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ORIGINAL ARTICLE

Improved salt tolerance of medicinal plant *Codonopsis pilosula* by *Bacillus amyloliquefaciens* GB03

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Abstract The plant growth promoting rhizobacterium (PGPR) strain *Bacillus amyloliquefaciens* GB03, an important soil-borne bacterium, was shown to promote growth and abiotic stress tolerance in *Arabidopsis thaliana* as well as in some crop plants. This study aimed to evaluate the effects of GB03 on salt tolerance in *Codonopsis pilosula,* a traditional Chinese medicinal herb that is sensitive to salinity. Twenty-day-old seedlings of *C. pilosula* were either inoculated with GB03 or without it (as a control). At the same time, plants were treated with NaCl (0, 50, 100, or 150 mM) for 40 days. Growth parameters, photosynthetic indexes, malondialdehyde concentration, and leaf osmotic potential were measured after treatments. The result indicated that GB03 improved plant biomass of *C. pilosula* under salt conditions and improved the photosynthetic capacity by increasing net photosynthetic rate and stomatal conductance and decreasing intercellular CO_2 concentration under both 0 and 50 mM NaCl. The bacterium strain also decreased leaf osmotic potential and peroxidation of membrane lipids that could help the plant adapt to saline environments. This study provides insights into the application of selected bacteria in the culture of important Chinese herbal plants under mild salinity.

Keywords *Bacillus amyloliquefaciens* (GB03) · *Codonopsis pilosula* · Salt tolerance · Photosynthesis · Membrane lipid peroxidation

Abbreviations

TBA	Thiobarbituric acid
MDA	Malondialdehyde
LB	Luria broth
PGPR	Plant growth promoting rhizobacteria
VOC	Volatile organic compounds
Ψ_s	Osmotic potential

Introduction

Soil salinity is a growing threat for agricultural productivity worldwide. It is a major abiotic stress, and frequently one of the serious limiting factors for growing crops (Zhu 2001; Zhang and Shi 2013). First, plants suffer from ion imbalance and osmotic stress caused by high soil salt concentration (Zhang et al. 2010b). Elevated soil salt concentration also reduces the capability of plants to absorb water and negatively affects plant growth by increasing osmotic stress inducing stomatal closure, and reducing leaf expansion and photosynthetic rate (Deinlein et al. 2014; Rahnama et al. 2010). Consequently, salt stress decreases crop yields and leads to continuous loss of arable land. Thus, how to face and deal with the

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challenges that salinity brings is extremely important for agricultural production under changing environmental conditions.

The rhizosphere is a complex zone around roots, in which metabolites from the soil, microorganisms, and roots are inter-mixed (Paré et al. 2011). The microorganisms include both deleterious and beneficial bacteria and fungi that can impact hormone-mediated plant growth and disease susceptibility (Zhang et al. 2007; Ryu et al. 2004). In addition, the identified role of PGPR in growth and resistance to disease, inducible salt, and alkali resistance has been reported (Dardanelli et al. 2008; Han et al. 2014; Zhang et al. 2008a). *Bacillus subtilis* strain GB03, recently renamed as *B. amyloliquefaciens* strain GB03, can be introduced into the soil at the time of planting via seed coating (Choi et al. 2014). GB03 emits a bouquet of volatile metabolites, devoid of classic phytohormones, that are capable of triggering plant growth promotion (Ryu et al. 2003; Paré et al. 2005). These volatile organic compounds (VOCs) were shown to activate differential expression of approximately 600 transcripts related to stress responses, hormone regulation, and other expressed proteins (Ryu et al. 2003; Zhang et al. 2007). GB03 was shown to increase the efficiency of light energy conversion and photosynthesis rate in *Arabidopsis* (Zhang et al. 2008b), which could explain the growth promotion that is observed. Recently, applied field studies utilizing GB03 have demonstrated elevated iron accumulation in cassava (Freitas et al. 2015) and improved wheat (Zhang et al. 2014), white clover (Han et al. 2014), and *Puccinellia tenuiflora* (Niu et al. 2016) tolerance to salinity. To probe GB03-inducible salt tolerance in other glycophytes, a traditional medicinal plant was selected for the current work.

