Endophytic bacterial diversity of *Avicennia marina* helps to confer resistance against salinity stress in *Solanum lycopersicum*

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**ABSTRACT**

The current study aimed to explore the endophytic bacterial diversity of *Avicennia marina* and the potential roles of these endophytes in countering saline conditions in tomato plants. Molecular analysis revealed strains from *Paeonia bacterium, Bacillus, Microbacterium, Citrobacter, Lysinibacillus, Halomonas, Vibraibacterium, Exiguobacterium*, and *Vibrio*. However, *Bacillus pumilus* AM11 and *Exiguobacterium* sp. AM25 showed significantly higher growth in saline media. In response to salinity stress, tomato plants treated with AM11 and AM25 showed significantly higher (∼15–23%) biomass, photosynthetic rate and pigment accumulation compared to controls. Salinity-exposed plants had significantly reduced growth and increased (threefold) lipid peroxidation, whilst glutathione, catalase, and peroxidase activities were significantly reduced. In contrast, AM11, AM25, and methionine improved these physiochemical attributes. The study concludes that the application of bacterial endophytes from plants growing in saline conditions can offer other plants similar stress-resistance potential. Such halophytic bacterial strains can be used to improve plant growth in saline conditions.

**INTRODUCTION**

Plants in arid and semi-arid areas of the globe are exposed to various environmental factors that can negatively affect plant growth and yield (Arbona et al. 2017). Among the various environmental stresses, salinity stress is a major stress condition that markedly suppresses plant growth and yield (Negrão et al. 2017). More than 800 million ha of land are now salt-affected. Approximately 45 million ha of agricultural land have been adversely affected by salinity, and 1.5 million ha have lost fertility, corresponding to 20% of the world’s irrigated lands, which produce an estimated 40% of the world’s food supply (Munns and Tester 2008). Salinity stress induces ionic imbalances and increases Na⁺, Cl⁻, Mg²⁺, K⁺, and Ca²⁺ concentrations in cells. Salinity stress induces a high accumulation of Na⁺ and Cl⁻ in cells via the symplastic pathway (Suzuki et al. 2016). The ionic flux caused by salinity stress results in oxidative stress due to the overproduction of reactive oxygen species (ROS) (Chojak-Közıewksa et al. 2017). Plants can adapt to various unfavorable environmental conditions and respond to stressful conditions by regulating defense signalling pathways, thereby improving their antioxidant systems and metabolism (Khan et al. 2017).

To counteract oxidative stress, plants recruit various enzymatic and non-enzymatic antioxidants, which defend against ionic fluxes to lessen cellular damage (Mittler 2002; Turkan and Demiral 2009). Under stress conditions, ROS such as superoxide anion (O²⁻) and hydrogen peroxide (H₂O₂) are generated. The production of O²⁻ and H₂O₂ are significantly higher in plants during abiotic stress conditions, while in antagonistically activated defense, mechanisms such as superoxide dismutase (SOD) and catalase (CAT) are also synthesized to avoid oxidative damage to the plant (Vranova et al. 2002). SOD converts superoxide anion into water and hydrogen peroxide components, while CAT catalyzes the dismutation of hydrogen peroxide into water and oxygen (Vranova et al. 1997; Mittler 2002). In addition to SOD and CAT, polyphenol oxidases (PPO) and peroxidase (POD) also help in the defense mechanism of plants during abiotic stresses. PPO cause polyphenols to oxidize to quinones, which can be important for the plant’s defense responses, while PODs are oxidoreductive enzymes that play a crucial role in the process of wall-building, such as suberization, oxidation of phenols, and lignification of host plant cells against biotic and abiotic stresses during the defense reaction (Mohammadi and Kazemi 2002).

Various approaches through conventional and transgenic breeding have been used to enhance plant tolerance against various environmental stresses. However, the utilization of plant-growth-promoting microbes is considered an ideal approach for plant growth development and stress mediation (Khan et al. 2017). Numerous previous studies have used plant-growth-promoting rhizobacteria (PGPR) for plant growth improvement and mitigation of salinity stress (Bashan et al. 2014; Egamberdieva and Lugtenberg 2014; Kang et al. 2014; Mayak et al. 2004). However, very few plant-growth-promoting endophytic bacteria (PGPEB) are known to resist salinity stress. PGPEB colonize healthy plant tissues without causing any disease symptoms in the host plants. These bacterial endophytes have important roles in resisting the negative effects of biotic and abiotic...
stress conditions (Khan et al. 2013). Endophytic symbiosis of PGPEB with host plants, particularly in the roots, can regulate mineral uptake, plant hormone balance, and defensive root exudates (Chojak-Koźniiewska et al. 2017). Recent studies have indicated that endophytic microbes isolated from extreme environments can possess significantly higher plant-growth-promoting traits. For example, some endophytes from Prosopis strombulifera were found to be active in countering salinity stress (Sgroy et al. 2009). Therefore, the present study was undertaken to explore the endosymbionts of the mangrove tree Avicennia marina, because these trees comprise one of the most productive ecosystems in the world and are considered the most important halophytes. The endophytic bacterial strains isolated from A. marina found in the current study could be helpful to mitigate salinity stress in crop plants such as tomato.

