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Research Article

Effect of endophytic bacteria from Algerian prickly pear roots on wheat under drought stress

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Abstract
Cactus are among the most drought tolerant plants. As Opuntia is able to grow under the stress of drought, this study aims to check if endophytic bacteria isolated from cactus roots have beneficial potential for crops such as wheat during drought. Two endophytic bacterial isolates were isolated from the roots of the cactus and screened for their plant growth promoting characteristics, such as N-free growth and auxin production. These bacteria have demonstrated their potential to promote the growth of durum wheat under *in-vitro* conditions and have been identified as *Pseudomonas putida* and *P.brassicacearum*, following the sequencing of the 16S rRNA gene and phylogenetic analysis, and significantly improved growth parameters such as seeding length compared to the unobstructed control. After 05 days of contact of the two bacteria, *P.putida* and *P.brassicacearum*, with sprouted wheat seeds, a root growth rate of (39.88% and 62.14%, respectively) was recorded. The same effect on the growth of wheat roots is caused by the volatile substances of these bacteria deposited separately, with a rate of (53.30% and 24.18%) respectively. Symptoms of drought stress were visibly reduced on seedlings inoculated with *P.putida* and *P.brassicacearum* bacteria, a result supported by a growth rate of root parameters in length (260.83% and 179.60%), surface (21.98% and 60.17%) and scope (59.46% and 62.67%), respectively. This work opens up many perspectives for the characterization and selection of endophyte bacteria of under-used drought-tolerant species such as cacti for the improvement of the growth of field crops. These results promote the deployment of *Pseudomonas sp* as an effective biofertilizer in wheat.

Keywords: Endophytes, *Opuntia ficus-indica* L., Plant growth-promoting rhizobacteria, Drought, Pseudomonas, Wheat

INTRODUCTION

Endophytic bacteria that inhabit the inside of plant tissues with no visible damage to the host and promote plant growth directly or indirectly by a combination of pathways are regarded as beneficial endophytes for plants (Rosenblueth and Martínez-Romero 2006; Compant *et al*. 2010).

All plants and their associated microbial have evolved together to adapt to a given ecosystem (Rodriguez and Redman, 2008). In stressed environments, plants establish favorable rhizospheric conditions by recruiting and attracting beneficial microbial communities by the production of root exudates (sugars, amino acids, organic acids and signaling molecules) (Singh, 2015; Tiwari and Singh, 2017). Drought is one of the most important abiotic stresses adversely affecting significantly agricultural sustainability. The morphophysiological and biochemical traits related to drought stress include leaf wilting, reduction in leaf area, chlorophyll content and root elongation (Lata *and Prasad,* 2011). Under such soil conditions, the ecological role of Plant growth-promoting rhizobacteria (PGPR) is more obvious due to their positive effects, such as enhanced nutrient use efficiency and tolerance to biotic or abiotic stress in plants.

Several PGPR which are in close association with the root zones are reported to induce drought stress tolerance in some plants such as wheat, maize, sunflower, sugarcane and green gram (Sandhya *et al*., 2009, 2010; Moutia *et al*., 2010; Vardharajula *et al*., 2011; Saravanakumar *et al*., 2011; Kasim *et al*., 2013). *Pseudomonas* sp. is one of the largest groups of PGPR, which naturally occur in agricultural soils and known to possess several phytobeneficial traits (Srivastava *et al*., 2012). Tiwari *et al*. (2016) has been repoted that *Pseudomonas putida* (MTCC5279) ameliorated drought stress in chickpea (*Cicer arietinum*) plants by modulating membrane integrity, osmolyte accumulation (proline, glycine betaine) and ROS (Reactive oxygen species) scavenging ability.

