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Evaluation of multi-trait plant growth promoting *Pseudomonas fluorescens* isolated from Constantine Wheat rhizosphere Soil (Algeria) and screening there antifungal activity against two species of *Fusarium*

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ABSTRACT

Fluorescent pseudomonas can suppress various soilborne diseases, and their efficacy related both; to their antagonistic activities and rhizosphere competitiveness. This study was designed to isolate antagonistic *Pseudomonas fluorescens* from Wheat rhizosphere and evaluate their Plant promoting traits. Fifty-five antagonistic strainswere isolated from wheat soil, cultivated in Constantine region (Algeria),characterized morphologically, biochemically and molecularly, and screened for their Plant growth promoting traits. These PGP traits were analyzed by phosphate solubilization, indol acetic acid (IAA), the production of siderophore, ammonia, hydrogen cyanide (HCN), and the production of enzyme involved in the suppression of the pathogen like cellulase, pectinase, chitinase, and protease.At the end, the biocontrol capacity ofthese strainsagainst two speciesof *Fusarium*: *F. culmorum* and *F. pseudograminearum*was evaluated*in planta*. All the isolates showing a biochemical and morphological of *Pseudomonas fluorescens*. Under in-vitro conditions,all isolates produced cellulase and pectinase, 90.9% produced siderophore; hydroxamates type, 96.43% produced IAA.A 96.36% of isolatesproduceda clear zonearound the colony, exhibiting different sorts of phosphate solubilizing index (PSI) and 76.36% solubilizedthephosphate in liquid medium, 51.78% produced protease, 48.21% produced lipase, 16.36% produced chitinase, and only 10.9% produced HCN. The selected strains inhibited *Fusarium*sp growth when tested in pot experiments. Nine bacterial strains, which showed a maximum plant growth promoting traits using the molecular identification (16S r DNA gene sequence), were identified as *Pseudomonas fluorescens.*This study concludes that strains of *Pseudomonas fluorescens*, isolated from wheat rhizospheric soil from the region of Constantine (Algeria), showed variation in their plant promoting characteristics production that can contribute to the ability of these isolates to suppress fungal diseases. Based on the positive results of the antagonistic effect of selected strains, it is interesting to use the PGPR *Pseudomonas fluorescens* as inoculants biofertilizers to replace chemical fertilizers and pesticides for Wheat.

KEYWORDS: Pseudomonas fluorescens,Plant Growth Promoting Rhizobacteria, Fusarium.

INTRODUCTION

Plant growth Promoting rhizobacteria(PGPR) are a group of soil microorganisms that can stimulate plant growth, protect plants from diseases, and increase crop yield [27]. For decades, varieties of PGPR have been studied and some of them have been commercialized, including the species Pseudomonas, Bacillus, Enterobacter, Klebsiella, Azobacter, Variovorax Azosprillum, and Serratia [28]. However, the successful utilization of PGPR is dependent on its survival in soil, the compatibility with the crop on which it is inoculated, the interaction ability with indigenous microflora in soil, and environmental factors [45]. Another challenge is that the modes of action of PGPR are diverse and not all rhizobacteria possess the same mechanisms [17,23]. PGPR used as biofertilizers and/or antagonists against plant pathogens are a promising alternative to chemical fertilizers and pesticides. The number of bacterial species identified as PGPR has recently increased due to many studies on a wider range of plant species, the progress in bacterial taxonomy had developed abetter understanding of the various mechanisms action of these rhizobacteria. Currently, PGPR include diverse bacterial taxa isolated from various rhizosphere [7]. Therefore, several workswere performed in order to isolate effective PGPR from the rhizosphere of wheat. [1;26;39] haveisolated and examined PGPR strains that can be applied in the rhizosphere of wheat, which aims to assess their potential use in enhancing growth by producing phytohormone and their nitrogen-fixing capabilities.

Bacteria belonging to the fluorescent *Pseudomonas*groupare among the most abundant in the rhizosphere. In some cases, they represent over 60% of the total bacterial soil microflora[24]. These bacteria are also usually found among the potential biological control agents, which have the effect of improving the health of plants, and are particularly known for their antagonistic effect against plant pathogens. The wide variety of mechanisms of action of these *Pseudomonas*spp, is mainly linked to their great ability to produce a wide range of secondary metabolites, usually auxin, plant pathogen antagonists, Cyanogenesis(HCN), phosphate solubilization, production of siderophore and ACC desaminase activity, and induced systemic resistance in plants [13;63;67; 68].

The aim of this study isnot only leading to isolation and characterization of *Pseudomonas fluorescens*strains from the rhizosphere of wheat grown in the Constantine region (Algeria),but also evaluating their potential to promote plant growth, and studying there interaction in vivo with two species of *Fusarium* to assess their effect on the incidence of the disease.

