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Soil bacteria conferred a positive relationship and improved salt stress tolerance in transgenic pea (*Pisum sativum* L.) harboring Na^+/H^+ antiporter

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Abstract: Among grain legumes, peas (*Pisum sativum* L.) are highly sensitive to salt stress. Acclimatization of plants to such conditions is mandatory. We provide improved salt stress tolerance response of transgenic pea plants overexpressing the Na^+/H^+ gene from *Arabidopsis thaliana* and a positive association with salt-tolerant plant growth-promoting rhizobacteria (PGPR). In addition to salt stress tolerance and phosphate solubilization, the selected rhizobacterial isolates were identified for indole acetic acid and proline production ability. Seed germination percentage in transgenic pea plants was significantly higher under NaCl challenge. The wild-type (WT) pea plants inoculated with known numbers of viable cells of salt-tolerant PGPR and transgenic pea plants without any inoculation showed better growth performance under salt stress. However, the PGPR-inoculated transgenic plants showed significant increase in growth and biomass compared to the WT counterpart. An increase in antioxidant enzymes, i.e. superoxide dismutase and peroxidases, was observed in PGPR-inoculated transgenic plants under salt stress. We could not see any negative effect of the transgene in pea plants on the growth of associated PGPR. The overall impact of microbe-mediated elicitation responses in transgenic plants, whether at the biochemical or molecular level, may lead to protection against salt stress.

Key words: Salt stress tolerance, transgenic plants, Na^+/H^+ antiporter, plant growth-promoting rhizobacteria, *Pisum sativum* L.

1. Introduction

Among abiotic stresses, salt stress is a widespread problem in crop production. There are several reasons for salt stress, which affects plant growth from the early development of flower meristems until the development of seeds in the pods and by interrupting different physiological mechanisms (Grieve et al., 2007; Shahid et al., 2011). Although the plant life cycle suffers severely due to salt stress, which process is the most affected still needs to be explored (Vadez et al., 2012). Among grain legumes, pea (*Pisum sativum* L.) is an important vegetable crop worldwide (Richter et al., 2006). It represents about 40% of the total trade in pulses (Oram and Agcaoili, 1994). Pea crops are ranked among salt-sensitive crops, like other leguminous crops, and they produce low yields even at mild salt stress (Francois and Mass, 1994). Detailed information on genetic variability for salt tolerance is still lacking in the literature (Noreen et al., 2007). Conventional plant breeding combined with improved agriculture practices and biotechnology has contributed to dramatic crop improvement against different stresses over the past 50 years and this continues to provide further benefits (Vadez et al., 2012). However, there is intense pressure to produce further improvements in crop quality and quantity. In this regard, rapid progress has been made in genetic engineering techniques to transfer and achieve stable integration and expression of useful genes against biotic and abiotic stresses (Somers et al., 2003; Ali et al., 2010; El-Banna et al., 2010). The genes have been introduced into plants, cell cultures, and protoplasts by different transformation methods (Ali et al., 2010; Chen et al., 2012). These successful applications are due to the development of techniques that allow plant regeneration from cells or tissue. These crop improvement techniques (traditional plant breeding and genetic engineering) are still being explored to develop salt-tolerant lines of vital commercial crops (wheat, maize, rice, etc.), although partial success has been attained (Ashraf and Foolad, 2007). On the other hand, in some crops high tolerance to abiotic stresses has been attained through exogenous application of various chemicals (inorganic and organic). The exogenous application of these compounds has been proposed as an effective and cost-affordable

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methodology to increase crop yield under stress situations (Ashraf and Foolad, 2007). In the last few decades, various kinds of inorganic and organic chemicals have been used to overcome the antagonistic effects of salt stress on a number of potential crops. However, the application of microorganisms, and especially bacteria, to improve stress tolerance in agriculture (de Zelicourt et al., 2013) is a new and emerging application of microorganisms. Living organisms have the ability to adapt to their environment, and microorganisms also develop different interactions to adapt to plant environments that may be beneficial or pathogenic. The ability of plant growth-promoting rhizobacteria (PGPR) to enhance crop water relations through increasing specific metabolite accumulations, although sustaining leaf turgor of salinized plants, did not prevent the long-term growth inhibition of salinized plants (Munns and Tester, 2008). Under drought stress, mycorrhizal (Auge, 2001) or PGPR (Creus et al., 2004) inoculation often shows improved osmotic adjustment. Moreover, soil microbes also enhance water uptake through roots under both salinity and drought stresses (Dodd and Perez-Alfocea, 2012). Nevertheless, plants provide an outstanding ecosystem for bacteria. The microbes interact with plant tissues and cells to different degrees (Montesinos et al., 2002), showing a positive impact on plant fitness and physiology in diverse ways by direct and indirect mechanisms (Kim et al., 2011). The interaction of PGPR with transgenic plants against abiotic stresses (salt stress in this study) has not yet been fully explored. The present study is designed is to confer salt stress tolerance in *Pisum sativum* through genetic engineering as well as examine the interaction of the transgenic pea plants with halotolerant soil PGPR under salt-stressed conditions.

2. Materials and methods

2.1. Transgenic pea development

Agrobacterium-mediated pea transformation of the cultivar was done according to the protocol described earlier (Richter et al., 2006; Ali et al., 2010). *Agrobacterium* strain LBA4044 harboring vector construct pGII0229MASnhx1luc (Ali et al., 2010) was used for transformation of immature pea embryos. Transgenic plants were grown in a glasshouse at 24 ± 2 °C with a 16/8-h light/dark photoperiod in 2-L pots. Fertilizer was provided along with irrigation water as required.

2.2. Germination and seedling emergence studies under salt stress

For germination and seedling emergence studies under salt stress, 36 mature and healthy seeds of one T1 transgenic line (12-07) and 108 seeds of wild-type (WT) pea were selected. The experiment was conducted in pots containing vermiculite in three replicates. One week prior to seed setting, pots were irrigated with water containing NaCl concentrations of 0, 50, and 100 mM independently. Seeds were dipped in sterile tap water overnight and the next day six seeds in each pot for the WT in three replicates and four seeds in each pot for the transgenic lines were set for germination. Germination count started 7 days after seed setting following incubation for up to 14 days under greenhouse conditions. The average germination rate was calculated using three independent experiments.

