

PLANT GROWTH PROMOTING AND ANTAGONISTIC TRAITS OF INDIGENOUS FLUORESCENT *PSEUDOMONAS* SPP. ISOLATED FROM WHEAT RHIZOSPHERE AND *A. HALIMUS* ENDOSPHERE

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Abstract

Fluorescent *Pseudomonas* spp. are an important group of plant growth promoting rhizobacteria (PGPR). They increase the growth of their host plant directly or indirectly. In this study, 3 Fluorescent pseudomonads were isolated from the wheat rhizosphere and one from the endophyte of the halophyte *Atriplex halimus*. Based on biochemical, physiological reactions and 16S rRNA gene sequences, the isolates were identified as *Pseudomonas putida* AF2, *P. aeruginosa* RB5, *P. fluorescens* RB13 and *P. aeruginosa* EH4. These strains and *P. fluorescens* CHA0 were screened for their PGPR activities. All the strains solubilized phosphate with a maximum of 187.9 µg / ml. *P. fluorescens* CHA0 produced a significant amount (88.37µg/ml) of IAA. The siderophores production by all the strains was proved and the percent of production varied from 38 to 46. The strains produced HCN, protease and amylase. Mycelial growth of *F. oxysporum* and *A. alternata* was strongly reduced in the presence of antagonistic fluorescent *Pseudomonas* spp., with the inhibition rate varying between 25 to 38% and 17 to 27%, respectively. On the basis of excellent growth promoter, biocontrol activities, the fluorescent *Pseudomonas* spp. tested could be applied as inoculants of wheat for sustainable agriculture in salty soils.

Keywords: PGPR, Phosphate solubilization, siderophores, Indole acid acetic, HCN

Introduction

The rhizosphere, fraction of soil rich in root exudates, is occupied by various bacterial groups named rhizobacteria. *Pseudomonas* fluorescent spp. are among the most important plant growth-promoting bacteria (PGPR). The majority of the species of this group enhances plant growth by number of direct and indirect mechanisms (Glick *et al.*, 2007). Some species can be considered as biofertilization agents, they provide plants with required nutrients like fixed nitrogen, phosphorus and iron. About 20% of the fixed atmospheric nitrogen are attributed to the non-symbiotic bacteria, such as *Pseudomonas* (Sexana and Tilak, 1989). Phosphorus is also a limiting element for plants. They can only absorb the soluble forms provided by rhizobacteria, like fluorescent *Pseudomonas* spp., either *via* the mineralization of organic phosphate (by phosphatases), or *via* the solubilization of inorganic phosphate (by the effect of acids) (Lemenceau, 1992) Iron is another essential element for plant nutrition; however, it is slightly soluble in the soil solution (Verma *et al.*, 2010). In order to sequester iron, the microorganisms release siderophores (molecules with an exceptionally high affinity for Fe^{+3}). Consequently, they make this element available in the rhizosphere and contribute to the stimulation of the plant growth (Lemenceau 1992). Fluorescent *Pseudomonas* spp. can also produce plant growth regulators (phytohormons) involved effectively in elongation of plants (Patten and Glick, 2002) such as: auxins, gibberellic acid, cytokinins and ethylene. Indole acetic acid (IAA) is the most important group of Auxin, it affects both plant growth promotion and root nodulation (Ashrafuzzaman *et al.*, 2009). Fluorescent *Pseudomonas* species occupy an important place as biological control agents. They produce a wide range of antifungal metabolites, which protect the plant against phytopathogens (Haas and Defago, 2005). Several mechanisms are solicited in this effect. The production of a variety of antibiotics is an essential characteristic of fluorescent pseudomonads. The best known are phenazine-1-carboxylic acid (Ownley *et al.*, 2003), 2 - 4 - diacetylphloroglucinol (Scharifi-Tehrani *et al.*, 1998), the pyoluteorin (Maurhofer *et al.*, 1995) and hydrogen cyanide (HCN) (Lemenceau, 1992). These species can control the growth of pathogens by competing for nutrients such as carbon (Lemanceau *et al.*, 1988) and iron chelated by siderophores, thus making it unavailable for other phytopathogens (Lemanceau *et al.*, 2009). Some biocontrol *Pseudomonas* produce lytic enzymes, which can lyse a part of cell walls of some phytopathogens fungi. The most known are: chitinases, cellulases, proteases, β -1,3 glucanases (Compant *et al.*, 2005). Some species may have a protective effect by stimulating inducible defence mechanisms that make the host plant less susceptible to a subsequent pathogen attack (Van Loon, 1998). In addition, PGPR may contribute to the improvement of the plant resistance to

biotic and abiotic stresses such as salinity, drought (Nadeem *et al.*, 2012; Ali *et al.* 2014), deficiency in nutrient elements (Sheng, 2005) and toxicity of heavy metals (Islam *et al.*, 2014).

