1 **Efficient rhizobacteria promote growth and alleviate NaCl-induced stress in**
2 **the plant species *Sulla carnosa***

3
4 Nizar HMAEID^{1,2}, Mariem WALI^{1*}, Ouissal METOUI-BEN MAHMOUD¹, José J. PUEYO²,
5 Tahar GHNAYA¹ and Chedly ABDELLY¹

6
7 ¹ *Biotechnology Centre of Borj Cedria, Laboratory of Extremophile Plants, P.O. Box 901, 2050,*
8 *Hammam-Lif, Tunisia.*

9 ² *Institute of Agricultural Sciences, ICA-CSIC, C/Serrano 115-bis, 28006, Madrid, Spain.*

10
11 **Running title:** PGPR enhance salt tolerance capacity of *Sulla carnosa*.

12
13 ***Corresponding author:** Mariem WALI, Biotechnology Centre of Borj Cedria, Laboratory of
14 Extremophile Plants, P.O. Box 901, 2050, Hammam-Lif, Tunisia

15 wali.meriem@gmail.com
16
17
18
19

Abstract

The present work aims to characterize native bacteria from the saline rhizosphere of *Sulla carnosa* and to identify promising rhizobacteria isolates able to ameliorate the salt tolerance of this species. Bacteria were screened *in vitro* for salt tolerance capacity and plant growth promoting characteristics (PGP). Selected NaCl-tolerant bacteria showing a high PGP potential were further characterized for plant promotion effects on the growth of *S. carnosa* under salt stress (200 mM NaCl). Three putative salt-tolerant strains that showed multiple PGP-traits identified as *Acinetobacter* sp. (Br3), *Pseudomonas putida* (Br18) and *Curtobacterium* sp. (Br20) were selected for inoculation study. In a greenhouse experiment, NaCl significantly disturbed physiological parameters in non-inoculated *S. carnosa*. In these plants, NaCl reduced growth, increased foliar proline and malondialdehyde concomitant to Na⁺ shoot concentrations. However, bacterial inoculation with selected PGP isolates ameliorated significantly plant growth and alleviated salt-induced physiological disturbances. Hence, as compared to non-inoculated plants, inoculation provided a significant increase in dry biomass and increased photosynthetic efficiency and chlorophyll leaf content under saline condition. Additional analysis showed that microbial inoculation also enhanced total soluble sugars content and antioxidant enzymes activities thereby preventing reactive oxygen species (ROS)-induced oxidative damage in plants. These results suggest that the inoculation of NaCl-stressed plants with selected salt-tolerant PGPR inocula exert beneficial effects on plant growth by alleviating salt-induced toxicity stress on plant growth and development.

Keywords: NaCl; Salt tolerance; PGPR; Plant growth promotion; *Sulla carnosa*.

1. Introduction

High soil salinity constitutes the most stringent abiotic factor that adversely affecting crop growth and development. Salt-induced disturbances are reported in all stages of plants growth and many physiological and biochemical parameters are vulnerable to NaCl such water status, photosynthesis capacity, mineral nutrition, vigour, stomatal closure, protein synthesis, lipid metabolism and membrane integrity (Parida and Das, 2005; Zhou et al., 2018).

Several biotechnological methodologies to ameliorate salt resistance in plants have been attempted, but these methods are both time and cost-intensive (Grover et al., 2010). Recently, a new ecological approach was carried out for improving soil fertility and alleviating effects of salinity on crop growth by using microbial inoculants such as mycorrhiza and plant growth promoting rhizobacteria (PGPR) (Dimkpa et al., 2009; Etesami and Beattie, 2018; Saghafi et al., 2018). Efficient microbial inoculants can significantly alleviate salinity induced phytotoxic effects and improve plant growth (Orhan, 2016). Previous studies report the beneficial effects of the halotolerant bacteria isolated from saline ecosystems on the alleviation of salt toxicity effects in crop growth. In these studies, the application of PGPR compensates the deleterious effects of salinity on the growth and development of various agronomic crops such as tomato, canola and wheat (Mayak et al., 2004; Ansari et al., 2018; Saghafi et al., 2018). Indeed, those PGPR increased significantly plant biomass production, enhanced chlorophyll content, soluble sugars and elevated the activity of antioxidant enzymes as compared to both control and NaCl-stressed plants. PGP bacteria can be beneficial by improving plant growth through: production of phytohormones such as auxins, cytokinins and gibberellins (Marques et al., 2010; Majeed et al., 2015; Etesami and Beattie, 2018); increasing asymbiotic N₂ fixation that improves root growth

and nutrient acquisition (Dobereiner et al., 2003; Etesami, 2018); solubilization of phosphate, which facilitate plant mineral resources uptake from the soil (Majeed et al., 2015; Karnwal, 2017), and induction of systemic resistance against pathogens by decreasing the inhibitory effects of various pathogens agents on plant growth through the secretion of metabolites, including antibiotics, enzymes and siderophores (Lucy et al., 2004, Etesami, 2018; Karnwal, 2017).

Previous studies showed that rhizobacteria isolated from plants grown in arid and semi-arid areas are often adapted to adverse detrimental conditions and have the potential to promote plant growth under various environmental stresses (Ashraf et al., 2010; Dodd and Perez-Alfocea et al., 2012). Biological methods, such as salt-tolerant plants and microbial bacterial strains, were reported to be effective in reclaiming salt-affected areas (Batarseh, 2017). The use of green manure crops such as salt-tolerant legumes as an alternative strategy to reclaim saline soils results in increase adsorption capacity and nutrient availability, and improved soil physico-chemical properties (Ashraf et al., 2010). One such species of interest is *Sulla carnosa* (*Hedysarum carnosum* Desf) which is an important legume grown as animal feed in salt-affected areas. It is considerate as a facultative halophyte species, and a short-lived perennial legume native to North Africa (McDonald et al., 1991). Native bacteria inhabiting the rhizosphere of *Sulla carnosa* seem to be a promising source for plant growth promoting tool under high salinity condition by exhibiting high salt tolerance capacity and displaying a multiple set of PGP activities.

Therefore, in this research, salt-tolerant bacteria with PGP abilities from a salt-affected rhizosphere were screened and selected, with the aim to identify promising bacteria for possible use to increase salinity stress tolerance of *S. carnosa* plants.

2. Materials and methods

2.1. Bacterial isolation, purification, culture and selection

Bacteria strains were isolated from the rhizosphere of *Sulla carnosa* plants growing in a salt-affected area of Tunisia Sebkha Soliman (35 km northeast of Tunis; latitude 36°42'47.8" N, longitude 10°27'50.3" E; altitude: 1 m above sea level; bioclimatic stage: semi-arid climate; Supplementary Fig. S1) in which plants and microorganisms are affected by natural abiotic stresses, with the aim to select the best performing microbial inoculants. The physico-chemical characteristics of rhizosphere soils were determined. The soil had a pH of 8.62 (1:5 w/v); ECe recorded 32.87 dS m⁻¹; 1.23% organic matter (OM); 0.93% organic carbon (OC); 0.08% Nitrogen (N); nutrient concentrations (g kg⁻¹): Na, 283.56; K, 115; Ca, 1136.43 and Mg, 672.64. Strains were isolated from tightly adhering soil to the roots by serial dilution plating technique on nutrient agar medium (NA) plates and then placed in an incubator (Incucell, Germany) at 28 ± 2°C for 24-48 h. Distinct colony morphologies were randomly selected and streaked onto NA plates to obtain pure cultures, and then stocked in 25% (v/v) glycerol for further studies.

2.2. Salt tolerance capacity

The salinity tolerance of isolates was assessed by evaluating their potential to grow on NA medium containing different concentrations of NaCl: 1% (0.172 M); 2.5% (0.43 M); 5.0 % (0.86 M); 7.5% (1.29 M); 10.0% (1.72 M); and 11.62% (2 M). The cultures were spot inoculated on the respective plates and maintained in an incubator (Incucell, Germany) at 28 ± 2°C for 2 days. Following incubation, the isolates that could grow on medium supplemented with ≥ 5.0% NaCl were considered salt-tolerant strains and were further characterized *in vitro* for PGP attributes.