2.3. Bacterial isolations from saline soil and morphological, biochemical, and molecular characterization

Soil samples were collected from the rhizosphere of different grasses present in the salt range of the Peshawar district of Pakistan. The samples were taken from 15.24 cm in depth from the rhizosphere of halophytes and transferred to the laboratory in sterile plastic bags. Following immediate processing, samples were stored at 4 °C in a cold room. Fresh bacterial isolations (seven in number) after 24 h of incubation on agar plates as described by Miller (1972) were used for identification on the basis of colony and cell morphology (color, shape, and Gram staining) (Vincent, 1970). The salt stress tolerance capability of the bacterial isolates was observed by growing them on Luria Bertani (LB) media supplemented with different NaCl concentrations (0 mM, 100 mM, 200 mM, 400 mM, 600 mM, 800 mM, and 1 M) for 24 h at 36 ± 2 °C and 180 rpm.

2.4. Bacterial proline content determination

Bacterial proline contents were determined by growing cultures in liquid M9 medium supplemented with 0.714 M (4%) NaCl on a shaker 180 rpm at 30 °C for 48 h. The cultures were harvested by centrifugation in 2-mL Eppendorf tubes at 10,000 rpm at 4 °C for 2 min. For extraction and determination of proline contents, the method of Bates et al. (1973) was followed with some modifications. A bacterial pellet of 100 mg was weighed in a 2-mL Eppendorf tube, and then 1200 µL of 3% aqueous sulphosalicylic acid was added and centrifuged at 13,000 rpm and 4 °C for 10 min. After centrifugation, 500 µL of supernatant was taken into a test tube and 500 µL of autoclaved distilled H_2O was added. Then 1 mL of glacial acetic acid and 1 mL of ninhydrin (2% in acetone) were added. The prepared samples were incubated in a water bath at 90 °C for 1 h. The samples were cooled on an ice bath and 2 mL of toluene was added and vortexed briefly for 2 min. The supernatant was used for absorbance at 520 nm on a spectrophotometer. Proline concentrations were estimated using the standard curve of proline prepared from serial dilutions of proline stock solutions and expressed as µg/g fresh weight of bacterial cells.

2.5. Bacterial catalase and oxidase test

For determination of oxidase enzyme in bacterial isolates, an oxidase test was performed according to Steel (1961).

2.6. Bacterial indole acetic acid production

The indole acetic acid (IAA) production by the rhizobacteria was tested using LB broth and the Salkowski colorimetric method according to Gutierrez et al. (2009) with some modification. PGPR isolates were grown in LB broth supplemented with L-tryptophan (500 µg mL^{-1}) at 36 ± 2 °C at 160 rpm for 5 days, and the supernatant was harvested by centrifugation on the 6th day. Then 2 mL of supernatant was mixed in 2 drops of orthophosphoric acid and added to 4 mL of Salkowski reagent (12 g L^{-1} $FeCl_3$ in 429 mL L^{-1} H_2SO_4) (Glickman and Dessaux, 1995). The samples were incubated for 30 min at room temperature and absorbance was measured at 530 nm on a spectrophotometer. Auxin concentrations were estimated using the standard curve of IAA, prepared from serial dilutions of IAA (Sigma-Aldrich, St. Louis, MO, USA) stock solutions.

2.7. Bacterial phosphorous solubilization index

Pikovskaya's medium (Pikovskaya, 1948), specific for determining phosphate solubilization ability of the rhizobacteria, was poured into sterilized petri plates under sterilized conditions; a pinpoint inoculation of the selected rhizobacteria was made on solidified agar plates under aseptic conditions. The plates were incubated at 28 °C for 7 days. The solubilization index was calculated using the following formula (Edi-Premono et al., 1996):

$$SI = \frac{\text{colony diameter (cm) + halo zone diameter (cm)}}{\text{colony diameter (cm)}}$$

2.8. Molecular characterization and phylogenetic analyses

For the confirmation of the integrated gene (*AtNHX*1) in transgenic plants, specific primers At-nhx (f) 138 5`-ATAGATGGATGAACGAAT-3´ and At-nhx (r) 437 5`-AGTCAAAGGTTCCAATGT-3´, forward and reverse respectively, were used for amplification with a PCR profile of initial denaturation at 94 °C for 5 min followed by denaturation at 94 °C for 40 s, annealing at 57 °C for 30 s, and extension at 72 °C for 2 min for 35 cycles with a final extension at 72 °C for 10 min (Ali et al., 2010)

From the seven bacterial isolates, two (PS2 and PS5) were selected for molecular characterization based on IAA production potential, phosphate solubilization efficiency, and NaCl stress tolerance ability. These two strains were further subjected to interaction study with transgenic pea plants under salt stress. For 16S rDNA-based molecular characterization of bacterial isolates, total DNA was isolated according to the standard protocol described by Sambrook et al. (1989). Partial *16S rRNA* gene was amplified by using universal forward primer 27F 5`-AGAGTTTGATCCTGGCTCAG-3` (Lane, 1991) and reverse primer 1492R 5`-GGTTACCTTGTTACGACTT-3` (Turner et al., 1999). The PCR products (~1400 bp) were extracted with the Gene Jet PCR Purification Kit (K0701) and custom sequenced (Macrogen, Korea).

The partial 16S rDNA sequences of bacterial strains PS2 and PS5 were submitted to GenBank and used for further analysis (accession numbers KF986215 and KF986216, respectively). The 16S rDNA sequences of *Paenibacillus* sp. obtained through a BLAST search were selected for phylogenetic analyses on the basis of maximum identity (up to 90%) and minimum expected value (e-value) (Jenkins et al., 2002). The sequences were aligned using Clustal W in MEGA6 software and the neighbor-joining method was used for the construction of a phylogenetic tree with a bootstrap value of 1000 (Tamura et al., 2011).