MATERIALS AND METHODS

Isolation and identification of Pseudomonas fluorescens:

Soil samples from durum wheat (*Triticumdurum*) rhizospherewere collected from Constantine region, and ten grams of this soil samples were used in serial dilution method[4]. *Pseudomonas fluorescens* were isolated on Kings B medium, containing per liter of distilled water: 20 g peptone, 10 ml glycerol, 1,5 g K_2HPO_4, 1,5 g $MgSO_4$ $7H_2O$, 18 g Agar [4]. The colonies showing fluorescent yellow to yellowish green coloration on Kings B were picked up and stored at 4°C.

Identification of isolates was performed using Bergey's Manual of Systematic Bacteriology, following every characterizing aspect, considering tests: macroscopic appearance(appearance of the colony on solid King B medium, form, and texture),Gram reaction, mobility, oxidase test,Gelatinhydrolisis, Arginine dihydrolaseand growth at 42°C and 4°C[12; 49].

Another selective test of our isolates, is the antagonistic effect against *Fusarium culmorum* and *Fusarium pseudograminearum*,the causal agents of wilt wheat, isolated from wilted plants cultivated in Constantine region(Algeria), and identified on the basic of 18S rDNA sequence analysis, and the access number are respectivelyKP726896,KP726902. The bacterial strains were screened against the phytopathogens by a dual culture method given by [38], and the index of inhibition was calculated.

Measurement of Plant growth promoting activities:

-Detection of microbial Siderophore production:

This test was conducted by qualitative and quantitative methods.

The qualitativesiderophore production was tested in Chrome AzurolSulfonateAgarmedium (CAS), described by [5]. CAS agar plates were spot inoculated with each bacterial strain and incubated for 72h at 30°C.Positive cultures for siderophoreproduction produced an orange halo around the colony. The size of this halo was measured.

For the quantitative siderophore production, 100µl of culture strains were inoculated in King B medium and incubated at 30°C for 48 h. The cells were removed by centrifugation at 5,000rpm for 20min,then500µl of the supernatant were mixed with 500µl of CAS solution and incubated for 30 minat room temperature and darkness.The color changed from blue to orange at the rate of production of siderophore. The OD was measured

by a spectrophotometer at 630 nm.The percentage ofsiderophorewas calculated using the following formula (29):

$\% = (S_t - S_e) / S_t \times 100$, where:

S_t: OD of CAS solution of intense blue color (control).

S_e: OD of sample solution.

For the detection ofsiderophore's nature, two tests were used (the strains were cultured in king B medium for 48h at 30°C):

Arnow test(6) for the detection of catechols. One ml of supernatant was amended with 0.1 ml of 5 M/l HCl and 0.5ml of ammonium molybdate (containing 10g of $NaNO_2$ and 10 g of $Na_2Mo\ 2H_2O$ diluted in 50ml of distilled water). When a yellow color appeared, 0.1 ml of 10 N NaOH is added. If a red pink color is observed, it indicates the presence of catechols.

$FeCl_3$ test(47)for the detection of hydroxamates. One ml of supernatant was amended with a solution of chloric iron (2% $FeCl_3$). Formation of a reddish purple color indicates the presence of hydroxamates.

-Production of Ammonia:

Isolates were inoculated into peptone water, and incubated for 4days at 30°C.Nessler's reagent (0.5ml) was added in each tube. The development of faint to yellow color indicates small amounts of ammonia and deep yellow to brownish color indicates maximum amounts of ammonia production [15].

-Indol acetic acid (IAA) production:

The production of IAA was estimated according to a method of [14], and modified by [2]. A 500 µl of 24 h old bacterial cultures were inoculated in tubes containing 5ml of King B medium amended with 0.1%of Tryptophan, and were incubatedin an incubator shaker at 30 °C and 180 rpm for 48 h in dark. One tube was kept uninoculated as control. After the incubation, bacterial cultures were centrifuged at 10,000 rpm for 15 min at 4 °C. Two ml of supernatant were mixed with 4 ml of Salkowsky'sReagent (1 ml of 0.5 M $FeCL_3$ in 50 ml of 35% $HCLO_4$) along with 2 drops of orthophosphoric acid,and the mixture was kept in the dark. After 30 min in dark incubation at 28°C, a developmentof pink color indicates the qualitative IAA production. For the quantitative estimation, the absorbance at 530nm was measured using UV visible spectrophotometer, and the concentration of IAAwas calculated using the standard curve. The result was expressed as µg/ml over control[30].

-HCN production:

Hydrogen cyanide (HCN) production was evaluatedaccording to[9]. Bacterialisolates were inoculated in King B agar medium amended with 4.4g/l of glycine. Awhatman filter paper N°:1 was impregnated with 0.5% picric acid and 2% of sodium carbonate, and was placed in the lid of each Petri dish. Then the disheswere sealed with parafilm and incubated at 30°C for 96h. Discoloration of the filter paper from deep yellow to orange and orange to brown indicates the production of HCN.

