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Effect of Plant Growth-Promoting Rhizobacteria on *Salicornia ramosissima* Seed Germination under Salinity, CO_2 and Temperature Stress

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Abstract: In a scenario of climate change and growing population, halophyte root microbiota interactions may be a sustainable solution to improve alternative crop production while combating abiotic stress. In this work, seeds of the cash crop halophyte *Salicornia ramosissima* were inoculated with five different plant growth-promoting rhizobacteria consortia, isolated from the rhizosphere of five halophytes in southwestern Spain salt marshes. For the first time, we recorded seed germination response to three interactive abiotic stressors, CO_2 (400 and 700 ppm), temperature (25 and 29 °C) and salinity (171, 510 and 1030 mM NaCl), all of them related to climate change. Salinity played a decisive role, as no significant differences were registered between treatments at 171 mM NaCl and no germination took place at 1030 mM NaCl. At 510 mM NaCl, one rhizobacterial consortium improved seed parameters notably, increasing up to 114% germination percentage and 65% seedlings biomass. These first findings encourage us to think that cash crop halophytes like *S. ramosissima* and halophyte root microbiota may be valuable resources for human or animal feeding in a future climate reality.

Keywords: halophytes; root microbiota; rhizomicrobiome; cash crop; climate change; Spain

1. Introduction

To guarantee the future demands of society and maintain the socio-economic system, potential solutions are being studied to adapt current agricultural practices to climate change [1]. Among them, alternative plant species may be used, with salinity tolerance and low water requirements, but also with agronomic potential. As a result, on one hand, fresh water would be reserved for the irrigation of lower salinity-tolerant crops and, on the other hand, marginal lands, currently unproductive, would be used for these alternative tolerant crops [2].

In line with this strategy, halophytes have been proposed as sustainable edible crops (cash crops) [3]. They inhabit brackish soils and have developed many morphological and physiological adaptations to survive under hostile environmental conditions and still maintain a high productivity [2,4–8]. This is allied to their content in bioactive and healthy properties [2,3,6,9,10]. In the Mediterranean basin, there is special interest in the chemical composition and health promoting components of wild halophytes, like those belonging to *Salicornia* genus. They may be promising functional food products and/or sources of bioactive compounds [11]. Indeed, there have been some successful cultivation cases of some *Salicornia* species, and they can be found in bibliography or media as "sea asparagus" [12–14].

The C3 halophyte *S. ramosissima* J. Woods was used in this work, as it has been recently considered as a food and pharmaceutical candidate with immediate commercial interest [15–17], thus playing an important role as a multifunctional cash crop. This fact is especially important in Mediterranean countries, including Spain, where *S. ramosissima* grow, bearing in mind that they will be among the most affected areas of the planet by climatic events because of their geographical location and their traditional agricultural production [1].

An important biotool related to these species are their rhizosphere and endorhiza microbiota. Mutualistic interactions with some of these bacteria, especially plant growth-promoting bacteria (PGPB), may positively affect the growth and health of host plants and reinforce their tolerance to stressors [18,19]. *Salicornia* and the rest of halophytes are highly salt–resistant plants that can grow in areas with high levels of salinity (from 200 mM NaCl), but it is well known that salt exposure over tolerance limits triggers physiological and biochemical alterations that affect plant growth, development and production [5]. Moreover, increases in atmospheric CO_2 concentration and temperature are predicted for future climate reality together with salinity [1]. Salt-tolerant plant growth-promoting rhizobacteria (PGPR) may reinforce halophytes growth and environmental stressors tolerance, as has been recently demonstrated in a work with *Salicornia* sp. under salinity stress [20]. However, screening of PGPR in halophytes are scarce to date [21–29]. Also, no studies of the effect of PGPR in *Salicornia* under NaCl, CO_2 and temperature stress have been carried out.

In view of the above, the present work is aimed at:

(1) the isolation and characterization of the cultivable rhizobacteria of several halophytes from southwestern Spain salt marshes;
(2) the selection of PGPR consortia;
(3) the analysis of the impact of PGPR inoculants on seed germination of *Salicornia ramosissima* under NaCl, CO_2 and temperature stressors.

