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# Characterization and evaluation of potential halotolerant phosphate solubilizing bacteria from *Salicornia fruticosa* rhizosphere

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Soil salinization is a significant abiotic factor threatening agricultural production, while the low availability of phosphorus (P) in plants is another worldwide limitation. Approximately 95–99% of the P in soil is unavailable to plants. Phosphate-solubilizing bacteria (PSB) transform insoluble phosphates into soluble forms that plants can utilize. The application of PSB can replace or partially reduce the use of P fertilizers. Therefore, selecting bacteria with high solubilization capacity from extreme environments, such as saline soils, becomes crucial. This study aimed to identify twenty-nine bacterial strains from the rhizosphere of *Salicornia fruticosa* by sequencing the 16S rDNA gene, evaluate their development in increasing concentrations of NaCl, classify them according to their salinity response, and determine their P solubilization capability. The bacteria were cultivated in nutrient agar medium with NaCl concentrations ranging from 0.5% to 30%. The phosphate solubilization capacity of the bacteria was evaluated in agar and broth National Botanical Research Institute (NBRI) media supplemented with calcium phosphate (CaHPO<sub>4</sub>) and aluminum phosphate (AlPO<sub>4</sub>), and increased with 3% NaCl. All bacterial strains were classified as halotolerant and identified to the genera *Bacillus*, *Enterobacter*, *Halomonas*, *Kushneria*, *Oceanobacillus*, *Pantoea*, *Pseudomonas*, and *Staphylococcus*, with only one isolate was not identified. The isolates with the highest ability to solubilize phosphorus from CaHPO<sub>4</sub> in the liquid medium were *Kushneria* sp. (SS102) and *Enterobacter* sp. (SS186), with 989.53 and 956.37 mg·Kg<sup>-1</sup> P content and final pH of 4.1 and 3.9, respectively. For the solubilization of AlPO<sub>4</sub>, the most effective isolates were *Bacillus* sp. (SS89) and *Oceanobacillus* sp. (SS94), which raised soluble P by 61.10 and 45.82 mg·Kg<sup>-1</sup> and final pH of 2.9 and 3.6, respectively. These bacteria demonstrated promising results in *in vitro* P

solubilization and can present potential for the development of bioinput. Further analyses, involving different phosphate sources and the composition of produced organic acids, will be conducted to contribute to a comprehensive understanding of their applications in sustainable agriculture.

#### KEYWORDS

salinity, tolerance, phosphorus, rhizobacteria, soil

## 1 Introduction

Soil salinization is one of the main abiotic factors that threaten agricultural production (Gamalero et al., 2020; Sagar et al., 2022). Saline soils naturally occur in arid and semi-arid climatic regions under limited drainage conditions, associated with the presence of a high water table, and in coastal regions (Pedrotti et al., 2015; Gamalero et al., 2020; Sagar et al., 2022). This process is aggravated by climate change, which causes increases in global temperature, rainfall regimes, and sea level (Ullah et al., 2021). Anthropogenic actions also cause the accumulation of salts in the soil, and it can occur in agricultural areas owing to incorrect management of irrigation water, use of low-quality water, poor drainage, and poor soil and fertilizer management (Pedrotti et al., 2015; Gamalero et al., 2020; Sagar et al., 2022). The occurrence of salinized arable land increases annually. It is estimated that more than 3.0% of the surface soils and 6% of the subsoils are salinized owing to natural or anthropogenic processes (Food and Agriculture Organization, 2021). Salinization has accelerated in coastal agricultural lands, with salinity increasing from 1 to 33% over the last 25 years (Ullah et al., 2021).

Most plant species cannot tolerate high salinity (Etesami and Beattie, 2018; Ondrasek et al., 2022). The harmful effects caused by excess salt in plants are related to osmotic and ionic stress, which globally affect the plant and impair its water balance and nutrition, leading to a reduction in the photosynthetic rate and the generation of reactive oxygen species that cause molecular damage (Hashem et al., 2016; Bulgari et al., 2019). Thus, soil salinization severely affects crop productivity and limits agricultural use in affected areas. However, some plant species, called halophyte plants, have adapted to environments with high salinity (Etesami and Beattie, 2018). These adaptations can be morphological, physiological, or biochemical but can also be related to symbiotic interactions with plant growth-promoting bacteria (PGPB) (Etesami and Beattie, 2018; Egamberdieva et al., 2022). Plants of the genus *Salicornia* are halophytes that develop in coastal regions and have ecological importance and commercial value, as they develop in areas where most plants cannot develop and produce a large amount of biomass that can be used for the production of vegetable salt (Furtado et al., 2019). Some studies have reported PGPB isolated from the rhizosphere of *Salicornia* spp., such as *Klebsiella pneumoniae*

from *Salicornia bigelovii* (Rueda-Puente et al., 2003), *Pseudomonas pseudoalcaligenes* from *Salicornia europea* (Ozawa et al., 2007) and *Brachybacterium saushtrense* and *Pseudomonas* spp. from *Salicornia brachiata* (Jha et al., 2012).

