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Article

Bioaugmentation Improves Phytoprotection in *Halimione Portulacoides* Exposed to Mild Salt Stress: Perspectives for Salinized Soil Restoration

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Abstract: Rhizosphere bacteria have a decisive influence on plant ionic adjustment, as well as in ameliorating plant growth under an array of stress situations. Plant growth-promoting rhizobacteria (PGPR) colonize the rhizosphere of plants and promote plant growth through mechanisms such as solubilization of mineral phosphates, biological N_2 fixation, production of siderophores and phytohormones, and can produce an amelioration of plant resistance. This can be of extreme importance when considering the restoration of salinized grounds by halophytic species. This present work aims to evaluate the physiological fitness and phytoprotection improvement by salt marsh PGPR in *Halimione portulacoides* under mild and severe salt stress. Plants inoculated with PGPR-consortium showed higher photochemical performances, improved antioxidant response, and promotion of osmotic balance traits, that boosted the individual's ability to cope with mild salt stress. All these changes are also in line with the differential elemental profiles (Na, K, and Ca) observed in the different plant tissues. Even under severe salt stress, some physiological traits were improved when compared to the non-inoculated individuals. The results developed under this work, point out an important role of bioaugmentation in promoting plant fitness and improving salt tolerance, with a great potential for applications in seawater agriculture, restoration, and bio-reclamation of salinized soils.

Keywords: halophyte; rhizobacteria; PGPR; root inoculation; osmotic stress

1. Introduction

It is understood that a rapid climate change is taking place worldwide with alarming environmental and economic consequences, mainly thru, sea-level and temperature rise, increase in periodicity and strength of extreme climate events, such as droughts, floods, and storms, resulting in water and land salinity variation [1]. Simultaneously, it is predicted that by the year 2050 the world population will reach 9.7 billion, consequently, global food demand is projected to grow by more than 35%, and freshwater demands for irrigation are predicted to increase 19% [2]. Moreover, an estimated 33% of the world's irrigated agricultural lands are suffering from high soil salinity causing a 1 to 2% agricultural soil loss every year [3,4]. Soil salinization reduces crop yields and can affect the performance of other ecosystems services. This happening often goes neglected and is exacerbated by inadequate agricultural and water management practices, consequently, lands affected by salinization are increasing due to changes in climate and land use [5]. All these aspects impose severe salinity-induced constraints on Earth's crop yields, carrying major socio-economic costs and the potential impact on future global food security [6].

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To guarantee the agricultural demands of the future, potential solutions are being studied and developed. Generally, salt stress is a powerful yield-limiting factor that disrupts every major crop's development and performance. When exposed to NaCl concentrations ranging from 40 to 200 mM, the majority of the world's cash crop plants suffer severe damage, wither and die. [7]. On the other hand, and in contrast, halophytes can not only survive but also thrive and be highly productive under these same saline conditions. Halophytes, which constitute about 1% of the Earth's flora, are defined as plant species that can grow and reproduce under salinity concentrations of over 200 mM NaCl [8]. These plants are known to play a key role in the stability and protection of coastal and wetland habitats, maintaining ecological stability, and providing a variety of globally relevant unique ecological and economic services, and also have enormous potential to aid soil restoration, water purification, and agricultural development [9]. Phytoremediation in saline-sodic soils was found to be an inexpensive solution to regulate environments and is found to be a potential substitute for chemical amelioration [10]. In line with this approach, several halophyte species can remove, transfer, or stabilize salt, metals, pharmaceutical products, pesticides, cyanotoxins, and nanoparticles from the soil and groundwater in an efficient, low-cost, easy-to-use, and eco-friendly way [11]. Furthermore, the freshwater increasing demand and growing scarcity for agricultural practices dictate a need for sustainable crop production. A strategy to cope with the loss of soil and freshwater availability is seawater agriculture, which proposes to grow halophytes plants as a sustainable edible crop (cash crops) [12]. *Halimione portulacoides* L. Allen (Amaranthaceae), is a halophyte that is being linked to both strategies, is one of the most widely spread and numerous species across the Mediterranean marshes, a C3 succulent that belongs to the Chenopodiaceae family [13,14].

Plant-growth promoting rhizobacteria (PGPR) are beneficial soil bacteria, which thru mutualistic interactions directly and/or indirectly facilitate and improve plant growth, nutrition, and development [15]. The mechanisms by which PGPR promote plant growth may include, atmospheric nitrogen fixation, phosphate solubilization, and iron acquisition by bacterial siderophore, biocontrol, and phytopathogens prevention, phytohormones, for example, gibberellic acid (GA3), abscisic acid (ABA), and indole-3-acetic acid (IAA) and bacterial enzymatic synthesis such as bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an ethylene regulator [16–19]. Moreover, endophytes, plant root colonizing bacteria, that establish direct interactions with the host root cells, in the same way as rhizobacteria, has been shown to stimulate not only plant growth but stress resistance and tolerance mechanisms [20,21]. Additionally, it is increasingly more common the usage of PGPR consortia instead of a single strain to achieve or elicit all the intended responses [22,23]. Effective PGPR usage is constrained by a number of variables such as climate, water, soil characteristics, target species, etc. [24], and it is understood that these bacteria have growth conditions where they can be more or less effective, for example, it was found that some PGPR with increasing salinity, and with no relation with bacterial death, did not display their plant-growth promoting attributes [25]. Nonetheless, several studies have not only identified salt tolerate PGPR but also associated PGPR consortia specific for salt-stressed plants [21,23]. Halophytes like *H. portulacoides* can inhabit high salinity areas, but it is well-known that even salt tolerate species have their NaCl limitations, salt exposure that exceeds the tolerance limit provokes physiological and biochemical alterations affecting growth and potentially causing death [8]. Despite this, most studies in the application of PGPR inducing salt-stress resistance have been undertaken in salt-sensitive plants and the information is lacking.

For this study, several salt marshes acquired PGPRs, specifically *Bacillus aryabhattai*, *Stenotrophomonas rhizophila*, *Pseudomonas oryzihabitans,* and *Salinicola endophyticus* have been selected in order to build a potentially effective consortium. Recent promising findings were taken into consideration in the identification and selection of those rhizobacteria with the purpose of salt stress amelioration in halophyte plants [26–29]. This present work aims to evaluate the physiological fitness and phytoprotection improvement by salt marsh

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PGPR in *H. portulacoides* under mild and severe salt stress. Thereby, plants were inoculated with a PGPB consortium composed of rhizobacteria with different plat-growth promoting traits and exposed to mild and severe salinity levels, whilst its photochemical performance and biochemical traits were analysed. Given the ongoing and increasing soil salinity problem, it is relevant to understand the capabilities that PGPR bioaugmentation has in *H. portulacoides* and if it can increase its biotechnological potential for re-vegetation of salt-affected soils.

2. Results

2.1. Photochemical processes

Halimione portulacoides, when subjected to a rise in salinity, displayed significant differences in key energy transduction flux per leaf cross-section due to rhizobacterial inoculation. Although some PGPR-induced variation was observed, with respect to absorbed (ABS/CS; Figure 1A) and trapped (TR/CS; Figure 1B) energy flux, no significant changes could be observed. On the other hand, inoculated samples exhibited higher electron transport energy flux (ET/CS; Figure 1C) and lower dissipation energy flux (DI/CS; Figure 1D) through the salt gradient, as well as higher oxidized reaction centres (RC/CS; Figure 1E) at 400 mM NaCl.