Codonopsis pilosula (Franch.) Nannf., a traditional medicinal herb in China, Korea, and Japan, has many bioactive components, such as polysaccharides, triterpenes, phytosterols, sesquiterpenes, phenolic glycosides, alkaloids, and essential amino acids for humans (Xin et al. 2012; Wang et al. 2013). The herbal tea prepared from the roots of *C. pilosula* is prescribed to fortify the immune, digestive, and hematopoietic systems in the traditional Chinese medicines (Kim et al. 2014; Wang et al. 2013). Nevertheless, *C. pilosula* is sensitive to saline conditions (Kim et al. 2014).

Although strong evidence shows PGPR influence plant performance and stress tolerance, their detailed effects on salt tolerance of traditional Chinese herbal crops have not been explored. Therefore, this study aimed to evaluate the effects of GB03 on *C. pilosula* salt tolerance. The study shows the potential application of beneficial bacterium strains in cultivation of Chinese herbal plants under mild salt conditions.

Materials and methods

Bacterial culture

Bacillus amyloliquefaciens strain GB03 was streaked onto Luria broth (LB) agar plates and incubated under 28 °C and dark for 24 h. Cells were then transferred to liquid LB and cultured under 28 °C with 250 rpm to yield 10^9 colony forming units (CFU) mL^{-1}, as determined by optical density and serial dilutions (Zhang et al. 2008a).

Plant growth and treatments

Codonopsis pilosula seeds were surface-sterilized (soaking for 10 s in 75% (v/v) ethanol followed by 10 min in 5% (v/v) sodium hypochlorite), and then seeds were rinsed with sterile water for five times and stored at 4 °C in a refrigerator overnight. After germination on filter paper, seeds were transferred to plugs (diameter 5 cm, depth 6 cm) on a tray containing autoclave-sterilized commercial vermiculite–soil mixture and watered with half-strength Hoagland solution (5 mM KNO_3, 1 mM $NH_4H_2PO_4$, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 60 μM Fe-citrate, 92 μM H_3BO_3, 18 μM $MnCl_2{\cdot}4H_2O$, 1.6 μM $ZnSO_4{\cdot}7H_2O$, 0.6 μM $CuSO_4{\cdot}5H_2O$, and 0.7 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) every 3 days to keep the soil water content at 60–70% (soil capacity). Each plug (one plant, 20 days old) was inoculated directly with 1 mL bacterial suspension or liquid LB as a control into the vermiculite. At the same time, seedlings were watered with the nutrient solution supplemented with 0, 50, 100, or 150 mM NaCl as salt treatments that were continued every 6 days and with water in the interval to keep the soil water content at 60–70% (Han et al. 2014). Plants were grown in a glass house with additional illumination from metal halide and high-pressure sodium lamps set to a 14/10 h for the light/dark cycle with a total light flux of 800 μmol m^{-2} s^{-1}, an average temperature of 28 ± 2 °C/23 ± 2 °C, and a relative humidity of 70 ± 10%.

Plant biomass and physiological index measurements

Sixty-day-old plants were harvested for plant growth and physiological index measurements. First, net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate of the three largest mature leaves for each plant (the average was calculated as one replication) were measured using a photosynthetic system (GFS 3000, ZQ-WALZ009; Walz, Effeltrich, Germany) in the greenhouse from 09:30 am to 11:30 am. Growth conditions were: photosynthetic available radiation of 800 μmol m^{-2} s^{-1} (saturated light intensity), relative humidity 65 ± 5%, leaf

