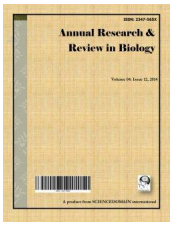

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Halotolerance of Indigenous Fluorescent Pseudomonads in the Presence of Natural Osmoprotectants

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Authors' contributions

This work was carried out in collaboration between both authors. Author MG designed the study. Author FA wrote the protocol and the first draft of the manuscript and managed the analyses of the study. Author FA managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Salinity is one of the major factors affecting agriculture. To grow in saline environments, bacteria and plants have to adjust their turgor pressure by accumulating compatible solutes as glycine betaine and proline. Inoculation of plants of economic interest, mainly wheat, by Plant Growth Promoting Rhizobacteria such as *Pseudomonas* species is an effective biological approach for the recovery of soils affected by salt.
Methodology: The halotolerance of indigenous *Pseudomonas* strains was tested in the presence of high salt concentrations. Under these stress conditions, the effect of natural osmoprotectant molecules elaborated by the halophyte *A. halimus* was observed.
Results: In this study, 3 Fluorescent pseudomonads were isolated from wheat rhizosphere and one from the endophyte of *Atriplex halimus*. They were identified as *P. putida* AF2, *P. aeruginosa* RB5, *P. fluorescens* RB13 and *P. aeruginosa* EH4; they exhibited good PGPR activities. The growth of

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the strains was stimulated in the presence of 100 and 300 mM of NaCl. *P. fluorescens* CHA0 was inhibited at 500 mM; the remaining strains were affected by 800 mM. Exogenous supply of glycine betaine and proline alleviated the stress. The extract of the halophyte *A. halimus* restored the growth of 3 strains. NaCl/ 900 mM was strongly inhibitor of all bacteria. The restoration of the growth of *P. aeruginosa* RB5 and *P. aeruginosa* EH4 by glycine betaine or proline was significant. No osmoprotectant molecule could overcome stress imposed by 1000 mM.

Conclusion: On the basis of their halotolrance and their ability to use natural osmoprotectant to restore their growth, the PGP fluorescent pseudomonads strains tested could be applied as inoculants of wheat for sustainable agriculture in salty soils.

Keywords: A. halimus; glycine betaine; PGPR; proline; osmoprotectants.

1. INTRODUCTION

Salinity is one of the major factors affecting agriculture; about 10% of the total area of the globe is affected by salt [1]. According to Flowers and Colmer [2], most (72 %) of the surface of the earth is covered in a salt solution dominated by Na+ and Cl-. In Algeria, saline soils occupy large areas: 3.2 million hectares of the total area [3]. In order to survive and grow in saline environments, bacteria and plants have to adjust their turgor pressure and to protect the enzymes and macromolecules against environmental osmolarity fluctuations [4,5]. The accumulation of compatible solutes is the most important strategy to cope with osmolarity [2]. These are organic molecules responsible for osmotic balance and do not interfere with cell function and metabolism even though accumulated in the cytoplasm at very high concentrations (above 1M) [6,7]. The organic molecules, glycine betaine (GB) and proline (Pro) are among the most potent compatible solute used by a wide variety of microbial and plant species [8,9]. The halophyte *Atriplex halimus* accumulates the both molecules [10] and the root exudation of these compatible solutes in a medium could ensure osmoprotective function to rhizobacteria. They could indeed contribute to the survival of halosensitive bacteria in these ecosystems as proven with marine macroalgae [11].

Fluorescent *Pseudomonas* spp. are an important group of plant growth promoting rhizobacteria (PGPR). They increase the growth of their host plant directly or indirectly [12,13]. In addition, they may contribute to the improvement of inoculated plant resistance to biotic and abiotic stresses such as salinity and drought [14,15,16,17]. This characteristic is attributed to the synthesis of phytohormons such as indole-3-actic acid (IAA) [18], the release of exopolysaccharides which reduce Na^+ uptake by plants and improve water holding capacity under unfavorable environmental conditions [19]. In addition, the PGPR could produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase to lower plant ethylene levels, often a result of various stresses [20,21]. Consequently, the use of osmotolerant PGPR strains like *Azospirillum* or *Pseudomonas* as inoculants in wheat result in improved yields under saline conditions [22,23].