Materials and methods

Collection of plant samples for the isolation of endophytic bacteria

Leaves from different parts of A. marina growing in the Al-Quram area (Muscat Governorate, Sultanate of Oman) were collected, placed in sterilized zip bags and kept in an ice box prior to experiments. The plant samples were cut into 0.5-cm fragments in order to separate the inner and outer bark. Then, surface sterilization of the samples was carried out in 2.5% sodium hypochlorite for 30 min in a shaking incubator at 120 rpm, followed by successive washing with autoclaved double distilled water to eliminate any ectomycorrhizae or epiphytic microbes (Bayman et al. 1997; Vazquez et al. 2000; Khan et al. 2015). Isolation of bacteria from roots was carried out on nutrient agar medium. The newly emerged bacterial colonies were isolated by reculturing on fresh nutrient agar plates (NA, 50 ppm).

Molecular identification and phylogenetic analysis

The selected bacterial endophytes (Bacillus pumilus AM11 and Exiguobacterium sp. AM25) were identified by PCR amplification and sequencing of their 16S ribosomal RNA (rRNA) genes using the primers 27F (5′-AGAGTTTGTATCAGGCTCAG-3′) and 1492R (5′-CGGCTACCTTGTTACGACTT-3′), as described by Shehzad et al. (2016). The sequences were aligned using MEGA (version 6.01) and were then subjected to BLASTn analysis to correlate them with highly homologous bacterial strains. The 16S rRNA gene sequences of isolates were submitted to NCBI GenBank. The analysis involved 145 nucleotide sequences. All positions containing gaps and missing data were eliminated. The final dataset comprised 234 positions. Evolutionary analyses were conducted using MEGA. Evolutionary history was inferred using the maximum parsimony, neighbor-joining, and UPGMA methods. Substitution patterns and rates were estimated using the Tamura et al. (2004) model and the Kimura (1980) two-parameter model. The estimated transition/transversion bias (R) was 1.03.

IAA production

The quantification of indole-3-acetic acid (IAA) produced by the endophytes in culture broth was carried out through colorimetric analysis. All endophytes were cultured in 20 mL Czapek broth without l-tryptophan and incubated at 30 ± 2°C in a shaking incubator at 200 rpm for 7 days, according to the method of Khan et al. (2015). The bacterial cultures were then centrifuged at 10,000 × g for 10 min at 4°C, and each cell-free culture was filtered through a 0.45-µm cellulose acetate filter (DISMIC®, Denmark). The filtrates were acidified to pH 2.8 with 1 N HCl and extracted three times with 20 mL ethyl acetate. The ethyl acetate fractions were combined and evaporated under vacuum at 45°C in a rotary evaporator. The residue was resuspended in 3 mL 50% methanol: water. One milliliters of the supernatant was mixed with 2 mL Salkowski’s reagent (12 g FeCl3/L in 7.9 M H2SO4) and maintained in the dark for 1 h. The resultant change in color was analyzed at 535 nm in an ELISA spectrophotometer (xMark; Bio-Rad, USA). The amount of IAA was calculated using a standard of pure IAA (Sigma-Aldrich, Korea, Ltd) prepared separately. On the basis of positive results, strains AM11 and AM25 were selected for further analysis.

Endophytic bacterial potential under saline conditions

The potential of the selected endophytes B. pumilus AM11 and Exiguobacterium sp. AM25 in a saline environment was investigated using the method described by Shahzad et al. (2017). In brief, B. pumilus AM11 and Exiguobacterium sp. AM25 were grown in nutrient broth supplemented with varying concentrations of sodium chloride, and the bacterial cell density (OD600) and biomass measurements were assessed. Then, the collection of bacterial cells was carried out by means of centrifugation (10,000 × g at 1°C for 20 min).

Plant growth conditions

In this study, we used tomato (Solanum lycopersicum) as a host plant. For the sterilization of seeds, NaOCl (5%) was used followed by thoroughly rinsing with deionized water. The sterilized seeds were germinated in plastic pots filled with soil growing medium [perlite (7%–11%), zeolite (6%–8%), peat moss (13%–18%) and coco peat (63%–68%)] under controlled conditions at a temperature of 30 ± 2°C (Kang et al. 2014). The growing medium contained the following macronutrients: K2O (100 mg/L), NH4+ (90 mg/L), P2O5 (350 mg/L), and NO3− (205 mg/L).

Bacterial inoculation and plant stress treatment

The experiment comprised eight treatments performed in triplicate: (i) control, (ii) B. pumilus AM11, (iii) Exiguobacterium sp. AM25, (iv) B. pumilus AM11 + salinity, (v) Exiguobacterium sp. AM25 + salinity, (vi) methionine + salinity, (vii) methionine, and (viii) salinity. Briefly, B. pumilus AM11 and Exiguobacterium sp. AM25 (20 mL with cell density of 108 CFU/mL) were applied to 14-day-old tomato seedlings. Because B. pumilus AM11 and Exiguobacterium sp. AM25 showed pronounced growth in response to salinity (NaCl; 250 mM) and methionine (1.0 mM) treatments, 20 mL of both strains at 108 CFU/mL were applied to the tomato plants daily for 7 days. Moreover, an equal volume of nutrient broth was used as control. Upon stress completion, all the plants were immediately harvested in liquid nitrogen and stored at −80°C. During harvesting, the growth
parameters, for example, length and biomass of root and shoot, as well as number of leaves, were documented.

