atmospheric N₂ was checked qualitatively by streaking the isolates on N-free malate media plates amended with 1.5 M of NaCl and incubated at 33 °C for 24 h.

2.2. Identification of Selected Isolates Using a 16S rRNA Gene Sequence

Selected strains were identified by partial sequencing of the 16S rRNA gene using the standard molecular identification method. A fragment of the 16S rRNA gene from the total genomic DNA was amplified using polymerase chain reaction (PCR) using universal forward (5'-GAGTTTGATCCTGCTCAG-3') and reverse (5'-GTTACCTGTTACGACTT-3') primers (BioSune Biotechnology Co. Ltd., China). The PCR product was purified using the Gel/PCR DNA Mini Kit (Real Biotech, Taiwan) and outsourced for sequencing (First Base Laboratories Pvt. Ltd., Selangor, Malaysia). The sequence data were aligned and analyzed to identify the bacterium and its closest neighbors by using BLAST (National Center for Biotechnology Information (NCBI) Bethesda, MD USA).

2.3. Glasshouse Trial

2.3.1. Soil Preparation and Fertilizer Application

The soils were collected from a farmer's plot in block D, Sawah Sempadan, Tanjong Karang, Selangor; the soil series was Bernam, pH 4.23. The available nutrient content of the initial soil was 0.62% of N, 0.08 mg kg⁻¹ of P, 0.29 mg kg⁻¹ of K, 0.23 mg kg⁻¹ of Ca, and 0.33 mg kg⁻¹ of Mg. The soil was air-dried for two weeks and sieved through a 2 mm sieve. Two kg of air-dried soil was weighed, sterilized, and transferred into non-drained plastic pots with the following dimensions: 20 cm diameter × 15 cm height. The fertilizers were applied to the soil following the recommendations by the Department of Agriculture, Kuala Selangor, Selangor, Malaysia. Inorganic fertilizers, namely Urea, Triple Super P, and Muriate of Potash were applied at the rates of 170, 80, and 150 kg ha⁻¹, respectively. Phosphate and potassium fertilizers were applied as basal dosages while nitrogen was applied in three split applications 15, 30, and 45 days after transplanting (DAT).

2.3.2. Inoculum Preparation and Application to Rice Seedlings

Tryptic soy broth (TSB) medium was inoculated with one loopful of each of the 24-h-old selected bacterial cultures and shaken for 24 h. Approximately 10⁸–10⁹ CFU mL⁻¹ of overnight-grown bacterial suspension or sterile distilled water (as a control) were inoculated onto germinated rice seedlings and left to settle for 1 h. Single seedlings of three rice varieties, namely BRR1 dhan67, Putra-1, and MR297, were transplanted into a plastic pot filled with 2 kg of sterilized soil. In the inoculated treatments, plants were given the second inoculation of 5.0 mL of washed bacterial cells at 14 DAT, whereas for the uninoculated treatment the cells were replaced with sterilized distilled water.

2.3.3. Soil Salinity Adjustment

The soil was salinized at 14 DAT using sodium chloride (NaCl) at the rate of 4 g/L to reach an electrical conductivity (EC) of 8 dSm⁻¹. The EC level was maintained throughout the experimental period.

2.3.4. Determination of Total Chlorophyll Content

The chlorophyll content of the leaves was determined at 30 DAT. Fresh leaf samples were cut by hand into small pieces and approximately 0.1 g was transferred into a test tube containing 10 mL of 80% acetone. The test tubes were covered using aluminum foil and stored in the dark at room temperature for 3–4 days. The test tubes were then shaken using a vortex mixer and the sediments were allowed to settle at the bottom. The spectrophotometric absorbance of the extract was recorded at 663.2 nm and 646.8 nm and 80% acetone was used as a blank. Total chlorophyll content was expressed as μmol g⁻¹ FW according to the methods by Lichtenthaler [29].

Calculations:

$$\text{Total chlorophyll } (\mu\text{mol g}^{-1} \text{ FW}) = (7.15 \times A_{663.2 \text{ nm}} + 18.71 \times A_{646.8 \text{ nm}}) \times \text{SW}$$

where $A_{663.2}$ = absorbance at 663.2 nm

$A_{646.8}$ = absorbance at 646.8 nm

SW = sample weight (g)

2.3.5. Relative Water Content

Relative water content (RWC) percentage was determined at 30 DAT following the protocol described by Teulat et al. [30]. Fresh fully-developed leaves were collected randomly from the rice plants. The fresh weight was measured and the leaves were submerged in a test tube filled with distilled water and incubated in a refrigerator for 24 h. The leaves were blotted dry using tissue paper and the fully turgid weight was measured. The leaves were oven-dried for 24 h at 72 °C. Eventually, the dry weight was recorded and the relative water content was determined using the equation below.

$$\text{RWC (\%)} = (\text{fresh weight} - \text{dry weight}) / (\text{fully turgid weight} - \text{dry weight}) \times 100$$

2.3.6. Electrolyte Leakage

Electrolyte leakage (EL) percentage was measured by cutting the leaf samples into discs and submerging them into test tubes consisting of 25 mL deionized water. The test tubes were shaken for 10 s and the initial electrical conductivity (EC_0) of the water was measured by using an EC meter. After overnight refrigeration at 4 °C, the second reading of the electrical conductivity of the solution was measured (EC_1). Finally, the test tubes were autoclaved at 121 °C for 20 min for the determination of EC_2 . The following formula was used to determine the EL percentage as described by Yang et al. [31].

$$\text{EL (\%)} = (EC_1 - EC_0) / (EC_2 - EC_0) \times 100$$

2.3.7. Measurement of Seedling Dry Matter

The plants were harvested at 45 DAT. The plant fresh weight was recorded and the plant tissue was oven-dried at 72 °C for 48 h before measuring the total dry matter (TDM).

2.3.8. Plant and Soil Analyses

The root and shoots of oven-dried plants were separated. The plant samples were ground and used for the measurement of Na and K content following the procedure stated by Awang et al. [32]. The Na/K ratio was calculated from the reading of these values. The Na and K contents in the soil samples were determined following the method by McGrath [33] with some modifications. Approximately 0.5 g of soil were digested with 3 mL of HCl and 1 mL of HNO₃ at 110 °C to obtain 1 mL of aliquot. After cooling, 10 mL of 1.2% HNO₃ was added and heated at 80 °C for 30 min. The volume was adjusted to 20 mL using distilled water and heated for 30 min. Then, the samples were filtered and sent for determination of Na and K using an atomic absorption spectrophotometer (ASS).