-Phosphate solubilization Test:

For the qualitative estimation, the bacterial strains were inoculated on plates of Pikovskaya agar medium [52], and incubated at 28°C for 7 days. Plates were observed for clearing zones around the bacterial colonies; that is a sign of phosphate solubilization activity.Phosphatesolubilization index (PSI) was calculatedaccording to this formula [64]:

PSI = [Colony diameter + halozone diameter]/Colony diameter.

The quantitative analysis of tricalcium phosphatesolubilizationwas carriedoutin liquid medium, by inoculating 100µl of the culture strains and incubating it at 30°C for 11 days. After incubation, the bacterial cultures were centrifuged at 3000rpm for 20min and the amount of soluble phosphate was measured by John method [37]. Two ml of supernatant were taken and placed in a test tube and 8ml of mixed reagent were added. To prepare the mixed reagent, 1.5g of ascorbic acid will be added to 100ml of the stock solution; (20 g of $(NH_4)_6Mo7O_{24}4H_2O$ were dissolved in 300 ml of distilled water, and 450ml of 10N H_2SO_4 will be added slowly with constant stirring to which 100 ml of 0.5 % antimony potassium tartrate will be added, the solution was diluted to one liter and stored in amber colored glass bottle).The mixture was shaken 10min to complete color development. The absorbance was determined at λ= 880 nm, andthe concentration of soluble phosphate was estimated using a standard curve,and expressed as equivalent phosphate in µg/ml[37].

- Enzymatic activity:

** Catalase production:*

Catalasewas performed qualitatively using the method described by [59]. Hydrogen peroxide $H_2O_2$was added on the colonies grown on nutrient agar medium plates; effervescences indicates catalase activity.

** Preparation of colloidal chitin:*

Colloidal chitin was prepared from shrimp shells (Sigma) according to the modified method of [10]. In brief, 40g of chitin powder were slowly dissolved in 400ml of concentrated HCl and kept at 30°C for 1h in chemical hood with vigorous stirring. Chitin was precipitated as a colloidal suspension by adding 2liter of cold distilled water and left overnight at 4°C. The supernatant was slowly decanted and the precipitate was collected on a filter paper and washed extensively with distilled water for four to five times until colloidal chitin became neutral(pH 7,0). The colloidal chitin was autoclaved at 121°C for 20min and stocked at 4°C for further use as a substrate.

**Chitinase production:*

The ability of the isolates to decompose colloidal chitin was performed on the colloidal chitin agar medium. The compositionper literwas:4g colloidal chitin, 1.1g Na_2HPO_4, 0.2g $MgSO_47H_2O$,0.7gKH_2PO_4, 0.001 g $FeSO_4$, 0.001g$MnSO_4$, 2g $(NH_4)_2SO_4$and 15g agar; pH was adjusted to 8± 0.2 and autoclaved for 15 min at 121°C [56]. The isolates were inoculated and incubated at 30°C for 10 days. The ability of chitinase production was shown by a clear halo around bacterial colonies [55].

** Amylase production:*

The amylase production was evaluated on nutrient agar amended with 1% of soluble starch [20]. Starch medium plates were inoculated with bacteria and incubated at 30°C for5 days. After incubation period, the plates were flooded with iodine solution, kept for a minute and then poured off. The appearance of clearzone surrounding the colony indicates positive for starch hydrolysis test [36].

**Cellulase activity:*

The isolates were inoculated on King B agar medium plates amended with 1%of CMC (Carboxyl Methyl Cellulose). The plates were inoculated and incubated at 30°C for 5 days, after they were flooded with Congo red solution (1% w/v)and kept for 20 min, and followed by washing the plates with solution of NaCl 1N. Formation of clear zone indicates cellulase degradation.

**Lipase activity:*

Bacteria were inoculated on nutrient agar amended with egg yolk [48]. After 48h of incubation, the plates were flooded with a saturated solution of$CuSO_4$ and dried for 15 to 20min at room temperature. The appearance of blue greenish color on the surface around the colony indicates the production of lipase.

**Pectinase activity:*

The pectinase activity wasscreened using King B medium amended with 0.5% pectin. The plates were incubated for 48h, flooded with iodine solution and kept for 30min. The appearance of clear halo around colonies indicates pectinase production[20].

**Protease activity:*

The assay for protease production was determined by a clear zoneon skim milk agar plates,obtained by mixing 1 g of agar suspended in 50 ml of distilled water, with 5 g of skimmed milk powder suspended in 50 ml of distilled water[16].