In the context of improving the productivity of wheat in Algeria - especially in soils affected by salt and drought-, the use of Fluorescent *Pseudomonas* species as seed inoculants, could promote growth by alleviating salt stress on plants. The ability to use natural osmoprotectant molecules coming from the halophyte(s) as *Atriplex halimus*, is a determining factor in the choice of PGPR. Thus, to determine the impact of the halophyte *A. halimus* extract on the restoration of the growth of Pseudomonas this study was carried out.

2. MATERIAL AND METHODS

2.1 Isolation of Fluorescent Pseudomonas spp.

The study was conducted on 2 samples of soil, the first belongs to the rhizosphere of durum wheat (*Triticum durum*) (fertile soil: conductivity 486 µS/cm at 20°C; pH 7.25), the second one is derived from the endophyte of the halophyte *Atriplex halimus* (saline soil conductivity: 4.09 mS/cm at 20°C; pH 7.30).

For a selective isolation of fluorescent *Pseudomonas* species, the selective Gould's S1 medium was used [24]. To isolate them from the rhizosphere of wheat, 10 g of soil strongly attached to the roots were stirred with 90 ml of sterile 0.05 M buffer phosphate (pH 7.0). Serial decimal dilutions were made from this suspension. The fluorescent Pseudomonas

species colonizing the endophyte of *Atriplex halimus* were isolated as described by Forchetti et al. [25]. An aliquot of 0.1 ml of each dilution (of each sample) was speared on Gould's S1 medium. After incubation (23°C, 3 days), the colonies with a green fluorescent pigmentation, under UV light (366 nm), were picked up and purified on King B medium.

2.2 Biochemical and Physiological Tests

Firstly, determination of macroscopic, microscopic, enzymatic characters was carried out. Different biochemical characters, allowing the selection of isolates belonging to the genus *Pseudomonas*, were determined according to the Bergey's Manual of Determinative Bacteriology [26]. These tests led to the selection of 3 isolates from the wheat rhizosphere and one from the endophyte of *A. halimus*.

2.3 Screening of PGPR Activities

The isolates were tested for their ability to promote plant growth [27] by phosphate solubilization [28], indole acetic acid production [29,30] and siderophores production [31]. The biocontrol traits were also highlighted as the production of hydrogen cyanide [32]; the antifungal capacity against two phytopathogens: *Alternaria alternata* and *Fusarium oxysporium*, was tested [33]. The reference strain *Pseudomonas fluorescens* CHA0 underwent the same tests. All the experiments were performed in triplicate.

2.4 Effect of Salt, Synthetic and Natural Osmoprotectants on Bacterial Growth

This step was designed to select the most halotolerant strains and those having a positive response to the exogenous supply of synthetic and natural osmoprotectants.

2.4.1 Detection of osmoprotectant molecules

Before testing the osmopprotectant effect of *A. halimus*, it was indispensable to demonstrate the presence of the interesting molecules namely GB and Pro. For this purpose, a thin layer chromatography (TLC) was performed. The hydro alcoholic extract was prepared as follows: 25 g of the plant dried and grounds were added to 100 ml of acetone; the mixture was stirred vigorously and then filtered through prefilter. The plant material was then recovered in 200 ml of ethanol (70%). After vigorous stirring (30 mn) and filtration, the extract was evaporated to dry under vacuum at 45°C, recovered in 5 ml of distilled water and used in TLC. The migration was carried out in two development systems: Isopropanol-water (3: 1) [34] and n-butanol-acetic acid-water (6: 2: 2, w/w) [35], for the detection of GB and proline, respectively.

Standard solutions of GB or Pro (100 mM) (Sigma) and halophyte extract (HE) were deposited at respective volumes of 5 µl and 50 µl on TLC plates. The plates were revealed by spraying the Dragendorff reagent for detecting the GB and ninhydrin to visualize the presence of Pro. The plates were dried using a hair dryer. The betaines were characterized by a yellow-orange color instant. After heating the plates at 110°C, proline, if present, gives a yellow coloration characteristic.