2.4. Observation of Root Colonization by Salt-Tolerant PGPR Using SEM and TEM

The inoculated seedlings were uprooted at 45 DAT, washed with sterile distilled water, and cut into 1 cm and 1 mm for SEM and TEM, respectively. The root samples were pre-fixed overnight using 4% glutaraldehyde and a 0.1 M sodium cacodylate buffer was used for washing three times for 30 min each. For post-fixation, osmium tetroxides buffer (1%) was used. After a series of dehydrations in acetone (35, 50, 75, 95, and 100%), the samples were dried in a critical point dryer and mounted on aluminum stubs, sputter-coated in gold and viewed under SEM. For TEM observation, the samples were infiltrated with

acetone and a resin mixture and embedded. After the polymerized steps, the samples were sectioned and then coated with gold and observed under TEM.

2.5. Statistical Analysis

The glasshouse experiment was laid out in a factorial RCBD. The recorded data were analyzed using analysis of variance (ANOVA) SAS 9.4 software. Means were compared using Tukey's test (HSD) at a probability level of 0.05.

3. Results

3.1. Salt-Tolerance and Plant Growth-Promoting Properties of Selected Bacterial Isolates

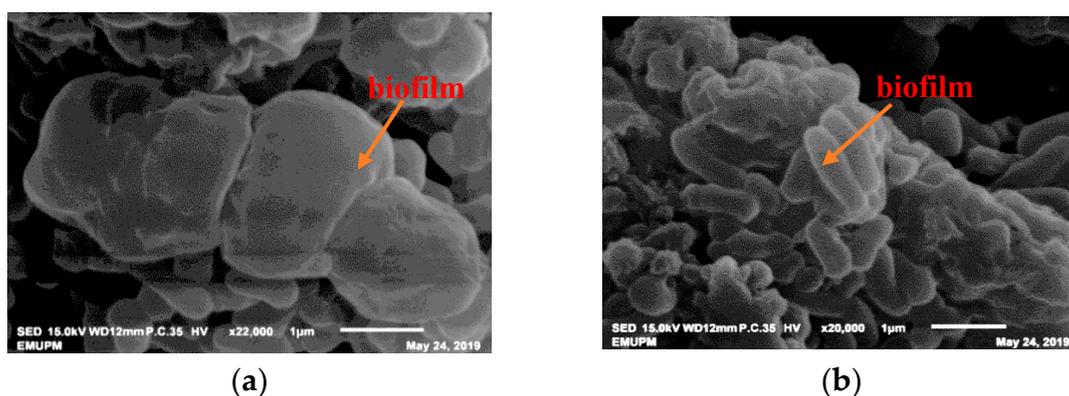
UPMRB9 produced the highest amount of exopolysaccharide (EPS) at 1.5 M of NaCl (30.50 g L⁻¹), which is significantly higher than UPMRG1, UPMRE3, and UPMRA4 (Table 1 and Scheme 1). This particular strain also produced significantly higher biofilm and Na uptake compared to other strains. Meanwhile, UPMRE6 recorded the highest floc yield production (19.67 g L⁻¹).

The plant growth-promoting characters showed that the UPMRB9 isolate produced significantly higher IAA (8.25 µg mL⁻¹) compared to UPMRA4, UPMRE3, and UPMRE6. The latter strain had the highest P (15.30 µg mL⁻¹) and K (2.97 mg L⁻¹) solubilizers. The qualitative test showed that UPMRB9, UPMRE6, and UPMRG1 were N₂ fixers.

Table 1. Salt-tolerance and plant growth-promoting traits of selected bacterial isolates at 1.5 M of NaCl concentration.

Bacterial Isolates	Salt-Tolerance Characteristics				Plant Growth-Promoting Characteristics			
	EPS (g L ⁻¹)	Floc Yield (g L ⁻¹)	Biofilm (590nm)	Sodium Uptake (mg L ⁻¹)	IAA (µg mL ⁻¹)	P Solubilization (µg mL ⁻¹)	K Solubilization (mg L ⁻¹)	Nitrogen Fixation
UPMRA4	7.36c	17.83a	0.44d	7.55cd	6.71b	9.21b	2.08ab	–
UPMRB9	30.50a	19.28a	1.14a	23.8a	8.25a	15.18a	2.17ab	+
UPMRE3	12.24c	9.73c	0.31e	6.40d	3.50c	9.34b	0.70c	–
UPMRE6	26.18ab	19.67a	0.95b	12.49bc	6.70b	15.30a	2.97a	+
UPMRG1	21.79b	12.90b	0.77c	13.51b	6.99ab	9.24b	1.72bc	+

Note: '+' indicates positive, '-' indicates negative. Means with the same letter in a column do not differ significantly using Tukey's test at $p > 0.05$. EPS: exopolysaccharide; IAA: indole-3-acetic acid.

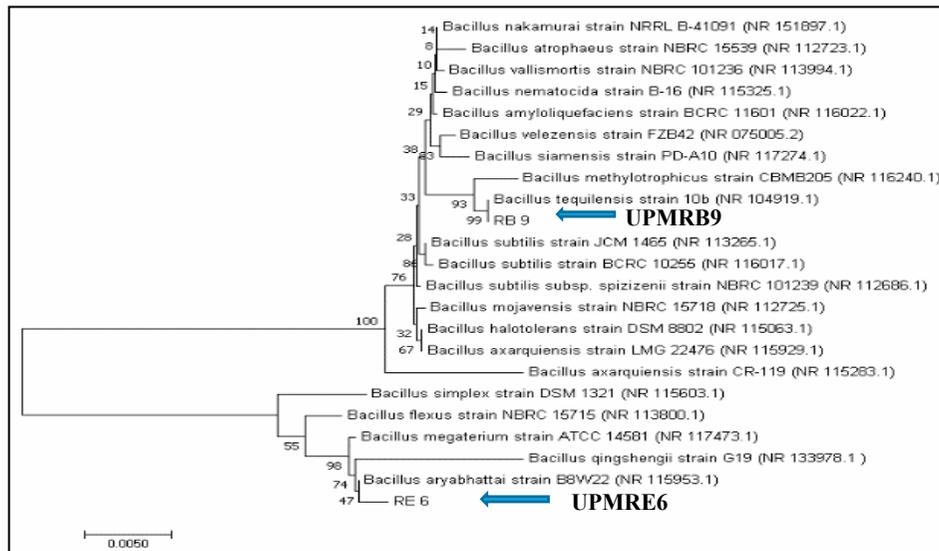


Scheme 1. Biofilm protects bacterial cells within EPS matrix of UPMRB9 (a) and UPMRE6 (b) at 1.5 M of NaCl concentration.

