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Consortia of Plant-Growth-Promoting Rhizobacteria Isolated from Halophytes Improve the Response of Swiss Chard to Soil Salinization

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Abstract: Inadequate fertilization or the indiscriminate use of water with high salt concentrations have led to salinization of agricultural soils. In this context, biofertilization with plant-growth-promoting rhizobacteria (PGPR) is an environmentally benign strategy to stimulate plant growth, even under salt stress. Thus, we studied the use of isolated PGPR consortia from halophytes to enhance Swiss chard growth under saline conditions. Growth, photosynthetic apparatus response, nutrient status, pigment concentrations, and secondary metabolites with antioxidant activity were determined in Swiss chard plants grown at 0 and 85 mmol L^{-1} NaCl. In general, inoculation of plants with PGPR has been shown to be an effective strategy to stimulate the growth of Swiss chard and improve its tolerance to salt stress. Inoculated plants watered with 85 mmol L^{-1} NaCl showed higher values of leaf dry weight than control plants. Furthermore, PGPR inoculation reduced electrolyte leakage and Na^+ uptake and improved chlorophyll *a* fluorescence parameters, chlorophyll and carotenoid concentrations, stomatal conductance, and antioxidant capacity of Swiss chard. Finally, our findings highlight the potential of isolated PGPR from halophytes to counterbalance the deleterious effect of salinity and stimulate crop growth.

Keywords: anthocyanins; *Beta vulgaris* L.; chlorophyll fluorescence; flavonoids; gas exchange; halophilic rhizobacteria; nutrient content; phenolics; pigment concentrations; soil salinization

Citation: Redondo-Gómez, S.; Romano-Rodríguez, E.; Mesa-Marín, J.; Sola-Elías, C.; Mateos-Naranjo, E. Consortia of Plant-Growth-Promoting Rhizobacteria Isolated from Halophytes Improve the Response of Swiss Chard to Soil Salinization. *Agronomy* **2022**, *12*, 468. https://doi.org/10.3390/agronomy12020468

Academic Editor: Dionisios Gasparatos

Received: 28 December 2021
Accepted: 11 February 2022
Published: 13 February 2022

1. Introduction

Our current highly intensive cultivation systems have led to soil salinity due to poor soil management practices through inadequate fertilization or the indiscriminate use of water with high salt concentrations [1,2]. The culture of crops in saline soils results in the development of ionic toxicities, the production of reactive oxygen species, and osmotic stresses that alter plant growth [3,4].

The inoculation of plant growth-promoting rhizobacteria (PGPR) is one of the potential strategies to decrease salinity stress in plants and stimulate plant growth, in addition to overcoming environmental threats posed by fertilizers [5,6]. Some direct mechanisms by which PGPR are known to promote plant growth are biological nitrogen fixation, phosphate solubilization, siderophore production for iron uptake, improvement of other plant nutrients uptake, or modulation of plant growth hormones such as indole-3-acetic acid (IAA) [7,8]. Furthermore, PGPR promote indirect mechanisms such as biofilm production, which improves bacterial adhesion to the surface of root tissue, facilitating the nutrient uptake and compartmentalization of toxic elements [8–10].

Swiss chard (*Beta vulgaris* L.) is a moderately salt sensitive leaf vegetable. It has a large leaf blade, thicker petiole, and no root enlargement [11]. The leaf is highly nutritious and is used mainly for human consumption [12]. Swiss chard is considered the richest

source of calcium, iron, phosphorus, and magnesium and is a good source of natural antioxidants, such as carotenoids, flavonoids, and other dietary phenols that have been shown to contribute significantly to the antioxidant defense system of the organism against oxidative stress and several chronic diseases (e.g., diabetes) [13].

There are no previous studies evaluating the interactive effect of salinity and PGPR inoculation on Swiss chard. Therefore, we tested the effect of PGPR consortia from halophytes on growth, nutrient status, pigment concentrations, secondary metabolites with antioxidant activity, and the response of the photosynthetic apparatus of Swiss chard under salinity conditions. Isolated PGPR from the halophytic rhizosphere were shown to be especially effective in improving the growth of agricultural crops under saline stress conditions [14]. Finally, a single microorganism does not always elicit all the mechanisms to promote plant growth, thus the use of PGPR consortia, instead of a single strain, is of current interest in research [8,15].

2. Materials and Methods

2.1. Plant Materials, Growth Conditions, and Treatments

Swiss chard seeds (*B. vulgaris* var. Bressane) were pre-hydrated for 24 h. Pre-hydrated seeds were placed in Perlite-covered trays, previously moistened with distilled water, and incubated at 21/25 °C, 40–60% relative humidity and natural daylight 250–1000 µmol m^{-2} s^{-1} light flux in a glasshouse (University of Seville Glasshouse General Services) for 1 week. Seedlings were then transferred separately to plastic pots (15 cm high × 18 cm diameter; 1 plant per pot) filled with organic commercial substrate (Gramoflor GmbH und Co. KG, Vechta, Germany) and perlite (3:1) and grown under previous conditions. The plants were watered daily with 100 mL of tap water. After 30 d of growth, the different treatments were established.

Four different treatments (n = 12 per treatment) were established: 2 biofertilization treatments (a rhizobacteria consortia + non-inoculated control) and 2 salinity concentrations (0 and 85 mmol L^{-1} NaCl). Rhizobacterial inoculation was carried out the day after salinity treatments were established. After the establishment of treatment, the pots were watered daily with 100 mL of tap water or tap water and 85 mmol L^{-1} NaCl according to treatment.

2.2. Rhizobacteria Selection

The rhizobacterial strains used in this study were *Pseudarthrobacter oxydans* SRT15 (GenBank ID MH304399, NCBI Taxonomy ID 1671) and *Bacillus zhangzhouensis* HPJ40 (GenBank ID MH304389, NCBI Taxonomy ID 1178540). They were isolated from *Salicornia ramosissima* J. Woods and *Atriplex portulacoides* L. halophytes, respectively, that inhabit salt marshes in SW Spain, as detailed in Mesa-Marín et al. [16]. They were selected to establish a bacteria consortium because both exhibited interesting properties to promote plant growth. SRT15 was able to fix nitrogen, solubilize phosphate, and demonstrate notable production of IAA (indole-3-acetic; 21 mg mL^{-1}). On the other hand, strain HPJ40 produced biofilms and siderophores, but also solubilized phosphate and fixed atmospheric nitrogen. To ensure bacterial salt tolerance, strains SRT15 and HPJ40 were seeded in TSA (Tryptone Soya Agar) plates with increasing concentrations of NaCl. The maximum tolerated concentration of NaCl for SRT15 was 102 mmol L^{-1}, while HPJ40 tolerated 170 mmol L^{-1} NaCl (maximum concentration assayed). To avoid antagonistic interactions between them, their strain compatibility with growing together was assessed by liquid growth and further plating.