Screening antagonistic activity in vivo againstF.culmorum andF.pseudograminearumand evaluation of promotion growth:

Two experiments have been performed:

-The first experiment was designed to test the interaction between bacterial strains and two fungal isolates (antagonistic activity). Eleven isolats were used to evaluate there ability for disease suppression. The selection of strains was based on the: phosphate solubilization index, production of siderophores, and production of IAA. Thestrains were grown at 30° C / 24 hours in nutrient broth thenthe cultures were adjusted to a density of 10^8 bacteria / ml for each strain. The durum wheat seeds were surface sterilized in ethanol for one minute and in sodium hypochlorite for 15 minutes, followed by ten times washing with sterile distilled water, after were allowed to grow in petri plates having sterile filter paper, at 20° C for 5 days. The germinated seeds were then transplanted in plastic pots containing sterilized soil, inoculated with 2 ml of bacterial suspension per plant, and placed in a growth chamber under standard conditions. The plants were watered regularly with distilled sterile water. One week after, the plants were infected with 5ml of *Fusarium* per plant (The inoculum of pathogen was prepared in two flasks containing a sterile liquid PDA inoculated with two Fusarium strains separately: *F.culmorum* and *F.pseudograminearum*). Ten plants per treatment were used in this test. Plants containing none pathogen were treated as positive control. The evaluation of the desease was carried out for 45 days based on a rating scale of symptoms proposed by [62], and included four values from zero to three: 0, no symptoms; 1,

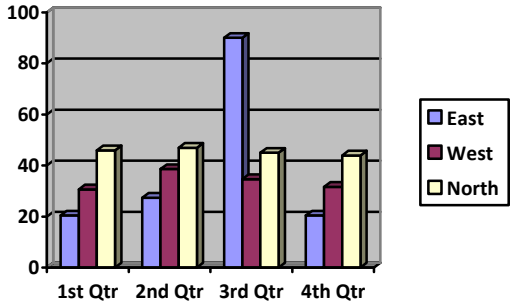
slight or moderate yellowing of the plant, slight collar rot; 2, moderate or severe yellowing of the leaves with browning of the rod,and important collar rot and secondary roots, and 3, death of the plant.Based on these ratings, the disease index was calculated.

-The second experiment evaluated the promotion growth of bacterial isolates, and included only the same bacterial treatments, and in the same conditions realized in the first experiment. Five replicates were performed for each treatment, with 2 plants per replicate. After 30 days of planting, morphological characteristics of each plant were recorded: plant height, root length, and the biomass as fresh materiel and after ovendriedit at 65°C overnight.

Molecular identification:

The isolates showing the best potential of PGPR activitieswere selected for molecular identification,based on 16S rDNA sequence analysis. The DNA of the isolates was extracted using the QIAGEN kit (DN easy Blood and Tissue Kit for purification of total DNA) according to the manufacturer's instructions. The amplification of the 16SrDNA gene (1.5 Kb)was carried out by PCR using the universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG- 3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3')according to[61].

The reaction was carried out with 25µl of solution containing 5µl of DNA, 1.5µl of each primer (5µM), 0.64 dNTPs (10mM), 1.5 µl $MgCl_2$(25mM), 6.66µl ultrapure sterile H_2O(LP), 0.2µl of Taqpolymerase (5U/ml), and 5µl of PCR buffer (x5 Gren Go taq). The amplification reaction was performed in a thermo-cycler (PCR System 9700, AppliedBiosystems),programmed foraninitial cycle of94ºC for 5s, followed by 35 cycles of 94ºC for 1s, 55ºC for 1s, 72ºC for 1s, followed by a final extension at 72°C for 7min.PCRfragments obtained were sequenced using the automatic sequencer at DNA (GeneticAnalyser 3500, Applied System,HITACHI). The nucleotide sequences of 16 rDNA were subjected to Blast Analysis with NCBI database(http://www.ncbi.nlm.nih.gov/Blast.cgi).



Statistical analysis:

Plant growth promotion data were analyzed statistically usinga software Minitab 13 program, by performing an analysisof variance (one way ANOVA). The significance of differences between mean values was evaluated by LSD.

Results:

A 60 isolates were obtainedon King B medium from wheat rhizosphere soil showing fluorescent colonies. Nearly all the strains were Gram-negative, except five isolates that were gram-positive, and were excluded from further testing (Figure 1).

The 55 strains were tested on the basis of cultural, morphological and biochemical characteristics as described in Bergey's Manual of Determinative Bacteriology [36]. They were characterized as fluorescent, Gram-negative, tested positively for oxidase test, mobility, Arginine dihydrolase, did not grow at 42°C, and showed antagonistic activity against *Fusariumculmorum* and *Fusarium pseudograminearum.* These isolateswere assumed to belong to fluorescent *Pseudomonas spp*. (Table 1).

The isolates showed a different response with both soilborn phatogen *Fusarium culmorum* (Fus1) and *Fusarium pseudograminearum* (Fus 7). The most potential isolates were the four isolates out 55, which have numbers of Ps4, Ps61, Ps50, and Ps43 with in vitro inhibition index of Fus1 39.22%, 32.94%, 32.16% and 31.37% respectively; and the isolates wich have numbers of Ps34, Ps15, Ps11, and Ps57 with in vitro inhibition index of Fus7 45.83%, 43.11%, 41.78% and 40% respectively (figure 2).

