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# Halotolerant bacteria isolated from extreme environments induce seed germination and growth of chia (*Salvia hispanica* L.) and quinoa (*Chenopodium quinoa* Willd.) under saline stress

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ABSTRACT

The aim of the study was to characterize halotolerant bacteria and to evaluate their plant growth promotion potential on chia and quinoa seedlings under saline stress. Isolated microorganisms were evaluated for nitrogen fixation, phosphate solubilization, and production of siderophores and indole acetic acid. Three strains and two consortia were selected: *Halomonas* sp. (SFS), *Micrococcus luteus* (SA211), *Bacillus* sp. (HX11), C1 (SA211 + SFS), and C2 (SA211 + HX11). *In vitro* assays using water agar and half-strength Murashige-Skoog plates showed that an increase in salinity led to an increased seedlings mortality and a decrease in germination (lower than 40%), in total length (varying between 16% and 87% decreases), root length (from 60% to 92% lesser length) and dry weight (from 7% to 86% lower weight). Also, the relative growth index (RGI) decreased for both crops in most treatments, except those with HX11 and C2. These treatments had the highest growth parameters and RGI values in presence of high salinity in chia (50 and 100 mmol/L NaCl) and quinoa (200 and 400 mmol/L NaCl). SA211, the highest producer of indole acetic acid, showed a detrimental effect and anomalous phenotype on plants. Our results suggest that *Bacillus* sp. HX11, with multiple plant growth promotion traits and tolerance to saline stress, has a great potential as a bioinoculant in saline conditions and could be used as a biofertilizer for crop production.

## 1. Introduction

The detrimental effects of salinity are evidenced on plants growth and in soil physical properties (Kibria and Hoque, 2019; Lavado, 2007). On one hand, the high osmotic pressure inhibits plant water uptake, and the presence of large amounts of soluble ions and molecules could cause toxicity from specific ions (Bensidhoum et al., 2019). As a consequence of ionic and osmotic stress, induced by salinity, plants experiment changes in their morphological, physiological and biochemical attributes. Morphological changes include a decrease in germination, root and shoot growth, less biomass production, decrease in crop productivity, nutritional and ionic homeostasis disorders (Kibria and Hoque, 2019). Physiological and biochemical changes include decreases in chlorophyll contents, increases in ROS generation, changes in antioxidant enzymatic activities, and osmolites production, among others (Bensidhoum et al., 2019; Kibria and Hoque, 2019). On the other hand, salinity affects the soil structure. Saturation of the soil exchange complex with sodium disrupts the soil aggregates, producing impermeable

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layers and alkalinization of the soil solution (Zaman et al., 2018). The negative effects on plant growth and soil structure affect crop yield, increasing dependence on chemical inputs and causing disruptions to the processes of nitrogen uptake, plant growth development, the biological activity of soils, and soil ecosystem services (FAO, 2018).

Traditional strategies used to solve or mitigate salinity effects on soils involve soil recovery by washing it or by adding calcium amendments that remove sodium in the exchange complex of the soil (Taleisnik and Lavado, 2016). Another alternative is the application of microorganisms, halophiles in particular, as bioremediation agents and plant growth promoters in salt-affected soils (Abbas et al., 2019; Alexander et al., 2019; Bensidhoum et al., 2019; Zahir et al., 2019).

Halotolerant bacteria with plant growth promotion (PGP) abilities can colonize plant roots and enhance plant growth by direct and indirect mechanisms (Arora et al., 2012), which include an increase in nutrients uptake, protection against pathogens, and delivery of phytohormones, among others (Prasad et al., 2019). They can also decrease the content of $Na^+$ available for plants and enhance soil properties through exopolymeric substances (EPS) and biofilm formation (Banerjee et al., 2019; Grover et al., 2011). Bacterial EPSs play a significant role in the soil ecology system improving soil aggregation and soil adhesion, enhancing water retention, and regulating the diffusion of carbon sources (Arora et al., 2012). Therefore, these bacteria could relieve salt stress by PGP and by leaving salts less available in the soil, becoming biotechnological tools for bioremediation. However, different plant species with diverse ranges of stress tolerance could show variable responses to specific PGP bacteria.

Chia (*Salvia hispanica* L.) and quinoa (*Chenopodium quinoa* Willd.) are crops with different environmental stress tolerance. Chia was rediscovered in the last decade for food and industry (Vázquez-Ovando et al., 2013) because of its nutritional (high proportion of the α-linolenic polyunsaturated fatty acid) and pharmacological properties (Ayerza, 1995). Although it is known as a sensitive crop for different types of stress, there is not much information about its behavior in saline soils even less with PGP bacteria. Quinoa is a stress-tolerant plant cultivated (Adolf et al., 2013; González et al., 2009; Ruiz et al., 2016) along the Andes for the last 7000 years. Its grains have higher nutritional value than traditional cereals as maize, millet, sorghum, or wheat, and it is a promising crop worldwide for human feeding (Vega-Gálvez et al., 2010). Although the positive interaction of quinoa with PGP bacteria in plant growth was already reported (Yang et al., 2016), to the best of our knowledge this is the first study that compares, under salinity conditions, the behavior of these two crops with different response to stress interacting with PGP bacteria.

The aim of this study was to characterize and select halotolerant bacteria with PGP abilities isolated from the Salar del Hombre Muerto (northwest of Argentina). We also evaluated their ability to increase the percentage of germination and to improve the initial growth of chia and quinoa seedlings at high salt concentrations.

## 2. Material and methods

### 2.1. Screening of plant growth promotion abilities

A total of 238 microorganisms were isolated from salt pan samples (soil and brine) from the Salar del Hombre Muerto in Catamarca, Argentina as described by Martinez et al. (2019a). Liquid samples were collected from a salt pond (brine), and a freshwater reservoir, while soil samples were collected from two different sites in the salt mine (Site 1-S1 and Site 2-S2) (Fig. S1, Supplementary material) (Martínez et al., 2019a). One hundred and eleven out of 238 strains were selected because they came from the soil and were able to grow in presence of salt in "*in vitro*" assays. These strains were evaluated for different Plant Growth Promotion (PGP) traits. First, the biological $N_2$ fixation was evaluated at three different salinity levels: without salt, and with 1 and 2 mol/L of NaCl. Then, the strains able to fix $N_2$ were selected and tested without salts and with 1 mol/L of NaCl to evaluate phosphate solubilization and production of siderophores and indole acetic acid (IAA). In all cases, experiments were conducted in triplicate using fresh bacterial inoculum (24 h growth at 30 °C and under agitation at 200 rpm in an orbital shaker-incubator, ES-20 Biosan) prepared in nutrient broth supplemented with salts (NB-Salt) (2.5% w/v of Salt Solution: NaCl 98 g/L, KCl 8.3 g/L, $MgSO_4$ 3.3 g/L) (Martínez et al., 2019a). All used chemicals were of analytical quality, reagents were from Cicarelli® labs and all used culture media were Britania®.

#### 2.1.1. Biological nitrogen fixation (BNF)

Bacteria were inoculated and cultured at 30 °C for 7 days in two different nitrogen-free media, *i.e.* tubes with 5 mL of semisolid NFb medium (Döbereiner et al., 1976) and plates with 20 mL $N_2$-free Ashby agar (Kryuchkova et al., 2014). Nitrogen fixation was revealed by veil formation and change of color from green to blue in NFb medium and by colony growth in $N_2$-free Ashby agar (Döbereiner et al., 1976; Kryuchkova et al., 2014).

#### 2.1.2. Phosphate solubilization

A qualitative assay was initially performed in two media with different phosphate sources: NBRIP agar (Shekhar Nautiyal, 1999) containing tri-calcium phosphate ($Ca_3(PO_4)_2$), and Muromtsev agar (Kryuchkova et al., 2014) containing dibasic sodium phosphate ($Na_2HPO_4$). In both media, after 7 days of incubation at 30 °C, the formation of clear halos surrounding the bacterial colonies indicated positive phosphate solubilization.