To prepare the bacterial suspension for inoculation, both strains were grown separately in 250 mL Erlenmeyer flasks containing 50 mL of TSB (Tryptone Soya Broth) medium and incubated on a rotary shaker for 18–24 h at 28 °C. The cultures were then centrifuged in 50 mL Falcon tubes at 7000 rpm (6300× g) for 10 min and the supernatant was discarded. The pellets were washed twice with sterile physiological saline solution (NaCl 0.9% w/v) (by resuspension and centrifugation) and finally resuspended in tap water to obtain a final volume of 600 mL. Each pot was watered with 25 mL of bacterial suspension. Thus, 24 pots were inoculated with the rhizobacterial suspension. The other 24 non-inoculated plants

were watered with 25 mL of tap water. Inoculations were performed at the beginning of the experiment and after 15 days of the first inoculation. To determine UFC/mL of the final bacterial suspension, dilutions and plating of overnight cultures were performed prior to inoculant preparation. Thus, 50 mL of overnight SRT15 culture showed $1 \cdot 10^9$ UFC mL^{-1}, while 50 mL of overnight HPJ40 achieved $1 \cdot 10^{12}$ UFC mL^{-1}. Then, after centrifugation and resuspension in 600 mL, the final inoculant solution was composed of approximately $1 \cdot 10^7$ UFC mL^{-1} of the SRT15 strain and $1 \cdot 10^{10}$ UFC mL^{-1} of HPJ40 strain.

To track the survival of the strains in the Swiss chard rhizosphere at the end of the experiment, spontaneous rifampicin resistant mutants of both strains were used. For that, 100 µL of high concentration bacterial overnight cultures in TSB at 28 °C and shaking were transferred to TSA plates amended with 100µg mL^{-1} of rifampicin. Mutant strains that showed growth comparable to wild-type strains were selected and conserved in 15% glycerol at −80 °C for further use. A pot of each treatment was inoculated with SRT15 Rif^R and HPJ40 Rif^R strains, in the same pattern as described above. At the end of the experiment, 1 g of rhizospheric soil attached to the roots was mixed with 25 mL of sterile SSF in a Falcon tube, the sediment was allowed to settle for 10 min, and 100 µL aliquots and dilutions were placed on TSA plates amended with 100µg mL^{-1} of rifampicin to verify the survival of the strains after the experimental period.

2.3. Electrical Conductivity and Growth Measurements

Substrate conductivity measurements (n = 6) were performed during the experiment with a conductivity meter (Probe GS3, Decagon, Pullman, WA, USA).

After 30 d of growth in the different treatments, the height of the plant and the percentage of alive leaves (n = 9) were determined. Furthermore, the length and width of the leaves (at the base) of the second pair of leaves at the base of the plant were measured (n = 18). The leaf area was calculated by applying the ellipse formula. Finally, the plants were harvested. All plants were divided into roots and leaves. The dry mass was determined after drying the samples at 80 °C for 48 h.

2.4. Leaf Relative Water Content

After 30 d of treatment, the relative water content (RWC; n = 9) was calculated as follows:

$$\text{RWC} = (FW - DW)/(TW - DW) \times 100 \quad (1)$$

where FW is the fresh mass of the leaf, TW is the turgid mass after rehydrating the leaf in tap water for 24 h at 4 °C, and DW is the dry mass after drying the leaf at 80 °C for 48 h [17].

2.5. Gas Exchange

One day before the plants were harvested, the gas exchange parameters were measured in fully expanded random leaves (n = 8) using an infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE, USA; equipped with a light leaf chamber LI-6400-02B) in an open system. Net photosynthetic rate (A), intercellular CO_2 concentration (Ci), stomatal conductance (Gs), and instantaneous water use efficiency (iWUE; ration between A and Gs) were determined at photon flux density (PPFD) of 1000 mmol photons m^{-2} s^{-1} (with 15% blue light to maximize stomatal aperture), CO_2 concentration surrounding the leaf of 400 mmol mol^{-1} air, air temperature 24 ± 1 °C, relative humidity of 45 ± 5%, and vapor pressure deficit of 2.0–3.0 kPa [18].

2.6. Chlorophyll Fluorescence

Chlorophyll fluorescence was measured in fully expanded random leaves (n = 9) using a portable modulated fluorimeter (FMS-2; Hansatech Instruments Ltd., Kings Lynn, UK) after 30 d of treatment. Measurements were made in 9 plants per treatment as described by Porcel et al. [19]. Thus, the maximum quantum efficiency of PSII photochemistry (Fv/Fm) was measured in 30 min of dark-adapted leaves. The same leaves were used to measure the light-adapted parameters: quantum yield of PSII photochemistry (ΦPSII) and quantum

yield of non-photochemical quenching (ΦNPQ). Fv/Fm reflects the potential maximum efficiency of PSII (i.e., the quantum efficiency if all PSII centers were open), ΦPSII indicates the proportion of absorbed energy that is used in photochemistry, and ΦNPQ provides an indication of the amount of energy that is dissipated in the form of heat [20,21].