2.9. Bacterial interaction with transgenic and WT pea under 100 mM NaCl stress

Following seedling emergence in tissue culture jars on filter paper M-shaped bridges with 35 mL of half-strength MS growth medium (Murashige and Skoog, 1962) with major and minor components, the transgenic and WT pea seedlings were inoculated with 2 mL of a known number of viable cells of each strain (PS2 and PS5) grown in LB media at 30 °C and 180 rpm to 0.8 OD at 600 nm. The bacterial cells were collected at 3000 rpm for 10 min and resuspended in half-strength MS medium for adjustment to 0.8 OD at 600 nm. For the control, plants were inoculated with LB media without any PGPR. Three days after inoculation, 100 mM NaCl stress was applied. All the treatments were replicated three times and each experiment was repeated three times. The plants were incubated in a growth room at 25 ± 2 °C with a 16/8-h light/dark photoperiod. The growth medium in tissue culture jars was replaced with fresh medium containing 100 mM NaCl after every 7 days. The plants were harvested 24 days after seedling emergence.

The following treatments were used for both transgenic and nontransgenic pea plants: 1) control (no stress), 2) NaCl stress (100 mM), 3) bacterial strain PS2, 4) bacterial strain PS2 + NaCl stress (100 mM), 5) bacterial strain PS5, 6) bacterial strain PS5 + NaCl stress (100 mM), 7) bacterial strain PS2 + bacterial strain PS5, 8) bacterial strain PS2 + bacterial strain PS5 + NaCl stress (100 mM).

2.10. Morphological and biochemical analysis of transgenic and WT plants under salt stress

Plant shoot and root length were measured at the time of harvesting. Fresh weights of shoots and roots were recorded. These samples were kept in an oven at 70 °C for 72 h and dry weight of shoot samples was recorded. The % change in water content was calculated as % change in water content = [(FW – DW) / FW] × 100 (Zhang and Blumwald, 2001), where FW = fresh weight and DW = dry weight.

2.11. Antioxidant enzyme extraction

For transgenic and WT pea plants, peroxidase (POD) activity was measured following the assay mixture containing 0.1 mL of enzyme extract, 1.35 mL of 100 mM MES buffer (pH 5.5), 0.05% H_2O_2, and 0.1% phenylenediamine. Change in optical density was recorded at 485 nm for 3 min with a spectrophotometer. The activity of POD was presented as ΔOD_{485nm} min^{-1} mg $protein^{-1}$ (Herzog and Fahimi, 1973).

Superoxide dismutase (SOD) activity was determined by calculating the inhibition of photochemical reduction of nitro blue tetrazolium according to Beauchamp and Fridovich (1971).

2.12. Statistical analysis

The data were analyzed with Statistix version 8.1 in Windows 7 using two-way analysis of variance (ANOVA) followed by Duncan's multiple range test to determine the significance of values in factorial design for pot experiments. The data in the graphs are presented with each sample corresponding to three independent replicates, and each replicate was the pool of the three plantlets of a jar. For germination studies, box-and-whisker plots were generated by SigmaPlot 9.0 software; the boxes indicate the range between the 25% and 75% percentiles as well as the median. The whiskers mark the 5% and 95% percentiles. Statistical data analysis was made with SigmaStat 3.1 in which all pairwise multiple comparison procedures were done according to the Holm–Sidak method.

3. Results

*AtNHX*1-expressing transgenic pea seeds exhibited a higher germination rate than the WT under NaCl stress as shown in Figure 1. The tolerance level for 100 mM NaCl was found for transgenic pea seedlings and they exhibited significant root growth as compared to WT plants.

3.1. Bacterial isolation from saline rhizosphere

In this study, seven bacterial isolates (halophytes) were obtained from saline rhizospheric soil. The purified isolates were named accordingly (Supplementary Table 1; on the journal's website). The isolates were characterized for their potential to promote plant growth.

3.2. Morphological characterization of the isolates

Six isolates (PS1 to PS6) were found to be gram-positive and one isolate (PS7) was gram-negative. The colony-forming units (CFUs) of the isolates were ranked as PS4 > PS1 > PS6 > PS7 > PS5 > PS2 > PS3. The morphology of the bacterial cultures was screened for form, elevation, margin, appearance, pigmentation, size, odor, and Gram test (Supplementary Table 1).

3.3. Salt stress tolerance of bacterial isolates

The salt stress tolerance ability of bacterial isolates was checked on different NaCl concentrations (from 0 mM to 1 M NaCl) supplemented in LB growth medium. The bacteria exhibited different salt tolerance capabilities. Isolates PS1 and PS2 showed better growth on 200 mM NaCl, while isolate PS6 showed better growth on 600 mM NaCl. The isolates were ranked for their salt tolerance on the basis of their average growth (OD) on all concentrations as PS3 > PS2 > PS4 > PS5 > PS6 > PS7 > PS1 (Supplementary Figure 1; on the journal's website).

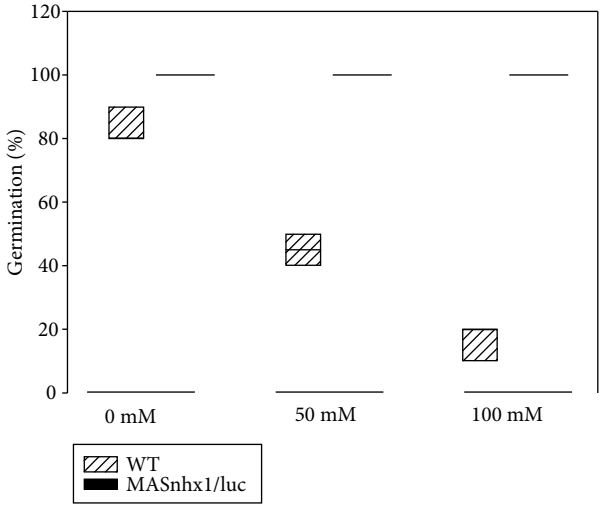

Figure 1. *AtNHX*1 transgenic seeds showed significantly higher germination rates than the wild type under 50 mM and 100 mM NaCl stress. Thirty-six mature and healthy seeds of transgenic pea line 12-07 and 108 seeds of the wild-type (WT) pea were compared. Germination count started 7 days after seed setting, following incubation for up to 14 days under greenhouse conditions. The average germination rate was calculated using three independent experiments.

3.4. IAA production by bacterial isolates

The bacterial isolates produced IAA differently on media containing L-tryptophan, which is an IAA precursor. Isolates PS4 and PS7 produced maximum IAA concentrations of 109.4 µg/mL and 61.19 µg/mL, respectively. The selected seven isolates were ranked for their IAA production ability as PS4 > PS7 > PS1 > PS3 > PS6 > PS2 > PS5 (Figure 2).