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 and increase the crop yield by alleviating salt stress on plants. For this purpose, indigenous bacteria isolated from wheat rhizosphere and the endosphere of the halophyte *Atriplex halimus* were screened for their plant growth promoting properties.

Materials and methods

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 $\mu\text{S}/\text{cm}$ at 20 ° C; pH 7.25), the second one is derived from the endophyte of the halophyte *Atriplex Halimus* (saline soil or Sebkh: conductivity: 4.09 mS/cm at 20°C; pH 7.30) (Fig.1). The roots were gently removed from soil on three different points and placed in plastic bags sterilized by UV and transported to the laboratory in a cooler and kept in the refrigerator (4 ° C) for later use.



Figure 1: Photo showing the appearance of *A. halimus*

For a selective isolation of fluorescent *Pseudomonas* species, the selective Gould's S₁ medium was used (Gould *et al.*, 1985). The medium contains (per liter): 18 g of agar, 10 g sucrose, 10 ml of Glycerol, 5 g of casamino acids, 1 g of NaHCO₃, 1 g MgSO₄, 7 H₂O, 2,3 g K₂HPO₄, 1.2 g sodium Lauroyl Sarcosine (SLS) ad 20 mg of trimethoprim. Sterile solution of trimethoprim (Millex filter, porosity 0, 22 μm) was added after autoclaving of the medium (121 ° C, 15 min) and cooling to 40 ° C. The final pH of the medium is 7.4 to 7.6; no adjustment was required.

To isolate the fluorescent *Pseudomonas* from the rhizosphere of wheat, non-rhizospheric soil was removed by shaking vigorously. Ten grams of soil strongly attached to the roots were stirred with 90 ml of sterile 0.05 M buffer phosphate (pH 7.0) for 30 minutes. Serial decimal dilutions were

made from this suspension in the same buffer. An aliquot of 0.1 ml of each dilution was spread on Gould's S1 medium.

The fluorescent *Pseudomonas* species colonizing the endophyte of the halophyte *Atriplex halimus* were isolated as described by Forchetti *et al.*, (2007). The rhizospheric soil was eliminated by washing with sterile distilled water. The roots were then surface disinfected with ethanol 70% for 1 min, then surface sterilized with 3 % sodium hypochlorite solution for 2 min. The roots were rinsed with sterilized distilled water, to eliminate all traces of disinfectant, then cut and added to sterile 0.05 M buffer phosphate and macerated with a sterile mortar and pestle. One gram of the homogenate was transferred in a tube containing 9 ml sterile 0.9% NaCl. From this solution, a series of decimal dilutions were made. A volume of 0.1 ml of each dilution was plated on Gould's S1 medium. After incubation at 23 °C for 3 days, the colonies with a green fluorescent pigmentation under UV light (366 nm) were picked up and purified on King B medium. Twenty isolates belonging to the rhizosphere of wheat and the endophyte of *Atriplex halimus* were maintained.

Biochemical and physiological tests

Determination of macroscopic, microscopic (Gram, cell morphology, mobility), enzymatic (oxidase, catalase, nitrate reductase) characters were carried in the first step. Different biochemical characters were highlighted in order to select isolates belonging to the genus *Pseudomonas* as described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). These preliminary tests have conducted to the choice of 3 isolates from the wheat rhizosphere of initially designated: 2, 5 and 13, and one isolate from the endophyte of *A. halimus* noted EH4.

Maintenance of isolates

To avoid any mutation in the genes, the isolates were kept at a temperature of 4 ° C in nutrient broth supplemented with 30% glycerol.