For a quantitative assay, bacterial pre- inoculum (24 h growth in NB-Salt at 30 °C and 200 rpm) was seeded and optical density at 600 nm ($OD_{600}$) was adjusted to 0.2 by dilution with NB-Salt. Then, 50 μL of each inoculum was seeded in 50 mL centrifuge tubes containing 10 mL of NBRIP liquid medium. After 7 days of incubation at 30 °C and 250 rpm in an orbital shaker-incubator (ES-20, Biosan), soluble phosphate was quantified using the Vanadomolybdophosphoric Acid Colorimetric Method (American Public Health Association (APHA) (APHA) (APHA) et al. (2005) Absorbance was measured in a spectrophotometer (model 752, BIOTRAZA) at 470 nm and the obtained values were related to a calibration curve of $KH_2PO_4$.

#### 2.1.3. Siderophores production

Pure bacterial inoculum was plated as spots on Chrome azurol-S (CAS) agar plates and incubated for 5 days at 30 °C. The formation of orange-yellowish halos surrounding the bacterial colonies indicated positive siderophore production. CAS agar (Schwyn and Neilands, 1987) was prepared with 60.5 mg of CAS dye dissolved in 50 mL of distilled water and mixed with 10 mL iron (III) solution (mmol/L$FeCl_3$ 1 mmol/L and mmol/LHCl 10 mmol/L). Under stirring, this solution was slowly added to 72.9 mg cetrimide dissolved in 40 mL distilled water. The CAS dye was slowly added to Plate Count Agar with salt (APCsalt) (Martínez et al., 2019a) which was used as base media for CAS agar plates. The siderophores production was estimated semi-quantitatively as the ratio of Halo diameter/Colony diameter (Searle et al., 2015).

#### 2.1.4. Indole-3-Acetic Acid (IAA) production

The production of IAA was determined by colorimetry (Glickmann and Dessaux, 1995). Bacteria were cultured in NB-Salt without and with the addition of tryptophan (0.5 g/L) in an orbital shaker-incubator (model ES-20, Biosan) at 150 rpm for 5 days at 30 °C. For color development, 1 mL of reagent R1 (12 g/L of $FeCl_3$ in 7.9 mol/L of $H_2SO_4$) (Pilet and Chollet, 1970) was added to 1 mL of sample solution, then mixed and left in the dark for 30 min at room temperature. Absorbance was measured in a spectrophotometer (model 752, BIOTRAZA) at 530 nm, and the measured values were compared to an IAA calibration curve.

### 2.2. Growth curves and biofilms production

Bacterial growth kinetics were performed by culturing in nutrient broth (without NaCl) and also supplemented with NaCl in 11 different concentrations: 10, 50, 100, 250, 500, 750, 1000, 1250, 1500, 1750, and 2000 mmol/L, in 96-wells microplates. Fresh 24 h-pre-inoculums adjusted to 0.1 optical density were used. Plates were incubated at 30 °C and absorbance was measured in a plate reader (Thermo Scientific® Multiskan FC) at 600 nm hourly until growth reached the stationary phase, and then every 8 h for three additional days.

Biofilms production was quantified following the protocol of Merritt et al. (2011). For that, bacteria were cultured in 96-wells microplates in the same conditions as stated before for the growth. Plates were incubated at 30 °C without agitation for six days. Absorbance was measured in a plate reader (Thermo Scientific® Multiskan FC) at 595 nm.

### 2.3. Antagonism assay

The evaluation of bacterium-bacterium antagonistic interactions was carried out in plates with APCsalt (Martínez et al., 2019a) following two plating procedures: in opposition and double-layer agar. For opposing trials, the method described by Bell et al. (1982) was used with slight modifications. One strain was streaked in the center and other bacteria were striated on both sides of the central strain. The lack of contact between strains indicated antagonism. For double-layer agar trials (Long and Azam, 2001), plates were filled with melted agar up to 50% of their final volume and left until partially gelled. At this point, one strain was inoculated in the solid medium and the remaining 50% agar was poured over the first semi-gelled layer. Once the second layer was gelled, other strains were streaked above. The appearance of a halo of inhibition of bacterial growth indicated antagonisms.

### 2.4. Salinity stress amelioration assay

According to the results obtained in the PGP activities and antagonism assays, three strains and two consortia were selected to evaluate their effect on seeds germination and growth attributes in water agar (WA) medium (Sadeghian and Yavari, 2004). The selected strains were *Halomonas* sp. SFS, *Micrococcus luteus* SA211, and *Bacillus* sp. HX11, whereas the consortia were C1 (formed by SA211 +SFS) and C2 (formed by SA211 + HX11) (Table 1). The WA medium was used for better discriminations of the selected treatments because of its nutrient restrictions. Then, the treatments with the best behavior in WA medium (strains SA211 and HX11, and the C2), were evaluated in half-strength Murashige-Skoog (MS/2) agar medium (Murashige and Skoog, 1962). The nutrients availability of MS/2 medium represents a situation close to a soil-crop system. The control treatment was liquid growth medium (NB-Salt) without bacteria (Table 1). Before seed treatment, bacterial strains were grown in NB-Salt at 30 °C in an orbital shaker incubator (model ES-20, Biosan) at 200 rpm for 24 h. Then, $OD_{600}$ was adjusted by dilution with NB-Salt to a final value of 0.6 to attain a bacterial suspension of approximately $10^6$-$10^8$ CFU $mL^{-1}$ cell density for the seed bacterization.

#### 2.4.1. Water agar (WA) assay

The growth medium WA used for seed germination was prepared by the addition of Agar-agar (1.5%) to different saline solutions of NaCl. In both cases (chia and quinoa), 15 mmol/L NaCl was used as a control condition since its electrical conductivity (EC) resembles that of a non-saline soil (Table 1). Previous to bacterization, chia and quinoa seeds were surface sterilized by consecutive washing (5 min at 150 rpm) with aqueous solutions (first with 70% ethanol and then with 3% NaClO solutions), followed by five minutes rinses with Milli-Q water (Millipore). Sterilized seeds were immersed in each bacterial suspension for 2 h, using NB-Salt without bacteria for the un-inoculated control.

For each treatment, 20 seeds were placed in a plate with their corresponding growth media (four replicates per treatment) with constant photoperiods of 8 h light: 16 h dark for chia and 10 h light: 14 h dark for quinoa. A constant temperature of 23 °C was used for both crops. The assay was conducted for seven days.

**Table 1**
Germination and growth conditions for the seed-bacteria interaction assays under induced saline stress in chia (*Salvia hispanica* L.) and quinoa (*Chenopodium quinoa* Willd.) using two growth media: water agar (WA) and half-strength Murashige-Skoog agar (MS/2). Bacterial treatments included the seed bacterization with cultures of single strains and two consortia (C1 and C2). The salinity levels of the growth media were characterized by sodium chloride (NaCl) concentration and electrical conductivity (EC).

| Growth medium | WA | | MS/2 | |
|---|---|---|---|---|
| Salinity variables | NaCl (mmol/L) | EC (dS/m) | NaCl (mmol/L) | EC (dS/m) |
| Chia | 15 | 1.50 | 0 | 2.82 |
| | 50 | 5.37 | 25 | 5.37 |
| | 100 | 9.57 | 75 | 9.57 |
| | 150 | 13.58 | – | – |
| Quinoa | 15 | 1.50 | 0 | 2.82 |
| | 200 | 17.11 | 175 | 20.00 |
| | 300 | >20 | 375 | >20 |
| | 400 | >20 | – | – |
| Bacterial treatment | Control (without bacteria) | | Control (without bacteria) | |
| | SFS (*Halomonas* sp.)[a] | | SA211 (*Micrococcus luteus*)[b] | |
| | SA211 (*Micrococcus luteus*)[b] | | HX11 (*Bacillus* sp.)[a] | |
| | HX11 (*Bacillus* sp.)[a] | | C2 (SA211 + HX11) | |
| | C1 (SA211 + SFS) | | | |
| | C2 (SA211 + HX11) | | | |

[a] Identity percentage >99% according to 16S rDNA sequencing (Martínez et al., 2019a).
[b] Identified by complete genome sequencing (Martínez et al., 2019b).