2. Materials and Methods

2.1. Isolation of Cultivable Rhizobacteria From Halophytes

Plants of *Halimione portulacoides* and *Salicornia ramosissima* were harvested in May 2017 from the Piedras (37°16'09.1'' N 7°09'36.4'' W) and Tinto (37°13'51.40" N 6°54'28.40" W) river estuaries, southwest Spain, and transported to the laboratory. Next, approximately 5 g of *H. portulacoides* and *S. ramosissima* rhizosphere with soil adhered to their roots were mixed in two separated 50 mL sterile Falcon tubes with 40 mL of physiological saline solution (NaCl 0.9% w/v) by shaking for 15 min using a vortex mixer. After that, big particles and sediments were allowed to settle for 10 min. Then, 100 µL of the supernatant suspensions, 10^{-1} and 10^{-2} dilutions were pour-plated onto tryptic soy agar (TSA) NaCl 0.2 M medium plates. This medium has been employed in our previous works [27,30,31], as it has similar NaCl concentration to estuarine sediments in southwest Spain. Following the incubation for 48 h at 28 °C, single colonies were individually plated on TSA NaCl 0.2 M according to differences in colony morphology. Then, they were subsequently re-isolated by plating on TSA NaCl 0.2 M and 48 h incubation at 28 °C in order to ensure purity of the culture. Pure bacterial liquid cultures were preserved in 15% glycerol at −80 °C for further use. TSA NaCl 0.2 M was prepared by replacing part of distilled water with a SW30 (salt water) solution when preparing TSA medium (SW30 solution: Per liter, NaCl 234 g, $MgCl_2 \cdot 6H_2O$ 39 g, $MgSO_4 \cdot 7H_2O$ 61 g, $NaHCO_3$ 0.2 g, NaBr 0.7 g, KCl 6 g, $CaCl_2$ 1 g, H_2O e.q. to 1 L), to finally autoclave at 121 °C during 20 min.

2.2. Rhizobacteria Characterization, Identification and Consortia Design

Phenotypic observation (Gram type, shape and motility) of rhizobacteria pure isolates was done by optical microscopy (Gram staining and wet mount). Next, bacterial growth was evaluated under salt and temperature stress. For that, rhizobacteria isolates were plated onto TSA medium plates amended with SW30 solution, with NaCl concentrations of 0.2 M, 1.2 M and 2 M and incubated

at 28 and 40 °C, for a maximum period of 4 days. Salt tolerance was expressed as the maximum tolerable concentration (MTC) of NaCl, namely the maximum concentration of NaCl not affecting bacterial growth. As for plant growth-promoting (PGP) traits of rhizobacteria, the same methodology as described in detail in [29] was used. Briefly, bacterial growth in NFb (nitrogen free bromothymol blue) medium was used to test nitrogen fixation [32]. Phosphate solubilization was observed on NBRIP (National Botanical Research Institute's phosphate) medium plates when bacterial growth caused the appearance of surrounding transparent halos [33]. In the same way, orange halos revealed production of siderophores on CAS (chrome azurol S) plates [34]. In all cases, plates were incubated during 72 h at 28 °C before analyzing the results. The synthesis of IAA (indole-3-acetic acid) was colorimetrically estimated as explained in [29]. In the same line, capability of biofilm formation was measured by an adhesion capacity assay in wells of 96-wellmicrotitre plates and further absorbance measures (detailed in [29]). Presence of bacterial ACC (1-Aminocyclopropane-1-Carboxylate) deaminase activity was detected following again a colorimetrically method that quantifies the amount of α-ketobutyric acid generated from the cleavage of ACC [35]. Total protein concentration of toluenized cells [36] was later determined and used to calculate bacterial ACC deaminase activity, represented as μmoles of released α-ketobutyrate per mg of protein per hour. NaCl concentration in all media was adjusted to 0.2 M by adding SW30 solution before autoclaving. Pure liquid cultures of strains with interesting properties were sent to StabVida Company (Portugal) using FTA cards (Flinders Technology Associates) for identification by 16S rRNA sequencing. Sequences were deposited in GenBank with unique accession numbers. Finally, the best-performing strains from each halophyte were selected to conform bacterial consortia. To avoid antagonistic interactions between them, their strain compatibility to grow together was assessed by liquid growing and further plating.

2.3. Rhizobacteria Used for Inoculation in This Study

Five rhizobacteria consortia, from the rhizosphere of five different halophytes inhabiting southwestern Spain salt marshes, were used in this experiment. Three have been isolated, identified and described in previous works, and the other two are characterized in this study (Table 1).

Table 1. Rhizobacterial consortia used in this study.

Consortia Number	Hosting Halophyte	Sampling Location	Bacterial Strains	Reference
1	*Spartina densiflora*	Tinto estuary, SW Spain	*Pseudomonas composti* SDT3 *Aeromonas aquariorum* SDT13 *Bacillus thuringiensis* SDT14	[30]
2	*Arthrocnemun machrostachyum*	Odiel estuary, SW Spain	*Vibrio kanaloae* RA1 *Pseudoalteromonas prydzensis* RA15 *Staphylococcus warneri* RA18	[27]
3	*Spartina maritima*	Tinto and Odiel estuaries, SW Spain	*Bacillus methylotrophicus* SMT38 *Bacillus aryabhattai* SMT48 *Bacillus licheniformis* SMT51 *Pantoea sp.* RSO7	[29,31]
4	*Halimione portulacoides*	Piedras estuary, SW Spain	Described in Section 3.2.	Present study
5	*Salicornia ramosissima*	Tinto and Piedras estuaries, SW Spain	Described in Section 3.2.	Present study

2.4. Salicornia ramosissima Seeds Source and Experimental Treatments

Seeds of *S. ramosissima* were collected in October 2017 from Odiel salt marshes (37°13'7.00" N 6°57'35.92" W, SW Spain) and transported to the laboratory. They were surface-disinfected prior to the experiment, by immersion and vigorous shaking in 5% (v/v) sodium hypochlorite for 1 min, followed by being washed five times with sterile distillated water. They were then randomly divided in 72 blocks of 100 seeds, as follows: Three NaCl concentrations (171 mM, 510 mM and 1030 mM), in

combination with two temperatures regimes (light/darkness: 25/14 °C (optimal) and 29/18 °C (optimal +4 °C), two CO_2 concentrations (400 and 700 ppm) and six inoculation treatments (non-inoculated and five rhizobacterial consortia).

