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Restoration of *Triticum aestivum* Growth under Salt Stress by Phosphate-Solubilizing Bacterium Isolated from Southern Algeria

Nadia Belkebla [1], Sylia Ait Bessai [1], Juliana Melo [2], Maria Filomena Caeiro [3], Cristina Cruz [2] and El-hafid Nabti [1,*]

[1] Laboratoire de Maitrise des Energies Renouvelables, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Bejaia 06000, Algeria
[2] Centre for Ecology, Evolution and Environmental Changes (CE3C), Faculdade de Ciências da Universidade de Lisboa, Edifício C2, Piso 5, Sala 2.5.03 Campo Grande, 1749-016 Lisboa, Portugal
[3] Centro de Estudos do Ambiente e do Mar (CESAM), Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal
* Correspondence: nabtielhafid1977@yahoo.com or el-hafid.nabti@univ-bejaia.dz

Abstract: Salinity causes significant agricultural losses in many areas in the world. Plant growth promoting bacteria (PGPB) are a promising solution to enhance plant growth and productivity under such stress conditions by different mechanisms, mainly phosphorous solubilization. This study aims to improve wheat seedling growth under salt stress by a halotolerant phosphorous-solubilizing bacterial strain. Soil sample was collected in the south of Algeria (Ghardaia), and bacterial isolation was carried out on nutrient agar (NA) at different NaCl concentrations (300; 600 and 900 mM). The ability of the halotolerant isolates to solubilize inorganic phosphorous at 0; 300; 600 and 900 mM NaCl was determined. The isolate that showed the highest solubilization indexes was selected and identified as *Pseudomonas azotoformans*. Sterile wheat (*Triticum aestivum*) seeds were inoculated by this strain and then sown in soil at different NaCl concentrations (0; 100; 200; 300 mM). Different growth parameters were measured after 15 days. The strain showed its highest capacity for phosphorous solubilization (255.152 ± 0.01 µg/mL) at 300 mM NaCl, and for phytate mineralization (0.168 ± 0.002 U/mL) at 100 mM NaCl. The highest amount of soluble phosphorous in the soil was 49.42 ± 0.36 ppm obtained at 100 mM NaCl. Seed germination percentage, shoot and root length and fresh and dry weights were found to be higher in bacterial inoculated seedlings compared to uninoculated ones. According to this study, the use of plant growth promoting bacteria represents an important biotechnological approach to restore phosphorous levels in saline soils and to promote plant growth in salt-affected agricultural land.

Keywords: biofertilization; PGPB; phosphorous; salt stress; *Triticum aestivum*; Algeria

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1. Introduction

With the demand for food supply, especially wheat production in developing countries, an increase in cereal production is required to meet the nutritional needs of these countries [1]. Chemical fertilizers are used to increase crop growth and yield but the increased use of chemical pesticides, herbicides and excessive irrigation has led to accumulation of toxic substances in soil and salinization of agricultural land [1]. More than 6% of the land surface is affected by salinity, which corresponds to 20% of irrigated land located particularly in arid and semi-arid areas. In Algeria, about 10–15% of irrigated land is affected, occupying 3.2 million hectares of the total area, located in the north of the country [2].

Salinity highly limits plant growth and development. It affects hormone production, transpiration, photosynthesis, nutrient translocation and increases ethylene levels in roots

and other metabolic processes [3,4]. Several strategies have been developed to improve plant tolerance to this abiotic stress such as selection of varieties known for their high salt tolerance, production of genetically modified crops and the exogenous application of compatible solutes known for their osmo-protective properties, but these approaches are expensive and not sustainable [5]. Alleviation of salt stress devastating effects on plants using halotolerant plant growth promoting bacteria (PGPB) is an appropriate solution for sustainable, less costly and healthy agriculture [6].

PGPB provide beneficial effects on soil quality and plant growth through several mechanisms such as phytohormone production [7], pest control [8], degradation of organic pollutants [9], increasing plant nutrient availability through atmospheric nitrogen fixation [10], solubilizing and mineralizing phosphorous for plant growth [11] and improving tolerance to abiotic stresses (drought and salinity) [12]. The use of PGPB in agriculture promotes plant growth and protects plants from the adverse effects of salt stress [6]. Several studies have reported that inoculation of wheat with PGPB strains such as *Azotobacter* sp., *Azospirillum* sp. and *Pseudomonas* sp. were shown to increase plant tolerance to salt stress and promoted plant growth under these conditions [2,13,14].

Phosphorous (P) is an essential limiting macronutrient for plant growth and development [15]. This element is implicated in several physiological processes such as enzyme activation, protein synthesis, photosynthesis, disease development and insect resistance and osmoregulation [16]. Phosphorous is mainly immobilized in the soil by chemical precipitation where it becomes less soluble and therefore unavailable to plant roots [17]. Several studies have demonstrated the ability of PGPB to solubilize inorganic phosphorous and/or mineralize organic phosphorous into accessible forms to plants [18,19] through the secretion of low molecular weight organic acids, such as gluconic acid and citric acid [20]; these organic acids consequently lower the pH and chelate the cations attached to insoluble phosphorous and convert them into soluble forms ($H_2PO_4^-$) [21], and by the release of extracellular enzymes: phosphatase and phytases catalyzing the hydrolysis of the phosphoric esters [22].

Soluble salts reduce soil fertility and affect plant growth and development. In this context, our study aims to isolate and characterize a halotolerant bacterial strain capable of solubilizing insoluble forms of phosphorous at different NaCl concentrations and being useful for improving wheat seedling growth under saline conditions.

2. Material and Methods

2.1. Soil Sampling and Bacterial Isolation

A soil sample was collected from an agricultural field in southern Algeria (Ghardaia, 32°15′21.9″ N 3°38′01.6″ E) using a sterile metallic corer from 10 to 30 cm depth. Physico–chemical soil properties such as ph, electrical conductivity, gravimetric moisture content and total organic matter were measured. The soil sample was sterilely homogenized and 1 g of the soil was added to 9 mL of phosphate-buffered saline (PBS), containing per 1 L of distilled water: NaCl (8 g); KCl (0.2 g); KH_2PO_4 (0.24 g); Na_2HPO_4 (1.44 g), and mixed to form the first dilution 10^{-1}. Then, 1 mL of this solution was serially diluted from 10^{-1} to 10^{-7}. A total of 1 mL of each dilution was used to inoculate three Petri dishes containing nutrient agar medium supplemented with different concentrations of NaCl (300, 600 and 900 mM). The inoculation was carried out in triplicate. After incubation at 30 ± 2 °C for 48 h, 10 colonies of different appearance were selected from the 3 different NaCl-containing media and then purified on the appropriate culture medium for each isolate.