In North Africa, *several programs involve direct alleviation of desertification by restoring vegetation cover with drought-resistant perennial forage species, among of them, Opuntia ficus-indica L. which is* commonly known as prickly pear, belongs to the plant family Cactaceae. This xerophytic species is highly water use efficiency through the development phenological, physiological and structural adaptations for growth and survival in arid environments (Nefzaoui *et al*., 2014; Mangalassery *et al*. 2017). Cacti develop an association with niche soil microbes that could also contribute to the ability to overcome these stressful conditions (Fonseca-García *et al*., 2016). Among them are actinobacteria belonging to Gram-positive bacteria (Govindasamy *et al*., 2022). Species such as cacti and their associated microbes are less explored, especially for PGPR activities of their bacteria. Therefore, the present study was conducted to isolate endophytic bacteria from the roots of cactus plants and to investigate the efficacy of PGPR strains on wheat under drought stress.

MATERIALS AND METHODS

Root samples of *O.ficus-indica* L. were collected from the USTOMB University campus (3542'28.6"N 034'45.6"W), located in Oran (north-west Algeria). In this region, average temperatures for the warmest and coldest month are 31°C and 5.1°C, respectively. The climate is semi-arid, with average precipitation ranging from 300 to 400 mm. Wheat caryopses (*Triticum durum*) were collected from the region of el Guettar W. de Relizane, West of Algeria (36°04'03.6"N 0° 48'28.0"E)

Isolation and molecular characterization of bacteria from *O. ficus indica*'s roots

After washing the roots of *O. ficus indica* L. with tap water, then with sterile distilled water. They are immersed in 70% ethyl alcohol for 20 to 30 minutes for sterilization, then in a 2.6% sodium hypochlorite solution for 5 min. The roots were then washed 8-10 times with sterile distilled water. Then cut, crushed with sterile pliers and spread on solid YEM medium (mannitol 10 gL 1; yeast extract 0.12 gL 1; NaCl 1 gL 1; $MgSO_4$0.2 gL 1; K_2HPO_4 0.5 gL 1; gelose 20 gL 1; pH 6.8) and incubated at 28°C.

Individual colonies were collected, purified by repetitive striations from YEM medium and stored in glycerol at 30–40% at -80°C for later molecular identification. DNA from the isolates (E1-3)and E2 was extracted and amplified using primer 16S-27f and 16S-1492r, then PCR was performed by a 96°C denaturation; and a final extension at 72°C for 10 minutes with primers: Primer 16S-27F and 16S 1492R. then purification of the amplicons was done using the ExoSap- It kit. After PCR analysis. The Big Dye V3.1 Dye Terminator Kit (Thermo Fisher Scientific) was used for sequencing, and the results were compared to existing sequences using the MEGA 11 program, with the WEB tool on the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov) to obtain the homology.

Preparation of the inoculum

For the preparation of the inoculum, both bacterial strains were streaked on a solid LB medium (10 g.L1 Bacto-tryptone, 5 $g.L^{-1}$ Yeast Extract, 5 $g.L^{-1}$ NaCl, 15 $g.L^{-1}$ Agar, pH 7). After 24 h at 28°C, bacterial cells were collected in 10 mM $MgSO_4$, washed twice with 50 mL of 10 mM $MgSO_4$ by centrifugation for 5 min at 5000 rpm, and resuspended in 50 mL of 10 mM $MgSO_4$.

The bacterial titer was adjusted to an OD600 nm of 0.002 to obtain an inoculum with a bacterial density of 2×106 Colony forming units.mL−1 (CFU.mL−1). This bacterial density was confirmed by counting the number of CFU on LB medium for all experiments.

Effects of bacterial strains on plant growth

Drought stress

The surface of the durum wheat caryopses was sterilized in 10% NaCl for 10 minutes. Then after several rinsing with sterile water, we launched the germination on filter paper sterilized in petri boxes at room temperature (25°C). After 24h, potting (12cm tall and 10cm in diameter, containing sterilized peat), sprouted seeds grown in a growth chamber for 07 days (30°C, 300µ mol/m²s fluorescent bulb and 12 hours of darkness). Then inoculated the seedlings with 80 µl of bacterial substance (inoculum) with water retention for 10 days (drought stress).