#### 2.4.2. Murashige-Skoog/2 agar assay

In this assay, we used half-strength Murashige-Skoog (MS/2) (Murashige and Skoog, 1962) medium with sugar (0.2% sucrose) gelled with 0.8% Plant Agar (Duchefa Biochemie). In this case, only two bacterial strains: SA211 and HX11 and one consortium: C2 (SA211 + HX11) were evaluated, accounting for a total of four treatments considering the control (NB-Salt without bacteria). Because of the composition of MS/2 medium, with a basal EC value (2.82 dS/m), no salt was added to the control treatment. The other salinity treatments were prepared by the addition of different saline solutions of NaCl to the MS/2 agar medium (Table 1). Chia and quinoa seeds were surface sterilized using vapor-phase sterilization. Seeds were placed in open Petri dishes inside a 10 L desiccator. Next to the seeds container, a 250 mL glass beaker with 100 mL household bleach (NaClO 5.75%) was added with 3 mL of hydrochloric acid (HCl 37%). The desiccator was closed and sealed hermetically, and the chlorine gas acted from 4 to 6 h (Romano Armada et al., 2019).

Ten seeds were sown in each MS/2 plate and placed under cold conditions, at 4 °C for 24 h for seed stratification. After that, plates (three replicates per treatment) were placed vertically in a growth chamber and incubated with the same photoperiod and temperature of the water agar assay, for seven days.

#### 2.4.3. Phenotypic measurements

For both salinity stress amelioration assays, germination percentage (G), mortality (cumulative number of dead seedlings), and growth attributes were measured after seven days. The following growth attributes were measured: total length (TL), from the root tip until cotyledons; primary root length (RL), from the base of the hypocotyl until the root tip, for each seedling. Also, dry weight (DW) was measured for each replicate.

### 2.5. Relative Growth Index (RGI)

The data collected from both assays (in WA and MS/2 agars) were combined and weighted according to the germination (%) through an index. All measured growth attributes (germination percentage, total length, root length, and dry weight) were related and expressed as a value of the Relative Growth Index (*RGI*) of the seedlings (Eq. 1)

$$RGI = \frac{G_t}{G_c} \times \frac{TL_t/TL_c + RL_t/RL_c + PW_t/PW_c}{a} \quad (1)$$

where the sub index *t* denotes the bacterial treatment assayed and the sub-index *c* corresponds to the reference control treatment (without bacteria and salt), *G* is the germination percentage, *TL* is the total length of the seedlings, *RL* is the root length, *PW* is the whole dry weight, and *a* is the total number of attributes (equals three).

### 2.6. Statistical analysis

Statistical differences on PGP-activities of selected strains were determined by variance analysis considering an alpha (α) level of 0.05, using the software InfoStat (Di Rienzo et al., 2008).

In the case of plant-bacteria interaction assays, treatments were arranged in a growth chamber in a completely randomized design with four (WA) or three (MS/2) replicates, depending on the assay. Plates were frequently rotated to avoid sub-environmental effects. Data were subjected to analysis of variance to test the effects of the treatments considering an alpha (α) level of 0.05.

## 3. Results

### 3.1. In vitro PGP activities

A total of 111 strains were screened for PGP activities. Regarding $N_2$ fixation in N-free Ashby agar, 100 strains (90%) were able to grow without salts, 94 (85%) at 1 mol/L NaCl and only six (5.4%) grew at 2 M NaCl. In NFb medium, 70 strains (63%) fixed atmospheric nitrogen in the absence of salts, and only one of the SFS was able to perform with 1 and 2 mol/L NaCl (Fig. S2, Supplementary material). Considering both media, seven strains were able to fix $N_2$ at the highest salinity condition: HA120a, HA120b, HA120c, SFS, HX11, M3, and M9. Therefore, they were selected for further evaluation of PGP activities. Besides, the other five strains were selected because they showed the fastest growth and produced the highest biomass at 1 mol/L NaCl in Ashby agar: HX12a, HX12b, SA211, SA129b, and M12. Tests for bacterial N-fixing capacity were carried out only qualitatively as a first screening.

From the 12 selected strains, only the seven that were able to solubilize $PO_4^{3-}$ (HA120a, HA120b, HA120c, HX11, M3, SA211, SFS) were evaluated for IAA and siderophore production (Table 2). Strains HX12a, HX12b, M9, M12, and SA129b did not solubilize $PO_4^{3-}$ under any salinity condition. The seven bacteria resulting from the final PGP screening were previously identified by 16 S rDNA sequencing (Martínez et al., 2019a, 2019b).

*In vitro* assays revealed that strains HA120c and HX11 showed the highest capacities in phosphate solubilization without salts, while SFS was the best under the saline condition ($p < 0.05$) (Table 2). Considering IAA production, SA211 produced significantly ($p < 0.05$) higher quantities than other strains without salts and slightly higher with 1 mol/L of NaCl. However, under saline stress, all strains showed small amounts of IAA with a significantly lower impact on HA120c, M3, and SA211 (Table 2). In the case of siderophores production, SFS was the strain with the highest production regardless of the presence of salt, followed by SA211 only under the saline condition (Table 2).

### 3.2. Bacterial growth curves and biofilm production

Considering bacterial growth curves, SFS was the strain with higher biomass production and tolerance to salinity. Compared to the other strains, it showed the highest growth (Fig. S3, Supplementary material), reached when grown between 1000 and 1750 mmol/L NaCl. Furthermore, M3 also showed a high salinity tolerance but in a lower concentration range than SFS, with the highest growth between 750 mmol/L and 1250 mmol/L. For HX11, HA120a, and HA120c, the highest biomass production was observed between 500 mmol/L and 1500 mmol/L, while HA120b showed optimal growth between 500 mmol/L and 1250 mmol/L, evidencing the four strains a wide range of tolerance at different salinity concentrations. However, HA120b and HA120c showed the lowest biomass production among them (Fig. S3, Supplementary material). On the other hand, SA211 was the strain with the lowest tolerance to salinity, with the maximum biomass production between 500 and 750 mmol/L of NaCl (Fig. S3, Supplementary material).

Regarding biofilms, SFS showed the highest production in presence of 1250 and up to 1500 mmol/L of NaCl, which coincides with the range of highest biomass production in the growth curve. Conversely, SA211 and HA120c showed the highest biofilm production in the lowest salinity concentrations, from 0 mmol/L to 250 mmol/L of NaCl. Strains HX11, HA120a, and HA120b showed low biofilms production under lower saline stress (from 0 mmol/L to 50 mmol/L NaCl). The strain M3 was the only strain that did not produce biofilms in any salinity condition (Fig. S3, Supplementary material).