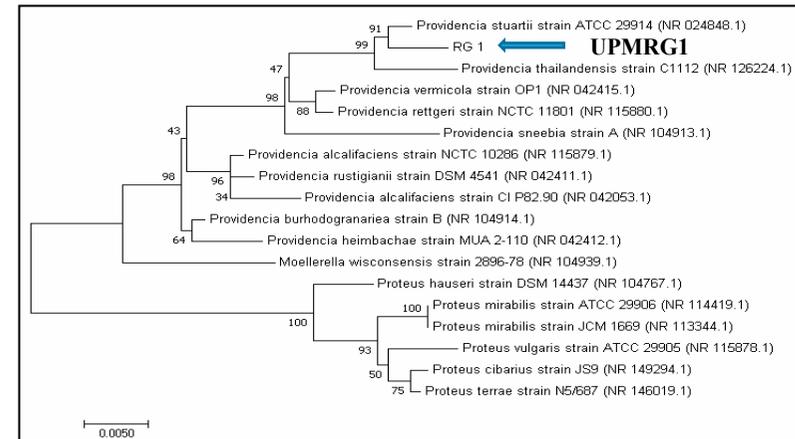
3.2. Identification of PGPR Isolates Based on Partial 16S rRNA Gene Sequences

The PGPR isolates UPMRB9, UPMRE6, and UPMRG1 were selected for molecular identification based on their salt-tolerance and plant growth-promoting properties. The 16S rRNA fragments were successfully amplified using PCR. Approximately 1413 bp for UPMRB9 and UPMRG1 and 1411 bp for

UPMRE6 were sequenced. The BLASTX comparison searches against the NCBI nucleotide database revealed 99% similarity of the isolates UPMRB9 with *Bacillus tequilensis*10b (NCBI accession number: NR 104919.1), UPMRE6 with *Bacillus aryabhatai*B8W22 (NCBI accession number: NR 115953.1), and UPMRG1 with *Providencia stuartii* strain ATCC 29914 (NCBI accession number: NR 024848.1). The phylogenetic analyses of the PGPR isolates were done based on neighbor-joining bootstrap analysis (Figure 1a,b).



(a)



(b)

Figure 1. (a): Neighbor-joining tree based on the 16S rRNA gene sequence showing the phylogenetic relationship of UPMRB9 and UPMRE6 to related isolates; bar represents the nucleotide divergence value. (b): Neighbor-joining tree based on the 16S rRNA gene sequence showing the phylogenetic relationship of UPMRG1 to related isolates; bar represents the nucleotide divergence value.

3.3. Effect of Bacterial Inoculation on Total Chlorophyll Content of Three Rice Varieties

The total chlorophyll concentration of the rice plants was varied under non-saline and saline conditions. Under non-saline conditions, the total chlorophyll content of BRRI dhan67 treated with UPMRE6 increased by 19%, that of Putra-1 inoculated by UPMRB9 increased by 10% and that of MR297 treated with UPMRE6 increased by 15% (Figure 2a). On the contrary, under salt stress conditions, UPMRB9 inoculation produced the highest total chlorophyll content of all rice varieties with increments of 22%, 28%, and 28% for rice varieties BRRI dhan67, Putra-1, and MR297, respectively, compared to uninoculated plants (Figure 2b).

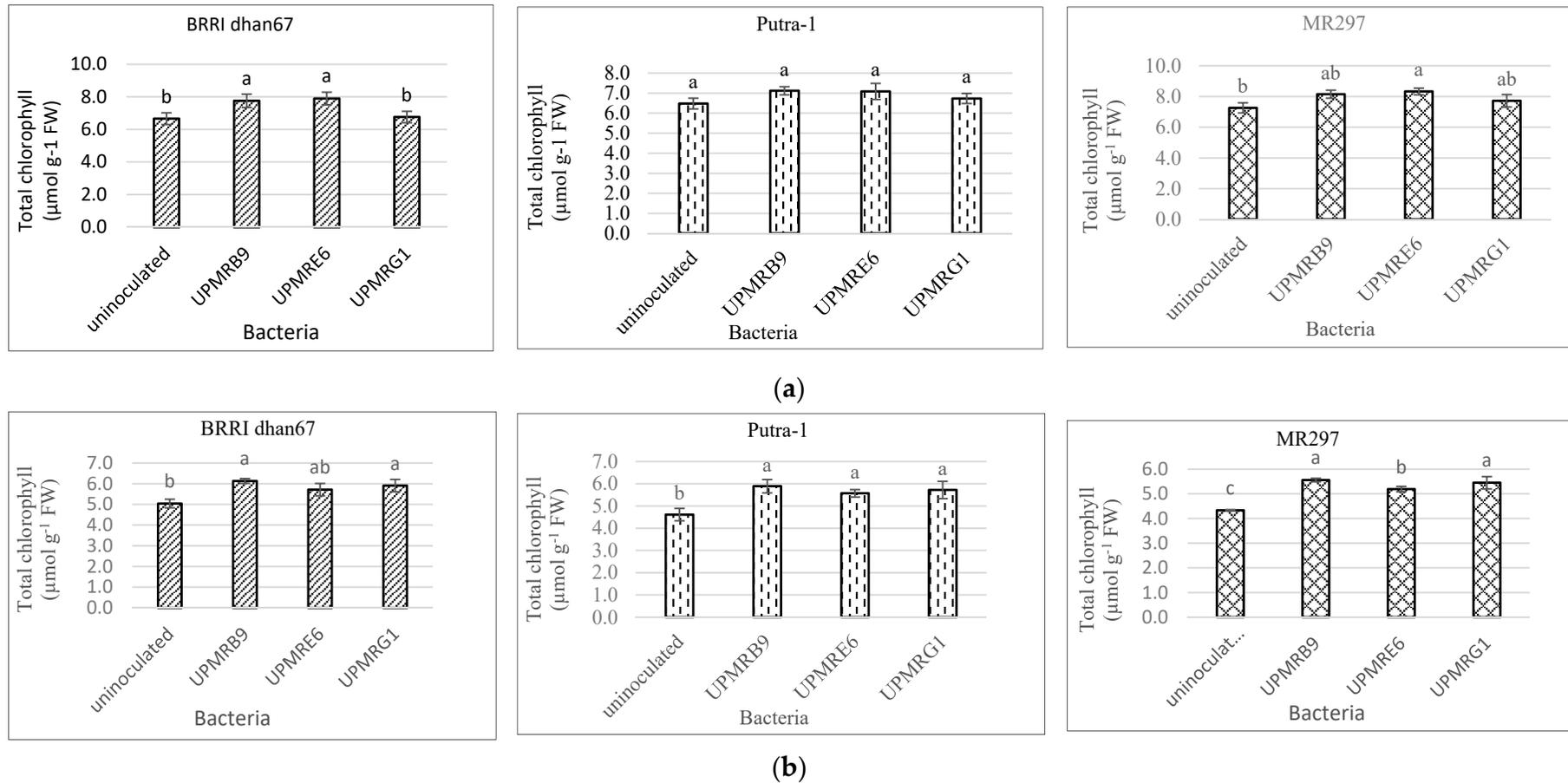


Figure 2. (a) Effect of bacterial inoculation on total chlorophyll content of three rice varieties under non-saline conditions. Means with the same letter in a column do not differ significantly using Tukey's test at $p > 0.05$. (b) Effect of bacterial inoculation on total chlorophyll content of three rice varieties under saline conditions. Means with the same letter in a column do not differ significantly using Tukey's test at $p > 0.05$.