The plants were examined against the control for symptoms of drought such as leaf wilt, curling and marginal necrosis.The plants have been carefully removed from the soil to preserve the root system intact. Then, the roots were rinsed with distilled water (removal of soil residues). To estimate their length, perimeter and surface, the roots were spread on a tray (40cmx50cm) to position them (05 roots per tray) using the IMAGEJ application and different measurements (Length, area and perimeter) were taken. The statistical study of the results was carried out with ANOVA with P=0.05.

Physical contact of bacteria

Using experimental in vitro analysis to study the effects of bacteria promoting plant growth of durum wheat caryopses, they were deposited on MS medium (1.36 g K_2HPO_4, 2.13g Na_2HPO_4, 0.2g $MgSO_4.7H_2O$ in 1l distilled water). After 05 days of germination, the seedlings were inoculated, and after 07 days of inoculation, root lengths were measured.

Physical contact of volatile substances

Using the same experimental system, bacteria strains were physically separated from wheat seedlings. After 05 days of germination, phenotypic effects could only be triggered by volatile compounds. Then, after 07 days, the root length was measured.

Effect of temperature and salinity

The influence of temperature on the growth of the two bacteria E1 and E2 was evaluated on solid LB medium (Tryptone: 10g, yeast extract, 5g, Sodium chlorure NaCl: 5g, Agar: 15g, 1l of distilled water), at a temperature of 7°C to 55°C, during 48 hours of incubation.

Different concentrations of NaCl were added: 2%-5%-7%-9% and 10% of the solid LB medium to assess the effect of salinity on the growth of bacterial strains, seeded and incubated for 72 hours at 28°C.

IAA production and nitrogen fixation

The bacterial strains were tested on MS media, adding tryptophan (5 mM), (Fluka) (Khalid *et al*., 2004).

Tryptophan was added after sterilization of the stock solution (100 mM) through a 0.22µm porosity membrane. The 10 ml tubes are inoculated in 100 µl with different bacterial suspensions, a fresh culture (24h). After incubation at 28°C/48h under agitation (120 rpm), the cultures are centrifuged at 3000 rpm/15min. 1 ml of each obtained supernatant is mixed with 2 ml of Salkowski's reagent (2% $FeCl_3$ (0.5 M) in a 35% solution of perchloric acid). The appearance of pink colour indicated the presence of IAA. The amount of the latter is determined by reading the absorbance at 530 nm against a control (1 ml of non-inoculated medium + 2 ml of Salkowski reagent). This test was performed more than 03 times.

RESULTS AND DISCUSSION

After alignment of the gene sequences with other gene sequences of bacterial species (Fig.1)and with an Outgroup Acinetobacter baumannii LN611374, a phylogenetic tree was realized, and the belonging of endophytic isolates was more than 99% homology with the sequence of *P.brassicacearum* (E2), and 98% with the *P.putida* (E1-3).

The Fasta file of the two (E1-3) and (E2) strains was submitted to the NCBI gene bank and accession numbers were assigned to them (E1-3:ON044991 and E2:ON044992) (Fig. 1). Inoculation of the wheat seedlings by the two bacterial strains (*P.putida* and *P.brassicacearum*) alleviated the symptoms of water stress. The qualitative evaluation of plants through repeated experiments compared to the control, proved that both strains tested were effective (Fig 2. Based on the results obtained by the ANOVA analysis, the root systems of wheat treated with both bacteria were more branched than the control Fig.3 (perimeter, area). Treatment with both strains contributed significantly to root growth compared to the control (Table 1, Fig 5.)

It is clear that the length of the roots was almost doubled. The most important result was obtained with the bacterium *P.putida* (30.18cm) and *P.brassicacearum* (24.65cm) compared to the control (8.81 cm) with $P=1.52x10^{-19}$.

The results obtained on the root systems inoculated by *P. putida* (260.83%) were significantly more important than those of the plants inoculated by *P.brassicacearum* (179.7%) , also visible on the root surface (21.98%, 60.17% with $P=2.15x10^{-03}$) and perimeter (59.46%, 62.67%, with $P=8.25x10^{-04}$) respectively for *P.putida* and *P.brassicacearum* (Table 2, Fig. 6).