2.3. Assays for detection of plant growth promoting ability

2.3.1. Indole acetic acid (IAA) production

The capacity of bacterial isolates to synthesize IAA was tested according to the method developed Bric et al. (1991). Bacteria were grown on Luria-Bertani broth and incubated on a rotary shaker with 150 rpm (Protech, Model SI-100D, Malaysia) at $28 \pm 2^\circ\text{C}$ for 24 h. After incubation, the broth was centrifuged, and 1 ml of supernatant was taken and mixed with 2 ml of Salkowski's reagent and kept in the dark for 30 min. The formation of pink colour in test tubes indicated IAA production.

2.3.2. Screening of ACC deaminase activity

Bacterial isolates were screened for 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity by plating on Dworkin and Foster (DF) salt minimal medium containing ACC as the sole nitrogen source (Penrose and Glick, 2003). Bacterial suspensions were spot onto DF plates and placed in an incubator (Incucell, Germany) at $28 \pm 2^\circ\text{C}$ for 3-4 days. The isolates growing well on the ACC-amended plates were considered positive for ACC deaminase activity.

2.3.3. Solubilization and quantitative estimation of inorganic phosphate

Bacterial isolates were assayed for phosphate solubilization ability according to the method of Gupta et al. (1994). Freshly grown cultures were spotted onto a Pikovaskaia's (PVK)-agar medium plates and incubated at $28 \pm 2^\circ\text{C}$ in an incubator (Incucell, Germany) for 5-7 days. Phosphate solubilization was confirmed by apparition a clear zone around a colony. The amount

of solubilized inorganic phosphate by isolates in PVK liquid medium was quantified according to the method described by Nautiyal (1999).

2.3.4. Screening of nitrogen-fixing activity

Nitrogen-fixing ability was detected in isolates by following the assay as described by Dobereiner et al. (1976). Bacterial suspensions were inoculated into test tubes containing N-free liquid medium, and then incubated at $28 \pm 2^\circ\text{C}$ on a rotary shaker (Protech, Model SI-100D, Malaysia) for 5 days. Following incubation, the formation of turbidity in broth indicated N_2 -fixing bacteria.

2.3.5. Siderophore detection

Siderophore activity was detected following the Chrome Azurol S (CAS) assay of Schwyn and Neilands (1987). Bacterial suspensions (10 μl) were spot inoculated on CAS-agar plates and incubated at $28 \pm 2^\circ\text{C}$ in an incubator (Incucell, Germany) for 48 h. Development of yellow orange halo zone around the colony indicated siderophore production.

2.4. Genetic characterization of isolates

Genomic DNA was extracted using the illustra Bacteria genomicPrep Mini Spin Kit (IllustraTM, Buckinghamshire, UK) according to the manufacturer's protocol. The polymerase chain reaction (PCR) was used to amplify DNA fragments (50 ng) from the isolates that showed high PGP activities in screening assays. The 16S rDNA gene was assayed using universal primer set: forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5'-

GGTTACCTTGTTACGACTT-3'). Amplified PCR products were separated by electrophoresis on 1.5% agarose gel in 1 X TAE buffer and stained using ethidium bromide (EtBr) for visualization of DNA fragments. The PCR products were excised and purified using a PCR purification kit (Illustra™, Buckinghamshire, UK) and then were sequenced using an automatic DNA sequencer (ABI 310 DNA, Applied Biosystems, USA). Sequence similarity data was obtained using the BLAST analysis tool of NCBI Blast servers (<http://www.ncbi.nlm.nih.gov>). The 16S rDNA sequences were deposited in the GenBank database of NCBI to receive accession numbers.

2.5. Planting assay and inoculation

A pot experiment was carried out to evaluate the effect of three promising isolates (Br3, Br18 and Br20) on growth and physiological and biochemical parameters of *Sulla carnosa* plants grown under saline and non-saline conditions. Selection of isolates was performed on the basis of their salt tolerance ability and PGP features. *Sulla* seeds were surface-sterilized with 30% hydrogen peroxide and 70% ethanol (1:1) for 10 min, followed by several rinses with demineralised sterile water. Sterilized seeds were then sown in plastic pots (15 cm diameter, 20 cm depth) each filled with 2 kg of autoclaved soil (100°C for 1 h on 3 consecutive days). Soil in the pot experiments used for plants growing was a loam-silt (20% clay, 58% silt, and 22% sand) in texture, and collected from the surface horizon (0-40 cm) in Borj Cedria region, Hammam-Lif, Tunisia (latitude 36°42'35" N; longitude 10°25'45" E). The chemical characteristics of pre-treated saline soil and the post-treated soil used in this study were determined (Table 3). At the early-four-leaf stage seedlings, pots were inoculated with the appropriate inoculums by adding 1 ml of

pure bacterial cultures (10^8 cfu ml⁻¹) close to the root system or with distilled water for the negative control plants. After a pre-treatment period of 21 days, plants were exposed to salt stress through the application of 50 ml of saline solution (200 mM NaCl) or 50 ml of sterilized tap water for control plants (normal condition). The experiment was performed using a completely randomized block design, with five replicates (pots) per treatment. Plants were grown in a controlled growth chamber 22-18°C with 16-8 h day/night photoperiod (relative humidity 70%; photon flux intensity 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Harvesting occurred after 4 weeks of salt treatment. The plant biomass, nutrition, photosynthetic pigments, photosynthetic efficiency, electrolyte leakage (EL), proline, malondialdehyde (MDA), total soluble sugars (TSS) and antioxidant (Superoxide dismutase: SOD; Catalase: CAT; Glutathione peroxidase: GPX and Ascorbate peroxidase: APX) activities were evaluated.

2.6. Plant analysis

2.6.1. Plant growth and mineral nutrition

After harvest, *Sulla* plants were divided into roots and shoots and fresh weights were recorded. Samples were then oven-dried at 60°C for 72 h for dry weight (DW) determination. To determine ions concentration, dry matter (100 mg) was homogenized and digested in 5% HNO₃ solution and then the obtained extract was filtered with Whatman filter paper N°1. The element concentrations sodium (Na⁺) and potassium (K⁺) were determined in the filtrate by flame photometry (Corning, Cambridge, UK).

2.6.2. Total chlorophyll and photosynthesis efficiency

Photosynthetic pigments were determined in fully expended young leaves. Chlorophyll was extracted from homogenised fresh leaves (100 mg) in 5 ml of 80% (v/v) acetone solution, incubated at 4°C for 72 h, and then the homogenate was centrifuged at 500 x g for 5min. The amount of total chlorophyll content (Chl T) was determined at 645 and 663 nm using a spectrophotometer (Model Cary 60; Agilent Tech., Santa Clara, CA, USA) and calculated according to the equation of Lichtenthaler and Welburn (1983).

$$\text{Total chlorophyll (mg g}^{-1}\text{ FW)} = ((20.2 \times A_{645} + 8.02 \times A_{663}) \times V) / (W \times 1000)$$

Where A_{663} = Absorbance of the extract at 663 nm; A_{645} = Absorbance of the extract at 645 nm; V = Volume of the extract (5 ml); W = Leave fresh weight (100 mg).

Chlorophyll a fluorescence was performed in dark-adapted leaves (30 min) using a portable fluorimeter (OS1-FL, Opti-Sciences Inc., Hudson, NH, USA) as described by González-Moro et al. (1997). Photosynthesis efficiency was calculated as: $F_v/F_m = (F_m - F_0)/F_m$; where F_0 = minimum fluorescence; F_m = maximum fluorescence; F_v = variable fluorescence ($F_m - F_0$).

