Article

Plant Growth-Promoting Rhizobacteria Isolated from the Jujube (*Ziziphus lotus*) Plant Enhance Wheat Growth, Zn Uptake, and Heavy Metal Tolerance

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Abstract: In recent years, the low Zn content of wheat has become critical. Consequently, solutions that can improve the Zn nutrition of wheat are highly researched. In the present investigation, we aimed to evaluate the potential benefits of phosphate-solubilizing bacteria isolated from *Ziziphus lotus* on wheat seedling growth. Based on the phosphate-solubilizing criteria, four isolated strains, J16, J143, J146, and J158, were identified by 16SrRNA gene sequencing as *Pseudomonas moraviensis*, *Bacillus halotolerans*, *Enterobacter hormaechei*, and *Pseudomonas frederiksbergensis*, respectively. Studies of the conventional properties of plant growth-promoting rhizobacteria (PGPR) showed that *E. hormaechei* J146 produced up to 550 mg·L^{-1} of indole-3-acetic acid (IAA). Siderophores and ammonia were produced by all strains but cellulase was restricted to *B. halotolerans* J143, whereas proteases were missing in *E. hormaechei* J146 and *P. frederiksbergensis* J158. *E. hormaechei* J146 tolerate up to 1.5 mg·L^{-1} of copper and cadmium, while *B. halotolerans* J143 withstood 1.5 mg·L^{-1} of nickel. Strains *B. halotolerant* J143, *E. hormaechei* J146, and *P. frederiksbergensis* J158 remarkably improved wheat seed germination, plant growth, and Zn absorption. Lastly, nutrient measurement revealed that a wheat plant inoculated with *E. hormaechei* J146 and *P. frederiksbergensis* J158 increased its nitrogen and potassium uptake by up to 17%.

Keywords: plant growth-promoting rhizobacteria; jujube plant; plant–rhizobacteria interactions; phosphate solubilization; Zn solubilization; heavy metal tolerance; halotolerance; wheat seed germination; wheat Zn uptake

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), the world's population will reach around 9.8 billion by 2050 [1]. To ensure food security, the current agricultural production must be at least doubled [2,3]. To achieve this goal, it is necessary to have very fertile soils or to supplement nutrients in low fertility soils by applying a large amount of fertilizers [4]. So far, chemical fertilizers have helped feeding the world by providing three main nutrients to plants: nitrogen, phosphorus, and potassium (N, P, and K). However, the extensive use of N and P chemical fertilizers is responsible for micronutrient deficiency in crops, adverse effects in soil, and the poor quality of grains. In recent years, the nutritional quality of wheat and especially its low Zn content has become critical [5].

Zinc (Zn) is a micronutrient needed in small amounts for the proper growth and development of plants [6]. It is involved in the metabolism of auxin and carbohydrates, and acts as a very important antioxidant [7,8]. It is an essential element for crop production and to ensure the optimal size of fruits; it is also required in the carbonic enzyme activitythat is present in all photosynthetic tissues and for chlorophyll biosynthesis [9–12]. In general, Zn has its main roles in protein synthesis, enzyme activation, oxidation and revival reactions, and metabolism of carbohydrates. The Zn solubilization property is important in plant nutrient cycling. So, emerging solutions that can improve grains' Zn content are highly desired. Indeed, wheat fields are deficient in micronutrients, notably in Zn, and wheat grown under such deficient conditions produces seeds with a low Zn content. Zn deficiency affects both crops and humans [13]. Diet Zn deficiency concerns millions of people throughout the world due to limited access to Zn-rich foods (oysters, shellfish products, etc.) and probably the abundance of Zn inhibitors, such as the phytic acid, that bind Zn and inhibits its absorption [14]. Furthermore, Zn deficiency is responsible for human illness and disease development and is considered as a major risk factor, ranked 5th among the 10 most important risk factors in developing countries (WHO: World Health Organization, 2020). In wheat plant growth, mineral nutrient reserves in the seed must be adequate to sustain growth until the root system can take over the nutrient supply function. During the early establishment phase, supply of mineral nutrients will come partly from seed reserves and partly from soil. Therefore, large seed reserves of mineral nutrients are especially important for crops grown on soils that are deficient in one or more nutrients [5].

Plant growth-promoting rhizobacteria (PGPR) represent a wide variety of soil beneficial bacteria that colonize the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate plant growth by various direct (P solubilization, N fixation, phytohormones production, etc.), or by indirect mechanisms (reducing pathogenic infection and/or mitigating abiotic stresses) [15]. A number of PGPR was isolated from the rhizosphere of several plants belonging mainly to the genera of *Bacillus*, *Pseudomonas*, *Enterobacter*, *Actinobacter*, *Rhodococcus*, etc. [16–18]. These PGPR are the best-known beneficial microorganisms associated with plants and the best performing bio-inoculants as they have shown promising performance under controlled conditions, such as phosphate solubilization, production of phytohormones, siderophores, and nitrogen fixation [19]. PSB (phosphate-solubilizing bacteria) are part of the PGPR and can solubilize inorganic P from a variety of compounds, such as dicalcium phosphate, tricalcium phosphate, or rock phosphate [20]. They play also an essential role in the cycling of macro- and micronutrients, such as iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn), by modifying the root morphology and consequently increasing the exchange surface area and nutrient uptake from the soil [21]. In this context, the use of microbial biotechnology bio-inoculants is a promising strategy to improve the absorption of macro- and micronutrients by plants and consequently promote their growth. These practices are spreading very quickly through the identification of new bacterial strains effective in improving plant growth, such as *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Enterobacter* [22].

Antibiotic resistance is a major issue whose emergence and spreading rates are increasing. It is one of the major concerns in using bacterial-based biofertilizers [23]. The resistance of PGPR to antibiotics is a double-edged sword: On the one hand, resistant bacteria can serve either as markers to monitor bacterial survival in vitro or in vivo [24,25] or to help them compete in native and open microbial niches [26]. On the other hand, their application in soil as bio-inoculants may represent potential risks by transferring antibiotic resistance genes (ARGs) to other bacteria [27]. However, the potential source of ARGs carried by PGPR and other derived biocontrol agents and/or bio-fertilizers is widely forgotten and ignored [23].

The wild jujube, *Ziziphus lotus* (L.), is a deciduous shrub belonging to the Rhamnaceae family. It can survive in arid climates and tolerates biotic and abiotic stresses. This plant reaches 2–6 m, with tightly branched stems and small flowers and some of them produce fruits [28]. This shrub also behaves as a weed in many crops, including winter and spring

cereals, legumes, and orchards in some regions of Morocco, such as Chaouia, Haouz, Zear, Rhamna, and the Middle Atlas [29]. The characterization of PSB colonizing the rhizosphere of jujube plants and their effects on plant growth remains unclear and poorly explored. In the present study, we isolated four PSB strains from jujube (*Ziziphus lotus*) and evaluated their PGP properties, assessed their effect on wheat plant growth and absorption of macro- and micronutrient, such as N, P, K, and Zn, and investigated their ability to withstand heavy metals and salt stress as well as antibiotic resistance.

2. Materials and Methods

2.1. Sampling and Screening of Phosphate Solubilizing Rhizobacteria

Five samples of rhizospheric soil of jujube plants (*Ziziphus lotus*) were randomly collected at a depth of 5 to 25 cm from the experimental farm of Mohammed VI Polytechnic University, Benguerir, Morocco. Rhizosphere samples were immediately transported in sterile plastic bags to the laboratory and then were serially diluted by dissolving 1 g of each soil sample in 9 mL of sterile deionized water. A total of 100 µL of the stock solution was mixed with 900 µL of sterile distilled water to obtain a 10^{-1} dilution, and so on until reaching a 10^{-6} dilution. Next, 100 µL of each dilution was plated on NBRIP (National Botanical Research Institute's Phosphate) agar medium containing 5 g·L^{-1} tricalcium phosphate as the sole source of phosphate [30]. The initial media pH was adjusted to 7.00 before autoclaving. Seven days post incubation at 30 °C, the P solubilization on plates was checked for the formation of transparent halos around the colonies. Selected PSB isolates were purified, stored, and selected for further studies.