**Analysis of antioxidants in tomato plants**

Antioxidants were estimated according to the method described by Shalata and Tal (1998). Using liquid nitrogen, the leaves of tomato plants were ground into powder using a mortar and pestle. The powder was resuspended in protein extraction buffer (50 mM Tris-HCl buffer (pH 7.0) with 1 mM EDTA, 3 mM MgCl₂, and 1.0% w/v PVP). Then, the debris was removed by centrifugation at 13,000 × g for 15 min at 4°C. The protein quantification was recorded using the method of Bradford (1976). The activities of all enzymes were recorded in U/mg proteins.

100 mM potassium phosphate buffer (pH 6.8), pyrogallol (50 µM), H₂O₂ (50 µM), and enzyme extract. The amount of malondialdehyde (MDA) formed was read on an ELISA meter. The level of lipid peroxides was expressed as µmol of MDA formed per gram fresh weight. The experiments were repeated three times.

 POD activity was assayed according to the method of Aebi (1984). The crude enzyme extract was treated with enzymes were recorded in U/mg proteins.

CAT (E.C1.11.1.6) activity was assayed by the method of Bradford (1976). The activities of all enzymes were recorded in U/mg proteins.

With some modification, the POD and PPO assays were analyzed as reported by Kar and Mishra (1976). The samples were homogenized in potassium phosphate buffer pH 6.8 (0.1 M) and centrifuged at 2°C for 15 min at 5000 × g in a refrigerated centrifuge. The assay mixture for PPO was as described, but for POD activity the assay mixture comprised 100 mM potassium phosphate buffer (pH 6.8), pyrogallol (50 µM), H₂O₂ (50 µM), and enzyme extract. The amount of malondialdehyde (MDA) formed was read on an ELISA reader at 420 nm for both activities. One unit of POD and PPO was defined as an increase of 0.1 absorbance units.

Reduced glutathione contents were assessed according to the protocol described by Ellman (1959). Samples were ground in 2 mL of 10% (v/v) trichloroacetic acid in a chilled mortar and pestle. The homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The resulting supernatant was added to 3 mL of 150 mM NaH₂PO₄ (pH 7.4). Five hundred milliliters of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) (75.3 mg of DTNB dissolved in 30 mL of 100 mM phosphate buffer, pH 6.8) was then added. The mixture was incubated at 30°C for 5 min. Absorbance was determined at 412 nm, and the GSH concentration was calculated with reference to a standard curve. All the experiments were performed three times.

By using the method of Ohkawa et al. (1979), the level of MDA to measure the extent of lipid peroxidation was recorded. For this assay, 1.5 mL of 20% acetic acid (v/v; pH 3.5), 0.2 mL of 8.1% (w/v) sodium dodecyl sulfate and 1.5 mL of 0.81% (w/v) aqueous solution of thiobarbituric acid were added in succession to a reaction tube. To this reaction mixture, 0.2 mL of one tissue homogenate extracted in 10 mM potassium phosphate buffer (pH 7.0) was added. After adding the phosphate buffer mixture, the samples were heated for 60 min in boiling water. The reaction mixture was immediately cooled on ice, and 5 mL of butanol:pyridine (15:1, v/v) solution was added. The upper organic layer was separated into a new tube, and the intensity of the resulting pink color was analyzed at 532 nm using a spectrophotometer. The level of lipid peroxides was expressed as µmol of MDA formed per gram fresh weight. The experiments were repeated three times.

**Statistical analysis**

The experiments were replicated three times (10 plants per replicate). All values are expressed as the mean ± standard deviation (SD). Data were presented and analyzed using GraphPad Prism software (version 7.0, California, USA). Furthermore, the mean values of the results were also subjected to Duncan’s multiple range (DMRT) tests at p < 0.05 (SAS 9.1, Cary, NC, USA).

**Results**

**Endophytic bacterial diversity of A. marina**

Endophytic bacteria were isolated from A. marina leaves. Briefly, a total of 10 trees were randomly selected, and approximately 212 leaf parts were sampled and sterilized for isolation of endophytic bacteria. These tissue segments yielded 113 bacterial endophyte isolates (Table 1). Morphological analysis revealed that 24 of the endophytic bacteria possessed different morphological characteristics, whereas some were found to be morphs of the same species. Simpson’s species richness and diversity indices were 0.329 (D) and 3.042 (1/D), respectively, whereas the Shannon-Wiener H and E indices were 1.3 and 0.808, respectively. These bacterial endophytes were identified by amplifying and sequencing the 16S rRNA region.

**Molecular identification and phylogenetic analysis**

The 16S rRNA sequences from the 28 bacterial isolates displayed fragment sizes ranging from 708 to 980 bp. The average T(U), C, A, and G ratios were 24.5, 26.5, 23.6, and 25.3, respectively. The homologous sequences were aligned with MEGA4 using the ClustalW method. The phylogenetic tree was constructed by MEGA4 using the maximum likelihood method. The sequences of the bacterial isolates were compared with those present in the GenBank database. The topologies of the phylogenetic trees were confirmed by the bootstrap test, and the level of sequence similarity was calculated.

