Endophytic bacterium \textit{Bacillus subtilis} (BERA 71) improves salt tolerance in chickpea plants by regulating the plant defense mechanisms

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\textbf{ABSTRACT}

Plant growth-promoting endophytic bacteria can stimulate the growth, nutrient acquisition, symbiotic performance and stress tolerance of chickpea plants under saline soil conditions. The aim of this study was to investigate the stress-adaptive mechanisms of chickpea plants mediated by \textit{Bacillus subtilis} (BERA 71) under saline conditions. Inoculation with BERA 71 enhanced plant biomass and the synthesis of photosynthetic pigments and reduced the levels of reactive oxygen species (ROS) and lipid peroxidation in plants under conditions of stress. Furthermore, the activities of ROS-scavenging antioxidant enzymes (superoxide dismutase, peroxidase, catalase and glutathione reductase), the levels of non-enzymatic antioxidants (ascorbic acid and glutathione) and the total phenol content were increased in stressed plants during bacterial association. The bacteria decreased sodium accumulation and enhanced the nitrogen, potassium, calcium and magnesium content in the plants. The suppression of ROS generation and of lipid peroxidation and the accumulation of proline in BERA-71-inoculated plants enhanced the membrane stability under salinity stress and non-stress conditions.

\textbf{Introduction}

It has been estimated that nearly 7% of all land is affected by high salinity (El-Beltagy and Madkour 2012; Ruiz-Lozano et al. 2012), which inhibits plant growth, resulting in considerable reduction of plant yields. High salinity is a devastating abiotic stress factor that severely affects the normal growth and development of plants (Hashem et al. 2016b). Increased salinity in arid and semiarid regions of the globe leads to serious problems with the fertility of agricultural lands, which is further aggravated by irrigation using hard, saline water. High salinity in soil induces ionic and osmotic stress, resulting in growth retardation through malfunctioning photosynthesis and ion homeostasis (Porcel et al. 2012; Alqarawi et al. 2014a, 2014b; Hashem et al. 2016b). Salinity-induced alterations in photosynthesis are often associated with perturbations in carbon and nitrogen assimilatory pathways and have negative effects on plant yield (Tejera et al. 2004). Some of the key deleterious products that hinder plant metabolism are reactive oxygen species (ROS). Excessive production of toxic ROS causes oxidative damage and affects the functional integrity of cells (Ahmad et al. 2010, 2012). ROS, including superoxide, hydroxyl, hydrogen peroxide and other free radicals, are detrimental to normal metabolism, and excessive accumulation of ROS in leaves leads to the oxidation of cellular molecules, including lipids, proteins and chlorophylls, which leads to programmed cell death (Mittler 2002; Ahmad et al. 2010).

To cope with the negative effects of high salinity, plants activate self-defense mechanisms to prevent oxidative damage. Among the several defensive strategies, the up-regulation of antioxidant systems, accumulation of compatible osmolytes (Vardharajula et al. 2011; Alqarawi et al. 2014a) and compartmentalization of toxic ions to less sensitive tissues (Ghorbanpour et al. 2013) significantly contribute to plant tolerance to environmental stress. In the antioxidant system, enzymatic or non-enzymatic components work in coordination to neutralize toxic ROS. Enzymatic components include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione reductase (GR), while non-enzymatic antioxidants include ascorbic acid, tocopherol, glutathione and secondary metabolites; all of these components are involved in the elimination of ROS to protect the cells against oxidative damage (Vardharajula et al. 2011; Abd_Allah et al. 2015). Osmolytes, including free proline, sugars and amino acids, help maintain the normal water level for regulating cellular metabolism and function (Agami et al. 2016).

Numerous studies have suggested that beneficial soil microorganisms promote plant growth and yield (Adesemoye et al. 2008; Berg 2009; Cho et al. 2015; Egamberdieva et al. 2016, 2017a). The important roles of beneficial microbes are as follows: bio-fertilization, plant growth promotion, restriction of pathogen growth and induction of plant tolerance against abiotic stresses (Emmerling et al. 2002; Bharti...
et al. 2016; Hashem et al. 2016b). Plant growth-promoting rhizobacteria (PGPR) are thought to promote plant growth directly as well as indirectly. Included among the direct beneficial effects of PGPR are mechanisms such as root growth promotion, rhizoremediation, stress amelioration and biological control of plant pathogens (Lugtenberg and Kamilova 2009; Pereira et al. 2016). In addition to affecting plant mineral nutrition, PGPR modulate host plant growth through their involvement in the metabolism of phytohormones, such as auxins, ethylene, gibberellins, abscisic acid and cytokinins (Turan et al. 2014; Shahzad et al. 2017). PGPR containing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase regulate the growth and development of plants by reducing the endogenous ethylene levels (Glick et al. 2007, Glick 2014).