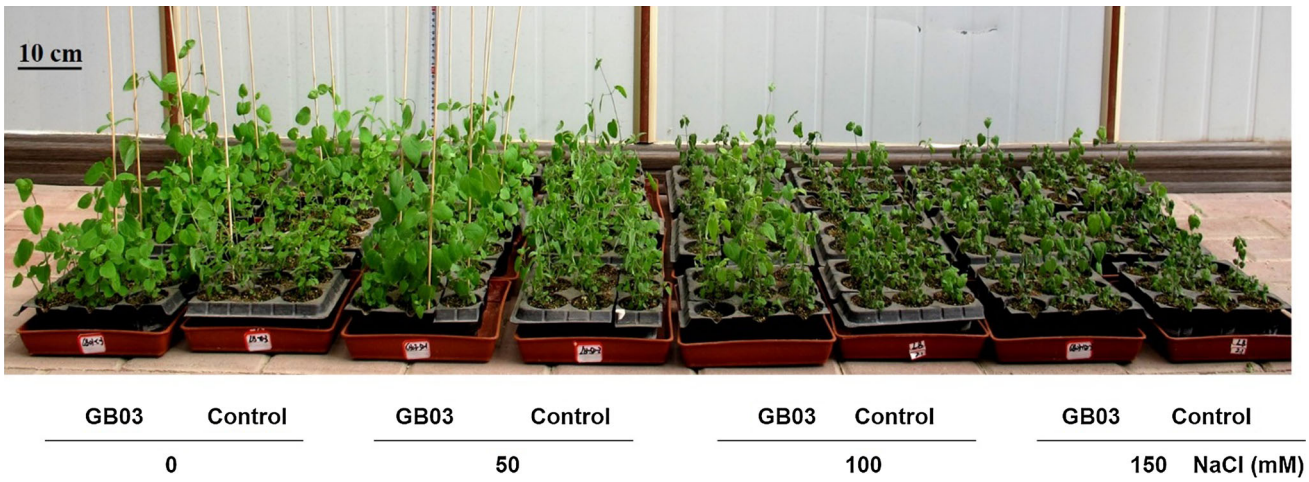

Fig. 1 Picture was taken on the day of measurement and harvest to show the effects of inoculation with GB03 on growth of *C. pilosula* plants under various concentrations of NaCl. Here, GB03 represents *Bacillus amyloliquefaciens* GB03 suspension in LB and 0, 50, 100, and 150 NaCl concentrations (mM)

temperature 28 ± 2 °C, and CO_2 concentration of 360 μmol mol^{-1} (Ma et al. 2012). Sixty-day-old plants were removed from the pots, roots rinsed with water to remove attached vermiculite, and shoot height and root length were measured. Roots (after blotting with tissue paper) and shoots were weighed fresh immediately and then oven-dried at 80 °C for 2 days for dry weight.

To probe oxidative stress, the biomarker malondialdehyde (MDA) was extracted and measured spectro-photometrically using a thiobarbituric acid (TBA) protocol (Bao et al. 2009; Han et al. 2014). Absorbance was detected at 450, 532, and 600 nm using a UV spectrophotometer (UV-2102C, Unico Instrument Co., Ltd, Shanghai, China).

Leaf osmotic potential (Ψs) was measured according to Ma et al. (2012). Fresh leaves were frozen in liquid nitrogen. Cell sap was collected by thawing slowly and then Ψs was measured using a cryoscopic osmometer (Osmomat-030, Gonotec GmbH, Berlin, Germany) under 25 °C. The readings (mmol kg^{-1}) were adopted to calculate the solute potential (Ψs) in MPa with the formula $\Psi s = -\text{moles of solute} \times R \times K$; here, $R = 0.008314$ and $K = 298.8$ (Ma et al.2012).

Data analysis

Means of growth, physiological indexes, and photosynthetic measurements were showed in figures with standard deviation ($n = 8$). Statistical analyses, one-way ANOVA, and Duncan's multiple range tests were performed using the software SPSS 17.0 (SPSS Inc, Chicago, IL, USA).

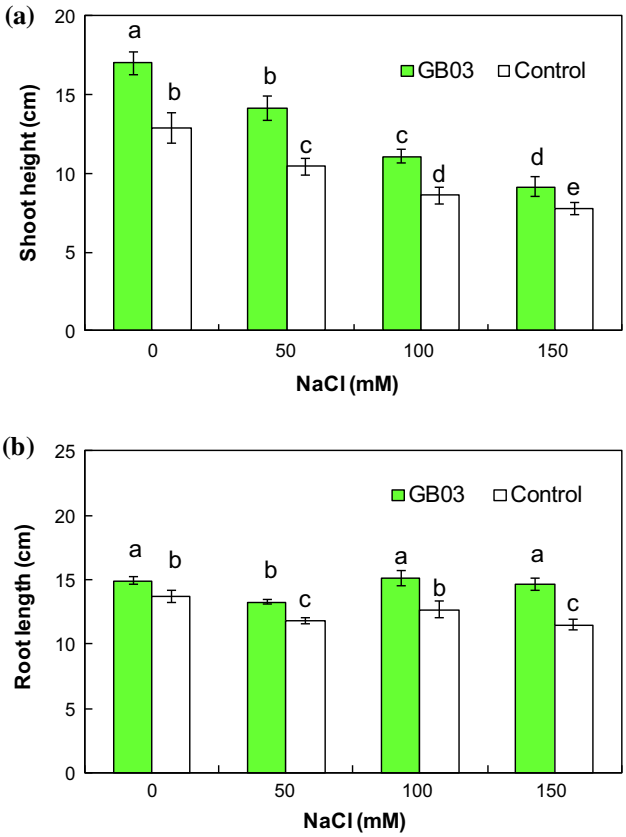

Fig. 2 Effects of GB03 inoculation on shoot height (**a**) and root length (**b**) of *C. pilosula* under various concentrations of NaCl. *Bars* with *different letters* indicate significant differences from the Duncan test ($P < 0.05$). *Bars* represent the mean values and *error bars* represent the standard deviations from eight repetitions