2.6.3. Shoot proline content, lipid peroxidation and electrolyte leakage

The method of Bates et al. (1973) was used to quantify the proline content. Fresh leaves (0.5 g) were homogenised with 3% sulfosalicylic acid (Sigma-Aldrich, Steinheim, Germany) and incubated at 100°C in a shaking water bath (Ratek, Model SWB20D, Australia) for 30 min, and then allowed for cool. Following incubation, 2 ml of extract was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid. The obtained reaction mixture was incubated at 100°C

in a boiling water bath for 1 h. After cooling to room temperature, 4 ml of toluene was added to reaction mixture tubes and stirred well until obtaining two phases. The amount of proline was quantified by measuring the absorbance of the chromophore phase at 520 nm using a spectrophotometer (Model Cary 60; Agilent Tech., Santa Clara, CA, USA).

Lipid peroxidation was estimated as thiobarbituric acid reactive substances (TBARS) following the method of Heath and Packer, (1968). Fresh root samples (0.5 g) were homogenised in 10 ml of 0.1% trichloroacetic acid (TCA), centrifuged at 15 000 x g for 15 min, and then 1 ml aliquot of the supernatant was mixed with 4 ml of 0.5% thiobarbituric acid (TBA). The reaction mixture was incubated at 95°C in shaking water bath (Ratek, Model SWB20D, Australia) for 30 min, cooled in an ice bath and centrifuged at 10000 x g for 10 min. The absorbance of supernatant was measured at 532 nm using a spectrophotometer (Model Cary 60; Agilent Tech., Santa Clara, CA, USA) to estimate the level of lipid peroxidation (MDA) in plant tissues.

Cell membrane integrity was determined by the electrolyte leakage (EL) level according to the method described by Shi et al. (2006). Fresh uniform leaf discs (10 mm) were incubated in vials filled with 30 ml of de-ionized water, and then incubated at room temperature for 24 h. The initial electrical conductivity of the solution (EC1) was recorded using a conductivity meter (Model EC-Metro Basic 30, Crison Instruments, SA., Barcelona, Spain), samples were incubated at 95°C for 20 min in a shaking water bath (Ratek, Model SWB20D, Australia), cooled to room temperature, and then the final conductivity (EC2) of the bathing solution was recorded. The electrolyte leakage (EL) was calculated according to the following formula:

$$EL (\%) = (EC1 / EC2) \times 100$$

2.6.4. Estimation of total soluble sugar (TSS) content

TSS content was performed by the anthrone reagent assay (Shields and Burnett, 1960). Freshly leaves (0.5 g) were homogenized in 5 ml of 95% (v/v) ethanol solution, and then extracts were centrifuged at 3500 x g for 10 min. An aliquot (0.1 ml) of supernatant was mixed vigorously with 3 ml of anthrone reagent, incubated at 100°C in a shaking water bath (Ratek, Model SWB20D, Australia) for 10 min, and then allowed to cool in an ice water bath. TSS content was proportional to the intensity of the developed green colour in test tubes and measured at 625 nm by a spectrophotometer (Model Cary 60; Agilent Tech., Santa Clara, CA, USA).

2.6.5. Antioxidant enzymatic activities

Antioxidant enzymatic activities were determined as described by Tejera et al. (2004). Frozen leaf samples (1 g) were homogenised with 4 ml mM of potassium phosphate buffer (pH 7.2). The homogenates were centrifuged at 18,000 x g for 10 min (4°C) and collected supernatants were used for the assays of enzymatic activities.

The activity of CAT (E.C. 1.11.1.6) was carried out by following the consumption of H₂O₂ according to the method described by Patterson et al. (1984). The amount of catalyzed enzyme was calculated at 240 nm using a UV-visible spectrophotometer (Model Cary 60; Agilent Tech., Santa Clara, CA, USA). The APX activity (E.C. 1.11.1.11) was determined as described earlier by García-Limones et al. (1980). The amount of ascorbate oxidized was estimated by the decrease in absorbance at 290 nm. The GPX activity (E.C. 1.11.1.7) was measured according to the procedure of Paglia and Valentine (1967) using H₂O₂ as a substrate. The activity of SOD

(E.C. 1.15.1.1) was assayed photochemically at 540 nm by inhibition of 50% of nitro blue tetrazolium (NBT) production according to the method of Spitz and Oberly (1989).

2.7. Statistical analyses

Statistical analysis was performed using SPSS Statistical Software Package (SPSS inc., V18.0). One-way analysis of variance (ANOVA) was employed followed by Duncan's Multiple Range Test (DMRT) to determine the significant difference among means of the treatment at $P < 5\%$. Different letters are used to indicate significantly different means.

3. Results

3.1. Isolation of bacteria and morphological characterization

A total of 26 rhizobacterial isolates were successfully isolated from the rhizosphere of the natural habitat of *Sulla carnosia* and characterized morphologically. Most of the isolates showed similar colony morphology which were white, yellow, or creamy on medium plates. The colony size of the isolates reached 1-5 mm in diameter. Microscopic examination revealed a heterogeneous bacterial collection which regrouped Gram-negative and Gram-positive bacillus and coccus bacterial genera.

3.2. Salt tolerance capacity and plant growth promoting abilities

To study the salt (NaCl) response of the different selected bacteria, all colonies were grown on nutritive agar-based medium added with different concentration of salt. Of the tested bacterial strains, most isolates could grow normally at 5% NaCl (w/v). However, only the high levels of

NaCl (10% = 100 g/L) repressed the growth of 11 bacteria isolates. Nevertheless, some isolates have the ability to tolerate this elevated salt concentration 1.72 M NaCl (Fig. 1). Based on the capability to grow at 10% of NaCl, the tested bacteria were classified as moderate tolerant ones (+), high tolerant (++) or strong tolerant ones (+++).

On the other hand, based on the NaCl screening, among the 26 salt-tolerant isolates, seven isolates showed salt tolerance up to 10% NaCl were selected and screened for PGP properties (Table 1). Phosphate solubilizing capacity screening data showed that only 3 isolates showed clear halo zones around the colonies growth of size ranging from 4.0 to 23.0 mm. For the range of tricalcium phosphate solubilization, the quantitative estimate reached 124.8, 421.6 and 673.3 $\mu\text{g ml}^{-1}$ respectively with Br10, Br20, and Br18 (Table 1). IAA synthesis of the isolates ranged from 0.025 to 5.145 $\mu\text{g ml}^{-1}$. The highest IAA production was recorded in isolate Br20 (5.145 $\mu\text{g ml}^{-1}$), whereas Br18, the most efficient P-solubilizer, produced only 0.157 $\mu\text{g ml}^{-1}$ IAA (Table 1). Siderophore detection assay revealed that 6 bacterial isolates are able to produce siderophores on CAS medium. Among them, the strain Br18 showed the maximum production. The characterization of N-fixing ability showed that only four strains had this capacity. Out of the seven isolates screened for ACC deaminase activity, only five strains were considered positive for ACC activity (Table 1).

For greenhouse experiment, three bacterial isolates Br3, Br18, and Br20 were selected based on both their high salt tolerance capacity and displaying a promising set of PGP attributes (Fig. 1, Table 1).

3.3. Molecular identification of bacterial inoculants

The obtained sequenced of 16S rDNA gene were analyzed by comparison with sequences in GenBank through Nucleotide BLAST search. Comparison data revealed that bacterial isolate Br3 showed 99% similarity with *Acinetobacter* species; whereas Br18 showed 100% similarity with *Pseudomonas putida* and strain Br20 showed 99% similarity with *Curtobacterium* species. The sequences of bacterial isolates Br3, Br18 and Br20 were deposited in the GenBank database and accessions numbers were assigned as MF59363, MF680672, and MF680834, respectively.

3.4. Plant growth, chlorophyll concentrations and photosynthesis efficiency

The bacterial isolates Br3, Br18, and Br20 were selected and further screened for plant promotion effects on the growth of *Sulla carnosa* plants under salt stress condition. The results indicated that salt stress significantly affected the growth of *S. carnosa*, with clear visual signs of plant damage. Salt-stress plants showed a significant reduction in biomass production (43.74%) as compared to non-stressed plants (Fig. 2A). Hence, the use of selected microbial inoculants enhanced significantly biomass production under both normal and saline conditions. Moreover, the data also show that all inoculated plants cultivated in the presence of salt stress produced significantly more biomass as compared to those cultivated under the same condition but without inoculation with any bacteria (Fig. 2A).

