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# Antifungal activity of *Pseudomonas* strains isolated from wheat Rhizosphere against *Fusarium* sp.

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## Abstract

*Fusarium* diseases of small grain cereals, especially wheat cause significant yield losses worldwide. In this study, the antagonistic effect of *Pseudomonas* isolated from wheat rhizosphere, was studied against *Fusarium* species. The solubilization activity of mineral phosphate was evaluated using Pikovskaya's (PVK) medium, PGP traits were checked and antagonistic activity was reached by dual culture technique in Potato Dextrose Agar (PDA) medium. A total of 71 *Pseudomonas* were isolated from the rhizosphere of tree varieties of wheat (Salama, Wafia and Rajae) cultivated in the Northwest of Morocco. Of which 52% were able to solubilize tri-calcium phosphate (TCP). On the basis of the (Solubilization index ≥ 1.44), 11 strains of *Pseudomonas* were screened for their plant growth promoting (PGP) traits and evaluated for the ability to suppress growth of *Fusarium* species. The selected bacteria were able to produce hydrogen cyanide (HCN) except PS11 and PR23. Only PS11 and PR9 isolates showed the production of siderophores. Production of indole acetic acid was observed only in two bacteria, PW9 and PR9. All the isolates were positive for the production of some researched hydrolytic enzymes (amylase, protease, cellulase and chitinase). Results showed that PR19 had maximum inhibition against *Fusarium* sp. (59.16±1.44%) whereas PW11 showed the least inhibitory effect (20.43±1.86%). These results make some *Pseudomonas* strains, as PR9 and PR19, attractive as PGP bacteria. However, it requires further studies under pot culture as well as field conditions before to be recommended as biofertilizers and biocontrol agents for wheat.

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## Introduction

Wheat is the most widely grown crop in the world and provides 20% of daily protein and food calories for 4.5 billion people. It is the second most important food crop in the developing world after rice. Phytopathogenic fungi are among the most important factors that cause annually serious losses to agricultural products (Ekundayo *et al.*, 2011). Several *Fusarium* species can infect small grain cereals (wheat, barley and oat); the predominant species can vary according to geographic region, environmental conditions and crop species involved (Logrieco *et al.*, 2002; Van der Lee *et al.*, 2015). *Fusarium* spp. can cause direct damages such as seedling foot and stalk rots, or indirect damages resulting from seedling blight or reduced seed germination; however, the most important diseases in cereals that may cause severe reduction in yield and quality are head blight of small cereals as wheat, barley and oat (Munkvold, 2003; Nganje *et al.*, 2004). *Fusarium* toxins are secondary metabolites produced by toxigenic fungi that naturally contaminate cereals. They represent a source of grave concern in cereals and cereal-based products, resulting in harmful contamination of foods and feedstuffs (Placinta *et al.*, 1999). Biological control of plant pathogens is considered as a key component of disease management and a viable alternative to chemical control. Management of fungal diseases using antagonistic microorganisms has been the focus of intense research worldwide (Killani *et al.*, 2011). A variety of antagonistic bacteria, including members of the genera *Azospirillum, Azotobacter, Pseudomonas, Bacillus* and *Enterobacter,* are known to colonize the rhizosphere of most of the cereals and act as biocontrol agents (Naureen *et al.*, 2005). Different mechanisms have been identified in bio-antagonism of fungal plant diseases including competition for space or nutrients (e.g iron), production of antifungal secondary metabolites (e.g HCN), and secretion of hydrolytic enzymes such as chitinases and glucanases (Mayak *et al.*, 2004). The present study was aimed to isolate and characterize the genus of *Pseudomonas* subspecies, for their PGP traits and evaluate their antifungal activity against *Fusarium* sp.

## Materials and methods

*Isolation of Pseudomonas spp.*

*Pseudomonas* strains were isolated from the rhizosphere soil of three varieties of wheat (Salama, Wafia and Rajae) grown in Northwest of Morocco. One gram of rhizospheric soil was suspended in 9 ml of sterile physiologic water. After 1h of agitation aliquots of 100 µl of each dilution ($10^{-1}$ to $10^{-7}$) were plated on King's B medium (King *et al.*, 1954). Plates were incubated at 30 °C for 24 to 48 h. Colonies were isolated and purified on the same medium.