**Table 1. Endophytic bacterial strains isolated from the leaf parts of A. marina.**

| Strain ID | Name                        | Host part | BLASTn homology (%) | Contigs length (Q20) | GC ratio |
|----------|-----------------------------|-----------|---------------------|----------------------|----------|
| AM 1     | Pseudomonas latus           | leaf      | 99                  | 1399                 | 54.44    |
| AM 2     | Bacillus pumilus            | leaf      | 93                  | 1411                 | 54.78    |
| AM 3     | Microbacterium sp.          | leaf      | 98                  | 1328                 | 55.85    |
| AM 4     | Bacillus sp.                | leaf      | 93                  | 1408                 | 55.67    |
| AM 5     | Bacillus licheniformis      | leaf      | 93                  | 1409                 | 54.88    |
| AM 7     | Bacillus endophytic         | leaf      | 98                  | 1404                 | 54.50    |
| AM 8     | Bacillus aquamaris          | leaf      | 98                  | 1408                 | 54.86    |
| AM 10    | Citrobacter sp.             | leaf      | 99                  | 1428                 | 54.97    |
| AM 11    | Bacillus pumilus            | leaf      | 96                  | 1424                 | 54.3     |
| AM 12    | Lysinibacillus sp.          | leaf      | 99                  | 1397                 | 53.62    |
| AM 13    | Halomonas sp.               | leaf      | 93                  | 1422                 | 55.69    |
| AM 14    | Lysinibacillus sphaericus   | leaf      | 98                  | 1406                 | 53.83    |
| AM 15    | Vibrio sp.                  | leaf      | 99                  | 1420                 | 55.30    |
| AM 17    | Lysinibacillus sp.          | leaf      | 98                  | 1400                 | 53.25    |
| AM 18    | Lysinibacillus sp.          | leaf      | 98                  | 1406                 | 55.704   |
| AM 19    | Halomonas sp.               | leaf      | 99                  | 1411                 | 54.74    |
| AM 20    | Bacillus megaterium         | leaf      | 99                  | 1406                 | 53.32    |
| AM 21    | Vibrio sp.                  | leaf      | 93                  | 1411                 | 54.46    |
| AM 23    | Vibrio sp.                  | leaf      | 98                  | 1394                 | 52.51    |
| AM 24    | Halomonas sp.               | leaf      | 99                  | 1393                 | 53.44    |
| AM 25    | Exiguobacterium sp.         | leaf      | 98                  | 1407                 | 56.26    |
| AM 26    | Uncultured bacterium        | leaf      | 93                  | 1354                 | 54.16    |
| AM 27    | Lysinibacillus sp.          | leaf      | 93                  | 1401                 | 54.21    |
| AM 28    | Bacillus sp.                | leaf      | 98                  | 1433                 | 54.124   |

Note: QC20: The number of bases in the sample sequence that give a Q20 score (>99% confidence) for each base call.
respectively. The sequences were aligned using MEGA 7.0 and subjected to BLASTn analysis to correlate the sequences with those of highly homologous bacterial strains. Most of the bacterial sequences showed 98–100% similarity to related bacteria in the NCBI database (Table 1). On the basis of the BLASTn analysis, the bacterial strains were identified as species belonging to the following genera: Paenibacillus sp. (1 strain), Bacillus sp. (8 strains), Microbacterium sp. (1 strain), Citrobacter sp. (1 strain), Lysinibacillus sp. (5 strains), Halomonas sp. (3 strains), Virgibacillus sp. (1 strain), Exiguobacterium sp. (1 strain), Vibrio sp. (2 strains), and uncultured bacterium clone (1 strain) (Table 1). Bacillus sp. and Lysinibacillus sp. were the most predominant phyllospheric bacterial endophytes.

The phylogenetic analysis of endophytic bacterial strains showed a similarity level of 79–100% to the 16S rRNA sequences of related species in the NCBI nucleotide database (Figure 1). The evolutionary history was inferred using the maximum parsimony method. For all sites, parsimony-informative indices such as consistency index (0.47), retention index (0.89), and composite index (0.59) were also calculated (Figure 1). The branch lengths in the phylogenetic tree were 2.1 and 3.1 using neighbor joining, maximum parsimony, and UPGMA methods, respectively, all with 1000 bootstrap replications. The maximum likelihood analysis estimated the transition/transversion bias (R) as 0.72, and a log value of −2312.1 was revealed for the 145 sequences with 159 positions in the final dataset. The nucleotide frequencies were A = 24.60%, T/U = 23.48%, C = 22.03%, and G = 29.89%. The average evolutionary divergence of all sequence pairs was 0.51 ± 0.01. The number of segregating sites was 0.790598, ps/a was 0.142437, nucleotide diversity was 0.148135, and the Tajima test statistic was 0.129611 for all sequences (Tables S1–S7).

Resistance against salinity conferred by isolated endophytic bacterial strains

The potential role of the endophytic bacterial microbiome in countering abiotic stresses has recently been demonstrated by Brader et al. (2014). The bacterial endophytes B. pumilus AM11 and Exiguobacterium sp. AM25 were grown in NaCl-supplemented media to investigate their responses to the presence of Na⁺ and Cl⁻ ions in the medium. The ability of particular bacterial strains to grow in high salinity is essential for plant adaptation in a stressful environment and can elucidate the survival mechanisms used by endophytes in natural environments with high salinity. We accordingly grew B. pumilus AM11 and Exiguobacterium sp. AM25 in 5 M NaCl and assessed their growth dynamics. The growth of B. pumilus AM11 and Exiguobacterium sp. AM25 was found to be remarkably prominent at 12.5 × 10⁸ CFU/mL under normal growth conditions. The supplementation of the medium with NaCl affected bacterial cell growth. At an NaCl concentration of 5 M, AM11 and AM25 growth was 2.34 × 10⁸ CFU/mL and 4.53 × 10⁸ CFU/mL, respectively, which was higher than for all other AM strains. This shows that the viability and cellular arrangements of AM11 and AM25 cells were not threatened under moderate NaCl stress. These endophytes may extend their beneficial impacts to plants in similar environmental conditions (Figure 2).
Effect of salinity and methionine on tomato plant growth under salinity stress