In our previous work, plant growth-promoting endophytic bacteria isolated from chickpea plants stimulated the growth, nutrient acquisition, symbiotic performance and stress tolerance of chickpea plants in saline soil (Egamberdieva et al. 2011). Therefore, the objectives of this study were to investigate the stress-adaptive mechanisms of chickpea plants mediated by Bacillus subtilis under saline conditions.

Materials and methods

Plant materials and endophytic bacteria

Seeds of chickpea (Cicer arietinum, cv. Giza 1) were provided by the Agriculture Research Center, Giza, Egypt. The endophytic bacterium (B. subtilis BERA 71) was previously isolated from roots of Talh trees (Acacia gerrardii Benth.) as described in detail by Hashem et al. (2016b). The 16S rDNA sequence of B. subtilis BERA 71 was submitted to the GenBank nucleotide sequence database under the accession number KX090253 (http://www.ncbi.nlm.nih.gov/nuccore/KX090253?report=GenBank) as described in detail in our previous work (Hashem et al. 2016b). The formulation of BERA 71 was carried out as described in our previous study (Hashem et al. 2016a), and the lyophilized material contained 3.6 × 10⁹ cfu (colony forming units) of BERA 71 g.

Plant growth and treatments

The seeds were disinfected with mercuric chloride (0.1%, v/v) for 5 min and subsequently rinsed five times with ion-free distilled water. The seeds were germinated on a wet filter paper for five days at 25°C in a seed germination incubator. The healthy and uniform-sized sprouted seedlings were transferred to plastic pots (5 l volume, one seed/pot) filled with 3 kg of soil mixture composed of autoclaved sand:peat (1:1:1, v/v/v). The pots were arranged in a completely randomized block design with five replicates. The pots factorially designed with the following treatments: (1) Control: Plants without salt stress and bacterial treatments; (2) Plants + BERA 71: Plants treated with B. subtilis without salt stress; (3) Plants + salt stress: Plants treated with salt stress (200 mM NaCl) in the absence of B. subtilis; and (4) Plants + BERA 71 + salt stress: Plants treated with B. subtilis under salt stress. The germinated seeds were coated with B. subtilis BERA 71 (15 g of formulated material/kg of germinated seeds). The soil was salinized with 200 mM NaCl along with Hoagland solution (Hoagland and Arnon 1950) after true leaves developed. Post-incubation, the plants were kept in a growth chamber at the Plant Production Department, College of Food and Agricultural Sciences, King Saud University, Saudi Arabia, for eight more weeks under control conditions (the temperature of the day/night cycle was set to 25°C/18°C, 350 μmol photon m⁻² s⁻¹ light intensity was provided by fluorescent tubes, and the humidity was 70–75%).

Plant growth and nutrient content

When ready for harvest, the plants were carefully removed from the pots, and the roots were separated from shoots. The third leaf from the top was harvested from plants of each treatment for biochemical analysis. Subsequently, root and shoot samples were oven-dried at 70°C for three days to measure their dry weight. Total nitrogen content was determined in dry leaves of chickpea plants by the Kjeldahl method (Ugrinovits 1982), and other elements such as Na⁺, K⁺, Ca²⁺ and Mg²⁺ were analyzed following the method described by Wolf (1982). The leaf powder was acid digested (H₂SO₄/HNO₃ mixture, 1/5, v/v,), and the concentration of each ion was measured using a flame photometer (Jenway Flame Photometer, Bibby Scientific Ltd-Stone-Stafs-St15 05A–UK).

For estimation of photosynthetic pigments, fresh leaves were extracted in dimethyl sulfoxide (DMSO) as described by Hiscox and Israelstam (1979). The absorbance was determined spectrophotometrically at 480, 510, 645 and 663 nm (T80 UV/VIS Spectrometer, PG Instruments Ltd, USA) to quantify chlorophyll a, chlorophyll b and carotenoids; DMSO was used as a blank.