### 3.3. Antagonism assays

In general, strains grew well together except M3, which was

**Table 2**
*In vitro* plant growth-promoting (PGP) activity of bacterial isolates. The PGP activities evaluated were $PO_4^{3-}$ solubilization, Indole acetic acid (IAA) production, and siderophores production (ratio between colony and chelation halos diameter). Bacterial isolates were identified by 16S rDNA sequencing (Martínez et al., 2019a, 2019b). Different letters indicate significant differences between bacterial treatments in the same salt condition ($p \leq 0.05$).

| Strain (Accession number)[c] | $PO_4^{3-}$ Solubilization (mg/L) | | IAA Production (mg/L) | | Siderophores Production | |
|---|---|---|---|---|---|---|
| | Without salts | With 1 mol/L NaCl | Without salts | With 1 mol/L NaCl | Without salts | With 1 mol/L NaCl |
| *Bacillus* sp. HA120a (MF990750) | 113.44 ± 2.96c | 41.93 ± 5.99a | 0.00[d] ± 0.09a | 0.00[d] ± 0.08a | 1.61 | 1.92 |
| *Bacillus* sp. HA120b (MF990751) | 112.44 ± 4.24c | 41.17 ± 2.46a | 1.18 ± 0.08a | 0.00[d] ± 0.08a | 2.00 | 1.92 |
| *Bacillus* sp. HA120c (MF990752) | 130.94 ± 4.81c | 40.52 ± 3.38a | 0.00[d] ± 0.48a | 1.13 ± 0.15b | 1.51 | 1.35 |
| *Bacillus* sp. HX11 (MF990753) | 135.44 ± 4.84c | 40.85 ± 2.61a | 0.40 ± 0.04a | 0.04 ± 0.40a | 1.20 | 1.75 |
| *Bacillus* sp. M3 (NR[b]) | 31.91 ± 7.55b | 33.02 ± 6.61a | 0.55 ± 0.29a | 0.99 ± 0.27b | Nh[a] | Nh[a] |
| *Micrococcus luteus* SA211 (MF990761) | 11.74 ± 2.26a | 33.42 ± 1.16a | 31.24 ± 3.70b | 2.16 ± 0.53c | Nh[a] | 2.67 |
| *Halomonas* sp SFS (MF990763) | 0.00[d] ± 4.95a | 46.72 ± 4.46a | 0.35 ± 0.04a | 0.39 ± 0.02a | 2.93 | 3.00 |

[a] Nh, No halo production in CAS medium.
[b] NR, No registered.
[c] Accession number in the NCBI database.
[d] Under the limit of detection.

completely inhibited when plated with any strain with exception of SA211 (Fig. S4, Supplementary material).

### 3.4. Bacterial effect upon seeds germination:3.4.1 Germination dynamic

Chia seeds did not germinate at the two highest saline conditions assayed (100 and 150 mmol/L NaCl) in WA. With 15 mmol/L of NaCl, a similar germination dynamic was shown by all treatments with exception of C1 (SA211 + SFS) and *Halomonas* sp. (SFS) which had a negative effect. The treatments HX11 and C2 achieved their maximum germination percentage 144 h after seeding, while Control and SA211 achieved this percentage at 96 h and 168 h, respectively (Fig. 1A). It is remarkable that since the fourth day after sowing seeds inoculated with C1 showed important mortality reaching more than 30% at the end of the experiment (Fig. 1A). In the case of 50 mmol/L NaCl, chia showed the highest germination percentage in the presence of SA211, 72 h after seed plating onwards (Fig. 1B). In general, all treatments showed increasing mortality since 96 h after seeding. At the end of the experiment, C1 showed 100% of mortality, whereas the Control, SFS, and SA211 had 30.0%, 26.25%, and 16.25% of mortality, respectively. HX11 and C2 showed the lowest mortality values with 12.5% and 10%, respectively (Fig. 1B).

Quinoa seeds germinated in all tested salinity conditions in WA (Fig. 1C–F). Except for HX11 ($p = 0.38$), all treatments showed significant differences among salinity levels in the final germination percentage ($p < 0.05$) (Fig. 1). There were no germination differences among treatments with 15 and 200 mmol/L NaCl (Fig. 1C; 1D). However, there was a delay higher than 24 h to reach maximum germination in the salinity level of 200 mmol/L NaCl with SFS, SA211, and C1 than for the treatments Control, HX11, and C2 (Fig. 1D). In the salinity level of 300 mmol/L NaCl, there was a germination decrease with SA211 and SFS compared to other treatments (Fig. 1E). Moreover, HX11 and C2 reached about 90% and 75% of germination, respectively, at 72 h; while the Control treatment surpassed 90% germination after 144 h (Fig. 1E). In the case of 400 mmol/L NaCl, the germination was significantly higher ($p \leq 0.0033$) with HX11 and C2 (the latter in lower proportion) during the whole germination process (Fig. 1F). Only these two treatments surpassed 80% of germination at 144 h. The other treatments

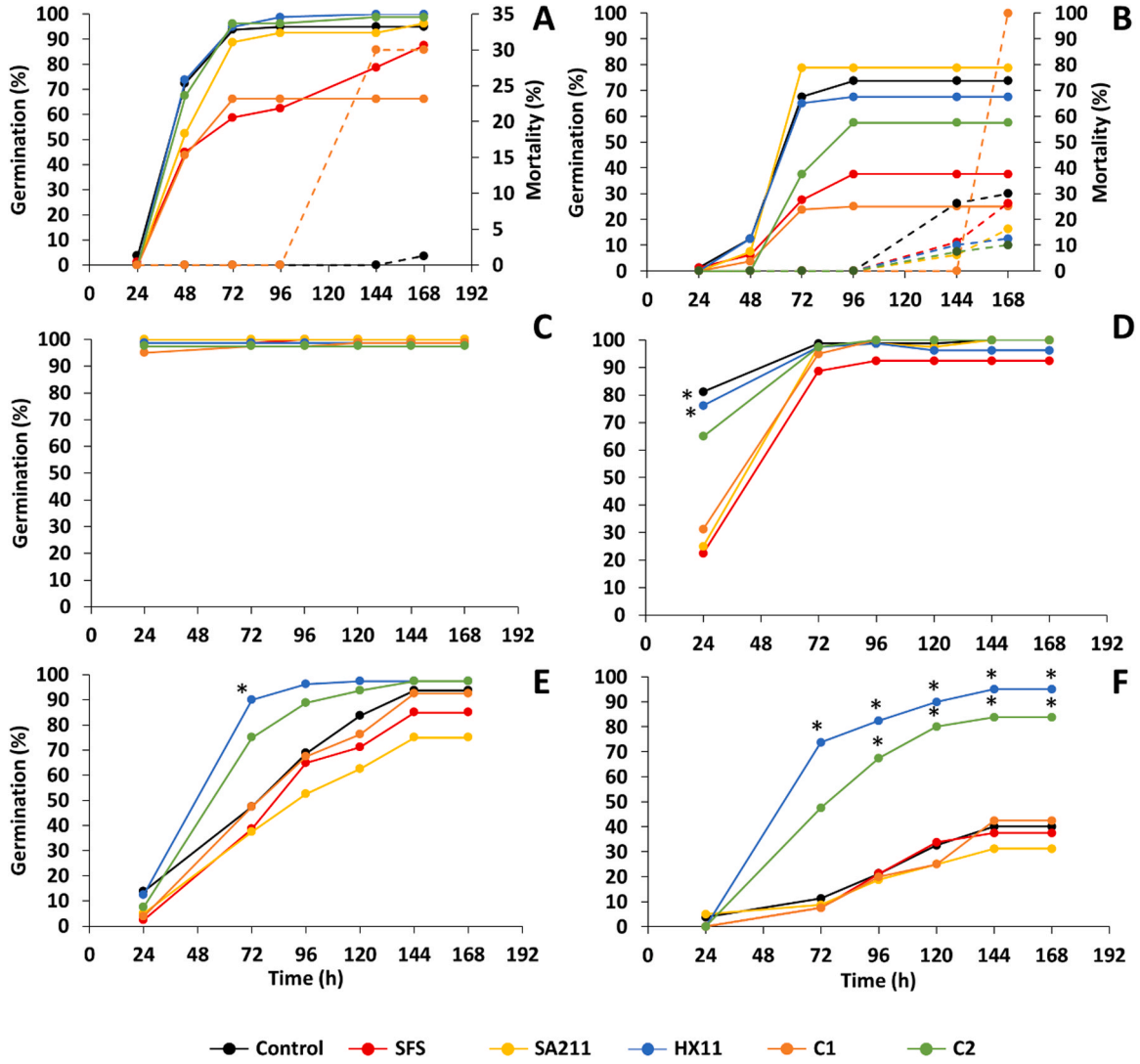


**Fig. 1.** Germination curves expressed as cumulative germination percentage (%) of chia (*Salvia hispanica* L.) and quinoa (*Chenopodium quinoa* Willd.) seeds in the presence of six treatments: Control (without bacteria), *Halomonas* sp. SFS, *Micrococcus luteus* SA211, *Bacillus* sp. HX11, C1 (Consortium 1: SA211 + SFS) and C2 (Consortium 2: SA211 + HX11). In chia curves, mortality percentage is also shown (dashed line). Two salinity conditions were assayed in chia: 15 mmol/L (A) and 50 mmol/L (B) NaCl in water agar medium at 23 °C and 8/16 h light/dark cycle. Four salinity conditions were assayed in quinoa: 15 mmol/L (C), 200 mmol/L (D), 300 mmol/L (E), and 400 mmol/L (F) of NaCl in water agar medium at 23 °C and 10/14 h light/dark cycle. Each point represents the average value of four replicates per treatment. Star marks (*) indicate significant differences with respect to the Control ($p \leq 0.05$).