3.5. Proline production by bacterial isolates

For proline production, strains PS1 and PS2 showed maximum proline production under both NaCl stress and control conditions; however, the other isolates, PS3, PS4, PS5, PS6, and PS7, showed less proline production ability. For proline production the isolates were ranked as PS1 > PS2 > PS5 > PS4 > PS3 > PS6 > PS7 (Figure 3).

3.6. Phosphate (P) solubilization by bacterial isolates

For P-solubilization on Pikovskaya's agar, isolate PS2 showed the maximum solubilization index of 2.1. Isolates

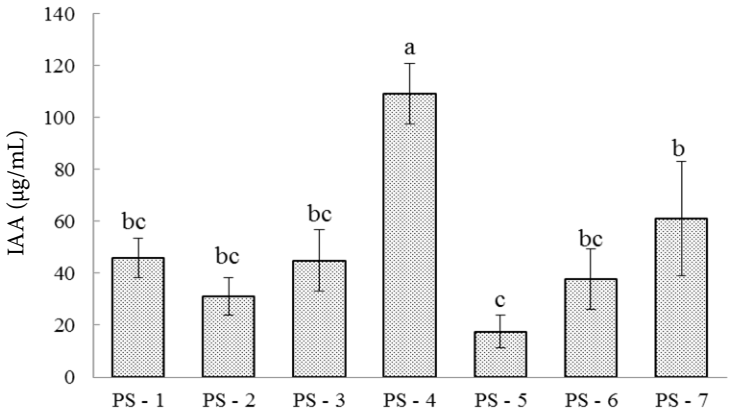

Figure 2. Indole-3-acetic acid (IAA) production ability of the isolates on Luria Bertani broth with Salkowski colorimetric assay. L-tryptophan was used as an IAA precursor. Absorbance was measured at 530 nm. Auxin concentrations were estimated using the standard curve of IAA, prepared from serial dilutions of IAA (Sigma-Aldrich, St. Louis, MO, USA) stock solutions. Statistical analysis was performed using one-way ANOVA. The data in the graph are presented as means of three replicates (n = 3), and bars show standard errors (mean ± SE). All means sharing a common letter are similar; otherwise, means differ significantly at $P < 0.05$.

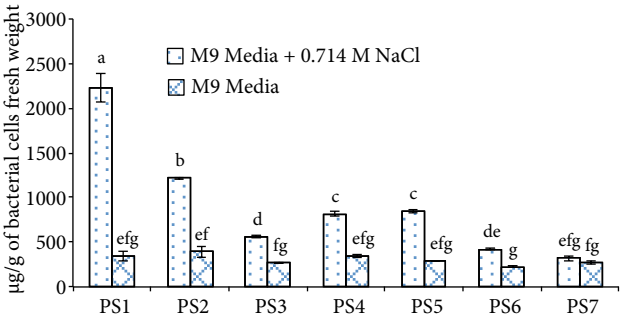

Figure 3. Proline production ability of the bacterial isolates under NaCl stress (0.714 M) and control on M9 media. Proline concentrations were estimated using the standard curve of proline, prepared from serial dilutions of proline stock solutions. The data in the graph are presented as means of three replicates (n = 3), and bars show standard errors (mean ± SE). All means sharing a common letter are similar; otherwise, means differ significantly at $P < 0.05$.

PS6 and PS7 did not show any activity of P-solubilization. The other isolates were ranked as PS5 > PS2 > PS4 > PS3 > PS1 (Supplementary Table 2; on the journal's website).

3.7. Catalase and oxidase tests

The test for catalase and oxidase showed that isolate PS7 was negative for catalase activity, whereas PS3 and PS6 were negative for the oxidase test (Supplementary Table 2).

3.8. Molecular characterization of bacterial isolates

The amplifications of partial *16S rRNA* genes from saline rhizospheric soil bacterial isolates were approximately 1400 bp. The phylogenetic analyses on the basis of the partial *16S rRNA* gene sequence of two *Paenibacillus* strains, PS2 (GenBank accession KF986215) and PS5 (GenBank accession KF986216), characterized in this study and other *Paenibacillus* strains isolated from distinct geographical locations in the world resulted in two lineages: clades I and II. The Pakistani *Paenibacillus* strains showed little diversity in 16S rDNA sequences. However, these strains were closely related to the *Paenibacillus ehimensis* strain from South Korea. Clade I contained isolates from China, Korea, Japan, and India, while some isolates from China and Japan were in clade II. Clades I and II were highly similar to each other. Clades I and II showed 80% similarity to South Korean strain HT228, which is closely related to Pakistani strains.

The two Pakistani *Paenibacillus* strains, PS2 and PS5, were very divergent and grouped alone in the phylogenetic tree (Figure 4). Thus, these *Paenibacillus* strains could be considered as atypical.

3.9. Transgenic plant inoculation study

The two prospective plant growth-promoter bacterial isolates (PS2 and PS5) characterized by partial 16S rDNA sequence analysis were selected for preliminary study of their interaction with transgenic (salt-tolerant) and WT (salt-sensitive) peas under 100 mM NaCl stress and without NaCl stress. Both of the bacterial strains showed promising results in plant root/shoot growth promotion under salt stress (Figure 5).

3.10. Plant biomass

The dry weight of the transgenic and WT pea plants increased when they were inoculated with the PGPR, especially under salt stress. The shoot dry weight of transgenic plants was significantly increased over the WT counterpart in all treatments. Maximum values were recorded when plants were inoculated with the PS2 or PS5 bacterial isolate separately under control conditions.