The exposure of crops to salinity stress suppresses both their growth and their biomass. In the present study, plants inoculated with *B. pumilus* AM11 and *Exiguobacterium* sp. AM25 showed significantly higher (*p < .01*) shoot lengths (~30–40%) under normal growth conditions compared to salinity stress conditions. Similarly, methionine-treated plants also revealed significantly increased shoot length (~15%) compared with inoculated salt-treated tomato plants. Overall, plants inoculated with *B. pumilus* strain AM11 and *Exiguobacterium* sp. AM25 showed more pronounced shoot elongation compared with methionine-treated and control plants (Table 2). In almost all the plants treated with methionine, *B. pumilus* AM11, and *Exiguobacterium* sp. AM25, positive effects were detected in the biomass of shoots and lengths of roots under normal growth conditions compared to non-inoculated control plants. Similarly, other growth parameters such as the number of leaves and internodes were also significantly higher (*p < .02*) in methionine, *B. pumilus* AM11, and *Exiguobacterium* sp. AM25 plants than in controls. However, under exposure to NaCl (250 mM), root and shoot length/biomass as well as leaf number of tomato was significantly reduced. Those plants which were treated with NaCl showed distorted and abnormally stunted growth.

Root analysis

Root weight was significantly (*p < .05*) lower (51% and 17%) in NaCl- and methionine-treated plants, respectively, whereas no significant difference was recorded between the control and AM25 + NaCl-treated plants. All other treated plants displayed statistically significant differences (*p < .05*) in root weight compared to those of controls: AM11 = 90%, AM11 + NaCl = 32%, AM25 = 252%, and methionine + NaCl = 40%. The root length was significantly decreased in all salt-stressed plants in comparison to controls. The root weight of plants treated with only NaCl was lower (5%) than that of the control plants. Similarly, plants treated with *B. pumilus* AM11 + NaCl, *Exiguobacterium* sp. AM25 + NaCl, and methionine + NaCl had lower root weights, 28%, 37%, and 14%, respectively, than did the control plants. The salinity stress also reduced the surface area of roots. Plants treated with NaCl, *B. pumilus* AM11 + NaCl, *Exiguobacterium* sp. AM25 + NaCl, and methionine + NaCl had lower root surface areas (7%, 42%, 36%, and 28%, respectively) than did their respective control plants. The average diameter of roots was also lower in all salt-stressed plants treated with salt only and with endophytes and methionine applied, the exceptions being *Exiguobacterium* sp. AM25 with and without salt, which showed no significant differences. Plants treated with NaCl, *B. pumilus* AM11 + NaCl, and methionine + NaCl had 1%, 20%, and 15% smaller average diameters, respectively, than those of control plants. The root volume was also lower in plants treated with salt only and with endophytes and methionine applied. Plants treated with NaCl, *B. pumilus* AM11 + NaCl, *Exiguobacterium* sp. AM25 + NaCl, and methionine + NaCl had 8%, 54%, 36%, and 39% lower root volumes compared with their respective control plants. Shoot weight was remarkably reduced (50%) in NaCl-treated plants, whereas in all other treated plants the shoot weight was higher (35–90%) than that of the control. The shoot lengths of plants treated with NaCl, AM11 + NaCl, and AM25 + NaCl were 50%, 5%, and 10%, lower, respectively, than those of the controls. Shoot length in the other treated plants was higher (5–10%) than that of the control plants.

Changes in chlorophyll pigments and photosynthesis caused by AM11 and AM25 during salinity treatment

Chlorophyll *a* content was significantly (*p < .05*) enhanced (~85–95%) in *B. pumilus* AM11 and *Exiguobacterium* sp. AM25-treated plants, with or without salinity stress. Conversely, the chlorophyll *a* content was detected to be statistically lower (*p < .05*) in response to exogenous methionine application under both salinity stress and normal growth conditions (Figure 3(A)). Chlorophyll *b* content was significantly (*p < .05*) higher (~75–80%) in plants treated with *B. pumilus* AM11 and *Exiguobacterium* sp. AM25, with or without salinity stress. However, the chlorophyll *b* content was significantly lower (*p < .05*) in response to exogenous methionine application under both salinity stress and normal growth conditions (Figure 3(B)). The carotenoid content was significantly (*p < .05*) higher (~65–80%) in plants treated with *B. pumilus* AM11 and *Exiguobacterium* sp. AM25 without salinity stress compared with salinity-stressed plants. However, the carotenoid content was significantly lower (*p < .05*) in response to exogenous methionine application under both salinity stress and normal growth conditions (Figure 3(C)). Photosynthesis was markedly (*p < .05*) boosted (15%, 50%, 80%) in AM11, AM25, and methionine-treated plants, respectively, under conditions of salinity stress. Only in the control plants treated with NaCl was the rate of photosynthesis lower (38%) than that in control plants under normal conditions (Figure 3(D)).
Mitigation of salinity-stress-induced oxidative stress by *B. pumilus* AM11 and *Exiguobacterium* sp. AM25