showed less than 40% of germinated seeds during the whole period of germination (Fig. 1F).

In MS/2 medium, the final germination percentage (seven days after seeding) did not show significant differences ($p > 0.05$) between treatments for any salinity condition in chia and quinoa seeds. In chia, all bacterial treatments achieved a similar final germination percentage at all salinity levels (above 90%) (Supplementary Table 1). However, in quinoa, while germination was similar in the condition without salts and with 200 mmol/L (above 90%), there were lower germination percentages in all treatments at 400 mmol/L NaCl. The highest germination reduction was found in the Control treatments (Supplementary Table 1).

### 3.4.1. *Bacterial growth promotion effects and Relative Growth Index (RGI) in water agar*

Considering chia, in 15 mmol/L all treatments showed similar values in germination except C1. Control presented higher values than bacterial treatments in total length, roots length, and dry weight. However, only considering bacterial treatments, SA211 and HX11 showed the heaviest seedlings, while SA211, HX11, and C2 showed a better performance than other treatments in root and total length. In all measured growth attributes, C1 displayed the lowest values (Fig. 2A). Under the saline condition (50 mmol/L of NaCl), treatments with SA211, HX11, and C2, showed higher germination with respect to the control (42.8%, 25.7%, and 9.1%, respectively). Furthermore, considering total length, seedlings inoculated with SA211, HX11, and C2 were 11.6%, 49.4% and 18.4% longer than control plants, respectively. The treatment with HX11 also showed a 21.4% increment compared to control seedlings and an overall highest roots length. The treatment C1 exhibited a detrimental effect leading to the mortality of all seedlings (Fig. 2B).

Considering the bacterial effect on quinoa, in 15 mmol/L bacterial treatments showed similar values than the control in all measured growth attributes with exception of dry weight, which was significantly higher with HX11 (Fig. 2C). In the case of 200 and 300 mmol/L, there was not a significant effect of bacterial treatments (Fig. 2D; E). However, in 400 mmol/L there was a significant increase in germination and dry weight with HX11 and C2 concerning the control. In total length and roots length, all treatments displayed similar values with exception of SA211 and SFS that showed shorter seedlings. In this case, C1 did not

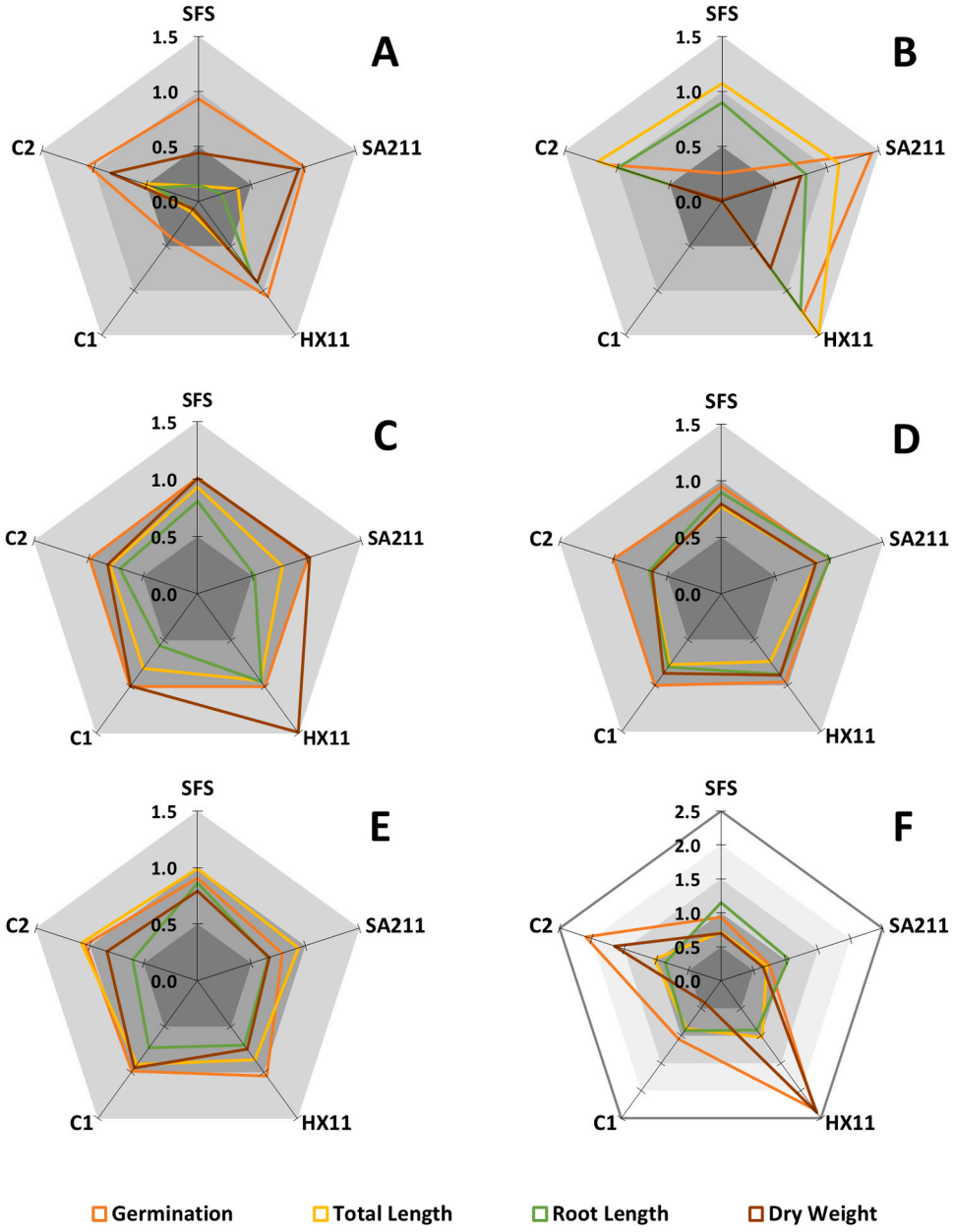


**Fig. 2.** Measured growth attributes: germination (G), total length (TL), roots length (RL), and dry weight (DW), in chia (*Salvia hispanica* L.) and quinoa (*Chenopodium quinoa* Willd.) seedlings in presence of six treatments: Control (without bacteria), *Halomonas* sp. SFS, *Micrococcus luteus* SA211, *Bacillus* sp. HX11, C1 (Consortium 1: SA211 + SFS), and C2 (Consortium 2: SA211 + HX11). Two salinity conditions were assayed in chia: 15 mmol/L (A) and 50 mmol/L (B) NaCl in water agar medium at 23 °C and 8/16 h light/dark cycle and four salinity conditions were assayed in quinoa: 15 mmol/L (C), 200 mmol/L (D), 300 mmol/L (E) and 400 mmol/L (F) of NaCl, in water agar medium at 23 °C and 10/14 h light/dark cycle. Gray zones represent the values of the growth attribute with respect to the reference Control value (without bacteria and NaCl) for each bacterial treatment: the darkest gray zone represents the 0.5 of the reference control value, the middle gray zone corresponds to the control value (1) and the lighter one to 1.5 of the reference value. In the case of 400 mmol/L (F) NaCl, the inner polygon represents 0.5 of the reference Control value, with outer concentrically gray areas showing increments every 0.5 units with respect to the control.

show a detrimental effect as in chia (Fig. 2F).