2.2. Quantitative Phosphate Solubilization Assay

Inorganic P-solubilizing activity was quantified using Erlenmeyer flasks (250 mL) containing 50 mL of modified NBRIP broth supplemented with 0.5 g·L^{-1} insoluble tricalcium phosphate (TCP) and inoculated by bacterial suspension (0.1 mL of OD$_{600nm}$ = 0.8). NBRIP medium (not inoculated) was used as a blank. Bacterial cultures were incubated on a rotary shaker at 28 ± 2 °C for five days under shaking conditions at 150 rpm. After the incubation period, the growth medium was harvested by centrifugation at 13,000 rpm for 10 min. The supernatant was filtered through a 0.22 µm sterile syringe filters to remove the insoluble materials [31] and then used as a matrix for the quantification of the soluble P released into the solution by colorimetric method using SKALAR [32]. The dissolved P concentration was determined by subtracting the P concentration of the blank from the final concentration of the soluble P in the inoculated broths. The final pH of the supernatant was also measured in each sample [33].

2.3. 16S rRNA Gene Sequencing and Phylogenetic Analysis

Four selected rhizospheric strains exhibiting the highest phosphate-solubilizing ability were characterized by 16S rDNA gene sequence analysis. The polymerase chain reaction (PCR) reactions were carried out directly on fresh bacterial suspension. The fragments of 16S rDNA were amplified using a pair of universal primers pA (5'-AGAGTTTGATCCTGG CTCAG-3') and 926R_Quince (degenerated one) (5'-CCG YCAATTYMTTTRAGTTT-3'), (Baker, G et al., 2003), and MyTaq Mix, 2X (ThermoFisher 10572014) containing (Taq DNA polymerase, dNTP, MgCl$_2$ and buffer). Amplification of 16S rDNA sequences was made in a 50 µL reaction mixture containing 25 µL of MyTaq mix, 1 µL of each primer (20 µM), 22 µL of DNase/RNase-free distilled water, and 1 µL of bacterial suspension as the DNA template. The reaction was performed in a VWR® thermal cycler using the following PCR optimized conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, elongation at 72 °C for 1 min, and final elongation at 72 °C during 10 min. The amplified 16S rDNA fragments (910-pb) were checked using gel electrophoresis. Next, the amplified products were sequenced by Genome Quebec. The resulting DNA sequences were aligned and compared with available standard sequences of bacterial lineage in the National Center for Biotechnology

Information GenBank (http://www.ncbi.nlm.nih.gov/, accessed on 9 August 2020) and the High-Quality Ribosomal RNA databases SILVA (https://www.arb-silva.de, accessed on 9 August 2020) using the BLAST algorithm to identify the isolates. The phylogenetic tree of the identified PSB was built using UGENE software [34]. Sequences were submitted to the NCBI GeneBank database and accession numbers were obtained.

2.4. Indole Acetic Acid Production Assay

Our four selected PSB were tested for the quantitative determination of indole acetic acid (IAA) production. In this context, 100 µL of each PSB isolate ($OD_{600nm} = 0.8$) was grown in 250 mL Erlenmeyer flasks containing 50 mL of Tryptic Soy Broth (TSB) supplemented with 0.1% L-tryptophan as IAA precursor at 28 ± 2 °C under shaking at 200 rpm for 7 days. After the incubation period, 2 mL of Salkowski reagent (0.5 M $FeCl_3$:70% perchloric acid/water (2:49:49 ratio)) [35] was added into test tubes containing 1 mL of culture supernatant filtrates and then the mixture was gently vortexed and left for 30 min in dark for the development of color at room temperature (26 ± 2 °C). The absorbance was spectrophotometrically determined at an OD_{535nm}. A standard curve was developed with known amounts of commercial IAA (Sigma Aldrich, Overijse, Belgium) using Salkowski reagent and sterile TSB broth. The concentration of IAA produced in each supernatant was estimated using the standard curve and expressed in $\mu g \cdot mL^{-1}$. Experiments were performed in triplicate.

2.5. Zn Solubilization Assay

The ability of bacteria to solubilize insoluble forms of Zn was evaluated in Tris-mineral agar medium amended with Zn oxide (ZnO) ($1.244 \text{ g} \cdot L^{-1} = 15.23$ mM), Zn phosphate ($Zn_3(PO_4)_2$) ($1.9882 \text{ g} \cdot L^{-1} = 5.0$ mM), and Zn carbonate ($ZnCO_3$) ($1.728 \text{ g} \cdot L^{-1} = 5.2$ mM) at a 0.1% Zn final concentration. The Tris-mineral agar medium contains D-glucose—$10.0 \text{ g} \cdot L^{-1}$; $(NH_4)_2SO_4$—$1.0 \text{ g} \cdot L^{-1}$; KCl—$0.2 \text{ g} \cdot L^{-1}$; K_2HPO_4—$0.1 \text{ g} \cdot L^{-1}$; and $MgSO_4$—$0.2 \text{ g} \cdot L^{-1}$. The pH was adjusted to 7.00 ± 0.25 before autoclaving [36]. Single colonies were spot inoculated on plates [16], and incubated 10 days at 30 °C. The appearance of the halo zone around the colonies is indicative of Zn solubilization.

2.6. Siderophores Production Assay

Production of siderophores by bacteria was qualitatively measured on a chrome-azurol S (CAS) medium, as previously reported [37]. Each strain was spot-inoculated separately on CAS agar plates and incubated for 3 days at 30 °C. Post the incubation period, the development of a yellow/orange halo around the colony indicated siderophores production.

2.7. Extracellular Enzyme Assays

Bacteria were qualitatively tested to produce two extracellular enzymes, namely, protease and cellulase, using the plate method. Protease activity (casein degradation) was checked according to the method of Kavitha et al. (2013) [38] by inoculating bacteria into nutrient agar medium containing casein $5 \text{ g} \cdot L^{-1}$, yeast extract $2.5 \text{ g} \cdot L^{-1}$, glucose $1 \text{ g} \cdot L^{-1}$, and agar $15 \text{ g} \cdot L^{-1}$, and adjusted to pH 7. Post cooling, the medium was amended with 10% of skim milk, seeded by each bacterial strain using the spot inoculation method. After 48 h of incubation at 30 °C, the proteolytic activity was indicated by a clear zone around the colonies. To monitor the cellulase activity, we used a mineral–salt agar medium containing 0.4% $(NH_4)_2SO_4$, 0.6% NaCl, 0.1% K_2HPO_4, 0.01% $MgSO_4$, 0.01% $CaCl_2$ with 0.5% carboxymethyl cellulose, and 2% agar. The plates were surface-inoculated with bacteria and then incubated for 48 h at 30 °C. After that, plates were stained during 15 min with 0.1% Congo Red and then destained for an additional 15 min using 1 M NaCl. The development of the halo zone around the colonies is indicative of CMC degradation, reflecting bacterial cellulase production [39].

2.8. Ammonia Production Assay

Bacteria were tested qualitatively and quantitatively for their ability to produce ammonia in peptone water according to Cappuccino et al. (1992) [40]. Freshly grown cultures (100 µL of OD_{600nm} = 0.8) were inoculated into 10 mL of peptone water in each tube and incubated for 48 h under shaking conditions (30 °C and 150 rpm). Uninoculated medium was used as a control. Next, 0.5 mL of Nessler's reagent was added to the supernatant of each bacterial culture. The development of brown to yellow colors correlated with ammonia production, whose quantification was calculated spectrophotometrically at OD_{450nm} based on a standard curve prepared using solutions of ammonium sulfate from 0 to 0.3 µmol·mL^{-1} [41].