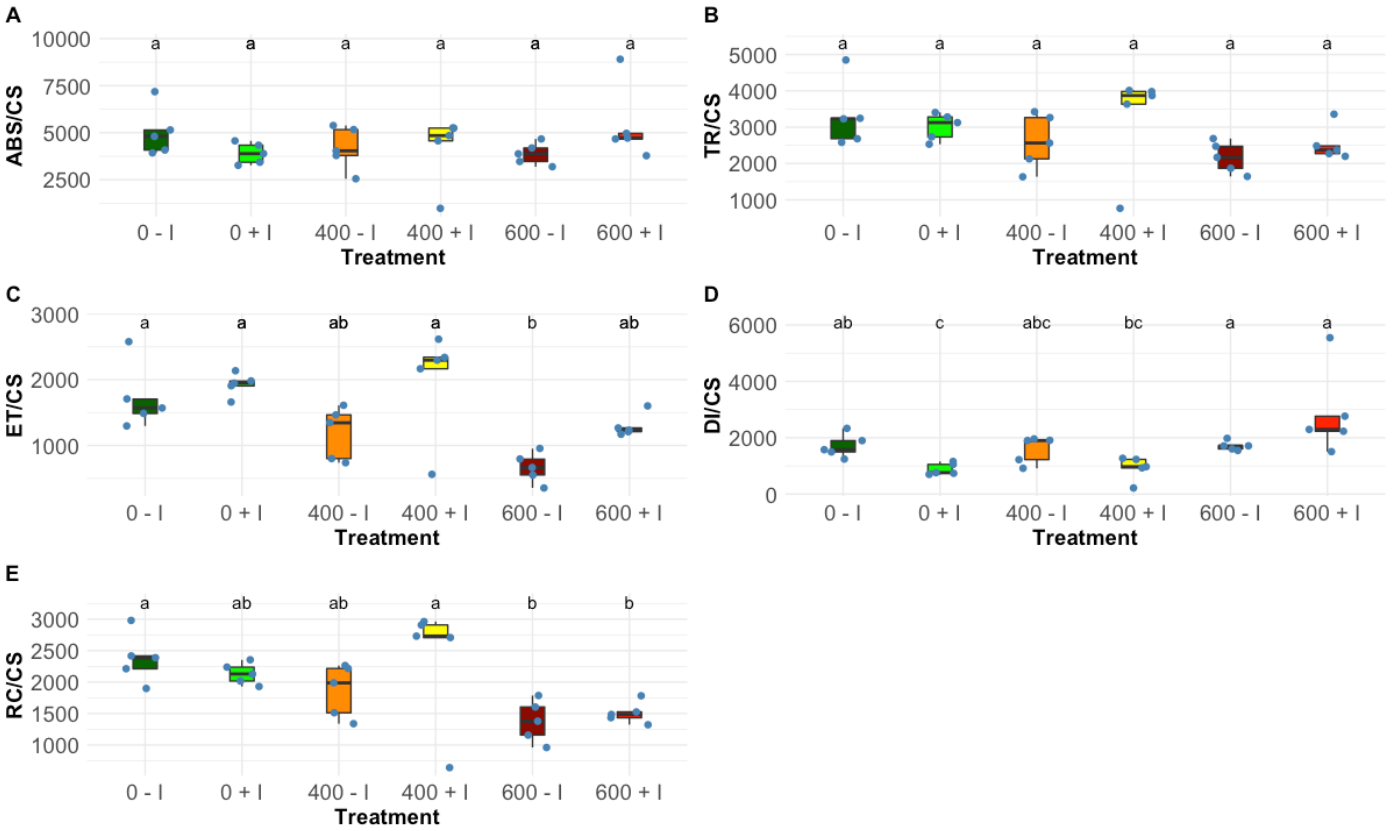

Figure 1. Phenomological energetic parameters, (**A**) absorbed energy flux (ABS/CS), (**B**) trapped energy flux (TR/CS), (**C**) electron transport energy flux (ET/CS), (**D**) dissipation energy flux (DI/CS) and (**E**) oxidized reaction centres (RC/CS) on a cross-section basis, in non-inoculated and inoculated *Halimione portulacoides* dark-adapted leaves (N=5), in conjunction with the NaCl treatments. Letters denote significant differences at p <0,05.

Observing the OJIP derived parameters, significant changes were found in the photosystem II (PS II) and photosystem I (PS I) photochemical traits. Inoculated plants, at 0

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and 400 mM NaCl, displayed significantly higher values in the contribution or partial performance of the light reactions for PS I (TR_0/DI_0; Figure 2A) and the contribution of dark reactions from Q_A^- to plastoquinone ($\psi_0/ (1 - \psi_0)$; Figure 2C), this variation was also found in the PS II/PS I redox equilibrium constant ($\psi_{E0}/ (1 - \psi_{E0})$; Figure 2D), favoring PS II. A similar trend was found in the reaction centre density within the PS II antenna chlorophyll bed but was significant only at 400 mM NaCl (RC/ABS; Figure 2B). Regarding the two integrative indexes, significant and distinct values were visible, when treated with 0 mM and 400 mM salt concentrations, structural and functional index (SFI; Figure 2E) was significantly higher in the inoculated plants and, as expected, lower in the non-photochemical or dissipation structural and functional index (SFI_{NPQ}; Figure 2F). At 600 mM NaCl, these variables did not show significant variation between non-inoculated and inoculated samples.