2.5. Preparation of Bacterial Inoculants and Inoculation of Salicornia ramosissima Seeds

To prepare the five suspensions for seed inoculation, all rhizobacteria were grown separately in 250 mL Erlenmeyer flasks containing 50 mL of TSB medium NaCl 0.2 M and incubated in a rotary shaker during 18 h at 28 °C. Then, cultures were centrifuged in 50 mL Falcon tubes at 7000 rpm during 10 min and the supernatant was discarded. Pellets were washed twice with sterile physiological saline solution (NaCl 0.9% w/v) (by resuspension and centrifugation) and finally resuspended in sterile physiological saline solution (NaCl 0.9% w/v) to get a suspension with an optical density OD_{600} = 1 (*ca.* 10^8 cells per ml) for each bacteria. Then, equal amounts of bacterial suspensions were adequately mixed to get the five final OD_{600} = 1 inoculant suspensions.

Disinfected seeds were divided into six groups and aseptically submerged at room temperature for 1 h under slightly shaking in 5 mL of the 5 different bacteria consortia suspensions and 5 mL sterile physiological saline solution (NaCl 0.9% w/v) as the non-inoculated control.

2.6. Salicornia ramosissima Gnobiotic Seed Germination Assay

After inoculation treatment, seeds were placed in 9% agar plates. A total of 25 seeds were used per plate and four plates per treatment as experimental replicates (n = 4, 100 seeds per treatment). Agar plates were amended with NaCl 171 mM, 510 mM and 1030 mM. These concentrations have been previously used for *Salicornia* and other halophytes by authors of this work. Plates with 0 mM NaCl were not used, as this concentration has been considered a suboptimal salinity for *Salicornia ramosissima* growth [37]. Plates with seeds were incubated in four different controlled-environment chambers (Aralab/Fitoclima 18.000 EH, Lisbon, Portugal). Chambers were programmed with alternating diurnal regime of 16 h of light (maximum photon flux rate, 300 μmol m^{-2} s^{-1}) and 8 h of darkness and relative humidity 50 ± 5%. Conditions of the chambers were the result of combining two temperature regimes (light / darkness): 25/14 °C (optimal) and 29/18 °C (optimal +4 °C); and two CO_2 concentrations: 400 and 700 ppm. The high level of CO_2 concentration and temperature were selected following the data from the IPCC (Intergovernmental Panel on Climate Change) for the next century (2014a). Therefore, the four chambers were programmed as follows (ppm CO_2 / temperature in °C): (1) 400/25, (2) 400/29, (3) 700/25, (4) 700 / 29. Atmospheric CO_2 concentrations in chambers were continuously recorded by CO_2 sensors (Aralab, Lisbon, Portugal) and maintained by supplying pure CO_2 from a compressed gas cylinder (Air liquid, B50 35 K).

Plates were daily inspected for 11 days (when there was no more germination) to record plant biomass and germination rate. Seed germination was considered after cotyledon appearance. Seedling biomass was recorded on day 12 through fresh weighting (FW). As for germination rate, three parameters were calculated at the end of the experiment: Final germination percentage, number of days to first germination (FGD) and mean time to germination (MTG) [38,39]. MTG was calculated using the equation:

$$\mathrm{MTG} = \sum_i (n_i \times d_i)/N \quad (1)$$

where n is the number of seeds germinated until day i; d is the incubation period in days, and N is the total number of seeds germinated in the treatment, which means that the lower the value, the more rapid the germination.

2.7. Statistical Analysis

Statistical analyses were done using 'Statistica' v. 6.0 (Statsoft Inc.). Generalized linear models (GLM) were used to analyze the interactive effects of PGPR, temperature, CO_2 and NaCl (as categorical

factors) on the biomass and germination parameters (as dependent variables) of *S. ramosissima* seeds. Significant test results were followed by Tukey tests for identification of important contrasts.

3. Results

3.1. Isolation, Characterization and Identification of Cultivable Bacteria from H. portulacoides and S. ramosissima Rhizosphere in Southwestern Spain Salt Marshes

A total of 43 bacterial strains from the rhizosphere of *H. portulacoides* and 44 from *S. ramosissima* were isolated (Supplementary Tables S1 and S2). Of these, 79% were able to grow in 1.2 M NaCl plates (Figure 1), so most of the isolates could be considered as halotolerant bacteria. In general, rhizobacteria growing at 0.2 M and 2 M NaCl plates had a better growth at 28 °C, while bacteria growing at 1.2 M NaCl plates had a greater growth at 40 °C (Figure 1).

