Phosphate solubilizing rhizobacteria isolated from jujube ziziphus lotus plant stimulate wheat germination rate and seedlings growth

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Jujube plant (*Ziziphus lotus* (L.) Desf.) can survive arid climates and tolerates both biotic and abiotic stresses. Here, we isolated nine phosphate solubilizing bacteria strains from jujube rhizosphere, designated J10 to J13, J15, & J153 to J156. Genotypic identification, based on 16S rDNA sequencing, revealed six strains that belong to *Pseudomonas* (J10, J12, J13, J15, J153, J154), two to *Bacillus (*J11, J156*),* and one to *Paenibacillus* J155*.* Siderophores were produced by all strains, proteases activity was missing in *Pseudomonas* sp. J153 & J154, whereas cellulase was restricted only to *Pseudomonas* sp. J10, *Paenibacillus xylanexedens* J155 and *Bacillus cereus* J156. Indole-3- acetic acid and ammonia were also produced by all strains, with a maxima of 204,28 µg .mL^{-1} in *Bacillus megaterium* J11 and 0.33 µmol.mL^{-1} in *Pseudomonas* sp. J153, respectively. *Pseudomonas* sp. J10 and *B. cereus* J156 grew on plates containing 1500 µg.mL^{-1} of nickel nitrate, while *Pseudomonas* sp. J153 withstood 1500 µg.mL^{-1} of either copper sulfate or cadmium sulfate. Lastly, the study of the potential of the isolates for promotion of early plant growth showed that wheat seeds inoculated with either *P. moraviensis* J12 or *B. cereus* J156 remarkably increased germination rate and seedlings growth.

Phosphate solubilizing rhizobacteria isolated from jujube *Ziziphus lotus* plant stimulate wheat germination rate and seedlings growth

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Abstract

Jujube plant (*Ziziphus lotus* (L.) Desf.) can survive arid climates and tolerates both biotic and abiotic stresses. Here, we isolated nine phosphate solubilizing bacteria strains from jujube rhizosphere, designated J10 to J13, J15, & J153 to J156. Genotypic identification, based on 16S rDNA sequencing, revealed six strains that belong to *Pseudomonas* (J10, J12, J13, J15, J153, J154), two to *Bacillus (*J11, J156*),* and one to *Paenibacillus* J155. Siderophores were produced by all strains, proteases activity was missing in *Pseudomonas* sp. J153 & J154, whereas cellulase was restricted only to *Pseudomonas* sp. J10, *Paenibacillus xylanexedens* J155 and *Bacillus cereus* J156. Indole-3- acetic acid and ammonia were also produced by all strains, with a maxima of 204,28 µg.mL^{-1} in *Bacillus megaterium* J11 and 0.33 µmol.mL^{-1} in *Pseudomonas* sp. J153, respectively. *Pseudomonas* sp. J10 and *B. cereus* J156 grew on plates containing 1500 µg.mL^{-1} of nickel nitrate, while *Pseudomonas* sp. J153 withstood 1500 µg.mL^{-1} of either copper sulfate or cadmium sulfate. Lastly, the study of the potential of the isolates for promotion of early plant growth showed that wheat seeds inoculated with either *P. moraviensis* J12 or *B. cereus* J156 remarkably increased germination rate and seedlings growth.

Subjects Soil Microbiology, Plant Bacteria Interaction, Plant Growth Promotion, Soil Biofertilization

Keywords Jujube plant, Plant growth promotion, Rhizobacteria, Phosphate solubilizing bacteria, Antibiotics resistance, Heavy metals tolerance, Wheat seeds germination

Introduction

Phosphorus (P) is considered one of the most important elements in plant nutrition after nitrogen. It is an essential macronutrient to all major metabolic processes in plants growth *e.g.*

photosynthesis, energy transfer, respiration, and signal transduction *(Khan et al., 2010; Rahman et al., 2017*). Phosphate solubilizing microorganisms including bacteria play an important role in enhancing soil fertility and plant growth (*Miransari & Mackenzie, 2010*). Therefore, it is paramount to explore management strategies which are considered as an environmentally friendly process and economically feasible procedure to improve crop production and maximize their yields in P-poor soils (*Zaidi et al., 2009*). Exploration of the biodiversity of rhizobacteria and the optimization/manipulation of microbial interactions in the rhizosphere represents an imperative step towards formulating more efficient microbial inoculants with high P-solubilizing ability (*Khan, Zaidi & Wani, 2007*). Although P is plentiful in soils in both organic and inorganic forms, it is in unavailable forms for root uptake (Sharma et al., 2013). Numerous soil microorganisms particularly those present in plant's rhizosphere can release the bound forms of P to a soluble form to increase its bioavailablity to plants *(Narayanasamy, Ghosh & Sarkar, 1981; Dubey et al., 1997; Dave & Patel, 2003*). PSB (phosphate solubilizing bacteria) belong to plant growth promoting rhizobacteria (PGPR) and are capable of solubilizing inorganic P from a variety of sources, such as dicalcium phosphate, tricalcium phosphate, or rock phosphate (*Khan et al., 2010*). Rhizobacteria are considered to be the best-known beneficial plant-associated bacteria and the most valuable bio-inoculants as they showed promising performances under controlled conditions such as the production of phytohormones, siderophores, phosphate solubilization, and nitrogen fixation *(Pérez-Montaño et al., 2014*).

A number of strategies have been documented to improve phosphorus availability in soils including, agronomic practices, organic amendments, composting, arbuscular mycorrhizal fungi, P efficient cultivars, and phosphate solubilizing microbes (*Kunwar, Lamichhane & Gauchan, 2018)*. This later strategy is one of the most promising, as it is more sustainable and considered to be eco-friendly (*Gyaneshwar et al., 2002*). In contrast to other strategies, microbes have multiples benefits to plants as they contribute directly and indirectly to the nutrition, biocontrol of pathogens, and mitigating abiotic stresses (Kunwar, Lamichhane & Gauchan, 2018). Strains belonging to the bacterial genera *Pseudomonas, Bacillus, Rhizobacterium, and Enterobacter* are known as potent P solubilizing microorganisms (*Whitelaw, 1999*).

Antibiotic resistance is a major concern whose emergence and spreading rates are increasing. It is one of the major problems in deploying bacterial-based biofertilizers (*Kang et al., 2017*). Antibiotic resistance genes (ARGs) harbored by PGPB can be an inborn or gained property. Intrinsic resistance may be due to the presence of multidrug efflux pumps. This is supported by the phylogenetic analysis of several genes involved in antibiotic resistance which could be due to an evolutionary pattern (*D'Costa et al., 2011; Van Goethem et al., 2018*). Acquired antibiotic resistance may also reflect the acquisition of new resistance genes from other organisms by horizontal gene transfer from bacteria. The resistance of PGPR to antibiotics is a double-edged sword, on the one hand, resistant bacteria can serve either as markers to monitor bacteria survival in vitro or in vivo (Kluepfel, 1993; Trivedi et al., 2004), and to help them competing in native and open microbial niches (Cray et al., 2013). On the other hand, their application in soil as bio-inoculants may represent potential risks by transferring ARG to other bacteria colonizing the same