2.2. Halotolerance of the Selected Isolates

The halotolerance of selected bacterial isolates was determined using Glucose Minimal Medium containing (per liter): glucose (5 g); NH_4Cl (1 g); KH_2PO_4 (3 g); Na_2HPO_4 (2.4 g); NaCl (0.5 g) and $MgSO_4.7H_2O$ (0.2 g); pH = 7 ± 0.2. This medium was prepared at different NaCl concentrations (0; 100; 200; 300; 400; 500; 600; 700; 800; 900; 1000; 1100; 1200; 1300; 1400; 1500 and 1600 mM). 20 µL of each bacterial fresh culture (OD_{600} = 0.2) (SPECTRONIC

20 D ChemLab Images and instruction, Hanovern, New Hampshire, USA) were used to inoculate 2 mL of NaCl-containing GMM, and uninoculated medium was used as control. The procedure was performed in triplicate. After 48 h incubation at 28 °C under shaking (100 rpm/min), the bacterial growth was estimated by measuring the optical density with spectrophotometer at 600 nm [23].

2.3. Inorganic Phosphorous Solubilization

2.3.1. On Solid Medium

Selected isolates were tested for their ability to solubilize inorganic phosphorous [Ca_3 $(PO_4)_2$] on Pikovskaya's [24] medium containing, per liter of distilled water, in g/L: glucose (10 g); Ca_3 $(PO_4)_2$ (5 g); $(NH_4)_2SO_4$ (0.5 g); NaCl (0.2 g); $MgSO_4.7H_2O$ (0.1 g); KCl (0.2 g); yeast extract (0.5 g); $MnSO_4.H_2O$ (0.002 g); $FeSO_4.7$ H_2O (0.002 g); and agar (15 g). This medium was prepared at different NaCl concentrations (0, 300, 600 and 900 mM). Three spots of 5 µL of each bacterial suspension were placed in a Petri dish containing the PVK prepared medium, and three Petri dishes were used for each isolate. The ability of an isolate to solubilize inorganic phosphorous was indicated by the appearance of a clear halo around the colonies [25]. After incubation for 21 days at 28 °C, 3 isolates were able to solubilize inorganic phosphorous at 0, 300, 600 and 900 mM NaCl. The solubilization index (SI) was calculated for each isolate at the 2nd; 7th; 15th and 21st days of incubation, according to the formula of Edi-Premono (1996) [SI] = diameter of (colony + halo zone)/colony diameter] [26]. The isolate with the highest solubilization index was selected for further tests.

2.3.2. Quantification of Solubilized Phosphorous in Liquid Medium

Quantitative estimation of inorganic phosphorous solubilization efficiency was performed in Erlenmeyer flasks (250 mL) containing 50 mL of PVK broth prepared at different NaCl concentrations: 0, 300, 600 and 900 mM. This medium was inoculated with the selected isolate (200 µL of bacterial suspension with approximately 6×10^8 CFU mL^{-1}), then incubated at 28 °C for 2, 7, 15 and 21 days under shaking (180 rpm/min). The experiment was carried out in triplicate. Sterile uninoculated broth served as a control. After each incubation period, the cultures were harvested by centrifugation (eppendorf 58/OR centrifuge) at 10,000 rpm/min for 20 min. The pH of supernatants was determined. Then, the supernatants were used for soluble phosphorous estimation.

To measure the amount of released phosphorous in the culture medium, two solutions were prepared separately:

Solution (1): chloromolybdic acid: 1.5 g of ammonium molybdate dissolved in 40 mL H_2O (45 °C) were added to 34.2 mL HCl (12N), and then we added H_2O to this solution until a final volume of 100 mL.

Solution (2): chlorostannous acid: 2.5 g of $SnCl_2.H_2O$ were dissolved in 10 mL of HCl (12N) and then completing with H_2O to a final volume of 100 mL.

To estimate the concentration of soluble phosphorous in the medium, 1 mL of supernatant was mixed with 4.5 mL of solution (1) and 25 µL of solution (2). After incubation at room temperature (23 ± 2 °C) for 15 min, the OD was measured at 600 nm. The experiment was carried out in triplicate. The amount of soluble phosphorous was estimated according to a standard curve prepared using different concentrations of KH_2PO_4: 50, 100, 150, 200, 250 and 300 µg.mL^{-1} [27].

2.4. Determination of Phytase Production

2.4.1. On Solid Medium

The ability of our isolate to mineralize phytate through the production of phytase was tested at different NaCl concentrations. A total of 5 µL of the bacterial suspension was inoculated on the medium containing (per liter of distilled water): glucose (15 g); sodium phytate (1 g); NH_4NO_3 (2 g); KCl (0.5 g); $MgSO_4.7H_2O$ (0.5 g); $MnSO_4.H_2O$ (0.003 g); $FeSO_4.7H_2O$ (0.003 g) and agar (20 g). This medium was prepared at 0, 300,

600 and 900 mM NaCl, and the pH was adjusted to 7.5. Sodium phytate was used as a substrate for phytase to release phosphorous. After 48 h of incubation at 28 °C, clear halos were observed around the colonies of our isolate at the different NaCl-containing media, indicating therefore that the isolate is able to mineralize phytate by phytase production. The solubilization index (SI) was measured and calculated according to the formula of Edi-Premono (1996) [26] after 2, 5 and 10 days of incubation at 28 °C [27].

2.4.2. Quantitative Analysis of Phytase Production

Quantitative analysis of extracellular phytase production was performed by inoculating 200 µL (6×10^8 CFU mL^{-1}) of the selected isolate's culture into Erlenmeyer flasks (250 mL) containing 50 mL of a medium containing (per liter of distilled water): sodium phytate (1 g); peptone (10 g); $(NH_4)_2SO_4$ (2 g); KCl (0.5 g); $MgSO_4.7H_2O$ (0.5 g); $MnSO_4.H_2O$ (0.003 g); $FeSO_4.7\ H_2O$ (0.003 g). This medium was amended with different NaCl amounts to get final concentrations of 0; 300; 600 and 900 mM at pH = 7.5. After incubation at 28 °C for 2; 7 and 10 days under agitation (200 rpm/min), the cultures were centrifuged at 10,000× *g* for 10 min at 4 °C and the supernatant was used for extracellular phytase estimation [28].