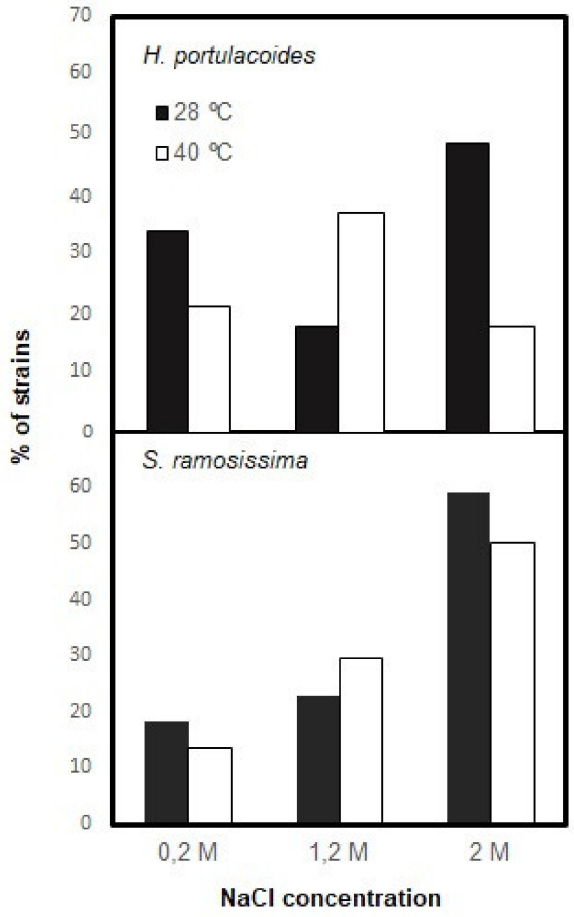

Figure 1. Percentage of rhizobacteria isolated from *Halimione portulacoides* and *Salicornia ramosissima* with visible growth in plates under different salinity and temperature conditions.

Moving to PGP properties, it is noteworthy that 81% of strains showed at least one out of the six PGP properties tested, and 39% showed a minimum of three (Figure 2). Within those isolated from *H. portulacoides* rhizosphere, 45% of strains showed nitrogen fixation capacity, 27% could solubilize phosphate, 32% produced siderophores and 23% formed biofilms (Figure 2). Regarding isolates from *S. ramosissima* rhizosphere, 65% had the ability to fix atmospheric nitrogen, 40% of the strains were able to produce siderophores and auxins, 31% could solubilize phosphate and 27% formed biofilms (Figure 2). Only two strains out of 87 produced the ACC deaminase enzyme.

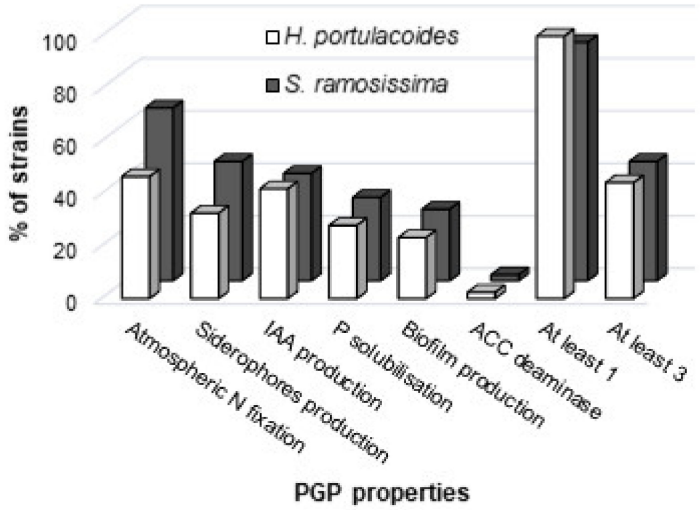

Figure 2. Percentage of rhizobacteria isolated from *Halimione portulacoides* and *Salicornia ramosissima* in southwest Spain showing the plant growth-promoting (PGP) activities tested. The last two bars indicate the percentage of strains with at least one or three PGP properties of the ones analyzed in this work.

The most interesting rhizosphere strains from *H. portulacoides* and *S. ramosissima* were identified by 16S rRNA sequencing. Sequences were deposited in GenBank with accession numbers shown in Table 2 (from MH304385 to MH304399, and MH917124).

Table 2. Closest species to the 16 isolates based on their 16S rRNA sequence. HPJ stands for strains isolated from *H. portulacoides* rhizosphere, while SRP and SRT for those from *S. ramosissima*.