In non-inoculated and inoculated plants, NaCl also affected the photosynthetic parameters. Therefore, salt-induced significant decrease of this parameter chlorophyll concentration in the leaves (Fig. 2B) as well as the Fv/Fm values (Table 2). However, our findings show also that the salt effect on chlorophyll content was more accentuated in non-inoculated plants in which this

decrease reached 50% as compared with control plants, but this decrease does not exceeded 30% in salt-stressed plants that were inoculated with selected microbial isolates. Moreover, inoculation alleviated also the negative effect of NaCl on the values of Fv/Fm (Table 2).

Hence we suggest that the positive effect of inoculation with *Acinetobacter* sp. (Br3), *Pseudomonas putida* (Br18) and *Curtobacterium* sp. (Br20) on plant growth and biomass production under saline condition could be directly related to the alleviation of NaCl-induced reduction of chlorophyll concentration and disturbances of photosynthesis machinery.

3.5. Sodium and potassium concentrations

The analysis of plant nutrient status showed that salt stress strongly decreased K⁺ uptake and increased Na⁺ content in plant tissues (Fig. 3). By contrast, inoculation of NaCl-stressed plants with PGP isolates Br3, Br18 and Br20, significantly reduced Na concentration but enhanced K accumulation in the shoots. These data suggested a selective effect of bacteria on the absorption of K under saline condition.

3.6. Lipid peroxidation, proline concentration, and electrolyte leakage

The effect of NaCl-stress on lipid peroxidation and membrane integrity was evaluated by the determination of MDA and electrolyte leakage. Data showed that saline condition, NaCl-induced oxidative stress damage by increasing MDA content concomitant with elevated EL percentages (Table 2). However, bacterial inoculation significantly reduced MDA and EL levels. This suggests a protective effect induced by bacteria against salt-induced membrane disturbances. The data presented in table 2 also show that NaCl-induced salinity significantly increased the

348 biosynthesis of proline. The high levels of EL, MDA, and proline accumulated during salt
349 treatment induced osmotic stress that caused cell damages by increasing the permeability of cell
350 membranes. However, bacterial inoculation with Br3, Br18, and Br20 was effective in alleviating
351 NaCl-induced stress in *Sulla* plants.

353 3.7. Total soluble sugars (TSS) content

354 Analysis of TSS assay revealed that untreated control plants presented the lowest content of
355 TSS (Table 2). In the absence of NaCl, inoculation with PGP bacteria induced a significant
356 increase in soluble sugars content in *Sulla carnos*a. However, under saline condition, TSS
357 contents were considerably higher in inoculated plants, as compared to stressed controls, and as a
358 result, plant growth and biomass production improved (Table 2).

360 3.8. Effects of microbial inoculation and saline condition on antioxidant enzyme activities

361 Data analysis showed that NaCl-stress induced an increase of the APX, CAT, SOD and
362 GPX activities respectively by 76%, 118%, 13% and 110% as compared to untreated control
363 plants (Fig. 4). However, under non-saline condition, the antioxidant enzyme activities of *Sulla*
364 plants inoculated with the isolates *Acinetobacter* sp. (B3), *Pseudomonas putida* (Br18), and
365 *Curtobacterium* sp. (Br20) were also increased. In NaCl-stressed inoculated plants, the
366 antioxidant enzymes showed the most important activities (Fig. 4). Our results suggest the
367 enhanced accumulation of antioxidant enzymes in the leaves of salt-stressed plants may increase
368 salt tolerance capacity, leading to improved plant growth and development.

3.9. Effect of microbial inoculation on soil property under saline condition

The chemical properties of pre-treated and post-treated soils used in pot experiment were determined (Table 3). Data showed that under non-saline condition, bacterial inoculation induced a slight reduction in EC and Na concentration in soil. Salt treatment induced significant and important increase in EC, pH and Na values in soil. These effects were essentially pronounced in the absence of inoculation because we showed an alkalization of soil by a 1.2 unit of pH and the EC increased from 1.14 to 18.54 dS⁻¹ at the end of experiment.

Indeed, under normal condition inoculation with *Acinetobacter* sp. (B3), *Pseudomonas putida* (Br18) and *Curtobacterium* sp. (Br20) did not induce a significant modification of pH values but it reduced the soil EC (Table 3). However, when plants were subjected to salt, inoculation was responsible to the maintain of pH at values close to those of non-saline conditions, essentially due to induced acidification by these bacteria. Also, inoculation induced a decrease in the EC of soil probably by the reduction of NaCl concentration in the rhizosphere. Taken together, these ameliorations of soil proprieties induced by the inoculation could be directly related to the mitigation of NaCl-induced damage in the plant test, *S. carnosus*.

4. Discussion

NaCl-induced salinity adversely affects plant growth and development, which leads to a gradual decline in crop productivity (Rockström and Falkenmark, 2000; Rahnesan et al., 2018; Saghaei et al., 2018). The use of PGPR is a potential biological tool for alleviating abiotic constraint including salinity, to ameliorate plant productivity under these constraints (Shrivastava and Kumar, 2015; Etesami and Beattie, 2018).

In the current study, we demonstrated that, although it was considered as halophyte species, NaCl-induced salinity significantly affected the growth of *S. carnos*, and salt-treated plants presented a significant decrease in biomass production and photosynthesis parameters as compared with the non-saline condition (Fig. 2, Table 2). The effect of salt was also related to an increase in foliar Na⁺ concentration (Fig. 3), which caused damage to cell membranes as measured by increased MDA contents (Table 2). In addition to this toxic effect of NaCl, we showed also that this salt-induced an osmo-perturbation leading to an important accumulation of free proline (Table 2), which suggests playing an important role in osmo-regulation process (Ahmad et al., 2012). However, our study demonstrates that following inoculation with hyper-tolerant strains to NaCl *Acinetobacter* sp. (Br3), *Pseudomonas putida* (Br18) or *Curtobacterium* sp. (Br20) and selected from the natural soil habitat of *S. carnos*, we showed a significant decrease of all detrimental effects of NaCl on the growth and physiological and biochemical parameters (Fig. 2, Table 2), suggesting that the use of salt-tolerant microbial inoculants alleviate the NaCl-induced growth disturbances in this species. In this context, previous studies reported that inoculation with PGPR mitigate salinity induced negative effects on growth of various agricultural crops (Ahmad et al., 2012; Karnwal, 2017; Ansari et al., 2018; Etesami, 2018). The Plant-Bacteria association depend on several parameters related to the adaptation of the introduced PGPR inoculants with the root environment (root exudates and metabolites), and environmental conditions (Egamberdieva et al., 2017). Hence, we cannot introduce salt-sensitive inoculants to ameliorate the productivity of plant in salt-affected soils or highly sensitive bacterium to heavy metals to remediate contaminated soils. In our work, we selected a highly salt-tolerant PGP rhizobacteria to ameliorate NaCl tolerance in *Sulla carnos* (Fig. 1, Table 1).

Hence, it has been demonstrated that salt-tolerant PGP bacteria isolated from alkaline soil, are physiologically adapted to abiotic stresses and could increase salt tolerance capacity and the growth of various crop plants, such as tomato, maize, and pepper in saline soil conditions (Mayak et al., 2004; Ferreira et al., 2018; Yasin et al., 2018). In fact, efficient PGPR used to alleviate NaCl-induced stress must be obligatory resistant to this constraint, or their development will be inhibited by salt (Sharma et al., 2015; Upadhyay and Singh, 2015; Chanratana et al., 2017).

In the first step of our work, we demonstrated the high tolerance of used bacteria selected from the natural salt habitat of *S. carnosus*. These bacteria were able to grow at salt concentration exceeding 5% (Fig. 1, Table 1). The possible mechanism that allows this tolerance to salt, halotolerant bacteria are able under elevated salt concentration to use microbial adaptive responses to high-osmolarity stress by the accumulation of compatible solutes either synthesis or uptake (Aslam and Ali, 2018; León et al., 2018).