Results

Plant growth

Bacillus amyloliquefaciens GB03 promoted plant growth of *C. pilosula* under various salt conditions (Fig. 1). Shoot height increased significantly by 31.8, 35.4, 28.9, and 17.9% ($P < 0.05$) under 0, 50, 100, and 150 mM NaCl treatments, respectively, compared with corresponding controls (Fig. 2a); GB03 significantly improved the length of roots by 9, 12.4, 19.4, and 27.7% ($P < 0.05$) with 50, 100, and 150 mM NaCl treatments, respectively (Fig. 2b).

GB03 improved plant biomass of *C. pilosula* under various saline conditions. Shoot fresh weight increased significantly by 26.2, 55.1, 34.9, and 46.9% ($P < 0.05$) with 0, 50, 100, and 150 mM NaCl treatments, respectively, compared with corresponding controls (Fig. 3a). Shoot dry weight increased significantly by 27.9, 37.8, 25.9, and 38.2% ($P < 0.05$) with 0, 50, 100, and 150 mM NaCl treatments, respectively, compared to corresponding controls (Fig. 3b). GB03 improved root fresh weight significantly by 66.0, 72.7, and 28.4% ($P < 0.05$) with 0, 50, and 150 mM NaCl, respectively, compared to corresponding controls (Fig. 3c). Root dry weight increased by 117, 69.9, 31.0, and 37.9% with 0, 50, 100, and 150 mM NaCl treatments, respectively, compared with corresponding controls (Fig. 3d).

Photosynthetic parameters

GB03 enhanced net photosynthesis rate by 84 and 61% (Fig. 4a), leaf stomatal conductance with GB03 treatment higher by 190 and 97% (Fig. 4b), and GB03 leaf transpiration rate higher by 70 and 58% ($P < 0.05$) with 0 and 50 mM NaCl treatments (Fig. 4d), respectively. Intercellular CO_2 concentration decreased with GB03 treatment by 39 and 45% ($P < 0.05$) with 0 and 50 mM NaCl, respectively (Fig. 4c).

MDA concentration

It is well known that soil salinity increases the level of reactive oxygen species (ROS) in the leaves of plants, which can be indicated by the concentration of malondialdehyde (MDA), one of the main products of membrane lipid peroxidation (Yazici et al. 2007). GB03 reduced leaf MDA concentration under various saline conditions. Compared with control, GB03 significantly reduced leaf MDA concentration by 41.5% with 100 mM NaCl treatment, although it had no significant effects on leaf MDA concentration with 0, 50, and 150 mM NaCl treatments (Fig. 5).