environment (Ramakrishna, Yadav & Li, 2019). However, a potential source of ARGs carried by PGPR and derived biocontrol agents and/or bio-fertilizers is widely forgotten and ignored (*Kang et al., 2017*). Hence, the urgency of large-scale introduction of the beneficial bacteria into soils can aggravate the situation leading to the spread of ARGs in the environment. In addition to antibiotic resistance, heavy metal pollution is an environmental concern that can have harmful effects on human health when they are taken up in amounts that cannot be processed by the organism (*Chauhan & Solanki, 2015*). Many toxic elements such as Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} and As^{3+} are generally found in detectable quantities in wastewater (*Pescod, 1992*). Some of these elements are necessary for plant growth, but a high concentration of them in wastewater becomes an obstacle (*Pescod, 1992*), which leads to the remediation of contaminated environments *via* sustainable methods (*Pizarro-Tobías et al., 2015*). Several methods are already being used to clean up the environment from these types of contaminants. The use of microorganisms capable of adsorbing heavy metal ions for bioremediation in contaminated soil is considered as an eco-friendly method and do not produce secondary pollution. Certain PGPR can reduce soil toxicity around plant roots. These PGPR also protect plants from being affected by toxic heavy metals (*Gamalero & Glick, 2011*). Various free-living rhizospheric PGPR can be used in contaminated soils to alleviate lethal effects of heavy-metals (*Belimov et al., 2004*). *Bacillus* and *Pseudomonas* exhibit vital role in the bioremediation of heavy metals (*Khan & Ahmad, 2006; Niu et al., 2011*).

The jujube tree (*Ziziphus lotus* (L.) Desf.) is a deciduous shrub belonging to the Rhamnaceae family. It is present in Morocco, in several biotopes of arid and semi-arid regions. It reaches 2 to 6 m, with tightly branched stems and smaller flowers and fruits (*Wang et al., 2016*). It grows on all soils: limestone, siliceous, clayey, and sandy, without human intervention, and it supports small amounts of salt (*Ionesco & Sauvage, 1969*). This shrub also behaves as a weed in several crops, including winter and spring cereals, food legumes and orchards in certain regions of Morocco such as Chaouia, Haouz, Zear, Rhamna and the Middle Atlas *(Rsaissi & Bencharki, 2012*).

The characterization of jujube rhizospheric PSB and their effects on plant growth is poorly understood and have not been extensively studied yet. The omnipresence of PGPR microorganisms in nature especially in rhizospheres, and their exceptional ability to enhance plant growth led us to undertake the present study. We isolated, for the first time in Morocco, PSB from rhizospheric soil of jujube plants (*Ziziphus lotus* (L.) Desf.). We subsequently identified their genotype, assessed their conventional PGPR properties and tolerance to heavy metals and resistance to antibiotics in vitro, and finally evaluated their effects on wheat seeds germination.

Materials & Methods

Sampling and bacterial isolation

The sampling site, located in the experimental farm of Mohammed VI Polytechnic University (UM6P), Benguerir-Morocco (32.219731E, -7.892268N), is characterized by a temperate continental monsoon climate. The annual rainfall is 290,6 mm and mainly occurs from October to January. Eight samples of roots and rhizospheric soil of jujube were collected from a 5 to 25 cm depth, packed and labeled in sterile plastic bags, then transported immediately to the laboratory in

cool boxes. Fractions from all soil samples were mixed and analyzed for some physicochemical properties at the Agricultural Innovation and Technology Transfer Center (AITTC) of UM6P. The results are shown in Table 1. Rhizospheric samples were serially diluted under aseptic conditions by suspending 1 g of rhizosphere soil in 9 mL of sterile deionized water. Next, 100 µL of serial dilutions were subsequently plated on Tryptic Soy Agar medium (TSA) (EMD Millipore, Berlin, Germany) and incubated at 28 ±2 °C till the appearance of bacterial colonies. Screening of individual colonies was carried out by repeated streaking.

Screening of phosphate solubilizing bacteria

All bacterial isolates were qualitatively screened for inorganic P solubilization by inoculating a single colony of each strain in National Botanical Research Institute's Phosphate growth medium (NBRIP) containing 10 $g.L^{-1}$ glucose; 0.1 $g.L^{-1}$ $(NH4)_2$ SO_4; 5 $g.L^{-1}$ $MgCl_2$ $6H_2O$; 0.2 $g.L^{-1}$ KCl, 0.25 $g.L^{-1}$ $MgSO_4,7H_2O$ and finally 5 $g.L^{-1}$ $Ca_3(PO_4)_2$ (TCP: insoluble tricalcium phosphate) as a sole source of phosphate (*Nautiyal, 1999*). The initial media pH was adjusted to 7.00 before use. Each bacterium was incubated on NBRIP plate at 30 °C for 7 days and only colonies surrounded by clear halos were selected for further studies as potential P solubilizer candidates. PSB were subsequently sub-cultured in TSB (Tryptic Soy Broth) (Professional lab, Casablanca, Morocco) liquid media and cryopreserved at −80 °C until use.

Quantification of phosphate solubilization by bacteria

Inorganic P-solubilizing activity was quantified using TCP (in a modified NBRIP liquid medium). Briefly, bacterial suspension (0.1 mL of OD_{600nm} = 0,8) was inoculated in a 100 mL flask containing 50 mL of NBRIP broth in triplicate. Non-inoculated medium was used as blank, while *Rhizobium tropici* CIAT 899 served as a positive control. Bacterial cultures were incubated at 28 ± 2 °C during five days under shaking condition at 150 rpm. The cultures were then harvested by centrifugation at 13.000 rpm for 10 min and the soluble P, contained in the supernatant, was quantified by colorimetric method using SKALAR (SKALAR SAN++ SYSTEM). Dissolved P concentration was determined by subtracting the P concentration of the blank from the final concentration of soluble P in the inoculated broths. The final pH of each culture supernatant was also measured. The experiments were performed in triplicate and the results are means of the replicates.

Bacterial antibiotic resistance and heavy metal tolerance

Antibiotic resistance profile of selected PSB was determined using TSA medium supplemented with selected antibiotics namely kanamycin (50 $\mu g.mL^{-1}$), streptomycin (100 $\mu g.mL^{-1}$), tetracycline (10 $\mu g.mL^{-1}$), ampicillin (100 $\mu g.mL^{-1}$), chloramphenicol (20 $\mu g.mL^{-1}$) and spectinomycin (60 $\mu g.mL^{-1}$).

Heavy metal tolerance of selected isolates was tested using the same method (TSA plates) with the addition of increasing concentrations (ranging from 0 to 1500 $\mu g.mL^{-1}$) of three heavy metals;

cadmium sulfate ($CdSO_4$), copper sulfate ($CuSO_4.5H_2O$) and nickel nitrate (N_2NiO_6). The plates were incubated at 30 °C for 24 h.

Strains genotyping using 16S rRNA gene sequencing

PSB identification was performed using 16S rRNA gene sequencing. The polymerase chain reactions (PCR) were carried out directly with fresh bacterial suspension, using a pair of universal primers pA (5'- AGAGTTTGATCCTGGCTCAG-3') and 926R_Quince (degenerated one) (5'-CCG YCAATTYMTTTRAGTTT-3'), and MyTaq Mix, 2X (ThermoFisher, Casablanca, Morocco) containing Taq DNA polymerase, dNTP, $MgCl_2$ and buffer. Amplification of 16S rDNA sequences was made in 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 overnight bacterial culture as 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, 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-bp) were sequenced by Genome Quebec, Canada. The generated DNA sequences were aligned to available standard sequences of bacterial lineage in the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov/) and the High-Quality Ribosomal RNA databases SILVA (https://www.arb-silva.de) using BLAST algorithm to carry out a taxonomic assignment of each isolate. The phylogenetic tree of identified PSB was built using Ugene software.