Table 1. Average length, area and perimeters of ten samples of wheat root

	Length (cm)	Surfaces (cm²)	perimeter (cm)
P. putida	31.80	8.55	102.43
P. brassicacearum	24.65	11.22	104.49
Control	8.813	7.005	64.234

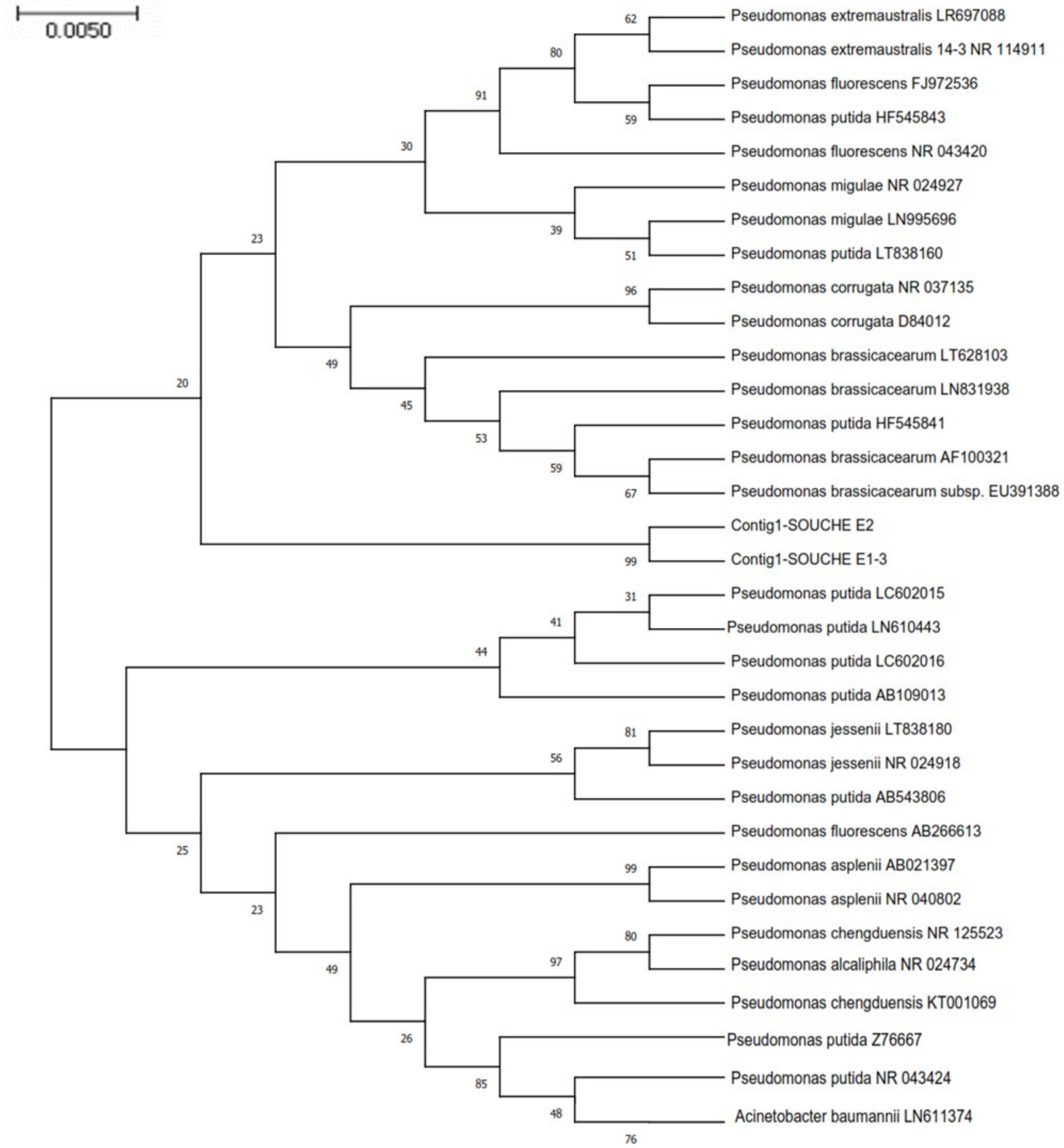


Fig. 1. *Phylogenetic tree based on a comparison of the 16S rRNA gene sequences of the isolated bacteria E1-3 and E2 and strains of related species. The tree was constructed by the neighbour-joining method and rooted using Acinetobacter baumannii LN611374 as an outgroup. Bootstrap values for 1000 replicates are shown. Bar, 5 nt changes per 100 nt.*