2.9. Biofilm Formation Assay

A crystal violet (CV) assay was used to investigate the biofilm formation by studied bacteria [42]. Briefly, each inoculant was diluted to 1/100 with fresh TSB medium and 200 µL of bacterial suspension were inoculated in the wells of a 48-well microtiter microplate and incubated overnight at 30 °C under shaking conditions (180 rpm). The uninoculated medium was used as a negative control. Next, the culture suspensions were removed using a *VACUSIP* system. The microplate was then washed three times with phosphate buffer saline (PBS at pH 7.3) to remove planktonic bacteria. Afterward, the microplate was stained with 2% crystal violet for 15 min at room temperature and then washed with distilled water. The bacterial biofilm was solubilized using 200 µL of 95% ethanol and the OD_{600nm} was measured using the *VICTOR NivoTM multimode* plate reader. The OD values were taken as an index of bacteria that adhere to the surface and form biofilms.

2.10. Antibiotics Resistance and Heavy Metal Tolerance

The ability of the studied bacteria to resist to selected antibiotics was monitored on TSA medium [43]. Six antibiotics were tested; ampicillin (100 µg·mL^{-1}), streptomycin (100 µg·mL^{-1}), chloramphenicol (20 µg·mL^{-1}), kanamycin (50 µg·mL^{-1}), tetracycline (10 µg·mL^{-1}), and spectinomycin (60 µg·mL^{-1}). The antibiotics resistance profile was associated to overnight bacterial growth on plates at 30 °C. Parallelly, bacterial tolerance to heavy metals was assessed also using the agar plate method. Briefly, increasing concentrations, ranging from 0 to 1500 µg·mL^{-1}, of three trace elements (copper ($CuO_4S\cdot5H_2O$), cadmium ($CdSO_4$), and nickel (N_2NiO_8)) were added to the medium after cooling [44]. Plates were next streaked with bacteria and the results were determined after an overnight incubation at 30 °C. *Escherichia coli* DH5α was used as a control in both experiments.

2.11. NaCl Tolerance and Temperature Growth Monitoring

To evaluate the tolerance of our selected PSB to NaCl, each isolate was streaked on TSA plates supplemented with different concentrations of NaCl, ranging from 0 to 5%, and then incubated at 30 °C for 24 h [45]. We also examined their ability to tolerate heat stress. In brief, we streaked each strain on TSA medium and the plates were incubated overnight at different temperatures 30, 37, and 42 °C. Post 24 h incubation, the thermotolerance was recorded for each bacteria [46].

2.12. Seed Germination Assay

Seeds of durum wheat (variety *Vitron*) were surface sterilized with 2% sodium hypochlorite solution for 1 min, rinsed thoroughly with sterile distilled water, soaked in 70% ethanol for 1 min, and washed 5 times in single-distilled water followed by air-drying. Inoculum for each PSB isolate was prepared in TSB liquid medium at 30 °C for 24 h (OD_{600nm} = 0.8), centrifuged at 10,000 rpm for 5 min, and then the supernatant was discarded and the cell bacterial cell pellets were resuspended in 5 mL of sterile distilled water, vortexed for 20 s, and used for seed inoculation. This step was applied by soaking fifteen seeds in 5 mL of the bacterial suspension for 30 min under a gentle shaking, air

dried, and then placed on sterile Petri dishes containing 0.7% agar medium and incubated at 25 °C. Triplicates were maintained for each treatment, and uninoculated seeds (treated only with sterilized water) were used as a negative control. For germination, seeds were incubated in a dark space for 48 h, and further left at room temperature in a day/night cycle. The germination rate was recorded at both 24 h and 48 h and the root length, shoot length, fresh weight, and dry weight was measured at Day 7. The germination rate and vigor index were calculated according to the following equations [47]:

$$\text{Germination rate (\%)} = (\text{Number of seeds germinated}/\text{Total number of seeds}) \times 100$$

$$\text{Vigor index} = \% \text{ germination} \times \text{total plant length}$$

2.13. Inoculation of Wheat and Plant Growth Monitoring

Bacteria were used as bioinoculants for wheat growth. Durum wheat (variety *Vitron*) seeds were surface sterilized and coated as described above for seedlings germination assay. The experiment was conducted in plastic pots (height 18 cm, diameter 20 cm) that were previously sterilized (sodium hypochlorite 5%) and filled with 5 kg of sterilized substrate mixture containing sand and agricultural soil (3:1). Bacterial treatments and uncoated seeds (negative control) were tested in a complete randomized design using six replicates for each strain. Eight inoculated wheat seeds were sown in each pot. Seven days later, the number of seedlings were reduced to five per pot. Pots were irrigated with sterile distilled water once a day and kept in sunlight. Each two weeks, plants were treated with 20 mL of freshly prepared bacterial suspension ($DO_{600nm} = 0.8$). After 8 weeks, plants were harvested and separated into shoots and roots. Soil was rinsed off from the roots under tap water. Plants were then analyzed by measuring the length and fresh and dry weight of the roots and shoots. The dry weights of the samples were recorded post-oven-drying at 70 °C for 48 h.

Dried plants were independently ground and used as matrices for determination of the plant macro- and micronutrients N, P, K, and Zn. The concentration of each element was determined using Optical Emission Spectrometry combined with Inductively Coupled Plasma (Agilent 5110 ICP-OES). The outcomes are expressed as percentage of dry matter (% DM). Quadruplicate sets were performed for each treatment.

2.14. Statistical Analysis

Results presented here are the mean of three replicates $\pm$ standard deviation. Statistical analysis was performed using IBM SPSS statistics 20 for windows. The differences between treatments were statistically analyzed using analysis of variance (ANOVA) and subsequently by Tukey's multiple range test at $p < 0.05$.

3. Results

3.1. Screening of the Four P-Solubilizing, Jujube-Associated Rhizobacteria (J16, J143, J146, and J158)

Jujube plant (*Ziziphus lotus*) rhizospheric bacteria were plated on NBRIP agar medium containing tricalcium phosphate (TCP) as the sole source of phosphate. Out of 31 colonies, based on the appearance of a clear halo zone around the colonies, four isolates, named J16, J143, J146, and J158, were selected for further studies (Figure 1A). To quantify P solubilization, the isolates were grown in liquid NBRIP broth (See Material and Methods: M&M). The values of released P ranged between 36 and 278.5 mg·L^{-1}; the highest was recorded in J146, while the lowest in J143 (Figure 2). P solubilization was accompanied by an acidification of the milieu as the pH dropped from 7.0 to 4.0.
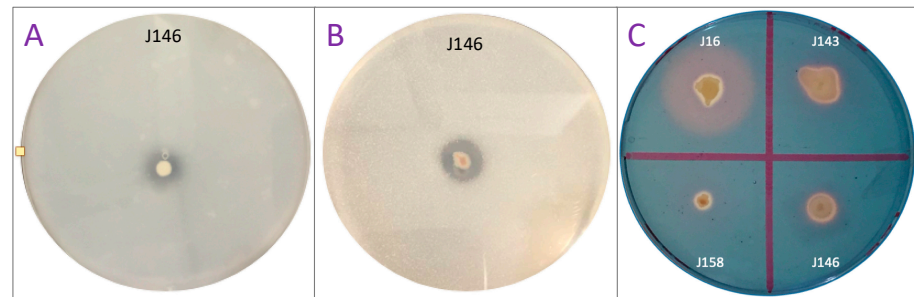
Figure 1. Illustration of some tests performed on plates for the evaluation of the plant growth-promoting (PGP) traits of the examined isolates: (**A**) phosphate solubilization; (**B**) Zn solubilization; (**C**) siderophores production.
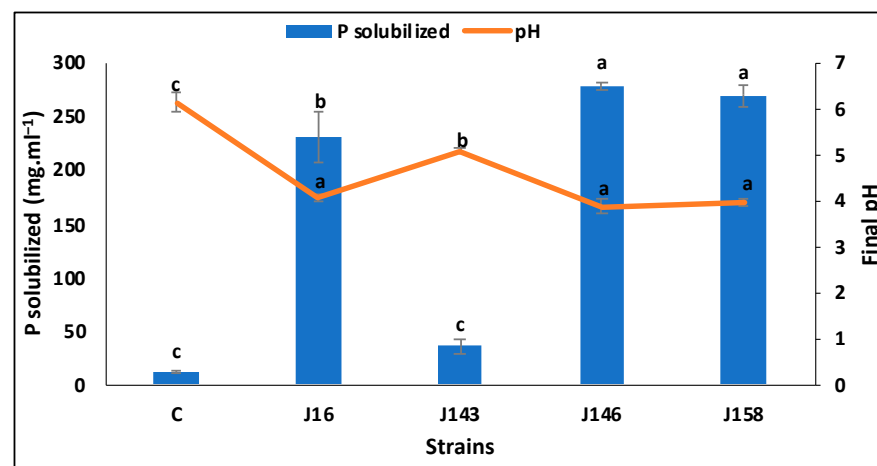