Screening of PGPR activities

All the isolates were screened for their PGPR activities. A reference strain: *Pseudomonas fluorescens* CHA0 was also tested. It was isolated from the rhizosphere of tobacco in soil resistant to disease rot roots in Switzerland (Keel and Défago, 1997). It was kindly provided by Professor Défago (Institute of plant sciences, Zurich, Switzerland). All the following tests were replicated three times

Phosphate solubilization

The ability of the isolates to solubilize the inorganic tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) was tested on National Botanical Research Institute's medium Phosphate (NBRIP) according to the method of Nautiyal (1999). A spot of 5 μl of the culture of each strain was deposited on the NBRIP medium. After incubation 28 ° C for 10 days, the diameter of clear zone (halo) surrounding the bacterial growth as well as the diameter of colony, were measured. The diameter of the solubilization halo was determined by subtracting the diameter of the colony from the total diameter. Quantitative estimation of phosphate solubilization was also carried out on NBRIP liquid medium inoculated with 100 μl of a culture of each isolate. Cultures were incubated for 10 days at 28 ° C with agitation and then centrifuged at 3000 rpm/min for 20 min. The amount of phosphate in the supernatant was estimated by the colorimetric method of Olsen and Sommers (1982). The absorbance of the resulting blue compound was measured at 620 nm, thereby expressing the concentration of phosphate soluble (P_2O_5). A standard calibration curve was performed with a solution of KH_2PO_4 .

Indole acetic acid (IAA) production

In order to demonstrate the production of IAA, a volume of 100 μl of each isolate was grown on 10 ml of liquid medium Luria Bertani supplemented with tryptophan (5 g/l). After 48 h of incubation at 30 ° C, bacterial solutions were centrifuged (5000 rpm/20 min). A volume of 1ml of each supernatant was mixed with 2 ml of the solution of Salkowski (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl_3 solution). Development of a red color indicates the production of IAA (Gordon and Weber 1951). The amount of AIA in the supernatant was estimated by colorimetric method (Loper and Schroth, 1968). The optical density was measured at 530 nm after 30 min of incubation. A standard calibration curve was performed with a solution of AIA (Fluka) in a range 0 to 10^{-3} M.

Ammonia production

Production of ammonia (NH_3) by all the isolates was tested qualitatively as described by cappuccino and Sherman (1992). A volume of 100 μl of freshly grown cultures were inoculated in 10 ml of peptone water and incubated for 96 h at 30 ° C. Nessler reagent (500 μl) was added. The production of ammonia is then revealed by the appearance of a yellow or orange color.

Siderophore production

The production of siderophores by isolates was determined in chrome azurol agar (CAS) by the method of Schwyn and Neilands (1987).

For this, the King B medium was inoculated with spots of a fresh culture of each isolate. After incubation at 28 ° C for 24 h, the medium was covered with 15 ml of CAS agar. Subsequently to a short contact, siderophores producing bacteria form an orange halo on the blue-green medium (Schwyn and Neilands, 1987; Sahu and Sindhu, 2011) The diameter of siderophore production was estimated by subtracting the diameter of the colony of the total diameter (halo + colony). Quantitative analysis was performed according to the method described by Payne (1994) using iron free succinate medium (SM) (Meyer and Abdallah, 1978) consisting of (g/l): K₂HPO₄, 6.0; KH₂PO₄, 3.0; MgSO₄ 7H₂O, 0.2; (NH₄)₂SO₄, 1; succinic acid 4.0, pH 7.0. It was inoculated with old cultures at the rate of 1 % (v/v) and incubated (30°C, 48 h) with constant shaking (150 rpm). After centrifugation of the cultures (5000 rpm / 20 mn), 500 µl of each supernatant were mixed with 500 µl of CAS solution and incubated (30 min, in the dark at room temperature). The color changed to orange according to the amount of produced siderophore. The absorbance of the resulting complex was measured at 630 nm against a blank containing 0.5 ml of uninoculated medium and 0.5 ml of CAS solution. Siderophores content was expressed in percentage according to formula proposed by Sayyed *et al.*, (2005):

$$\% \text{ siderophore units} = \frac{Ar-As}{Ar} \times 100$$

Where, Ar: absorbance of the blank at 630 nm (CAS reagent)

As: absorbance of the sample at 630 nm.

Production of hydrocyanic acid (HCN)

The method described by Bakker and Shippers (1987) was adopted to evaluate the ability of fluorescent *Pseudomonas* strains to produce hydrogen cyanide. Bacterial cultures were streaked on Trypticase soy agar supplemented with glycine (4.4 g/l). Inside the lids of the plates, a disc of Whatman filter paper previously immersed in 0.5% picric acid solution (in 2% sodium carbonate), was placed. Plates were then sealed with parafilm and incubated in an inverted position for 4 days at 28 ° C. Appearance of light brown to dark brown color indicated HCN production.