2.7. Photosynthetic Pigments

At the end of the experiment, the leaf samples randomly collected (n = 6) were flash frozen in liquid N_2 and freeze dried for 48 h in the dark to avoid photodegradation processes [22]. The samples were then ground in pure acetone and pigments extracted at −20 °C for 24 h in the dark, centrifuged at 4000 rpm for 15 min at 4 °C and the resulting supernatant was scanned on a dual beam spectrophotometer (Hitachi Ltd., Tokyo, Japan) from 350 to 750 nm at 0.5 nm steps. Sigma Plot Software with a Gauss-Peak Spectra algorithm fitting library was used to determine all target pigments [23]. The De-Epoxidation State (DES) was calculated, from the resulting pigment concentrations, as follows [22]:

$$DES = [Antheraxantin] + [Zeaxanhin]/[Violaxanthin] + [Antheraxantin] + [Zeaxanthin] \quad (2)$$

2.8. Nutrient Content

The dry leaves of Swiss chard were ground; 0.5 g subsamples were taken from the leaves of the 12 replicate plants to establish a pull per treatment. Subsamples were digested with 6 mL of HNO_3, 0.5 mL of HF and 1 mL of H_2O_2. Phosphorus (P), sodium (Na), magnesium (Mg), manganese (Mn), calcium (Ca), potassium (K), iron (Fe), and zinc (Zn) were measured (n = 3 per treatment, replicates obtained from sample pull) by inductively coupled plasma spectroscopy (ICP; ARL-Fison 3410, Glasgow, UK) in leaves.

2.9. Biochemical Assays in Swiss Chard Leaves

The samples were harvested 30 days after the first inoculation. For quantification of flavonoids, phenolics, anthocyanins, and total antioxidant capacity, leaves from 3 plants from each treatment were collected in liquid nitrogen, freeze-dried for 72 h, and ground, first with a coffee grinder and then to a fine powder with a TissueLyser (Qiagen, Hilden, Germany).

For anthocyanin quantification, the advantage of their characteristic behavior in acidic media was taken. A total of 10 mg of leaf powder were mixed with 10 mL of methanol acidified at 1% with HCl (37%) and vortexed. The samples were stored at −20 °C for 24 h in the dark for extraction. The extracts were then centrifuged at 4000 rpm for 15 min at 4 °C and the absorbance of the supernatant was read at 530 nm. Taking into account the molar extinction coefficient of the major anthocyanin present, the formula Abs 530 nm/98.2 was used to calculate the total anthocyanins in μg g^{-1} DW. All determinations were made in triplicate (n = 9, 3 replicates × 3 plants).

For the analysis of flavonoids, phenolics, and total antioxidant capacity, 10 mg of powder were mixed with 10 mL of pure methanol, vortexed, and stored at −20 °C for 24 h in the dark for extraction. The extracts were then centrifuged for 15 min at 4000 rpm and 4 °C, and the supernatant was stored at −20 °C for further analysis. All determinations were made in duplicate (n = 6, 2 replicates × 3 plants). For the quantification of flavonoids, a standard calibration curve with quercetin ranging from 0.01 to 0.08 mg mL^{-1} in methanol was performed. Then 1 mL of the ethanolic solution of $AlCl_3 \cdot 6H_2O$ 2% (w/v) in ethanol 80 % was added to 1 mL of methanolic extracts and standards. The mixtures were kept at room temperature for 1 h in darkness, the absorbance was read at 420 nm and compared with the calibration curve for quercetin. The flavonoid content was expressed as milli-equivalents of quercetin per gram of dry weight.

Phenolics were quantified using the method of Folin and Ciocalteau [24]. For that, a standard calibration curve with gallic acid was performed that ranged from 10 to 80 mg L^{-1} in methanol. Then, 500 μL of methanolic extracts and standards were mixed with 2.5 mL of 10 times diluted Folin–Ciocalteu phenol reagent (Sigma) (1/10 in distilled water). After 5 min of incubation at room temperature and darkness, 2 mL of Na_2CO_3 7.5% (w/v in distilled water) was added. The mixtures were kept for 1 h in darkness at room temperature and finally the absorbance readings were performed at 750 nm and compared with the calibration curve for gallic acid. The phenolic content was expressed as milliequivalents of gallic acid per gram of dry weight.

The total antioxidant capacity was evaluated by the ability of plant extracts to react to 2.2-diphenyl-1-picrylhydrazyl (DDPH) and quench free radicals. For that, 1 mL of DDPH solution (Glentham Life Sciences; 0.05 mg/mL in methanol) was added to 1 mL of methanol and extracts and

incubated for 30 min in darkness at room temperature. After that, the absorbance was measured at 515 nm and the percentage of inhibition was calculated as follows:

$$\text{Inhibition (\%)} = [(A_R - A_S)/A_R)] \times 100 \quad (3)$$

where A_R represents the reference absorbance (methanol + DDPH) and A_S the sample absorbance (methanolic extract + DDPH).

2.10. Statistical Analysis

Statistical analysis was performed using the SPSS 26.0 statistical program (SPSS Inc., Chicago, IL, USA). Data were analyzed using analysis of variance (ANOVA). Duncan's test was applied to establish the significance between treatments ($p < 0.05$).

3. Results

3.1. Electrical Conductivity and Growth Measurements

The electrical conductivity of the substrate of the salt-treated Swiss chard plants ranged from 0.95 to 2.36 dS m^{-1} during the experiment, since these plants were watered daily with 85 mmol L^{-1} NaCl, while the mean conductivity of the substrate of the plants grown in the absence of salt was 0.1 dS m^{-1} (Figure 1). However, the rhizobacterial strains survived in the rhizosphere of all plants at the end of the experiment.

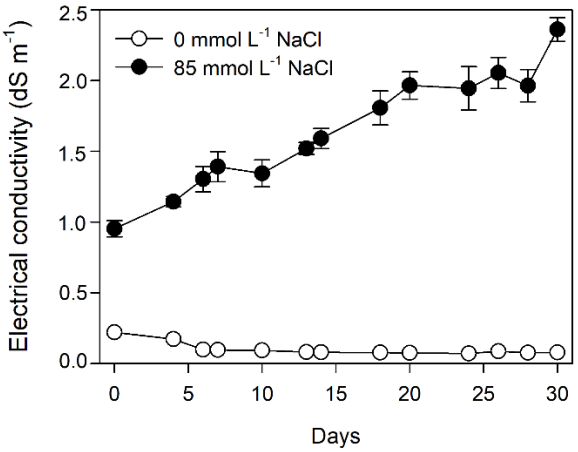

Figure 1. Electrical conductivity of Swiss chard plants grown at 0 and 85 mmol L^{-1} NaCl. Each value represents the mean of six replicates ± SE.