Determination of lipid peroxidation and hydrogen peroxide levels

Fresh leaves (5 g) were extracted in 1% trichloroacetic acid (TCA), and the homogenate was centrifuged at 10,000 rpm for 5 min. One milliliter of supernatant and 4.0 ml of 0.5% (w/v) thiobarbituric acid (TBA) were heated at 95°C for 30 min. Samples were cooled in an ice bath and centrifuged at 5000 rpm for 5 min for clarification. Then, the absorbance of the supernatant was measured at 340 and 500 nm (Heath and Packer 1968). MDA was calculated according to the following equation: MDA equivalents (μg/g FW) = 1000 [(Abs₅₃₂ − Abs₆₀₀ nm)]/155.

For estimation of hydrogen peroxide (H₂O₂), leaf tissue (0.5 g) was homogenized with 0.1% TCA followed by centrifugation at 12,000 rpm for 15 min. The supernatant (0.5 ml) was treated with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of potassium iodide (1 M). The absorbance of the supernatant was measured at 390 nm, and the H₂O₂ concentration was determined from a standard curve (Sergiev et al. 1997). The results are expressed as n mol g⁻¹ FW.

Determination of proline and total soluble phenolics

Leaf tissue (0.5 g) was extracted by using 3% (w/v) aqueous sulphasalicylic acid, and the extract was subjected to centrifugation at 3000 rpm for 20 min. The obtained supernatant was mixed with acetic acid and ninhydrin reagent, and then, the
mixture was boiled for 1 h. The reaction was terminated by placing the mixture in an ice bath, and the absorbance was read spectrophotometrically at 520 nm using toluene as a blank (Bates et al. 1973).

Total phenolics were extracted in 80% ethanol (v/v) and estimated using Folin and Ciocalteu’s phenol reagent, and the absorbance of the mixture was read at 750 nm (Slinkard and Singleton 1977). The total phenolic content was measured using a pyrogallol standard curve.

**Assay of antioxidant enzymes**

Fresh leaf tissue (10 g) was macerated in 50 mM sodium phosphate buffer (pH 7.0) containing 1% soluble polyvinyl pyrrolidine (PVP). The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C, and the supernatant was used for the enzyme activity assays. Proteins in the enzyme extract were estimated as described by Lowry et al. (1951).

Superoxide dismutase (SOD, EC 1.15.1.1) was estimated as described by Bayer and Fridovich (1987) following the photo-reduction of nitroblue tetrazolium (NBT). The activity of SOD was expressed as enzyme unit (EU) mg⁻¹ protein, and one unit of SOD was defined as the amount of protein causing a 50% inhibition of NBT reduction. For estimation of catalase (CAT, EC 1.11.1.6) activity, the Luck (1974) method was adopted, and the activity was calculated by using an extinction coefficient of 36 × 10³ M⁻¹ cm⁻¹ and was expressed as EU mg⁻¹ protein. Peroxidase (POD, EC 1.11.1.7) activity was determined by following the method described by Kar and Mishra (1976). The assay mixture comprised 125 μM phosphate buffer (pH 6.8), 50 μM pyrogallop, 50 μM H₂O₂ and 1 ml of the 20 x diluted enzyme extract in a final volume of 5 ml. The amount of purpuragolin formed was determined by measuring the absorbance at 420 nm, and the enzyme activity was expressed as EU mg⁻¹ protein.

**Determination of non-enzymatic antioxidants**

Ascorbic acid levels were estimated following the method described by Mukherjee and Choudhuri (1983). The total phenolic content was measured using a pyrogallol standard curve.

**Statistical analysis**

The experimental design was completely randomized and repeated three times. The obtained data were given as the mean with standard error values by using SPSS-21 software, and the differences in the means were determined by the least significant difference (LSD) (P = .05) test.

**Results**

**Plant growth, nutrients and photosynthetic pigments**

The beneficial effect of *B. subtilis* BERA 71 on chickpea plant growth under saline soil (200 mM NaCl) conditions was determined by measuring the dry weight of the root and shoot, and the results are shown in Table 1. Chickpea plants exposed to saline conditions exhibited a considerable reduction in the dry weight of the root and shoot. On the other hand, *B. subtilis* significantly enhanced the dry weight (28.7%) of the shoot compared to the control plants. *B. subtilis* inoculation resulted in increased mineral uptake under non-stressed and NaCl-stress conditions (Table 1). During favorable control conditions, *B. subtilis* inoculation resulted in improvements of 29.5%, 17.6%, 42.3% and 29.0% for nitrogen, potassium, calcium and magnesium uptake, respectively, in chickpea plants. Soil salinity caused an accumulation of sodium (69.14%) and reduced the uptake of nitrogen (54.2%), potassium (54.7%), calcium (55.8%) and magnesium (55.6%). The soil-salinity mitigating effect of *B. subtilis* on chickpea plant growth was confirmed by the reduced uptake of sodium (44.8%) and reduced sodium/potassium ratio in NaCl-treated plants; the accumulation of other ions and the potassium/calcium ratio were enhanced in NaCl-treated plants.