Figure 2. Quantification of released P by the phosphate-solubilizing bacteria (PSB) strains. The pH of the culture supernatants is indicated by the orange line. The bacterial isolates used are J16, J143, J146, and J158. C is the negative control: non-inoculated medium. The values represent the means of 3 replicates ($n = 3$) $\pm$ standard deviations. Letters a, b, and c highlight significant differences at $p < 0.05$.

3.2. The Isolates J16, J143, J146, and J158 Belong to the Pseudomonas, Enterobacter, and Bacillus genera

To identify the genotype of our four isolated PSB strains, DNA sequences corresponding to 16S rRNA genes were determined and aligned using GenBank and SILVA database. We found that J16 is related to *Pseudomonas moraviensis* (99.77% sequence identity), J143 corresponds to *Bacillus halotolerans* (99.65% sequence identity), J146 is 99.42% identical to *Enterobacter hormaechei*, and strain J158 shares 100% sequence identity with *Pseudomonas frederiksbergensis* (Figure 3 and Table 1).

Table 1. Molecular identification of the selected PSB using 16S rRNA gene sequencing.

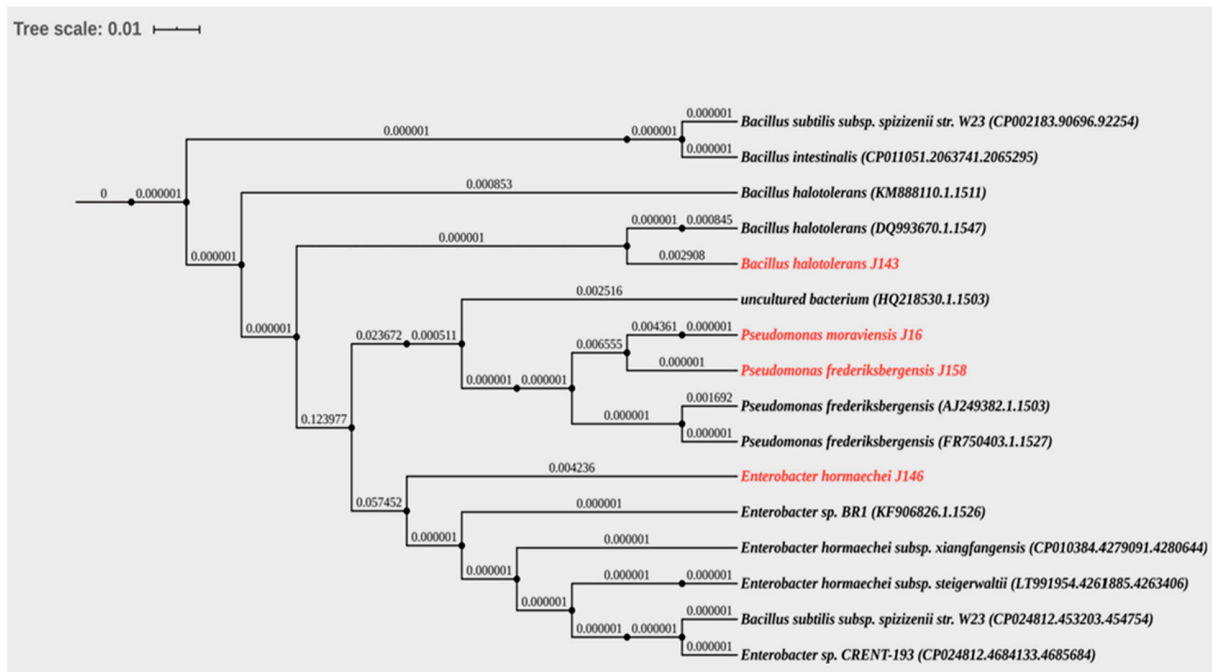| Isolates	Strains	Identity	Accession Number
J16	*Pseudomonas moraviensis*	99.77%	MW420981
J143	*Bacillus halotolerans*	99.65%	MW420982
J146	*Enterobacter hormaechei*	99.42%	MW420983
J158	*Pseudomonas frederiksbergensis*	100%	MW420984

Figure 3. Neighbor-joining phylogenetic tree showing the relationship between the four studied strains indicated in red and their representative species from the NCBI database, built using UGENE software.

3.3. E. hormaechei J146 Overproduced Indole-3-Acetic Acid (IAA)

Production of IAA by bacteria is one of the remarkable PGPR features. We tested the ability of the four strains to produce IAA by monitoring the appearance of a pink color after the addition of Salkowski reagent to the culture (see Materials and Methods). The quantity of IAA was determined based on a standard curve. All strains produced various amounts of IAA, ranging from 99.48 to 550.44 $\mu g \cdot mL^{-1}$ (Figure 4). The highest value was detected in *E. hormaechei* J146, while the lowest one was recorded in *B. halotolerans* J143 (Figure 4).
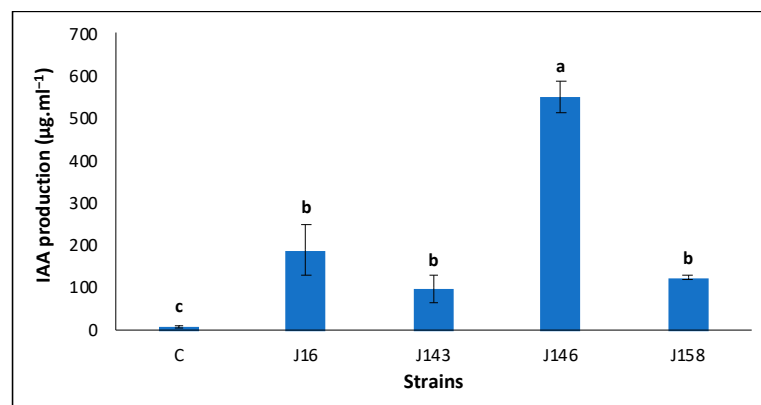
Figure 4. Indole acetic acid (IAA) production by PSB strains grown on media amended with 0.1% of L-tryptophan. Strains used are *P. moraviensis* J16, *E. hormaechei* J146, *P. frederiksbergensis* J158, and *B. halotolerans* J143. C is the negative control: non-inoculated medium. Values represent the means of 3 replicates ($n = 3$) $\pm$ standard deviations. Letters a, b, and c point the significant differences at $p < 0.05$.

3.4. Zn Solubilization from Zinc-Oxide by P. moraviensis J16, E. hormaechei J146, and P. frederiksbergensis J158

Zinc solubilization on plates showed that, except for strain *B. halotolerans* J143, the three remaining strains induced the formation of clear halo zones around colonies using

ZnO as a substrate. The maximal size of halo was seen in *E. hormaechei* J146 (Figure 1B). However, none of the tested strains was able to solubilize $Zn_3(PO_4)_2$ or $ZnCO_3$.