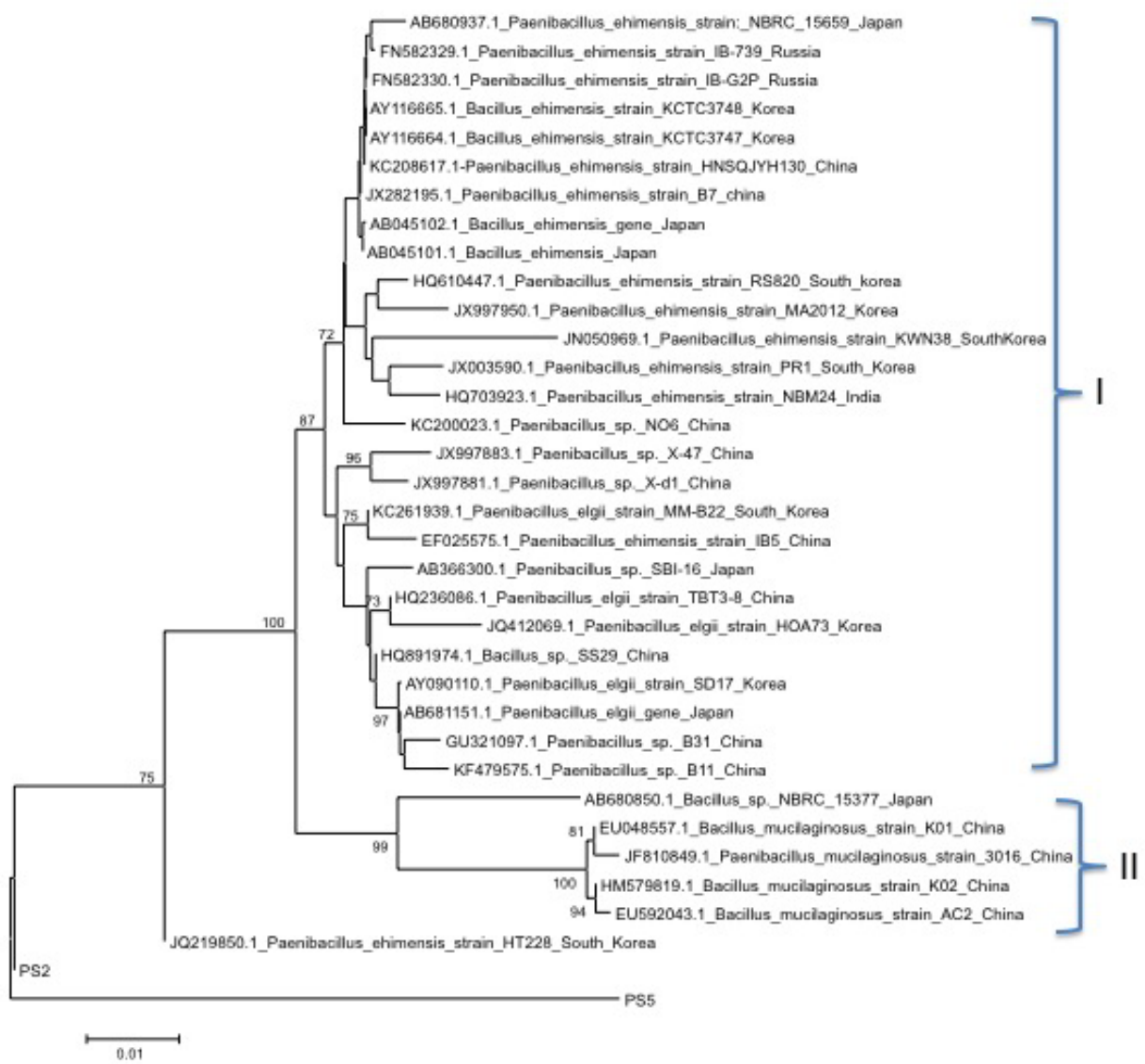

Figure 4. Neighbor-joining tree representing phylogenetic relationships of *Paenibacillus* strains PS2 and PS5 from Pakistan. Partial 16S rDNA sequence and other related sequences selected via BLAST search. The tree is based on pairwise comparisons using the Jukes–Cantor parameter on nucleotide sequences. Upper and lower branch points show bootstrap values (1000 replicates) supporting a particular phylogenetic group. The scale bar represents nucleotide substitutions per site. The *Paenibacillus* strains from this study are PS2 and PS5 (Pakistani origin). All other nucleotide sequences from different geographical locations are identified according to the GenBank accession number, isolate name, and country of origin.

Under salt stress conditions, the PGPR-inoculated transgenic and WT shoot dry weight was significantly ($P < 0.05$) increased (Figure 6A).

The root dry weight measurements showed promising results in salt stress conditions for inoculated plants. The transgenic pea roots' dry weight in all treatments was significantly ($P < 0.05$) higher than that of the WT pea. However, WT peas also showed good results when inoculated with bacterial isolates under salt stress. The highest values for transgenic peas were recorded when they were inoculated with bacterial isolates PS2 + PS5 under stress and without stress. For WT pea, bacterial inoculation without any other stress increased root dry weight compared to the control and under salt stress alone. All the inoculated treatments showed better results than noninoculated treatments (Figure 6B).

3.11. Percentage of water content

The percentage water contents of shoots were nonsignificant between treatments, while there were significant differences between transgenic and WT peas