Considering *RGI* in chia, the highest values were obtained by the Control condition and HX11 in 15 mmol/L NaCl and by SA211 and HX11 in 50 mmol/L. The treatments SFS and C1 showed the lowest index values, showing a detrimental effect in the seedlings in both salinity conditions (Fig. 3A). In the case of quinoa, with 15 mmol/L NaCl, the treatment with HX11 showed the highest *RGI* among all treatments. With 200 and 300 mmol/L, all bacterial treatments reached similar index values, while with 400 mmol/L HX11 showed a significantly higher value than all other treatments (Fig. 3B).

### 3.4.2. *Bacterial growth promotion effects and Relative Growth Index (RGI) in MS/2*

Considering chia seedlings, under the condition without salts, all bacterial treatments showed lower values than the Control (1.0) in the four measured growth attributes. Moreover, among all treatments, that with SA211 produced the lowest values (Fig. 4A). However, with 100 mmol/L of NaCl, there was an increase in total length, roots length, and dry weight with C2 and with HX11 in a lower proportion (Fig. 4B). Moreover, the inoculation with HX11 showed homogenous and similar growth in chia seedlings compared with the Control. In the case of SA211, while there was an increase of dry weight with respect to the control, the other growth attributes remained below it (Fig. 4B).

In the case of quinoa, under the condition without salts, all bacterial treatments showed lower or equal values than the Control in germination and root length. However, seedlings inoculated with HX11 and C2 showed an increase in total length and dry weight, respectively, when comparing with the control (Fig. 4C). Moreover, in the saline condition (200 mmol/L), the treatment with HX11 produced the highest values in total and roots length ($p < 0.05$), showing homogenous growth of the seedlings (Fig. 4D). Regarding germination and dry weight, similar values to the control were obtained in all treatments. No growth attributes other than germination percentage could be measured in the condition of 400 mmol/L NaCl because during the evaluated time the seedlings only showed an emerging radicle.

For both crops under saline conditions, SA211 showed a negative effect with the lowest values in total length and roots length. Also, in the presence of SA211, the seedlings showed a different phenotype, with small and twisted development.

Regarding chiaś *RGI* in MS/2 medium without salts, HX11 and C2 revealed similar values with respect to the Control treatment, while SA211 was significantly lower than it (Fig. 5A). However, with 100 mmol/L NaCl, HX11 and C2 showed the highest index values. In addition, a positive trend of HX11 and C2 can be observed under salinity conditions. In both saline conditions, SA211 showed a detrimental effect, giving rise to the lowest *RGI* values and small twisted seedlings as can be seen in the photographs (Fig. 5A).

Considering index values of quinoa, without salt, the highest values were obtained by Control and HX11 treatments, while SA211 showed the lowest one. With 200 mmol/L NaCl, there were no clear differences among treatments. Although HX11 showed a slightly higher RGI than the Control, differences were not significant. In this case, SA211 treatment also showed a detrimental effect on seedlings growth only in the saline condition where the roots were negatively affected by the bacteria (Fig. 5B). In addition, and as observed on plates, the treatment with HX11 showed a beneficial effect on the seedlings for both chia and quinoa, being more homogenous among themselves in terms of size and length (Fig. 5).

## 4. Discussion

Based on the knowledge that halophile and halotolerant bacteria with Plant Growth Promotion (PGP) abilities can benefit plants development under saline stress, we began our research evaluating PGP traits and *in vitro* plant-bacteria interaction with bacteria isolated from a hypersaline environment and two crops of different tolerance to abiotic stress. While all the PGP activities are important when selecting bacterial strains as bio inoculants, the capability of fixing atmospheric N is of particular interest.

Recent research showed that increasing rates of nitrogen fertilization leads to significant increases in seeds production, number of leaves and inflorescence per plant, and dry biomass on chia plants (Sosa and Ruiz-Ibarra, 2018; Souza et al., 2017) Moreover, it is known that salinization affects the normal transformations of N in soil by retarding or inhibiting several biological/microbial processes as the nitrification, causing an accumulation of nitrite or by increasing $NH_4$ volatilization rates (Akhtar et al., 2012). In this sense, we considered that the nitrogen fixation PGP property was extremely important and therefore it was selected to start the stepwise screening of PGP traits. However, because it was precisely the first activity evaluated, it was only measured qualitatively.

The response of crops to saline stress was different, as expected. Quinoa is a crop with high tolerance to different types of abiotic stress (Adolf et al., 2013; González et al., 2009; Shabala et al., 2012), while chia is a crop with low tolerance to abiotic stresses (Paiva et al., 2018; Raimondi et al., 2017; Stefanello et al., 2015). Our *in vitro* results with WA medium confirmed these reports as quinoa germinated with up to 400 mmol/L NaCl, while chia did so with saline concentrations lower than 100 mmol/L NaCl. However, growth parameters increased with bacterial inoculation under salinity conditions, opening a new possibility for extending chia cultivation to saline soils.

In general, the increase in salinity led to a decrease in germination

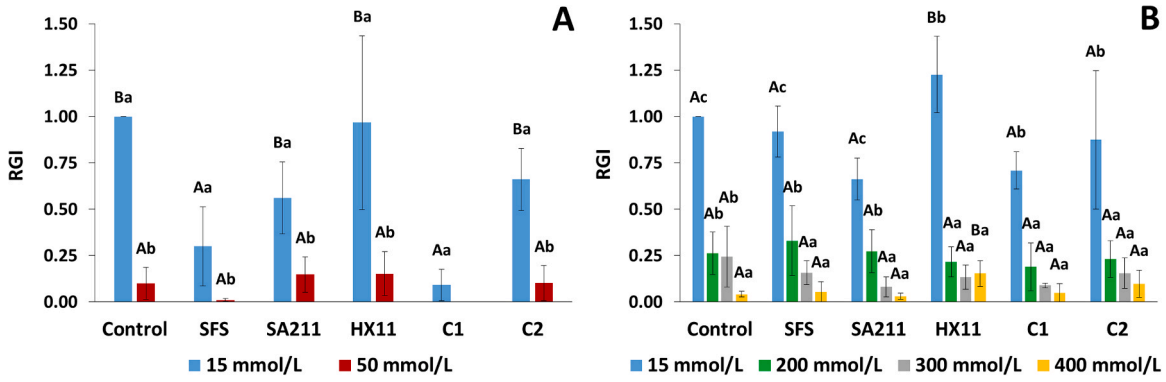


**Fig. 3.** Relative growth index (*RGI*) values of chia (*Salvia hispanica* L.) (A) and quinoa (*Chenopodium quinoa* Willd.) (B) seedlings in water agar medium (WA) in presence of six treatments: Control (without bacteria), *Halomonas* sp. (SFS), *Micrococcus luteus* SA211, *Bacillus* sp. HX11, C1 (Consortium 1: SA211 + SFS) and C2 (Consortium 2: SA211 + HX11) and with different levels of salinity characterized by the sodium chloride concentration (mmol/L of NaCl). Values are the means of three replicates of each treatment ± SD. Uppercase letters indicate differences between bacterial treatments of the same salt level, while lower case letters indicate differences between saline conditions within the same treatment ($p \leq 0.05$).