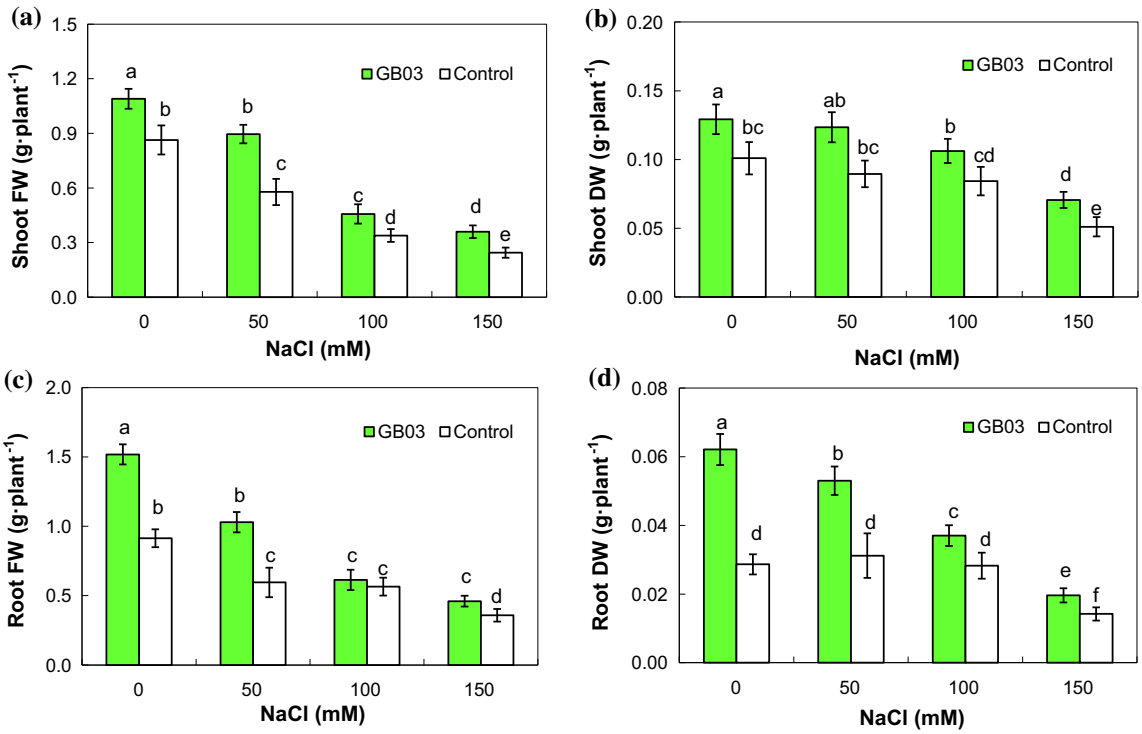

Fig. 3 Effects of GB03 inoculation on plant growth of *C. pilosula* under various concentrations of NaCl: **a** shoot fresh weight, **b** shoot dry weight, **c** root fresh weight, and **d** root dry weight. *Bars* with *different letters* indicate significant differences from the Duncan test ($P < 0.05$). *Bars* represent the mean values and *error bars* represent the standard deviations from eight repetitions