Siderophore production and ammonia production:

All 55 isolates showed no positive result for Arnow's assay, while $FeCL_3$ assay showed a positive result for Hydroxamates type of siderophore. The CAS assays showed orange halos color around 90.9% colonies, with the largest diameter observed in two strains, Ps9 and Ps17 (17 mm), followed by 15 mm of diameter in strains Ps7,

Ps8, Ps4 and Ps53 (figure 1 and table 1). The quantitative estimation of siderophore production showed that 42 strains (76.36%) produced siderophore in liquid medium, ranging from 2.56% to 93.46%.

Another important trait of PGPR, that may indirectly influence plant growth, is theproduction of ammonia. All isolates were able to produce ammonia, this was determined in peptone water after the addition of Nissler's reagent; development of brown to yellow color indicates ammonia production (Table 2).

IAA production, Catalase and HCN production:

IAA is the phytohormone known to enhance plant growth. All 55 bacterial strains were tested for qualitative IAA production, showing a pink color after addition of Salkowsky's reagent, except strain Ps11 showed no color. For the quantitative estimation, 96.43% of strains produced an IAA ranging from 1.12 µg/ml to 28.87 µg/ml, with the highest amount recorded for strain Ps6. All isolates were found to be catalase positive, strong effervescences of O_2 evolved when 6% of H_2O_2 solution was flooded on the colonies grown on King B medium. This indicates a positive result for catalase production.HCN production was observed in color change of filter paper from deep yellow to orange, in only six strains, andthe higher ability was appeared on with strains Ps18, Ps26 and Ps 65 (Table 2).

Phosphate solubilization:

The 55 selected strains were tested for their ability to solubilize inorganic phosphate on a solid medium containing $Ca_3(PO_4)_2$as the sole source of phosphorus. After 7 days of incubation, 96.36% of isolates produced a clear zone around the colony translated qualitative solubilization of phosphate. The isolates exhibited different sorts of phosphate solubilizing index (PSI) ranging from 1 to 7.66. This variation in substrate's utilization by these strains could be due to the difference in their organic acids production (Figure1 and table 2). The quantitative estimation of soluble phosphate on liquid medium was determined after 11days of incubation. A 76.36% of isolates solubilize the phosphate in ranges from 4.5 µg/ml to 723.3 µg/ml. Despite that no amount of solubilizing was observed on liquid medium of the strain Ps10, though it showed the highest index of solubilization (PSI=7.66) (table 2).

Enzymatic activities:

The improvement of biocontrol efficiency and plant growth promoting was also observed in production of different enzymes. All strains showed a positive result with catalase test, and only one strain (Ps11) showed amylase activity. While chitin activity was detected only in nine strains with the observation of a large halo zone (25 mm) on strain Ps66. Production of plant polymer hydrolytic enzymes involved in pectinase, and cellulase, was observed in all strains with variable quantities that were visible by formation of clearance zone.

The results of lipase test revealed that 48.21% (27 strains) of isolates produced lipase enzyme. Proteolytic enzyme production was detected in 51.78% of isolates (29 strains) by formation of a clear zone around cells on skim milk agar medium (Figure 1, Table. 3).

Based on the above results, 11 isolates were selected; Ps7, Ps8, Ps12, Ps14, Ps17, Ps47, Ps52, Ps53, Ps65, Ps66, and Ps68 for the following experiments.

Disease suppression in plants and growth promotion:

The inoculation of wheat plants with a mixture of bacterial isolates and fungal strains, generated a weak attack of pathogens. However, the percentage of plants inoculated only by *F.culmorum* having a score of symptoms ≥2 was observed on 90% compared to those inoculated with *F.pseudograminearum* only; the percentage observed was 70%. When bacterial cultures were applied to the soil, they improved differently the attack of the plant against the pathogen (*F. culmorum* or *F. pseudograminearum*) (Table 4).

The selected bacterial strains significantly enhanced all growth parameters compared with the infested control (Table 5). At 30 days, the height of plants in all treatments was better and taller than the non-inoculated except one treatment with strain Ps52; which was smaller than the control. And the same treated plants showed a variation in the fresh and dry weight comparedto the control. As might be expected, the reduction of disease by PGPR treatments was accompanied by an increase in plant growth. However, growth measurements on un-infected plants indicated that the PGPR strains under test also directly promote the growth of wheat.

Bacterial identification using 16s rDNA Gene Sequence:

Based on the maximum positive results of plant promoting growth traits of the isolates, 9 of strains were selected, and were the aim of a molecular characterization using the sequencing of the 16s rDNA gene. Two strains were identified as *P.fluorescens* strain DmBR 2; Two strains were identified as *P.fluorescens* strain NITDPY, One strain was identified as *Pseudomonas* sp. EP_S_49, One strain was identified as *P.fluorescens* strain dqe01,One strain was identified as *Pseudomonas fluorescens* A506, One strain was identified as *Pseudomonas fluorescens* strain B-Exp9, and One strain was identified as *P.geniculata* strain MD 05.All results were shown in (Table 6) with accession number and percentage of similarity.