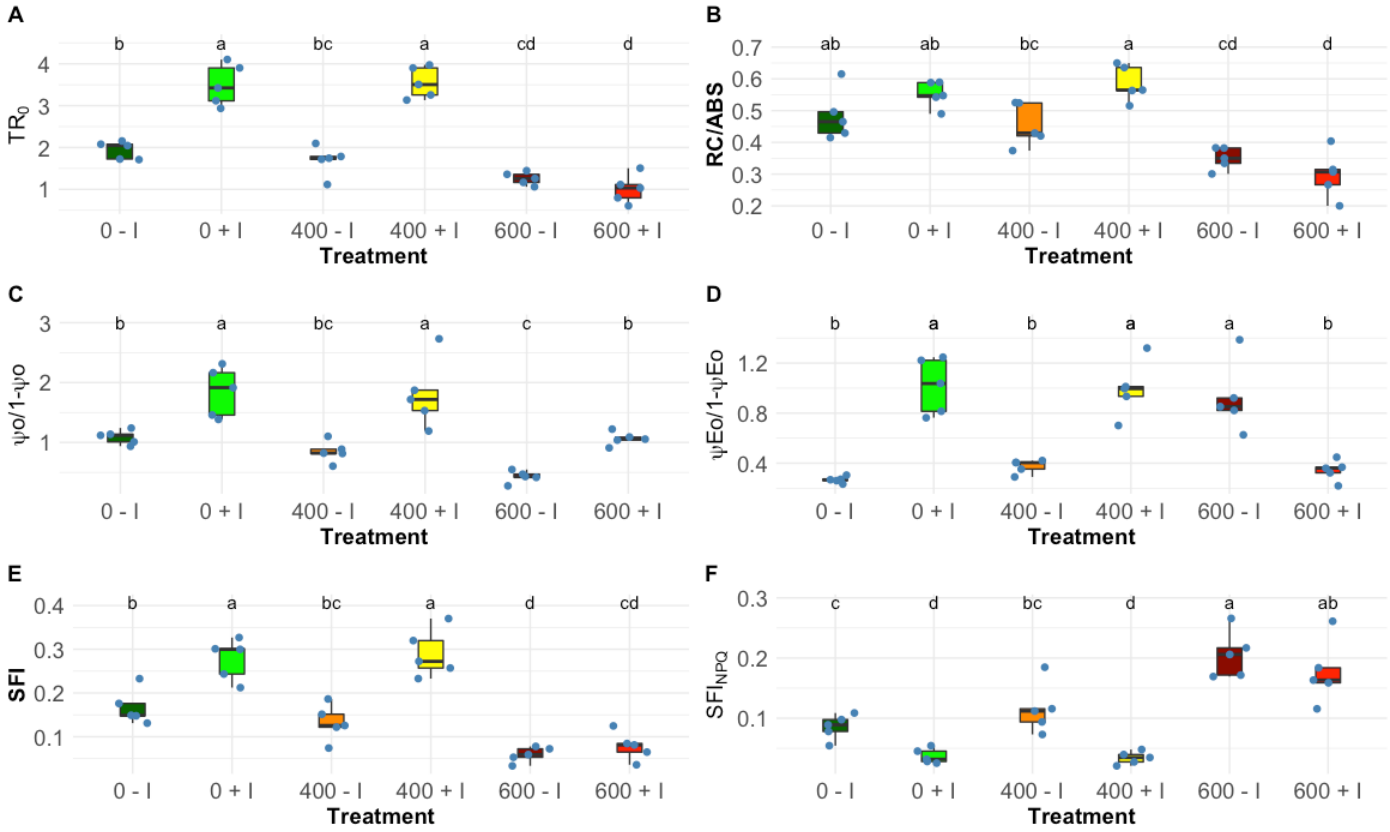

Figure 2. OJIP derived parameters, (**A**) contribution or partial performance due to the light reactions for primary photochemistry (TR_0/DI_0), (**B**) reaction centre II density within the antenna chlorophyll bed of PS II (RC/ABS), (**C**) the contribution of the dark reactions from quinone A to plastoquinone ($\psi_0/ (1 - \psi_0)$), (**D**) the equilibrium constant for the redox reactions between PS II and PS I ($\psi_{E0}/ (1 - \psi_{E0})$), (**E**) structural and functional index for photosynthesis (SFI) and (**F**) non-photosynthetic or dissipation structural and functional index (SFI_{NPQ}) in non-inoculated and inoculated *Halimione portulacoides* dark-adapted leaves (N=5), in conjunction with the NaCl treatments. Letters denote significant differences at $p < 0,05$.

2.2. Na, K, Ca, and Cl accumulation in leaf, stem, and root tissues

Elemental concentration inside the plant tissues exhibited differential allocation upon the application of the tested salt regimes, displaying some expected significant variation amongst NaCl concentration and between PGPR treated plants. Nonetheless, in the stem tissues, there were no significant differences found between non-inoculated and inoculated *H. portulacoides*, leaving the intra-salt treatment variation to be found only in leaf

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and root tissues (Figure 3B, E, H, K). Ion accumulation in this halophyte appears to have a similar tendency within salt treatments except at 400 mM NaCl, where inoculated plants, when compared to non-inoculated plants, show a significantly lower quantity of Na and a significantly higher Cl in the leaf tissues (Figure 3A, G), and displayed significant lower values of Na and Cl in the root tissues (Figure 3C, L). Additionally, at 400 mM NaCl, it was observed a significant increase in the K and Ca in the leaves of the inoculated plants (Figure 3D, G), whereas root Ca was found to be significantly higher in inoculated plants at 0 mM NaCl (Figure 3I), nonetheless in the root K accumulation was not affected by inoculation treatment (Figure 3F).

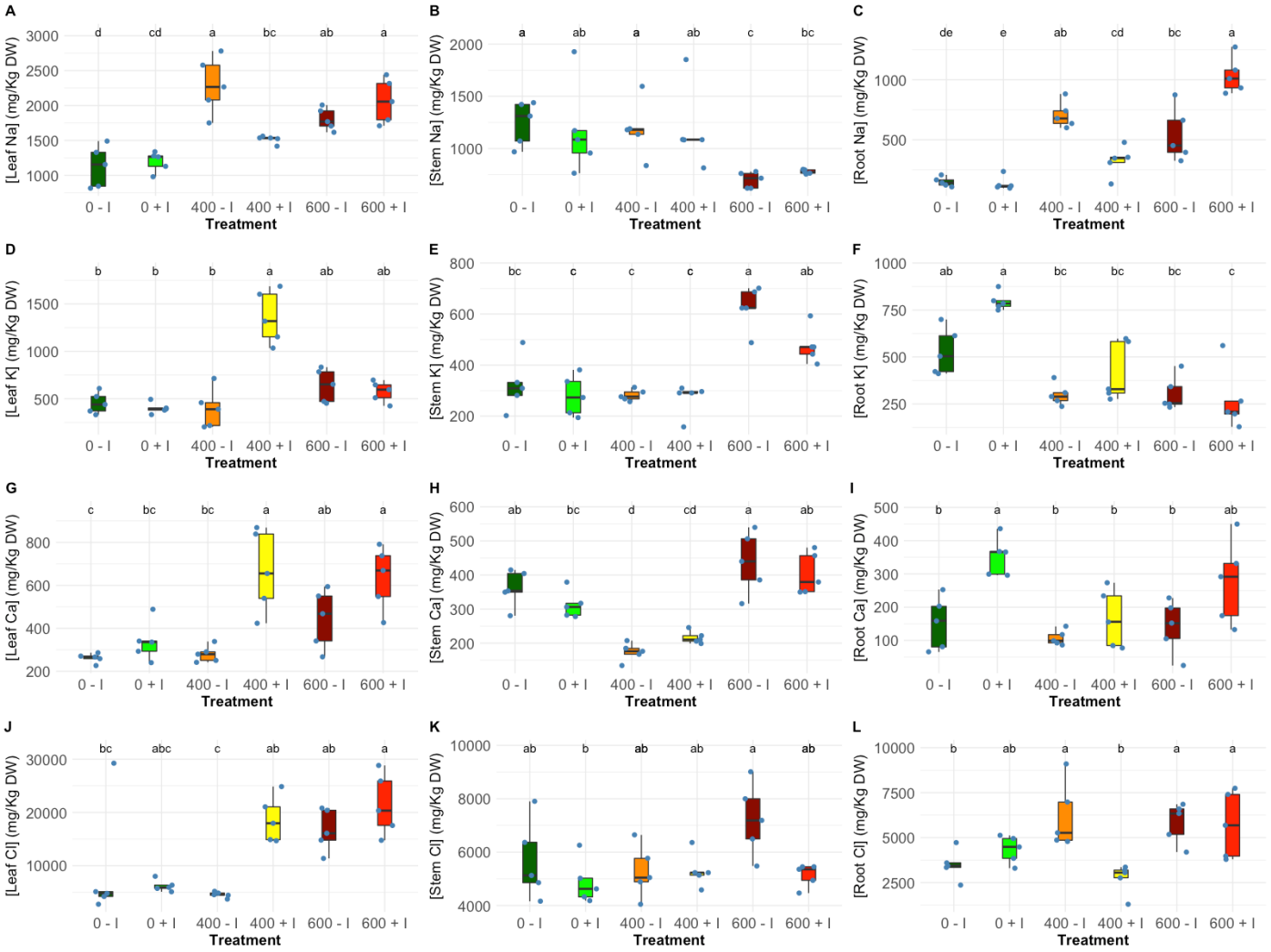

Figure 3. (**A, B, C**) Sodium (Na), (**D, E, F**) potassium (K), (**G, H, I**) calcium (Ca), and (**J, K, L**) chloride (Cl) concentration (mg/Kg) in non-inoculated and inoculated *Halimione portulacoides* (N=5) leaf, stem and root tissues (N=5), in conjunction with the NaCl treatments. Letters denote significant differences at $p < 0,05$.