3.4. Effect of Bacterial Inoculation on the Relative Water Content of Three Rice Varieties

Bacterial inoculation had a significant effect on an increasing percentage of relative water content both under non-saline and saline conditions (Figure 3a,b). Under non-saline conditions, the inoculation of UPMRE6 increased the relative water content of BRRI dhan67 and MR297 by 28% and 34%, respectively, while UPMRB9 increased the relative water content of Putra-1 by 28%. Under saline conditions, the relative water content of BRRI dhan67 and Putra-1 inoculated with UPMRB9 increased by 39% and 49%, respectively, while the relative water content of MR297 inoculated with UPMRE6 increased by 62%.

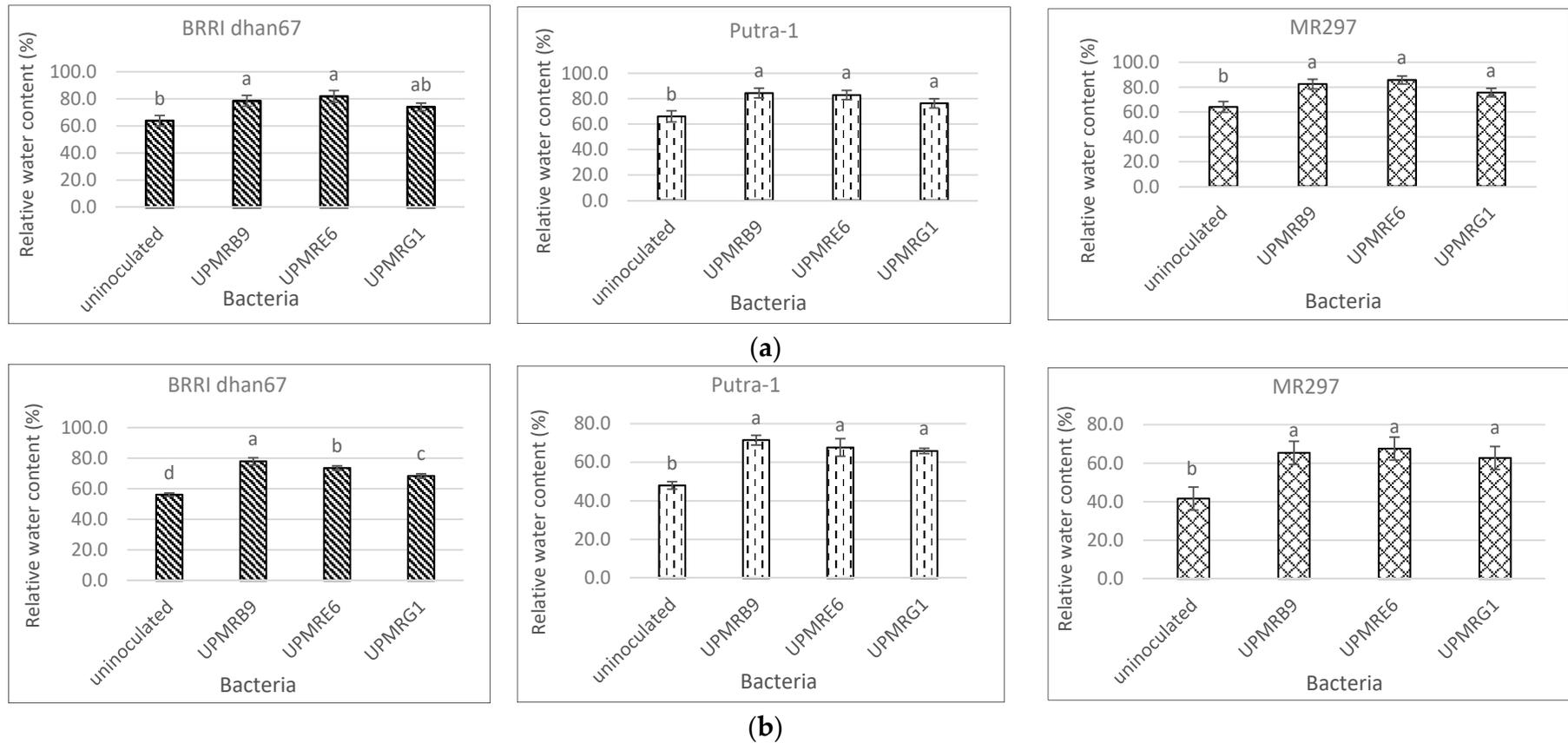


Figure 3. (a) Effect of bacterial inoculation on the relative water content of three rice varieties under non-saline conditions. Means with the same letter in a column do not differ significantly using Tukey’s test at $p > 0.05$. (b) Effect of bacterial inoculation on the relative water content of three rice varieties under saline conditions. Means with the same letter in a column do not differ significantly using Tukey’s test at $p > 0.05$.

3.5. Effect of Bacterial Inoculation on Electrolyte Leakage of Three Rice Varieties

Under non-saline conditions, electrolyte leakage of three rice varieties was not significantly varied by bacterial inoculation (data not shown), whilst under saline conditions, these values were significant across all inoculations (Figure 4). Leaf electrolyte leakage was significantly reduced with bacterial inoculation as compared to uninoculated plants. The highest reductions of electrolyte leakage in BRRI dhan67 and MR297 treated with UPMRB9 were 61% and 92%, respectively, while Putra-1 treated with UPMRG1 reduced the leakage by 55%.