*B. subtilis* inoculation resulted in a significant increase in the synthesis of photosynthetic pigments, including chlorophyll a, chlorophyll b and carotenoids (Table 2). However, soil salinity led to a significant decrease in the chlorophyll a, chlorophyll b and total chlorophyll content, which decreased by 41.3%, 47.5% and 28.3%, respectively, compared to the controls. While soil salinity reduced the ratio of chlorophyll a/b, carotenoids and total chlorophyll, *B. subtilis*

| Treatments | Root dry weight (g) | Shoot dry weight (g) | N (mg/g) | Na (mg/g) | K (mg/g) | Ca (mg/g) | Mg (mg/g) |
|------------|---------------------|---------------------|----------|-----------|----------|-----------|-----------|
| Control    | 1.4±0.03            | 2.1±0.12            | 28.5±0.12| 3.4±0.09  | 10.8±0.07| 1.5±0.01  | 5.1±0.15  |
| BERA71     | 1.8±0.02            | 3.0±0.05            | 40.5±0.47| 3.9±0.07  | 13.1±0.07| 2.6±0.02  | 7.3±0.12  |
| SS         | 0.7±0.01            | 1.3±0.02            | 13.0±0.20| 11.0±0.13 | 4.9±0.05 | 0.6±0.01  | 2.3±0.08  |
| SS + BERA71| 1.3±0.02            | 1.8±0.14            | 33.0±0.11| 6.1±0.18  | 8.7±0.23 | 1.1±0.07  | 3.9±0.10  |
| LSD at: 0.05 | 0.08                | 0.32                | 0.42     | 0.43      | 0.08     | 1.08      | 5.04      |

Note: Data presented are mean of five replicates ± SE. SS: salt stress; BERA71: *Bacillus subtilis* BERA71

Table 1. Plant biomass and nutrient content of chickpea treated with NaCl (200 mM) in presence and absence of *Bacillus subtilis* BERA 71.
supplementation in soil alleviated the NaCl-induced decline in pigment synthesis and led to an increase in the levels of chlorophyll a (17.3%), chlorophyll b (34.1%) and total photosynthetic pigments (21.0%) compared with the salt-affected uninoculated plants.

**Physiological parameters**

The effect of *B. subtilis* and NaCl on lipid peroxidation (measured in terms of MDA production) and hydrogen peroxide levels in plants is shown in Figure 1(A,B). Salinity increases the production of hydrogen peroxide, which has a direct impact on the membrane integrity. The percent increase in hydrogen peroxide due to NaCl was 62.9%, resulting in a 41.05% increase in MDA. In contrast, *B. subtilis* decreased the hydrogen peroxide (23.5%) and MDA (27.5%) levels in plants under non-stress conditions, and it significantly mitigated the detrimental effects of salinity by reducing the hydrogen peroxide and MDA levels by 23.7% and 25.2% respectively.

Chickpea plants inoculated with *B. subtilis* showed an increase in the production of proline and in the total phenol content (Figure 2). NaCl-induced stress stimulated the accumulation of proline (68.9%), while it decreased the total phenol content (41.3%). *B. subtilis* treatment ameliorated the negative impact of salinity on the total phenol content in the plants by decreasing (26.3%) their synthesis in salt-treated plants.

The activities of antioxidant enzymes and the levels of non-enzymatic antioxidants were measured to determine the beneficial effect of *B. subtilis* under salinity stress and non-stress conditions (Figure 3). The activities of antioxidant enzymes, including SOD, CAT, POD and GR, were stimulated by 12.2%, 8.7%, 36.8% and 12.5%, respectively, in plants due to the effect of *B. subtilis*. Salinity-stressed chickpea plants exhibited enhancement of SOD (63.7%), POD (74.4%) and GR (53.9%) activities when compared to their controls. However, the inoculation of NaCl-treated plants with *B. subtilis* led to a further increase in the activities of SOD (5.4%), POD (15.7%) and GR (11.7%). CAT activity was severely decreased under salinity stress, while bacterial interactions in plants stimulated CAT activity. In addition, the ascorbic acid content and the reduced-glutathione content were increased by 22.5% and 9.2%, respectively, due to *B. subtilis* inoculation, and those levels were further increased by 13.3% and 7.8%, respectively, during salinity stress.