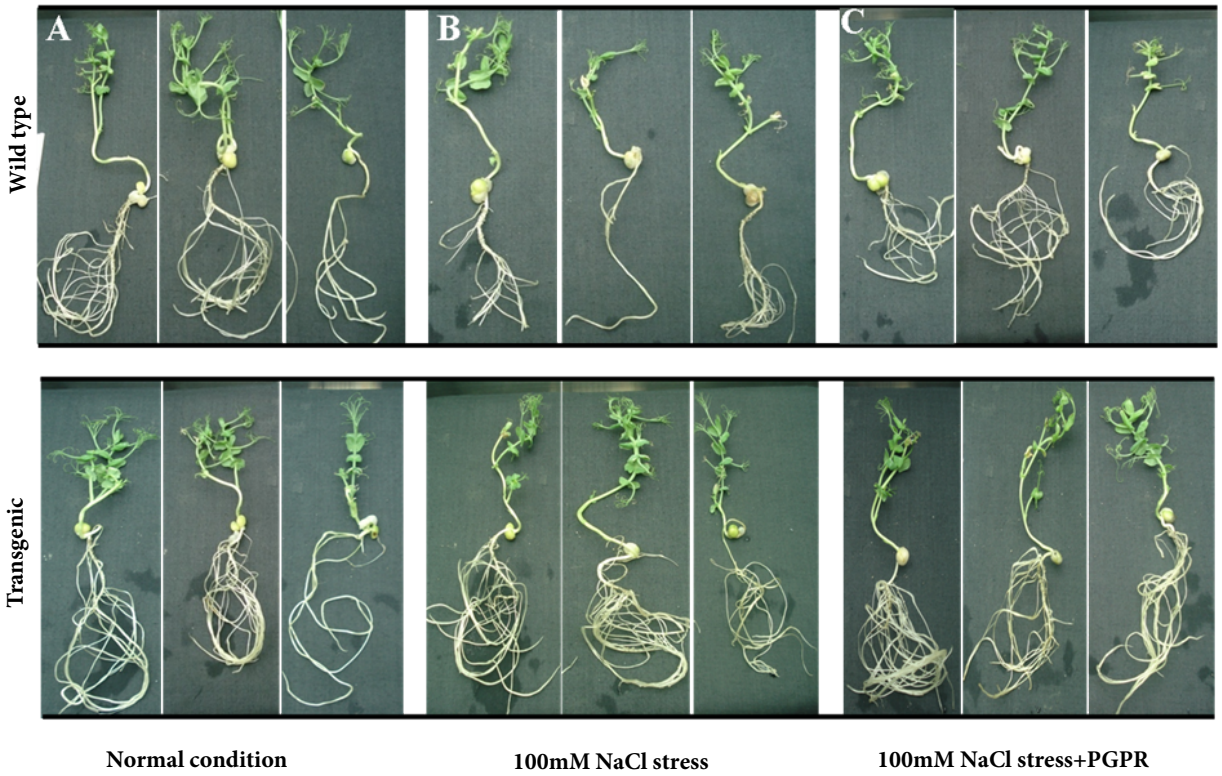

Figure 5. Seedling growth of transgenic and WT pea plants with 100 mM NaCl stress and with PGPR inoculation. A) WT and transgenic pea plants without NaCl stress. B) WT and transgenic pea plants with 100 mM NaCl stress. C) WT and transgenic pea plants with 100 mM NaCl stress and with PGPR interaction. The experiment was conducted in glass jars containing filter paper bridges. Plants were inoculated with half-strength MS salt liquid when needed. The pictures were taken 24 days after seedling emergence.

($P < 0.05$). Although PGPR-inoculated plants showed promising increases in shoot/root water contents, the transgenic plants showed significantly higher ($P < 0.05$) percentage of water contents in shoots and roots than the WT in all treatments (Supplementary Figures 2 and 3; on the journal's website).

3.12. Antioxidant analysis

Antioxidant enzymes are a key factor in every type of stress for scavenging reactive oxygen species (ROS). The results for antioxidant production were promising. A significant ($P < 0.05$) increase in the transgenic pea line was observed compared to WT pea plants in all treatments. The SOD results showed a considerable increase in bacteria-inoculated plants under salt stress and without stress compared to uninoculated ones both in transgenic and WT peas. SOD was significantly increased under salt stress when plants were inoculated with bacterial isolates. The transgenic pea plants' SOD concentration significantly ($P < 0.05$) increased in comparison to WT peas in all treatments, except when PS5 was inoculated under salt stress, which resulted in higher SOD concentrations in WT peas than transgenic ones (Figure 6C).

POD was significantly ($P < 0.05$) increased under salt stress. When the plants were inoculated with PGPR, it significantly increased the POD contents in both transgenic and WT pea plants. The maximum increase in POD concentration was observed in transgenic plants when inoculated with PS2 or PS2 + PS5 in combination under salt stress (Figure 6D)

4. Discussion

Genetic engineering of transgenic plants and their interaction with beneficial microflora for salt stress tolerance is a promising approach. We observed positive effects of the prospective PGPR association with transgenic peas (*Pisum sativum* L.) against salt stress. The transgenic plants were harboring the Na^{+}/H^{+} antiporter gene from *Arabidopsis thaliana* for salt stress tolerance (Apse et al., 1999) and in divergent orientation the *bar* gene for phosphinothricin herbicide tolerance. Our findings show that the beneficial bacteria can be influenced by specific transgenic plants against salt stress. However, the major focus of our research is whether transgenic plants harboring the Na^{+}/H^{+} antiporter gene and interaction of salt-tolerant beneficial bacteria (PGPR) can influence the