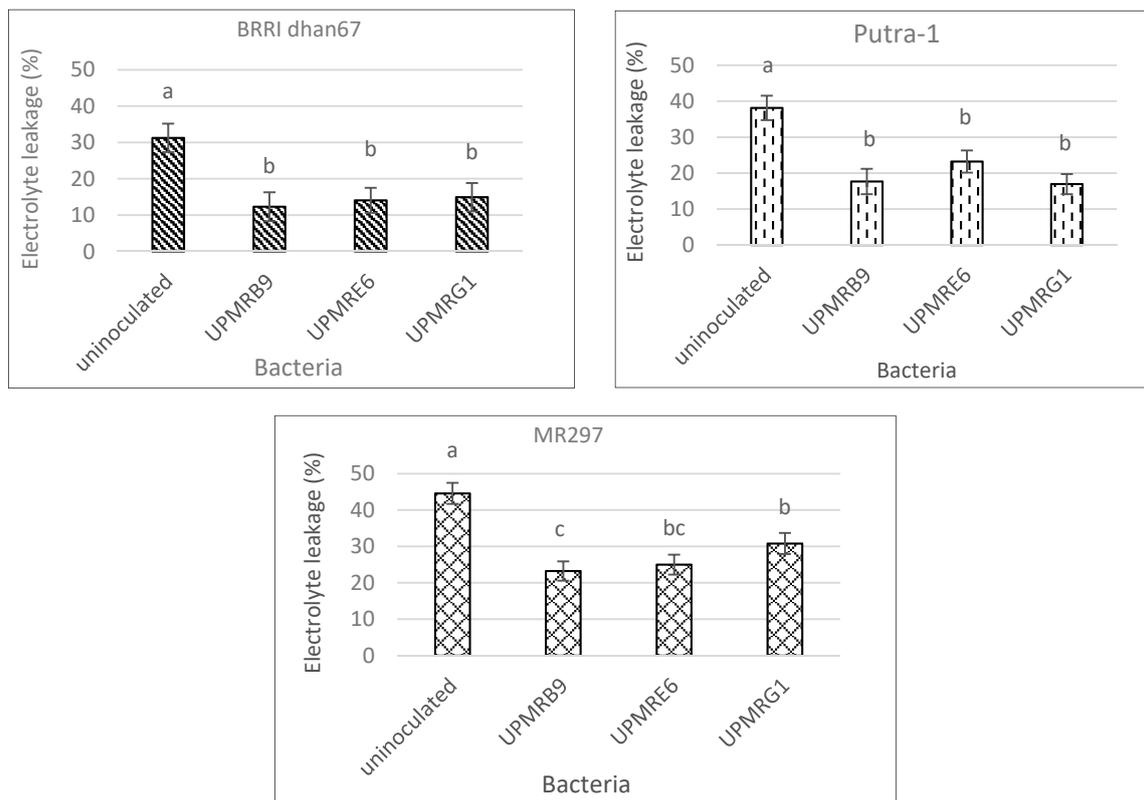


Figure 4. Effect of bacterial inoculation on electrolyte leakage of three rice varieties under saline conditions. Means with the same letter in a column do not differ significantly using Tukey's test at $p > 0.05$.

3.6. Effect of Bacterial Inoculations on Total Dry Matter Production of Three Rice Varieties

Total dry matter (TDM) of the three rice varieties varied significantly due to the bacterial inoculations under both the non-saline and saline conditions. Bacterial inoculation increased TDM under non-saline conditions as compared to the uninoculated control, except for the inoculation of UPMRG1 on MR297 (Figure 5a).

Under saline conditions, UPMRB9 significantly produced higher TDM compared to other treatments for the BRRI dhan67, Putra-1, and MR297 varieties with increments of 56%, 156%, and 50%, respectively, compared to the uninoculated control (Figure 5b).

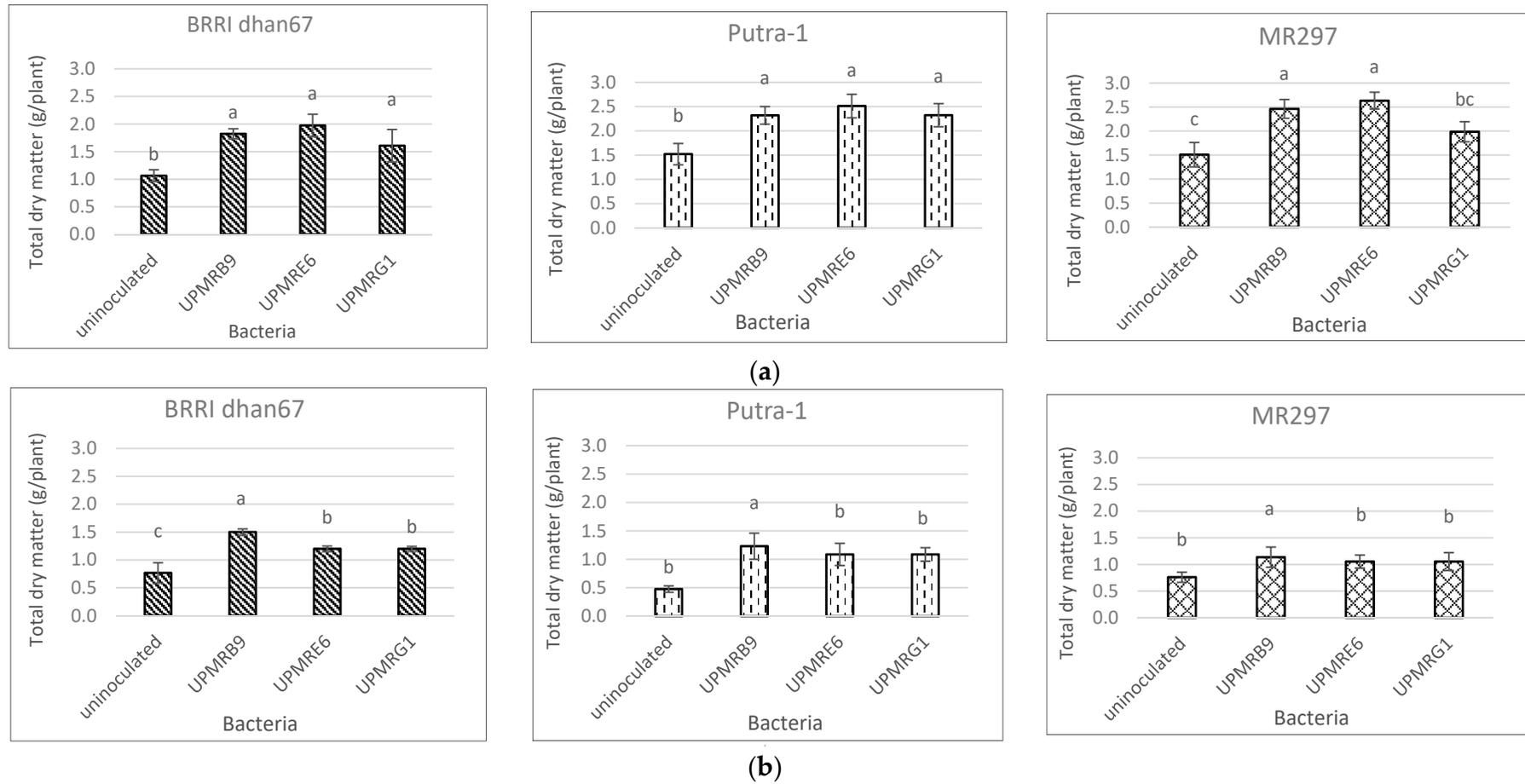


Figure 5. (a) Effect of bacterial inoculation on total dry matter production of three rice varieties under non-saline conditions. Means with the same letter in each variety do not differ significantly using Tukey’s test at $p > 0.05$. (b) Effect of bacterial inoculation on total dry matter production of three rice varieties under saline conditions. Means with the same letter in each variety do not differ significantly using Tukey’s test at $p > 0.05$.