Hydrolytic enzymes

The antagonistic activity by elaboration of some bacterial enzymes such as protease and amylase was tested. Protease activity, shown by casein degradation, was determined in skim milk agar. Starch degradation was studied on starch agar. Bacteria were streaked onto the plates and incubate at 27 ° C for 5 days. Enzyme activity was identified by the development of a clearing zone (halo) around the colonies.

Antifungal activity

The antagonistic activity of selected *Pseudomonas* isolates against two phytopathogens: *Alternaria alternata* and *Fusarium oxysporium*, was tested. An agar disc of 3 mm diameter was cut from an actively growing (96 h) of fungal culture and placed in the center of the Petri plates containing Potato Dextrose Agar (PDA). A loopful of each actively growing fluorescent *Pseudomonas* isolates was spotted (5 µl) in opposite to the fungal disc. Plates inoculated with fungal strain and without bacteria were used as control. After an incubation of 10 days at a temperature of 25 ° C or 28 ° C, the diameter of inhibition zone was recorded and the inhibition percentage was calculated according to the formula established by Kumar *et al.* (2002):

$$I = [(C-T)/C] \times 100. \text{ Where:}$$

I: Percent inhibition of the fungus tested

C: Radial growth of fungus in control plates (mm)

T: Radial growth of fungus on the plate inoculated with bacteria (mm).

Data analysis

All the Data concerning phosphate solubilization, siderophores and the AIA production 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 data was the mean of three replicates.

Molecular identification of strains

Molecular identification of the isolates (2, 5, 13 and EH4) was based on the amplification and sequencing of genes encoding 16S rRNA. The procedure consists of amplifying the gene in the presence of a primer recognizing the region on the DNA which is subsequently sequenced. 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 "<http://blast.ncbi.nlm.nih.gov/Blast.cgi>" 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, and in the form of phylogenetic trees that show the taxonomic position of each isolate. Sequences were submitted to the GenBank.

Results and discussion

Physiological, biochemical and molecular characterization

The Belonging of the 4 bacterial isolates to fluorescent *Pseudomonas* sp. was confirmed on the basis of cultural, morphological and biochemical tests as described in Bergey's manual of determinative bacteriology (Holt *et*

al., 1994). A common characteristic of all the isolates was the production of pigments that fluoresce in short wavelength (254 nm). Microscopic observation reveals that all isolates are rods slightly curved gram negative and mobile. Oxidase and catalase tests are positive. All the isolates strains were aerobic strict with oxidation of glucose. They produced a thick veil on Luria Bertani or Trypticase soy broth. The smell of Jasmine was also a dominant feature. All strains liquefied gelatin and possessed a nitrate reductase. They were able also to use mannitol, arabinose, trehalose, glycerol, citrate as a carbon source. The characteristics studied are illustrated in table 1.

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 ₂ S	-	-	-	-	-
Indole	-	-	-	-	-

+: Positive

-: Negative

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 designated by the numbers 2, 5, 13, EH4 have been identified and named, respectively as: *Pseudomonas Putida* AF2, *Pseudomonas aeruginosa* RB5, *Pseudomonas fluorescens* RB13 and *Pseudomonas aeruginosa* EH4. They were submitted to GenBank under the accession no: KM592940, KM592942, KM592939 and KM592941, respectively.

Characteristics of plant growth promoter

Phosphate solubilization:

Isolates identified as fluorescent *Pseudomonas* spp. namely: *P. putida* AF2, *P. aeruginosa* BR5, *P. fluorescens* RB13, *P. aeruginosa* EH4 and the reference strain, *P. fluorescens* CHA0 were tested for their ability to