The phytase activity determines the amount of inorganic phosphorous released in the medium, hence one unit [29]. To determine the rate of phosphorous released, two mixtures were prepared:

Mixture (1): 0.2 mL of the previously obtained supernatant were mixed with 0.5 g of sodium phytate dissolved in 100 mL sodium acetate buffer (0.2 M, pH = 5.5). After incubation at 28 °C for 30 min, the reaction was stopped by adding 102 mL of 15% trichloroacetic acid.

Mixture (2): 100 mL of sulphuric acid (1 M) were mixed with 100 mL of ascorbic acid (10%) and 4 mL of ammonium molybdate (3:1:0.1) at 2.5%.

For the phytase estimation, 100 µL of mixture (1) were mixed with 900 µL of mixture (2) and incubated at 28 °C for 20 min, and then the absorbance of the final mixture was measured at 700 nm [28].

2.5. Molecular Identification of the Selected Isolate

The selected strain was identified based on the 16S rDNA sequence. DNA extraction was followed by amplification of this gene using the forward primer (356F) 5′ACWCCTACGGGWGGCWGC and the reverse primer (1064R) 5′AYCTCACGRCACG AGCTGAC.

Polymerase chain reaction (PCR) reactions were carried out in a final volume of 20 µL. The reaction mixture consisted in 10 µL of MyTaq Red Mix 2x (Bioline, Paris, France), 1 µL of each primer F and R (at 10 µM/each), 1 µL of DNA sample, and 7 µL of ultrapure water. The mixture was placed in Tpersonal cycler (Whatman Biometra, Göttingen, Germany), programmed as follows: initial denaturation at 95 °C for 5 min, then 35 cycles each one consisting in three steps: denaturation at 95 °C for 10 s, annealing at 55 °C for 10 s, elongation at 72 °C for 60 s, and then a final elongation at 72 °C for 7 min.

Aliquots of the PCR reactions were resolved on 0.7% agarose gels stained with ethidium bromide. PCR products were purified by Zymoclean DNA kit (Zymo Research, Irvine, CA, USA), following the manufacturers' instructions. Purified PCR products were sequenced in both directions at StabVida (Caparica, Portugal) using the primers previously cited.

The obtained sequences were compared with the sequences available from the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov) accessed on 12 November 2021, using the BLAST algorithm in order to identify our isolate.

2.6. Wheat Germination and Growth Improvement by the Selected Isolate under Salt Stress

2.6.1. Germination Improvement

Wheat seeds (*Triticum aestivum*) were rigorously chosen according to their morphology and size, and then surface sterilized by soaking them in ethanol (70%) for 1 min and in

sodium hypochlorite solution (12%) for 3 min. the sterilization was followed by 6 times successive wash with sterile distilled water [30]. Sterile wheat seeds were soaked in washed bacterial suspension (OD = 0.8) for 1 h at room temperature. Control seeds were soaked in sterile distilled water [31].

Sterile filter paper discs with a diameter equal to that of the Petri dishes were placed in Petri dishes (1 filter paper/box). The filter paper was soaked (2 mL/dish) with sterile saline solutions (sterile distilled water + NaCl) at different NaCl concentrations (0, 50, 100, 150, 200, 250 and 300 mM). Afterwards, the bacterial inoculated seeds and the control seeds were placed in the boxes at a rate of 15 seeds/box/NaCl treatment (the experiment was performed in triplicate) which means 3 Petri dishes/NaCl treatment [32].

The experiment was carried out in the dark at an average temperature of 25 °C. The number of germinated seeds was counted every 2 days until the 14th day (no more germinating seeds). During this essay, there was no need to extra seed watering. At the end of the experiment, the final percentage of germination was calculated.

2.6.2. Growth Promotion of Wheat Seedlings

The soil used in the experiment is a soil with a low mineral content. It was provided by the Centre for Ecology, Evolution and Environmental Change (CE3C), Faculty of Science, University of Lisbon. It was mixed with vermiculite (50/50: *v*/*v*) and autoclaved.

The germinated seeds (bacterial inoculated seeds and the control seeds) were sowed in pots (7 cm × 12 cm) containing the soil-vermiculite mixture at a rate of 2 seeds/pot, at a depth of about 1 cm. The pots were watered with 20 mL/pot every 3 days with saline solutions (water with NaCl at concentration of 0, 100, 200 and 300 mM) according to the NaCl treatments in the germination phase. The experiment was carried out with 6 replicates (pots) per treatment. The pots were placed in a growth chamber with a light/dark photoperiod of 16/8 h, at temperature of 25/20 °C and a relative humidity (RH) of 70%/50%. After 15 days, the shoot and root length as well as the shoot and root fresh and dry weights of the samples in each treatment were determined. In addition to these plant growth parameters, the amount of soluble phosphorous in the soil where the wheat seedlings were growing was quantified according to [33]. This experiment was stopped at the 15th day because after this period the control seedlings were completely damaged.

The ability of the isolate to release phosphorous into the soil was tested by measuring the soluble phosphorous amounts in the soil before and after each treatment (bacterial and NaCl treatments). These amounts were determined according to a standard curve prepared using different concentrations of KH_2PO_4 (Sigma, Madison, Wisconsi, USA) [33].

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7. The wheat germination results and the wheat seedling growth parameters data were analyzed by analysis of variance, subject to a 95% confidence interval.

3. Results and Discussion

3.1. Selection of the Isolate N76

The performed physico–chemical analysis of the soil sample showed that it is characterized by the following parameters: pH = 8.25, electrical conductivity (EC) = 3.8 ds/m, gravimetric moisture content (θg) = 0.243 and total organic matter was = 1.2%

The soil sample was found to be alkaline (pH > 7), non-saline (EC < 4 ds/m) and rich in organic matters.

From this soil sample, a total of 10 bacterial isolates were obtained on NA medium at different NaCl concentrations (300, 600 and 900 mM). Among these isolates the isolate N76 was selected on the basis of its high salt tolerance and its ability to solubilize inorganic phosphorous at 0; 300; 600 and 900 mM NaCl, showing the highest solubilization index comparing to the other isolates.