Salinity and inoculation had a significant effect on Swiss chard plants (Figure 2). Salinity reduced leaf and root dry weights by about 30 and 53%, respectively, compared to plants grown without NaCl. However, inoculation increased these parameters to values similar to those of the non-saline treatment (Figure 3A,B). Thus, inoculated plants at 85 mmol L^{-1} NaCl showed the highest values of dry leaf weight, although no significant differences were recorded with respect to inoculated plants developed in the absence of salt (Figure 3A). The plants at 85 mmol L^{-1} NaCl had a lower height ($p < 0.0001$), but the same percentage of alive leaves as the rest of the treatments (Figure 3C,D). Furthermore, salt-inoculated plants showed leaf area and leaf length similar to plants grown in the absence of salt, while those non-inoculated recorded the lowest values of both parameters ($p < 0.0001$ for both; Figure 3E,F) and leaf width (10.3 cm versus 11.7 cm of the rest of the treatments; $p < 0.01$).

3.2. Leaf Relative Water Content and Gas Exchange

The relative water content of the leaf (RWC) showed a significant response to salinity and inoculation (ANOVA, $p < 0.0001$). RWC values were 97%, for both non-saline treatments, 95 and 93% for plants at 85 mmol L^{-1} NaCl, non-inoculated and inoculated, respectively.

Inoculated plants recorded the highest values of net photosynthetic rate (A), although there were no significant differences (Figure 4A). On the other hand, the values of stomatal conductance (Gs) and intercellular CO_2 concentration (Ci) were significantly higher for inoculated plants, regardless of saline treatment ($p < 0.01$ for both parameters; Figure 4B,C). On the contrary, the instantaneous water use efficiency (iWUE) values were higher for non-inoculated plants ($p < 0.05$; Figure 4D).

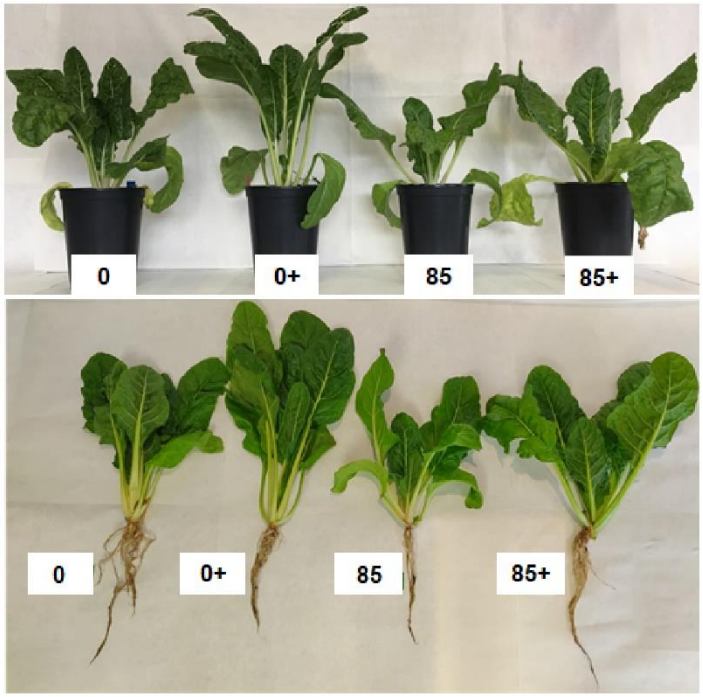

Figure 2. Swiss chard plants grown with 0 and 85 mmol L^{-1} NaCl, non-inoculated and inoculated (+). In the upper image, the plants are shown in their pots, and in the lower image, the same plants can be observed after they are removed from the pots and washing their roots.

3.3. Chlorophyll Fluorescence

Swiss chard inoculated plants recorded higher values of maximum quantum efficiency of PSII photochemistry (Fv/Fm), although significant differences were only recorded between inoculated plants without salt and non-inoculated plants at 85 mmol L^{-1} NaCl ($p < 0.05$; Figure 5A). The quantum yield of PSII photochemistry (ΦPSII) showed a pattern similar to that of Fv/Fm, but in this case there were no significant differences (Figure 5B). Finally, the quantum yield of non-photochemical quenching (ΦNPQ) of the inoculated plant was affected by salinity ($p < 0.05$; Figure 5C), it increased the amount of energy that was dissipated in the form of heat (higher NPQ values).

3.4. Photosynthetic Pigments

In Table 1 we can see how salinity and inoculation affected chlorophyll *a* and neoxanthin concentrations (ANOVA; Duncan test, $p < 0.05$ and $p < 0.0001$, respectively). Furthermore, the inoculated plants at 85 mmol L^{-1} NaCl showed the highest chlorophyll *b* concentration ($p < 0.01$; Table 1). On the other hand, salinity affected the concentrations of pheophytine *a*, β-carotene, violaxanthin, and zeaxanthin, as well as the De-Epoxidation State (DES) in Swiss chard leaves ($p < 0.0001$ for all these pigments and DES). Salinity increased the content of pheophytine *a* but decreased the rest of the pigments and DES. Finally, lutein concentration ranged from 27 to 29 μg g^{-1}, there were no significant differences between treatments (data not presented).

3.5. Nutrient Content

Macro and micronutrients were affected by salinity and inoculation (ANOVA; Duncan test, $p < 0.0001$ for all nutrient concentrations; Table 2). Inoculated plants showed higher leaf Mg and Zn concentrations compared to non-inoculated plants, regardless of saline treatment. Inoculation also increased Ca levels in the presence of salt and reduced Na concentration. On the other hand, salinity increased the P content of the leaf, without a significant effect of inoculation. The highest Mn concentration was recorded for inoculated plants grown with 85 mmol L^{-1} NaCl. In contrast, this treatment caused the lowest K content. Finally, the Fe concentration of the leaves was improved by salinity and inoculation, thus the highest Fe values were recorded for the combination of both treatments.