Another limiting factor in agricultural production worldwide is the low availability of phosphorus (P) in plants. Plants absorb P in the form of monobasic ( $\text{H}_2\text{PO}_4$ ) and dibasic ( $\text{HPO}_4^{2-}$ ) ions (Gomes et al., 2010). However, approximately 95–99% of the P in soil is unavailable to plants. It is associated with the mineral fraction, mainly calcium in calcareous soils, iron and aluminum in acidic soils, and organic compounds in soils rich in organic matter (Gomes et al., 2010; Rasul et al., 2019). In acidic soils, the most common forms are aluminum phosphates variscite ( $\text{AlPO}_4 \cdot 2\text{H}_2\text{O}$ ), followed by strengite ( $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$ ) (Bashan et al., 2013). In alkaline soils with high availability of  $\text{Ca}^+$ , phosphate is associated with calcium (P-Ca), which are, in decreasing order of solubility, dihydrated dicalcium phosphate (brushite)  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  > anhydrous dicalcium phosphate (monetite)  $\text{CaHPO}_4$  > octacalcium phosphate  $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$  > tricalcium phosphate  $\text{Ca}_3(\text{PO}_4)_2$  > hydroxyapatite  $\text{Ca}_5(\text{PO}_4)_3\text{OH}$  > fluorapatite  $\text{Ca}_5(\text{PO}_4)_3\text{F}$  (Bashan et al., 2013). It is estimated that more than 40% of agricultural land has limited productivity due to P deficiency (Bailemi and Negisho, 2012). In recent years, the use of phosphate fertilizers to maintain agricultural production has increased. For example, in Brazil, the total annual use of phosphate fertilizers has increased from an annual average of 0.04 T in 1960 to 2.2 T in 2016 (Withers et al., 2018). However, P added to the soil is quickly immobilized and becomes inaccessible to plants, resulting in the low utilization of phosphate fertilizer. In addition, the application and accumulation of phosphate fertilizers promote the eutrophication of water bodies and contamination by metals in the soil, causing damage to plants, animals, and humans (Katherine, 2010; Azzi et al., 2017). Additionally, the high salinity may induce competition between  $\text{H}_2\text{PO}_4^-$  and  $\text{Cl}^-$  ions (Maksimovic and Žarko, 2012). Therefore, the consequences of saline stress can lead to even greater difficulties in phosphorus uptake by plants. Moreover, plants under salt stress are most affected in crucial systems for nutrient absorption, cellular membrane stability, and transport pathways, and this also affects the absorption of phosphorus by the plant (Muhammed et al., 1987; Cruz et al., 2018; Roy and Chowdhury, 2021).

Phosphate-solubilizing microorganisms play a crucial role in the dynamics of P cycling in the soil (Alori et al., 2017; Zhu et al., 2018). These microorganisms can act as plant growth promoters by making P available to plants (Oliveira et al., 2009). The mobilization of P by microorganisms occurs through a variety of mechanisms such as a) acidification of the medium by the extrusion of H<sup>+</sup> and/or organic acids, b) metal complexation, c) metal reduction, d) extrusion of phosphatases, and e) indirect dissolution of phosphate through the stimulation of acid production by plants (Bashan et al., 2013; Krishnaraj and Dahale, 2014). Various studies have sought to isolate and identify phosphate-solubilizing bacteria as bioprospecting strains with the potential to develop sustainable alternatives for P management in agriculture (Oliveira et al., 2009; Zhu et al., 2011; Jiang et al., 2018; Suleman et al., 2018; Chen and Liu, 2019; Wan et al., 2020; Jiang et al., 2022). Many soil microorganisms can mobilize P; however, their transformation capacity may be associated with ecological conditions.

Environmental stressors lead to a decrease in various microbial activities, such as respiration, nitrogen mineralization, and the functioning of various enzymes (Zhu et al., 2011; Singh, 2016). An increase in salinity can profoundly impact the efficacy of phosphate-solubilizing microorganisms due to the inhibition of certain enzyme activities (Sritongon et al., 2022). For instance, the activities of enzymes such as dehydrogenases, which play a crucial role in acid synthesis, and phosphatases, involved in the mineralization of organic phosphorus, can be inhibited (Silva et al., 2007; Suleman et al., 2018). As a result, the overall performance of microorganisms in phosphate solubilization can be severely compromised. Silva et al. (2007) reported that continuous soil irrigation with reject water from a salt mine decreased the activity of several enzymes, including dehydrogenases and phosphatases. On the other hand, Sritongon et al. (2022) observed high enzymatic activity in the rhizosphere soils of *O. sativa* grown in saline fields.

The prospecting of phosphate-solubilizing bacteria (PST) as an environmentally friendly alternative to improve phosphorus uptake by plants is promising. However, these bacteria may experience a reduction in their solubilization activity due to an increase in salinity. Therefore, studies aiming to bioprospect microorganisms that are tolerant to higher salt concentrations and possess attributes for plant growth are promising. Gil et al. (2023) isolated and identified bacteria from hypersaline and hypergypsic soils that exhibit traits promoting plant growth. They were capable of increasing root size under osmotic stress in *Medicago* sp. plants. In light of these considerations, this study aimed to identify and evaluate the tolerance of bacteria isolated from the rhizosphere of *Salicornia fruticosa* to increasing amounts of salt (NaCl) and their ability to solubilize calcium (CaHPO<sub>4</sub>) and aluminum phosphate (AlPO<sub>4</sub>).

## 2 Materials and methods

### 2.1 Origin of the isolates

The bacterial strains characterized in this study were isolated from the rhizosphere of *S. fruticosa* in two different saline environments: hypersaline plains (Rio de Janeiro, RJ) (23°00'10" S,

43°34'30" W) and a deactivated salt mine (São Pedro da Aldeia, RJ) (22°49'59" S, 42°05'15" W) by Xavier (2021). The bacteria were isolated in Nutrient Agar medium supplemented with 5%, 10%, 15%, and 20% NaCl. The bacteria, identified with the 'SS' acronym representing 'saline soils' from where they were isolated, have been stored in agar stock at -20°C in the Molecular Genetics Laboratory of Microorganisms at the Federal Rural University of Rio de Janeiro.