3.2. Halotolerance of the Isolate N76

The halotolerance of N76 was studied on GMM medium prepared at (0, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500 and 1600 mM of NaCl). The isolate N76 was found able to tolerate up to 1.4 M NaCl, with an optimum growth at 300 mM NaCl. The impact of salinity on the growth of the isolate N76 is shown in Figure 1.

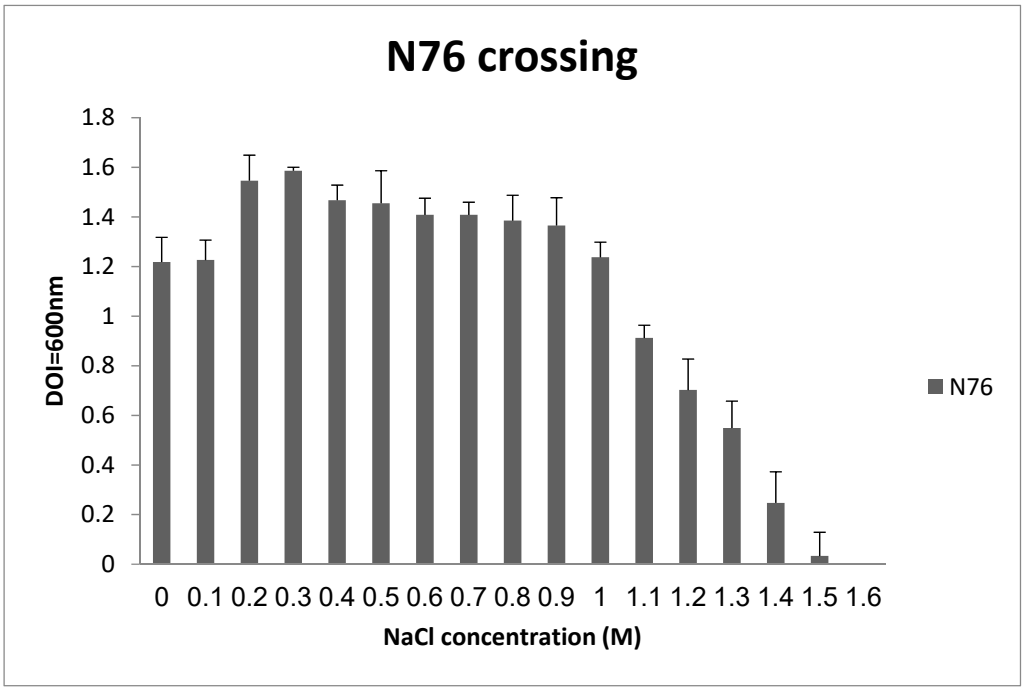

Figure 1. Impact of NaCl concentration (0–1.6 M) on the growth of isolate N76.

3.3. Molecular Identification of the Selected Isolate

Comparison of the partial 16S rDNA sequence of the strain N76 with those deposited in the GenBank database was performed by multiple alignments using the BLAST heuristic algorithms. The resulting sequence of the strain N76 (GenBank accession numberON810356) has shown 98% identity and 93% coverage with *Pseudomonas azotoformans* strain Seq323_Culture2_NC120620.

Pseudomonas sp. strains play an important role in maintaining soil fertility [34]. Their potential as plant growth promoting bacteria is well known and these microorganisms have been reported for their applications as biofertilizers [35]. Several studies have highlighted the ability of *Pseudomonas* strains to solubilize phosphorous such as *P. fluorescens* and *P. aeruginosa* [27], *P. palleroniana* and *P. jessenii* [36], and *P. azotoformans* and *P. proteolytica* [37].

3.4. Phosphorous Solubilization on Solid and Liquid Medium

Phosphorous is one of the most important mineral nutrients for plant growth and development. PGPB improve plant growth via enhancement of plant nutrient acquisition through transformation and solubilization of insoluble phosphorous in the soil [38]. The use of halotolerant phosphorous-solubilizing PGPB is a sustainable alternative strategy to the use of chemical fertilizers for salt-affected soil fertilization with phosphorous and agricultural production improvement [39].

In this study, our strain was able to solubilize tricalcium phosphorous on PVK medium supplemented with 0, 300, 600 and 900 mM NaCl (Table 1). The solubilization indices (SI) varied from 2.2 $\pm$ 0.08 to 4 $\pm$ 0.02, accordingly to the days of incubation. The highest SI (4 $\pm$ 0.02) was recorded in PVK medium at 300 mM NaCl after 15 days of incubation at 28 °C. This result may be explained by the fact that the optimum growth of *Pseudomonas azotoformans* under salinity conditions was at 300 mM NaCl (Table 1). Our results are

superior to those reported by [18]. The study carried out by [18] revealed the ability of a halophilic bacterial strain to solubilize phosphorous in saline medium (30 g/L); after 10 days of incubation, the phosphorous solubilization zone was about 2.5–3.0 cm.

Table 1. Inorganic phosphorous solubilization by *Pseudomonas azotoformans* N76 on both solid and liquid media.

Strain	NaCl (mM)	2 Days			7 Days		
		SI	**Medium′ pH**	**Soluble—P µg/mL**	**SI**	**Medium′ pH**	**Soluble—P µg/mL**
N76	0	2.87 ± 0.05	6.22 ± 0.02	136.625 ± 0.01	3.5 ± 0.05	6.05 ± 0.03	142.875 ± 0.23
	300	2.66 ± 0.03	6.66 ± 0.02	85.375 ± 0.03	3 ± 0.05	6 ± 0.01	141.625 ± 0.1 2
	600	2.41 ± 0.02	6.77 ± 0.04	59.225 ± 0.06	2.42 ± 0.05	6.11 ± 0.01	117.875 ± 0.19
	900	2.2 ± 0.08	6.79 ± 0.05	54.125 ± 0.02	2.33 ± 0.05	6.75 ± 0.02	59.425 ± 0.08
		15 Days			**21 Days**		
	NaCl (mM)	**SI**	**Medium′ pH**	**Soluble—P µg/mL**	**SI**	**Medium′ pH**	**Soluble—P µg/mL**
	0	3.87 ± 0.06	5.36 ± 0.02	242.125 ± 0.25	3.5 ± 0.01	5.22 ± 0.03	242.875 ± 0.34
	300	4 ± 0.02	5.24 ± 0.04	255.125 ± 0.13	3.5 ± 0.03	5.16 ± 0.02	255.152 ± 0.11
	600	3.1 ± 0.03	5.74 ± 0.05	216.875 ± 0.12	3.14 ± 0.02	5.58 ± 0.05	219.125 ± 0.31
	900	3.3 ±0.02	5.85 ± 0.02	201.625 ± 0.32	3 ± 0.01	5.61 ± 0.02	205.375 ± 0.25