2.4.2 Halotolerance of fluorescent pseudomonads

Tests were conducted on Glucose Mineral Medium (GMM) prepared according to Bonaterra et al. [36] containing (g/l): glucose 5, NH_4Cl 1, KH_2PO4 3, Na_2HPO_4 2.4, NaCl 0.5, $MgSO_4$, 7 H_2O 0.2. NaCl was added to final concentrations 0, 100, 300, 500, 800, 900 and 1000 mM. The medium was supplemented or no with one type of osmoprotectant. GB and Proline were sterilized (filtration, millipore 0.22 µm) and added to the sterile GMM medium at a final concentration of 1 mM [37,38]. The HE was diluted to 1/100th.

All the prepared solutions were inoculated with 100 µl of different washed suspensions (D.O 600 nm: 0.01 – 0. 05) and incubated in a shaking water bath at 25 ° C for 7 days. Bacterial growth was revealed by measuring the OD at 600 nm [39]. The osmoprotectrice effect of GB, Pro or HE was estimated by comparing the values of the D.O with those of controls without osmoprotectant. The test was performed in triplicate.

2.4.3 Data analysis

All Data were subjected to an analysis of variance by One way ANOVA (Turkey test) procedures to a value of $P \leq 0.05$ by using the software GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA). Each datum was the mean of three replicates.

2.5 Molecular Identification of Strains

Molecular identification of the 4 isolates was based on the amplification and sequencing of genes encoding 16S rRNA. The sequences obtained were compared with homologous sequences contained in the database computer international "GenBank" using Blast (Basic Local Alignment Search Tool) on the web site of Genbank in order to accurately determine their phylogenetic affiliation. The results were expressed as a percentage of similarity of the strain to identify with related species.

3. RESULTS AND DISCUSSION

3.1 Physiological, Biochemical and Molecular Characterization

The cultural, morphological and biochemical tests confirmed that the isolates belonged to *Pseudomonas* genus, as described in Bergey's manual of determinative bacteriology [26]. A common characteristic of all the isolates was the production of pigments that fluoresce in short wavelength (254 nm). Under the Microscopic, all isolates were rods slightly curved gram negative and mobile. The characteristics studied are illustrated in Table 1.

3.2 Sequencing Analysis

The alignment of the nucleotide sequences of strains chosen with genes encoding 16S rRNA databases showed a strong similarity with genes coding for the 16s RNA of the genus *Pseudomonas*. The isolates have been identified and named, respectively as: *Pseudomonas Putida* AF2, *Pseudomonas aeruginosa* RB5, *Pseudomonas fluorescens* RB13 (isolated from wheat rhizosphere) and *Pseudomonas aeruginosa* EH4 (isolated from the endophyte of the halophyte *A. halimus*). They were submitted to GenBank under the accession no: KM592940, KM592942, KM592939 and KM592941, respectively.

3.3 PGPR Activities

The plant growth promoting traits of the Fluorescent Pseudomonas spp. studied are reported in the Table 2. PGPR colonize roots of plant and promote plant growth and development by several mechanisms such as activation of phosphate solubilization, production of phytohormones (IAA), and siderophores. They protect plant by suppression of deleterious organisms through the HCN production, and antibitics synthesis [40], According to Mayak et al. [41], PGPR associated with plants growing under chronically stressful conditions have been shown to promote plant growth better than those growing in normal sites. Zahir et al. [42] have shown that auxin production by *Rhizobium phaseoli* improved the growth and yield of *Vigna radiata* L. under salt stress conditions. The fluorescent *Pseudomonas* spp. tested could be used as wheat inoculants to alleviate saline stress in salt affected soils.