Strain	16S rDNA Sequenced Fragment (bp)	Accession n.	Related Species	Identity (%)
HPJ2	1377	MH304385	*Vibrio spartinae*	99.85
HPJ9	1368	MH304386	*Bacillus siamensis*	99.93
HPJ15	1400	MH304387	*Marinobacter sediminum*	99.21
HPJ21	1397	MH304388	*Bacillus aryabhattai*	100
HPJ40	1398	MH304389	*Bacillus zhangzhouensis*	99.79
HPJ43	1410	MH304390	*Bacillus zhangzhouensis*	99.79
HPJ49	1328	MH304391	*Bacillus subtilis*	100
HPJ50	1390	MH304392	*Vibrio parahaemolyticus*	99.78
SRP14	1396	MH304393	*Bacillus paralicheniformis*	100
SRP15	1420	MH304394	*Bacillus aryabhattai*	100
SRT1	1397	MH304395	*Vibrio neocaledonicus*	99.93
SRT8	1334	MH304396	*Thalassospira australica*	99.55
SRT12	1071	MH917124	*Halomonas taeanensis*	99.81
SRT13	1411	MH304397	*Vibrio alginolyticus*	99.65
SRT14	1405	MH304398	*Vibrio alginolyticus*	99.57
SRT15	1377	MH304399	*Pseudarthrobacter oxydans*	99.49

3.2. Design of Two Rhizobacteria Consortia from H. portulacoides and S. ramosissima Cultivable Isolates

Among the bacteria isolated from *H. portulacoides* rhizosphere, *Vibrio spartinae* HPJ2, *Marinobacter sediminum* HPJ15 and *Vibrio parahaemolyticus* HPJ50 were selected to establish a rhizobacterial *consortium*, which we numbered as consortium 4 (Table 3). HPJ2 presented all the properties studied. HPJ15 produced more auxins than the other strains and showed a high tolerance to NaCl and temperature. HPJ50 had all the PGP properties tested except for ACC deaminase activity (Table 3). The three bacterial isolates were cultivated together and none of them showed antagonistic activity against each other (not shown).

As for *S. ramosissima* rhizobacteria isolates, *Vibrio neocaledonicus* SRT1, *Thalassospira australica* SRT8 and *Pseudarthrobacter oxydans* SRT15 were selected regarding their performance as PGPR and used as *consortium* 5 (Table 3). SRT1 exhibited five out of sx PGP properties and a high salinity tolerance, whereas SRT8 was able to produce ACC deaminase. Finally, SRT15 was the largest auxins producer among all the isolates (Table 3). The three rhizobacteria were cultivated together and there was no antagonistic activity against each other (not shown).

Table 3. NaCl tolerance at 28 and 40 °C and PGP traits analyzed in this study for consortia isolated from *Halimione portulacoides* rhizosphere, labelled as number 4; and from *Salicornia ramosissima* rhizosphere, as number 5.

Hosting Halophyte	Strain	NaCl Tolerance [a]		PGP Properties					
		28 °C	40 °C	Nitrogen Fixation [b]	Phosphate Solubilization [c]	Siderophores Production [c]	IAA Production (mg/mL)	Biofilm Production [b]	ACC Deaminase Activity (μmol α-cetog h^{-1} mg $prot^{-1}$)
Halimione portulacoides	HPJ2	1.2	1.2	+	12	30	4.12	+	1.86
	HPJ15	2	2	-	-	12	15.41	-	-
	HPJ50	1.2	1.2	+	9	20	7.48	+	-
Salicornia ramosissima	SRT1	2	1.2	+	10	20	5.65	+	-
	SRT8	1.2	1.2	-	-	-	-	+ -	1.24
	SRT15	0.2	0.2	+	9	-	20.99	-	-

[a] Maximum tolerable concentration in M NaCl; [b] (+) presence or (-) absence of growth; [c] Halo diameter in mm.

3.3. Effect of CO_2, Temperature, NaCl and PGPR Inoculation on Salicornia ramosissima Seed Germination Parameters

There were significant effects of CO_2, temperature, salt and PGPR inoculation on seed germination percentage, both independently (Table 4, GLM: CO_2, $p < 0.01$; T, $p < 0.01$; NaCl, $p < 0.01$, PGPR, $p < 0.01$) and combined together (GLM: $CO_2 \times T \times NaCl \times PGPR$, $p < 0.01$).

Table 4. Generalized linear model (GLM) significance as *p*-values for the growth and germination parameters of *Salicornia ramosissima* seeds under conditions of CO_2, T, NaCl and PGPR inoculation (as categorical variables) and their interaction. * Significance level 95% and ** Significance level 99%.

Parameter	Biomass	% Germination	FGD	MTG
CO_2	0.01 **	0.00 **	0.87	0.00 **
T	0.12	0.00 **	0.08	0.00 **
NaCl	0.00 **	0.00 **	0.00 **	0.00 **
PGPR	0.00 **	0.00 **	0.19	0.00 **
CO_2 x T	0.00 **	0.20	0.26	0.02 *
CO_2 x NaCl	0.64	0.00 **	0.63	0.03 *
CO_2 x PGPR	0.47	0.00 **	0.63	0.00 **
T x NaCl	0.71	0.01 **	0.17	0.03 *
T x PGPR	0.00 **	0.28	0.62	0.41
NaCl x PGPR	0.00 **	0.00 **	0.81	0.00 **
CO_2 x T x NaCl	0.05 *	0.26	0.08	0.45
CO_2 x T x PGPR	0.06	0.00 **	0.62	0.00 **
CO_2 x NaCl x PGPR	0.02 *	0.79	0.12	0.10
T x NaCl x PGPR	0.00 **	0.16	0.28	0.61
CO_2 x T x NaCl x PGPR	0.01 **	0.00 **	0.89	0.00 **