### 2.2 DNA extraction, amplification, and sequencing of the 16S rDNA gene

DNA was extracted as described by Tito et al. (2015). The 16S rDNA gene was amplified using the polymerase chain reaction (PCR) technique, using primers 338F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1378R 5'-CGGTGTGTACAAGGCCCGGAACG-3'. PCR assays were performed in 25 µL volumes containing the following reagents: reaction buffer (1X), 1 U of Taq DNA polymerase, 2.5 mM of MgCl<sub>2</sub>, 0.2 mM of dNTP, and 0.4 µM of each primer. The reaction was performed in a thermocycler (Bio-Rad, Hercules, CA, USA), with an initial step of denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 40 s; extension at 72°C for 1 min and 30 s; and a final extension step at 72°C for 7 min (Weisburg et al., 1991). The PCR products were separated using electrophoresis on a 1.5% agarose gel, stained with SYBR Green I (Life Technologies, Carlsbad, CA, EUA), and visualized under ultraviolet light using an L-PIX EX photodocumentation system (Loccus Biotechnology, Cotia, SP, Brazil).

The PCR products were purified using Exo-Sap (USB Corporation, Cleveland, Ohio, USA), as recommended by the manufacturer. The purified PCR products were sequenced using BigDye<sup>TM</sup> Terminator v3.1 Cycle Sequencing Kit. The reaction was performed in a thermocycler (Bio-Rad, Hercules, CA, USA), with initial denaturation at 94°C for 1 min; followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 15 s; and extension at 72°C for 4 min. Samples were purified by precipitation using 3M sodium acetate, 125 mM EDTA, and 70% ethanol. Sequencing of the samples was performed using the 3500 Genetic Analyzer equipment (Applied Biosystems®).

The sequences were edited using BioNumerics software (v. 7.6) and compared with the sequences deposited in the NCBI database using the BLASTn algorithm (Altschul et al., 1997). Sequence alignment was performed using the ClustalW algorithm (Thompson et al., 1994) in MEGAX software (v.11.0.8). Phylogenetic relationships were determined using the neighbor joining (NJ) algorithm and p-distance model. The strength of each branch was determined using a nonparametric bootstrap test with 1000 repetitions (Felsenstein, 1985). A sequence derived from *Arthrobacter oryzae* (NR 041545.1) was used as the external group.

### 2.3 Salinity test and classification of bacterial isolates

Bacterial isolates were seeded onto nutrient agar (meat extract 1.0 g.L<sup>-1</sup>; yeast extract 2.0 g.L<sup>-1</sup>; peptone 5.0 g.L<sup>-1</sup>; sodium chloride

5.0 g.L<sup>-1</sup>; agar 15.0 g.L<sup>-1</sup>) with increasing concentrations of NaCl (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 5%, 10%, 15%, 20%, 25%, and 30%). Plates containing the cultures were placed in an incubator at 30°C and analyzed over 10 days after inoculation. The growth interval was determined based on the NaCl concentration at which the isolate grew. The bacteria were inoculated into the medium in triplicates.

The isolates were classified according to their growth intervals at different NaCl concentrations: non-halophilic (0.5% to 3% NaCl), halotolerant (0.5 to > 5% NaCl), and halophilic (5% to 25%). The classification range was adapted from Oren (2013) and Daoud and Ben Ali (2020).

## 2.4 Evaluation of phosphorus-solubilizing ability in solid medium

The bacterial isolates were inoculated into test tubes containing 5 mL of DYGS medium and incubated for 24 h at 150 rpm. Subsequently, the optical density (OD) of each culture was adjusted to 0.9–1.0 by spectrophotometry at 600 nm. An aliquot of 7 µL of the cultures was inoculated onto 6 mm paper discs arranged on modified National Botanical Research Institute's phosphate (NBRIP) medium (glucose 10 g.L<sup>-1</sup>; MgCl<sub>2</sub>.6H<sub>2</sub>O 5 g.L<sup>-1</sup>; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.25 g.L<sup>-1</sup>; KCl, 0.2 g.L<sup>-1</sup> and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 g.L<sup>-1</sup>; plus 3% NaCl and containing 5g.L<sup>-1</sup> of CaHPO<sub>4</sub> or AlPO<sub>4</sub> as a phosphate source. The bacteria were inoculated into the medium in triplicates. The solubilization halo formed around the colonies was determined on the seventeenth day after inoculation. The solubilization index (SI) was calculated using the following formula:

$$SI = \frac{\text{halo diameter (mm)}}{\text{colony diameter (mm)}}$$

The bacterial isolates were classified according to their solubilization capacity as low (SI<2), medium (2≤SI<4), and high (SI≥4) (Berraquero et al., 1976).

## 2.5 Evaluation of phosphorus-solubilizing ability in liquid medium

The bacterial isolates were cultured and their optical densities were adjusted as previously described. The NBRIP liquid culture

medium was supplemented with 3% NaCl containing CaHPO<sub>4</sub> or AlPO<sub>4</sub> and the pH was adjusted to 7.0. The isolates were inoculated into the medium in triplicates. Three hundred microliters of each culture was inoculated into 50 mL falcon tubes containing 35 mL of NBRIP medium and cultivated under agitation at 150 rpm for 14 days. The concentration of soluble phosphate was determined on the day of inoculation (day 0) and at the end of the incubation period (final 14 days). For this, a 10 mL aliquot of each sample was transferred to a 15 mL falcon tube and centrifuged at 6000 rpm for 10 min. The supernatant was filtered with the aid of a syringe filter with a 0.22-µm membrane. The filtrate was used to determine the soluble phosphate content with adapted methods described by Teixeira et al. (2017), and the final pH of the medium after cultivation. For quantification, the filtrates were diluted 1:150 (v/v) or 1:200 (v/v) for CaHPO<sub>4</sub> and 1:50 for AlPO<sub>4</sub> using deionized water. Quantification is based on the formation of a blue-colored molybdic phosphorus complex obtained after the reduction of molybdate with ascorbic acid and measured by spectrophotometry at 660 nm. A phosphate standard curve was constructed using anhydrous KH<sub>2</sub>PO<sub>4</sub>. Available P values were determined according to the equation:

$$P = \frac{L - b}{a} \times d$$

In which:

- P – concentration of available phosphorus, in mg kg<sup>-1</sup>.
- L – sample absorbance reading.
- a – angular coefficient of the standard curve (intercept).
- b – linear coefficient of the standard curve.
- d – dilution factor of the filtrate.