Table 1. Biochemical characterization of the isolates

Isolates	2	5	13	EH4	CHA0
Fluorescence on king B	+	+	+	+	+
Fluorescence on king A	-	+	-	+	-
Growth at 42°C	-	+	-	+	-
Growth at 4°C	+	-	+	-	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Aerobic	+	+	+	+	+
Gelatine hydrolysis	-	+	+	+	+
Glucose	+	+	+	+	+
Citrate	+	+	+	+	+
Arabinose	+	+	+	+	+
Trehalose	+	+	+	+	+
Mannitol	+	+	+	+	+
Glycerol	+	+	+	+	+
H_2S	-	-	-	-	-
Indole	-	-	-	-	-

+: Positive -: Negative

Table 2. Plant growth promoting activities of bacterial strains *In vitro*

Bacterial strain	Accession n°	P. solub. (µg/ml)	IAA (µg/ ml)	Sidero. (%)	HCN	Inhib. *A. alternate* (%)	Inhib. *F. oxysporum* (%)
P. putida AF2	KM 592940	108.85	30.69	42	+	38	19
P. aeruginosa RB 5	KM 592942	169.86	30.39	38	+	38	20
P. fluorescens RB13	KM 592939	187.9	50, 95	43	+	28	19
P. aeruginosa EH4	KM 592941	168.45	36.88	46	+	25	17
P. fluorescens CHA0	x	50.08	88.37	39	+	25	27

(P solub.: Phosphate solubilization; IAA: Indole acetic acid; sidero: Siderophores; Inhib: Percentage of inhibition)

3.3 Detection of Natural Osmoprotectants

Chromatography on silica gel of HE has confirmed the presence of positive Dragendorff-compounds. An important spot corresponding to the GB was observed (Fig. 1). A yellow color characteristic of proline was detected after ninhydrin spray.

3.4 Halotolerance of Fluorescent Pseudomonas spp.

After 7 days of incubation at 25°C on medium GMM, fluorescent Pseudomonas strains showed different behaviors according to the applied treatment (Fig. 2).

The growth of all strains was very significantly ($P \leq 0.001$) stimulated in media containing 100 mM NaCl, except *P. fluorescens* CHA0 which exhibited the lesser degree ($P \leq 0.05$).

The same positive effect was observed in the presence of 300 mM NaCl, except for *P. fluorescens* CHA0, indifferent to this change. All strains respond in the same way to both concentrations (100 and 300 mM) without a significant difference between the two values. The growth of the strains was repressed beyond 300 Mm. At a concentration equivalent to 500 mM, *P. putida* AF2, *P. fluorescens* RB13 and *P. fluorescens* CHA0 were very affected ($P \leq 0.001$). *P. aeruginosa* RB5 and *P. aeruginosa* EH4 were significantly more tolerant ($P \leq 0.01$). NaCl 800 and 900 mM drastically inhibited ($P \leq 0.001$) the development of all the strains examined (Fig. 2).

3.5 Effect of Osmoprotectant Molecules

In the presence of 100 and 300 mM, the exogenous supply of GB, proline or *A. halimus* extract (separately) has no promoting effect on the proliferation of the majority of strains: *P. putida* AF2, *P. aeruginosa* RB5 and EH4.

GB restored the growth of all the strains stressed by the salt concentration 500 mM. This effect is very important ($P \leq 0.001$) with *P. putida* AF2 and *P. fluorescens* CHA0 and significant ($P \leq 0.01$) with *P. fluorescens* RB13 and *P. aeruginosa* EH4. Proline exerted the same effect as GB with slight non-significant difference. At this same salt stress value, the use of *A. halimus* carried no benefit to bacteria, except *P. aeruginosa* EH4.