Pseudomonas azotoformans was able to solubilize tricalcium phosphorous in PVK broth medium supplemented with 0, 300, 600 and 900 mM NaCl. The maximum phosphorous solubilization was recorded at 300 mM with 255.152 ± 0.01 µg/mL of solubilized phosphorous followed by 219.125 ± 0.03 µg/mL and 205.375 ± 0.05 µg/mL obtained at 600 and 900 mM NaCl respectively. Our isolate showed maximum phosphorous solubilization under all NaCl concentrations at 21 days. Our results about the maximum amount of soluble phosphorous (255.152 ± 0.01 µg/mL) are a bit lower than those reported by [18] which were 283.16 µg/mL, and higher to those obtained by [37] which were 109.47 µg/mL. Therefore, our strain may be considered having a good potential as phosphorous-solubilizing bacterium.

In this study, solubilization of inorganic phosphorous in liquid medium by *P. azotoformans* was accompanied by a pH decrease from an initial pH = 7.0 to pH = 5.16 ± 0.02 after 21 days incubation. A pH of 5.16 ± 0.02 with 255.15 ± 0.01 2 µg/mL of soluble phosphorous was recorded in the culture supernatant, while the pH of the control was constant (pH = 7). The significant decrease in pH (from 7 to 5.16 ± 0.02) in the culture supernatant observed during inorganic phosphorous solubilization indicates the production and release of acids. During phosphorous solubilization, the pH of the growth medium decreases due to the production of several low molecular weight organic acids which facilitate phosphorous solubilization through their hydroxyl and carboxyl groups by chelating the phosphorous-bound cation, thereby converting it to a soluble form [34,37,40]. According to [39], several organic acids are applied in phosphorous solubilization such as gluconic, citric, lactic, 2-ketogluconic, oxalic, tartaric, acetic, fumaric, malic, glyoxalic, isobutyric, isovaleric, itaconic, propionic, succinic, aspartic, maleic, glutamic, glycolic, maloni and α-ketobutyric ά acids. The release of oxalic and malic acid, lactic acid, citric acid and succinic acid into the growth media during phosphorous solubilization by *Pseudomonas azotoformans* has been reported by [37].

Several studies have reported the possible correlation between phosphorous solubilization rates and a decrease in the pH of growth medium [18,27,41]. However, Singh et al., 2014 [42] reported that there is no correlation between pH and the amount of solubi-

lized phosphorous, and reported also that phosphorous solubilization is not necessarily correlated with the acidity of medium.

3.5. Detection and Quantification of Phytase Activity

Phytate is the most abundant form of organic phosphorous (organic phosphorous represents 10 to 50% of total phosphorous) in the soil [39]. Phytase transforms phytate into soluble phosphorous (phosphorous esters) available to plants roots [39]. Our strain *Pseudomonas azotoformans* was able to mineralize phytate by the production of phytase in agar medium containing phytate as the only source of phosphorous. This agar medium was prepared at 0, 300, 600 and 900 mM NaCl. The incubation was performed at 28 °C during 2, 5 and 10 days. The solubilization indexes were determined. They were ranged from 3.25 ± 0.02 to 6.5 ± 0.03. The highest IS = 6.5 ± 0.03 was recorded at 300 mM NaCl and 10 days incubation (Table 2).

Table 2. Organic phosphorous mineralization by *Pseudomonas azotoformans* on solid and liquid media.

Strain	NaCl (mM)	2 Days		5 Days		10 Days	
		SI	Soluble—P U/mL	SI	Soluble—P U/mL	SI	Soluble—P U/mL
N76	0	3.62 ± 0.01	0	4.33 ± 0.02	0.027 ± 0.001	5.28 ± 0.04	0.168 ± 0.002
	300	3.28 ± 0.03	0	5.28 ± 0.02	0.014 ± 0.004	6.5 ± 0.03	0.097 ± 0.004
	600	3.57 ± 0.05	0	3.9 ± 0.03	0.002 ± 0.0001	5 ± 0.01	0.012 ± 0.001
	900	3.25 ± 0.02	0	4.25 ± 0.01	0	4.85 ± 0.02	0.004 ± 0.001

The quantification of the amount of produced phytase by our isolate in the liquid medium amended with the different NaCl concentrations showed that until 2 days incubation the amount of soluble phosphorous was 0 µg/mL, which means that our strain did not produce the phytase yet. The maximum amount of soluble phosphorous is 0.168 ± 0.002 U/mL (0.168 ± 0.002 µg/mL.min) at 0 mM/28 °C/10 days incubation (Table 2).

The phytase has been extracted and characterized from several *Pseudomonas* isolates, including *Pseudomonas simiae* and *Pseudomonas putida* [43], *Pseudomonas palleroniana*, and *Pseudomonas proteolytica* and *Pseudomonas azotoformans* [37]. Production of phytase by bacterial isolates could promote plant growth in phosphorous -deficient agricultural soils [29,44]. In our study, we focused on the solubilization of inorganic phosphorous (tricalcium phosphorous) more than on the mineralization of organic phosphorous (phytate) because the isolate N76 is more efficient in the solubilization of inorganic phosphorous than in the mineralization of organic phosphorous.

3.6. Effect of P. azotoformans on Wheat Seed Germination and Wheat Seedling Growth

Phosphorous is the second limiting nutrient for plant growth. This nutrient is involved in several processes such as cell division, new tissue growth, nucleic acid structure, protein synthesis, photosynthesis, glycolysis, respiration, membrane synthesis and stability, enzyme activation/inactivation, redox reactions, signaling, carbohydrate metabolism, nitrogen fixation, energy generation and transfer [45].

The availability of phosphorous in soils is about 0.05% (*w*/*w*), but only 0.1% of it is available for plant absorption. Plants need elevated available phosphorous concentrations to reach their maximum productivity [46]. Phosphorous deficiency leads to 5–15% reductions in crop yields [47]. Phosphorous availability in soil depends on several factors, including pH and ion concentrations in the soil [48]. Salinity significantly reduces phosphorous uptake by plants because phosphorous ions precipitate in such conditions and become unavailable to plant roots [49], so plants require additional phosphorous to maintain growth and productivity under such stress conditions [50].