3.5. Strain P. moraviensis J16 Is the Best Siderophores Producer

Here, we tested the ability of the studied strains to produce siderophores in vitro using the CAS-agar plate assay. Although at various extends, all strains produced siderophores as judged by the size of the halo zone and the intensity of the color change of the Cas-agar plates (Figure 1C). *P. moraviensis* J16 was found to be the efficient producer of siderophores, *B. halotolerans* J143 and *E. hormaechei* J146 produced intermediate levels, whereas *P. frederiks-bergensis* J158 was the lowest producer one (Figure 1C).

3.6. Lack of Proteases and Cellulase in E. hormaechei J146 and P. frederiksbergensis J158

Selected strains were checked for their capacities to produce cellulase and proteases. Both *P. moraviensis* J16 and *B. halotolerans* J143 developed clear halo zones around their colonies, while *E. hormaechei* J146 and *P. frederiksbergensis* J158 strains did not (Table 2). Besides, the cellulase activity, revealed by the yellow/whitish zones around the colonies, were only seen in *B. halotolerans* J143 (Table 2).

Table 2. Relevant phenotypic traits observed for the PSB strains on the plate assays.

Strains	Antibiotic Resistance	NaCl Tolerance	Temperature Tolerance	Siderophores Production	Extracellular Enzymes		Zn Solubilization		
					Proteases	Cellulases	ZnO	$Zn_3(PO_4)_2$	$ZnCO_3$
E. coli DH5α	−	3%	37 °C	−	−	−	−	−	−
P. moraviensis J16	Amp R, Cm R, Spect R	<5%	42 °C	+++	++	−	+	−	−
B. halotolerans J143	−	5%	42 °C	++	++	+	−	−	−
E. hormaechei J146	Tet R, Amp R, Cm R	5%	42 °C	++	−	−	+	−	−
P. frederiksbergensis J158	Amp R, Cm R	<5%	42 °C	+	−	−	+	−	−

The '+' and '−' signs indicate efficiencies as follows: −, negative result; +, weakly positive; ++, moderately positive; +++, highly positive. The 'R' means resistance to antibiotics; *E. coli* DH5α was used as the negative control.

3.7. Strain E. hormaechei J146 Overproduced Ammonia

Regarding ammonia production, all tested strains produced it in various concentrations, ranging from 0.23 to 0.33 $\mu mol \cdot mL^{-1}$. The highest value was measured *in E. hormaechei* J146, whereas the lowest was seen in *B. halotolerans* J143 (Figure 5A).
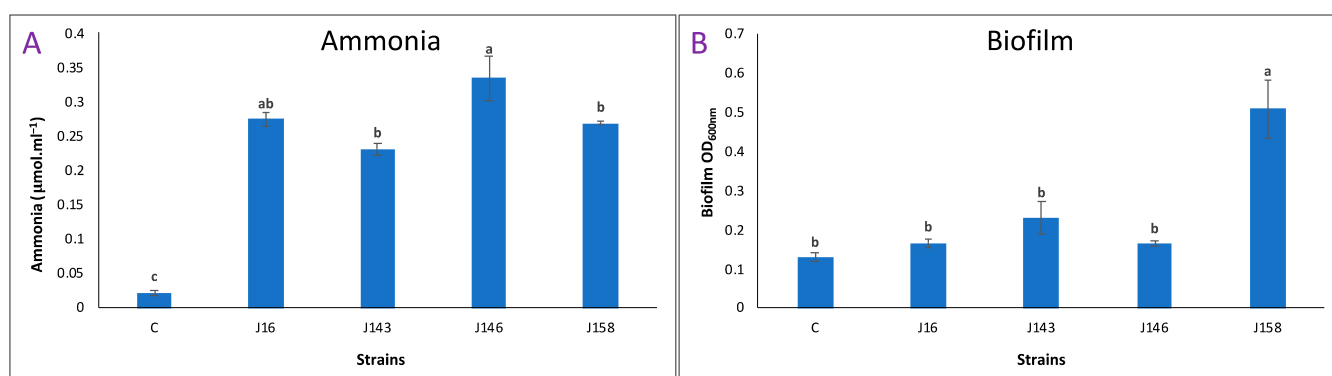

Figure 5. Ammonia and biofilm production by the selected PSB strains: (**A**) ammonia production; (**B**) biofilm formation. C is the negative control: non-inoculated medium. The values represent means of 3 replicates (*n* = 3) ± standard deviations. Letters a, b, and c highlight significant differences at *p* < 0.05.

3.8. Strain P. frederiksbergensis J158 Induced a Strong Biofilm

Here, we assessed the biofilm formation by the studied strains using the crystal violet binding assay (see Materials and Methods). When compared to the control, a strong biofilm formation was seen in *P. frederiksbergensis* J158 but not by the other strains (Figure 5B).

3.9. PSB Strains Displayed Partial Antibiotics Resistance

Soil is considered as a reservoir of antibiotic-resistant bacteria (ARB). We tested for the ability of the four strains to grow on plates supplemented with six selected antibiotics frequently used for medical uses (see Materials and Methods). As summarized in Table 2, strains *P. moraviensis* J16, *E. hormaechei* J146, and *P. frederiksbergensis* J158 resisted both ampicillin and chloramphenicol, strains *P. moraviensis* J16, *E. hormaechei* J146 resisted spectinomycin and tetracycline, while all the tested strains were sensitive to kanamycin and streptomycin. Lastly, strain *B. halotolerans* J143 was sensitive to all the tested antibiotics.

3.10. E. hormaechei J146 and B. halotolerans J143 Withstood Copper/Cadmium and Nickel, Respectively

Heavy metals, such as cadmium, copper (Cu), and nickel (Ni), are exceptionally toxic and dangerous environmental pollutants. In this part, we investigated the ability of the four strains to grow on TSA plates supplemented with increasing concentrations of these trace elements. We found that *E. hormaechei* J146 supported up to 1.5 mg·L^{-1} of either copper or cadmium, and *B. halotolerans* J143 tolerated up to 1.5 mg·L^{-1} of nickel. In contrast, *P. frederiksbergensis* J158 was the least tolerant—0.3 mg·L^{-1} of cadmium or copper and 0.5 mg·L^{-1} of nickel (Table 3). Besides, *E. coli* DH5α was found to tolerate 0.3, 0.5, and 1 mg·L^{-1} of cadmium, nickel, and copper, respectively.

Table 3. Heavy metal tolerance by the four studied strains.

Strain	CuO$_4$S·5H$_2$O (µg·mL^{-1})				CdSO$_4$ (µg·mL^{-1})				N$_2$NiO$_8$ (µg·mL^{-1})			
	300	500	1000	1500	300	500	1000	1500	300	500	1000	1500
Pseudomonas moraviensis J16	+	+	+	−	+	−	−	−	+	+	+	−
Bacillus halotolerans J143	+	+	+	−	−	−	−	-	+	+	+	+
Enterobacter hormaechei J146	+	+	+	+	+	+	+	+	+	+	−	−
Pseudomonas frederiksbergensis J158	+	−	−	−	+	−	−	−	+	+	−	−
E. coli DH5α	+	+	+	−	+	−	−	−	+	+	−	−

+ for tolerance, − for sensitivity.

3.11. E. hormaechei J146 and B. halotolerans J143 Tolerated Salt Stress

To test for bacterial optimal growth temperature, the four studied strains were grown on TSA plates and incubated at 30, 37, and 42 °C. All of them grew up to 42 °C (Table 2). We checked the ability of our strains to grow on TSA plates containing increasing concentrations of NaCl, ranging from 0 to 5%. We found that the growth of both *P. moraviensis* J16 and *P. frederiksbergensis* J158 halted at 5% NaCl, whereas, under the same concentration, the growth of either *B. halotolerans* J143 or *E. hormaechei* J146 was not affected (Table 2).