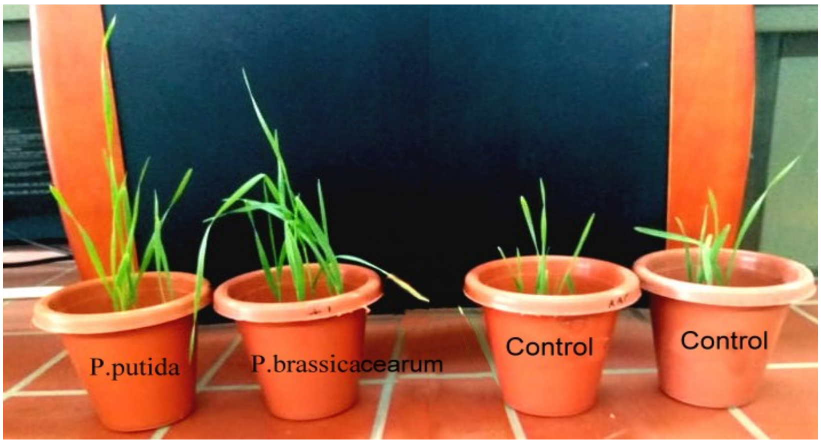
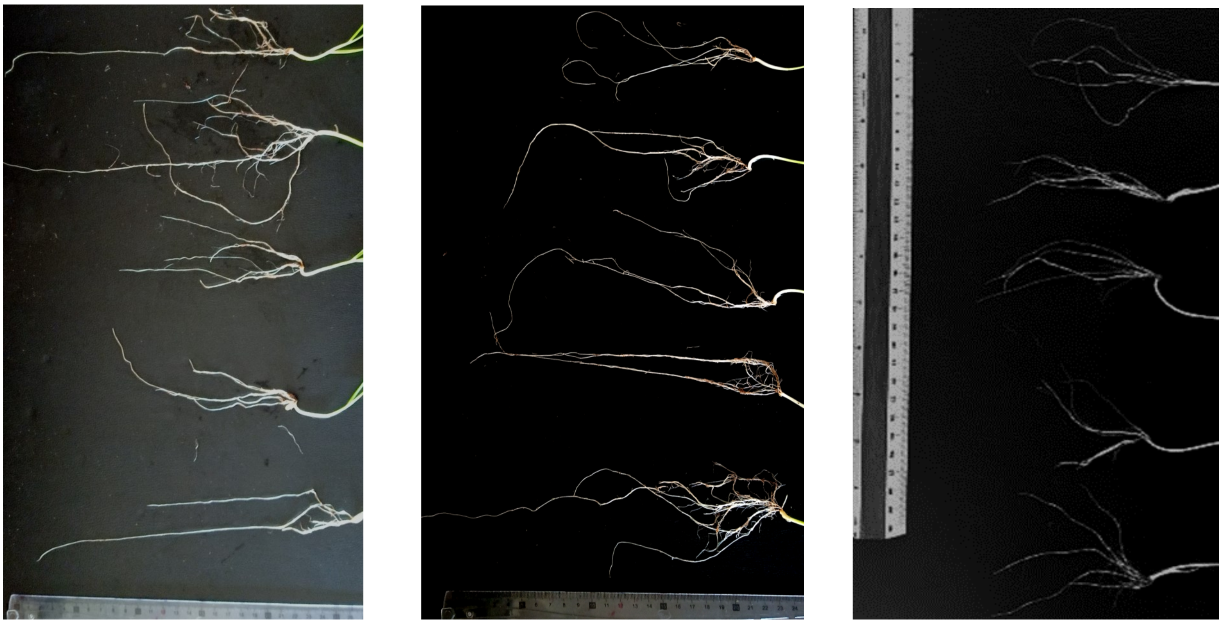