CAT content was significantly \((p < .05)\) lower (80–90%) in all treated plants in comparison with their controls (Figure 4(A)). The glutathione peroxidase content was significantly \((p < .05)\) higher (45%) in plants treated with *B. pumilus* AM11 without salinity stress compared with the salinity stress plants, but only 1% higher in plants treated with *Exiguobacterium* sp. AM25 with salinity stress compared with plants without salinity stress. However, the glutathione peroxidase content was significantly lower \((p < .05)\) in response to exogenous methionine application under salinity stress compared with normal growth conditions (Figure 4(B)). The POD content was significantly \((p < .05)\) lower (70%) in salt-applied tomato plants in comparison with control plants. However, all the other treated plants showed no significant differences in POD contents between salinity stress and normal growth conditions (Figure 4(C)). Similarly, the PPO content was significantly \((p < .05)\) lower (11%) only in plants treated with *Exiguobacterium* sp. AM25 under salinity stress compared with plants without salinity stress. The difference in PPO content was insignificant between salinity stress and normal growth conditions (Figure 4(D)).

Lipid peroxidation was significantly \((p < .05)\) higher (15%) in plants treated with *B. pumilus* AM11 with salinity stress than in plants without salinity stress and 16% lower in plants treated with *Exiguobacterium* sp. AM25 without salinity stress than in plants with salinity stress. However, the MDA content was significantly \((p < .05)\) higher (90%) in salt-stressed control plants than in control plants under normal conditions, whereas the MDA content was 28% lower in response to exogenous methionine application under salinity stress than under normal growth conditions (Figure 5(A)). The superoxide anion content showed no significant

### Table 2. Growth parameters of plants under various treatment conditions.

| Parameters                  | Control          | NaCl  | AM11         | AM11 + NaCl | AM25  | AM25 + NaCl | Methionine | Methionine + NaCl |
|-----------------------------|------------------|-------|--------------|-------------|-------|-------------|-------------|-------------------|
| Root weight (g)             | 1.3 ± 0.2c       | 0.6 ± 0.12d | 2.6 ± 0.16b  | 1.8 ± 0.9   | 4.8 ± 0.11a | 1.3 ± 0.8c  | 1.1 ± 0.3c    | 1.9 ± 0.4c        |
| Root length (cm)            | 27.7 ± 0.23b     | 26.1 ± 0.23b | 34.4 ± 0.21a | 24.7 ± 0.18c | 27.6 ± 0.22b | 17.3 ± 0.17d | 33.3 ± 0.23a | 28.5 ± 0.26b      |
| Root surface area (cm²)     | 337.3 ± 0.39c    | 312.7 ± 0.32d | 310.1 ± 0.34d | 177.5 ± 0.27f | 372.5 ± 0.37b | 235.5 ± 0.3e | 391.7 ± 0.37a | 281.4 ± 0.35e      |
| Root average diameter (mm)  | 38.7 ± 0.24b     | 38.07 ± 0.25b | 28.6 ± 0.22c  | 22.7 ± 0.19c | 42.9 ± 0.21a | 43.2 ± 0.0a  | 37.3 ± 0.2b   | 31.4 ± 0.24bc     |
| Shoot weight (g)            | 5.2 ± 0.17c      | 2.5 ± 0.16d  | 9.3 ± 0.18a   | 7.7 ± 0.11b  | 10.7 ± 0.16a | 6.9 ± 0.9b   | 6.6 ± 0.7b    | 5.1 ± 0.16c        |
| Shoot length (cm)           | 20 ± 0.19c       | 15 ± 0.14d   | 24 ± 0.2a     | 19 ± 0.18c   | 23 ± 0.25ab  | 18 ± 0.19    | 22 ± 0.21b    | 21 ± 0.21c         |

Note: The different letter(s) in each row indicates that the values are significantly different from each other as evaluated by DMRT \((p < .05)\).

**Figure 3.** Changes in chlorophyll pigments and photosynthesis in plants treated with AM11 and AM25 during salinity stress. Each data point is the mean of at least three replicates. Error bars represent standard errors. The bars marked with an asterisk indicate that the values are significantly different from the control/NFC as evaluated by two-way ANOVA analysis with multiple mean comparison using Sidak’s test. *\(p < .05\) and ***\(p < .001\).
difference in plants treated with *B. pumilus* AM11 and *Exiguobacterium* sp. AM25 with or without salinity stress. However, it was significantly (*p* < .05) lower (∼20–35%) in plants treated with methionine and salt with salinity stress compared with plants without salinity stress (Figure 5(B)). The reduced glutathione content was significantly (*p* < .05) lower (40%) in plants treated with *Exiguobacterium* sp. AM25 with salinity stress than in those without salinity stress, whereas the contents of reduced glutathione in plants treated with *B. pumilus* AM11 were only 8% higher without salinity stress than with salinity stress (Figure 5(C)).