3.7. Effect of Salinity, PGPR and Rice Varieties on Na/K Ratio in the Above-Ground (Shoot) and Below Ground (Root and Soil) Parts of Three Rice Varieties

Under non-stress conditions, the Na/K ratio was not varied significantly (data not shown). Under saline conditions, the BRRI dhan67 rice plant treated with UPMRB9 had the greatest reduction, of 81%, in Na/K in its above-ground parts (Table 2).

A similar trend was observed for the Na/K ratio in the roots of BRRI dhan67 treated with UPMRB9, with a 53% reduction (Table 3). Interestingly, the soil sample showed the highest increment of the Na/K ratio in the MR297 rice plants treated with UPMRB9 (119%).

Table 2. Effect of bacterial inoculation on the ratio of Na/K (% decrease) in the above-ground parts (shoots) of three rice varieties under saline soil conditions.

Variety	Shoot			
	Uninoculated	UPMRB9	UPMRE6	UPMRG1
BRRI dhan67	0.73a	0.14c (81%)	0.22b (70%)	0.26b (64%)
Putra-1	1.13a	0.39c (66%)	0.57b (50%)	0.60b (47%)
MR297	1.37a	0.81c (41%)	1.04b (24%)	1.09b (20%)

Means having the same letter in each variety do not differ significantly using Tukey's test at $p > 0.05$.

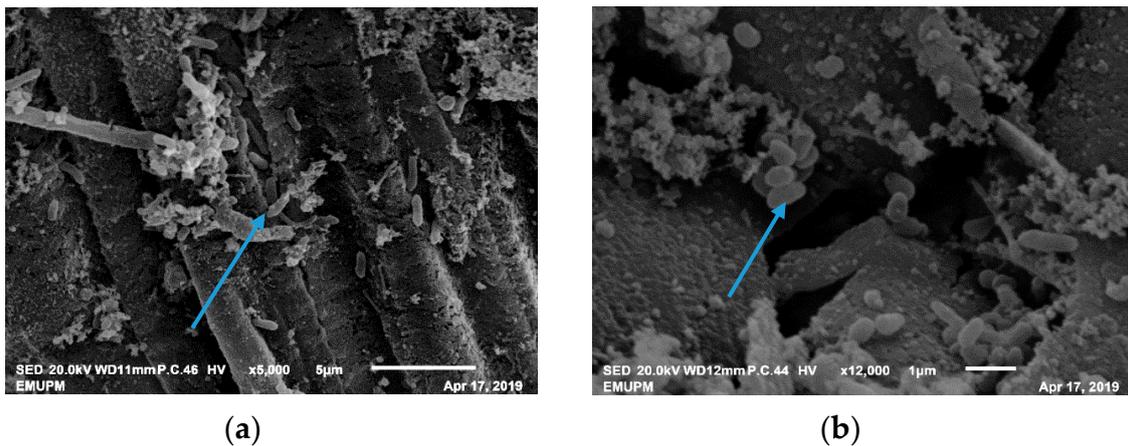
Table 3. Effect of bacterial inoculation on the Na/K ratio (% decrease/increase) in the below-ground parts (roots and soil) of three rice varieties under saline soil.

Variety	Root				Soil			
	CONTROL	UPMRB9	UPMRE6	UPMRG1	CONTROL	UPMRB9	UPMRE6	UPMRG1
BRRI dhan67	0.61a	0.29c (53%)	0.50b (18%)	0.53b (13%)	0.50b	0.81a (62%)	0.67ab (34%)	0.63b (26%)
Putra-1	0.79a	0.90a (14%)	0.42b (47%)	0.45b (43%)	0.46b	0.64a (39%)	0.48b (4%)	0.46b (0%)
MR297	1.11a	1.04a (6%)	0.60b (46%)	0.56b (50%)	0.27b	0.59a (119%)	0.30b (11%)	0.37b (37%)

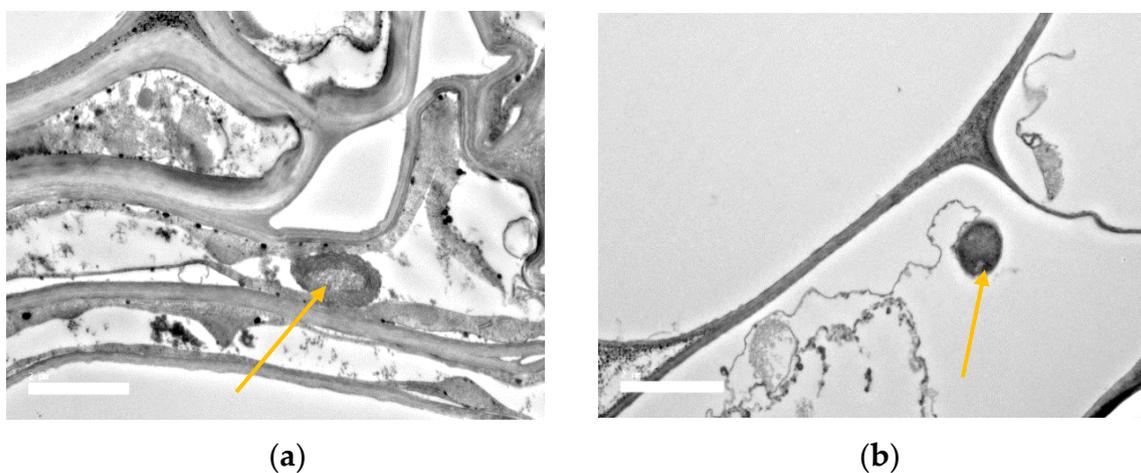
Means having the same letter in each variety do not differ significantly using Tukey's test at $p > 0.05$.

3.8. SEM and TEM Observations of Bacterial Root Colonization

Electron micrograph observations revealed that the rice roots of BRRI dhan67 were successfully colonized with the inoculum under salt stress conditions. SEM showed that the UPMRB9 isolates were abundant around roots' rhizosphere. The bacterial presence was observed (a) in between cell walls and the root hair zone and (b) in aggregate formation near root crevices (Scheme 2). The transverse sections of the root observed under TEM showed that UPMRB9 also inhabit in (a) the intercellular spaces within the epidermis and (b) near the vascular bundle along the cell wall within the epidermis (Scheme 3).



Scheme 2. SEM micrograph showing the colonization of UPMRB9 on roots of a BRRI dhan67 plant. The blue arrow shows UPMRB9 (a) in between cell walls and (b) near the crevices under saline conditions.