S. ramosissima seed germination was not appreciated in 1030 mM NaCl plates. Therefore, final analysis was conducted comparing 171 mM and 510 mM NaCl plates. All seeds germinated within two days after plating. In general, *S. ramosissima* seed germination percentage was higher in 171 mM NaCl plates than in 510 mM NaCl (Figure 3A,D). However, differences between inoculation patterns were only notable in 510 mM NaCl (Figure 3D1–4). Inoculum 3 emphatically facilitated *S. ramosissima* seed germination in the four conditions of temperature and CO_2 tested, compared to the other inocula and the control (Figure 3D1–4). These increments in percentage ranged from 68% to 114% compared to

the control. This was also confirmed when data was represented as accumulated seed germination per days (Figure 4). Inoculation with consortium 4 slightly improved germination rate only at high temperature and CO_2 values (Figure 4F–H). On the other hand, germination percentage of inoculated seeds in some cases did not differ from the control or was significantly lower, as the case of consortia 2 (Figure 4D2) or 5 (Figure 4D1).

As for seed mean time germination (MTG), there were also significant effects of CO_2, temperature, salt and inoculation, both independently (Table 4, GLM: CO_2, $p < 0.01$; T, $p < 0.01$; NaCl, $p < 0.01$, PGPR, $p < 0.01$) and combined together (GLM: CO_2 x T x NaCl x PGPR, $p < 0.01$). Figure 3 shows that MTG differences between treatments were only remarkable in seeds growing in 510 mM NaCl (Figure 3B,E), but there was not a clear difference between inoculation treatments, as occurred for seed germination percentage (Figure 3E).

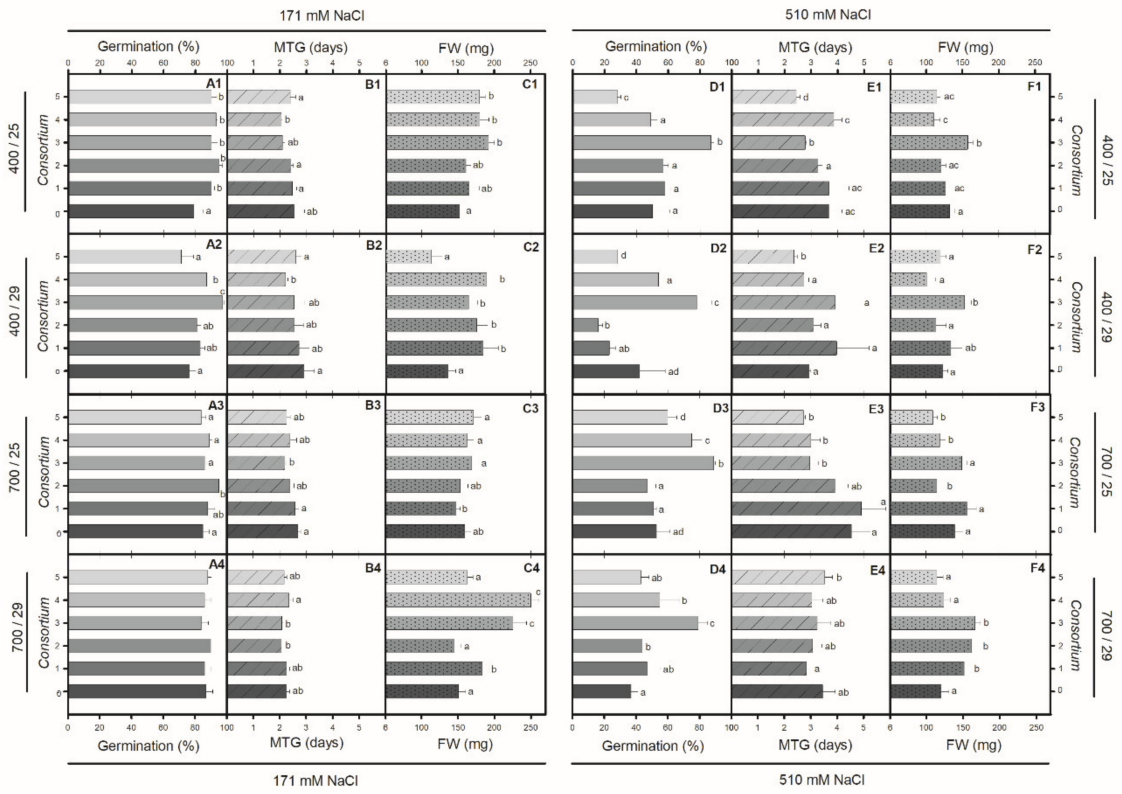

Figure 3. Germination percentage (columns **A** and **D**), mean time to germination (MTG) (columns **B** and **E**) and seed biomass (columns **C** and **F**) response of *Salicornia ramosissima* seeds growing in 171 mM (columns **A, B** and **C**) and 510 mM NaCl plates (columns **D, E** and **F**). For every parameter, each row (from 1 to 4) represents different conditions of CO_2 and temperature. Rhizobacteria consortia used are displayed by different columns and are numbered from 1 to 5, according to the halophyte rhizosphere they were isolated from: (1) *Spartina densiflora*, (2) *Arthrocnemum macrostachyum*, (3) *Spartina maritima*, (4) *Halimione portulacoides* and (5) *Salicornia ramosissima*. Number 0 was used for non-inoculated control seeds. Values are means ± S.E. of four replicates, with 25 seeds each (n = 4, total = 100 seeds per treatment). Different letters indicate means that are significantly different from each other.