When inoculated with selected halotolerant bacteria, *Acinetobacter* sp. (Br3), *Pseudomonas putida* (Br18) or *Curtobacterium* sp. (Br20), *S. carnosus* plants showed a better resistance to the presence of 200 mM NaCl. This amelioration of plant resistance to salt by these bacteria was evident when evaluating the impact of salt on growth and photosynthesis. We demonstrated that the three bacteria alleviated significantly the negative impact of NaCl on these parameters (Fig. 2, Table 2).

Several studies have tried to analyze the mechanisms by which bacteria can improve the resistance in plants. Hence, some research shows that the use of PGP bacteria can promote plant growth and enhance salt tolerance capacity by inducing synthesis of osmolytes (Siddique et al., 2011; Chanratana et al., 2017; Karnwal, 2017; León et al., 2018). Our results revealed that after

evaluation, we selected *Acinetobacter* sp. (Br3), *Pseudomonas putida* (Br18), and *Curtobacterium* sp. (Br20) because they exhibited multiple PGP features, such as N₂-fixation, phosphate solubilization, IAA synthesis, ACC deaminase and siderophores production (Table 1). Our findings are consistent with previous studies that demonstrating the beneficial effects of inoculation by PGP bacterial isolates in inducing salt tolerance capacity and improving the biomass production of various crops, such as barley, pepper and maize (Hmaeid et al., 2014; Maxton et al., 2017; Aslam and Ali, 2018).

NaCl-induced soil salinity causes many adverse effects on plant growth, due to a low osmotic and specific ion injury as well as to nutritional disorders that result in reduced plant growth (Essa et al., 2002; Zhou et al., 2018). In this study, 200 mM NaCl increased Na⁺ and decreased K⁺ concentrations in *Sulla* plants (Fig. 3). However, with the bacterial application, K⁺ concentration increased significantly (Fig. 3C and 3D), whereas Na⁺ concentration decreased significantly (Fig. 3A and 3B). Similar results were also reported by Kohler et al. (2006), Yildirim et al. (2011), Etesami and Beattie (2018), and Yasin et al. (2018) that inoculation with PGP isolates improved the mineral uptake, chlorophyll content and plant growth under saline conditions. PGP microbial inoculants enhanced plant growth under high-NaCl condition by the production of plant growth regulators in the vicinity of rhizosphere, thereby improving root system development and nutrient acquisition (Dimkpa et al., 2009; Li and Jiang, 2017).

Therefore, our study suggests that bacterial inoculants Br3, Br18 and Br20 (*Acinetobacter* sp., *Pseudomonas putida*, and *Curtobacterium* sp., respectively) were selected on the basis of their salt tolerance capacity and exhibiting multiple PGP features might positively affect the growth of plant under high salinity condition. In addition, this study indicates that PGP isolates

458 have the potential to promote plant growth under NaCl-stress through direct mechanism, and also
459 improve saline soil properties. We showed that all bacterial strains induced reduction in EC_e, Na
460 and alkalized the soil pH as compared with that serving to plant culture without inoculation
461 (Table 3). A lowering of pH is the direct result of soil acidification by PGP-induced secretion of
462 organic acids in the rhizosphere (Rodriguez et al., 2004; Wu et al., 2006; Hassan et al., 2017).
463 Phosphate solubilization and mineralization by bacterial strain can also helps plant to absorb
464 mineral because it induce more available pool of nutrient as phosphate and maintain adequate pH
465 around neutral by proton extrusion (Wu et al., 2006; Tao et al., 2008). Furthermore, Ashraf et al.,
466 (2004) and Ilangumaran and Smith, (2017) noted that efficient microbial inoculants could protect
467 their host plants against toxic accumulation of ions. Hence, PGP-bacteria can reduce Na and Cl
468 available for plant uptake by increasing the synthesis of osmoprotectors in plant tissues and
469 producing exopolysaccharides in roots that bind sodium in soil. Previous studies have also
470 reported that bacterial inoculants release metal-chelating substances in the host plant rhizosphere,
471 such as iron-chelating siderophores may promote growth and enhance stress tolerance capacity
472 by influencing the plant the uptake and bioavailability of various metal ions (Dimkpa et al.,
473 2009). The use of ACC deaminase producing PGP-bacteria could be effective in promoting the
474 growth of a variety of plants under various stressful conditions, such as flooding, heavy metals,
475 high salinity and drought (Ghnaya et al., 2015; Chanratana et al., 2017; Maxton et al., 2017) by
476 reducing the levels of the stress-hormone ethylene (Khan et al., 2018; Yasin et al., 2018).
477 Selected isolates appear to have several PGP activities (Table 1), such as the production of IAA,
478 siderophores, nitrogen fixation, P-solubilization, and ACC deaminase activity which might
479 explain its ability to mitigate salt stress on *Sulla carnosa* plants.

The inoculation with bacterial isolates induced significant improvement of photosynthetic pigments, photosynthesis efficiency and TSS production under salt constraint (Fig. 2, Table 2). Similar results reported that photosynthetic pigments accumulation was enhanced in PGPR-inoculated plants, such as barley, soybean, and maize under saline stress conditions (Hmaeid et al., 2014; Kang et al., 2014; Aslam and Ali, 2018). TSS accumulation constitutes an adaptive response by plants modulating the redox balance in cells, chloroplasts integrity, vacuole membrane function and stomatal opening (Nadeem et al., 2007; Desale et al., 2013; Li and Jiang, 2017). High-NaCl induces cellular accumulation of reactive oxygen species (ROS), which induces oxidative damage of membrane lipids, proteins and nucleic acids (Khan et al., 2017; Li and Jiang, 2017; Yasin et al., 2018). Plants have evolved an efficient antioxidant defense system comprising enzymes, which play an important role in protecting plants against oxidative damage (Apel and Hirt, 2004; Hichem et al., 2009; Zhou et al., 2018). This present research showed enhanced activities of antioxidant enzymes (APX, CAT, SOD, and GPX) in leaves of inoculated *Sulla* plants as compared to control NaCl-stressed plants (Fig. 4). Our funding was supported by those of Gururari et al.(2013), Etesami and Beattie (2018), and Santos et al. (2018), who reported that the activities of ROS-scavenging enzymes, such as APX, CAT, GPX, GR, and SOD, were significantly enhanced in various agricultural crops inoculated by PGP microbial isolates under saline conditions.

NaCl-induced oxidative stress was evident in our experiment since plants subjected to salt showed elevated concentration of MDA and high percentage of EL (Table 2). However, the use selected microbial strains reduced significantly the MDA and EL levels in NaCl-stressed *Sulla* plants (Table 2). Similarly, other findings showed that PGPR enhanced plant salt resistance by

reducing the damage induced by ROS and protect the integrity and stability of cellular tissues from the detrimental effects of salt stress (Garg and Manchanda, 2009; Hmaeid et al., 2014; Singh and Jha, 2017; Yasin et al., 2018). Furthermore, the induction of proline in salt-stressed *Sulla* plants, which represent oxidative stress, might indicate cell membrane damage (Table 2). However, bacterial inoculation significantly decreased proline content (Table 2). Our results are similar to other findings who reported that the enhanced salt tolerance capacity of plants was correlated with PGP bacterial inoculation that decreases proline and MDA concentrations concomitantly with increased levels of ROS-scavenging enzymes, as well as the accumulation of TSS, which function as osmoregulants, and as a result, reduced cell membrane damage and enhanced biomass production (Li and Jiang, 2017; Etesami and Bettie, 2018; Yasin et al., 2018).

5. Conclusion

Based on these findings, selected salt-tolerant isolates *Acinetobacter* sp., *Pseudomonas putida* and *Curtobacterium* sp. used for inoculation present a good PGP potential in enhancing growth and inducing salt tolerance capacity of *Sulla carnosia* through direct or indirect mechanisms. Thus, these microbial inoculants could be an effective bio-source for promoting the growth of salt-sensitive crops.