Indole-3- acetic acid (IAA) measurement assay

Bacteria were analyzed for the quantitative determination of indole acetic acid (IAA) production. For this purpose, 100 µL of each PSB strain (OD_{600nm} = 0.8) was grown in 50 mL Tryptic Soy Broth (TBS) supplemented with 0.1% L-tryptophan as IAA precursor at 28 ± 2 °C in a shaking incubator at 200 rpm. After 7 days, 2 mL of Salkowski reagent [0.5M $FeCl_3$: 70% perchloric acid/water (2:49:49 ratio)] (*Glickmann & Dessaux, 1995*), was pipetted into test tubes containing 1 mL of culture supernatant filtrates. The tubes containing the mixture were gently vortexed and left for 30 min in dark for the development of color at room temperature (26 ± 2°C). The absorbance was determined at an $OD_{535\,nm}$. The quantity of IAA produced in each supernatant was estimated in ($\mu g.mL^{-1}$) from a calibration curve using a standard IAA (Sigma Aldrich, Overijse, Belgium).

Siderophores production assay

Qualitative production of siderophores by selected strains was detected on the chrome-azurol S (CAS) medium as previously described (*Schwyn & Neilands, 1987*). Briefly, each bacterial culture was spot-inoculated separately on CAS agar plates. The plates were kept at 30 °C for 3 days. After the incubation period, the appearance of orange halo (blue to yellow/orange) around the colony was considered as a positive result for siderophores production.

Extracellular enzymes production assay

Bacteria were qualitatively analyzed for the production of protease and cellulase by the plate method (*Kavitha, Nelson & Jesi, 2013*). Protease activity (casein degradation) was tested by inoculation of selected strains into nutrient agar medium containing casein 5 $g.L^{-1}$, yeast extract 2.5 $g.L^{-1}$, glucose 1 $g.L^{-1}$, and agar 15 $g.L^{-1}$ and amended with 10% of skim milk. After 48 h incubation at 30 °C, a clear zone around colonies indicated positive proteolytic activity. For cellulase activity, a mineral–salt agar plate 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 were surface-inoculated with each strain and incubated 48 h at 30 °C. Plates were stained with 0.1% Congo Red (Sigma Aldrich, Casablanca, Morocco) for 15 min. Following de-staining during 15 min, using 1 M NaCl, the development of the halo zone around the colonies reflects cellulase production.

Ammonia production assay

Bacteria strains were tested, qualitatively and quantitatively, for ammonia production in peptone water as previously described (*Cappuccino & Sherman, 1992*). Briefly, freshly grown cultures were inoculated into 10 mL peptone water and incubated for 48 h at 30 °C on a shaker (150 rpm). Post incubation period, 0.5 mL of Nessler's reagent was added to each tube. Ammonia production is proportional to the brown color intensity. It was measured spectrophotometrically at $OD_{450\ nm}$ using the VICTOR NivoTM Multimode Plate Reader (PerkinElmer, Casablanca, Morocco) and determined using a standard curve prepared with 0.1–1 $\mu mol.mL^{-1}$ ammonium sulfate.

Wheat seeds germination assay

Our selected strains were assessed for their effect on seed germination. 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. PSB cell pellets were obtained by centrifuging an overnight culture (OD_{600nm}=0,8) at 10. 000 rpm for 5 min, the supernatant was removed, and the pellets were resuspended in 5 mL of sterile distilled water, vortexed and used for seed treatment. Fifteen sterilized seeds were treated with 5 mL of bacterial suspension for 30 min, air-dried, and then placed on sterile Petri dishes containing 0.7% agar medium and incubated at 25 ºC. Triplicates were maintained for each treatment. Seeds were surface sterilized with 2% sodium hypochlorite solution for 1 min, rinsed thoroughly with sterile distilled water. Next, seeds were incubated in a dark incubator for 48 h, then left at room temperature in a day/night cycle. The germination rate was recorded after 24 h and 48 h. Root length, shoot length, fresh weight, and dry weight were measured after 7 days. The germination rate and vigor index were calculated using the following formula *(Islam et al., 2016)*:

$$Germination\ rate(\%) = \frac{Number\ of\ seeds\ germinated}{\text{Total number of seeds}} \times 100 \qquad (1)$$

$$\text{Vigor index} = \%\ Germination \times Total\ plant\ lenght \quad (2)$$

Statistical analysis

Results presented here are the mean of triplicates (n=3) ± 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 $p< 0.05$.

Results

Bacteria screening identified nine best phosphate solubilizing strains

The screening of P solubilizing bacteria from different rhizospheric soil samples of jujube on NBRIP led to the isolation of forty-one bacterial isolates. This microbial population has different aspects, but all exhibited a common character of tricalcium phosphate ($Ca_3(PO_4)_2$) solubilization on solid medium. Indeed, bacterial isolates were able to form a clear zone (halo) around their colonies on the NBRIP medium, indicating positive solubilization of P from tricalcium phosphate (TCP). Nine isolates were selected as being the best performers on plates and named J10 to J13, J15, and J153 to J156 (J for Jujube). Next, we tested their ability to solubilize inorganic phosphorus ($Ca_3(PO_4)_2$) in NBRIP liquid medium. The amount of soluble P and growth media's pH were measured 5 days post incubation. Eight strains were found to release P from TCP with concentrations ranging from 20,5 $mg.L^{-1}$ to 264 $mg.L^{-1}$ (Fig. 1). Remarkably, the highest solubilization was recorded for strain J153, while the lowest one (20,5 $mg.L^{-1}$) was measured for strain J11. The amount of P solubilization by the referenced strain, *Rhizobium tropici* did not exceed 67,5 $mg.L^{-1}$. As expected, P solubilization was accompanied by a significant drop in pH, of the culture media, from 7.0 to 4.0 (Fig. 1).

Strains J10-13, J15 and J153-156 belong to the genera of *Pseudomonas*, *Bacillus*, and *Paenibacillus*

Characterization of the nine PBS strains to the genus level was performed by 16S ribosomal DNA gene partial sequencing. Generated sequences of 900-bp length were aligned to available 16S rDNA sequences using GenBank and SILVA databases. As summarized in Table 2, three strains (J12, J13, and J15) show 98% identity to the 16S rRNA gene sequences of *Pseudomonas moraviensis*; three strains J10, J153, and J154 share 98-99% identity to *Pseudomonas* sp.; two strains J11 and J156 exhibit 98 and 99% identity to *Bacillus megaterium* and *Bacillus cereus*, respectively. Lastly strain J155 shares 98% identity to *Paenibacillus xylanexedens* (Fig. 2).

Except for *Pseudomonas* sp. J11 and *B. cereus* J156, remaining strains displayed resistance at least to one antibiotic

PGPB tend to harbor genes that confer resistance to antibiotics (*Kang et al., 2017*). To assess bacterial resistance to antibiotics, we checked our strains for growth on plates supplemented with a set of different antibiotics, frequently encountered among bacteria isolated from soils. As

reported in Table 3, out of the nine tested strains, seven presented resistance at least to one antibiotic. Strains *P. moraviensis* J12 & J15, *Pseudomonas* sp. J153 & J154 resist to chloramphenicol and ampicillin, while *Pseudomonas* sp. J10 & J13 confer resistance to chloramphenicol, ampicillin, and spectinomycin. Strains *Pseudomonas* sp. J11 and *Bacillus cereus* J156 are sensitive to all tested antibiotics. Strain *P. xylanexedens* J155 is resistant to both kanamycin and spectinomycin. Lastly, none of the tested strains are resistant to neither streptomycin nor tetracycline **(**Table 3). In the next steps, to avoid any potential contamination, we took advantage to these resistances to grow bacteria on selective media.