*Selection of phosphate solubilizing bacteria*

Phosphorus solubilizing activities of each isolate was assayed by spotting 10 µl of cultures on PVK media plates (Pikovskaya, 1948) containing 5 g of tri-calcium phosphate [$Ca_3$ $(PO_4)_2$] as sole phosphorus source. The plates were incubated at 30°C for 7 days. The ability of the bacteria to solubilize insoluble phosphate was described by the solubilization index (SI) = the ratio of the total diameter (colony + halo zone) to the colony diameter (Edi-Premono *et al.*, 1996).

*Plant growth-promoting traits*

*Hydrogen Cyanide (HCN) Production*

HCN production was checked by streaking the culture on nutrient agar medium supplemented with glycine (4.4 g /l). Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed on the top of the plate (Bakkerand Schippers, 1987). Plates were sealed with parafilm and incubated at 30°C for 48hrs. After incubation if whatman filter paper becomes orange to brown coloured it indicates production of HCN.

*Siderophores production*

The plates of TSA were spot inoculated with test bacteria and incubated at 30 °C for two days. A layer of chrome azurol S medium (CAS) (Schwyn and Neilands, 1987) was poured on the surface of each plate. After 24 h in the dark, development of orange halo around the bacteria was considered as positive for siderophores production.

*Quantitative assay of indole acetic acid (AIA) production*

The tested bacterial strains were cultured for 2 days in sucrose-minimal salts (SMS) medium (sucrose 1%; $(NH_4)_2SO_4$ 0.1%; $K_2HPO_4$ 0.2%; $MgSO_4$ 0.05%; NaCl 0.01%; yeast extract 0.05%; $CaCO_3$ 0.05%; pH 7.2) supplemented with 0.05% of L-tryptophan. After incubation, a 1 mL of supernatant was mixed with 2 mL of Salkowski reagent (Gordonand Weber, 1951) and the development of a pinkish color indicated the production of IAA. After 25 min incubation at room temperature, the absorbance was read at 535 nm.

*Quantitative assay of P Solubilization*

Solubilization activity was quantified using $Ca_3(PO_4)_2$ in PVK broth medium. Bacterial suspension (0.5ml of $10^8$ CFU $ml^{-1}$) was inoculated in a 100mL flask containing 50 ml PVK's broth. After incubation at 150 rpm at 30°C for 7 days, the culture was centrifuged at 1300rpm for 20 min. The supernatant was used to measure the soluble P content colorimetrically as described by Ames (Ames, 1966). Uninoculated flasks were established as the controls. The solubilized P content was estimated by subtracting the control P from the final P concentration.

*Production of hydrolytic enzymes*

The selected isolates were characterized biochemically for the production of amylase, protease, cellulase and chitinase. Amylase activity was assayed by soluble starch-yeast extract medium (Tsai *et al.*, 2007; Kammoun *et al.*, 2008). Protease activity was assayed by skim milk medium (Tsai *et al.*, 2007; Mahanta *et al.,* 2008). Cellulase activity was determined by Mandels–Reese medium with carboxymethylcellulose as the sole carbon source (Tsai *et al.*, 2007; Lee *et al.,* 2008). Chitinase activity was determined in chitin agar plates according to (Chernin *et al.,* 1995). Colloidal chitin was prepared as described by (Roberts and Selitrinnikoff, 1988). The halo ratios of clear zone (CZ) / colony size (CS) were calculated as enzyme activity indices (EAI).

*Isolation of Fusarium sp.*

Fungi were isolated from wheat grains, in Laboratory of Biotechnology and Biomolecular Engineering (LBBE), Faculty of Sciences and Technologies of TangierbyEl Aaraj *et al.* (2015). *Fusarium* species were isolated by direct plating method (Pitt and Hocking, 1997), and macroscopic and microscopic identifications were determined according to Nelson *et al.*, (1983).

*Antagonistic assay*

The selected *Pseudomonas* were screened for their *in-vitro* antagonistic ability against *Fusarium* sp.by dual culture technique (Rangeshwaran and Prasad, 2000). Bacterial isolates was streaked at the side of petri dish (1.5 cm away from the edge) containing PDA. Five mm mycelial plug from seven-day-old PDA cultures of *Fusarium* sp. was placed at the opposite side of petridishes perpendicular to the bacterial streak. Petri dishes were then incubated at 28±2°C for 7 days. Plates inoculated with phytopathogen alone were served as control. There were three replications for each isolate against pathogen. Percent of radial growth inhibition (PIRG) were recorded by the following formula (Naureen *et al.*, 2009):

PIRG (%) = (R1-R2/ R1) ×100

R1 = Radial growth of fungus in control plate

R2 = Radial growth of fungus interacting with antagonistic bacteria.