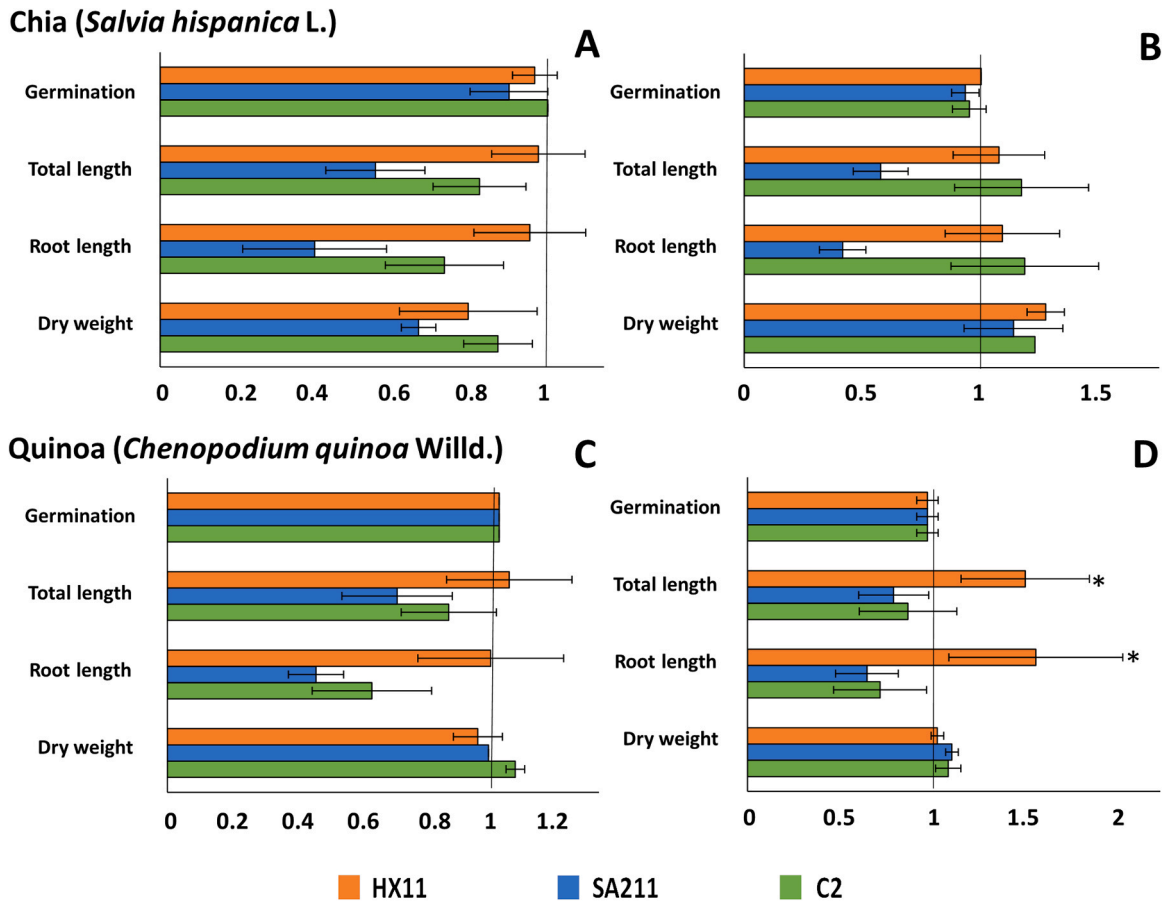


**Fig. 4.** Measured growth attributes in chia (*Salvia hispanica* L.) seedlings without salts (A) and 100 mmol/L of NaCl (B) and in quinoa (*Chenopodium quinoa* Willd.) seedlings without salts (C) and 200 mmol/L NaCl (D) in agar MS/2 medium, in presence of four treatments: Control (without bacteria), *Bacillus* sp. HX11, *Micrococcus luteus* SA211 and C2 (Consortium 2: SA211 + HX11). Measured growth attributes were germination (G), total length (TL), roots length (RL), and dry weight (PW). Values were transformed considering the control (without bacteria and salt) and represent the means of three replicates of each treatment ±SD. The vertical line in 1.0 corresponds to the reference control value. (*) indicates significant differences ($p \leq 0.05$) between treatments.

and/or in growth parameters for both crops in most treatments, excepting those with *Bacillus* sp. (HX11 and C2). On the other hand, *Halomonas* sp. SFS, a bacteria known for its tolerance to salts and PGP activities (Cherif, 2018; Martínez et al., 2019a; Mukherjee et al., 2019), negatively affected growth parameters and germination of both, chia and quinoa. This could be explained by the environmental conditions required by the strain *Halomonas* sp. SFS, which showed an optimal growth range between 1000 and 1750 mmol/L NaCl concentration, salinity values much higher than those tolerable for crops and higher than the maximum saline levels selected for trials on chia and quinoa. It is feasible that the strain was stressed by the low salinity levels. Moreover, SFS showed the highest biofilms production in the higher salinity concentrations. This is in line with the results of Ozturk and Aslim (2010) and Moshabaki et al. (2018), who reported an increased EPS production associated with NaCl tolerance. Furthermore, the combination of SFS with SA211 (C1) showed the worst performance in germination and growth attributes under the salinity conditions.

In the case of HX11 and C2, bacterial beneficial effects were found in the presence of high salinity in chia (100 mmol/L NaCl) and quinoa (with 200 and 400 mmol/L NaCl,) seedlings. These benefits could be due to high nutrient availability or by mechanisms of salt capture or exclusion, which enhance seeds germination and plant development. The latter mechanisms are usually seen in halophile extracellular polymeric substances (EPSs)-producing bacteria. Biofilm formation and bacterial EPS help to mitigate salinity stress by reducing the content of $Na^+$ available for plant uptake, which enhances plant growth, increases biomass and crop productivity, and improves yield in saline soil, among others. Also, EPSs contribute to bacterial colonization of plant roots and soil particles, improving soil structure (Banerjee et al., 2019; Upadhyay et al., 2011). Interestingly, strains HX11 and SA211 demonstrated the ability to produce biofilms and showed tolerance to lithium chloride (Martínez et al., 2019a). Particularly, SA211 showed high biofilms production from 0 mmol/L to 250 mmol/L, a range that includes the salinity levels selected for the stress amelioration assays. Therefore, the inoculation with EPS-producing bacteria, like the ones tested in this study, could serve as a useful tool for alleviating salinity stress in salt-sensitive plants due to a reduced passive flow of $Na^+$.

The presence of the strain SA211 alone showed a detrimental effect which altered the seedling phenotype (the whole seedling grew curled up) but, did not decreased their biomass production. Since SA211 is a great producer of IAA, which has main effects in rooting, growth of secondary roots, and root hairs, the anomalous growth could be caused by an overproduction of this auxin. It is known that the effect of IAA depends on its concentration: low concentrations can promote seeds germination and root growth, whereas high concentrations can inhibit these processes (Jangu and Sindhu, 2011; Tabatabaei et al., 2016). In this sense, SA211 showed a deleterious effect in quinoa seedlings in MS/2 media by causing an overproduction of lateral roots and short twisted plants. The same effect was seen with other bacteria as *Enterobacter taylorae, Klebsiella planticola, Alcaligenes faecalis, Xanthomonas maltophila, Pseudomonas* sp. and *Flavobacterium* sp. (Malik and Sindhu, 2011; Sarwar and Kremer, 1995; Suzuki et al., 2003). These bacteria produced high quantities of IAA and showed an inhibitory effect decreasing the root elongation zone and length and causing an