2.3. Photosynthetic pigments profile

Regarding *H. portulacoides* leaf pigments, significant changes were promoted by the distinct salt applications in both treated groups. Non-inoculated samples displayed lower chlorophyll *a* and *b* (Chl *a*, Chl *b*, respectively) relative concentration, at 0 and 400 mM NaCl (Figure 4A and B). The same was observed in the total chlorophyll and total carotenoids, only here the variation was significant (Figure 4K, L). Analysing the auroxanthin leaf concentration, we found some variation but no significance within salt treatments

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(Figure 4C). In addition, noteworthy variations due to consortium inoculation were also detected in the concentration of lutein (Figure 4F), violaxanthin (Figure 4G), and zeaxanthin (Figure 4H). When subjected to 0 mM and 400 mM NaCl, although comparatively the concentration was found to be higher in both these carotenoids, a significant difference was only expressed in the zeaxanthin. Bioaugmented plants, at 400 mM NaCl, showed a significant decrease in β-carotene concentration (Figure 4E). Antheraxanthin did not exhibit observable differences between inoculate treated plants (Figure 4D). Regarding the pigment ratios in the leaves exposed to the different salinities, bacterial inoculation led to a lower chlorophyll *a*/*b* ratio (Chl *a*/*b* ratio) at 0 mM NaCl (Figure 4M). On the other hand, and despite the significant variation found along with the tested NaCl concentrations total carotenoid to total chlorophyll ratio (Figure 4L, Car/Chl ratio) and de-epoxidation state (Figure 4I, DES) did not display significance when comparing non-inoculated to inoculated *H. portulacoides* individuals.

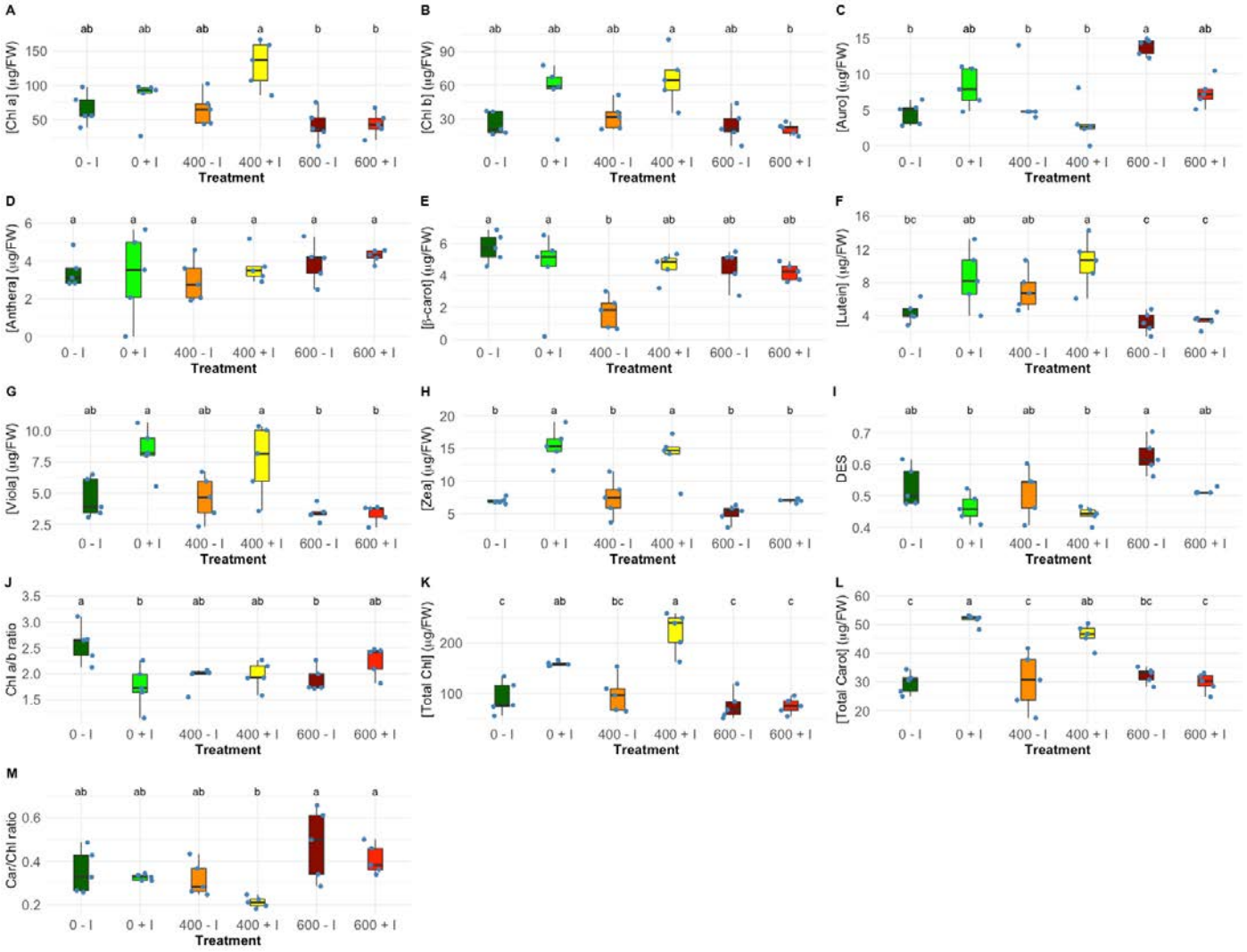

Figure 4. Leaves pigment relative concentrations (µg g-1 FW), (A) chlorophyll a (Chl a), (B) chlorophyll b (Chl b), (C) auroxanthin (Auro), (D) antheraxanthin (Anthera), (E) β-carotenoids (β-carot), (F) lutein (G) violaxanthin (Viola) and (H) zeaxanthin (Zea), (I) de-epoxidation state (DES), (J) chlorophyll a/b ratio (Chl a/b ratio), total (K) chlorophyll and (L) carotenoid (µg g-1 FW), and (M) total carotenoid to total chlorophyll ratio (Car/Chl ratio) in non-inoculated and inoculated *Halimione portulacoides* (N=5), in conjunction with the NaCl treatments. Letters denote significant differences at $p < 0,05$.

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2.4. Antioxidant enzymatic activities

Considering antioxidant enzyme activity in the leaf, PGPB inoculation led to a dissimilar response to salinity changes. Catalase activity (CAT) presented a highly significant decrease when salt concentrations increase, nevertheless at 400 mM NaCl the non-inoculated plants did not display a significant reduction (Figure 5A). Contrarily ascorbate peroxidase activity (APx) exhibited an increasing trend through salt treatments (Figure 5B). Guaiacol peroxidase activity (GPx) and superoxide dismutase activity SOD) did not show any significant variations between the non-inoculated and the inoculated groups (Figure 5C, D). Analysing the thiobarbituric acid reactive substances (TBARS) concentration, a significant difference was found in the non-inoculated plants then exposed to 600 mM NaCl (Figure 5E). Finally, regarding the total protein content of the leaves, an increasing tendency across the increasing salinities was observed in both sample groups and no significance was found in intra-salinity treatments.