3.6. Flavonoid, Phenolic, and Anthocyanin Concentrations and Total Antioxidant Capacity

Salinity reduced the number of flavonoids in Swiss chard, but inoculation increased its concentration such as that of the non-saline treatment ($p < 0.01$). In the absence of salt, inoculation also reduced the flavonoid content (Figure 6A). Although the presence of phenolic compounds was greater in inoculated plants, no significant differences were recorded between treatments (Figure 6B). However, anthocyanin quantification was affected by both salinity and inoculation (Figure 6C). Finally, the antioxidant capacity decreased with inoculation, regardless of saline treatment (Figure 6D).

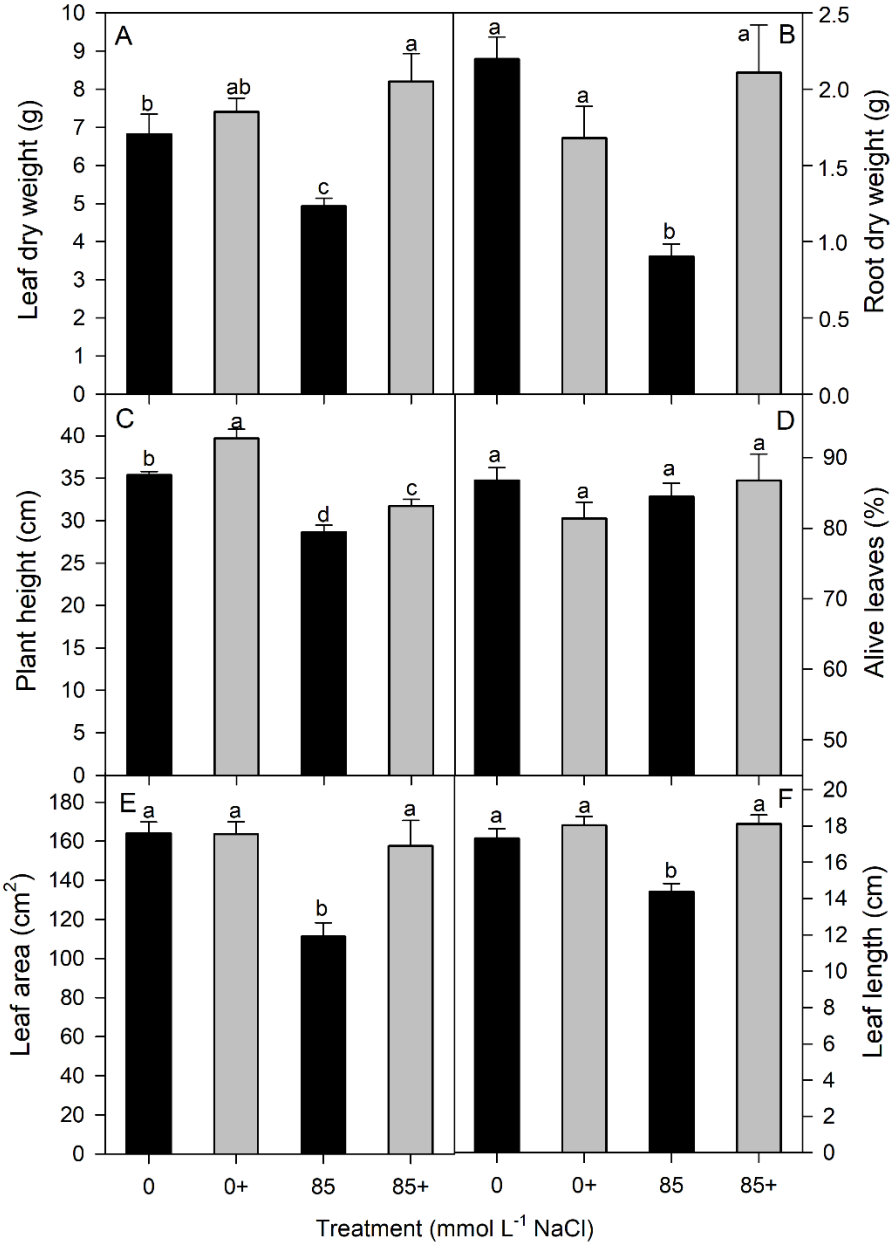

Figure 3. Leaf (**A**) and root (**B**) dry weight, plant height (**C**), percentage of alive leaves (**D**), mean leaf area (**E**) and mean leaf length (F) of Swiss chard after 30 d of treatment with 0 and 85 mmol L^{-1} NaCl, non-inoculated and inoculated (+). Each value represents the mean of nine replicates (n = 18 for leaf area and leaf length) ± SE. Different letters indicate means that are significantly different from each other (ANOVA; Duncan test, $p < 0.05$).