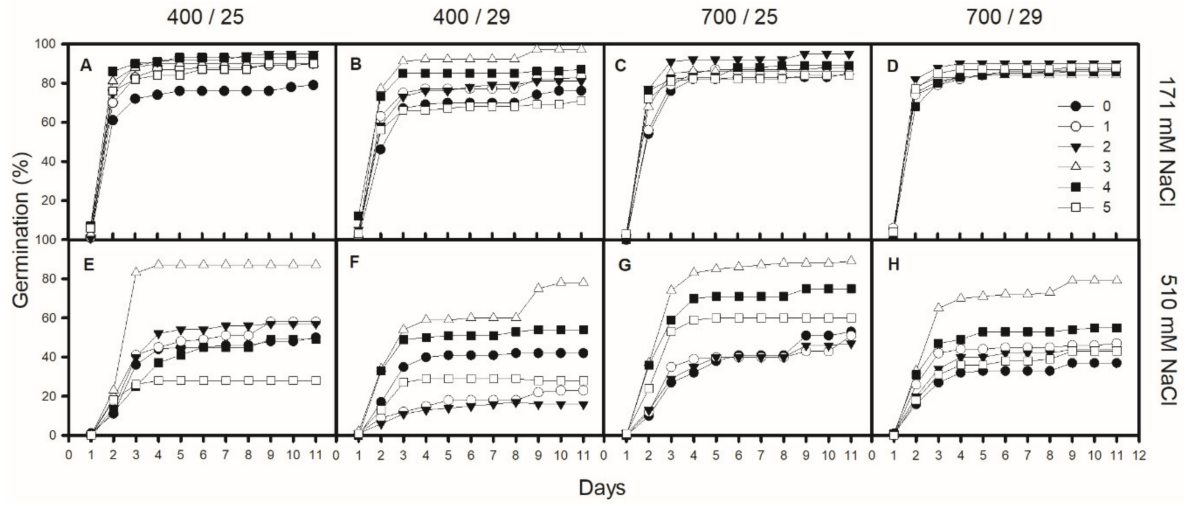

Figure 4. Accumulated seed germination for *Salicornia ramosissima* seeds growing in 171 mM (**A**–**D**) and 510 mM NaCl plates (**E**–**H**) in different conditions of CO_2 and temperature. Rhizobacteria consortia were isolated from (1) *Spartina densiflora*, (2) *Arthrocnemum macrostachyum*, (3) *Spartina maritima*, (4) *Halimione portulacoides* and (5) *Salicornia ramosissima* rhizosphere in southwestern Spain salt marshes. Values are means of four replicates, with 25 seeds each (n = 4, total = 100 seeds per treatment).

3.4. Effect of CO_2, Temperature, NaCl and PGPR Inoculation on Seedling Biomass

There were significant effects of CO_2, salt and PGPR inoculation on *S. ramosissima* seedlings fresh weight (FW) at the end of the experiment (Table 4, GLM: CO_2, $p < 0.05$; salinity, $p < 0.01$; inoculation, $p < 0.01$). Temperature had no significance, unless it was combined with other parameters (GLM: $CO_2 \times T$, $p < 0.01$; $T \times PGPR$ $p < 0.01$; $NaCl \times PGPR$, $p < 0.01$; $CO_2 \times T \times NaCl$, $p < 0,05$; $T \times NaCl \times PGPR < 0.01$). Also, combination of all the parameters studied had significant effect on FW (GLM: $CO_2 \times T \times NaCl \times PGPR$, $p < 0.05$).

In the same line with germination percentage, *S. ramosissima* seedlings fresh weight was lower in 510 mM NaCl plates than in 171 mM NaCl (Figure 3C,F). In 171 mM NaCl plates, *S. ramosissima* seedlings inoculated with consortia 3 and 4 demonstrated an increased biomass at the end of the experiment compared to the control, especially under combined high temperature and CO_2 (29 °C/700 ppm), with FW increments of 49.5% for consortium 3 and 65.6% for consortium 4 (Figure 3C4). In 510 mM NaCl plates, in most of the cases consortium 3 was the most effective improving *S. ramosissima* seedling FW during the experiment (Figure 3F1–4).

4. Discussion

For the first time, in this work we studied the effect of PGPR inoculation treatments on *S. ramosissima* seed germination under the combination of three environmental stressors: CO_2, temperature and salinity.