*Statistical analysis*

The data are reported as means ± SD (standard deviation) for three replicates. The results were compared by analysis of variance (ANOVA) according to Fisher protected LSD test ($p < 0.05$) using the Statgraphics Plus version 4.0.

**Results and discussion**

*Isolation and Selection of Phosphate Solubilizing Bacteria*

A total of 71 *Pseudomonas* spp. were isolated of which 52% were able to solubilize TCP. Based on the (SI≥ 1.44), 11 strains of *Pseudomonas* (PS4, PS5, PS11, PW9, PW11, PW14, PW17, PW18, PR9, PR19 and PR23) were screened for further studies. The highest SI was showed by PS11 with SI=2.83 (Table 1).

**Table 1.** PGP traits of the selected bacterial isolates.

| Isolates | SI | HCN Production | SID Production | AIA (mg/l) | P (mg/l) |
|---|---|---|---|---|---|
| PS4 | 1.57 | + | - | - | 60.10$^{b}$ (±5.00) |
| PS5 | 1.50 | + | - | - | 42.45$^{a}$ (±3,50) |
| PS11 | 2.83 | - | + | - | 41.24$^{a}$ (±7.23) |
| PW9 | 1.71 | + | - | 2.79$^{b}$ (±0.41) | 103.34$^{d}$ (±2.87) |
| PW11 | 1.83 | + | - | - | 44.56$^{a}$ (±5.50) |
| PW14 | 1.57 | + | - | - | 64.18$^{b}$ (±8.58) |
| PW17 | 1.83 | + | - | - | 132.63$^{e}$ (±0.55) |
| PW18 | 1.43 | + | - | - | 142.20$^{e}$ (±5.75) |
| PR9 | 2.33 | + | + | 2.03$^{a}$ (±0.53) | 69.33$^{c}$ (±7.01) |
| PR19 | 2.80 | + | - | - | 108.57$^{d}$ (±7,60) |
| PR23 | 2.16 | - | - | - | 46.67$^{a}$ (±1.65) |

Values indicate the mean ± SE for three replications. Letter indicate ranking order obtained by Fisher protected LSD test ($p < 0.05$).

*Plant growth-promoting traits*

Screening results of PGP activities of the selected bacteria are shown in Table 1. All phosphate solubilizing bacteria were able to produce hydrogen cyanide (HCN) except two strains PS11 and PR23. HCN plays an inhibition role in the electron transport and the energy supply to the cell causing death of the organisms; it inhibits the proper functioning of enzymes and natural receptor's reversible mechanism of inhibition, and it is also known to inhibit the action of cytochrome oxidase (Dowlingand O'Gara, 1994). Toyoda and Utsumi, (1991) reported that *P. solanacearum* were able to produce HCN and hydrolyzefusaric acid. This compound is the causative agent of the damage to plant that occurs upon *Fusarium* infection. Consequently, the bacterial strains can prevent the damage that is caused by various species of the fungus *Fusarium* (Toyoda and Utsumi, 1991).

Only two isolates (PS11 and PR9) showed the production of siderophores (Table 1).

It has been shown that disease reduction involving siderophore-mediated competition is an important antagonistic interaction that results in the exclusion of fungal pathogens from the rhizosphere due to reduction in the availability of iron for spore germination and hyphal growth (Rachid and Ahmed, 2005). Siderophores directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria, would suppress the growth of pathogenic organisms *viz., F. oxysporum* and *R. solani*, function as stress factors in inducing host resistance (Haas and Defago, 2005; Joseph *et al.,*2007;Wahyudi *et al.,*2011). Suppression of *Fusarium* wilt of radish by *Pseudomonas* strain WCS358 through siderophore-mediated competition for iron is another example (Costa and Loper, 1994).

Indole acetic acid (IAA) production was detected only in two bacteria, PW9 and PR9 getting to 2.79±0.41 and 2.03±0.53 mg/l respectively.