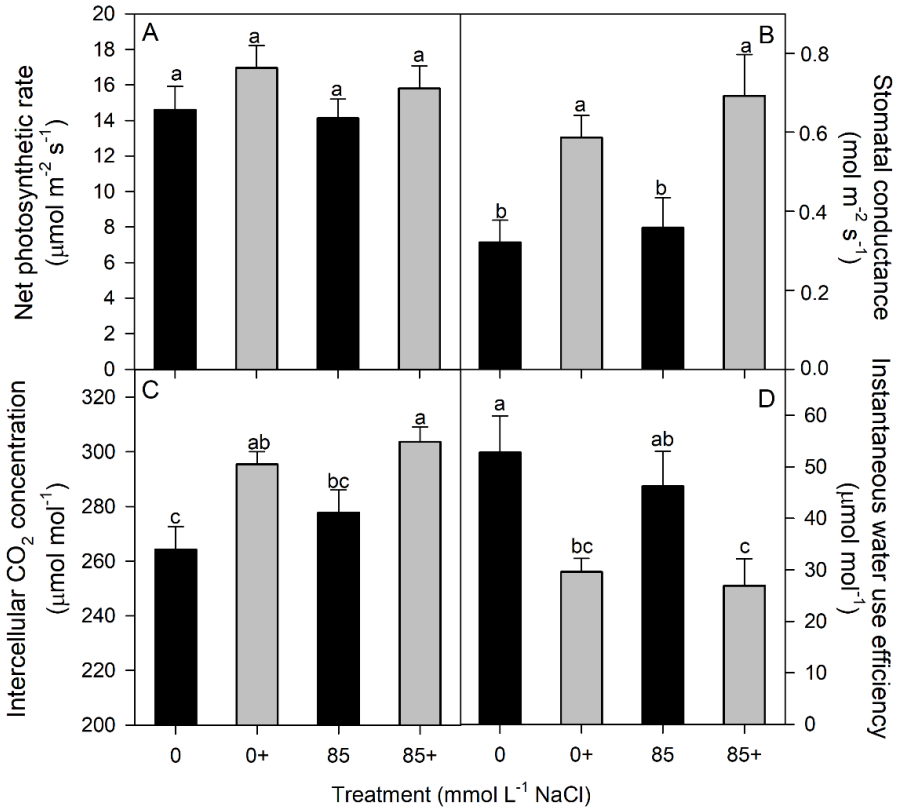

Figure 4. Net photosynthetic rate (**A**), stomatal conductance (**B**), intercellular CO_2 concentration (**C**), and instantaneous water use efficiency (**D**) of Swiss chard after 30 d of treatment with 0 and 85 mmol L^{-1} NaCl, non-inoculated and inoculated (+). Each value represents the mean of eight replicates ± SE. Different letters indicate means that are significantly different from each other (ANOVA; Duncan test, $p < 0.05$).

Table 1. Concentrations of chlorophylls *a* and *b*, pheophytine *a*, β-carotene, lutein, neoxanthin, violaxanthin, and zeaxanthin (µg g^{-1}) and De-Epoxidation State in Swiss chard leaves after 30 d of treatment with 0 and 85 mmol L^{-1} NaCl, non-inoculated and inoculated (+). Each value represents the mean of six replicates ± SE. Different letters indicate means that are significantly different from each other (ANOVA; Duncan test, p <0.05).

	Chl *a*	**Chl *b***	**Phe *a***	**β-carotene**	**Neoxanthin**	**Violaxanthin**	**Zeaxanthin**	**DES**
0 mmol L^{-1}	227 ± 9.9 ^{c}	75 ± 3.8 ^{b}	13 ± 0.7 ^{c}	13 ± 0.4 ^{a}	15 ± 0.5 ^{c}	12 ± 0.3 ^{a}	14 ± 0.4 ^{a}	0.46 ± 0.005 ^{a}
0 mmol L^{-1}+	265 ± 6.5 ^{a}	89 ± 2.6 ^{b}	16 ± 0.7 ^{c}	12 ± 0.2 ^{a}	18 ± 0.5 ^{a}	12 ± 0.1 ^{a}	13 ± 0.3 ^{a}	0.47 ± 0.005 ^{a}
85 mmol L^{-1}	236 ± 11.8 ^{bc}	90 ± 5.2 ^{b}	45 ± 8.4 ^{b}	9 ± 0.4 ^{b}	11 ± 0.7 ^{d}	6 ± 0.3 ^{b}	10 ± 0.4 ^{b}	0.38 ± 0.016 ^{b}
85 mmol L^{-1}+	269 ± 25.1 ^{ab}	109 ± 10 ^{a}	65 ± 6.3 ^{a}	10 ± 0.3 ^{b}	16 ± 0.6 ^{b}	6 ± 0.6 ^{b}	11 ± 0.3 ^{b}	0.37 ± 0.022 ^{b}

Table 2. Calcium, iron, potassium, sodium, phosphorus, magnesium, manganese, and zinc concentrations in Swiss chard leaves after 30 d of treatment with 0 and 85 mmol L^{-1} NaCl, non-inoculated and inoculated (+). Each value represents the mean of three replicates ± SE. Different letters indicate means that are significantly different from each other (ANOVA; Duncan test, p <0.05).

	Ca **mg g^{-1}**	**Fe** **mg kg^{-1}**	**K** **mg g^{-1}**	**Na** **mg g^{-1}**	**P** **mg g^{-1}**	**Mg** **mg g^{-1}**	**Mn** **mg kg^{-1}**	**Zn** **mg kg^{-1}**
0mmol L^{-1}	1.20 ± 0.004 ^{a}	98 ± 1.6 ^{c}	3.2 ± 0.01 ^{b}	1.7 ± 0.00 ^{b}	1.6 ± 0.01 ^{b}	0.88 ± 0.004 ^{b}	1126 ± 2 ^{a}	212 ± 1 ^{b}
0 mmol L^{-1}+	1.11 ± 0.019 ^{b}	103 ± 0.2 ^{b}	3.5 ± 0.06 ^{a}	1.7 ± 0.03 ^{b}	1.7 ± 0.03 ^{b}	0.95 ± 0.032 ^{a}	1155 ± 58 ^{a}	259 ± 5 ^{a}
85 mmol L^{-1}	0.86 ± 0.009 ^{c}	116 ± 2.9 ^{a}	3.2 ± 0.08 ^{b}	5.6 ± 0.11 ^{a}	2.1 ± 0.03 ^{a}	0.81 ± 0.004 ^{c}	1210 ± 24 ^{a}	215 ± 2 ^{b}
85 mmol L^{-1}+	1.05 ± 0.032 ^{d}	135 ± 5.5 ^{d}	2.6 ± 0.08 ^{c}	5.3 ± 0.14 ^{c}	2.1 ± 0.07 ^{b}	0.95 ± 0.035 ^{b}	1565 ± 50 ^{b}	251 ± 9 ^{c}