Acknowledgments

This work was supported by the Tunisian Ministry of Higher Education and Scientific Research LR15CBBC02 and by the Spanish Institute of Agricultural Sciences (ICA-CSIC). All authors thank scientific and technical staff of the two collaborating research groups for valuable advice and technical help.

Conflict of interest

The authors declare no conflicts of interest.

References

- Ahmad, M., Zahir, Z.A., Asghar, H.N., Arshad, M., 2012. The combined application of rhizobial strains and plant growth promoting rhizobacteria improves growth and productivity of mung bean (*Vigna radiate* L.) under salt-stressed conditions. *Annals of Microbiology* 62(3), 1321-1330.
- Ansari, F.A., Ahmad, I., 2018. Plant growth promoting attributes and alleviation of salinity stress to wheat by biofilm forming *Brevibacterium* sp. FAB3 isolated from rhizospheric soil. *Saudi Journal of Biological Sciences*. <https://doi.org/10.1016/j.sjbs.2018.08.003>. In Press
- Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* 55(1), 373-99.
- Ashraf, M., Hasnain, S., Berge, O., Mahmood, T., 2004. Inoculating wheat seedlings with exopolysaccharide-producing bacteria restricts sodium uptake and stimulates plant growth under salt stress. *Biology and Fertility of Soils* 40(3), 157-162.
- Ashraf, M.Y., Ashraf, M., Mahmood, K., Akhter, J., Hussain, F., and Arshad, M.: Phytoremediation of saline soils for sustainable agricultural productivity, in: *Plant Adaptation and Phytoremediation*, Springer, 335-355.
- Aslam, F., Ali, B., 2018. Halotolerant Bacterial Diversity Associated with *Suaeda fruticosa* (L.) Forssk. improved growth of maize under salinity stress. *Agronomy* 8(8), 131.
- Batarseh, M., 2017. Sustainable management of calcareous saline-sodic soil in arid environments: The leaching process in the Jordan valley. *Applied and Environmental Soil Science* 2017, 1-9.
- Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil* 39(1), 205-207.

Bric, J.M., Bostock, R.M., Silverstone, S.E., 1991. Rapid *In Situ* Assay for Indoleacetic Acid Production by Bacteria Immobilized on a Nitrocellulose Membrane. *Applied and Environmental Microbiology* 57(2), 535-538.

Chanratana, M., Han, G.H., Roy Choudhury, A., Sundaram, S., Halim, M.A., Krishnamoorthy, R., Kang, Y., Sa, T., 2017. Assessment of *Methylobacterium oryzae* CBMB20 aggregates for salt tolerance and plant growth promoting characteristics for bio-inoculant development. *AMB Express* 7(1), 1-10.

Desale, P., Patel, B., Singh, S., Malhotra, A., Nawani, N., 2013. Plant growth promoting properties of *Halobacillus* sp. and *Halomonas* sp. in presence of salinity and heavy metals. *Journal of Basic Microbiology* 54(8), 781-791.

Dimkpa, C.O., Merten, D., Svatoš, A., Büchel, G., Kothe, E., 2009. Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively. *Journal of Applied Microbiology* 107(5), 1687-1696.

Dobereiner, J., Marriel, I.E., Nery, M., 1976. Ecological distribution of *Spirillum lipoferum*, Beijerinck. *Canadian Journal of Microbiology* 22(10), 1464-1473.

Dobbelaere, S., Vanderleyden, J., Okon, Y., 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Critical Reviews in Plant Sciences* 22(2), 107-149.

Dodd, I.C., Perez-Alfocea, F., 2012. Microbial amelioration of crop salinity stress. *Journal of Experimental Botany* 63(9), 3415–3428.

Egamberdieva, D., Davranov, K., Wirth, S., Hashem, A., Abd Allah, E.F., 2017. Impact of soil salinity on the plant-growth-promoting and biological control abilities of root associated bacteria. *Saudi Journal of Biological Sciences* 24(7), 1601-1608.

605 Essa, A.M.M., Macaskie, L.E., Brown, N.L., 2002. Mechanisms of mercury
606 bioremediation. *Biochemical Society Transactions* 30(4), 672-674.

607

608 Etesami, H., 2018. Can interaction between silicon and plant growth promoting rhizobacteria
609 benefit in alleviating abiotic and biotic stresses in crop plants? *Agriculture, Ecosystems &*
610 *Environment* (253), 98-112.

611

612 Etesami, H., Beattie, G.A., 2018. Mining halophytes for plant growth-promoting halotolerant
613 bacteria to enhance the salinity tolerance of non-halophytic crops. *Frontiers in Microbiology*, 9.

614

615 Ferreira, N.C., Mazzuchelli, R.D.C.L., Pacheco, A.C., Araujo, F.F.D., Antunes, J.E.L., Araujo,
616 A.S.F.D., 2018. *Bacillus subtilis* improves maize tolerance to salinity. *Ciência Rural* 48(8).

617

618 García-Limones, C., Hervás, A., Navas-Cortés, J.A., Jiménez-Díaz, R.M., Tena, M., 2002.
619 Induction of an antioxidant enzyme system and other oxidative stress markers associated with
620 compatible and incompatible interactions between chickpea (*Cicer arietinum* L.) and *Fusarium*
621 *oxysporum* f.sp. *ciceris*. *Physiological and Molecular Plant Pathology* 61(6), 325-337.

622

623 Garg, N., Manchanda, G., 2009. Role of Arbuscular mycorrhizae in the alleviation of ionic,
624 osmotic and oxidative stresses induced by salinity in *Cajanus cajan* (L.) Millsp.
625 (pigeonpea). *Journal of Agronomy and Crop Science* 195(2), 110-123.

626

627 Ghnaya, T., Mnassri, M., Ghabriche, R., Wali, M., Poschenrieder, C., Lutts, S., Abdelly, C.,
628 2015. Nodulation by *Sinorhizobium meliloti* originated from a mining soil alleviates Cd toxicity
629 and increases Cd-phytoextraction in *Medicago sativa* L. *Frontiers in Plant Science* 6, 863.

630

631 González-Moro, M., Lacuesta, M., Iriberri, N., Muñoz-Rueda, A., González-Murua, C., 1997.
632 Comparative effects of PPT and AOA on photosynthesis and fluorescence chlorophyll parameters
633 in *Zea mays*. *Journal of Plant Physiology* 151(6), 641-648.

- Grover, M., Ali, S.Z., Sandhya, V., Rasul, A., Venkateswarlu, B., 2010. Role of microorganisms in adaptation of agriculture crops to abiotic stresses. *Biotechnology* 27(5), 1231-1240.
- Gupta, R.R., Singal, R., Shankar, A., Kuhad, R.C., Saxena, R.K., 1994. A modified plate assay for screening phosphate solubilizing microorganisms. *Journal of General and Applied Microbiology* 40(3), 255-260.
- Gururari, M.A., Upadhyaya, C.P., Baskar, V., Venkatesh, J., Nookaraju, A., Park, S.W., 2013. Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS-scavenging enzymes and improved photosynthetic performance. *Journal of Plant Growth Regulation* 32, 245-258.
- Hassan, T.U., Bano, A., Naz, I., 2017. Alleviation of heavy metals toxicity by the application of plant growth promoting rhizobacteria and effects on wheat grown in saline sodic field. *International Journal of Phytoremediation* 19(6), 522-529.
- Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. *Archives of Biochemistry and Biophysics* 125(3), 850-857.
- Hichem, H., Mounir, D., Naceur, E.A., 2009. Differential responses of two maize (*Zea mays* L.) varieties to salt stress: Changes on polyphenols composition of foliage and oxidative damages. *Industrial Crops and Products* 30(1), 144-151.
- Hmaeid, N., Metoui, O., Wali, M., Zorrig, W., Abdelly, C., 2014. Comparative effects of rhizobacteria in promoting growth of *Hordeum maritimum* L. plants under salt stress. *Journal of Plant Biology Research* 3, 37-50.