solubilize the inorganic phosphate on solid medium NBRIP containing tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ as the sole source of phosphorus. After an incubation for 10 days at 28 ° C, *P. putida* AF2 and *P. fluorescens* RB13 produced a clear zone around their colonies, reflecting a solubilization of $\text{Ca}_3(\text{PO}_4)_2$ (Figure 2) with a halo of 15 and 13 mm, respectively. They seem to be the most effective (Figure 3). *P. aeruginosa* RB5 and *P. fluorescens* CHA0 moderately solubilized phosphate and the diameter of the halo was 4 and 2 mm, respectively. *P. aeruginosa* EH4 was unable to carry this activity. The amount of solubilized phosphate on liquid medium NBRIP varied with strains (Figure 4). *P. fluorescens* RB13 solubilized a significant amount (187.9 $\mu\text{g} / \text{ml}$). Despite the absence of a halo of solubilization on solid medium, *P. aeruginosa* EH4 proved a significant activity; the quantity solubilized was 168.45 $\mu\text{g}/\text{ml}$, statistically equivalent to that of *P. aeruginosa* RB5 (169.86 $\mu\text{g}/\text{ml}$). *P. putida* has a moderate activity (108.85 $\mu\text{g} / \text{ml}$) followed by *P. fluorescens* CHA0 (50.08 $\mu\text{g} / \text{ml}$) thus demonstrating low phosphate solubilizing activity.

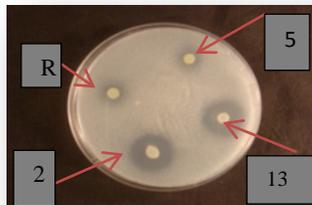


Figure 2: Areas of phosphate solubilization by fluorescent *Pseudomonas* spp.
 2: *P.putida* AF2; 5: *P.aeruginosa* RB5; 13: *P. fluorescens* RB13; R: *P. fluorescens* CHA0

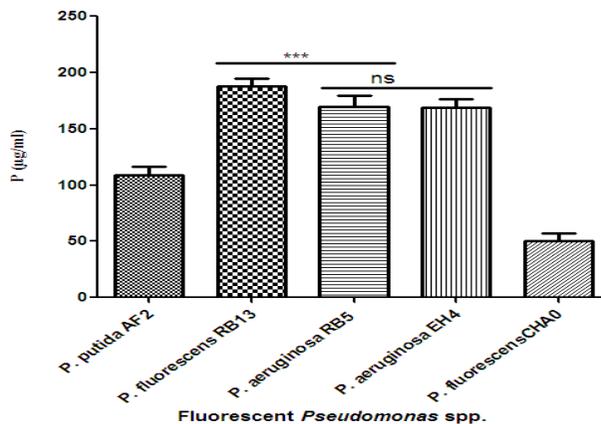


Figure 3: Phosphate solubilization of on liquid medium NBRIP by fluorescent *Pseudomonas* spp.

The results confirm clearly the ability of fluorescent *Pseudomonas* spp. to use $\text{Ca}_3(\text{PO}_4)_2$ available in NBRIP medium. This reflects the same function carried out by these species in their natural habitat. Indeed, rhizobacteria can solubilize organic phosphorus of soil by the action of phosphatase, or inorganic phosphorus by the release of organic acids with low molecular weight (Kloepper *et al.*, 1989) such as citric acid (Reys *et al.*, 2001) and gluconic acid (Fenice *et al.*, 2000). The solubilized quantity is consistent with that obtained by Chen *et al.*, (2006): *Bacillus megaterium* (140.6 $\mu\text{g} / \text{ml}$), and *Rhodococcus erythropolis* (151.2 $\mu\text{g} / \text{ml}$). *P. fluorescens* isolated from Wheat rhizosphere solubilized phosphate in the range of 89-93 $\mu\text{g}/\text{ml}$ (Schoebitz *et al.*, 2013).

Despite the absence of a halo of solubilization around the colonies, *P. aeruginosa* EH4 could efficiently solubilize phosphorus in liquid medium (168.5 $\mu\text{g} / \text{ml}$). This result supports the findings of Nautiyal (1999). In fact, organic acids secreted in order to solubilize phosphates circulate with difficulty on solid medium. Hence the choice of strains to be inoculated on the basis of the formation of a solubilizing halo on solid medium is not an evident criterion. It has been found by Cherif- silini *et al.*, (2013) that solid media NBRIP and Piskovskaya (PVK) (Pikovskaya, 1948) showed the same efficiency in detecting the solubilization of phosphates by *Bacillus* strains. However, the liquid medium NBRIP was 3 times more effective than PVK.