Fig. 2. *Morphological appearance of wheat treated by both bacteria (P.putida , P.brassicacearum).*



P.brassicacearum **—** ***P.putida*** **—** **Control**

Fig. 3. *Morphological appearance of the roots of wheat treated by the two bacteria (P.putida , P.brassicacearum).*

In present study, wheat experienced a delay in the onset of drought symptoms; when treated with both bacterial strains, visual evaluation of plant performance suggests that *P. putida* is more effective in alleviating drought symptoms. Larger root systems in terms of length and number of branched roots have been previously proven with better tolerance to water stress and improvements in maintaining plant productivity (wheat and maize), in times of drought (Michael *et al*., 2019).

Previous research has shown that root diameter reduction can allow for faster relative growth rates and rapid resource acquisition through root system expansion coupled with less investment in dry biomass (Birouste *et al*., 2014; Wahl and Ryser, 2000). Production of significant length and surface area has been shown to be better tolerant to water stress (Comas *et al*., 2013). The proliferation of higher-order roots results in significant water absorption capacity (Naseem and Bano, 2014; Bernawal *et al*., 2017). Tolerance to water stress by PRMPs depends on effective root colonization (Drogue *et al*., 2012, Klopper et al., 1996).

For IAA production and nitrogen fixation, both strains produced indole acetic acid with different ranges for each strain (35.1µg/l) for *P.putida* , and (48.2 µg/l) for *P.brassicacearum*, and gave positive results for nitrogen fixation (Table 3).

In similar studies, it is assumed that more than 80% of PGPR bacteria isolated from the rhizosphere can produce IAA and N_2 fixation (Patten and Glic 1996, Duca *et al*. 2004). Plants treated with *P.putida* GR12-2 PGPR-producing plants have been shown to have longer roots than untreated plants (Ngumbi, E., and Klopper 2016).

After inoculation of wheat caryopses (*Tritticum durum*) by the two bacterial strains *P.putida* and *P.brassicacearum* in the MS medium-containing petri boxes, in this experimental condition (the two strains in physical contact with the roots of the seedlings, which included the supposed action of diffusible and volatile substances, the two strains showed properties favouring the growth of plants but to a degree differ (Fig.4). *P.putida* and *P.brassicacearum* improved the roots with a length ratio of (39.88%, 62.13%) respectively, compared to the control, with positive and significant results (P<0.0001) on the length of the roots compared

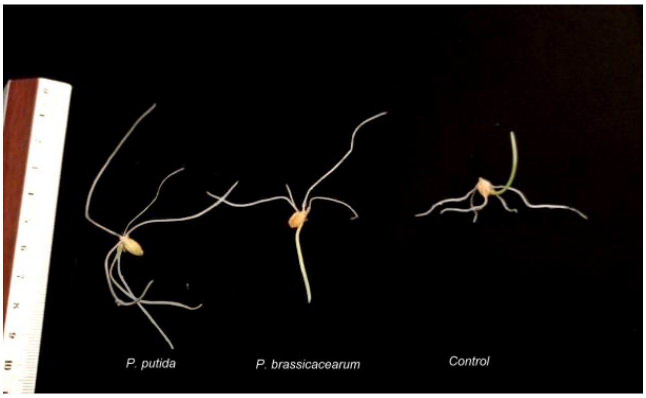


Fig. 4. *Physical contact of the inoculum with the sprouted seeds of the wheat*

Table 2. Growth rate of measurement parameters

Growth rate	Length (%)	Surfaces (%)	Perimeter (%)
P. putida	260.83	21.98	59.46
P. brassicacearum	179.7	60.17	62.67

Table 3. Growth of bacteria under different parameters and production of IAA

	T°	growth	salinity %	growth	N_2 Fixation	IAA Production (µg/ml)
P.putida	5	+	2	+++		
	10	++	5	+++		
	15	+++	7	+++		
	20	+++	9	+	+	35.1
	28	+++	10	-		
	45	+++				
	50	++				
	52	--				
P.brassicacearum	5	+	2	+++		
	10	++	5	-		
	15	+++	7	-		
	20	+++	9	-	+	48.2
	28	+++	10	-		
	45	+++				
	48	+				