Pseudomonas sp. J153 and _B. cereus_ J156 withstand high concentrations of copper sulfate/cadmium sulfate and copper sulfate/nickel nitrate, respectively

Heavy metals such as lead (Pb), cadmium (Cd), chromium (Cr), and mercury (Hg) are exceptionally toxic and dangerous environmental pollutants (*Tangahu et al., 2011*). We investigated the capacity of our strains to grow under various concentrations of nickel nitrate (N_2NiO_6), copper sulfate pentahydrate ($CuSO_4.5H_2O$) and cadmium sulfate ($CdSO_4$). The nine strains exhibited various tolerance characteristics (Table 4). In the copper assay, *Pseudomonas* sp. J153 grows up to 1500 µg.mL^{-1}, *Bacillus cereus* J156 to 1000 µg.mL^{-1}, while strains *Pseudomonas* sp. J10, *P. moraviensis* J12, *P. moraviensis* J13, and *P. moraviensis* J15 supported a maximum of 500 µg.mL^{-1}. The lowest tolerated concentration, 300 and 200 µg.mL^{-1} were seen in *B. megaterium* J11 and *Pseudomonas* sp. J154 strains, respectively. When tested for cadmium sulfate, only *Pseudomonas* sp. J153 grows up to 1500 µg.mL^{-1}, whereas 300 µg.mL^{-1} was the maximal concentration tolerated by strains *Pseudomonas* sp. J10, *P. moraviensis* J12, *P. moraviensis* J13, *P. moraviensis* J15, *Pseudomonas* sp. J154 and *B. cereus* J156. Lastly, low tolerance at 100 µg.mL^{-1} and 10 µg.mL^{-1} were detected in strains *P. xylanexedens* J155 and *B. megaterium* J11, respectively (Table 4). In the nickel nitrate assay, *Pseudomonas* sp. J10 and *Bacillus cereus* J156 strains, grow up to 1500 µg.mL^{-1} and strain *P. xylanexedens* J155 tolerated the lowest concentration of 300 µg.mL^{-1}, however, the remaining strains tolerate growth up to 500 µg.mL^{-1}. Taking together, our data highlighted the remarkable capacity of strains *Pseudomonas* sp. J153 and *B. cereus* J156 to withstand abnormal high concentrations of both copper sulfate/cadmium sulfate and copper sulfate/nickel nitrate, respectively.

B. megaterium J11 is the best indole-3-acetic acid producer

The production of indole acetic acid (IAA) is a major property shared by numerous rhizospheric bacteria that stimulate plant growth (*Mohite, 2013*). Seven days post incubation, all tested strains produced IAA although at various levels, ranging from 57,1 to 204,28 µg.mL^{-1} (Fig. 3**)**. The highest concentration was produced by *B. megaterium* J11, whereas the lowest one was measured for *P. xylanexedens* J155 (Fig. 3).

P. moraviensis J13 is the best siderophores producer

Siderophores are best known for binding iron (Fe) and for mobilizing soil-immobilized Fe, although they may also contribute to improve the availability of P to plants (*Sharma et al., 2013*),

by solubilizing minerals and chelating heavy metals, which in turn increases nutrient uptake and plant growth (*Gontia-Mishra et al., 2016*). The ability, *in vitro*, of selected PSB, to produce siderophores was qualitatively estimated using the CAS-agar plate assay. All tested strains were able to produce siderophores, although at various levels as deduced by the size of the halo zone and the intensity of the color change of the CAS-Agar (Table 3). *P. moraviensis* J13 was the most efficient siderophores producer, the six strains (*Pseudomonas* sp. J10, J153 &J154, *P. moraviensis* J12 & J15, and *P. xylanexedens* J155 produced intermediate level, whereas the lowest production was seen in both *B. megaterium* J11 and *B. cereus* J156 strains.

Pseudomonas sp. J153 is the best ammonia producer

Ammonia is a chemical compound having indirect plant health benefits, primarily by acting as metabolic inhibitor against phytopathogens(*Kumar et al., 2012*). All tested strains were able to produce ammonia with various concentrations. The highest value of 0.33 µmol.mL^{-1} was detected in *Pseudomonas* sp. J153, while the lowest one, 0.1 µmol.mL^{-1}, was measured in *P. xylanexedens* J155 (Fig. 4).

Proteases are not produced by _Pseudomonas_ sp. J153 and J154 and cellulase activity is restricted to _Pseudomonas_ sp. J10, J155 and _B. cereus_ J156

Bacterial extracellular enzymes such as proteases and cellulases play a dual important role in the biological control of phytopathogens and in soil fertilization (*Mitchell & Alexander, 1963*). The nine strains were tested for their ability to produce proteases and cellulases. Results of both proteases and cellulase assay are shown in Table 3. As for proteases production, except for *Pseudomonas* sp. J153 & J154, the remaining seven strains developed halo zone around the colonies. As a control, no halo zone was seen using *E. coli* strain DH5α, used here as a negative control. Cellulase activity was solely detected in three strains: *Pseudomonas* sp. J10, *P. xylanexedens* J155, and *B. cereus* J156, each of which formed a yellow/whitish zone around their colonies and were considered as cellulase positive. No cellulase activity was observed in the remaining six other strains.

Inoculation with _P. moraviensis_ J12 and _B. cereus_ J156 promote the highest rate of wheat seeds germination and seedlings growth

The treatment of wheat seeds by the nine PSB strains had a significant effect ($P < 0.05$) on the germination rate and wheat vigor index, as compared to the control (Fig. 5 A et E). However, these effects varied depending on the PSB isolates. For instance, both *P. moraviensis* J12 and *B. cereus* J156 strains were the most efficient in promoting wheat germination as represented by vigor index (Figure 5 E). Results revealed that, compared to non-inoculated control, seeds inoculated by each of the nine strains showed a considerable impact on different growth parameters (Fig. 6).

Regarding shoot and root length after 7 days of growth, seeds inoculated with all strains, especially *B. megaterium* J11 significantly enhanced shoot and root length ($p < 0.05$). Maximum root length was seen upon inoculation with *P. moraviensis* J12 (Fig. 5 B). We also noticed,

whatever the nature of the inoculum was, seeds root dry weights remain unchanged. In contrast, a significant increase in shoot dry weight was detected (Fig. 5 D). Furthermore, wheat seeds inoculation significantly affected shoot fresh weight, but not root dry weight except for *B. megaterium* J11, *P. moraviensis* J12, and J15 strains (Fig. 5 C).