3.12. E. hormaechei J146 Strain Is the Best Inducer of Seeds Germination

In this part, we investigated the effect of the four strains on wheat seed germination. Compared to non-inoculated seeds, bacterization of the wheat seeds by the four strains significantly enhanced the germination rate (Figure 6A & E). The highest result was seen in seeds coated with *E. hormaechei* J146, while the lowest was recorded for *P. moraviensis* J16 (Figure 6A). The calculated germination vigor index values varied between 968, for the negative control, and 1992, for *P. frederiksbergensis* J158 (Figure 6E). At Day 7, a significant increase in shoot length ($p < 0.05$) was recorded using the four strains. In addition, except for strain *E. hormaechei* J146, all the others significantly increased the root length (Figure 6B).

Remarkably, although at various levels, all strains significantly improved the weight of either the fresh shoots or roots (Figure 6C). Meanwhile, *E. hormaechei* J146 was the best weight performer in dry shoots (Figure 6D).
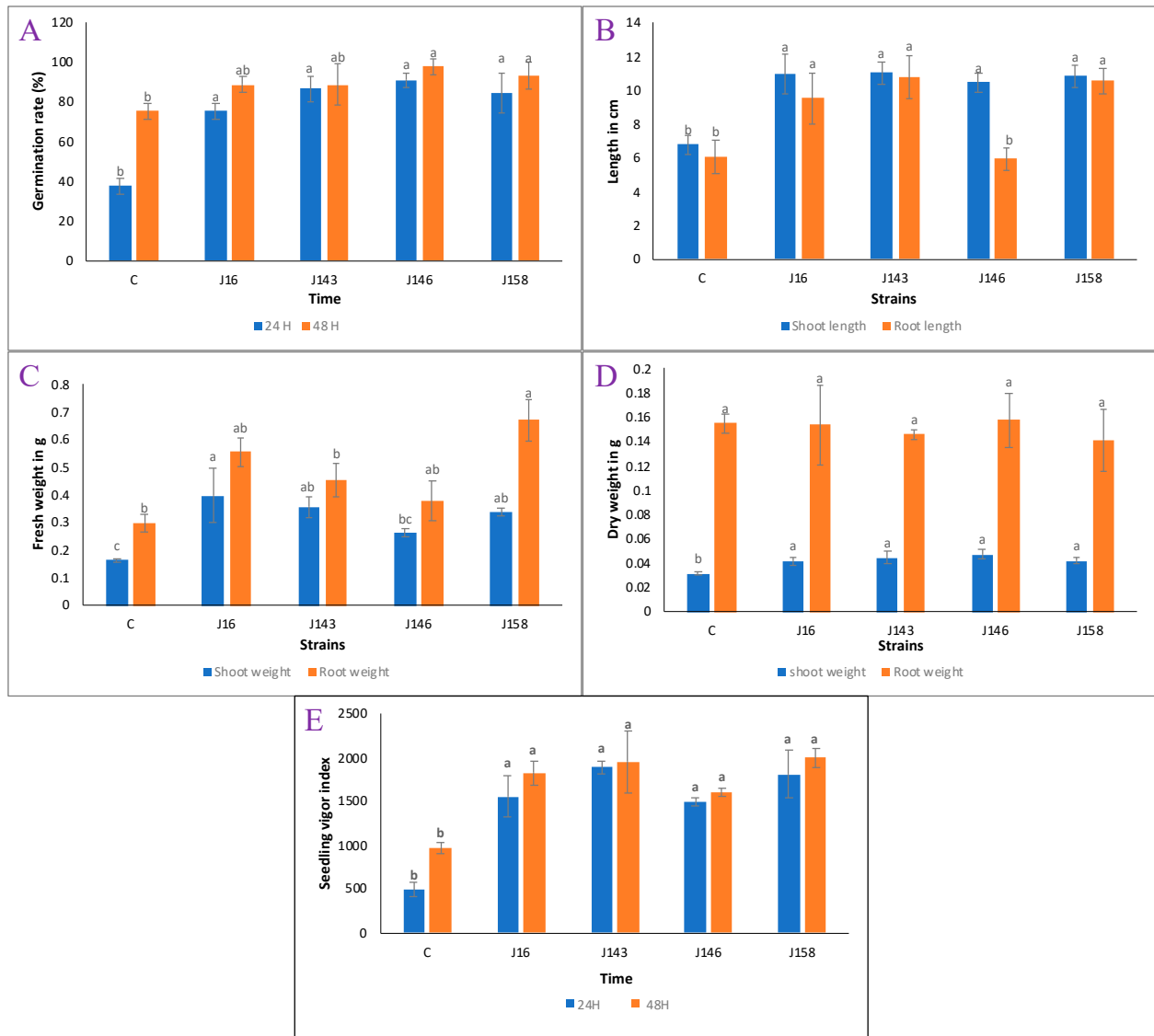

Figure 6. Effect of bacterial inoculation on wheat seed germination: (**A**) germination rate; (**B**) total lengths of shoots and roots; (**C**) fresh weight of shoots and roots; (**D**) dry weight of shoots and roots. (**E**) Seedling vigor index. C is the negative control: seeds treated with sterile distilled water. Letters a, b, and c highlight significant differences at $p < 0.05$. Strains used are: *P. moraviensis* J16, *B. halotolerans* J143, *E. hormaechei* J146, and *P. frederiksbergensis* J158.

3.13. P. frederiksbergensis J158 Induced the Best Wheat Plant Nutrients Uptake

To further investigate the role of the four strains on plant growth, we conducted a pot experiment in five replicates using seeds of durum wheat (see Materials and Methods). Individual application of the four PSB strains significantly increased the wheat plant parameters in normal sterilized soil. The vegetative growth parameters were recorded after harvest at 60 DAS (days after sowing) and the plant nutrient and ionic analyses were performed on dried and crushed shoots and roots. Compared to the non-inoculated seeds, biometric analysis of the wheat shoots and roots length shows that all strains increased significantly in shoot and root length (Figure 7A). We noticed that the highest shoot length was measured for the *P. frederiksbergensis* J158 treatment, while the highest root length was

recorded for the *B. halotolerans* J143 treatment, which was much higher than the negative control (uninoculated seeds).
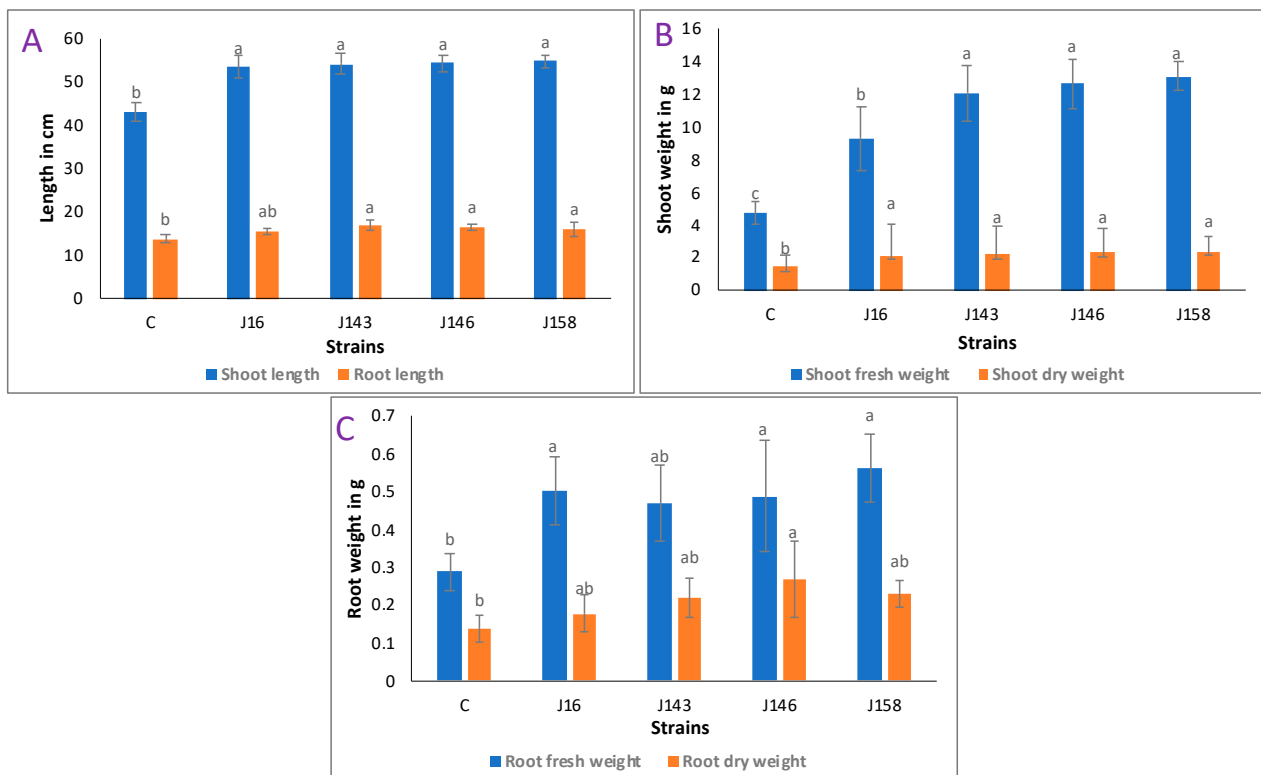