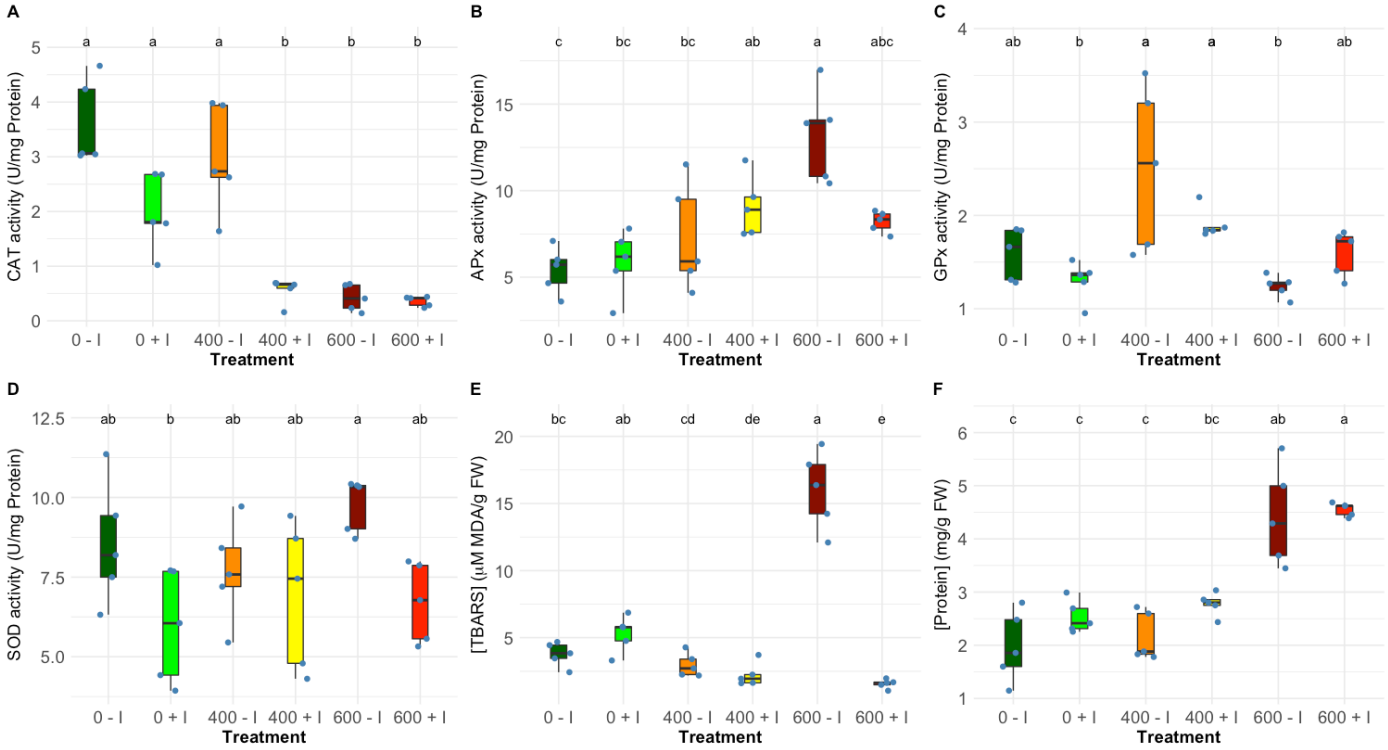

Figure 5. Oxidative stress biomarkers, (**A**) catalase (CAT), (**B**) ascorbate peroxidase (APx), (**C**) guaiacol peroxidase (GPx), and (**D**) superoxide dismutase (SOD) activities (U mg^{-1} protein) and (**E**) thiobarbituric acid reactive substances (TBARS; µM MDA g^{-1} FW) and (**F**) total protein content (Protein; µg Protein g^{-1}FW) in non-inoculated and inoculated *Halimione portulacoides* individuals (mean ± s. d., N=5), in conjunction with the NaCl treatments. Letters denote significant differences at p <0,05.

2.5. Proline quantification

Leaf water content was very similar in all tested treatments with the exception of the non-inoculated plants exposed to the highest salinity level, which showed comparatively low leaf water content when compared to the remaining treatments (Figure 6A). Worth noticing that under the highest salinity level, inoculated plants maintained their leaf water content. Proline content in both *H. portulacoides* groups exhibited an upward trend following the NaCl raise (Figure 6B). However, when comparing non-inoculated plants to PGPR having plants it was observable a significantly higher proline concentration at every salt treatment (0, 400, and 600 mM NaCl).

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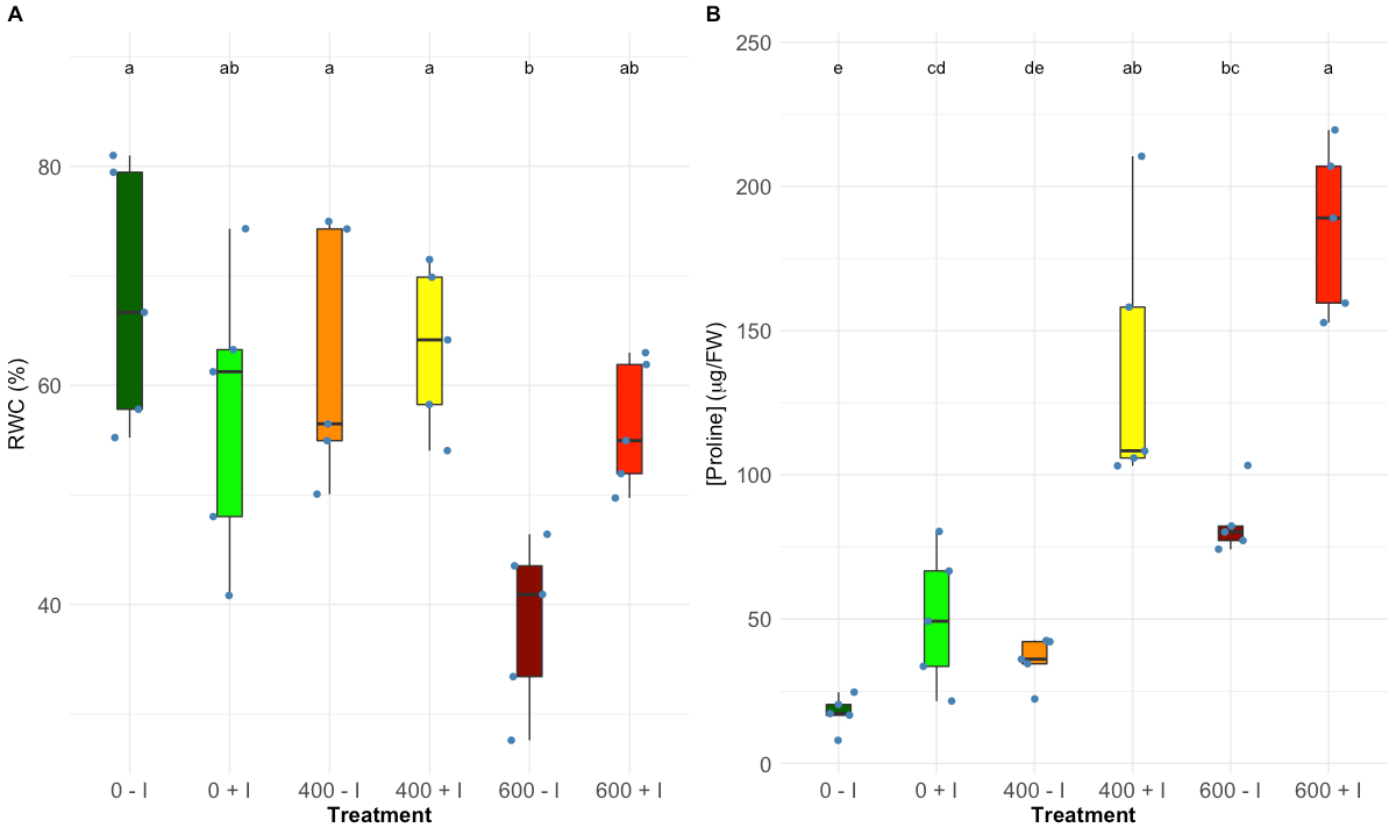

Figure 6. Leaves (**A**) relative water content (RWC) and proline concentration (**B**) of non-inoculated and inoculated *Halimione portulacoides* individuals (mean ± s. d., N=5), in conjunction with the NaCl treatments. Letters denote significant differences at p <0,05.

3. Discussion

According to the data assembled, across this and the last century, the Intergovernmental Panel on Climate Change (IPCC) report points to a global environmental change with alarming social, economic, and environmental consequences [1]. This will be particularly felt in coastal regions, with the temperature rise, expanding drought seasons, intensification of storm surges, sea-level rise, and coupled with the XXI century intensive agricultural practices it can be presumed that water and soil salinization is a major problem and one of the most impacting plant's abiotic stressors [30]. Plant growth-promoting bacteria (PGPR) has been widely recognized to be a great tool for the augmentation of crop productivity and the improvement of the plant's stress coping ability [31,32]. On the other hand, very little attention has been given to the enhancement of the innate tolerances found in the halophytes. The augmentation of the tolerance to excess salt is of great value considering the potential halotolerant species have in the bio-reclamation of salt-damaged soils [33]. In this work, bioaugmentation was achieved by the root inoculation of a consortium formed by competent salt marsh PGB-bacteria that were obtained and evaluated in a previous study [26].