At 800 mM, the deleterious effect of NaCl was remarkably mitigated by the different O.P used. Thus, GB acted significantly ($P \leq 0.01$) on *P. putida* AF2, *P. aeruginosa* RB5 and EH4. The growth of *P. fluorescens* RB13 was largely restored by GB ($P \leq 0.001$).In the majority of cases, Pro "osmoprotected" bacteria with the same amplitude as the GB. *A. halimus* extract did not help the bacteria to overcome the stress of 800 mM, except the strain *P. aeruginosa* EH4. It should be noted, finally, that *P. fluorescens* CHA0 was totally inhibited by this saline concentration, no molecule seems effective. At 900 mM / NaCl, all strains

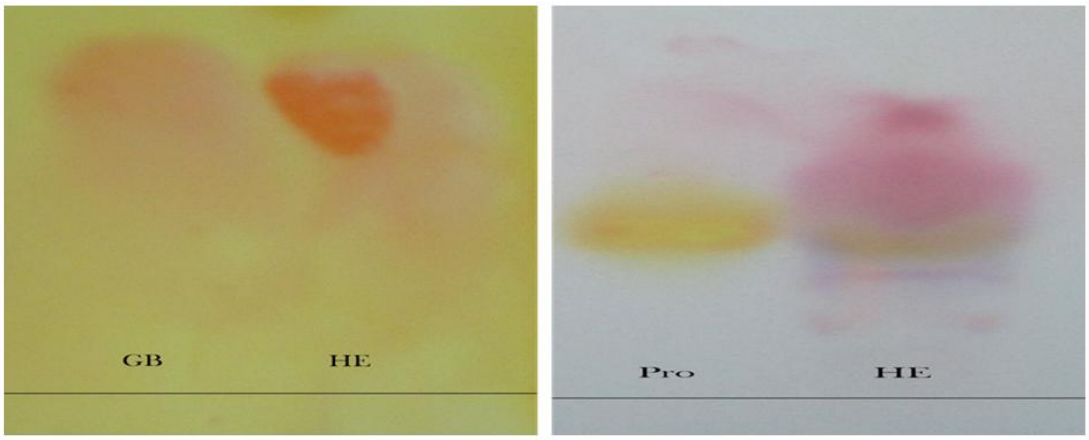


Fig. 1. Detection of compatible solutes [proline (Pro) and glycine betaine (GB)] inhalophyte extract (HE) by thin layer chromatography

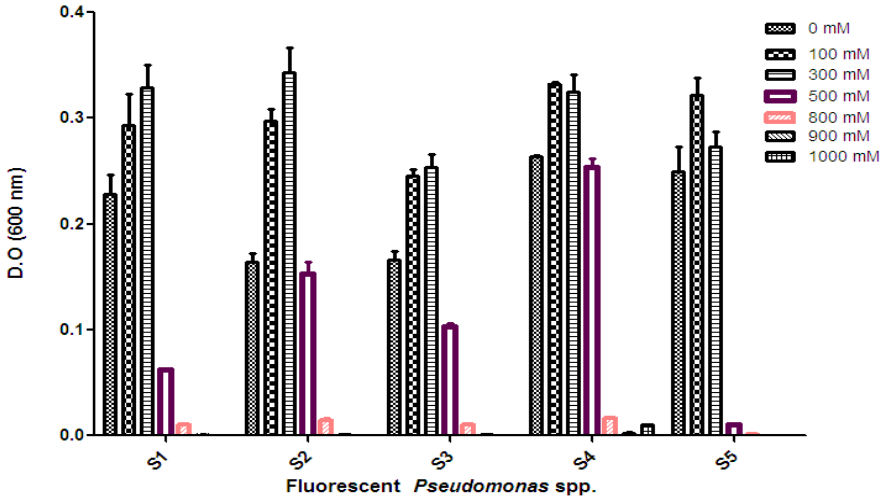
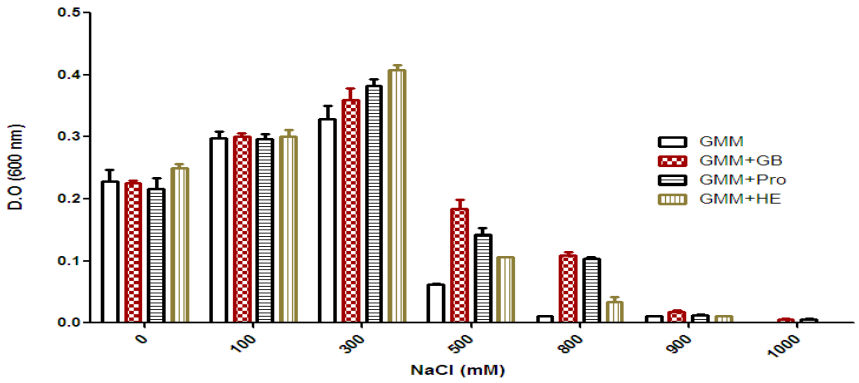