3.6.1. Effect of *P. azotoformans* on Wheat Seed Germination

The impact of our strain in improvement of wheat seed germination was tested under different salinity conditions (0, 50, 100, 150, 200, 250 and 300 mM). The results showed that salt stress has negatively affected the germination rates of both inoculated and uninoculated seeds. Increasing NaCl concentrations decreased wheat seed germination. Inoculated seeds with the *P. azotoformans* strain showed a significant ($p < 0.005$) increase in germination rates compared to the control seeds at 100 mM. The final germination percentage has been improved from 78.33% to 96.11% at 0 mM NaCl due to seed inoculation with *P. azotoformans*. The germination rates increased from 62.66% to 81.66%; 45.33% to 68.88%; 43.88% to 55.55%; 37.66% to 44.66%; 24.88% to 34.66%; 10 to 29.33% under 50, 100, 150, 200, 250 and 300 mM NaCl, respectively (Figure 2).

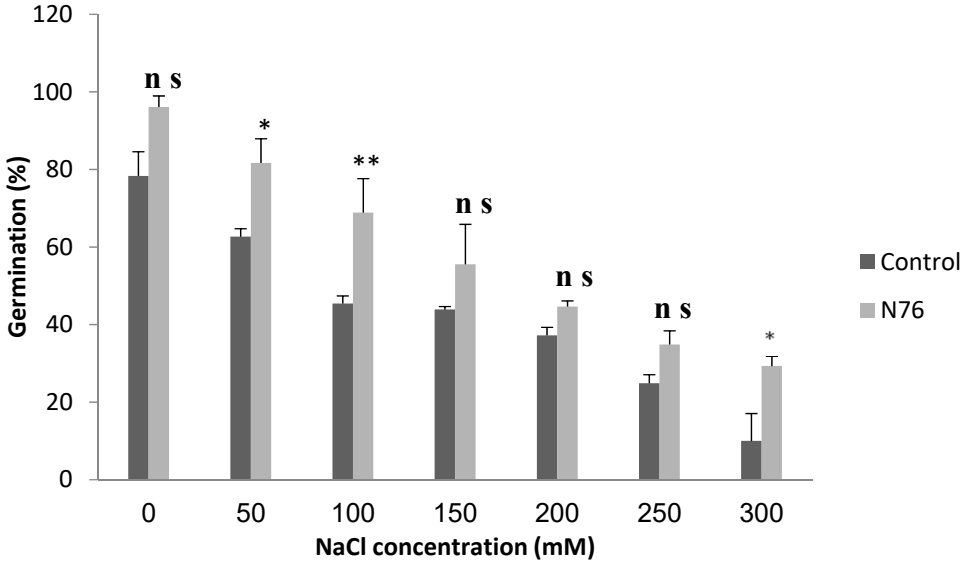

Figure 2. Effect of *P. azotoformans* on wheat seed germination under different NaCl concentrations. ns: no significant difference; *: significant difference ($p < 0.05$); **: significant difference ($p < 0.005$).

Salinity has a negative impact on seed germination and plant growth by affecting various metabolic processes from germination to maturity of the plant. Seed germination and early seedling growth are the stages that are the most susceptible to salinity and mitigating its effect at these early stages will increase the chance of establishing a successful crop under salt stress [51]. In our study, inoculation of wheat seeds with *P. azotoformans* strain significantly increased the germination rate compared to the control seeds; this improvement was observed under both stress and non-stress conditions. Since, PGPB application is beneficial in salt-affected soils, it is important to ensure that the used inoculum is able to resist and survive under these particular conditions. Several studies have reported that inoculation with PGPB results in increased seed germination rates, improves plant tolerance and promotes plant growth under salt stress [52,53].

3.6.2. Effect of *P. azotoformans* on Wheat Seedling Growth

The results of our study showed that wheat seedling growth was affected by NaCl at different concentrations (0, 100, 200 and 300), but seed priming with *P. azotoformans* N76 strain has mitigated the salt stress and promoted all the growth parameters of wheat seedlings. Fresh weights, dry weight, shoot and root lengths were improved in bacterial inoculated plants compared to the controls.

At 0 mM, fresh weight of the wheat seedlings has been increased from 99.68% due to bacterial inoculation. Under different concentrations of NaCl (100, 200 and 300 mM) the fresh weight of wheat seedlings has also increased from 92.95%, 96.30%, 70.10% respectively (Figure 3). Several studies have reported the positive effect of halotolerant PGPB is

decreasing the adverse effect of salt stress and enhancing wheat growth parameters (shoot length, fresh and dry weight of plants) under salt stress conditions [15,54]. Bhise et al., 2017 [55] reported that fresh weight of wheat plant inoculated with halotolerant PGPB was 2.92 ± 0.02 g after 15 days of growth at 100 mM NaCl, these results are higher than our results.

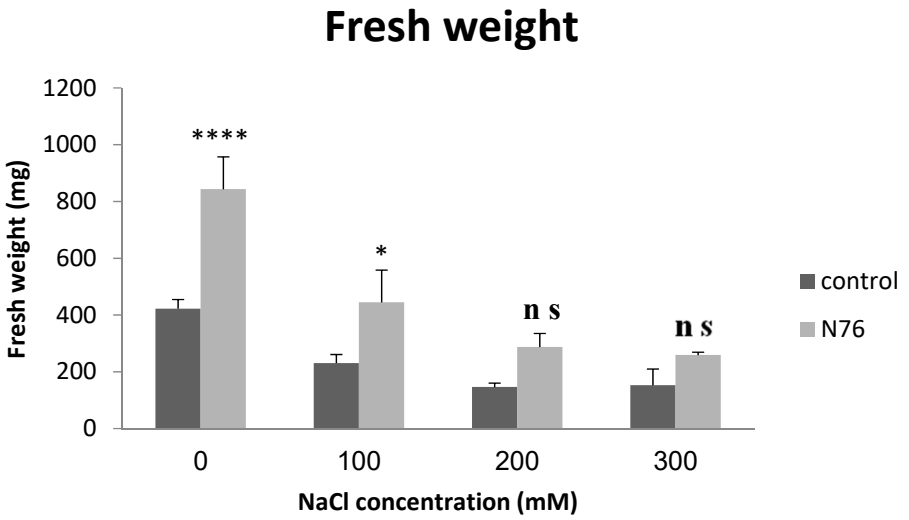

Figure 3. Effect of *P. azotoformans* on fresh weight of wheat seedlings under different NaCl concentrations. ns: no significant difference; *: significant difference ($p < 0.05$); ****: significant difference ($p < 0.0001$).