Figure 7. Effect of wheat plant growth inoculated by the four studied strains after 8 weeks of cultivation: (**A**) shoot/root length; (**B**) shoot fresh/dry weight; (**C**) root fresh/dry weight. The values represent means of replicates (*n* = 4) ± standard deviations. The different letters in superscript (a, b, and c) indicate a statistically significant difference at the 95% level between treatments. The strains used are *P. moraviensis* J16, *B. halotolerans* J143, *E. hormaechei* J146, and *P. frederiksbergensis* J158.

Bacterization of wheat seeds revealed also a significant improvement in shoot fresh and dry weight. Here, the greatest fresh weights of both the shoots and roots were seen in the *P. frederiksbergensis* J158 strain (Figure 7B).

Next, we further analyzed the macro- and micronutrient content of the inoculated wheat plants. The highest values of nitrogen (3.4%), phosphorus (0.31%), and potassium (3.94%) were measured in plants inoculated with *E. hormaechei* J146, *P. frederiksbergensis* J158, and *P. moraviensis* J16, respectively (Figure 8A–C). As for Zn acquisition by plants, the best performance was recorded in strain *P. frederiksbergensis* J158 (Figure 8D).
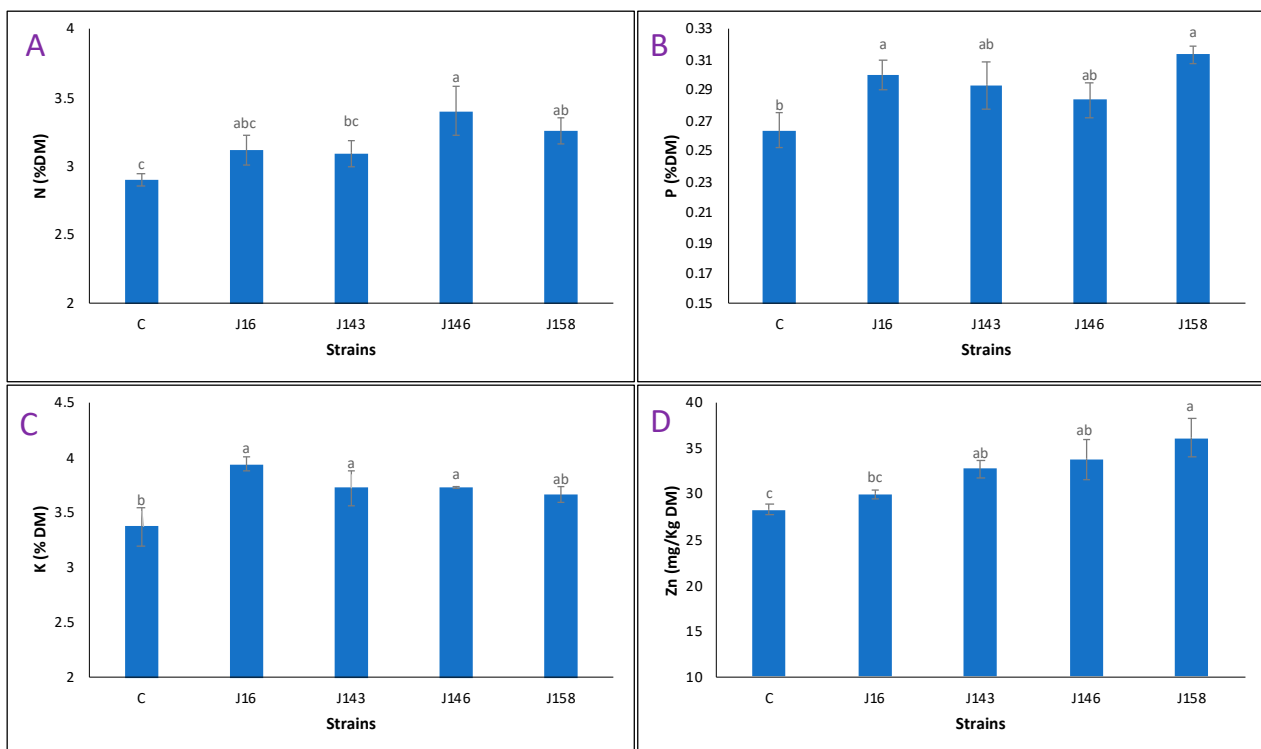
Figure 8. Wheat plant macronutriments and Zn uptake upon cacterization: (**A**) Na⁺; (**B**) P; (**C**) K⁺; (**D**) Zn. The values represent the means of four replicates ($n = 4$) ± standard deviations. Letters in superscript (a, b, and c) indicate a statistically significant difference at the 95% level between bacterial treatments using the four PSB strains: *P. moraviensis* J16, *B. halotolerans* J143, *E. hormaechei* J146, and *P. frederiksbergensis* J158.

4. Discussion

Because of the scarcity of studies on jujube-associated PGPR, its ecological importance, and its abundance in Morocco, this study aimed to isolate and screen PSB from the rhizosphere of jujube plants grown at Bengueir, Morocco. Based on P solubilization screening, four strains were selected from the rhizosphere of the jujube plant (*Ziziphus lotus*). Phylogenetic analysis revealed that two of them belong to genus of *Pseudomonas*, one to *Bacillus*, and the last one to *Enterobacter*. We studied conventional PGP properties by monitoring the production of either IAA, siderophores, extracellular enzymes, ammonia, or biofilm. Next, we evaluated the effect of the four strains on wheat seed germination, plant growth, and macro- and micronutrients uptake, Parallelly, we conducted studies aiming to evaluate their capacity to withstand intrinsic and extrinsic stresses.

4.1. Effect of Studied PSB Strains on Wheat Growth

Strains *P. moraviensis* J16, *B. halotolerans* J143, *E. hormaechei* J146, and *P. frederiksbergensis* J158 induced different degrees of wheat seedling growth. All strains enhanced the germination rate, results that are in line with reported studies on *Bacillus*, *Enterobacter*, and *Pseudomonas* [48–50]. In pot experiments, wheat plant growth parameters were increased significantly. Indeed, shoot length increased by 27.06%, root length by 22.94%, shoot fresh weight by 175.05%, shoot dry weight by 63.19%, root fresh weight by 132.14%, and finally root dry weight by 100%. It appears clearly that the best enhancement of plant shoot and root weight and height, as well as the vigor index, was mainly recorded in the *P. frederiksbergensis* J158 strain. This strain released up to 278.5 mg·L⁻¹ of P and induced a strong biofilm formation, more often associated with bacterial survival, nutrition, and abiotic stress resistance [51,52].