**Table 2. Photosynthetic pigments of chickpea treated with NaCl (200 mM) in presence and absence of Bacillus subtilis (BERA 71).**

| Treatments      | Chl a (mg/g fwt) | Chl b (mg/g fwt) | Chl a/b | Carotenoids (mg/g fwt) | Total PP (mg/g fwt) |
|-----------------|------------------|------------------|---------|------------------------|---------------------|
| Control         | 1.38±0.02        | 0.52±0.01        | 2.62±0.05 | 0.24±0.01              | 2.15±0.02           |
| BERA71          | 1.68±0.01        | 0.69±0.01        | 2.43±0.05 | 0.38±0.01              | 2.75±0.00           |
| SS              | 0.81±0.01        | 0.27±0.01        | 2.94±0.14 | 0.45±0.01              | 1.54±0.01           |
| SS + BERA71     | 0.98±0.01        | 0.41±0.01        | 2.40±0.07 | 0.36±0.01              | 1.95±0.02           |
| LSD at: 0.05    | 0.05             | 0.04             | 0.30     | 0.04                   | 0.05                |

Notes: Chl a: Chlorophyll a; Chl b: Chlorophyll b; Total PP: Total photosynthetic pigments.

Data presented are mean of five replicates ± SE.

![Figure 1](image1.png)

**Figure 1.** A, Hydrogen peroxide (*H₂O₂*) and B, lipid peroxidation (MDA) in chickpea (*Cicer arietinum*) treated with NaCl in presence and absence of *Bacillus subtilis* (BERA 71). Data presented are mean of three replicates.

![Figure 2](image2.png)

**Figure 2.** A, Total phenol and B, proline in chickpea (*Cicer arietinum*) treated with NaCl in presence and absence of *Bacillus subtilis* (BERA 71). Data presented are mean of three replicates.
Discussion

In our study, we observed that plants inoculated with *B. subtilis* BERA 71 exhibited different growth patterns compared to non-inoculated controls. High salinity stimulates chlorophyllase activity, which degrades pigment proteins. Lower levels of chlorophyll due to NaCl treatment have been detected in *C. arietinum* (Rasool et al. 2013), *Ephedra alata* (Alqarawi et al. 2014b) and *Sesbania sesban* (Abd_Allah et al. 2016). Inoculation with *B. subtilis* ameliorated the NaCl-induced detrimental effects on plant growth. PGPR have the potential to enhance crop resilience to stresses and are believed to regulate key growth-promoting pathways in the host plants, thereby resulting in proper growth maintenance (Stefan et al. 2013). Our results correlated with those of Mohamed and Gomaa (2012) and Stefan et al. (2013), who reported a significant improvement in the growth of *Raphanus sativus* and runner bean due to inoculation with PGPR, which was caused by its stimulatory effect on the synthesis of chlorophylls. The present study also suggested that an increase in the uptake of magnesium can support pigment synthesis and might be one of the prime reasons for the PGPR-induced increase in chlorophyll content. *B. subtilis* protected *C. arietinum* plants from the deleterious effects of NaCl on chlorophyll pigments, which might contribute to the enhancement of the efficiency of photosynthesis by maintaining the function of the pigment–protein complex (Rasool et al. 2013). A high salt concentration in growth medium leads to stunted plant growth due to the de novo synthesis of proteins and chlorophyll components (El-Tayeb 2005), while *B. subtilis* interactions increase chlorophyll synthesis through enhanced metabolism and through the synthesis of photoassimilates. Dawwam et al. (2013) reported that the stimulation of pigment synthesis in potato plants by PGPR might be due to the changes in N, P and K uptake. The results of the present study revealed that the inoculation of *C. arietinum* with *B. subtilis* significantly mitigated the toxicity of saline conditions on the photosynthetic machinery through the stimulation of the synthesis of pigments and their associated components.