Discussion:

The increasing importance of beneficial bacteria in agriculture has resulted in many efforts to isolate and identify bacteria associated with the rhizosphere of plants, in order to trace their roles in plant growth promotion and protection against phytopathogens. The aims of this study was the screening of*Pseudomonasfluoresens* strains in the rhizosphere of Wheat from Constantine region , the measurement of their plant growth promotion and their antagonism activities against two soil-borne fungal pathogens: *Fusarium culmorum* and*Fusarium pseudograminearum*. A total of 55 strains were isolated and identified as fluorescent *Pseudomonas* according to morphological and biochemical characteristics as described in Bergey's Manual of Determinative Bacteriology [36]. The antagonistic action of these same strains did not seem to be specific for the pathogenic agent in some cases, but a broad-spectrum efficacy has been observed, acting at the same time on several fungal isolates of the same genera (*F. culmorum* and *F. pseudograminearum*).

Additionally, the beneficial effects of *Pseudomonas fluoresens* are associated with their mechanism and metabolites. Moreover, certain PGPR possess more than one plant growth promoting mechanism [33]. Upon the examination of their siderophore production, all isolates were able to produce hydroxamates type of siderophore, with about 90.9% of isolates and the maximum of production reachs 93.46%. Similar result was obtained by [11] and [48], who concluded that *Pseudomonas fluorescens* showed formation of hydroxamate type. Other reports stated that the production of siderophore sequester iron in the root environment, and making it less available to the competitive deleterious microflora [8;11;21]. Chlorosis is a condition in which leaves produce insufficient chlorophyll. Siderophore producing microorganisms significantly increase chlorophyll concentration in leaf. Jurkevitch et al, [38] observed that siderophore producing *Pseudomonas* improve chlorophyll content and concluded that siderophore producing bacteria may have a potential role in controlling lime-induced iron deficiency in plants. Indole-3-acetic acid (IAA) is a member of the auxin family of phytohormones that influence many cellular functions in plants and therefore are important regulators of plant growth and development. In addition to production in plant tissues, IAA synthesis is widespread among plant-associated bacteria and provides bacteria with a mechanism to influence plant growth [50]. Almost the majority of isolates producedgrowth-promoting hormone IAA. Similar results were reported by [31], showed that *Pseudomonas* sp. Strain OG produced 29 µg/ml, and *Pseudomonas fluorescens* CHAO can produce up to 32 µg/ml of IAA [32]. HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens [19]. The production of HCN by certain strains of fluorescent *Pseudomonas* has been involved in the suppression of soil borne pathogens [65]. For instance, in the previous experiment, six strains only showed a production of HCN. Lanteigne et al, [43] isolated HCN producing *Pseudomonas* and observed their biological control activity. Other report suggests that HCN has antimicrobial activity and effectively controls the growth of plant pathogenic fungi. Genus *Pseudomonas* is one of the leading bacteria which inhibit the growth of pathogenic fungus in agriculture fields.

Another important plant growth promoting trait of PGPR is the phosphate solubilization, where bacteria expected to promote plant growth by increasing phosphorous uptake [34]. Theisolates obtained from the rhizosphere of wheat were tested for their efficiency of Phosphate solubilization, and approximately 76% of strains showed a positive result, both in solid or liquid medium.Ruchi et al, [57] had a similar observation, among 26 of the *Pseudomonas fluorescens* isolates, only 10 isolates showed a diameter of clear zone ranging between 17-22 mm. Others results have also been reported by [60];when the largest phosphate solubilizing index was created by *Pseudomonas* sp. with PSI= 2.98. It is evident from the in vitro tests that both solubilization of inorganic P and phosphatase activity (mineralization) can coexist in the same bacteria. Tao et al, [61] reported the coexistence of both capabilities in a single bacterium. These results are consistent with those of [60].

Pseudomonas fluorescens has the ability to produce the cellulose enzyme that degrades the fungal cell wall. This is an important mechanism of fungal inhibition, with pectinase production; which is known to catalyze the pectic substance through the depolymerisation's reaction. These enzymes have the role in preventing plant from infection caused by pathogens [54]. Extracellular lipase and protease can contribute to the ability of bacteria to suppress fungal diseases. Meanwhile, the production of these components by many of isolates demonstrated a valuable potential of PGPR for biological control. Some of *Pseudomonas* species as a group of PGPR can be involved in the control of plant diseases [3].