Discussion

Phosphorus is an important limiting factor in agriculture production and microbial P solubilization seems to be an effective process to release the precipitated P in soil. In the present work, we isolated and screened nine P solubilizing bacteria from Jujube roots. Genotyping analysis revealed that these strains belong to the genera of *Pseudomonas*, *Bacillus,* and *Paenibacillus*. A recent study based on ACC-deaminase-producing screening criteria, reported that *Pseudomonas* and *Bacillus* genus are among species that are associated with jujube rhizospheric plant with the dominance of *Pseudomonas* genus (*Zhang et al., 2020*). Our isolated bacteria were characterized as being P solubilizers with values ranging from 20,5 to 264 $mg.L^{-1}$. Comparatively, *Zhang et al., (2020)*, reported that *Pseudomonas lini* (KM349410) isolated from jujube rhizosphere, was the best P solubilizing strain at 69 $mg.L^{-1}$. We found here that the highest concentration of dissolved P in the medium was recorded by genus *Pseudomonas* followed by *Paenibacillus*, and then by our two *Bacillus* strains. Not surprisingly given, the pH of all bacterial cultures dropped significantly from 7.0 to 4.0, likely due of organic acids production *(Pandey & Maheshwari, 2007; Khan, Zaidi & Ahmad, 2014; Otieno et al., 2015*). Indeed, different genera of bacteria uses multiple P solubilization mechanisms, in addition to the production of acids, such as chelation and siderophores (*Pandey & Maheshwari, 2007*).

Indole-3-acetic acid has been associated with plant growth promoting effect of several rhizospheric microorganisms that stimulate elongation and proliferation of root systems *(Glick, 2012; Shilev, 2013; Otieno et al., 2015; Thomas, Murphy & Murray, 2016; Mazumdar, Saha & Ghosh, 2019*). IAA is also frequently used as a marker to select beneficial bacteria *(Went & Thimann, 1937*). Auxin production stimulates root development resulting in a higher nutrients and water absorption from the soil (Höflich, Wiehe & Kühn, 1994). *B. megaterium* J11 is the best IAA producer (204,28 $\mu g.mL^{-1}$), and the lowest in-term of P solubilization (20,5 $mg.L^{-1}$), but significantly stimulates wheat seedling. Recent genome sequencing of *Bacillus megaterium* TRQ8 isolated from wheat (*Triticum turgidum* subsp. durum) rhizosphere, revealed the presence of genes specifying factors associated with bacteria–plant interactions, *i.e*., auxin biosynthesis (indoles), phosphate metabolism, siderophores production, and osmotic/oxidative stress response (*Montoya, Cota & de los Santos Villalobos, 2019*).

Our isolated strains produced various levels of siderophores but *P. moraviensis* J13 was the best producer. During plant-bacteria association, siderophores production is beneficial to plant and are considered as an important trait of PGPR (*Bal et al., 2013*), that may influence plant growth as they mobilize different metal ions and play also an important role in bio-control (*Matthijs et al., 2007*). The *P. moraviensis* strain J12 exhibits a good production of siderophores in addition to be the highest P solubilizer, promotes shoot and root length, root fresh weight and stimulates wheat

seeds germination. Compared to *P. moraviensis* J13, strain *P. moraviensis* J12 increases root fresh weight. The third strain of *P. moraviensis* J15 increases root and shoot length, shoot dry weight and stimulates wheat seedling.

Ammonia production is an essential PGPR trait often associated with plant growth (*Yadav, Verma & Tiwari, 2010*). We found that *Pseudomonas* sp. J153 is the best ammonia producer. Remarkably, both *Pseudomonas* sp. J153 and J154 strains are lacking both proteases and cellulase activities, usually required to degrade the cell walls of phytopathogens (*Hameeda et al., 2008; Nagpure, Choudhary & Gupta, 2014*). These activities are required during plant-microbe interactions and in roots intercellular colonization (*Ma et al., 2011*). The cellulase activity was restricted only to *Pseudomonas* sp. J10, *B. cereus* J156 and *P. xylanexedens* J155. Despite that *P. xylanexedens* J155 is the lowest in terms of IAA and ammonia production and only intermediate in term of P solubilization, it stimulates wheat seedling, root and shoot development. This finding is in-lane with a report showing that *Paenibacillus* significantly increased both dry and fresh weight of inoculated wheat (*Zhao et al., 2015*). Interestingly, *B. cereus* J156 strain promotes wheat germination, seedlings growth, shoot length, shoot dry weight and seedling vigor index, a finding in-line with other studies (*Raju et al., 1999; Raj et al., 2003; Raj, Shetty & Shetty, 2004; Kamran, Shazia & Shahida, 2010*), in which Triticum aestivum's seeds germination was increased following inoculation with either *Pseudomonas* or *Bacillus* strain (*Kamran, Shazia & Shahida, 2010; Bal et al., 2013*).

As the soil is a heterogeneous habitat and represents a broad spectrum of different ecological niches, it is well admitted that bacterial resistance to antibiotics facilitates their survival among the microbiome communities. Except for *B. megaterium* strain J11 and *B. cereus* J156, all other strains were resistant towards at least one of the six tested antibiotics. Soils are important reservoirs of diverse antibiotic resistance genes that can increase rapidly in clinical settings through horizontal gene transfer. Therefore, agricultural soils could play a major role in antibiotic resistance transmission. Additionally, a previous report has provided evidence for the exchanges of antibiotic resistance genes between soil (environmental) bacteria and clinical pathogens (*Ramakrishna, Yadav & Li, 2019*). To enable a sustainable agriculture and an effective antibiotic policy, it become necessary to unravel the conditions modulating the abundance of resistance genes in their microbial environment (*Kang et al., 2017*). Based on our results, and to avoid future use of bacteria conferring multi-resistance to antibiotics, the two *bacillus* strains *B. megaterium* J11 and *B. cereus* J156 that are sensitive to antibiotics, may represent potential safer use as biostimulants candidates.

Here, we also addressed the capacity of our strains to grow under increasing concentrations of heavy metals such as cadmium sulfate, nickel nitrate and copper sulfate. Pollution by heavy metals is considered as the main contaminants of our food supply, especially crop production *(Chauhan & Chauhan, 2014*). Contamination of plants occurs through the absorption of heavy metals from the soil, air, and water (*Sharma, Agrawal & Marshall, 2008; Singh et al., 2010*). Consequently, several adverse effects due to heavy metals pollution are considered as a great concern to public health, environmental health, and agricultural production *(Fergusson, 1990; Msaky & Calvet, 1990; Ma et al., 1994; Goyer, 1997*). Compared to literature (Chauhan & Solanki, 2015), our

strains *Pseudomonas* sp. J10, *B.cereus* J156, and *Pseudomonas* sp. J153 tolerate higher concentration of heavy metals. The ability to adapt to heavy metal stress by developing various resistance mechanisms is partly mediated by bacteria production of intracellular metal binding proteins (*Hashem & Abed, 2002*). Both *Pseudomonas* sp. J10 and *B. cereus* J156 exhibit an adaptive response against nickel nitrate at 1500 µg.mL^{-1} whereas *Pseudomonas* sp. J153 tolerated up to 1500 µg.mL^{-1} of either copper sulfate or cadmium sulfate. Moreover, these values are remarkably high compared to 200 µg.mL^{-1} defined as the minimal inhibitory concentration of Cd and Ni in relation to previously isolated soil-bacteria (*Chauhan & Solanki, 2015*). However a recent study reported that *Bacillus cereus* tolerates up to1500 µg.mL^{-1} of cadmium sulfate (*Khan et al., 2018*). Collectively, it appears clearly that *Pseudomonas* sp. J10 & J153 and *B. cereus* J156 strains possess the ability to withstand higher concentrations of heavy metals. Therefore, they are potential candidates suitable as bioremediatory agents for contaminated soils.