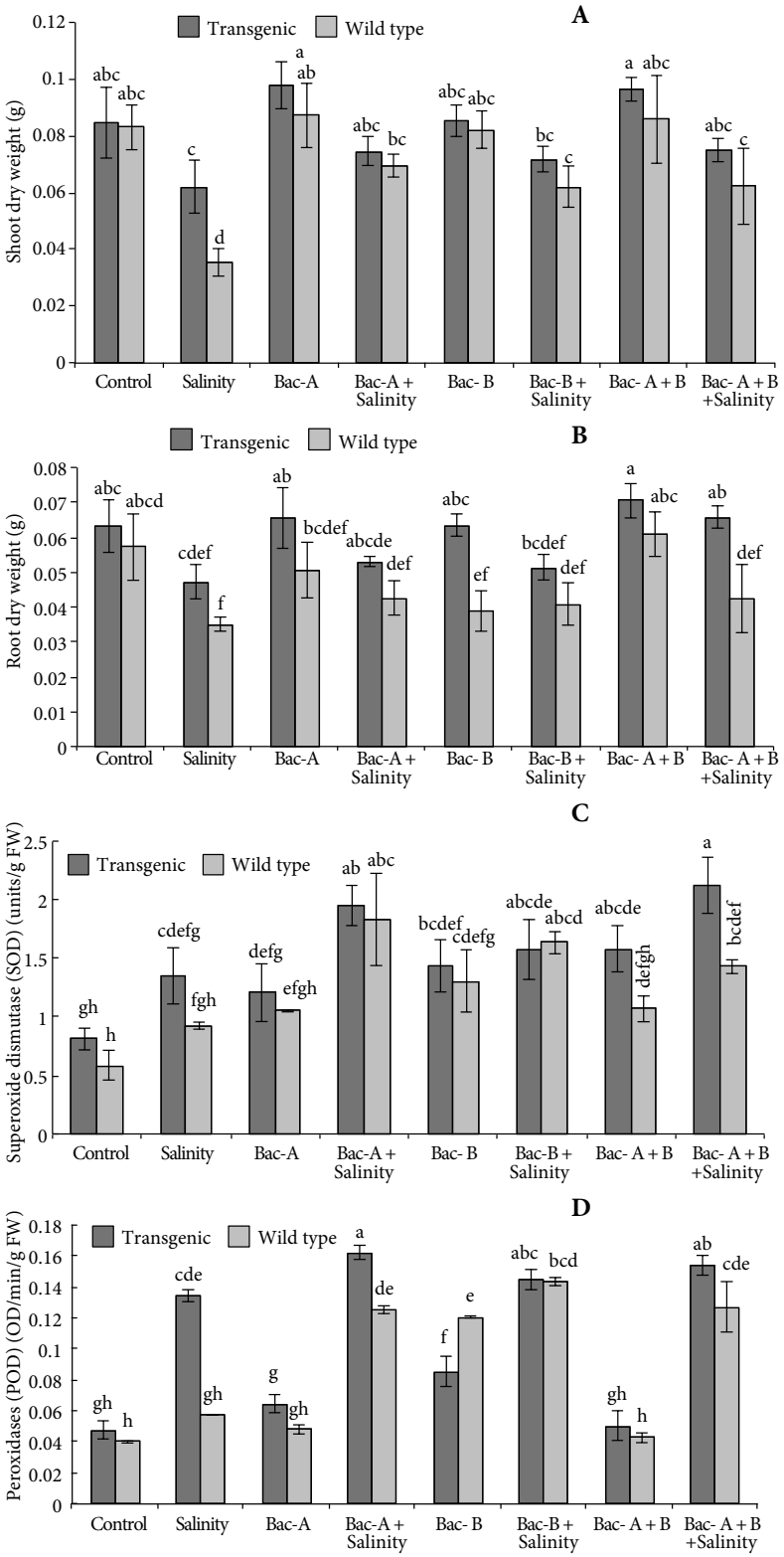

Figure 6. Transgenic and WT plants' interactions with PGPR under NaCl stress (100 mM). A) Shoot dry weight (g), B) root dry weight (g), C) antioxidant superoxide dismutase (SOD) production, D) peroxidase (POD) production. Data were recorded at 24 days after seedling emergence. Bac-A and Bac-B stand for isolate PS2 and PS5, respectively. The data in the graph are presented by each sample corresponding to three independent replicates, and each replicate was the pool of the three plantlets of the jars. The bars show standard errors (mean ± SE). All means sharing a common letter are similar; otherwise, means differ significantly at $P < 0.05$.

tolerance level of salt stress as well as seedling growth promotion. Rhizobacteria and free-living bacteria have been used as promising inoculants to enhance plant productivity (Kloepper, 1992). These root-colonizing PGPR implement positive effects on plant vigor and development (Somers et al., 2004; Dimkpa et al., 2009). The results of this study for total plant length (cm), shoot length (cm), and root length (cm) showed significant differences between PGPR-inoculated and uninoculated plants, and significant differences were also found between transgenic and WT pea plants. The inoculated WT pea plants and transgenic pea plants without any inoculation under salt stress showed similar results on average. There are different reports available that showed enhanced shoot and root lengths in inoculated plants compared to controls under salt stress (Naz and Bano, 2010; Shukla et al., 2012). We could not observe any negative effect of transgenes on the growth of PGPR. However, the interaction resulted in an increase in plant height, root length, biomass, and antioxidant production. The selected isolates are IAA producers and the IAA interaction with plants enhances root/shoot length (Patten and Glick, 2002). The isolates, individually as well as in combined application, increased the root/shoot fresh weight under salt stress compared to uninoculated plants. However, in the case of transgenic plants without any PGPR inoculation, root and shoot fresh weight increased compared to PGPR-inoculated WT plants under salt stress. Golpayegani and Tilebeni (2011) reported that, under salt stress, the fresh weight increased more in inoculated treatments than in the control in basil (*Ocimum basilicum* L.). On the other hand, NaCl stress reduces the mean dry weight of shoot and roots (Shukla et al., 2012). In our study, the dry weight was higher in inoculated plants than uninoculated plants under salt stress. However, the transgenic pea line showed significant increase in dry weight over the WT in all treatments because transgenic pea plants were harboring the Na^+/H^+ antiporter gene that provides tolerance against salt stress. Therefore, transgenic plants showed even higher salt stress tolerance when inoculated with PGPR. These findings for increase in dry weight with PGPR inoculation under salinity were previously reported by Mayak et al. (2004), Zahir et al. (2009), and Shukla et al. (2012). The salinity decreases the plant's osmotic potential in the rhizosphere, altering water availability by initiating physiological drought in plants (Shukla et al., 2012). The shoot and root percentages of water content were also higher in inoculated plant under salinity than in uninoculated plants. Transgenic plants showed increased percentages of water contents compared to the WT in all treatments. Higher hydration from the interactions of the PGPR-inoculated plants compared to control plants was also reported by Shukla et al. (2012). The higher hydration in PGPR-inoculated plants leads to better water use efficiency and as a result increases photosynthesis and biomass (Mayak et al., 2004). Higher salinity enhanced an imbalance of cellular ions causing ion toxicity, osmotic stress, increased assembly of ROS (Alscher et al., 1997; Mandhania et al., 2006), oxidative damage, and a change in concentrations of antioxidants (Bor et al., 2003; Gao et al., 2008). However, activity of antioxidant enzymes in wheat leaves treated with PGPR strains was reduced in comparison to uninoculated plants growing under salinity stress (Upadhyay et al., 2012). Han and Lee (2005) also reported similar results for lettuce, where decreased ascorbate peroxidase (APX) and glutathione reductase (GR) activity under salinity stress was observed when plants were inoculated with PGPR (*Serratia* sp. and *Rhizobium* sp.). On the other hand, the POD activity was higher in PGPR (*Pseudomonas* and *Bacillus* sp.)-inoculated soybean plants under salt stress (Kumari et al., 2015). The production of SOD and POD was also increased in PGPR (*Pseudomonas* and *Azospirillum*)-inoculated sunflower plants to tolerate salt stress (Naz and Bano, 2013). The salt stress tolerance of plant cells is correlated to an efficient oxidative system (Mittova et al., 2002). In the current investigation antioxidant enzymes (SOD and POD) increased in PGPR-inoculated plants (both transgenic and WT) compared to uninoculated ones, both in salinity and control conditions. The antioxidant enzymes play an important role in resistance to various abiotic stresses (Upadhyay et al., 2012). Our hypothesis is strongly supported by an increase in antioxidant status in transgenic peas inoculated with PGPR compared to WT peas (salt-sensitive) inoculated with isolates under salinity stress. PGPR inoculation induced a higher activity of ROS scavenging enzymes (SOD, CAT, APX, etc.) in *Solanum tuberosum* (Gururani et al., 2013). The higher levels of antioxidant enzymes in the rhizobacteria-inoculated plants can be associated with mitigation of the salinity stress (Bianco and Defez, 2009).