According to the literature, diverse bacterial species possess the ability to produce IAA (Ashrafuzzaman *et al.*, 2009; Saharan and Nehra, 2011). Besides, Yadav *et al.* (2010), reported that the bacterial strain *Pseudomonas putida* BHUPSB04 showed maximum significant concentration of IAA (25.65 µg $ml^{-1}$) followed by *Pseudomonas aeruginosa* BHUPSB02 (21.35 µg $ml^{-1}$). It functions as an important signal molecule in the regulation of plant development and indirectly by influencing bacterial amino cyclopropane-1-carboxylate (ACC) deaminase activity (Ryu and Patten, 2008; Wahyudi *et al.,* 2011).

**Table 2.** Enzyme activity indices and antagonistic effect of tested isolates against *Fusarium* sp.

| Isolates | Amylase | Protease | Cellulase | Chitinase | Antagonistic effect |
|---|---|---|---|---|---|
| PS4 | - | - | $0.61^{a}$ (±0.10) | - | $29.02^{b}$(±3.23) |
| PS5 | $0.14^{ab}$(±0.02) | - | $1.12^{b}$ (±0.23) | 0.76 (±0.16) | $48.38^{c}$(±5.60) |
| PS11 | $0.70^{d}$ (±0.08) | $2.33^{de}$(±0.14) | $1.65^{c}$ (±0.25) | - | $44.16^{c}$(±1.44) |
| PW9 | - | $2.03^{cd}$(±0.30) | - | - | $27.96^{b}$(±9.86) |
| PW11 | - | - | - | - | $20.43^{ab}$(±1.86) |
| PW14 | - | $1.80^{bc}$(±0.20) | - | - | $26.88^{a}$ (±4.93) |
| PW17 | - | $1.63^{b}$ (±0.20) | $0.44^{a}$ (±0.10) | - | $30.11^{b}$ (±3.72) |
| PW18 | - | $2.44^{e}$ (±0.20) | - | - | $26.88^{ab}$(±4.93) |
| PR9 | $0,10^{a}$ (±0.01) | $1.50^{b}$ (±0.17) | $0.56^{a}$ (±0.02) | - | $44.16^{c}$ (±1.44) |
| PR19 | $0.31^{bc}$(±0.15) | $2.23^{de}$(±0.25) | $2.28^{d}$ (±0.40) | - | $59.16^{d}$ (±1.44) |
| PR23 | $0.34^{c}$ (±0.15) | $0.90^{a}$ (±0.19) | $1.26^{b}$ (±0.13) | - | $44.10^{c}$ (±1.86) |

Values indicate the mean ± SE for three replications. Letter indicate ranking order obtained by Fisher protected.

It has been observed that the role of bacterial IAA in different plant-microbe interactions highlights the fact that bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms (Ahmad *et al.,* 2008; Samuel and Muthukkaruppan, 2011). The concentration of dissolved P was between 41.24 and 142.20 mg/l. Statistically, the maximum P solubilization was recorded by PW18 (142.20 mg/l).

*Production of hydrolytic enzymes and antagonistic effect*

The enzyme activities of tested isolates are shown in Table 2. The isolates PS5, PS11, PR9, PR19 and PR23 exhibited positive for the production of amylase. The highest EAI (0.70±0.08) was registered by the isolate PS11 while the lowest values were presented by 0.10±0.01 and 0.14±0.02 obtained by PR9 and PS5 respectively as per statistical analysis (Table 2). Protease activity was detected in all the selected bacteria except PS4, Ps5 and PW11.

Statistically the maximum production of protease was recorded by PW18, PR19 and PS11 with EAI 2.44 ±0.20, 2.23±0.25 and 2.33±0.14 respectively. Production of cellulase was positive for the bacteria isolates PS4, PS5, PS11, PW17, PR9, Pr19 and PR23. Statistical analysis showed that the highest EAI was recorded by PR19 isolated strains with EAI=2.28±0.40. Chitinase activity was detected only in PS5 isolate with EAI= 0.76 (±0.16).