Fig. 2. Effect of different NaCl concentrations on fluorescent Pseudomonas strains
S1: P. putida AF2; S2: P. aeruginosa RB5; S3: P. fluorescens RB13, S4: P.aeruginosa EH4; S5: P. fluorescens CHA0.



a)

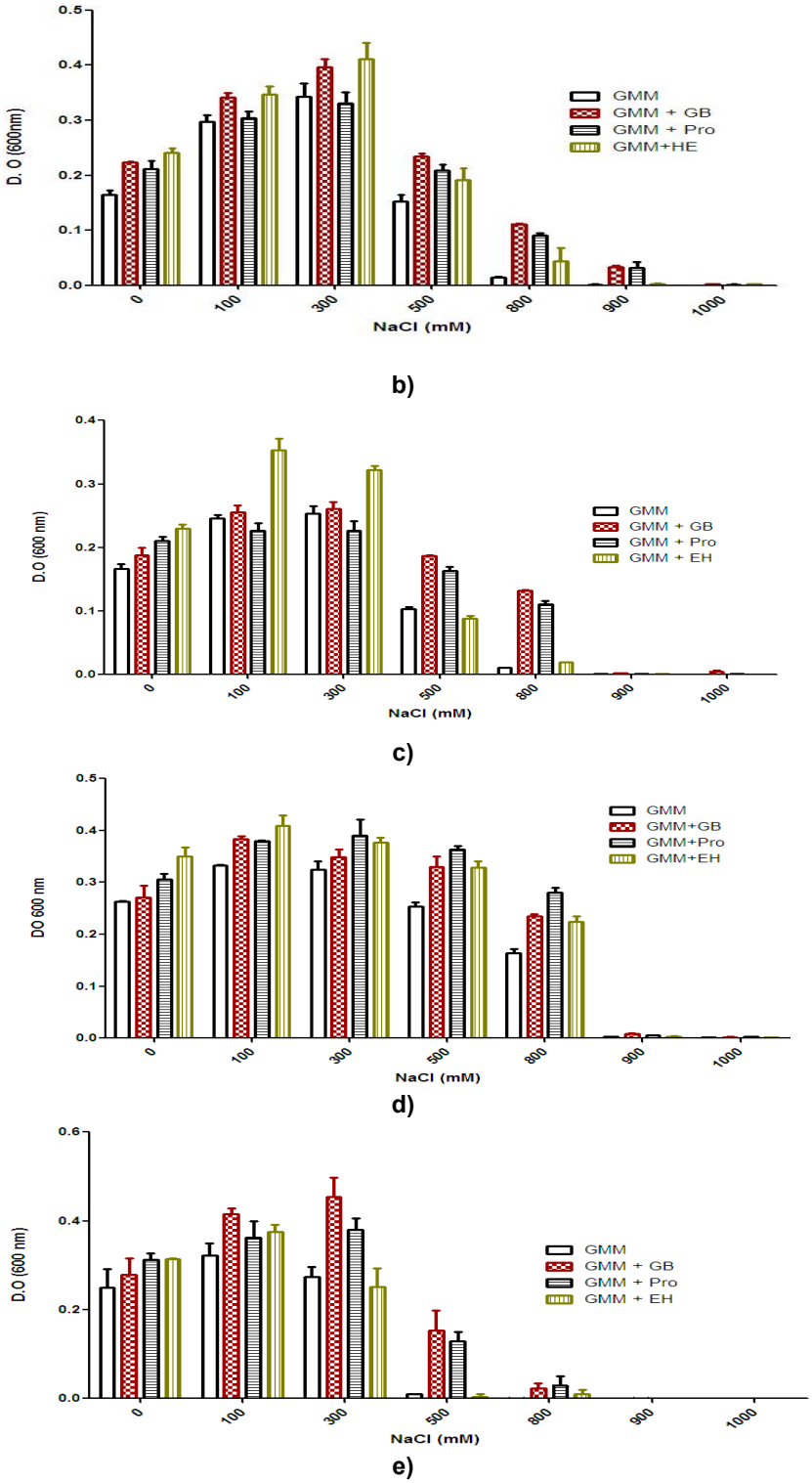


Fig. 3. Effect of different concentrations of NaCl and osmoprotectors on the growth of the 5 strains of Pseudomonas