The traits evaluated from the chlorophyll fluorescence measurements confirmed that *H. portulacoides* is highly salt-tolerant, as expected from the findings in previous studies [34,35], although at the high tested concentration (600 mM NaCl) already some signs of stress could be found. Nevertheless, the most noticeable differences were detected when comparing the non-inoculated and inoculated plants' responses to the NaCl treatments. The photochemical analysis showed that the inoculated plants exhibited a substantial improvement in their photosystem II efficiency at mild salt stress. Inoculated *H. portulacoides*,

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at 400 mM NaCl, displayed higher trapped (TR/CS) and transport energy flux (ET/CS) per cross-section, the main flux responsible for chemical energy production, and available reaction centres (RC/CS) per cross-section. In addition, bioaugmentation significantly increases the reaction centre II density within the PS II antenna, this could explain the greater energy translated at the electron transport chain, from quinone A to plastoquinone, suggesting a PGPR-induced improvement in the photon capture capability as well as a more efficient light-harvesting mechanism [36]. Plastoquinone is an efficient water-soluble electron carrier and functions as the primary electron acceptor of PS II [37]. Consequently, plastoquinone activity augmentation appears to have had a role in the significantly higher contribution or partial performance due to the light reactions for primary photochemistry and in the enhancement of the equilibrium constant for the redox reactions between PS II and PS I transport. [38–41]. Overall, these features are reflected in the significant increase shown by the structural and functional index for photosynthesis (SFI) in the inoculated plants at 0 mM a 400 mM NaCl, and, as expected, a significantly lower non-photosynthetic or dissipation structure functional index (SFI_{NPQ}) [42]. Additionally, the typical higher dissipation energy flux observed in stressed individuals [43], was also ameliorated in the plants inoculated with PGPB. These results revealed the PGPR consortium's significant enhancement of the photochemical performance of the plants at 0 mM NaCl but with a special impact at 400 mM NaCl.

These differences found at the photosynthetic level can be explained, in part, by the influence exercised by the PGPR in the ionic uptake and osmotic regulation mechanisms. As expected, the increase of NaCl in the environment led to an increase in the content of Na in the *H. portulacoides* leaves and roots, which is normally associated with salt stress biomarkers due to the direct correlation between the accumulation of Na in the cell and plant salt toxicity [44,45]. Besides, the increasing Na concentration in the medium will directly compete with other essential ions like K, resulting in the impairment of cellular functions regulated by potassium, such as enzyme activation, protein production, and, photosynthesis [46,47]. Nonetheless, our results, at mild salinity stress, showed a PGPR induced a significant reduction of the Na and Cl present in the roots, as well as a significant increase of the K and Ca content in the leaves tissues which could explain the significantly lower Na found in the inoculated plants leaves. This reveals superior maintenance of ionic homeostasis, indicated by the K: Na ratio in the inoculated plants tissues. The increase of the Ca concentration in the leaves could serve as an ionic balance mechanism, allowing the cell membrane to maintain the K/Na selectivity [43]. In fact, the uptake of K and Ca due to PGPR inoculation has been demonstrated to result in the up-regulation of the antioxidant system and mitigation of salt stress oxidative effects [48]. Furthermore, it has been described that rhizosphere inoculation by this halotolerant bacteria may confer salt tolerance through sequestering Na^+ into vacuoles, expelling Na^+ from roots, and, thru the exertion of a modulating effect in the ion transporters [49,50].

Leaf osmotic regulation is of crucial importance in salt stress plants [51]. The increased presence of organic osmolytes is a widespread plant strategy to prevent stress-induced damages to cellular organelles [52]. In halophyte plants, in order to counteract soil salinity, its commonly witnessed an accumulation of L-proline in the leaf, well known for its osmoprotectant potential [53,54]. This amino acid is considered to play a pivotal role in the protection of cellular membrane structures, the function of ROS scavenging enzymes, and the maintenance of leaf water content [55]. *Halimione portulacoides* presented a proline elevation in its leaves, through the increasing NaCl concentrations. Nonetheless, bioaugmented plants displayed a greater increase, exhibiting a significantly higher proline concentration in every NaCl treatment. The effect of the greater proline availability was clearly expressed in the leaf relative water content, at 600 mM NaCl. It is also known that, as a nitrogen-containing amino acid, proline expression is enhanced when nitrogen is available, this relationship could be considered a factor in the higher proline concentration found in the inoculated samples [56,57].

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Regarding the pigment profiles, it was clear that *H. portulacoides* plants were affected by the rise in salt concentration, revealing a shift photoharvesting to photoprotection in the higher salinities. The role of the xanthophyll cycle in the photoprotection of PSII is a well-characterized energy dissipation mechanism commonly observed in halophyte plants [58,59]. The de-epoxidation state (DES) of luminal violaxanthin cycle pigments (violaxanthin, to antheraxanthin, to zeaxanthin) was found to be one of the most effective dissipation of excess light energy mechanisms, reducing the overload of energy within light-harvesting complexes (LHCs) [60–62]. Even though, an increase of the de-epoxidation occurred in the highest test NaCl concentration, significant differences induced by bacterial inoculation were not found, however, the concentration of zeaxanthin was found to be significantly higher in the bio-augmented plants, at 0 mM and 400 mM NaCl. Zeaxanthin is characterized by having an important photoprotective role, it was found to enhance high-light stress mechanisms thru non-photochemical quenching (NPQ) of excitation energy [63,64]. Lutein also plays a role in the PSII photoprotection, has been described as functioning as a structural stabilization of antenna proteins and light-harvesting contributor, relocating excitation energy to Chl [60,65]. At 400 mM, the higher lutein concentration found in the inoculated plants coupled with the significantly higher zeaxanthin associated with the significantly higher RC II density within the antenna chlorophyll bed of PS II and antenna size of an active PS II shown by fluorometric analysis can be suggested that at 400 mM NaCl concentrations PGPR augmentation improves photoprotection. Moreover, auroxanthin, a noteworthy carotenoid due to its well-described role as an effective energy quencher under salinity stress conditions in isolated LHC IIb, showed a significant increase at 600 mM NaCl in the non-inoculated *H. portulacoides* [39,66,67]. This can indicate an alleviation of the potential photoinhibitory conditions due to bacterial inoculation, and thus a reduction of the photoprotective countermeasures. Chlorophyll a/b leaf pigment ratio displayed a significant decrease through NaCl concentrations, only in non-inoculated individuals, this could be an indication that the absorbed light exceeds the photochemical capability [43]. Additionally, at 0 mM and 400 mM NaCl, inoculation plants suffered an amelioration effect thru the total chlorophyll concentration significant increase, which can be explained by the nitrogen fixation capability found in the PGPR inoculate, and thus higher amounts of nitrogen available for the production of this photoreceptor pigment [26,68,69].

It is known that salinity-induced stress can give rise to a buildup of harmful molecules in plant tissues. Excess of Na^+ ion in the cytoplasm has a negative influence on the uptake of other ions that can ultimately lead to the impairment of various metabolic pathways and the increased production of reactive oxygen species (ROS) [68,70]. In order to counteract ionic changes, halophytes build up effective antioxidative mechanisms, just as proficient enzyme-based systems as well as mechanisms driven by pigment conversions [71,72]. When assessing the oxidative stress biomarkers equilibrated response tendencies to the salt gradient were found, but some noteworthy PGPR induced discrepancies were seen between the treated groups. Non-inoculated and inoculated plants showed a catalase (CAT) activity and ascorbate peroxidase (APx) activity reduction through increasing salinities, however at 400 mM NaCl, catalase displayed significantly higher activity in the non-inoculated samples, possible due to the also significantly higher uptake of Na^+ in the leaves [73,74]. On the other hand, at 600 mM NaCl, superoxide dismutase (SOD) and ascorbate peroxidase activity, in non-inoculated individuals, were shown to be higher, pointing out to higher H_2O_2 production by SOD and subsequent metabolization by APx [75]. Nevertheless, the significant increase of lipid peroxidation products (TBARS) found in the same group, suggests that in non-inoculated *H. portulacoides* when exposed to 600 mM NaCl, the peroxidasic metabolization might not be sufficient with a consequent higher ROS production and lower ROS scavenging mechanisms [74]. In sum, thru this data analysis, it can be claimed that the PGPR consortium root inoculation had a significant role in the amelioration of the phytoprotection and overall fitness of *H. portulacoides* when exposed to mild NaCl stress.