In the present study, exposure of *C. arietinum* to salinity stress resulted in greater production of hydrogen peroxide, which ultimately affected membrane structural integrity and led to the peroxidation of lipids. Our results corroborated with those of Ahmad et al. (2012), who demonstrated membrane damage in mustard cultivars as a result of NaCl treatment, which led to altered membrane functioning. Han et al. (2014) reported that the inoculation of white clover with *B. subtilis* (GB03) reduced the production of ROS, leading to significant reduction in membrane lipid peroxidation. The present study confirmed the protective role of *B. subtilis* in the...
strengthening of the membrane structures in C. arietinum under saline and non-saline conditions. Soil-salinity-induced membrane damage due to the production of ROS and peroxidation of lipids is well documented by several studies. For example, NaCl-treated mustard, O. basilicum and S. sesban showed higher levels of ROS and lipid peroxidation (Ahmad et al. 2012; Hashem et al. 2015; Abd_Allah et al. 2016), which inhibited plant growth. In addition, Rasool et al. (2013) observed a considerable reduction in lipid peroxidation in tolerant cultivars of chickpea, which exhibited less membrane damage than sensitive cultivars. Salinity affects the polyunsaturated fatty acid composition, leading to membrane dysfunction (Alqarawi et al. 2014b), and the present study suggests that B. subtilis might regulate the membrane functions by maintaining the optimal ratio of polyunsaturated to saturated fatty acids and by reducing the generation of ROS, such as hydrogen peroxide. Excess production of ROS triggers the peroxidation of unsaturated lipid components, leading to the loss of membrane integrity and causing leakage and desiccation.

The ameliorative role of B. subtilis against salt stress in chickpea might be due to the reduction of ROS and the up-regulation of antioxidant systems and nutrient uptake (Han et al., 2014; Janahiraman et al. 2016). Higher activity of SOD in response to salinity has been reported in C. arietinum (Rasool et al. 2013) and S. sesban (Abd_Allah et al. 2016). Though the activity of antioxidant enzymes was stimulated by NaCl treatment, B. subtilis inoculation further stimulated the antioxidant system, leading to accelerated elimination of toxic ROS (Agami et al. 2016; Hashem et al. 2016b). SOD is a key antioxidant enzyme involved in the scavenging of superoxide radicals and H2O2 for the reduction of the Haber–Weiss reaction and formation of hydroxyl (OH–) radicals. The up-regulation of SOD, CAT, POD and GR protected chickpea from free-radical-induced membrane dysfunction. The acceleration of POD activity in B. subtilis-inoculated chickpea might be due to improved salinity stress tolerance caused by enhancement of the biosynthesis of lignins and other related protective compounds for reducing oxidative stress (Boerjan et al. 2003). Chen et al. (2007) reported that higher POD activity in Vigna unguiculata ameliorated the effects of salt stress. Increased activity of H2O2-neutralizing enzymes, including CAT and POD, in B. subtilis-inoculated plants regulates plant growth by protecting delicate organelles, such as chloroplasts, wherein key metabolic processes occur (Han and Lee 2005; Hashem et al. 2016b). Increased CAT activity in chickpea plants under saline soil conditions might be due to decreased H2O2 production in apoplasts (Mutlu et al. 2009). Mittal et al. (2012) demonstrated that higher activity of CAT in Brassica juncea led to tolerance against high salinity. CAT plays a crucial role during stress and eliminates H2O2 (which is a signalling molecule that rapidly diffuses through membranes) to prevent membrane and organelle damage (Bienert and Chau-mont 2014). Habib et al. (2016) observed that inoculation of okra plants with ACC deaminase-producing PGPR increased the expression of antioxidant-coding genes, leading to enhanced salt stress tolerance. Similarly, PGPR-induced enhancement of antioxidant systems has been reported by Ghobarapour et al. (2013) and Younesi and Moradi (2014). The maintenance and accumulation of redox components have been reported in plants that have the ability to withstand stress (Rasool et al. 2013; Younesi and Moradi 2014; Agami et al. 2016; Hashem et al. 2016b). The interactions of B. subtilis in plants increased the production of redox components, i.e. ascorbic acid and reduced glutathione, which are electron donors of antioxidant enzyme-mediated reactions. Ascorbic acid, glutathione and GR are key components of the important ROS-scavenging pathway, which includes the ascorbate-glutathione cycle (Noctor and Foyer 1998). During salt stress, a series of redox reactions that occur in the ascorbate-glutathione cycle lead to the elimination of H2O2 in chloroplasts and the cytosol to ameliorate the effects of oxidative stress on plants. B. subtilis inoculation accelerated GR activity and increased ascorbic acid and glutathione contents in salt-affected plants, which might be due to the protection of the photosynthetic electron transport chain by maintenance of the NADP+/NADPH ratio for continuous flow of electrons to molecular oxygen, resulting in reduced superoxide radical generation (Noctor and Foyer 1998).