**Discussion**

Mangroves comprise a unique microbiome containing a variety of endophytes and are known to ameliorate various detrimental effects of numerous stresses. However, as a consequence of urban development and the rapid increase in world population, mangrove ecosystems have been subjected to substantial threats from contaminant input. Nearly 200 endophytic fungal species have been reported from the mangrove biome; however, the endophytic bacterial community of this biome has been less well explored. Li et al. (2016) isolated four endophytic fungi from the leaves and twigs of mangrove. Therefore, the present study was conducted to explore the bacterial endophytes of mangrove trees. We accordingly isolated and identified 28 bacterial endophytes, among which AM11 and AM25 were selected based on their tolerance to salinity stress.

*B. pumilus* AM11 is a gram-positive, rod-shaped, endospore-forming bacterium. It is closely related to some important industrially exploited bacteria such as *B. subtilis* and *B. licheniformis*. *B. pumilus* AM11 also has the potential to act as an alternative host for the production of certain industrial enzymes. *B. pumilus* is of importance not only with respect to salt stress but also for its general resistance to stresses (e.g. heat, UV radiation, and oxidative stress during fermentation processes) (Stadtman and Levine, 2003; Schweder and Hecker 2004; Gioia et al. 2007). *Exiguobacterium* is a widely distributed genus, found in plant rhizospheres and extreme and harsh environments (Rodrigues and Tiedje 2007; Vishnietskaya et al. 2011; White et al. 2011; Jiang et al. 2013). To date, 14 species of this genus have been characterized and reported for potential application in the agricultural industries (Hoper et al. 2006; White et al. 2011; Jiang et al. 2013).

Abiotic stresses, particularly salinity stress, affect plant growth and agricultural production worldwide, and an excess amount of salt in the soil has severe effects on plant growth, development, and crop productivity. High amounts of salt in soil cause perturbations at the cellular and whole-plant levels, which cause growth retardation as well as reductions in crop production. Na⁺ and Cl⁻ stress is also attributable to high salinity in plants. As a consequence of high salinity, numerous physiological and biochemical changes occur in plants, which hinder plant growth, development, and protein synthesis, disturb nucleic acid metabolism, and decrease photosynthesis and respiration (Zhang and Blumwald 2001; Sairam et al. 2002; Yang et al. 2014).
Bacterial cells possess an energy-dependent ionic transpor- tation system to control the influx of ions, for example, during salinity stress, where a high rate of antioxidant enzymatic activities can contribute to mitigating the negative effects (Halverson et al. 2000; Ali et al. 2014). In addition to ions, H₂O₂ is converted into water with the help of oxidative-stress enzymes such as CAT (Jincy et al. 2017). The results of the present study also support the regulation of excessive salt ions via the activation of glutathione, POX, and CAT, whereas methionine did not induce suppressive responses to bacteria during growth. This could promote the endophytes to mitigate oxidative damage by producing either extra- or intracellular enzymes (White and Torres 2010).

Previously, the negative and harmful effects of salt stress on various plants have been stated by Wang et al. (2003), Hasegawa et al. (2000), Munns and Tester (2008), Khan et al. (2011, 2012, 2013), and Kang et al. (2014) in various crop plants such as cucumber, pepper, soybean, and rice. These studies suggested that high NaCl concentrations negatively affected plant growth and development, and a similar observation was found in tomatoes subjected to salinity stress in this study. However, plant growth attributes were significantly \( (p < .05) \) improved by treating the tomato plants with *B. pumilus* AM11 and *Exiguobacterium* sp. AM25 and methionine when compared with NaCl treatments alone. Inoculation of *B. pumilus* AM11 and *Exiguobacterium* sp. AM25 has significantly increased the measured plant growth attributes (lengths of shoot and root, biomass of shoot and root, root diameter, root area, and root volume) when compared to salinity treatment alone. The increase in plant growth was significantly higher in methionine treatments, which have been recently shown to act as osmoprotectants for crop plants during stress (Gong et al. 2014; Ludmerszki et al. 2014).

In a comparative assessment of methionine with *B. pumilus* AM11 and *Exiguobacterium* sp. AM25, only methionine application resulted in significantly higher shoot lengths, whereas other attributes such as root length and weight were either significantly or somewhat higher in plants inoculated with *B. pumilus* AM11 or *Exiguobacterium* sp. AM25 under salinity stress. This shows that endophytic bacterial strains play a regulatory role in salinity stress. Plants with a combined application of methionine, *B. pumilus* AM11, or *Exiguobacterium* sp. AM25 with saline conditions showed higher plant growth and resistance against salt stress. Tomato is a sensitive plant to salinity stress. Previous studies have shown that tomatoes are sensitive to NaCl at concentrations as low as 50–200 mM (Zhang and Blumwald 2001; Santa-Cruz et al. 2002; Mayak et al. 2004; Kang et al. 2014; Khan MIR et al. 2014). However, we present herein that combined and individual application of endophytic bacteria and methionine can enhance the salinity tolerance of tomato crops. Furthermore, the exogenous application of methionine to crop plants not only improves plant growth but also contributes to plant fitness and enhances tolerance of salinity stress, as reported by Antoniou et al. (2017) and Zhang et al. (2014).

Endophytic microbes inhabiting plant tissues can adjust the ionic imbalance (Khan AL et al. 2014; Mercado-Blanco and Lugtenberg 2014) that enters via passive transport through the roots (Sattelmacher 2001). Because the endophytes grew well in saline media with little damage to their cellular oxidative systems, the endophyte-inoculated tomato plants showed increased growth and biomass compared to controls when subjected to salinity stress. Similar plant-growth-promoting and stress-mediated responses of bacterial endophytes have also been noted by Ali et al. (2014), Mercado-Blanco and Lugtenberg (2014), and Egamberdieva and Lugtenberg (2014).