Scheme 3. TEM micrograph showing the colonization of UPMRB9 on roots of BRRI dhan67. The yellow arrow shows UPMRB9 (a) in intercellular spaces and (b) within the epidermis under saline conditions.

4. Discussion

This study has successfully demonstrated the salt-tolerance and plant growth-promoting traits of selected PGPR isolates and their effect on increasing the salinity reduction of three rice varieties at the seedling stage under glasshouse conditions. Salt-tolerance traits revealed UPMRB9 as the highest EPS producer at 1.5 M of NaCl-amended medium. This might be due to the higher survivability rate of this strain, which maintained a considerably high population at 1.5 M of NaCl. It was reported that the cementing properties of EPS strengthen the aggregate formation of the bacteria with the soil particles and binds Na ions, thereby reducing their toxicity in the soil [26]. These findings are in line with Qurashi and Sabri [22], who found the highest dry weight of bacterial exopolysaccharides at 1.5 to 2 M of NaCl. Ghosh et al. [34] found that the EPS production of *B. tequilensis* J12 was not varied under salt stress conditions and that the osmotic stress of *Arabidopsis thaliana* was reduced by its inoculation as well as by augmented fresh weight, dry weight, and water content in plants over the uninoculated plants. Another survival strategy of bacteria in salt-stressed environments is floc yield or aggregate formation, which increase with the increased concentration of NaCl. Bacterial flocculation or aggregate formation correlate with EPS production, where the highest value in this study was recorded by *Bacillus aryabhatai* (UPMRE6). Likewise, Hong et al. [26] isolated a high floc yield producer identified as *B. iodinum* RS16 at 1.5 M of NaCl concentration that protects the host plant against various stresses. Furthermore, biofilm protects the bacterial cells within the EPS layer and acts as a boundary between

cells and the surrounding environment under salt stress conditions. In this study, the highest biofilm formation was produced by UPMRB9, which is positively correlated with EPS production. Previously, Kasim et al. [35] reported that the biofilm formation of *Bacillus spp* was higher with increasing NaCl concentrations and attained its highest peak at 500 mM and gradually decreased at 1000 mM of NaCl. The EPS and biofilm formations by UPMRB9 led to higher absorption of Na from the saline media. The results were supported by Arora et al. [36], who stated that under salinity stress, bacteria can bind Na⁺ ions through the secretion of EPS, which consequently reduces their toxicity in the soil. Therefore, a higher population of EPS-producing bacteria in the root zone will reduce the concentration of Na⁺ available for uptake, thereby alleviating salt stress effect on the plants.

The production of IAA is an important plant growth-promoting trait in PGPR, as it is a signal molecule in the regulation of plant development. In the current study, UPMRB9 was the highest IAA producer whereas UPMRE6 was the highest P and K solubilizer. This has been reported previously by Kang et al. [37], who stated that *B. tequilensis* SSB07 has a strong ability to produce biologically active metabolites, such as gibberellins, indole-3-acetic acid, and abscisic acid. Similarly, Bhattacharyya et al. [38] found a strain of *Bacillus aryabhattai* (AB211) that forms a clear zone on Pikovskaya's agar plate, which indicates P solubilization. Earlier, Chookietwattana et al. [39] identified *Bacillus megaterium* A12 as an efficient halo-tolerant P solubilizing bacteria under saline conditions. In another study, Singh et al. [40] observed significantly higher mobilization of K, biomass accumulation, chlorophyll content, and crude protein in wheat and maize inoculated with K-solubilizing *Bacillus mucilaginosus*. Moreover, the three bacterial isolates in this study, UPMRB9, UPMRE6, and UPMRG1, were able to fix atmospheric N₂ under saline conditions of up to 1.5 M of NaCl. A review by Yan et al. [41] found that salt-tolerant N₂-fixing PGPRs can produce osmolytes to maintain cell turgidity and metabolism to survive against osmotic stress in saline soils.

Chlorophyll concentration is an index of tissue tolerance to salt under saline conditions. Under normal growing conditions, the highest increment of chlorophyll was recorded in BRR1 dhan67 inoculated with UPMRE6, which is a 19% increase over the uninoculated plants. Even though BRR1 dhan67 is short-statured and low in grain yield compared to Putra-1 and MR297, the plant growth-promoting properties of UPMRE6, such as N-fixation and P and K solubilization, have enabled this bacterium to increase the chlorophyll content of BRR1 dhan67, possibly by improving the rooting structure and increasing the availability of essential nutrients. This statement can be supported by Bhattacharyya et al. [38], who showed an increase in total chlorophyll content (136%) of maize over the uninoculated control by the inoculation of *Bacillus aryabhattai*. Sapre et al. [42] reported an increase in chlorophyll content in oat seedlings by the application of PGPR. On the other hand, the highest increment of total chlorophyll content under saline conditions was measured by the inoculation of a MR297 rice plant with UPMRB9 (28%). This increment in the salt-susceptible rice plant MR297 might be due to the salt-tolerant properties of UPMRB9, which played a vital role in lowering the harmful effect of salinity through exopolysaccharides production and biofilm formation. Besides, UPMRB9 can inhibit the upward translocation of Na⁺ for normal physiological function and protects the chloroplast organelles of these high yielding rice varieties. PGPR enhancement of chlorophyll content in plants grown under abiotic stress conditions has been previously described by Habib et al. [43]. Moreover, Kang et al. [44] also reported that a higher photosynthetic rate and starch production in cucumber plants under saline conditions were related to increased chlorophyll content and had an influence on plant growth. Similarly, it was also documented that the application of *Brevibacterium sp.* (FAB3) aided to mitigate abiotic stress conditions via enhanced chlorophyll content, which lead to an improvement of plant yield attributes [45–47].

The status of water in plants is particularly expressed as relative water content (RWC). The reduction of RWC in plants is a common phenomenon under salinity and hence RWC could be a powerful indicator for evaluating salt or dehydration tolerance in plants [48]. A higher concentration of salt decreases the osmotic potential of a growth medium, leading to a reduction of water availability for plants. The loss of cell turgidity, which involves a reduction in leaf RWC, results in inadequate

water accessibility for cell extension processes [49]. In this study, the inoculation of MR297 rice plants with UPMRE6 increased the relative water content under both non-saline and saline conditions with increments of 34% and 62%, respectively. This might be due to the IAA production by the strain as stated by Baldan et al. [50], in which among the phytohormones, auxins and in particular indole-3-acetic acid (IAA) can modify root morphology and enhance root surface to enable the plants to acquire more nutrients and water from the soil. In another work, it was also noted that the decrease of RWC is more rapid in salt-sensitive varieties compared to resistant varieties [51]. This statement can be supported by Nunkaew et al. [52], who noted that EPS-producing salt-tolerant PGPR promotes soil aggregation and enhances soil structure, which resulted in higher water-holding capacity and an improved supply of nutrients to plants. Previous studies reported by several scientists also illustrated a remarkable increase of RWC in wheat and mung bean inoculated with PGPR under saline conditions [42,53,54].