Production of IAA

After 48 h of incubation at 28 ° C, all strains tested produced IAA (Figure 4). Analysis of variance showed a significant difference in production ($p < 0.01$) between *P. fluorescens* CHA0 (88.37 $\mu\text{g}/\text{ml}$), *P. fluorecens* RB13 (50.95 $\mu\text{g}/\text{ml}$) and *P. aeruginosa* EH4 (36.88 $\mu\text{g}/\text{ml}$). No significant difference was noted between the amounts produced by *P. putida* AF2 and *P. aeruginosa* RB5 (30.69 and 30.39 $\mu\text{g}/\text{ml}$, respectively). The results show some similarities with those obtained by other researchers such as Ahmed *et al.* (2005) who reported that *Pseudomonas* isolate Ps₁ produced 53.20 $\mu\text{g}/\text{ml}$. It has been noted that IAA production by rhizobacteria vary among different species and strains, and it is influenced by culture condition, growth stage and substrate availability (Mirza *et al.*, 2001). The IAA, auxin produced by a large number of rhizobacteria (Patten and Glick, 2002), is considered as a product of L-tryptophan metabolism; a significant amounts were produced on a nutrient broth supplemented with 2 and 5 mg / ml of tryptophan (Bharucha *et al.*, 2013). The production of this hormone by strains of *Pseudomonas* is beneficial to plant growth and root elongation and low amounts (10^{-9} to 10^{-12} M) are needed for the primary growth of the roots (Patten and Glick, 2002). The rates obtained in this study are sufficient to stimulate plant growth. IAA stimulates stress tolerance in plant (Marulanda

et al., 2009), thus, Rhizobacteria producing IAA participate effectively in the adaptation of plant during osmotic stress by lowering their energy demand (Zahir *et al.*, 2010; Ahmad *et al.*, 2013). The studied strains could be used as bioremediation agents.

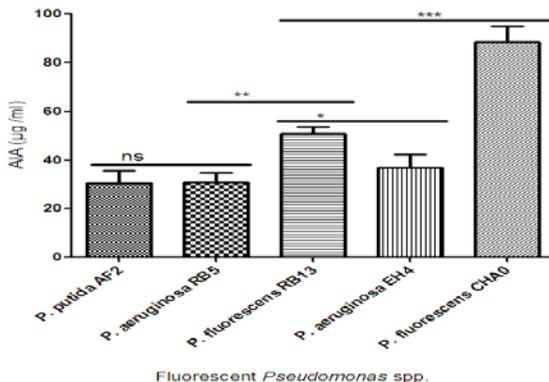


Figure 4: Production of IAA by fluorescent *Pseudomonas* spp.

Ammonia production

After addition of Nessler's reagent, a yellow-orange color has appeared, thus marking the production of NH_3 by all *Pseudomonas* strains tested. Ammonia production is considered as an important feature of rhizobacteria by improving indirectly the plant growth (Joseph *et al.*, 2007) and by ensuring a role in signaling during plant-rhizobacteria interaction (Becker *et al.*, 2002). It is well established that the ammonia released by bacteria accelerates the activity of glutamine synthetase (Sood *et al.*, 2002).

Siderophores production

Qualitatively, the 5 fluorescent *Pseudomonas* strains produced siderophores, they formed orange halos around the colonies (Figure 5). The diameter of the halo differs from one strain to another. It is maximum with *P. fluorescens* RB13 and *P. aeruginosa* EH4 (20 mm and 19 mm) respectively, followed by *P. aeruginosa* RB5 (16 mm). *P. putida* AF2 and *P. fluorescens* CHA0 gave the same value (12 mm). The presence of orange halo surrounding the colonies on CAS medium (blue) reflected the transfer of ferric ions from the medium to the siderophores (Schwyn and Neilands, 1987). The solid medium King B was favorable to the detection of siderophores production of by *Pseudomonas*.

On the liquid medium SM, the color of the complex bacterial supernatant - CAS solution turns from blue to orange depending on the rate of siderophores produced by each strain. Statistical analysis of data has shown a non-significant difference between the 5 strains tested. The % of production was as follow: *P. aeruginosa* EH4 (46), *P. fluorescens* RB13

(43), *P. putida* AF2 (42), *P. fluorescens* CHA0 (39), *P. aeruginosa* RB5 (38) (Figure 6). The liquid medium SM was favorable to the siderophores production; this observation was confirmed by Sharma and Johri (2003) and Sayyad *et al.*, (2005). They attributed an important role to succinate in increasing siderophore yields. The rate of production of siderophores is affected by the carbon source, trace elements and the pH of the medium (Sharma and Johri, 2003).