The use of genetic engineering and PGPR for salt stress tolerance is an established phenomenon. Every year, new genes and isolates (PGPR) are reported with potential for salinity stress tolerance around the world. There is a need for using both phenomena in combination for increased salt stress tolerance. The current study is based on this phenomenon, using transgenic peas (against salt stress) and halotolerant PGPR interaction to maximize salt stress tolerance. This is the first report in which transgenic pea plants harboring the Na^+/H^+ antiporter gene and salt-tolerant PGPR interaction have been evaluated for improved salt stress tolerance. However, further field experiments are recommended.

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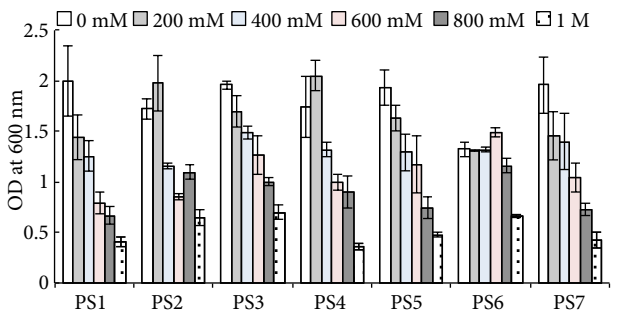
Supplementary Table 1. Morphological characterization of bacterial isolates from saline soils.

Strains	Form	Elevation	Margin	Appearance	Pigmentation	Size (μm)	Odor	CFUs	Gram test
PS1	Irregular	Flat	Undulate	Shiny	White	1.3	Odorless	7.72×10^7	+ve
PS2	Circular	Raised	Entire	Shiny	White	0.2	Odorless	4.96×10^7	+ve
PS3	Circular	Raised	Entire	Shiny	Yellow	0.1	Odorless	3.88×10^7	+ve
PS4	Punctiform	Raised	Entire	Shiny	White	0.1	Bad odor	8.44×10^7	+ve
PS5	Circular	Raised	Entire	Dull	White	1.8	Odorless	5.28×10^7	+ve
PS6	Punctiform	Raised	Entire	Dull	White	1.7	Odorless	6.67×10^7	+ve
PS7	Punctiform	Raised	Entire	Shiny	White	0.2	Odorless	6.28×10^7	-ve

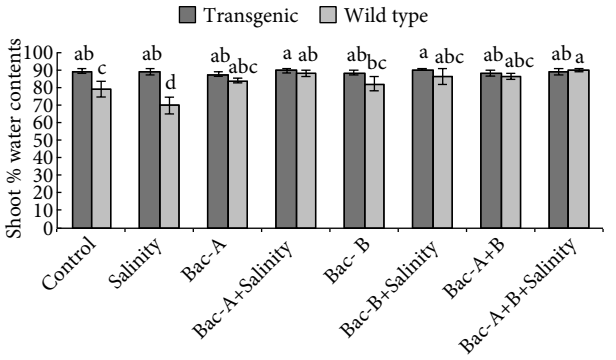
Supplementary Table 2. Phosphate solubilization and biochemical tests of bacterial isolates.

Isolates	P-solubilization		Biochemical tests	
	Qualitative	Index	Catalase	Oxidase
PS1	+	1.44	+	+
PS2	+	2.1	+	+
PS3	+	1.5	+	-
PS4	+	1.6	+	+
PS5	+	…	+	+
PS6	-	…	+	-
PS7	-	…	-	+

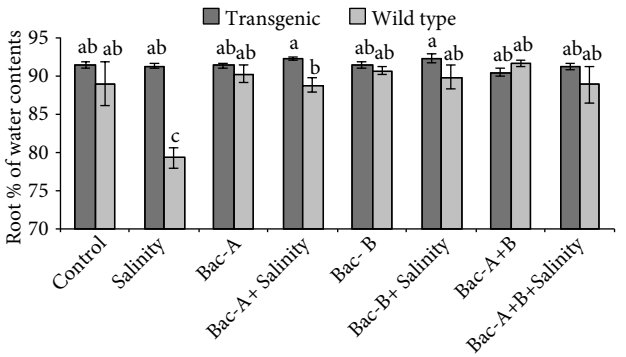
+ (positive), - (negative), … (not detectable).



Supplementary Figure 1. Growth of bacterial isolates (PS1 to PS7) in liquid LB media containing different NaCl concentrations ranging from 0 mM to 1M. Growth was observed at 600 nm after 24 h. The data are presented as means of three replicates, and bars show standard errors (mean ± SE).



Supplementary Figure 2. Effect of NaCl stress (100 mM) and PGPR interaction on shoot percentage of water contents of transgenic and wild-type pea plants. The experiment was performed in vitro in tissue culture jars containing half-strength MS media in a culture room at 25 ± 2 °C with a 16/8-h light/dark cycle. Bac-A and Bac-B stand for isolate PS2 and PS5, respectively. The data were analyzed by two-way ANOVA for treatments and varieties. Data are presented for each sample corresponding to three independent replicates, and each replicate was the pool of the three plantlets of the jars. Bars show standard errors (mean ± SE). All means sharing a common letter are similar; otherwise, means differ significantly at $P < 0.05$.



Supplementary Figure 3. Effect of NaCl stress (100 mM) and PGPR interaction on root percentage of water contents of transgenic and wild type pea plants. The experiment was performed in vitro in tissue culture jars containing half-strength MS media in a culture room at 25 ± 2 °C with a 16/8-h light/dark cycle. Bac-A and Bac-B stand for isolate PS2 and PS5, respectively. The data were analyzed by two-way ANOVA for treatments and varieties. Data are presented for each sample corresponding to three independent replicates, and each replicate was the pool of the three plantlets of the jars. Bars show standard errors (mean ± SE). All means sharing a common letter are similar; otherwise, means differ significantly at $P < 0.05$.