- Ilangumaran, G., Smith, D.L., 2017. Plant Growth Promoting Rhizobacteria in Amelioration of Salinity Stress: A Systems Biology Perspective. *Frontiers in Plant Science* 8.
- Kang, S.M., Khan, A.L., Waqas, M., You, Y.H., Kim, J.H., Kim, J.G., Hamayun, M., Lee, I.J., 2014. Plant growth-promoting rhizobacteria reduce adverse effects of salinity and osmotic stress by regulating phytohormones and antioxidants in *Cucumis sativus*. *Journal of Plant Interaction* 9(1), 673-682.
- Karnwal, A., 2017. Isolation and identification of plant growth promoting rhizobacteria from maize (*Zea mays* L.) rhizosphere and their plant growth promoting effect on rice (*Oryza sativa* L.). *Journal of Plant Protection Research* 57(2), 144-151.
- Khan, W.U., Ahmad, S.R., Yasin, N.A., Ali, A., Ahmad, A., Akram, W., 2017. Application of *Bacillus megaterium* MCR-8 improved phytoextraction and stress alleviation of nickel in *Vinca rosea*. *International Journal of Phytoremediation* 19(9), 813–824.
- Khan, W.U., Yasin, N.A., Ahmad, S.R., Ali, A., Ahmad, A., Akram, W., Faisal, M., 2018. Role of *Burkholderia cepacia* CS8 in Cd-stress alleviation and phytoremediation by *Catharanthus roseus*. *International Journal of Phytoremediation* 20(6), 581-592.
- Kohler, J., Caravaca, F., Carrasco, L., Roldán, A., 2006. Contribution of *Pseudomonas mendocina* and *Glomus intraradices* to aggregate stabilization and promotion of biological fertility in rhizosphere soil of lettuce plants under field conditions. *Soil Use and Management* 22(3), 298-304.
- León, M.J., Hoffmann, T., Sánchez-Porro, C., Heider, J., Ventosa, A., Bremer, E., 2018. Compatible Solute Synthesis and Import by the Moderate Halophile *Spiribacter salinus*: Physiology and Genomics. *Frontiers in Microbiology* 9.

- Li, H.Q., Jiang, X.W., 2017. Inoculation with plant growth-promoting bacteria (PGPB) improves salt tolerance of maize seedling. *Russian Journal of Plant Physiology* 64(2), 235-241.
- Lichtenthaler, H.K., Wellburn, A.R., 1983. Determinations of total carotenoids and chlorophylls and of leaf extracts in different solvents. *Biochemical Society Transactions* 11(5), 591-592.
- Lucy, M., Reed, E., Glick, B.R., 2004. Applications of free living plant growth-promoting rhizobacteria. *Antonie van Leeuwenhoek*. 86(1), 1-25.
- Majeed, A., Abbasi, M.K., Hameed, S., Imran, A., Rahim, N., 2015. Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. *Frontiers in Microbiology* 6, 198.
- Marques, A.P.G.C., Pires, C., Moreira, H., Rangel, A.O.S.S., Castro, P.M.L., 2010. Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea mays* as indicator plant. *Soil Biology and Biochemistry* 42(8), 1229-1235.
- Maxton, A., Singh, P., Masih, S.A., 2017. ACC deaminase-producing bacteria mediated drought and salt tolerance in *Capsicum annuum*. *Journal of Plant Nutrition* 41(5), 574-583.
- Mayak, S., Tirosh, T., Glick, B.R., 2004. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiology and Biochemistry* 42(6), 565-572.
- McDonald, J.H., Seed, R., Koehn, R.K., 1991. Allozymes and morphometric characters of three species of *Mytilus* in the Northern and Southern Hemispheres. *Marine Biology* 111(3), 323-333.
- Nadeem, S.M., Zahir, Z.A., Naveed, M., Arshad, M., 2007. Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. *Canadian Journal of Microbiology* 53(10), 1141-1149.

- Nautiyal, C., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters* 170(1), 265-270.
- Orhan, F., 2016. Alleviation of salt stress by halotolerant and halophilic plant Growth promoting bacteria in wheat (*Triticum Aestivum*). *Brazilian Journal of Microbiology* 47(3), 621-627.
- Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine* 70(1), 158-169.
- Parida, A., Das, A., 2005. Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety* 60(3), 324-349.
- Patterson, B.D., MacRae, E.A., Ferguson, I.B., 1984. Estimation of hydrogen peroxide in plant extracts using titanium (IV). *Analytical Biochemistry* 139(2), 487-492.
- Penrose, D.M., Glick, B.R., 2003. Methods for isolating and characterizing ACC deaminase containing plant growth-promoting rhizobacteria. *Physiologia Plantarum* 118(1), 10-15.
- Rahnesan, Z., Nasibi, F., Moghadam, A.A., 2018. Effects of salinity stress on some growth, physiological, biochemical parameters and nutrients in two pistachio (*Pistacia vera* L.) rootstocks. *Journal of Plant Interactions* 13(1), 73-82.
- Rockström, J., Falkenmark, M., 2000. Semiarid crop production from a hydrological perspective: Gap between potential and actual yields. *Critical Reviews in Plant Sciences* 19(4), 319-346.
- Rodriguez, H., Gonzalez, T., Goire, I., Bashan, Y., 2004. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. *Naturwissenschaften* 91(11), 552-555.

Saghafi, D., Ghorbanpour, M., Lajayer, B.A., 2018. Efficiency of *Rhizobium* strains as plant growth promoting rhizobacteria on morpho-physiological properties of *Brassica napus* L. under salinity stress. *Journal of Soil Science and Plant Nutrition*, (ahead), pp.0–0. Available at: <http://dx.doi.org/10.4067/s0718-95162018005000903>

Santos, A.D.A., Silveira, J.A.G.D., Guilherme, E.D.A., Bonifacio, A., Rodrigues, A.C., Figueiredo, M.D.V.B., 2018. Changes induced by co-inoculation in nitrogen-carbon metabolism in cowpea under salinity stress. *Brazilian Journal of Microbiology*. <https://doi.org/10.1016/j.bjm.2018.01.007>. In Press

Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* 160(1), 47-56.

Sharma, A., Singh, P., Kumar, S., Kashyap, P.L., Srivastava, A.K., Chakdar, H., Singh, R.N., Kaushik, R., Saxena, A.K., Sharma, A.K., 2015. Deciphering diversity of salt-tolerant bacilli from saline soils of Eastern Indo-gangetic plains of India. *Geomicrobiology Journal* 32(2), 170-180.

Shi, Q., Bao, Z., Zhu, Z., Ying, Q., Qian, Q., 2006. Effects of different treatments of salicylic acid on heat tolerance, chlorophyll fluorescence, and antioxidant enzyme activity in seedlings of *Cucumis sativa* L. *Plant Growth Regulation* 48(2), 127-135.

Shields, R., Burnett, W.W., 1960. Determination of protein-bound carbohydrate in serum by modified anthrone method. *Analytical Chemistry* 32(7), 885-886.

Shrivastava, P., Kumar, R., 2015. Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi Journal of Biological Sciences* 22(2), 123-131.