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4. Materials and Methods

4.1. Sampling sites and plant material collection

Samples of *Halimione portulacoides* were collected from Tagus estuary salt marsh located on the western coast of Portugal (38°46'N 9°05'W). In the laboratory, plant samples were rinsed and gently cleaned to eliminate dust and debris. The roots and portion of the stems *H. portulacoides* samples were cut to generate stem cuttings, at least two nods were left in the stem below the lowest branch for graft development. The props were hydroponically cultured in dark-walled vases filled with a nutritive solution (N:P:K 4:5:7, Bo 0,01 %, Cu 0,002 %, Fe 0,02 %, Mn 0,01 %, Mo 0,001 %, Zn 0,002 %) and placed in a phytoclimatic chamber with a sinusoidal function programmed to mimic a natural light environment (maximum PAR 300 µmol photons m^2 s^{-1}, 16/8 h day/night rhythm, 20/18 °C day/night temperature amplitude). Plants were kept in this condition for 48 days to allow acclimatization and root biomass growth.

4.2. Rhizobacteria Used for Inoculation in This Study

The rhizobacteria consortium was made with rhizobacteria obtained from different halophytes inhabiting the Portuguese west coast salt marshes: *B. aryabhattai* and *S. rhizophila* were collected from Horta dos Peixinhos, Aveiro (40°45'N 8°56'W) and *S. rhizophila* and *S. endophyticus* from Tagus estuary, Lisbon (38°46'N 9°05'W). Sediments adjacent to halophyte plants were collected by hand spade into sterile plastic bags, transported to the laboratory, and kept at 4 °C until analyses. Interstitial (pore) water was gathered using Rhizon samplers (Rhizosphere Research Products, Wageningen, The Netherlands). The rhizobacteria used in the consortium formation were isolated, identified, and described in a previous study [26].

Table 1. Summary of the bacteria isolates and their enzymatic properties of the rhizobacterial consortia used in this study [26].

		Plant-Growth Promoting Traits						
Bacterial Strains	**Sampling Site**	**Limit Salt Tolerence (mM)**	**P-Solubilization**	**Siderophore**	**ACC Deaminase (nm mg^{-1} h^{-1})**	**IAA (µg mL^{-1})**	**N-Fixation**	**EPS (OD_{540})**
Bacillus aryabhattai SP20	Horta dos Peixinhos, Portugal	856	+	+	-	-	-	0.80 ± 0.01
Stenotrophomonas rhizophila EH7	Horta dos Peixinhos, Portugal	856	+	+	-	15,02 ± 0.31	+	-
Pseudomonas oryzihabitans RL18	Tagus Estuary, Portugal	1711	+	+	+* (26.9 ± 14.13)	39.55 ± 1.01	-	0.40 ± 0.01
Salinicola endophyticus EL13	Tagus Estuary, Portugal	1711	+	+	+*	71.35 ± 6.86	-	0.54 ± 0.01

– negative; +* visible growth on solid DF +ACC medium.

4.3. Preparation of Bacterial Inoculants

All four rhizobacteria were cultivated individually in 250 mL Erlenmeyer flasks containing 50 mL of tryptic soy broth (TSB) medium modified by the addition of NaCl 25 gL^{-1} in a rotary shaker for 24 h (150 rpm, 30 °C). Then, cultures were centrifugated in 50 mL sterile Falcon tubes at 5000g, for 10 min at room temperature, and the supernatant was discarded. Pellets were washed twice with sterile 0.9% (w/v) saline solution and adjusted to a final concentration of 10^8 CFU mL^{-1} (OD600 = 1)[21]. Bacterial suspensions were then

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mixed, in equal amounts and diluted, achieving the final concentration of 10^7 CFU mL^{-1}, in order to produce the consortium suspension for root inoculation, as described in [76].

4.4. Experimental setup and root inoculation

After the abovementioned plant adaptation period, samples were arranged in 6 groups with 5 replicates. The first 3 of the groups were inoculated with the previously prepared rhizobacterial consortium solution (Table 1). Plants were inoculated by soaking the roots in the rhizobacteria consortium suspension for 6 h at room temperature (25 ± 1 °C) [76]. Control plants were soaked simultaneously in sterile physiological saline solution (NaCl 0.9% w/v). After 10 days of the adaptation period, *H. portulacoides* individuals from both inoculated and non-inoculated groups were separated into 3 sets with 5 replicate individuals and kept under the same described conditions, the nutritive solution was replaced, with NaCl treated nutritive solution in order to attain the desired target salinities (0, 400 and 600 mM NaCl):

0 mM NaCl non-Inoculated (**0 – I**)
0 mM NaCl Inoculated (**0 + I**)
400 mM NaCl non-Inoculated (**400 – I**)
400 mM NaCl Inoculated (**400 + I**)
600 mM NaCl non-Inoculated (**600 – I**)
600 mM NaCl Inoculated (**600 + I**)

Exposure trials lasted for 7 days after which chlorophyll fluorescence measurements were made and subsequently, plants were harvested. Leaf samples for biochemical measurements were immediately flash-frozen in liquid-N_2 and stored at -80 ºC until analysis.

4.5. Pulse amplitude modulated (PAM) fluorometry

In order to perform modulated chlorophyll fluorescence measurements, *H. portulacoides* attached leaves were dark-adapted for at least 30 min. The OJIP transient (or Kautsky curves) measurements were made using FluoroPen FP100 PAM (Photo System Instruments, Czech Republic). OJIP, assess the chlorophyll fluorescence induction kinetics of PS II. This is attained when a dark-adapted leaf is irradiated with the saturating light intensity of 3500 μmol m^{-2} s^{-1} then it exhibits a polyphasic rise in fluorescence (OJIP): level O represents all the open reaction centres at the onset of illumination with no reduction of Q_A (fluorescence intensity lasts for 10 ms); O to J transient indicates the net photochemical reduction of Q_A (the stable primary electron acceptor of PS II) to Q_A^- (lasts for 2 ms); the J to I transition is due to all reduced states of closed RCs such as $Q_A^- Q_B^-$, $Q_A Q_B^{2-}$ and $Q_A^- Q_B H_2$ (lasts for 2–30 ms); P-step coincides with a maximum concentration of $Q_A^- Q_B^2$ with plastoquinol pool maximally reduced and also reflects a balance between the light incident at the PS II side and the rate of utilization of the chemical (potential) energy and the rate of heat dissipation [77]. Table 2 summarizes all the parameters that were calculated from the fluorometric analysis.

Table 2. Summary of fluorometric analysis parameters and their description.

Ψ0/(1 - Ψ0)	Contribution of the dark reactions from quinone A to plastoquinone
ΨEo/(1 - ΨEo)	The equilibrium constant for the redox reactions between PS II and PS I
RC/ABS	Reaction centre II density within the antenna chlorophyll bed of PS II
TR0/DI0	Contribution or partial performance due to the light reactions for primary photochemistry
SFI	Structure functional index for photosynthesis
SFI (NO)	Non-photosynthetic or dissipation structure functional index
ABS/CS	Absorbed energy flux per cross-section.
TR/CS	Trapped energy flux per cross-section
ET/CS	Electron transport energy flux per cross-section.