Pseudomonas fluorescens has been shown to increase seed germination, root and shoot length, and seedling vigour in several instances [25;39;53]. Manikandan and Raguchander, [44] indicated that Pf1 liquid formulation reduced *Fusarium* wilt disease, and at the same time Pf1 liquid formulation triggers activity of defence enzymes in tomato roots during the infection of *F. oxysporum* f. sp. *lycopersici*. In fact, different wheat pathogens play a direct role in the destruction of natural resources in agriculture. Traditional use of chemical pesticides to suppress these pathogens is currently under revision due to public concern about the impact on human health and on the environment. For this reason, the interest in biological control has been increased recently [55]. Diverse PGPR produce anti-fungal metabolites such as DAPG [46], siderophores and secretion of lytic enzymes that may reduce the growth of phytopathogens present in the rhizosphere [19]. Mavrodi et al, [46] have isolated

new strains of *Pseudomonas* from agricultural soils, river silt, and soils from herbarium specimens that show the ability to reduce disease symptoms of both *R. solani* and *Pythium ultimum*; two wheat soilborne fungal pathogens; correlated with growth promotion of wheat seedlings at the same time. Plant growth promotion may reflect the phyto-stimulatory properties of these bacteria, including IAA synthesis and P-solubilisation. Sari et al, [58] also observed that all the *Pseudomonas* isolates significantly reduced diseases incidence of wheat take-all compared to *Ggt* only control. *Pseudomonas fluorescens* CHA and *P. fluorescens* bioIII (21p) were more effective in reducing take-all than the other isolates tested, and seed inoculation with *Pseudomonas fluorescens* bioIII (21P) sinificantly promoted root fresh, and shoot dry weight.

Table 1: Morphological and Biochemical characters

Biochemical characters	Reaction
Number of isolates	55strains
Gram reaction	Negative
Cellshape	Rods
Fluorescent pigment	yellow to greenish
Oxidase test	+
Catalase	+
Arginine Dihydrolase	+
Gelatin hydrolisis	94.64% (of strains)
Growth at 4ºC	+
Growth at 42°C	-
Mannitol	73.21% (of strains)

Table 2: Characterization of plant growth promoting traits

Isolates	Phosphate solubilization		Siderophore production	IAA Production			
	Index	Production of P (µg/ml)	% of sidro production	Catechols	Halo zone (mm)	Hydroxamates	µg/ml
Ps1	2,5	4,5	17,37	-	6	+	2,25
Ps2	2,5	298,8	36,76	-	10	+	0
Ps4	4	127,5	19,96	-	8	+	11
Ps6	0	64,8	62,39	-	13	+	28,875
Ps7	2,6	0	65,89	-	15	+	7,25
Ps8	2,33	0	59,93	-	15	+	18,4583333
Ps9	2,83	154,8	72,93	-	17	+	3,75
Ps10	7,66	0	4,25	-	3	+	4,375
Ps11	0	0	0	-	5	+	0
Ps12	3,5	251,7	3,46	-	5	+	10,875
Ps13	0	0	25,83	-	10	+	6,16666667
Ps14	5,25	100,5	0	-	2	+	7,33333333
Ps15	4	500,1	0	-	3	+	11,0416667
Ps16	4,25	419,7	0	-	-	+	1,75
Ps17	2,66	173,3	91,9	-	17	+	10,4583333
Ps18	2	329,9	0	-	2	+	5,41666667
Ps19	2,66	0	73,79	-	13	+	7,16666667
Ps22	0	29,8	56,94	-	10	+	7,375
Ps23	1,5	108,4	80,03	-	13	+	3,91666667
Ps25	0	183,3	56,33	-	11	+	7,5
Ps26	2	0	0	-	3	+	10,5833333
Ps29	3,2	313,4	71,14	-	13	+	10,7916667
Ps30	1,4	269	0	-	-	+	1,125
Ps31	4,66	173,7	24,25	-	7	+	5,75
Ps34	3,33	367,7	23,24	-	8	+	5,83333333
Ps35	3,4	37,6	22,93	-	9	+	10,5
Ps37	4	0	0	-	3	+	3,375
Ps40	3	479,7	34,32	-	10	+	13,2916667
Ps42	1,5	495,4	78,39	-	15	+	10,6666667
Ps43	3,75	686,3	63,95	-	11	+	10,5833333
Ps43 1	3,2	0	0	-	4	+	0,91666667
Ps45	2,33	37,2	0	-	-	+	14,5
Ps47	0	0	92,06	-	13	+	19,75
Ps50	2,75	0	17.74	-	8	+	10,1666667
Ps52	1,5	212,7	85.64	-	10	+	9,83333333
Ps53	2,75	0	93.46	-	15	+	4,58333333
Ps56	3	120,3	30.30	-	8	+	8,66666667
Ps57	1	0	35.75	-	-	+	7,375
Ps60	3,4	458,4	20.70	-	9	+	8,29166667
Ps61	1,66	388,5	17.88	-	8	+	10,9166667
Ps62	1,5	0	39.38	-	9	+	7,33333333
Ps63	0	0	35.47	-	11	+	13,625
Ps64	1	548,9	0	-	2	+	11,875