## 3 Results

### 3.1 Identification of bacteria and classification according to salinity

Among the 29 bacterial strains, 19 belonged to the phylum Proteobacteria and 10 belonged to the phylum Firmicutes. Bacteria in Proteobacteria belonged to the genera *Enterobacter*, *Halomonas*, *Kushneria*, *Pantoea*, and *Pseudomonas*. Isolate (SS145) grouped

TABLE 1 Identification of isolates, collection area, percentage of salt in the isolation medium, growth range, classification according to salinity, solubilization index (SI) in solid culture medium with CaHPO<sub>4</sub>, and quantification of solubilized P and final pH value in liquid culture medium containing CaHPO<sub>4</sub> and AlPO<sub>4</sub>.

Isolate ID	Genus	Collection area	%NaCl in the isolation medium	Growth interval	Classification according to salinity	SI	CaHPO <sub>4</sub>		AlPO <sub>4</sub>	
							P (mg kg <sup>-1</sup> )	pH	P (mg kg <sup>-1</sup> )	pH
SS85	<i>Bacillus</i> sp./CP049019.1	Deactivated salt mine	5%	0.5% - 10%	Halotolerant	Medium	474.798	4.2	2.50	2.8
SS89	<i>Bacillus</i> sp./CP049019.1	Deactivated salt mine	5%	0.5% - 10%	Halotolerant	Low	210.651	4.5	61.10	4.3

(Continued)

TABLE 1 Continued

Isolate ID	Genus	Collection area	%NaCl in the isolation medium	Growth interval	Classification according to salinity	SI	CaHPO <sub>4</sub>		AlPO <sub>4</sub>	
							P (mg kg <sup>-1</sup> )	pH	P (mg kg <sup>-1</sup> )	pH
SS231	<i>Bacillus</i> sp./CP115738.1	Hypersaline plains	5%	0,5% - 5%	Halotolerant	Medium	500.476	4.5	1.84	2.9
SS294	<i>Bacillus</i> sp./KX456341.1	Hypersaline plains	5%	0,5% - 10%	Halotolerant	Medium	351.641	4.7	8.02	3.8
SS186	<i>Enterobacter</i> sp./KR189294.1	Hypersaline plains	5%	0,5% - 15%	Halotolerant	Medium	956.372	3.9	12.36	4.7
SS97	<i>Enterobacter</i> sp./MK872311.1	Deactivated salt mine	15%	0,5% - 15%	Halotolerant	Medium	327.984	4.8	5.29	5.0
SS164	<i>Enterobacter</i> sp./MT613378.1	Hypersaline plains	5%	0,5% - 5%	Halotolerant	Medium	648.122	4.2	7.01	4.7
SS148	<i>Halomonas</i> sp./KP715923.1	Hypersaline plains	15%	0,5% - 15%	Halotolerant	Low	553.733	4.1	3.27	3.5
SS149	<i>Halomonas</i> sp./KY436502.1	Hypersaline plains	15%	0,5% - 15%	Halotolerant	Medium	213.861	4.7	7.91	3.2
SS157	<i>Halomonas</i> sp./MT760104.1	Hypersaline plains	5%	0,5% - 15%	Halotolerant	Low	401.450	4.5	9.75	3.6
SS151	<i>Kushneria</i> sp./AB970650.1	Hypersaline plains	15%	0,5% - 15%	Halotolerant	Low	266.286	4.4	9.75	3.8
SS162	<i>Kushneria</i> sp./KF560351.1	Hypersaline plains	5%	0,5% - 10%	Halotolerant	Medium	587.732	4.3	5.41	3.7
SS104	<i>Kushneria</i> sp./LR655847.1	Deactivated salt mine	20%	0,5% - 20%	Halotolerant	Low	345.340	4.3	2.50	3.8
SS99	<i>Kushneria</i> sp./NR_044001.1	Deactivated salt mine	15%	0,5% - 20%	Halotolerant	Medium	274.251	5.0	13.02	4.0
SS102	<i>Kushneria</i> sp./NR_044001.1	Deactivated salt mine	20%	0,5% - 20%	Halotolerant	Medium	989.539	4.1	10.88	3.0
SS88	<i>Oceanobacillus</i> sp./MH118526.1	Deactivated salt mine	5%	0,5% - 10%	Halotolerant	Medium	310.390	4.1	4.22	3.4
SS94	<i>Oceanobacillus</i> sp./MH118526.1	Deactivated salt mine	5%	0,5% - 20%	Halotolerant	Low	218.141	5.1	45.83	3.6
SS150	<i>Pantoea</i> sp./MH915636.1	Hypersaline plains	15%	0,5% - 20%	Halotolerant	Medium	436.044	4.6	23.89	3.5
SS141	<i>Pseudomonas</i> sp./KY072850.1	Deactivated salt mine	5%	0,5% - 10%	Halotolerant	Low	410.010	4.7	6.30	3.2
SS183	<i>Pseudomonas</i> sp./MN625859.1	Hypersaline plains	5%	0,5% - 15%	Halotolerant	Low	376.486	4.2	21.28	3.0
SS134	<i>Pseudomonas</i> sp./OP737584.1	Deactivated salt mine	15%	0,5% - 15%	Halotolerant	Medium	485.616	3.9	9.21	4.8
SS140	<i>Pseudomonas</i> sp./OP737584.1	Deactivated salt mine	5%	0,5% - 15%	Halotolerant	Low	365.430	4.4	8.98	4.6
SS161	<i>Pseudomonas</i> sp./OP737584.1	Hypersaline plains	5%	0,5% - 5%	Halotolerant	Medium	292.796	4.7	3.39	4.6
SS197	<i>Pseudomonas</i> sp./OP737601.1	Hypersaline plains	5%	0,5% - 5%	Halotolerant	Medium	687.589	5.0	4.22	3.4