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DI/CS	Dissipated energy flux per cross-section.
RC/CS	The number of available reaction centres per cross-section.

4.6. Ion analysis

Leaf, stem, and root tissues were washed with ultra-pure water to eliminate surface residues. Afterward, plant tissues were dried at 60 °C until the achievement of constant weight, then 100 mg of ground tissue was taken for acid digestion. This procedure was performed in Teflon reactors using an acid mixture of HNO_3:$HClO_4$ (7:1, v/v) at 110 °C for 3 hours and then allowed to cool down. Elemental measurements were done using a total reflection X-ray fluorescence analysis (TXRF) instrument (S2 PICOFOX™ spectrometer, Bruker Nano GmbH), 5 µL of the digestion product was placed in the middle of the quartz carriers. The cleaning and preparation of the quartz glass sample carriers were made according to [78]. Three quartz carries with mono and multi-elemental standards (Bruker Nano GmbH, Germany), were placed in each sample carrier in order to calibrate, considering measurement sensitivity, detection limits, and precision. For each trace element analysis digested tissue was irradiated for 800 seconds. Elemental analytical methodology accuracy and precision were evaluated by the replication of the certified reference material BCR-146. The TXRF spectra and data translation was performed using the Spectra 7.8.2.0 software.

4.7. Pigment profiling

Leaves from the differently treated samples were weighted before (FW) and after (DW) freeze-drying and this data were then used to calculate the leaf relative water content as RWC (%) = (FW-DW) / FW. For pigment extraction, freeze-dried leaf samples were grounded in an agate mortar and 100% acetone was subsequently added and introduced to a 2 min ultra-sound bath, to ensure complete leaf breakdown. Extraction was carried out for 24 h at -20 °C in the dark. After extraction, samples were centrifuged at 4.000g, 15min at 4 °C. Supernatants were scanned from 350 nm to 750 nm in 1 nm steps, using a dual-beam spectrophotometer (Shimadzu UV/VIS UV1601 Spectrophotometer). Finally, absorption spectra were analysed employing Gauss-Peak Spectra (GPS) method fitting library, using SigmaPlot Systat Software [79]. This method allowed the pigment recognition and quantification detected in the spectrum, ascertaining the leaf pigment profile, chlorophyll *a*, chlorophyll *b*, auroxanthin, antheraxanthin, β-carotene, lutein, violaxanthin, and zeaxanthin. For a better evaluation of the light-harvesting and photoprotection mechanisms, the De-Epoxidation State (DES) was calculated as:

$$DES = \frac{([\text{Antheraxanthin}] + [\text{Zeaxanthin}])}{([\text{Violaxanthin}] + [\text{Antheraxanthin}] + [\text{Zeaxanthin}])}$$

4.8. Oxidative stress biomarkers

Regarding enzyme extractions, *H. portulacoides* leaf samples were retrieved from -80 °C storage, and extractions were performed according to Tiryakioglu et al. [80], at 4 °C. In a ceramic mortar, 4 ml of 50 mM sodium phosphate buffer (pH 7,6) supplemented with 0,1 mM Na-EDTA was added to each 250 mg (FW) leaf sample, for homogenization. The homogenate was centrifuged at 8.890g, 20 min at 4 °C, and the supernatant was transferred to an eppendorf and put on ice, to be used in the following analyses.

The enzyme activity measurements of catalase (CAT, EC.1.11.1.6.), Ascorbate peroxidase (APx, E.C. 1.11.1.11), Guaiacol peroxidase (GPX, E.C. 1.11.1.7), and Superoxide dismutase (SOD, E.C. 1.15.1.1) were performed in a dual-beam spectrophotometer (Shimadzu UV/VIS UV1601 Spectrophotometer) using quartz cuvettes. Catalase activity assays were assayed according to the method of Teranishi et al. [81], by evaluating the H_2O_2

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consumption thru 240 nm absorbance decline (molar extinction coefficient of 39,4 mM^{-1} cm^{-1}). Ascorbate peroxidase was performed according to Tiryakioglu et al. [80], by monitoring the ascorbate oxidation thru 290 nm absorbance decline (molar extinction coefficient of 2,8 mM^{-1} cm^{-1}). Guaiacol peroxidase measurement was performed according to Bergmeyer et al. [82], by observing guaiacol oxidation products formation thru 470 nm absorbance rise (molar extinction coefficient of 26,6 mM^{-1} cm^{-1}). Superoxide dismutase total activity was measured according to the method of Marklund and Marklund [83], by calculating the oxidation rate of pyrogallol thru the 325 nm absorbance evaluation. Protein quantification was performed according to the Bradford method [84].

Membrane lipid peroxidation in leaf samples quantification was determined according to Heath & Packer [85]. Leaves were homogenized in a solution of fresh 0,5% (w/v) thiobarbituric acid (TBA) and 20 % (w/v) trichloroacetic acid solution, in a 100 mg (FW) to 2 mL proportion. Extraction was performed thru a 30 min incubation at 95 °C, after which, the samples were cooled in ice to stop the reaction and centrifuged at 4.000g, 5 min at 4 °C. The absorbance was read at 532 nm and 600 nm in a Shimadzu UV-1601 spectrophotometer. Malondialdehyde (MDA) concentration was calculated using the molar extinction coefficient, 155 mM^{-1} cm^{-1} when applying the following equation:

$$A_{532\,nm} - A_{600\,nm} = [MDA]mM \times \varepsilon MDA$$

4.9. Proline quantification

Proline content was estimated according to [86]. The plant material was homogenized in 3% (w/v) sulfosalicylic acid solution and centrifuged at 10.000 rpm, 15 min at 0 °C. The supernatant was kept and then used for proline quantification. The reaction comprised in the 1 h at 100 °C incubation of the 2 ml glacial acetic acid, 2 ml acid ninhydrin, and 2 ml extract solution. After which the reaction was stopped in an ice bath. The reaction mixture was extracted thru the application of 4 mL of toluene and its absorbance was read at 520 nm and compared with a standard curve of proline and expressed in µmol g^{-1} FW.

4.10. Statistical analysis

Boxplots with probability density of the data at different values smoothed by a kernel density estimator were computed and plotted using the ggplot2 package in R-Studio Version 1.4.1717. Non-parametric Kruskal–Wallis with Bonferroni posthoc tests, for comparisons between the variables and samples exposed to different exudate concentrations, were performed in R-Studio Version 1.4.1717 using the agricolae package [87].

5. Conclusions

Global soil salinization is an undeniable ongoing process, either due to exhaustive agricultural practices or increased marine flooding in coastal areas due to sea level rise. Thus, becomes of utmost importance to develop eco-friendly strategies to restore salinized soils. Halophytes are naturally adapted to salinized conditions nevertheless its tolerance is not unlimited. Thus, the bioaugmentation of these species with marine PGPR appears as a new solution for this problem. The inoculation by salt marshes PGPR consortium has a positive and significant impact in salt-stressed *H. portulacoides*, with the most remarkable enhancements between non-inoculated and inoculated individuals found in mild or suboptimal salt stress conditions. This is mainly achieved through the improvement of the photochemical efficiency of the plants, with higher energy use efficiencies and lower energy dissipation. Even though, under severe salinity stress the PGPR amelioration was less clear and their effects less significant, it had still a crucial impact on the maintenance of leaf water content, thru the endorsement of organic osmolytes production, seen in the

significantly higher proline concentration. Additionally, a mitigation of the levels of oxidative stress of the plants was also observed. In sum, bioaugmented *H. portulacoides* plants appear as a candidate species to be used in the restoration of salinized soil and seawater agriculture cash crop, being the studied PGPR key components of this process. Nonetheless, more research is needed to the exploration of the effectiveness, specificity, and limitations of this plant-growth promoting rhizobacteria.

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