a): P. putida AF2 b): P. aeruginosa RB5 c): P. fluorescens RB13 d): P.aeruginosa EH4 e): P. fluorescens CHA0, (GB: glycine betaine, Pro: proline, HE: halophyte extract)

was inhibited, the osmoprotective molecules tested had no effect.

The results of this study were consistent with those published by several researchers. It was observed that the addition of 100 and 300 mM to the culture medium stimulated the growth of all strains. Thus, a certain content of ions Na+ would be required for better bacterial growth in the medium. The supplementation of media with GB, Pro and EH (as osmoprotectants) was pointless because the stress was absent. It is evident that the GB improves the growth of stress cells, but not those unstressed [43]. At this concentration, it was a nutritional effect of the ions Na^+, Cl^- and not an osmotic pressure; the osmoprotectants added would be used instead of carbon, nitrogen and energy source.

It is well-known that the presence of NaCl at a final concentration of 800 mM in a medium inhibits completely *E. coli* [44], *Salmonella typhimurium* [45] and *P. mendocina* [46].

G.B is the most effective osmoprotectant for bacteria as *P.mendocina* [46, *P. aeruginosa* [38], *P. fluorescens* [47]. It is generally accumulated in the cytoplasm of cells undergoing osmotic stress exceeding 800 mM [48,45]. Several bacterial species respond positively to an exogenous supply of GB, even at low concentrations (1 mM) [49,50,48]; a maximum effect was observed even with 20 µM with *P. aeruginosa* [36] or with nanomolar concentrations with *E. coli* (a real rate found in nature) [51]. In this study it was noted that GB alleviated, but could not completely overcome the osmotic stress on bacterial cultures (800 or 900 mM depending on species).

Proline can be accumulated at molar concentrations, and it contributes to the stimulation of the growth of several stressed bacteria like *S. typhimurium* and *E. coli* [52,45]. Our results are inconsistent with those Miller and Wood [53] who noted that the *Pseudomonas* group does not accumulate proline as a compatible solute, and with those of Pocard et al. [46] announcing that this imino acid has no beneficial effect on *P. mendocina*. This difference may be due to the use of different culture media and different strains. Tests conducted by our care showed a significant improvement of the growth of *Pseudomonas* strains tested, even at high salt concentrations, in contrast to those obtained by Le Rudulier [54] which limited proline effectiveness to moderate osmotic stress. Low concentrations (< 1 mM) increased the tolerance of the Gram-negative bacteria [55].

The halophyte *A. halimus* is known for its ability to accumulate GB and Pro in response to stress exerted by the saline soil on which it grows [10]. The HE has not ensured osmoprotection of bacterial growth beyond 500 mM for the most strains. It is quite possible that some halophytes extracts could convey substances inhibiting the growth of bacterial strains. Cytotoxic alkaloids, isoflavonoids having antibacterial activity, as well as tannins (polyphenols), known to be able to precipitate proteins and inhibit bacterial respiration, have been described in the halophytes plants [56]. Other methods of preparation must be carried out in order to highlight the positive effect of *A. halimus* extracts and it is necessary to screen other halophytic plants in order to explore their beneficial effects on restoration of bacterial growth in saline soils.

4. CONCLUSION

On the basis of their excellent growth promoter, their biocontrol activities, their halototolerance, and their positive response to the exogenous supply of natural osmoprotectant molecules, the fluorescent Pseudomonas tested should be used as inoculants for sustainable agriculture in salty soils.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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