(Continued)



TABLE 1 Continued

Isolate ID	Genus	Collection area	%NaCl in the isolation medium	Growth interval	Classification according to salinity	SI	CaHPO <sub>4</sub>		AlPO <sub>4</sub>	
							P (mg kg <sup>-1</sup> )	pH	P (mg kg <sup>-1</sup> )	pH
SS96	<i>Staphylococcus</i> sp./LC511705.1	Deactivated salt mine	15%	0,5% - 15%	Halotolerant	Medium	344.270	4.7	7.55	3.6
SS101	<i>Staphylococcus</i> sp./MT353655.1	Deactivated salt mine	15%	0,5% - 15%	Halotolerant	Low	466.358	4.8	11.12	4.8
SS100	<i>Staphylococcus</i> sp./MT550814.1	Deactivated salt mine	15%	0,5% - 15%	Halotolerant	Low	532.573	4.7	14.68	4.3
SS308	<i>Straphylococcus</i> sp./MT550814.1	Hypersaline plains	5%	0,5% - 15%	Halotolerant	Medium	240.490	4.6	12.60	3.7
SS145	Uncultured bacterium/HQ143327.1	Hypersaline plains	15%	0,5% - 10%	Halotolerant	Low	783.286	5.6	1.84	4.3

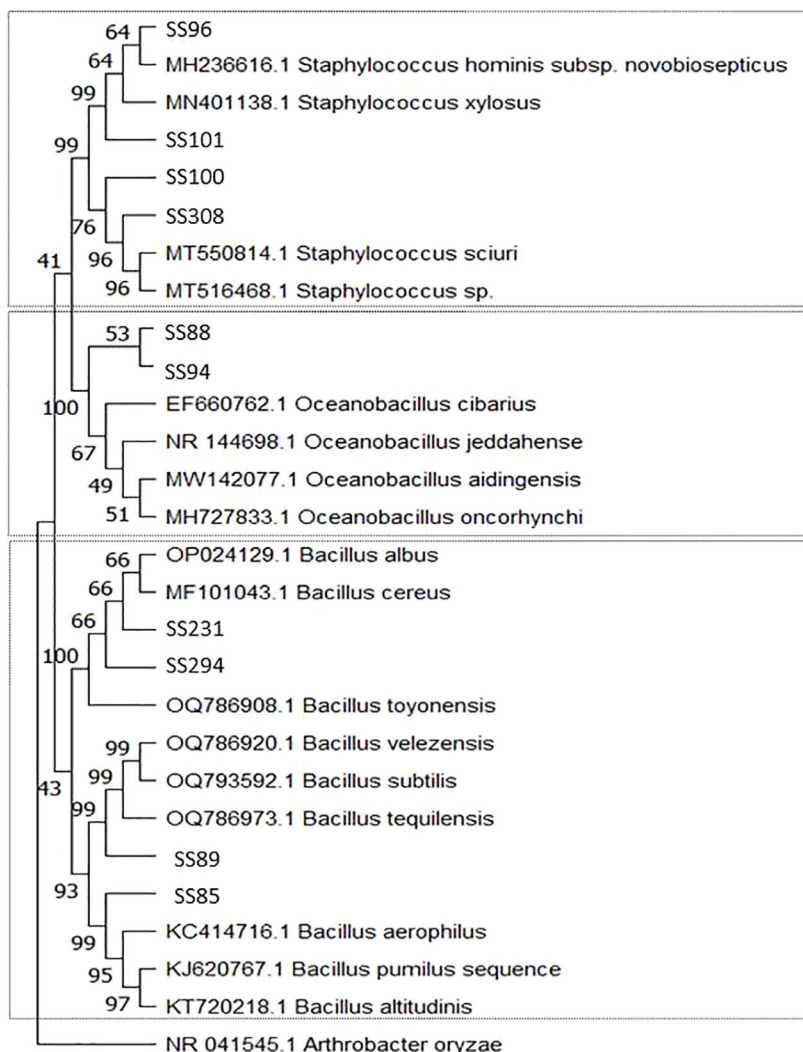


FIGURE 1

Phylogenetic tree constructed by the Neighbor-Joining method and Tajima-Nei model based on the 16S rDNA gene sequences of Proteobacteria phylum bacteria isolated from the rhizosphere of *Salicornia fruticosa*. The numbers at the nodes indicate the bootstrap values from 1,000 replicas.

with isolates from the phylum Proteobacteria but was not associated with any clade (Table 1, Figure 1). Isolates from the phylum Firmicutes belonged to the genera *Bacillus*, *Oceanobacillus*, and *Staphylococcus* (Table 1, Figure 2).