Strain *E. hormaechei* J146 displayed both the highest P solubilization and IAA production, a finding in line with previously reported data [53,54]. *E. hormaechei* J146 increased the

wheat germination rate and induced a good seedling vigor index. In addition, it improved the length and weight of wheat plant but not that of seedling roots. It is well known that PSB not only provide P to plants, but also stimulate plant growth by enhancing the uptake of nutritive elements such as N, by nitrogen fixation and IAA biosynthesis [55]. The IAA phytohormone is synthesized by plants and by a few microbes involved in root initiation and cell division [56,57]. Ammonia is a chemical compound exerting multiple plant health benefits, fundamentally by acting as metabolic inhibitors towards phytopathogens [58]. Here, strain *E. hormaechei* J146 was the best ammonia producer, which may explain the improved N absorption by wheat plant.

P. moraviensis J16 and *B. halotolerans* J143 improved early wheat seed germination at 24 h but not at 48 h. These strains improved the seedling vigor index, root and shoot length, shoot fresh weight, and shoot and root dry weight in seedlings. In pots experiments, inoculation of plant wheat with *P. moraviensis* J16 increased the length of the shoots but not of the roots, whereas *B. halotolerans* J143 induced the highest root length. Strain *P. moraviensis* J16 also improved shoot fresh weight and the weights of the dry and fresh roots. This strain produced the greatest amount of siderophores. The latter are known for their capacity to enhance the availability of phosphorus [16], through minerals solubilization and heavy metals chelation, which in turn enhance nutrient uptake [59]. Under limiting iron conditions, siderophores mediate biocontrol properties and act as solubilizers for iron from minerals or organic compounds [60,61].

Bacterial extracellular enzymes, namely, cellulase and proteases, are involved in several biological processes, such as soil fertilization and biological control of phytopathogens [62]. In this study, cellulase activity was only detected in *B. halotolerans* J143. Even though the latter is the lowest phosphate solubilizer, it remarkably improves wheat plant seedling and growth. This finding suggests that to induce plant growth, *B. halotolerans* J143 likely involves additional mechanisms rather than P solubilization.

4.2. Effect of the Four PSB Strains on Nutrient Uptake and Stress Tolerance

PGPR can improve plant growth by improving macro- and micronutrient absorption. In terms of macronutrients, an increased uptake of N, P, and K by wheat plant was observed; N by up to 17.24%, P by up to 19.23%, and K by up to 16.91%. The uptake of macro (N, P, and K) and micro (Zn) nutrients by plants from soil is mutually dependent [63]. They undergo complex processes in the soil and exhibit a dynamic balance between insoluble and soluble forms under the influence of soil pH. This balance could be affected by the secretion of organic acids and other activities of the soil microbiota, thereby improving their bioavailability to plant roots [64–66]. Strain *E. hormaechei* J146 was the best ammonia producer and interestingly improved N absorption by wheat plant and increased K and Zn uptake. The latter is an essential micronutrient needed in small amounts for healthy plant growth [9–12]. As for the ability of the four strains to mobilize Zn from three components, ZnO, $Zn_3(PO_4)_2$, and $ZnCO_3$, none of the tested strain was able to solubilize Zn from $Zn_3(PO_4)_2$ or $ZnCO_3$. In contrast, ZnO was solubilized by *P. moraviensis* J16, *E. hormaechei* J146, and *P. frederiksbergensis* J158, but not by *B. halotolerans* J143. Not surprisingly, given ZnO is considered as the most mobilizable form of Zn by PSB [67]. The solubilization of Zn is mediated by various mechanisms, including the production of either organic acids, siderophores, and proton oxido-reductive systems [68–70]. The highest Zn mobilization was seen in *E. hormaechei* J146.

In 2016, Joonu et al. described CZC (cobalt–Zn–cadmium) genes in the genome of *Enterobacter asburiae* [68]. The products of these genes permit resistance to Cd^{2+}, Zn^{2+}, and $Co^{2}+$ by a metal-dependent efflux driven by the proton motive force. It is worth noting that the enhancement of N, P, and Zn uptake by wheat plant was remarkably higher in strains *P. frederiksbergensis* J158 and *E. hormaechei* J146. We analyzed the published genomic sequence of the *E. hormaechei* strain MS7884A (GenBank: CP022532.1). We revealed the conservation of genes specifying a Zn ABC transporter permease subunit, which is the counterpart of ZnuB in *E. coli* [71], *ZnuC*, encoding for a predictive Zn ABC transporter periplasmic

Zn-binding protein. We also detected the presence of a Zn/cadmium/mercury/lead-transporting ATPase (encoded by the *zntA* gene). Finally, we also observed the conservation of a copper-exporting ATPase (locus_tag) and a copper-binding protein (encoded by *pcoE_2* gene). It is likely that the products of these two hypothetical genes may explain the remarkable capacity of *E. hormaechei* J146 to solubilize Zn and to tolerate high concentrations of copper, cadmium, and nickel. Further genomic studies should address the functional analysis of these genes.

In the rhizosphere, tolerance of extrinsic and intrinsic stresses is among the most important characteristics in the growth, establishment, and survival of microorganisms. Although PSB are the natural habitat of many soils, their performance is strongly dependent on various environmental factors, including soil composition, salinity, temperature, cation exchange capacity, organic matter, pH, and nutrient substance availability in the soils [72,73]. One of which is biofilm, which participates in plant biocontrol [74], and in the amelioration of soil fertility [75]. Salinity is one of the major abiotic stresses that affects bacterial development and limits the productivity of cultivated plants [76]. In this context, *B. moraviensis* J143 and *E. hormaechei* J146 tolerated up to a 5% NaCl final concentration, whereas the growth of the two *Pseudomonas* strains was halted at 5%.

It is well admitted that bacterial resistance to antibiotics enable bacterial survival among the microbiome communities. Except for *B. halotolerans* J143, the three other studied strains displayed resistance at least to one antibiotic, while all strains were sensitive to both kanamycin and streptomycin. Both *P. moraviensis* J16 and *E. hormaechei* J146 exhibited resistance to three antibiotics. In agriculture, the use of PGPR as inoculants is widely applied but only limited studies addressed their antibiotic resistance. Thus, the best practice is to do that systematically, to limit ARG dissemination into the environment. According to our findings, *P. moraviensis* J143, which is sensitive to the tested antibiotics, may be a suitable, safe biofertilizer candidate.

5. Conclusions

The present work allowed the characterization of four PSB strains isolated from the rhizosphere of jujube plant (*Ziziphus lotus*). We demonstrated that the inoculation of wheat seeds by all strains significantly improved the germination rate and plant growth of the seedling. Furthermore, a remarkable increase in macro- and micronutrient uptake was observed in the inoculated wheat plants. As there is an urgent need to improve the wheat Zn content, we suggest the potential application of our selected strains in the field. Besides, the ability to solubilize ZnO is of a great interest as it may be applied to bioremediate lands where Zn is abnormally abundant. In addition, our work contributes to the identification of strain *P. frederiksbergensis* J158, suitable as potential biofertilizer for wheat crops, and strains *B. halotolerans* J143 and *E. hormaechei* J146 as potential bioremediatory agents for contaminated soils.

Author Contributions: Conceptualization, L.B., A.A. and N.F.; funding acquisition, A.A.; investigation, N.F. and L.B.; methodology, N.F. and I.M.; project administration, A.A.; supervision, A.A. and A.M.; validation, A.A and L.B; writing—original draft, N.F. and L.B.; writing—review and editing, A.A. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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