778 Siddiquee, M.A., Glick, B.R., Chauhan, P.S., Yim, W.J., S
779 salt tolerance of red pepper seedlings (*Capsicum annuum* L.) by regulating stress ethylene
780 synthesis with halotolerant bacteria containing 1-aminocyclopropane-1-carboxylic acid
781 deaminase activity. *Plant Physiology and Biochemistry* 49(4), 427-434.
782
783 Singh, R.P., Jha, P.N., 2017. The PGPR *Stenotrophomonas maltophilia* SBP-9 augments
784 resistance against biotic and abiotic stress in wheat plants. *Frontiers in Microbiology* 8.
785
786 Spitz, D.R., Oberley, L.W., 1989. An assay for superoxide-dismutase activity in mammalian
787 tissue homogenates. *Analytical Biochemistry* 179(1), 8-18.
788
789 Tao, G.C., Tian, S.J., Cai, M.Y., Xie, G.H., 2008. Phosphate-solubilizing and -mineralizing
790 abilities of bacteria isolated from soils. *Pedosphere* 18(4), 515-523.
791
792 Tejera, N.A., Campos, R., Sanjuan, J., Lluch, C., 2004. Nitrogenase and antioxidant enzyme
793 activities in *Phaseolus vulgaris* nodules formed by *Rhizobium tropici* isogenic strains with
794 varying tolerance to salt stress. *Plant Physiology* 161(3), 329-338.
795
796 Upadhyay, S.K., Singh, D.P., 2015. Effect of salt-tolerant plant growth-promoting rhizobacteria
797 on wheat plants and soil health in a saline environment. *Plant of Biology Journal* 17(1), 288-293.
798
799 Wu, S.C., Cheung, K.C., Luo, Y.M., Wong, M.H., 2006. Effects of inoculation of plant growth-
800 promoting rhizobacteria on metal uptake by *Brassica juncea*. *Environmental Pollution* 140(1),
801 124-135.
802
803 Yasin, N.A., Akram, W., Khan, W.U., Ahmad, S.R., Ahmad, A., Ali, A., 2018. Halotolerant
804 plant-growth promoting rhizobacteria modulate gene expression and osmolyte production to
805 improve salinity tolerance and growth in *Capsicum annum* L. *Environmental Science and*
806 *Pollution Research* 25(23), 23236-23250.

807 Yildirim, E., Turan, M., Ekinci, M., Dursun, A., Cakmakci, R., 2011. Plant growth promoting
808 rhizobacteria ameliorate deleterious effect of salt stress on lettuce. *Scientific Research and Essays*
809 6(20), 4389-4396.

810
811 Zhou, Y., Tang, N., Huang, L., Zhao, Y., Tang, X., Wang, K., 2018. Effects of salt stress on plant
812 growth, antioxidant capacity, glandular trichome density, and volatile exudates of *Schizonepeta*
813 *tenuifolia* Briq. *International Journal of Molecular Sciences* 19(1), 252.

814

Figure 1

Figure 2

Figure 3

Figure 4

Figure captions

Figure 1. Salt tolerance capacity of some isolates on nutrient agar (NA) medium amended with 10% NaCl. (+) moderate tolerance; (++) high tolerance; (+++) strong tolerance.

Figure 2. Effect of NaCl and inoculation with Br3, Br18 and Br20 on plant biomass (A) and total chlorophyll content (B). Data are presented as the mean of 5 replicates. Values with different letters denote significant differences among treatment groups according to Duncan's test ($P \leq 0.05$).

Figure 3. Bacterial inoculation and salt influence on Na^+ and K^+ content: Na^+ in shoots (A) and roots (B), and K^+ in shoots (C) and roots (D). Data are presented as the mean of 5 replicates. Values with different letters denote significant differences among treatment groups according to Duncan's test ($P \leq 0.05$).

Figure 4. Effects of microbial inoculation and saline condition on antioxidant enzyme activity of *Sulla carnosa* plants: (A) Ascorbate Peroxidase (APX) activity, (B) Glutathione peroxidase (GPX) activity, (C) Superoxyde Dismutase activity and (D) Catalase (CAT) activity. Data are presented as the mean of 5 replicates. Values with different letters denote significant differences among treatment groups according to Duncan's test ($P \leq 0.05$).

Fig. S1. GPS data and geographic location of the study area: Sebkhia Soliman located in the Nabeul region, northeast of Tunis capital.

Table 1. Plant growth promoting properties of salt-tolerant rhizobacteria isolated from rhizosphere soil of *Sulla carnos*.

Isolate code	IAA synthesis	ACC	N-fixation ability	P-solubilization zone (mm)	P-solubilization ($\mu\text{g ml}^{-1}$)	Siderophore production
Br1	++	-	+	-	-	+
Br3	++	+	+	-	-	+
Br9	+	+	-	-	-	+
Br10	+	+	-	4.00 ± 0.2	124.8 ± 0.2	-
Br14	-	-	+	-	-	++
Br18	+	+	+	23.00 ± 0.1	673.3 ± 0.3	+++
Br20	+++	+	-	18.00 ± 0.1	421.6 ± 0.5	+

Table 2. PGPR inocula and salt influence on physiological parameters of *Sulla carnosa* plants: malondialdehyde (MDA), free proline content, electrolyte leakage (EL), photosynthesis efficiency (PE), and soluble sugars production.

	Salt (mM NaCl)	MDA ($\mu\text{mol g}^{-1}$ FW)	Free proline ($\mu\text{mol g}^{-1}$ DW)	EL (%)	PE (Fv/Fm)	Soluble sugars ($\mu\text{mol g}^{-1}$ DW)
Controls	0	28.51 $\pm$ 3.67 ^c	3.87 $\pm$ 0.67 ^d	26.41 $\pm$ 2.86 ^c	0.80 $\pm$ 0.03 ^a	5.98 $\pm$ 1.30 ^d
	200	88.43 $\pm$ 5.76 ^a	39.22 $\pm$ 3.32 ^a	73.74 $\pm$ 4.70 ^a	0.43 $\pm$ 0.01 ^d	13.07 $\pm$ 1.72 ^c
Br3	0	27.48 $\pm$ 2.26 ^c	2.79 $\pm$ 0.49 ^d	23.69 $\pm$ 3.02 ^c	0.81 $\pm$ 0.02 ^a	21.92 $\pm$ 2.53 ^b
	200	51.78 $\pm$ 4.23 ^b	13.16 $\pm$ 2.04 ^c	42.81 $\pm$ 5.12 ^b	0.60 $\pm$ 0.01 ^c	35.36 $\pm$ 2.33 ^a
Br18	0	29.80 $\pm$ 3.91 ^c	2.35 $\pm$ 0.46 ^d	24.64 $\pm$ 3.74 ^c	0.77 $\pm$ 0.02 ^a	21.17 $\pm$ 3.74 ^b
	200	45.84 $\pm$ 3.44 ^b	17.88 $\pm$ 1.44 ^b	41.30 $\pm$ 2.65 ^b	0.59 $\pm$ 0.05 ^c	33.65 $\pm$ 1.79 ^a
Br20	0	25.41 $\pm$ 3.07 ^c	2.46 $\pm$ 0.61 ^d	23.50 $\pm$ 2.08 ^c	0.80 $\pm$ 0.02 ^a	20.01 $\pm$ 3.24 ^b
	200	51.01 $\pm$ 2.55 ^b	12.86 $\pm$ 1.09 ^c	41.18 $\pm$ 4.89 ^b	0.66 $\pm$ 0.01 ^b	35.50 $\pm$ 2.03 ^a

Note: Data are presented as the mean $\pm$ SD ($n = 5$). Values followed by different letters are significantly different (Duncan's test; $P \leq 0.05$).

Table 3. Effect of microbial inoculation and saline condition on chemical properties of soil used in the pot experiment.

Treatment	EC (dS m ⁻¹)	pH	Soil Na (ppm)
Control	1.14 ± 0.07 ^d	7.5 ± 0.41 ^b	8.60 ± 0.56 ^e
Control *	18.54 ± 0.80 ^a	8.7 ± 0.14 ^a	226.47 ± 5.69 ^a
Br3	0.85 ± 0.13 ^d	7.1 ± 0.22 ^{b,c}	5.72 ± 0.53 ^e
Br3 *	13.87 ± 1.67 ^b	7.3 ± 0.34 ^b	158.12 ± 6.25 ^c
Br18	0.82 ± 0.04 ^d	7.2 ± 0.37 ^b	5.46 ± 0.52 ^e
Br18 *	12.32 ± 0.92 ^c	6.8 ± 0.84 ^c	143.26 ± 2.80 ^d
Br20	0.96 ± 0.13 ^d	7.6 ± 0.21 ^b	6.58 ± 0.38 ^e
Br20 *	14.68 ± 1.25 ^b	7.4 ± 0.12 ^b	167.94 ± 4.22 ^b

*: Saline condition