All isolates grown in both media containing 0.5% and above 5% NaCl, than were classified as halotolerant (Table 1) (Oren, 2008; Daoud and Ben Ali, 2020). Two isolates of *Pseudomonas* spp., one of *Enterobacter* sp., and one of *Bacillus* sp. developed at a maximum concentration of 5% NaCl. Three isolates of *Bacillus* spp., one of *Kushneria* sp., one of *Pseudomonas* sp., one of *Oceanobacillus* sp., and isolate (SS145) grew in 10% NaCl. Four isolates of *Staphylococcus* spp., three of *Halomonas* spp., three of *Pseudomonas* spp., two of *Enterobacter* spp., one of *Bacillus* sp.,

and one of *Kushneria* sp. accounted for growth up to 15% NaCl. Finally, three isolates of *Kushneria* spp., one of *Pantoea* sp., and one of *Oceanobacillus* sp. were grown in media containing up to 20% NaCl.

### 3.2 Phosphate solubilization in solid medium

All isolates grew in a solid culture medium containing dibasic calcium phosphate ( $\text{CaHPO}_4$ ) as a phosphate source (Figure 3). Ten isolates (31.0%) showed a low solubilization and 18 (58.6%) showed a medium SI (Table 1). Despite colony development, halo formation

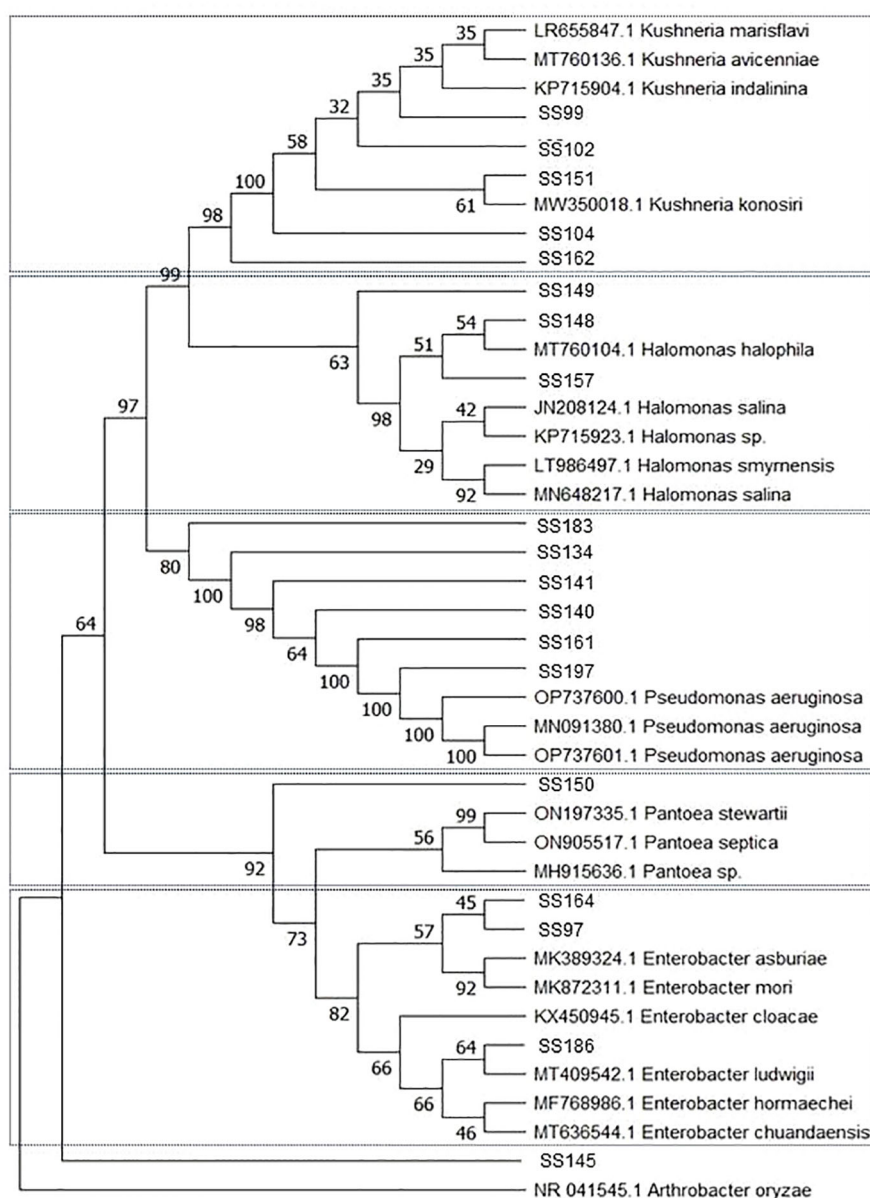


FIGURE 2

Phylogenetic tree constructed by the Neighbor-Joining method and Tajima-Nei model based on the 16S rDNA gene sequences of Firmicutes phylum bacteria isolated from the rhizosphere of *Salicornia fruticosa*. The numbers at the nodes indicate the bootstrap values from 1,000 replicas.



FIGURE 3  
NBRIP medium containing  $\text{CaHPO}_4$  inoculated with bacteria, displaying solubilization halos.

was not observed in the isolates of *Pseudomonas* sp. (SS140) and *Halomonas* sp. (SS157). The isolates also grew in a culture medium containing aluminum phosphate ( $\text{AlPO}_4$ ) as a P source, but no halo formation was observed.