Conclusions

The present study aimed to isolate and to characterize PSB isolated from the jujube plant (*Ziziphus lotus* (L.) Desf.). Among the nine tested strains, *Pseudomonas* sp. J12 and *Bacillus cereus* J156, emerged as potential bioinoculants as they share multiple beneficial conventional PGP traits and promote seedlings wheat growth. In addition to *Bacillus cereus* J156, strains *Pseudomonas* sp. J10 and J153 emerged as potential candidates suitable to bioremediate heavy metals contaminated soils. Future agronomic studies on the field, using various plants, are required to explore elected bacterial strains dual role in biofertilization and in the bioremediation processes.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests
The authors declare there are no competing interests.

Author Contributions
Nidal Fahsi designed and performed the experiments, analyzed the data, and wrote the first draft of the paper and drawed figures and tables.
Ismail Mahdi designed and performed some experiments and analyzed the data.
Abdelhalem Mesfioui supervised the administrative Nidal Fahsi thesis' work.
Latefa Biskri conceived the experiments, authored and reviewed drafts of the paper.
Abdelmounaaim Allaoui authored and reviewed drafts of the paper and approved the final draft.

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665

Figure 1

Phosphate solubilization by isolated strains.

Concentrations of solubilized P released in NBRIP broth of isolated PSB strains and the resulting pH of culture supernatants indicated by the blue line. C (Negative control: non-inoculated medium), RT (*Rhizobium tropici*: positive control). The values represent means of 3 replicates (n=3) ± standard deviations. Different letters indicate significant differences at $p < 0.05$.

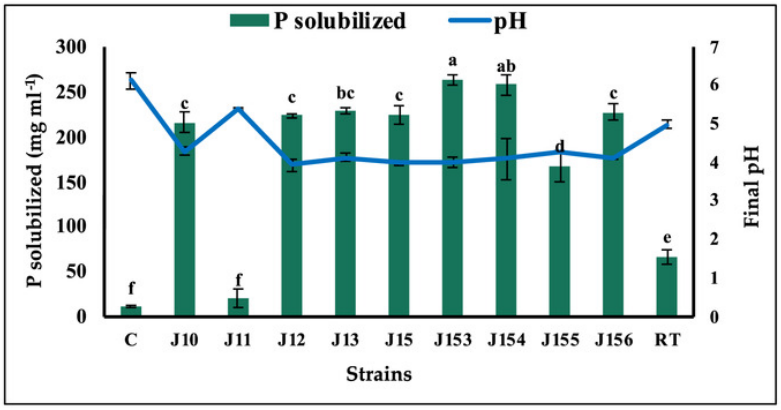
P solubilized
pH
P solubilized (mg ml-1)
Final pH
300
250
200
150
100
50
0
7
6
5
4
3
2
1
0
f
c
f
c
bc
c
a
ab
d
c
e
C
J10
J11
J12
J13
J15
J153
J154
J155
J156
RT
Strains

Figure 2

Phylogenetic tree of isolated strains.

Neighbor-joining phylogenetic tree showing relationship between the selected PSB from jujube soil and their representative species from NCBI database, built using UGENE Software. Multiple alignment of 16S rRNA gene sequences was performed using NCBI database

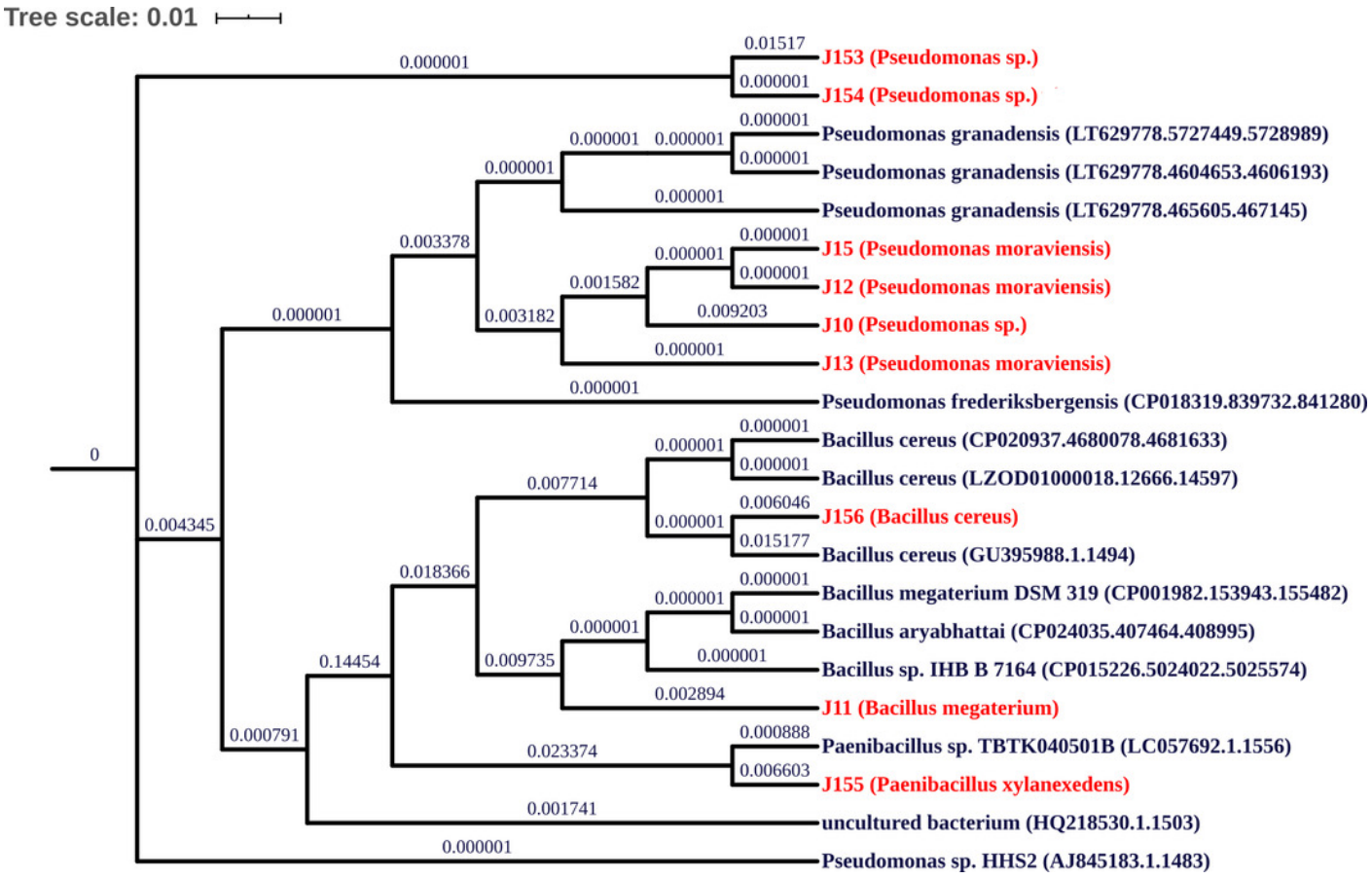

Figure 3

Indole acetic acid production by isolated strains.

Indole acetic acid production by selected PSB in the TSB broth amended with 0,1% of L-tryptophan. C (Negative control: non-inoculated medium). The values represent means of 3 replicates (n=3) ± standard deviations. Different letters indicate significant differences at $p < 0.05$.