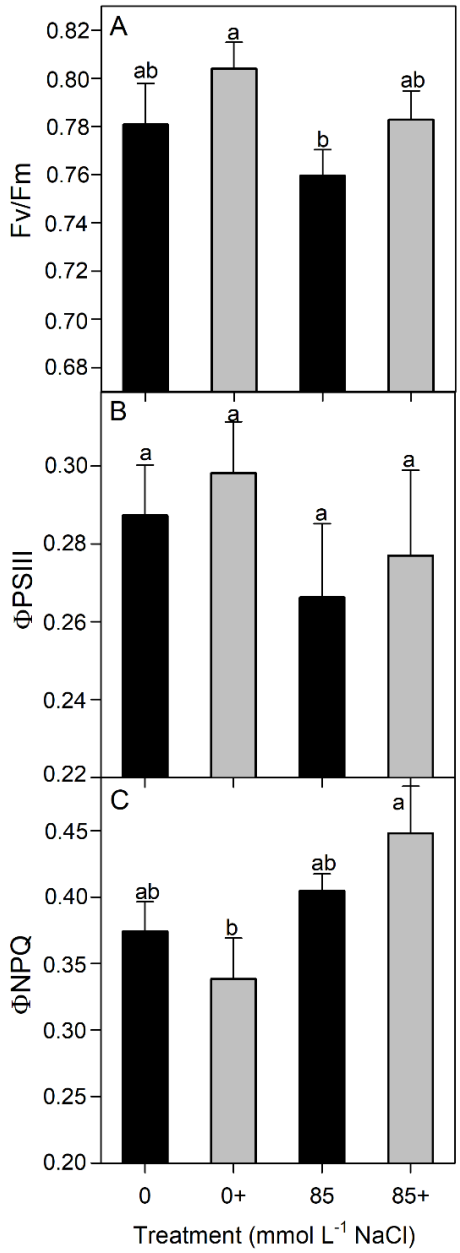

Figure 5. (**A**) Maximum quantum efficiency of PSII photochemistry (Fv/Fm), (**B**) quantum yield of PSII photochemistry (ΦPSII) and (**C**) quantum yield of non-photochemical quenching (ΦNPQ) of Swiss chard after 30 d of treatment with 0 and 85 mmol L^{-1} NaCl, non-inoculated and inoculated (+). Each value represents the mean of nine replicates ± SE. Different letters indicate means that are significantly different from each other (ANOVA; Duncan test, p <0.05).

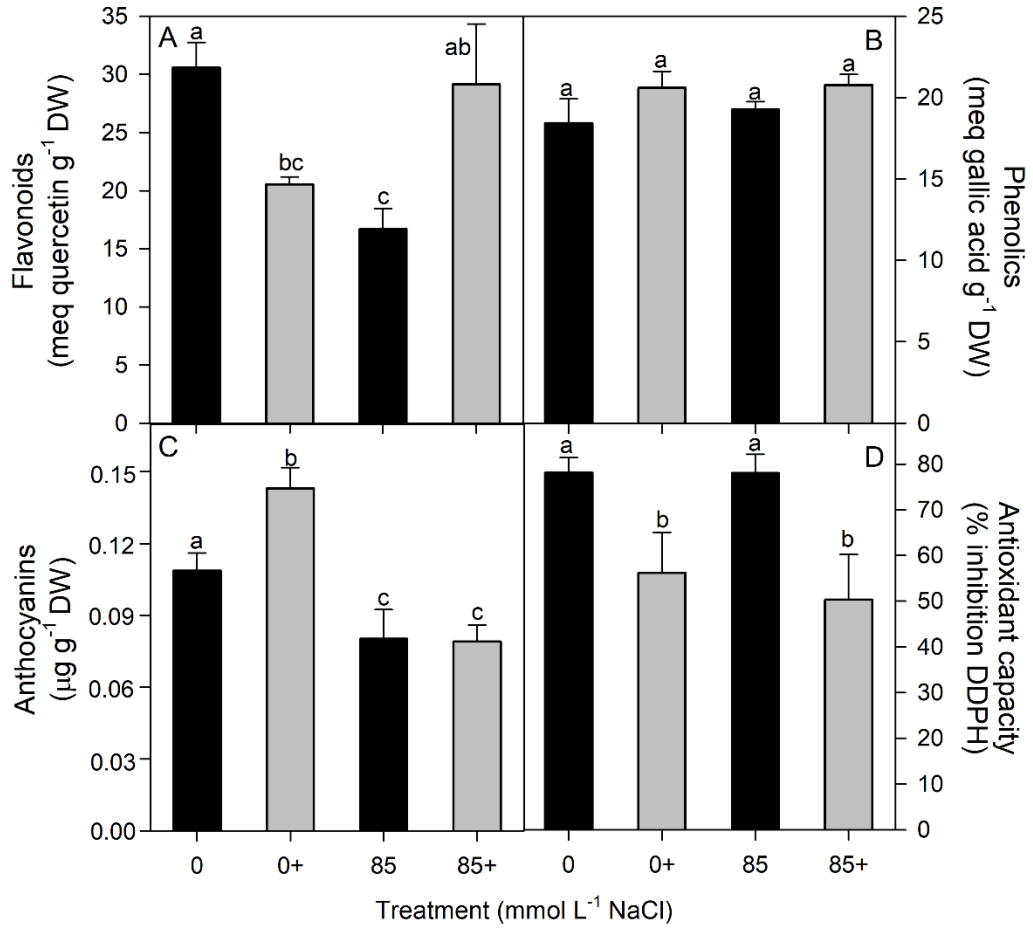

Figure 6. Flavonoids (**A**), phenolics (**B**), anthocyanins (**C**), and antioxidant capacity (**D**) of Swiss chard after 30 d of treatment with 0 and 85 mmol L^{-1} NaCl, non-inoculated and inoculated (+). Each value represents the mean of six replicates ± SE (nine replicates for anthocyanins). Different letters indicate means that are significantly different from each other (ANOVA; Duncan test, p <0.05).

4. Discussion

Inoculation with plant growth-promoting rhizobacteria (PGPR) isolated from halophytes had a positive effect on Swiss chard growth. Salinity reduced leaf dry weight after 30 d of treatment, but inoculated plants at 85 mmol L^{-1} NaCl showed higher leaf dry weight values than control plants (i.e., non-inoculated plants grown without salt). However, the height of the former plant was lower because the plants had shorter stalks. Furthermore, this treatment, 85 mmol L^{-1} NaCl and inoculation recorded similar values of root dry weight, percentage of alive leaves, leaf area, and leaf length to control plants.