### 3.3 Phosphate solubilization in liquid medium

The solubilization of the two sources of phosphorus,  $\text{CaHPO}_4$  and  $\text{AlPO}_4$ , by the bacterial isolates was accompanied by a reduction in pH. The content of P solubilized from  $\text{CaHPO}_4$  varied from 210.65 to 989.54  $\text{mg}\cdot\text{Kg}^{-1}$  and the pH of the culture media at the end of cultivation varied from 5.4 to 3.9 (Table 1, Figure 4). The *Kushneria* sp. (SS102) demonstrated the highest  $\text{CaHPO}_4$  solubilization, reaching 989.54  $\text{mg}\cdot\text{Kg}^{-1}$ , resulting in a final pH of 4.1. An increase in available P was also observed in media containing  $\text{AlPO}_4$ , varying from 1.84 to 61.10  $\text{mg}\cdot\text{Kg}^{-1}$ . The decrease in pH in media containing  $\text{AlPO}_4$  was higher than that in media containing  $\text{CaHPO}_4$ , varying from 5.0 to 2.8 (Table 1, Figure 4). The *Bacillus* sp. (SS89) and *Oceanobacillus* sp. (SS94) isolates showed the highest soluble P contents for  $\text{AlPO}_4$ , 61.10 and 45.83  $\text{mg}\cdot\text{Kg}^{-1}$ , with a final pH of 2.9 and 3.6.

## 4 Discussion

The inoculation of cultivated plants with PGPB is considered a promising practice for promoting the development of agriculture under adverse conditions, such as saline soils (Gamalero et al., 2020). The main objective of this study was to evaluate the efficiency of halotolerant bacterial isolates from the rhizosphere of the

halophyte *S. fruticosa* in the inorganic phosphates solubilization in solid and liquid culture media. The isolation and identification of bacteria associated with halophyte plants with growth-promoting attributes can contribute to the identification of strains with the potential for the development of bioinputs (Oliveira et al., 2009).

The isolates analyzed in this study came from media containing 5–20% NaCl and were all classified as halotolerant, exhibiting growth both at a concentration of 0.5% and above 5% NaCl (Oren, 2013; Daoud and Ben Ali, 2020). The salt content in saline soils is quite variable because of the influence of environmental factors, such as rain and tidal variations, which provide microhabitats with different salt concentrations (Quesada et al., 1982; Ventosa et al., 2008). Heterogeneity in the habitat can favor the selection of halotolerant microorganisms, as they are capable of adapting to fluctuating saline conditions in these environments, which gives them an advantage over specialized microorganisms, such as halophiles. The variation in salt content may explain why halotolerant bacteria were predominant among the evaluated bacteria. Halotolerant bacteria may offer increased benefits in agriculture owing to their superior adaptability to variations in salinity compared with halophilic bacteria.

The selection of phosphate-solubilizing bacteria in solid culture media has served as a universal indicator of phosphate solubilization for over half a century because it is a simple and inexpensive technique (Bashan et al., 2013). This method is based on the formation of a translucent halo around colonies. Halo formation occurs due to the dissolution of insoluble phosphate (Katznelson et al., 1962). The exudation of organic acids is one of the main mechanisms by which bacteria mobilize mineral phosphates (Alori et al., 2017). These acids can form metallic complexes or chelates with Ca, Al, and  $\text{Fe}^{+3}$  ions associated with phosphates without leading to the formation of translucent halos in the culture media (Merbach et al., 2009; Bashan et al., 2013). Therefore, plate tests must be complemented using quantitative tests in a liquid culture medium. Among the isolates studied, 27 showed solubilization activity of  $\text{CaHPO}_4$  from the formation of translucent halos (Table 1, Figure 3). No solubilization halos were observed in media containing  $\text{AlPO}_4$  despite colony development.

All isolates showed variations in the amount of available P and a reduction in pH in the liquid medium tests with  $\text{CaHPO}_4$  and  $\text{AlPO}_4$  (Table 1, Figure 3). Thus, solid culture media may be more suitable for the isolation of phosphate-solubilizing bacteria, as the ability to develop colonies in media containing insoluble P sources may indicate solubilization capacity even in the absence of halo formation. The levels of P mobilized from  $\text{CaHPO}_4$  were higher than those mobilized from  $\text{AlPO}_4$ . The solubility of P-Ca was strongly influenced by pH, increasing rapidly with a decrease in pH from 6.5. However, the solubility of P-Al increases because of acids at pH levels below 3, which is rarely observed in soils (Fankem et al., 2008). Wan et al. (2020) observed similar results in tests carried out with 18 phosphate-solubilizing bacteria isolated from soil at the Laiyang Experimental Station, Shandong, China, which were able to solubilize from 47.08 to 250.77  $\text{mg L}^{-1}$  of P from  $\text{Ca}_3(\text{PO}_4)_2$  and from 14.99 to 81.99  $\text{mg L}^{-1}$  from  $\text{AlPO}_4$ . Fankem et al. (2008) tested strains of *P. fluorescens* to solubilize different inorganic phosphate sources:  $\text{Ca}_3(\text{PO}_4)_2$ ,  $\text{AlPO}_4$ , and  $\text{FePO}_4$ . Solubilization of Ca-P was possible by simply acidifying the