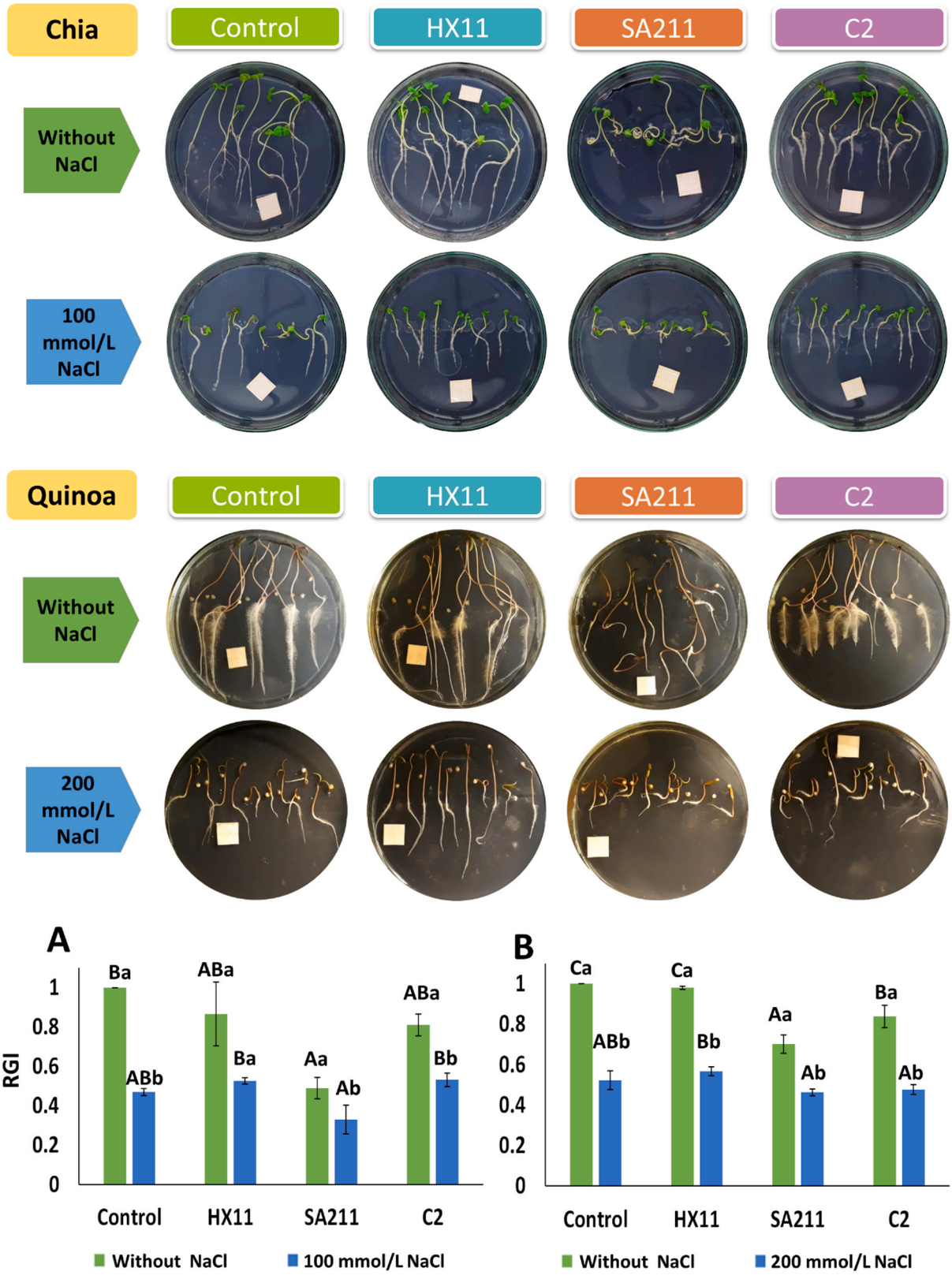


**Fig. 5.** Relative growth index (*RGI*) in MS/2 medium of Chia (*Salvia hispanica* L.) seedlings without salts (WS) and with 100 mmol/L NaCl (23 °C and 8/16 h light/dark cycle) (A) and quinoa (*Chenopodium quinoa* Willd.) seedlings without salts (WS) and with 200 mmol/L NaCl (23 °C and 10/14 h light/dark cycle) (B). Four treatments were assayed: Control (without bacteria), *Bacillus* sp. HX11, *Micrococcus luteus* SA211 and C2 (Consortium 2: SA211 + HX11). Values are the means of three replicates of each treatment ±SD. Uppercase letters indicate differences between bacterial treatments of the same salt level, while lower case letters indicate differences between saline conditions within the same treatment ($p \leq 0.05$). Photographs of seedlings grown vertically on agar plates containing MS/2 medium seven days after sowing are shown on the right side of the panels. White squares in the Petri dishes are 1 $cm^2$.

anomalous increase in root hairs and secondary roots formation (Jangu and Sindhu, 2011).

HX11 was the most promising strain for both crops showing the best values in measured growth attributes and RGIs under salt stress. Moreover, it showed an optimal growth and biomass production in a wide range of salinity concentrations, from 250 mmol/L to 1500 mmol/L, with biofilm production in the lower saline levels until 50 mmol/L. These features represent an advantage for HX11′s agricultural and biotechnological application and the possibility to apply and evaluate the strain in multiple conditions. This strain, identified as *Bacillus* sp. with a 99% of identity, was more closely related with *Bacillus atrophaeus* in the phylogenetic tree obtained by the analysis of its sequence with some bacteria from the NCBI database (Martínez et al., 2019a). Interestingly, *B. atrophaeus* is known for its PGP abilities and shows these properties without salts and under saline stress conditions (Karlidag et al., 2013). These authors reported, in strawberry plants, that *B. atrophaeus* produced a significant increase in shoot fresh and dry weights, root dry weight, chlorophyll content, and leaf relative water content with EC ranged from 1.30 $dS{\cdot}m^{-1}$ to 3.50 $dS{\cdot}m^{-1}$. Without salt stress, this strain also showed potential. Köberl et al. (2014) found *B. atrophaeus* as one of the most promising bacteria for field application because of its outstanding antagonistic, PGP, and stress tolerance capabilities. Yolcu et al. (2012) obtained, in vetch, the greatest stem diameter and a higher plant height, and leaves number with *B. atrophaeus* than those of the Control. However, there are not previous researches about its performance in chia or quinoa, where we concluded that the effect of HX11 is magnified when it is applied individually and that it decreases the detrimental effect of SA211 when is applied in a consortium. Its beneficial effect was particularly remarkable in quinoa, which has natural tolerance to salinity. When the saline stress increased, HX11 and C2 helped the seedling to overcome the stressful condition and improve germination. It is also remarkable that HX11 was the only treatment in with seeds germination was not affected by salinity, achieving the same germination percentages in all salinity levels, from 15 mmol/L to 400 mmol/L NaCl.

## 5. Conclusions

In conclusion, regarding plant growth promotion traits, three strains were the most promising in the evaluated activities. The strain *Bacillus* sp. HX11 showed the highest values in Phosphate solubilization without salts while *Halomonas* sp. SFS did so in the saline condition. Moreover, the strain SFS was the best siderophores producer in both tested salinity conditions. Finally, the strain *Micrococcus luteus* SA211 was the best producer of IAA without salts and with 1 mol/L NaCl.

Furthermore, our results suggest that the strain *Bacillus* sp. HX11, with multiple PGP traits and tolerance to saline stress, has great potential as a bioinoculant in saline conditions. The possibility of using this strain as a biofertilizer for crops in saline soils should be further studied. Considering the adverse risks and negative environmental effects associated with chemical fertilizers, the use of bacterial inoculants with PGP properties is a sustainable and interesting approach for further studies.

## CRediT authorship contribution statement

**María Florencia Yañez-Yazlle:** Conceptualization, Methodology, Formal analysis, Writing - original draft. **Neli Romano-Armada:** Formal analysis, Data curation, Writing - review & editing. **Martín Moises Acreche**: Visualization, Writing - review & editing, Supervision. **Verónica Beatriz Rajal:** Visualization, Writing - review & editing, Supervision. **Verónica Patricia Irazusta:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112273.

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