T° Temperature, N_2 Nitrogen fixation, IAA Indol Acetic Acid, + Tolerance, - No tolerance

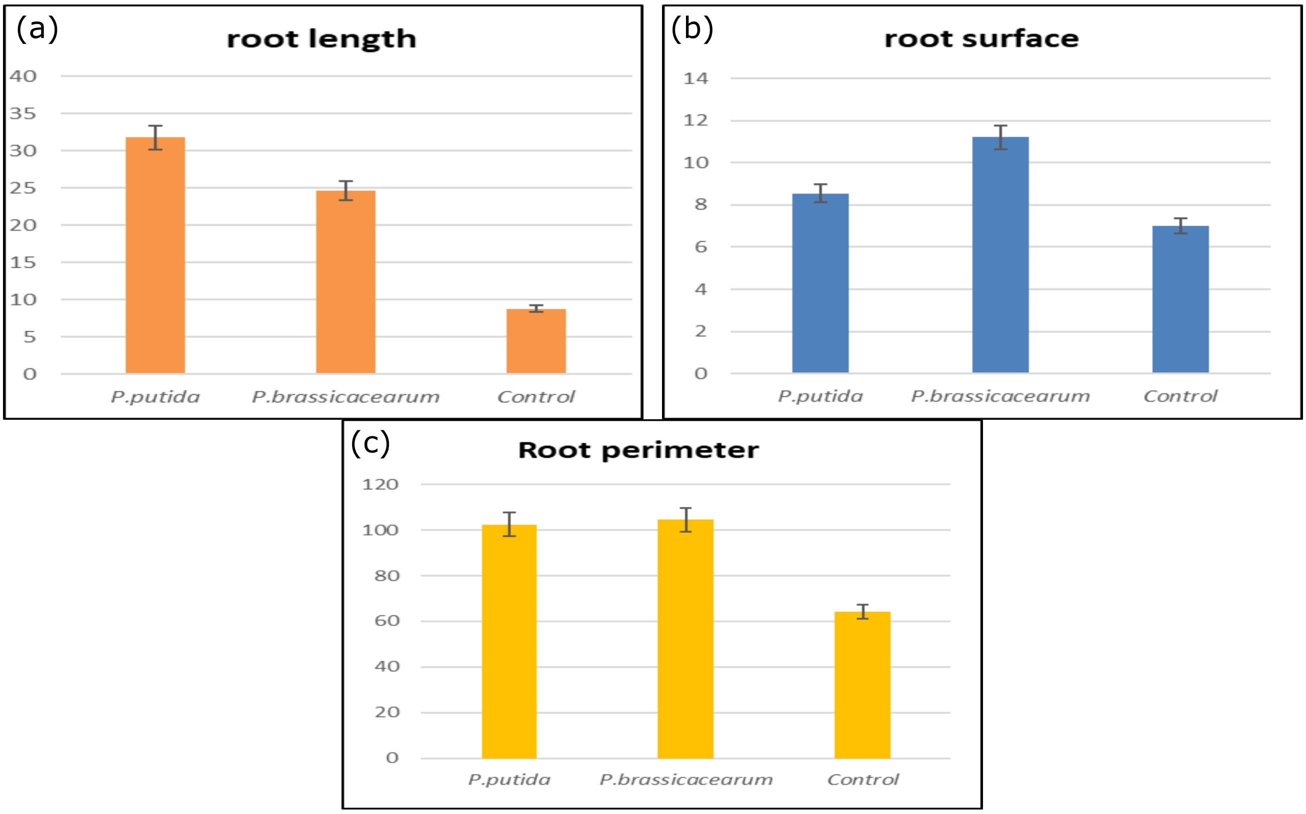


Fig 5: Effect of water stress on length (a), surface (b) and perimeter of durum wheat roots (*Triticum durum*).

to the control (Fig.7),

In the second *in vitro* experimental system in which the two bacterial strains were physically separated after seven days of germination, in this condition where the phenotypic effects observed could only be triggered by the volatile substances of the bacteria, these two strains led to beneficial effects on root growth rate (53.29%, 24.17%) by *P.putida* and *P.brassicacearum*, respectively compared to control, with (P<0.0056) Finally, both strains emit volatile substances to improve the wheat root system (Fig.7).