According to our results, our strain *P. azotoformans* N76 has increased the wheat seedlings' dry weight under 0, 100 and 200 mM NaCl, from 116.41%, 100.55% and 125.61% respectively. However, at 300 mM the strain did not bring any positive effect on the seedlings' dry weight (Figure 4). Our results are similar to those reported by [55]. Bharti et al., 2016 [56] showed a significant increase in wheat dry weight after inoculation with halotolerant PGPB strain compared to non-inoculated wheat grown in non-saline and saline soil. Emami et al., 2020 [40] reported improved growth parameters when wheat cultivars were inoculated with phosphorous-solubilizing bacteria compared to the non-inoculated control, the dry weight of wheat plants was 10 g after 120 days of planting.

Our results clearly showed a decrease in shoot length of the uninoculated seedlings in increased NaCl concentrations. The *P. azotoformans* strain was found to increase the shoot length of inoculated seedlings compared to uninoculated ones under both salt stress and non-stress conditions. The shoot length was found to be increased from 47.82%, 97.74%, 85.43% and 93.6% at 0; 100; 200; and 300 mM NaCl. The obtained results are shown in Figure 5. Our results are much higher to those obtained by Bharti et al., 2016 [56], who reported wheat shoot length of 11.46 ± 0.37 cm obtained after bacterial inoculation at 100 mM. Pande et al., 2017 [57] showed that maize seeds inoculated with phosphorous-solubilizing bacteria had the best growth compared to the non-inoculated treatment, which may be due to the production of organic acids (such as gluconic, formic and citric acids) by these strains. Iyer et al., 2017 [58] reported that shoot length and weight were improved when chickpea seeds were inoculated with PSB.

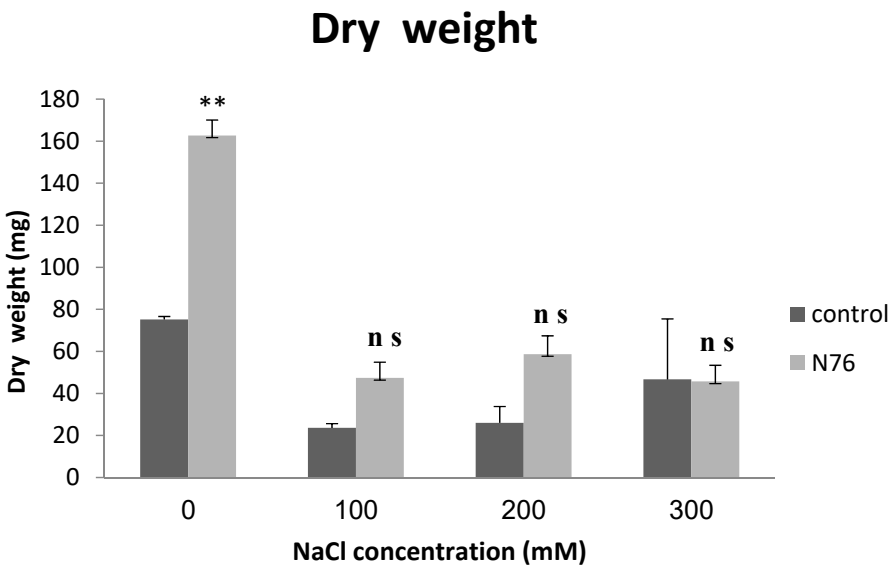

Figure 4. Effect of *P. azotoformans* on the dry weight of wheat seedlings under different NaCl concentrations. ns: no significant difference; **: significant difference ($p < 0.005$).

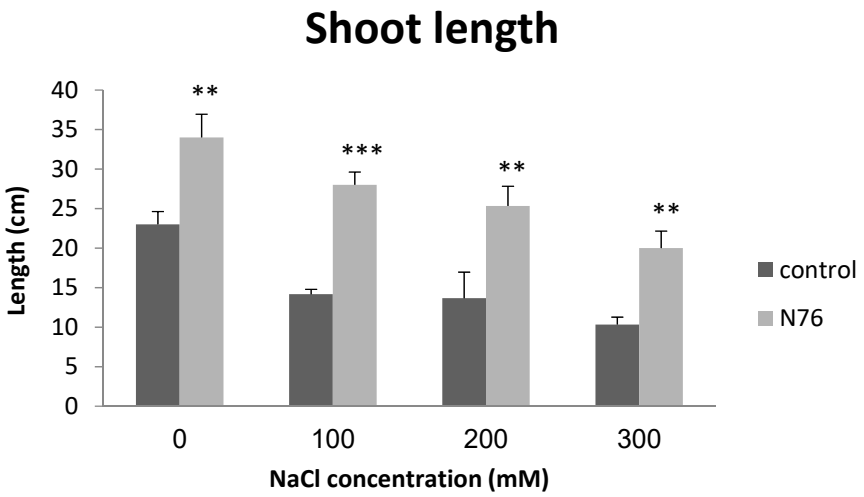

Figure 5. Effect of *P. azotoformans* on the shoot length of wheat seedlings under different NaCl concentrations. **: significant difference ($p < 0.005$); ***: significant difference ($p < 0.001$).