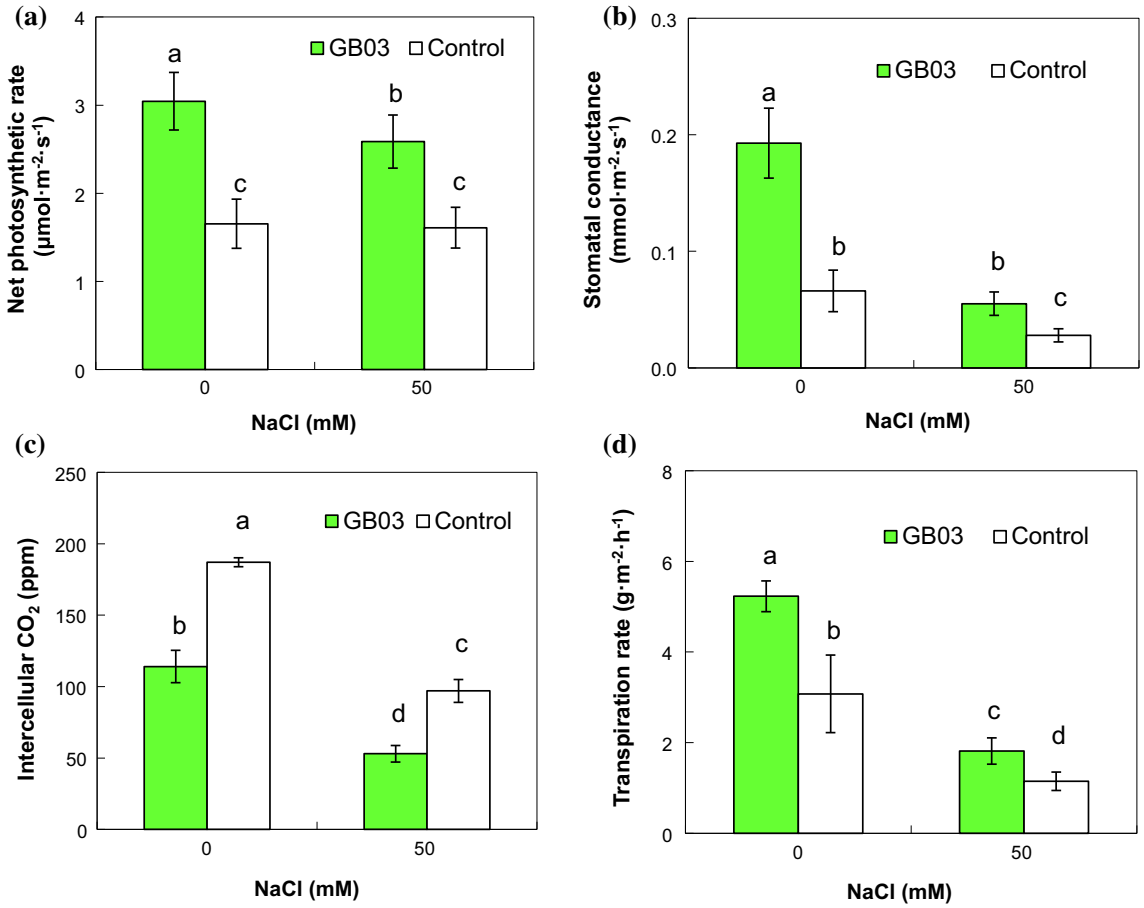

Fig. 4 Effects of GB03 inoculation on photosynthetic parameters of *C. pilosula* under various concentrations of NaCl: **a** net photosynthetic rate, **b** stomatal conductance, **c** intercellular CO_2, and **d** transpiration rate. *Bars* with *different letters* indicate significant differences from the Duncan test ($P < 0.05$). *Bars* represent the mean values and *error bars* represent the standard deviations from eight repetitions

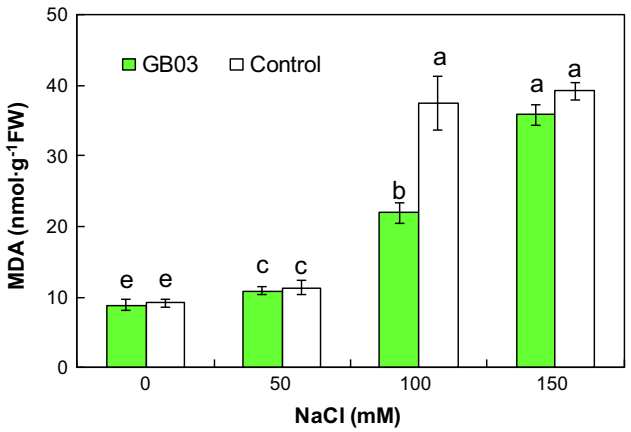

Fig. 5 Effects of GB03 inoculation on leaf malondialdehyde (MDA) concentration in *C. pilosula* grown under various concentrations of NaCl. *Bars* with different letters indicate significant differences from the Duncan test ($P < 0.05$). *Bars* represent the mean values and *error bars* represent the standard deviations from eight repetitions

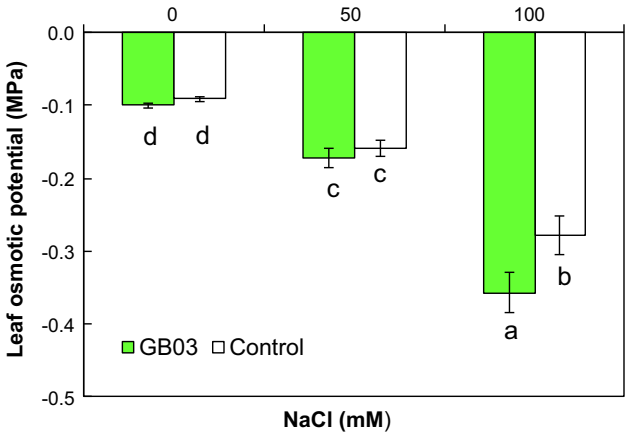

Fig. 6 Effects of GB03 inoculation on leaf osmotic potential of *C. pilosula* grown in various concentrations of NaCl. *Bars* with *different letters* indicate significant differences from the Duncan test ($P < 0.05$). *Bars* represent the mean values and *error bars* represent the standard deviations from eight repetitions

Leaf osmotic potential

Under lower salt concentration (0 and 50 mM NaCl), compared with the controls, GB03 had no significant effects on leaf osmotic potential of *C. pilosula*, although it was decreased by 9.5 and 8.6%. Nevertheless, GB03 decreased leaf osmotic potential significantly by 28% ($P < 0.05$) with 100 mM NaCl treatment (Fig. 6).