The growth decline caused by salinity was not due to photosynthetic capacity, as chlorophyll contents, gas exchange, and chlorophyll fluorescence parameters were not significantly affected by salinity. On the contrary, Liu et al. [13] explained that Na^+ stress (c. 7 mg g^{-1} Na FW) reduced the chlorophyll *a* and *b* contents, Fv/Fm, and NPQ values of Swiss chard under sodic-alkaline conditions. Mushtaq et al. [25] confirmed that NaCl (150 mmol L^{-1}) in soil reduced the content of photosynthetic pigments of *Cicer arietinum*, but little or no reduction was observed at low doses of salt (50 mmol L^{-1}). However, inoculation with PGPR enhanced Fv/Fm, ΦPSII, chlorophyll, and carotenoid concentrations, stomatal conductance (Gs), and intercellular CO_2 concentration, with a consequent improvement of photosynthetic rate. Pan et al. [26] conducted a meta-analysis to evaluate the effect of PGPR-inoculation on salt-sensitive and salt-tolerant plants under salt stress. They also reported that PGPR inoculation increased the concentration of chlorophylls and carotenoids and the photosynthetic rate. Bacterial inoculation probably reduced chlorophyll degradation, leading to improved photosynthetic activity and plant growth [27]. Aamir et al. [28] observed that the RWC of mung bean improved with PGPR inoculation due to longer roots, which ultimately resulted in increased water uptake. On the contrary, we found that the RWC values of inoculated Swiss

chard plants grown at 85 mmol L^{-1} NaCl were slightly lower, although this treatment showed a dry weight of the roots similar to that of the control. This discrepancy could be explained by the lower instantaneous water use efficiency (iWUE) of the inoculated plants, which was due in turn to the higher Gs values. According to Yasin et al. [29], stress alleviation encourages plant growth by increasing leaf area and stomatal conductance.

The reason behind the decline in growth parameters may be the toxic effects imposed by salt stress of Na^+ [30]. Continued irrigation with saline water increased the sodium content of the soil and, therefore, the uptake of Na cation by Swiss chard plants. Consistent with our results, Abdulmalek et al. [12] recorded 35 and 52% drops in the growth of Swiss chard plants treated with 50 and 100% leachate irrigation from landfill (approximately 2 and 2.7 dS m^{-1} conductivity, respectively) after four weeks. They concluded that the decline could only be attributed to the elevated Na content in the leachate used, which replaced Ca and Mg in solution, thus limiting their uptake and affecting structural growth in Swiss chard. In the same way, the ionic imbalance under salinity stress decreased the fresh and dry weight of spinach, lettuce, cabbage, strawberry, and tomato [6,31–34]. Therefore, the improvement in the growth of plants inoculated with PGPR under salt stress depends on the enhancement of the acquisition of nutrients [26]. Thus, PGPR showing IAA production (*Pseudarthrobacter oxydans* SRT15 in our study) reduced electrolyte leakage and Na^+ uptake, resulting in increased biomass production and improved salt stress mitigation in chickpea plants [35]. We found that inoculation increased leaf Ca, Mg, and Zn contents, and inoculation combined with salinity improved leaf Fe and Mn concentrations. Mabasa et al. [36] found similar patterns for the Mg and Fe content of Swiss chard leaves grown in amended soil with *Trichoderma* and watered with water of approximately 3 dS m^{-1} conductivity for 16 weeks. On the contrary, the addition of *Trichoderma* as a soil amendment resulted in a higher leaf K content and lower leaf Mn and Zn levels, while Ca and P contents were not affected. In our experiment, the P content was only affected by the presence of salt, although the two strains used in the inoculation were able to solubilize phosphate, and the rhizobacteria reduced the leaf K level of plants watered with NaCl. This lower K content could partially explain the reduced total antioxidant capacity of plants treated with salt and PGPR, since K^+ is involved in enzyme activation and reduced generation of reactive oxygen species (ROS) by decreasing the activity of NADPH oxidases, which are responsible for ROS production in the photosynthetic electron transport chain [37]. ROS leads to the production of H_2O_2, which is consequently converted to the hydroxyl radical ion, and overproduction of this radical degrades the plant cell membrane [38]. In this regard, carotenoid may promote plant growth by protecting photosynthesis and reducing oxidative damage in salt-sensitive plants after inoculation with PGPR under salt stress [26]. Thus, we found that pheophytine *a* and neoxanthin concentrations were higher for inoculated plants.

Phenolics and flavonoids can also act as radical scavengers or radical chain breakers, thus extinguishing strongly oxidative free radicals [39,40]. In our study, total phenolics were slightly higher in plants treated with PGPR regardless of saline treatment, although salinity reduced anthocyanins, inoculation in the absence of salt improved this phenolic compound by up to 24% more than in the control.

However, the De-Epoxidation State (DES) of Zeaxanthin indicates the degree of activation of the xantophyll cycle, which in turn correlates with non-photochemical quenching (ΦNPQ). This cycle is related to the capacity of the plant to reduce ROS generation by dissipating excess energy from photosynthesis [41]. In our study, the DES and ΦNPQ values suggested that there was no activation of the xantophyll cycle with inoculation.

5. Conclusions

On the basis of the results obtained in our experiment, we can generalize that there is a positive effect of PGPR consortia from halophytes on the growth of Swiss chard under salt stress. Inoculation improved the concentrations of chlorophylls and carotenoids, the photosynthetic capacity, and reduced the ionic toxicity. The alleviation of stress of plants inoculated with PGPR under salt stress was mediated by the acquisition of nutrients and the enhancement of antioxidant capacity. These findings are encouraging to think that PGPR from halophytes may be valuable natural resources for the cultivation of crops in saline soils.

Author Contributions: Conceptualization, S.R.-G.; methodology, S.R.-G., E.M.-N. and J.M.-M.; software, S.R.-G. and E.M.-N.; validation, S.R.-G. and J.M.-M.; formal analysis, S.R.-G. and E.M.-N.; investigation, S.R.-G., E.R.-R., C.S.-E. and E.M.-N.; resources, S.R.-G. and J.M.-M.; data curation, S.R.-G. and E.M.-N.; writing—original draft preparation, S.R.-G.; writing—review and editing, S.R.-G.;

visualization, S.R.-G.; supervision, S.R.-G.; funding acquisition, S.R.-G. and E.M.-N. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by MCIN/AEI/10.13039/501100011033 and UE "NextGenerationEU/PRTR" (PDC2021-120951-I00) and Junta de Andalucía (Programa I+D+i FEDER Andalucía 2014-2020, US-1262036).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank the University of Seville Greenhouse General Services (CITIUS) for its collaboration, as well as Pedro Valle and Rosario Armijo for their technical support. J.M.-M acknowledges the European Social Fund and Junta de Andalucía for personal funding (Talento Doctores DOC_00725).

Conflicts of Interest: The authors declare that they have no conflict of interest.

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