Under the normal growing conditions, the vigorous growth of rice seedlings proves that plants were not significantly affected by the leakage of electrolytes. Under salt stress conditions, plants probably suffered from oxidative stress leading to lipid peroxidation in the cell membrane, which increases the permeability of the cell membrane, and thus the electrolytes stored within the membrane outflows (leak) into the surrounding tissues. In this experiment, the highest reduction of electrolyte leakage was observed by the inoculation of MR297 plants, a salt-susceptible rice variety, with UPMRB9 (92%). High EPS production by UPMRB9 under salt stress conditions helps bind Na^+ ions through rhizosheaths (biofilm) formation around the roots and thereby reduce the availability of Na^+ for plants. It was reported by another researcher that *Arabidopsis thaliana* treated with *Bacillus subtilis* decreases the inflow of Na^+ by down-regulating the expression of the HKT1/ K^+ transporter [54]. Thus, PGPR could help protect the turgidity of plant cell membranes from the harmful effect of NaCl. Similar findings were reported earlier by Ghorai et al. [55], where remarkably low electrolyte leakage was observed by the inoculation of *Pseudomonas aeruginosa* AMAAS57 and *Pseudomonas fluorescens* BM6 in groundnut plants under saline conditions.

UPMRB9 is considered a high salt-tolerant strain as it can produce a high amount of biofilm, which is positively associated with EPS production. Apart from reducing the high salinity effect, this strain possesses multiple plant growth-promoting properties, which increased Putra-1's seedling dry matter by 156%. Although Putra-1 is a moderate salt-tolerant variety, it is a high yielding variety with taller plant height. Thus, the potential yield of the Putra-1 rice plant could be achieved by the inoculation of UPMRB9. Apart from EPS production, the high IAA production of this strain is vital for the initiation of adventitious roots and lateral root enlargement, which subsequently aided to increase the availability of nutrients and absorption of water [5]. Banerjee et al. [56] concluded that the inoculation of rice plants with IAA-producing isolate leads to a remarkable increase of root and shoot length. The increased plant dry matter production is due to bacterial inoculation, which is similar to the findings of Mahmood et al. [57], who inoculated mung bean plants with *Bacillus drentensis* and *Enterobacter cloacae*, resulting in significant improvements to plant biomass. A similar study by Viscardi et al. [4] found that tomato plants injected with selected plant growth-promoting strains of *A. chroococcum* (67B and 76A) had increased growth and biomass accumulation under salinity stress. Further study revealed that the injection of PGPR, including *Bacillus subtilis*, *Bacillus atrophaeus*, *Bacillus sphaericus*, *Staphylococcus kloosii*, *Kocuria erythromyxa*, and *Pseudomonas* increased both the shoot and root weights (fresh and dry) of strawberry and finger millet plants grown under abiotic stress conditions [58,59].

The uptake of Na^+ and K^+ for the different rice varieties was significantly affected under saline soil conditions. The lowest Na^+ translocation in shoots and roots was measured in the BRRI dhan67 rice plant inoculated with UPMRB9. The highest reduction of Na/K ratio was measured for this treatment combination at the rate of 81% and 53%, respectively. Besides, the increment of the Na/K ratio in the soil is a desirable trait, and in this study UPMRB9 was the highest accumulator of Na in the soil across all rice varieties. The incorporation of this strain into the rice plant leads to the binding of Na^+ ion in the rhizosphere region through the production of exopolysaccharides. Moreover,

bacterial biofilm production, which acted as a mechanical barrier for the upward translocation of Na^+ , resulted in a low Na/K ratio in shoots under saline conditions, which is associated with a general increase in the Na/K ratio in roots and a decrease in the soil Na/K ratio. The UPMRB9 isolate also has a significant influence on lowering the upward movement of Na^+ in the moderately tolerant genotype Putra-1 and the susceptible genotype MR297 in comparison to the uninoculated plants. The higher exopolysaccharide production of UPMRB9 might be the cause of the limited salt uptake in plants since the exopolysaccharide matrix can trap cations (Na^+) and produce extensive biofilm by altering root structure and increasing the expression of ion affinity transporters. In comparison to that, salt-susceptible species are unable to express such a mechanism, resulting in higher quantities of Na^+ translocation into the shoot tissue, which consequently leads to plant death [60,61]. The mechanism of salinity tolerance in plants involves the overexpression of AKT1 transporters to maintain a higher amount of K^+ in plant roots. Although some resistant plants uptake more Na^+ , they can maintain the balance of other important ions, such as K^+ . In addition to this, several reports have stated that under salt stress conditions, rhizobacteria can modulate the ionic balance in plants. Previous findings by Rojas-Tapias et al. [62] have shown an enhanced K^+ absorption and Na^+ rejection in maize plants treated with *Azotobacter* strains C5 and C9 under salt stress conditions. In another study, *Puccinellia tenuiflora*, known as a halophyte grass, exposed lower Na^+ deposition by the inoculation of *B. subtilis* GB03, which was confirmed by the upregulation of PtHKT1 and PtSOS1 genes and downregulation of PtHKT2 in roots under high salt concentrations (200 mM NaCl) [63].

The SEM observation showed a successful colonization of UPMRB9 around the roots of BRRI dhan67 under saline conditions. Moreover, an observation by TEM proved the endophytic characteristics of this strain (*Bacillus tequilensis*). This might be due to the hydrolyzing enzyme-producing abilities of UPMRB9, which help them enter intercellular spaces in plants and ensure a more efficient plant–microbe interaction. The endophytic characters of *Bacillus tequilensis* have also been previously supported by Kushwaha et al. [64] and Li et al. [65].

5. Conclusions

In this study, endeavors were made to explore the beneficial effects of locally isolated salt-tolerant PGPR strains on the growth of rice plant under glasshouse conditions. UPMRB9, identified as *Bacillus tequilensis*, consistently showed significant improvements, namely in total chlorophyll content, relative water content (%), total dry matter, reduction of electrolyte leakage (%), and Na/K ratio, when inoculated with three rice varieties with different levels of salt-tolerance and grown under saline conditions. The biggest significant effect of the bacterial inoculation was on the relative water content, electrolyte leakage and Na/K ratio of the BRRI dhan67 rice variety under saline conditions, suggesting a synergistic effect on the mechanisms of plant salt-tolerance. These improvements might be due to the salt-tolerance and plant growth-promoting traits of the selected bacterial strain. Hence, this potential isolate could be used as a source of biofertilizer to improve current rice cultivation systems and mitigate salinity problems in coastal salt-affected areas. This initial finding should be complemented with extensive field trials for future research prior to large-scale application.

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