Discussion

Plant growth-promoting rhizobacteria were shown to enhance plant performance and increase stress tolerance for *Arabidopsis* and a variety of traditional agricultural crops (Lugtenberg and Kamilova 2013). The mechanism by which GB03 increased growth in *C. pilosula* could be proposed to be analogous to that found in *Arabidopsis*, where GB03 increases cell expansion in shoots by regulating auxin transport (Zhang et al. 2007). Furthermore, Zhang et al. (2008a) and Niu et al. (2016) reported that GB03 conferred growth promotion under salinity through tissue-specifically regulating *AtHKT1;1* in *Arabidopsis*, and *PtHKT1;5*, *PtHKT2;1,* and *PtSOS1* in *Puccinellia tenuiflora*, respectively.

GB03-induced increases in photosynthesis and chlorophyll content in *Arabidopsis* are controlled by decreasing glucose sensing and ABA levels (Zhang et al. 2008b). Although GB03-induced iron and sulfur acquisition may also augment growth in *Arabidopsis* (Zhang et al. 2009; Aziz et al. 2016), as well as in *C. pilosula,* a direct role of increases in these elements in the growth of either species has yet to be established.

Soil salinity decreases plant photosynthetic rates caused by elevated Na^+ inside plants (Zhang and Shi 2013). Net photosynthetic rate, stomatal conductance, and intercellular CO^2 concentration, as important photosynthesis traits, are closely related to photosynthetic capacity (Ma et al. 2012). GB03 augmented photosynthetic capacity by enhancing photosynthetic efficiency and chlorophyll content in *Arabidopsis* (Zhang et al. 2008b). Here, we found that soil inoculation with GB03 conferred an increased net photosynthetic rate, stomatal conductance, and decreased intercellular CO_2 concentration under both 0 and 50 mM NaCl.

Soil salinity increases the levels of reactive oxygen species in plants (Zhang and Shi 2013). MDA, one of main products of peroxidation of membrane lipids, was closely linked to the degree of cell membrane damage (Yazici et al. 2007), and it could be adopted as a physiological indicator for evaluation of plant stress tolerance (Luna et al. 2000). Drought-tolerant *Malus prunifolia* had a better chloroplast structure under drought stress with lower levels of H_2O_2 and MDA than drought-sensitive *Malus hupehensis* (Wang et al. 2012). The decrease in MDA concentration recorded in the PGPR-treated plants links management of lipid peroxidation to better stress tolerance (Miao et al. 2010). In current work, GB03 significantly decreased leaf MDA concentration in *C. pilosula* under saline condition (100 mM NaCl), but had no significant effects under 0, 50, and 150 mM NaCl. We proposed that 0 and 50 mM NaCl are not stressful for *C. pilosula,* but 150 mM NaCl damaged cell membrane very seriously for both GB03 treatment and control plants.

When plants suffer saline or drought conditions, osmotic stress occurs rapidly (Munns and Tester 2008). Plants could adapt to osmotic stress through physiological and biochemical adjustments, like enhancing osmolytes and antioxidant systems (Ma et al. 2012). To enhance stress tolerances and reduce toxic effect of salinity, leaves accumulate osmoprotectants and adjust their osmotic potential ($\approx$leaf water potential) below that in apoplast and soil to ensure continued absorption of water from the soil (Janz and Polle 2012; Ma et al. 2012). GB03 enhanced *Arabidopsis* choline and glycine betaine synthesis that associated with increased osmolyte content, thus increased plant osmotic stress tolerance ability (Zhang et al. 2010a). In current work, GB03 reduced leaf osmotic potential significantly under salt stress condition (100 mM NaCl).

In summary, the results presented in this work established that soil inoculation of *B. amyloliquefaciens* strain GB03 increases plant growth and biomass of the important Chinese medicinal plant *C. pilosula* significantly under various saline soil conditions. GB03 improved photosynthesis under both 0 and 50 mM NaCl. GB03 also decreased leaf osmotic potential and peroxidation of membrane lipids. This study provides physiological evidence that beneficial–bacterial inoculation of medicinal plants grown in soils with moderate salt contamination can protect against salt toxicity.

Author contribution statement The work presented here was carried out by collaboration between all authors. JLZ, PWP, HS, and YQW designed the study. YNW, QQH, RX, QZ, and HRL collected test data, analyzed the data, and drafted the manuscript. JLZ, QQH, and HJG were responsible to interpret the results and complete the paper. PWP, SAK, and SMW revised the manuscript. All the authors read and approved the final manuscript.

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