Bacteria have been largely used in many works to improve different crops production [18,40–42] and even under environmental stress conditions [43,44], gaining the consideration as biofertilizers [45]. Nowadays, the current biofertilizer PGPR-based market represents only about 5% of the total chemical fertilizers market for agricultural practices [46,47]. This fact presents the need to increase the selection of PGP strains with biological activities adapted to specific agronomic situations [18]. In this context, the study of PGPR and halophile plants is a relatively unexplored field that presents a great opportunity of exploitation. There are no works analyzing the response of PGPR-inoculated plants to interactive temperature, salinity and CO_2 increments, which become relevant for the future in a scenario of global climate change.

In our previous studies, we observed that halophytes in southwestern Spain marshes harbored salt-tolerant rhizobacteria with PGP properties. Those studies were carried on (1) *Spartina densiflora* [28,

48], (2) *Arthrocnemum macrostachyum* [25,49] and (3) *Spartina maritima* [27,31,50]. Continuing this research line, the present work describes cultivable rhizomicrobiome of two other important halophytes from southwest Spain, (4) *Halimione portulacoides* and (5) *Salicornia ramosissima*.

A total of 43 bacteria from the rhizosphere of *H. portulacoides* and 44 from *S. ramosissima* were isolated. A high proportion of Gram-positive bacteria was recorded, especially from the *Bacillus* genera, as occurred in our previous works [25,27,28]. This is not surprising, as Tinto estuary is highly polluted, and this bacteria genus is well known as metal resistant, salt tolerant and commonly present in other hazardous scenarios [51,52]. This may be an advantage with a view to commercialization, as Gram-positive microorganisms possess heat-resistant spores that are exploited in order to easily formulate stable and dry powder products [47,53]. When discussing about PGP properties, *S. ramosissima* rhizosphere hosted a higher proportion of PGPR than *H. portulacoides* rhizosphere. Atmospheric nitrogen fixation was broadly present among the isolates and the rarest property to be found was the ACC deaminase activity (two strains out of 77), which is consistent with the existing bibliography [35]. It is worth stressing that most of the rhizoisolates showed at least one PGP property (81%). This data is similar to that studied for the other three halophytes previously mentioned [25,27,28], which suggests the potential of halophytes as a source of interesting and beneficial salt-tolerant bacteria.

The five PGPR inocula, isolated from the five previously mentioned halophytes, were tested on *S. ramosissima* seeds to study germination rates under a combination of three different abiotic stressors: temperature (25 and 29 °C), CO_2 (400 and 700 ppm) and NaCl (171, 510 and 1030 mM).

In general, *S. ramosissima* seeds scarcely germinated in 1031 mM NaCl plates, and seed germination parameters and seedlings biomass were lower in 510 mM NaCl than in 171 mM NaCl. These results were in line of the ones obtained by [54], who obtained better germination responses of *S. ramosissima* seeds at salinities under 257 mM NaCl. Indeed, soil salinity of southwestern Spain salt marshes moves within those thresholds [27]. The high number of experimental blocks made difficult to assess a general effect of PGPR inoculation of seeds, as we obtained improvements but also decreases compared to the control. However, a consistent result we found was that PGPR-inoculated seed germination percentage and seedling biomass improved under high temperature, salt and CO_2 for seeds treated with inoculum number 3. This effect may be due to the high IAA production capacity of the strains, improving seed development [55]. Also, all bacteria from consortium number 3 were able to fix atmospheric nitrogen and t is known that seedling vigor is likely to be enhanced by the increase of seed nitrogen content [56,57]. Another inoculum that obtained good results in some cases was inoculum number 4, maybe attributed to seed stress relief through the production of ACC deaminase [58], altogether with bacterial IAA synthesis [55]. As saline-induced stress in plants is partially the result of the plant production of stress ethylene, it is reasonable that lowering ethylene levels using ACC deaminase-containing plant growth-promoting bacteria might afford some protection against this stress [58]. However, despite the fact that germination is a crucial stage in the life cycle of any halophilous plant, there is a need of further experiments in grown *S. ramosissima* plants. They might facilitate that other bacterial PGP properties, not visible in seed gnotobiotic assays in agar plates, come into play in soil, as phosphate solubilization or siderophores production.

5. Conclusions

In our experiment, we found different germination responses of *S. ramosissima* seeds to PGPR inoculation under combined CO_2, temperature and salinity stress. Salinity played a decisive role, as the most remarkable differences between treatments were observed at 510 mM NaCl. PGPR inoculation did not improve germination parameters in all cases compared to the control. However, among the five consortia tested, number 3 significantly improved seedling parameters compared to the control in most cases. These first findings are encouraging to think that cash crop halophytes like *S. ramosissima* and PGPR may be valuable natural resources for human or animal feeding in a future climate reality.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/10/655/s1, Table S1: Gram staining, motility, NaCl tolerance and Plant Growth Promoting properties of the 43 rhizobacteria isolated from *Halimione portulacoides* in Piedras salt marsh (SW Spain). Strains selected to stablish the consortium appear highlighted in grey colour; Table S2: Gram staining, motility, NaCl tolerance and Plant Growth Promoting properties of the 44 rhizobacteria isolated from *Salicornia ramossisima* in Tinto (SRT strains) and Piedras (SRP strains) salt marshes (SW Spain). Strains selected to stablish the consortium appear highlighted in grey colour.

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