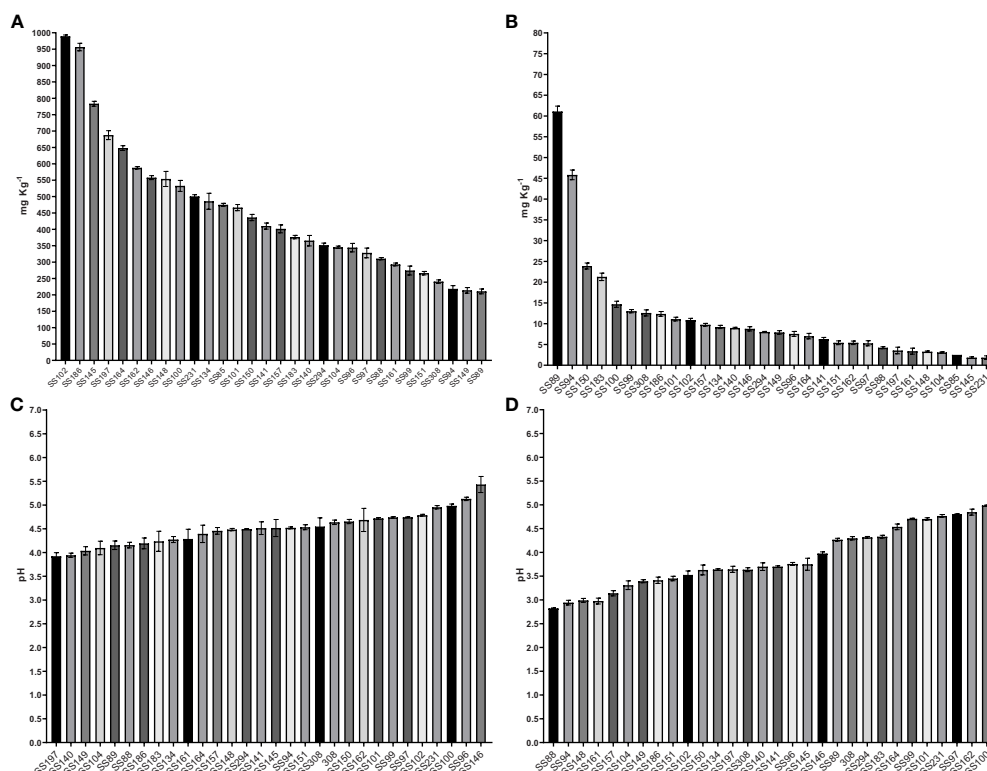


FIGURE 4

The P solubilizing levels, in  $\text{mg Kg}^{-1}$ , of bacteria in liquid NBRIP medium containing  $\text{CaHPO}_4$  (A),  $\text{AlPO}_4$  (B), and final pH on medium containing  $\text{CaHPO}_4$  (C) and  $\text{AlPO}_4$  (D). The results are the mean value of three replicates, error bars represent standard error.

medium. In addition, the authors analyzed the action of different organic acids produced by bacteria on  $\text{Ca}_3(\text{PO}_4)_2$  at pH 7 and 4 and found that acidity contributed to the solubilization of Ca-P by carboxylic acids. In contrast, the solubilization of  $\text{AlPO}_4$  and  $\text{FePO}_4$  seems to be related to the stability constants of the bond between the Fe or Al complex and different organic acids exuded by the bacteria (Fankem et al., 2008). Merbach et al. (2009) observed the production of various organic acids by bacteria capable of solubilizing different sources of mineral phosphate. These authors pointed out that bacterial strains that were more efficient in mobilizing P from calcium phosphate produced large amounts of citrate and tartarate, and to a lesser extent, malate. Furthermore, strains that solubilize iron and aluminum phosphates have tartarate and malate as their most important carboxylates, respectively (Merbach et al., 2009).

In our analyses, *Kushneria* sp. (SS102), *Enterobacter* sp. (SS186), and isolate (SS145) solubilized the highest amount of P from  $\text{CaHPO}_4$ , with 989.53, 956.37, and 783.82  $\text{mg Kg}^{-1}$ , respectively. The solubilization of phosphate from  $\text{AlPO}_4$  was more pronounced for the isolates *Bacillus* sp. (SS89) and *Oceanobacillus* sp. (SS94), which increased the soluble P to 61.10 and 45.82  $\text{mg Kg}^{-1}$ , respectively. These two isolates showed relatively low calcium phosphate solubilization capacity compared to the others, at 210.65 and 218.14  $\text{mg Kg}^{-1}$  of P. Similar results were observed by Wan et al. (2020) for a strain of *Bacillus* sp., which solubilized about 60  $\text{mg Kg}^{-1}$  of P from calcium phosphate but showed considerably high capacity for iron and aluminum phosphate, at 30  $\text{mg Kg}^{-1}$  and

50  $\text{mg Kg}^{-1}$ , respectively. The isolates *Pantoea* sp. (SS150) and *Pseudomonas* sp. (SS183) also showed superior aluminum phosphate solubilization activity to the others, raising the soluble P in the medium to 23.99 and 21.7  $\text{mg Kg}^{-1}$ , respectively. They showed intermediate values for calcium phosphate, at 436.04 and 376.49  $\text{mg Kg}^{-1}$  of soluble P. In acidic soils, there is an increase in the relative distributions of cations as  $\text{H}^+$  and  $\text{Al}^{3+}$ , which may lead to negative effects such as the depletion of nutrients and the high solubility of Al, Fe, and Mn, causing toxicity in plants (Tian and Niu, 2015). Furthermore, soil acidification reduces P availability for plant due to fixation with acidic cations such as Al and Fe (Gomes et al., 2010; Xiao et al., 2013; Qaswar et al., 2020). Therefore, the exploration of PSB capable of solubilizing aluminum phosphate can be particularly promising for Brazilian soils.

## 5 Conclusion

Bacteria isolated from the rhizosphere of *S. fruticosa* were classified as halotolerant. Some isolates demonstrated promising results in *in vitro* P solubilization and hold potential for the development of bioinputs or for the bioprocessing of rock phosphates. Analyses involving higher salt concentrations, different phosphate sources and the composition of produced organic acids will be conducted to contribute to a comprehensive understanding of their applications in sustainable agriculture.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

TE: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. XJ: Conceptualization, Data curation, Methodology, Writing – review & editing. AF: Investigation, Methodology, Writing – review & editing. GJ: Investigation, Methodology, Writing – review & editing. AR: Investigation, Methodology, Writing – review & editing. RL: Conceptualization, Project administration, Resources, Writing – review & editing. ZE: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. CI: Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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