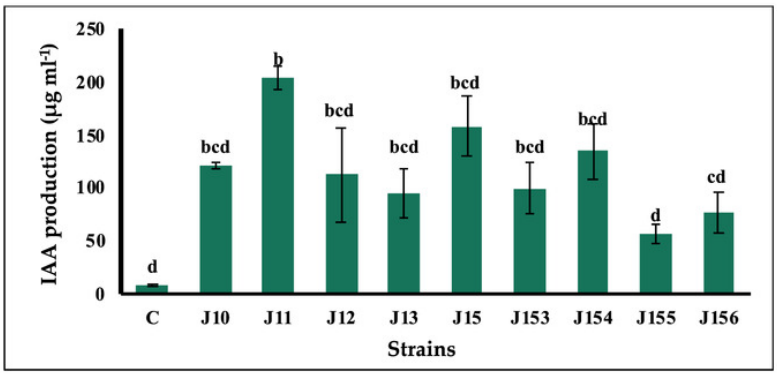
IAA production (µg ml-1)
250
200
150
100
50
0
d
bcd
b
bcd
bcd
bcd
bcd
bcd
d
cd
C
J10
J11
J12
J13
J15
J153
J154
J155
J156
Strains

Figure 4

Ammonia production by isolated strains.

Ammonia production by selected PSB. C (Negative control: non-inoculated medium). The values represent means of 3 replicates (n=3) ± standard deviations. Different letters indicate significant differences at $p < 0.05$.

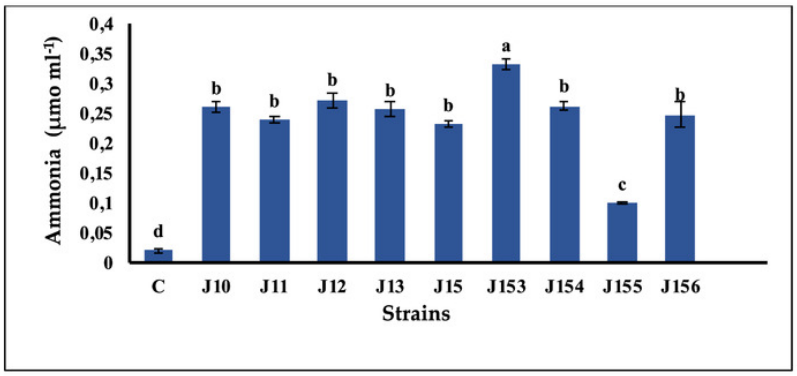
Ammonia (µmo ml-1)
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
d
b
b
b
b
b
a
b
c
b
C
J10
J11
J12
J13
J15
J153
J154
J155
J156
Strains

Figure 5

Effect of bacterial inoculation of wheat seed germination parameters.

Effect of bacterial inoculation of wheat seed germination parameters. (**A**) Germination rate after 24 and 48 h of incubation (**B**) Total length of shoots and roots after 7 days, (**C**) Fresh weight of shoots and roots after 7 days, (**D**) Dry weight of shoots and roots after 8 days, (**E**) Seedling vigor index. c (Negative control: no-inoculated seeds). Different letters indicate significant differences at $p < 0.05$.

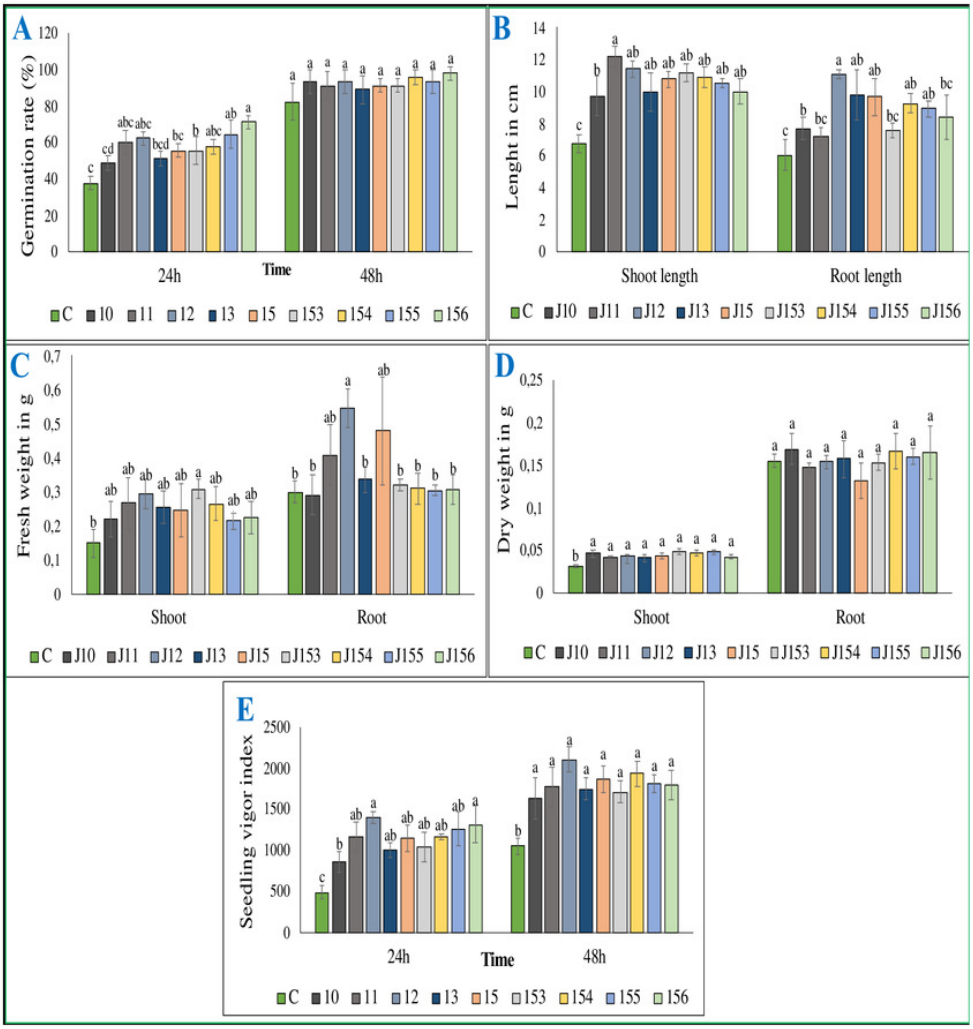
A
Germination rate (%)
Time
24h
48h
C J10 J11 J12 J13 J15 J153 J154 J155 J156
B
Lenght in cm
Shoot length
Root length
C J10 J11 J12 J13 J15 J153 J154 J155 J156
C
Fresh weight in g
Shoot
Root
C J10 J11 J12 J13 J15 J153 J154 J155 J156
D
Dry weight in g
Shoot
Root
C J10 J11 J12 J13 J15 J153 J154 J155 J156
E
Seedling vigor index
Time
24h
48h
C J10 J11 J12 J13 J15 J153 J154 J155 J156

Figure 6

Effect of studied strains on shoots and roots growth.

Effect on shoots and roots growth after seven days of inoculation of wheat seeds by the four *pseudomonas* strains: *P. moraviensis* J13, *Pseudomonas* Sp. J10, J153 and J154 (C-:control non-iniculated seeds).