The results of our investigation showed that under different concentrations of NaCl the *P. azotoformans* strain has a positive effect on the root length of the wheat seedlings compared to the control. Thus, the root length was improved from 120.70%, 53.2%, 34.69% and 39.42% at 0, 100, 200 and 300 mM, respectively (Figure 6). According to these results, the increase in root growth may be due to increased P uptake in wheat seeds inoculated with the *P. azotoformans* strain. These results are similar to those obtained by [32] whereby wheat inoculation with halotolerant bacterial strains improved wheat growth under salt stress (80 mM, 160 mM and 320 mM) and root lengths increased by 90% compared to non-inoculated controls at 320 mM NaCl. Upadhyay and Singh (2015) [59] also showed that wheat inoculation with PGPB increased root length at 200 mM. Similarly, several studies have reported increased phosphorous uptake in inoculated plants as a result of phosphorous solubilization by PGPB [17,40,60]

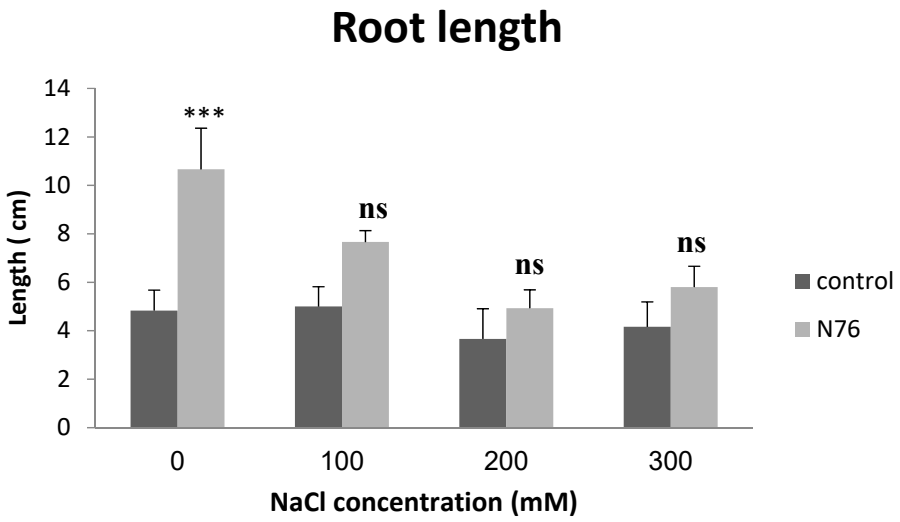

Figure 6. Effect of *P. azotoformans* on the root length of wheat seedlings under different NaCl concentrations. ns: no significant difference; ***: significant difference ($p < 0.001$).

3.7. Quantitative Estimation of Soluble Phosphorous in the Soil

Pseudomonas strains play an important role as plant growth promoters under salt stress via the expression of several PGP traits [53,61]. In our study, inoculation of wheat seeds with *P. azotoformans* improved all the seedlings' growth parameters; this improvement may be due to bio-solubilization of phosphorous in the soil.

The quantity of soluble phosphorous in the soil, where the wheat seedlings were growing, was measured to determine the effect of *P. azotoformans* strain on phosphorous solubilization in soils watered with saline solutions of different NaCl concentrations (0, 100, 200 and 300 mM).

Phosphorous content in the soil before sowing the wheat seed was 18.51 ± 0.25 ppm. After 15 days of uninoculated wheat seedlings' growth, the phosphorous concentrations were considerably decreased in the soil watered with saline solutions of 0 and 100 mM NaCl. This decrease may be explained by the fact that the plant has absorbed soluble phosphorous and used it in its metabolism during its growth. The amounts of phosphorous in soils watered with saline solutions of 200 and 300 mM NaCl has slightly decreased from 18.51 ± 0.28 to 17.82 ± 0.56 and 17.52 ± 0.62 ppm (Table 3). These results may show the negative effect of salt stress on phosphorous uptake by the wheat seedlings.

Table 3. The phosphorous availability (ppm) in control soil and soil containing inoculated seeds with *Pseudomonas azotoformans*.

	0 mM	**100 mM**	**200 mM**	**300 mM**
Control soil	10.56 ± 0.33	12.6 ± 0.28	17.82 ± 0.56	17.52 ± 0.62
Soil containing N76-inoculated-seeds	23.3 ± 0.74	49.42 ± 0.36	19.56 ± 0.45	19.5 ± 0.51

The soils where *P. azotoformans* inoculated seedlings were growing showed increased soluble phosphorous content. The highest amount of soluble phosphorous was 49.42 ± 0.36 ppm, recorded in soil irrigated with saline solution of 100 mM NaCl. In soils watered with saline solutions of 200 and 300 mM, the phosphorous contents were slightly increased (Table 3). Our results confirm what was reported by Srinivasan et al., 2012 [61] where the amount of phosphorous released by phosphorous-solubilizing fungi decreased with increasing NaCl concentration in the soil. Phosphorous availability is enhanced by microbial solubilization of insoluble phosphorous in the soil, which increases phosphorous availability to the roots. Thus, these phosphorous-solubilizing bacteria play a key role in restoration of soluble phosphorous

levels in the salt-affected and non-affected soils. The strain *Pseudomonas azotoformans* can also be used to produce smart fertilizers as described by Raimondi et al., 2021 [62].

4. Conclusions

Salt stress is a significant constraint for plant growth and productivity. The use of halotolerant-PGPB is a promising solution for salt stress alleviation and plant growth promotion under such conditions. Phosphorous-solubilizing and phosphorous-mineralizing halotolerant-PGPB can be used as important biofertilizers to increase the availability of soluble phosphorous for plant absorption in salinity-affected soils.

The results of our investigation highlighted the ability of the halotolerant bacterial isolate N76, identified as *P. azotoformans*, to solubilize inorganic phosphorous and to mineralize organic phosphorous at different NaCl concentrations. Inoculation of wheat seeds with this strain has resulted in increased germination rates and improved growth parameters of the wheat seedlings, both under salt stress and non-stress conditions. In addition, this strain was able to restore the amounts of soluble phosphorous in saline and non-saline soil. Therefore, our strain may be used as a biofertilizer for wheat growth improvement under salt stress.

According to the results of our study, the *P. azotoformans* strain N76 is highly recommended to be applied as a biofertilizer in salt-affected soil. This strategy can replace the massive use of chemical fertilizers in agricultural fields, thus promoting healthy and sustainable agriculture. Future study of the genetic mechanisms involved in inorganic phosphorous solubilization and organic phosphorous mineralization by this strain is required for a better understanding of how PGPB improve plant growth.

Author Contributions: The first author (N.B.) wrote the draft and realized the different experiments in the laboratory; the second author (S.A.B.) completed the draft until the final manuscript stage; the third author (J.M.) participated in the experimental design and several analyses; M.F.C. realized the molecular sequencing of the bacteria gene and the registration in the gene bank; C.C. revised checked the manuscript and corrected it; E.-h.N. made the conception and all the design of the paper, and after checking the final version, he added some necessary details. All authors have read and agreed to the published version of the manuscript.

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