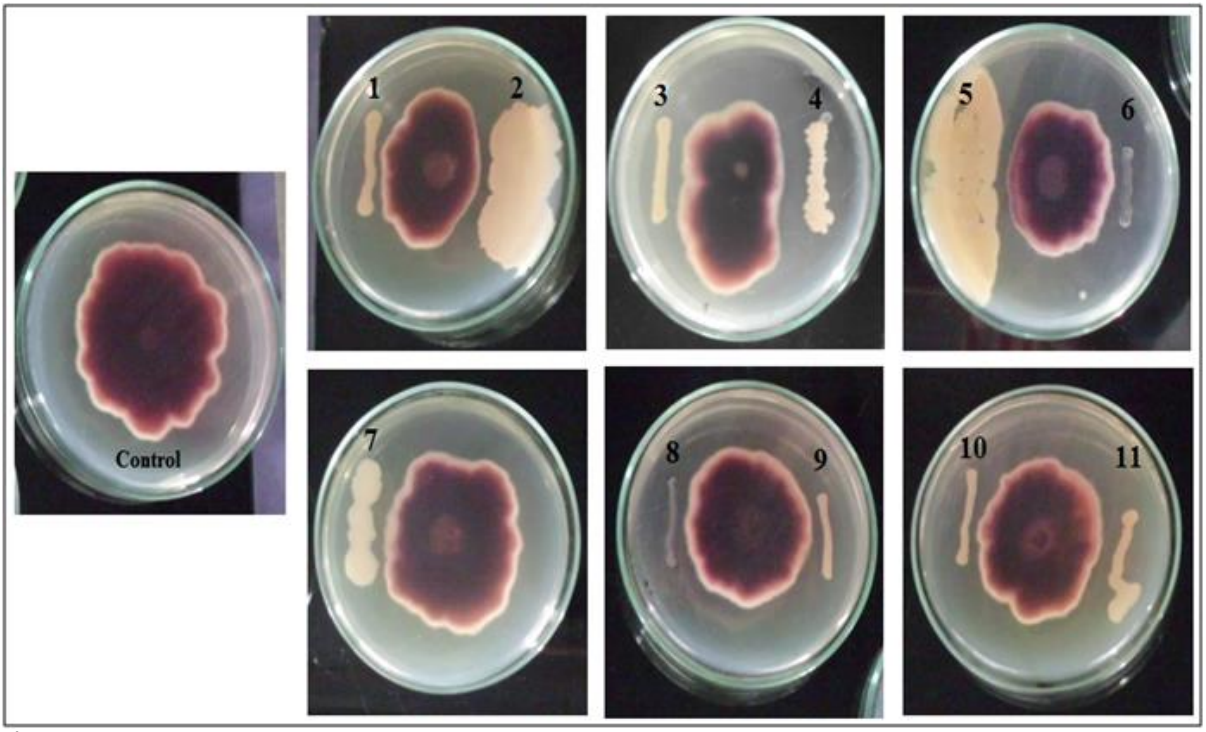


**Fig. 1.** Antagonistic activity of the selected *pseudomonas* spp. against *Fusarium* sp. on PDA agar medium.1: PS4; 2: PS5; 3: PS11; 4: PR19; 5: PR23; 6 PR9; 7: PW9; 8: PW11; 9: PW14; 10: PW17; 11: PW18.

The hydrolytic enzymes get great attention because they play a significant role in controlling diseases by excreting cell wall hydrolases (Chernin and Chet, 2002). Several rhizobacteria, including genera of *Pseudomonas*, are known to produce a battery of hydrolases such as chitinase which help in the maceration of cell walls of those plant pathogens (Lim *et al.*, 1991; Singh *et al.*, 1999; Huang *et al.*, 2004; Hoster *et al.,* 2005; Aktuganov *et al.*, 2007; Bogas *et al.*, 2007).

The selected isolates were evaluated for their antagonistic effect against *Fusarium* sp. (Fig. 1, Table 2). Statistical analysis showed that PR19 had maximum inhibition (59.16 ±1.44 %), whereas PW11 showed the least inhibitory effect (20.43 ±1.86 %) (Table 2).

The potential antagonistic activity of the bacteria PR19 could be correlated with the highest protease, chitinase and amylase activity of these bacteria isolate, also with its ability to produce HCN. Conversely, the isolate PW11 did not synthesize any hydrolytic enzyme and showed the modest antagonistic effect against *Fusarium* sp.

**Conclusion**

The results obtained in this study pointed out the possible use of strains of *Pseudomonas* as phosphate solubilizers and biocontrol agents against *Fusarium* sp.. However, further research is needed under pot culture as well as field conditions to elucidate the mechanism of action of the potential antagonistic bacteria in detail.

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