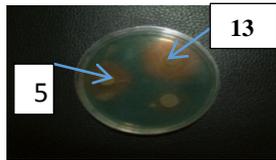


Figure 5: siderophores-production by: 13: *P. fluorescens* RB 3 and 5: *P. aeruginosa* RB5.

The Strains tested belong to the Pseudomonads known by their ability to produce siderophores such as pyoverdines and pseudobactines which gives a yellow-green color and fluorescence under UV light (Sharma and Johri, 2003). Due to their high affinity for ferric ions, siderophores chelate iron, making it inaccessible to the phytopathogenic microorganisms around the roots (Sreedevi *et al.*, 2014). Furthermore, they inhibit the spores germination of pathogenic fungi (Raaijmakers *et al.*, 1995) such as *Fusarium oxysporium*, *Rhizoctonia solani* and *phytium aphanidermatum* (Sahu and Sindhu, 2011). The involvement of these compounds in plant growth promotion of wheat was also demonstrated. Sayyed *et al.*, 2005 have reported an increase in the rate of germination, in the root and shoot length of wheat inoculated with *Pseudomonas* strains producing siderophores. Thus these indigenous species could be exploited in agriculture to improve plant growth and to preserve plant health.

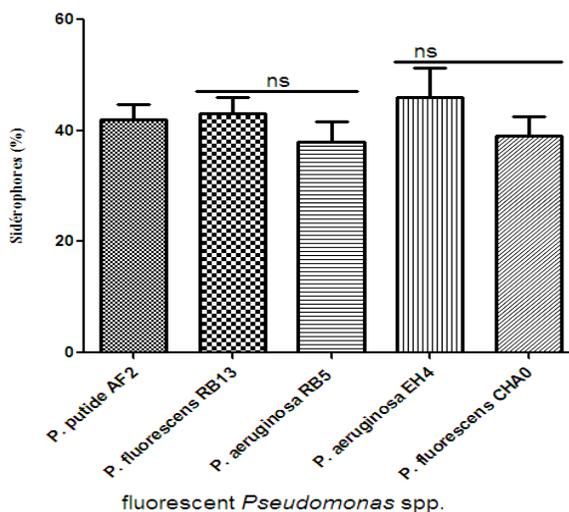


Figure 6: Siderophores production by *Pseudomonas* fluorescent spp. on liquid medium

Production of HCN

The appearance of light brown to dark brown color indicated HCN production by all the strains tested (Figure 7). Production was weak in *P. aeruginosa* RB5 and *P. aeruginosa* EH4 while it was strong in *P. putida* AF2, *P. fluorescens* RB13 and *P. fluorescens* CHA0. Variability of expression of genes (*hcnA*, *hcnB* and *hcnC*) would explain the difference in production of HCN between *Pseudomonas* strains tested (Ramette *et al.*, 2003). This variability allows the classification of bacteria into two groups: weak and strong producers. The enzyme HCN synthetase is a membrane flavoprotein which catalyses the formation of HCN and CO₂ from glycine (Ramette *et al.*, 2003). The production of HCN by *Pseudomonas* is involved in the suppression of various pathogens in particular fungi (Verma *et al.*, 1989). This volatile compound acts directly on cells by blocking the cytochrome c oxidase in the respiratory chain. It also contributes to the capture of some metal ions by forming complexes with them (Blumer and Haas, 2000) and can act as an inducer of plant resistance (Kumar *et al.*, 2012).



Fig. 7: Production of HCN by *P. fluorescens* CHA0 (in the left). In the right: a negative control

Production of hydrolytic enzymes

All fluorescent *Pseudomonas* strains tested have a protease and an amylase; they may be used as a biocontrol agent. Indirect mechanisms of action of PGPR involve the elimination of harmful microorganisms *via* the production of antibiotics or (and) lytic enzymes (Protease, amylase), HCN or catalase (Twisha and Desai, 2014). Dunne *et al.* (1997) have shown that the suppression of phytopathogenic fungus, *Pythium ultimum*, in the rhizosphere of sugar cane was due to the elaboration of extracellular protease by strains of *Stenotrophomonas maltophilia* W81. *Pseudomonas fluorescens* CHA0 synthesizes an extracellular protease active against the phytopathogenic nematode *Meloidogyne incognita* (Siddiqui *et al.*, 2005). Bacterial strains possessing a catalase are assumed to be highly resistant to various environmental, mechanical and chemical stress (Kumar *et al.*, 2012).