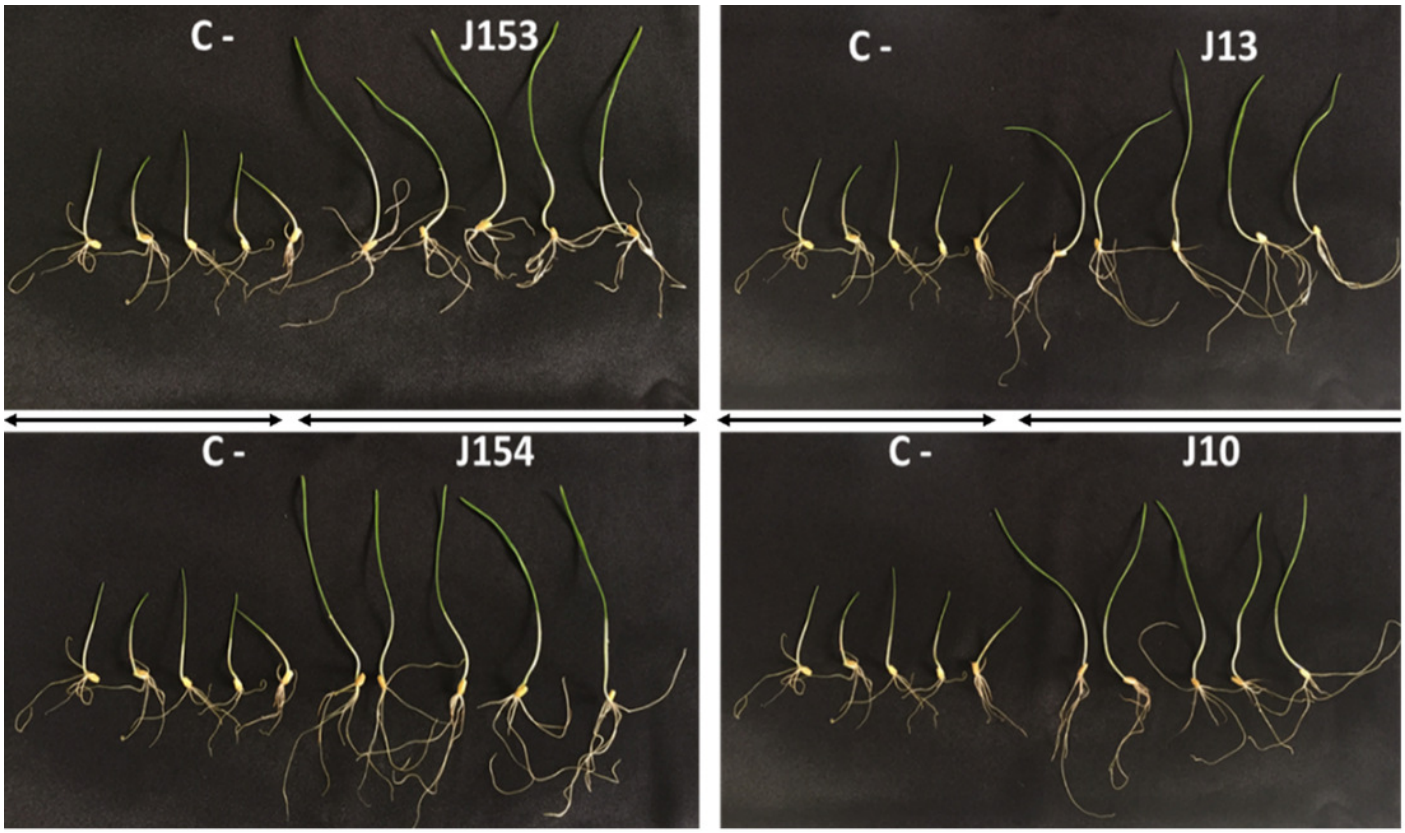

Table 1(on next page)

Selected physicochemical properties of the studied Jujube plant soil samples.

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2 **Table 1**. Physicochemical properties of the studied soil samples.
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Parameters		Results
Granulometry (%) (AFNOR NF X31-107)	Clay	21,00
	Fine silt	21,30
	Coarse silt	11,50
	Fine sand	21,50
	Coarse sand	22,60
Exchangeable elements (mg/kg) (AFNOR NF X31-108)	K_2O	749
	MgO	431
	CaO	8071
	Na_2O	208
pH-H_2O (NF ISO 10390)		8,7
Electrical conductivity mS/cm (NF ISO 11265)		0,15
Total limestone (%) (NF ISO 10693)		2,50
Organic matter (%) (NF ISO 14235)		2,92
Oslen Phosphorus (mg/kg) (NF ISO 11263)		17

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Table 2(on next page)

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

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

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

Isolate	Closest species	Identity (%)	Accession No.
J10	*Pseudomonas* sp.	98	MT771625
J11	*Bacillus megaterium*	98	MT771626
J12	*Pseudomonas moraviensis*	98	MT771627
J13	*Pseudomonas moraviensis*	98	MT771628
J15	*Pseudomonas moraviensis*	98	MT771629
J153	*Pseudomonas* sp.	99	MT771630
J154	*Pseudomonas* sp.	98	MT771631
J155	*Paenibacillus xylanexedens*	98	MT771632
J156	*Bacillus cereus*	99	MT771633

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Table 3(on next page)

Summary table of relevant phenotypic traits observed in selected PSB using plate assay.

The '+' and '-' signs indicate efficiencies as follow: -, negative result; +, weakly positive; ++, moderately positive; +++, highly positive. The 'R' means resistance to antibiotic. Final concentrations: chloramphenicol (Cm) 20 µg.mL-1; kanamycin (Kan) 50 µg.mL-1 ampicillin (Amp) 100 µg.mL-

Table 3. Summary table of relevant phenotypic traits observed in selected PSB using plate assay.

Strain	Antibiotic Resistance	Siderophores production	Extracellular Enzymes	
			Proteases	Cellulase
Pseudomonas sp. J10	Amp^R, Cm^R, $Spect^R$	++	+	+
B. megaterium J11	-	+	+	-
P. moraviensis J12	Amp^R, Cm^R	++	+	-
P. moraviensis J13	Amp^R, Cm^R, $Spect^R$	+++	+	-
P. moraviensis J15	Amp^R, Cm^R	++	+	-
Pseudomonas sp. J153	Amp^R, Cm^R	++	-	-
Pseudomonas sp. J154	Amp^R, Cm^R	++	-	-
Paenibacillus xylanexedens J155	Kan^R, $Spect^R$	++	+	+
B. cereus J156	-	+	+	+

Table 4(on next page)

Heavy metals tolerance of selected PSB strains

+ for tolerance, - for sensitivity

Table 4. Heavy metals tolerance of selected PSB strains

Strain	$CuSO_4.5H_2O$ (mg.L-1)				$CdSO_4$ (mg.L-1)				N_2NiO_6 ($mg.L^{-1}$)			
	0.3	0.5	1	1.5	0.3	0.5	1	1.5	0.3	0.5	1	1.5
Pseudomonas sp. J10	+	+	-	-	+	-	-	-	+	+	+	+
B. megaterium J11	+	-	-	-	-	-	-	-	+	+	-	-
P. moraviensis J12	+	+	-	-	+	-	-	-	+	+	-	-
P. moraviensis J13	+	+	-	-	+	-	-	-	+	+	-	-
Pseudomonas sp. J153	+	+	+	+	+	+	+	+	+	+	-	-
Pseudomonas sp. J154	-	-	-	-	+	-	-	-	+	+	-	-
Paenibacillus xylanexedens J155	+	-	-	-	-	-	-	-	+	-	-	-
B. cereus J156	+	+	+	-	+	-	-	-	+	+	+	+
E. coli DH5α	+	+	+	-	+	-	-	-	+	+	-	-

+ for tolerance, - for sensitivity