In our experiments, both strains showed marked activities of promoting plant growth both in physical contact with the roots of wheat caryopses and through the production of volatile substances.

The *P. putida* strain showed relatively light plant growth -promoting effects when the roots were in contact with the volatile substances of this bacterium, in agreement with previous studies carried out on different plants inoculated and grown *in vitro* (Ledger *et al*., 2016); with

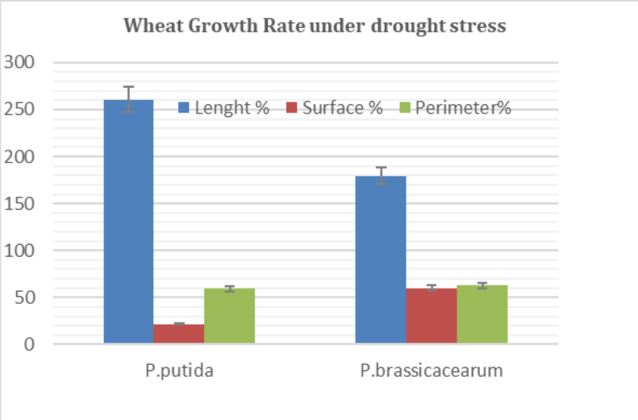


Fig. 6. *Growth rate of measurement parameters*

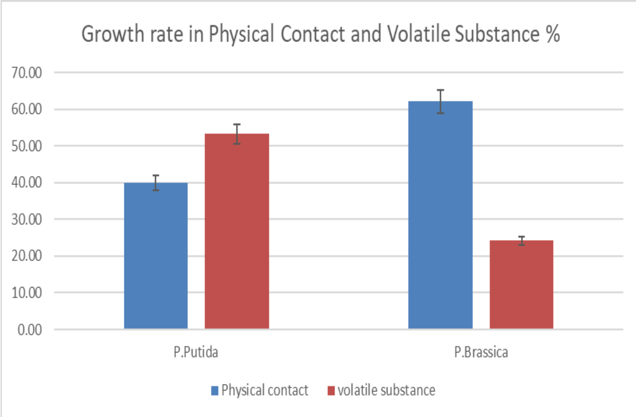


Fig. 7. *Effect of Bacteria in Physical Contact with Wheat Roots and in contact with their volatile substances*

rhizobacteria, *P.simiae* PICF7 and *Burkholderia phytofirmans* PSJN, improved the growth and development of Arabidopsis seedlings. The present study could allow to develop effective PGP screening tools for their ability to reduce the water stress symptoms of plants treated with the two bacterial strains *P. putida* and *P. brassicacearum* when applied at the beginning of the conditions of water deficit.

Conclusion

In this study, two bacterial strains were isolated from the roots of *O. ficus indica* L., and selected for molecular identification by rRNA sequencing. Genetic analysis revealed that these bacteria belonged to the genera *P. putida* and *P. brassicacearum* with accession numbers ON044991 and ON044992, respectively. The study helped advance knowledge of the PGP (Plant Growth Promoter) trait of these bacteria on wheat growth and their ability to alleviate symptoms of drought stress. These findings could also assist regulators and industry in assessing these bacteria as biofertilizers. The application of chemical fertilizers has a negative impact on the environment in addition to an ever-increasing cost, the use of biofertilizers could be a promising alternative. These increases are generally due to better nutrient removal and absorption and the production of phytohormones. Therefore, it would be desirable to improve our knowledge of the possibility of the persistence of these bacteria in wheat, which could affect the growth of the edible part (seeds).

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Conflict of interest
The authors declare that they have no conflict of interest.

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