Antifungal activity

After 10 days of incubation at 28 ° C, the antagonistic activity of the *Pseudomonas* strains toward 2 phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporium*, was checked. This action was revealed by the appearance of a zone of inhibition of the fungus growth around the bacterial

colony. All fluorescent *Pseudomonas* strains tested inhibited the growth of the 2 fungi; the inhibition rate varied according to the bacterial strain and the fungus.

Mycelial growth of *F. oxysporum* was strongly reduced in the presence of antagonistic fluorescent *Pseudomonas* strains, compared with the uninoculated control (Figure 8) the higher percentage of Mycelial growth inhibition was observed with *P. putida* AF2 and *P. aeruginosa* RB5 38% strains. *P. fluorescens* CHA0 and *P. aeruginosa* EH4 represent the lowest antagonistic action (25%) (Figure 9)

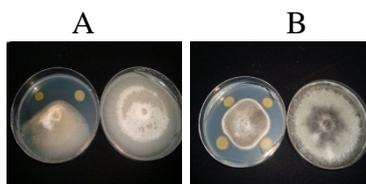


Figure 8: Antibiosis against *Fusarium oxysporium* (A) and *Alternaria alternata* (B)

Pseudomonas strains showed less inhibitory activity against *A. alternata* compared to that observed against *F. oxysporum* except *P. fluorescens* CHA0 that has similar potency against the 2 fungi. The highest antagonistic effect was recorded with *P. fluorescens* CHA0 (27%). The analysis of variance shows a non-significant difference concerning the antagonistic effect of the 4 remaining strains.

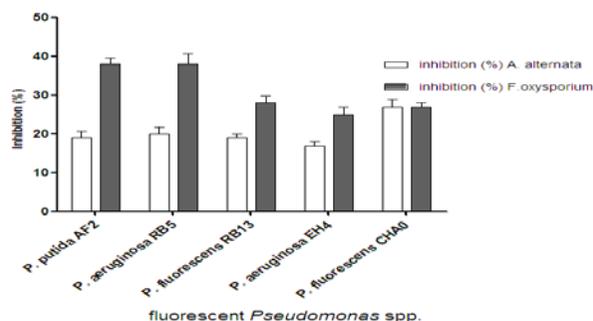


Figure 9: Inhibitory effect of fluorescent *Pseudomonas* spp. on *A. alternata* and *F. oxysporium*

Antifungal activity of fluorescent *Pseudomonas* against *F. oxysporum* and *A. alternata* strains could be strongly linked to the inhibitory substances such as siderophores, HCN and protease produced by the strains tested. The siderophore produced by *Pseudomonas* strains tested accentuate the iron deficiency in the vicinity of bacterial colonies, resulting in the inhibition of Mycelial growth. The production of HCN by the 5 strains tested contributes widely to the antagonistic effect. Yuan *et al.* (2012) noted that the volatile

compounds produced by the bacteria reduced the Mycelial growth and inhibited spore germination of *F. oxysporum*. The bacterial strains showed high activity against this fungus, although Notz *et al.*, (2002) reported that fusaric acid alters the gene responsible for the synthesis of 2, 4-diacetylphloroglucinol (DAPG) by *P. fluorescens* CHA0, in vitro and in the rhizosphere of wheat. The antagonistic effect may be due to the synthesis of other antibiotics such as acid carboxylic phenazine, the pyrrolnitrin and pyoluteorin (Haas and Defago, 2005). Variability in inhibition, observed between the strains, can be explained by the amount and effectiveness of the antibiotic produced.

Conclusion

On the basis of their excellent growth promoter (Phosphate solubilization, IAA and ammonia production) and their biocontrol activities (siderophores production, HCN, and antagonistic effect), the fluorescent *Pseudomonas* tested should be used as wheat inoculants to enhance growth and improve yield. They could be exploited as biofertilizer and as biocontrol agents to replace chemical fertilizers and fungicides that impair to human health. The use of the producing IAA strains is an efficient approach to alleviate osmotic stress imposed on plants in defined situations. They could be, therefore, exploited for sustainable agriculture in arid and salty soils.

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