Methionine (Met) treatments improve plant growth and stress tolerance by different methods. One of the recent
methods reported (Khan AL et al. 2014) was via suppression of ethylene formation by inhibiting 1-aminocyclopropane carboxylic acid synthase (ACS) activity. A report from (Gong et al. 2014) also showed that the overexpression of methionine increased alkali tolerance in tomato plants. Among osmoprotectants, methionine has been little understood; however, a limited number of studies show that it contributes to resistance against the negative effects of drought stress (Noreen et al. 2015). A similar conclusion is drawn from the current study, in which tomato plants were treated with methionine during salinity stress. To reduce the negative effects of salinity-induced stress, plant cells recruit methionine to alleviate the oxidative imbalance. The main role of methionine is to defend cells from the toxic burst of H$_2$O$_2$ and its prerequisites such OH- radicals (Ruiz and Blumwald 2002; Supian et al. 2017). The results of the current study showed that, under normal growth conditions, endophyte and methionine treatments increased the production of glutathione compared to control plants. Under conditions of salinity stress, glutathione levels were significantly higher in endophyte- and methionine-treated plants compared to those treated only with NaCl. This is in agreement with our latest finding in Khan et al. (2015) that the level of glutathione increased during cadmium stress. Moreover, combined treatments of endophytes and methionine significantly lower the glutathione levels in tomato plants under saline conditions. This suggests the alleviation of NaCl-induced toxicity by endophytes. In the case of individual applications, both endophyte- and methionine-treated plants must activate the synthesis of glutathione to counter the toxic effects of salinity. However, these levels were altogether reduced in tomato plants treated with NaCl only. A similar finding from Gill et al. (2013) showed that glutathione can help plants in abiotic stress.

To normalize the sodium-chloride-caused oxidant burst, antioxidants such as CAT, POD, and PPO are significantly activated at the onset of stress. Our results showed CAT activity in the endophytes + methionine and control treatments, but CAT activity was significantly lower in endophytes + methionine + NaCl, methionine, and endophytes. POD activity was significantly higher in plants inoculated with *B. pumilus* AM11 and *Exiguobacterium* sp. AM25, whereas in other treatments its activity was significantly lower. With the exception of the *B. pumilus* AM11 and *Exiguobacterium* sp. AM25 treatments, the controls and other treatments had a similar response (non-significant). In the case of PPO, the enzyme activity was significantly higher in *B. pumilus* AM11 and *Exiguobacterium* sp. AM25-treated plants as well as in *B. pumilus* AM11 + NaCl and *Exiguobacterium* sp. AM25 + NaCl-treated plants. A similar activity of antioxidant enzymes has also been shown by Cavalcanti et al. (2004) and Khan et al. (2012, 2015) under stressful conditions.

Salinity stress causes oxidative stress, which induces development and thus disturbs the role and function of cellular organs by peroxidization of the lipid bilayer (Munns and Tester 2008). However, ROS removal from cells occurs either directly (by CAT and POD) or indirectly (by redox antioxidants such as glutathione). In the current study, the tomato plants did not show any sign of lipid peroxidation when grown under normal conditions. The level of MDA production was not significantly different in plants treated with *B. pumilus* AM11, *Exiguobacterium* sp. AM25 or methionine versus in control plants. In contrast, the application of saline conditions resulted in a higher level of lipid peroxidation in plants treated only with saline compared to other treatments. The individual applications of *B. pumilus* AM11, *Exiguobacterium* sp. AM25, and methionine counteracted the sodium chloride toxicity by reducing the level of lipid peroxidation. The treatments of *B. pumilus* AM11 and *Exiguobacterium* sp. AM25 + methionine resulted in significantly reduced levels of lipid peroxidation. Because membrane-bound lipid hydroperoxides are hard to record owing to their lability, we noted the extent of lipid peroxidation by quantifying the secondary breakdown products such as MDA. Increased amounts of ROS autocatalyze the peroxidation of lipid membranes and affect membrane semi-permeability under high stress (Khan et al. 2013). Antioxidant activation can improve membrane stability and help to scavenge ROS before they induce injury, whereas the MDA content could be assessed for the evaluation of stress injury (Rivero et al. 2014). Thus, our results indicate less membrane injury in plants treated with *B. pumilus* AM11, *Exiguobacterium* sp. AM25, and methionine and with *B. pumilus* AM11 and *Exiguobacterium* sp. AM25 + methionine plants than in plants treated only with NaCl. Endophytic bacteria have recently been shown to mitigate stress by lowering the lipid oxidation level (Kang et al. 2014), which is consistent with the findings of the present study. The results of this work also indicate that the use of bacterial endophytes may help counter the adverse effects of salinity stress. The isolated endophytes showed the ability to grow effectively under saline conditions without compromising their potent cellular machinery for antioxidant production, as was seen from the activities of POD, PPO, and CAT. The results also suggest that *B. pumilus* AM11 and *Exiguobacterium* sp. AM25 may also stabilize ROS-based adverse effects by regulating antioxidants and related enzymes. Inoculation of bacterial endophytic strains may improve plant-growth-promoting effects, similar to the well-known effects of PGPR and osmoprotectant application to crop plants.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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