In addition, higher accumulation of proline and phenols in B. subtilis-inoculated chickpea plants clearly led to stress tolerance. In addition to the important role of proline in maintaining the water balance of cells, proline ameliorates salinity-induced toxic effects by mediating the scavenging of ROS to protect proteins and other important bio-molecular structures (Abd_Allah et al. 2016). The proline synthesis observed in PGPR-inoculated plants in the present study is corroborated by a previous study. Agami et al. (2016) demonstrated a significant improvement in proline accumulation due to PGPR (Azotobacter chroococcum A101), resulting in enhanced water uptake, water use efficiency and photosynthetic efficiency. B. subtilis-inoculated accumulation of proline might be due to alteration of the expression of proline-meta-bolizing enzymes, and it has been established that the accumulation of proline results from up-regulation and down-regulation of biosynthetic and degradative enzymes, respectively (Ahmad et al. 2010). Proline maintains protein turnover and regulates stress-protective proteins (Hashem et al. 2015). The accumulation of phenols in PGPR-inoculated chickpea plants correlated with a report by Bahadur et al. (2007), which suggested that greater accumulation of phenolics in pea seedlings led to higher tolerance to fungal infection. However, PGPR have the potential to release certain volatile compounds (Liu and Zhang 2015) that might directly influence the growth of chickpea plants.

B. subtilis-inoculated amelioration of salinity stress in plants was directly linked with efficient nutrient uptake and extrusion of toxic ions, including sodium. The excess salt concentration impeded the uptake of essential mineral elements, including nitrogen, phosphorous and potassium, while B. subtilis was helpful in enhancing the nutrient uptake in chickpea plants. Recently, Bharti et al. (2016) demonstrated that the application of Dietzia natronolimnaea to wheat seedlings grown under salinity stress led to ameliorate oxidative damage by improving the gene expression of important transport proteins, including SOS, NHX, HKT and HAK, which are involved in the exclusion and compartmentalization of toxic ions; the bacterial inoculation also led to increased uptake of essential ions by the seedlings. The higher accumulation of sodium induces osmotic and ionic stress, leading to oxidative damage and affects the regular uptake of potassium, leading to alterations in the K/Na ratio. However, C. arietinum plants inoculated with B. subtilis exhibited an increase in the K/Na ratio, thereby preventing excess sodium accumulation, which might have an impact on the expression of
transport-protein-coding genes. A significant improvement in the uptake of mineral ions, such as nitrogen, phosphorus, potassium and magnesium, in PGPR-treated chickpea plants resulted in higher plant growth through the activation of enzymes and maintenance of transmembrane voltage. Singh and Jha (2016) revealed that B. licheniformis HSW-16 interacts with Triticum aestivum, leading to improved systemic acquired resistance against salinity and to enhanced nitrogen fixation, ammonium assimilation, and potassium and phosphate uptake. The higher accumulation of Na⁺ ions in plants is deleterious to growth and disturbs potassium and calcium mobility within the plant cells, while several reports suggest that the higher K/Na ratio mitigates the salinity-induced stress effects (Wu et al. 2010; Abd_Allah et al. 2015; Hashem et al. 2015; Singh and Jha 2016). In addition, low values of the K/Na ratio due to salt stress enhance the susceptibility of plants and cause alterations in the tissue osmotic potential (Wu et al. 2010). Potassium is an important macro-element involved in several processes, including enzyme activation, stomatal movements and stress tolerance (Ahmad et al. 2014).

Conclusion
The growth and nutrient acquisition of chickpea plants were significantly affected under salinity stress, resulting in the alteration of several physiological and biochemical characteristics. The application of B. subtilis BERA 71 significantly enhanced the growth of chickpea plants by modulating the antioxidant system, leading to the amelioration of salinity-induced oxidative damage. The suppression of ROS generation and of lipid peroxidation and the accumulation of proline in B. subtilis-inoculated plants enhanced membrane stability under salinity stress and non-stress conditions.

Disclosure statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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