Ps65	4	0	0	-	3	+	17,9583333
Ps66	1	697,6	26.73	-	11	+	25,125
Ps67	2	573,3	20.10	-	7	+	2,58333333
Ps68	1,4	632,9	14.20	-	11	+	8
Ps69	4	0	24.46	-	9	+	13,625
Ps70	1,4	0	22.24	-	10	+	9,25
Ps70 2	1,5	0	0	-	2	+	1,66666667
Ps71	1,75	0	02.56	-	2	+	9,95833333
Ps73	1	233,4	33.03	-	7	+	4,70833333
Ps74	3,5	723,3	83.65	-	14	+	11,4166667
Ps77	1	503,6	24.64	-	8	+	9,20833333
Ps78	3,5	577,1	37.68	-	11	+	10,2083333

Table 3: Enzymes production results of selected isolates.

Strains	Lipaseactivity	NH3 production	Amilaseactivity	HCN production	Chitinase Halo (mm)	Cellulase	Pectinase	Protease	Gelatinase
Ps1	-	+	-	-	-	+	+	+	+
Ps2	-	+	-	-	-	+	+	-	+
Ps4	-	+	-	-	-	+	+	-	+
Ps6	-	+	-	-	-	+	+	-	+
Ps7	+	+	-	-	-	++	+	-	+
Ps8	+	+	-	-	-	++	++	+	+
Ps9	-	+	-	-	-	++	+	-	+
Ps10	-	+	-	-	-	+	+	-	+
Ps11	-	+	+	-	-	+++	+++	+	+
Ps12	-	+	-	+	-	+	+	-	+
Ps13	+	+	-	-	-	++	+	+	+
Ps14	-	+	-	-	-	+	++	-	+
Ps15	-	+	-	-	-	+++	+++	-	+
Ps16	-	+	-	+	-	+	+	+	+
Ps17	+	+	-	-	-	+++	+++	+	+
Ps18	+	+	-	+++	-	+	+	+	+
Ps19	+	+	-	-	-	+++	+++	+	+
Ps22	-	+	-	-	-	+++	+++	-	+
Ps23	+	+	-	-	-	+++	+++	+	+
Ps25	+	+	-	-	-	++	++	-	+
Ps26	+	+	-	+++	-	+	+	+	+
Ps29	+	+	-	-	+ (18)	+++	+++	+	+
Ps30	-	+	-	+	-	+	+	+	+
Ps31	-	+	-	-	-	+	+	-	+
Ps34	+	+	-	-	-	+	+	+	+
Ps35	+	+	-	-	+ (20)	+++	+++	+	+
Ps37	-	+	-	-	-	+	+	-	+
Ps40	+	+	-	-	+ (16)	+++	+++	-	+
Ps42	+	+	-	-	-	+++	+++	+	+
Ps43	+	+	-	-	-	++	++	+	+
$Ps43_1$	-	+	-	-	+ (17)	++	++	+	+
Ps45	-	+	-	-	+ (21)	+	+	-	+
Ps47	+	+	-	-	+ (15)	++	++	-	+
Ps50	-	+	-	-	-	++	++	-	+
Ps52	-	+	-	-	-	+	+	-	+
Ps53	-	+	-	-	-	+	+	+	+
Ps56	-	+	-	-	-	+	+	-	+
Ps57	-	+	-	-	-	+	+	-	+
Ps60	+	+	-	-	-	++	++	+	+
Ps61	+	+	-	-	-	+	+	-	+
Ps62	-	+	-	-	-	+	+	-	+
Ps63	+	+	-	-	-	++	++	+	+
Ps64	-	+	-	-	-	+	+	-	+
Ps65	+	+	-	+++	-	++	+	+	+
Ps66	+	+	-	-	+ (25)	++	+	+	+
Ps67	-	+	-	-	-	+	+	-	+
Ps68	+	+	-	-	-	+	++	+	+
Ps69	+	+	-	-	-	++	+	+	+
Ps70	+	+	-	-	-	+++	++	+	+
$Ps70_2$	-	+	-	-	-	+	+	+	+
Ps71	-	+	-	-	-	+	+	-	+
Ps73	+	+	-	-	+ (16)	+	+	-	+
Ps74	+	+	-	-	-	++	+	+	+
Ps77	-	+	-	-	-	+	+	+	+
Ps78	+	+	-	+	-	+	+	+	+

Note: +++ High production, ++ Medium production, + Low production, - No production

Table 4: Antagonistic effect of *Pseudomonas fluorescens* strain against *F.culmorum* and *F.pseudograminearum.*

	Disease Index	
Treatements	With F.culmorum	With F.pseudograminearum
Control	2.3 (90)	1.9 (70)
With Ps 7	1.5 (50)	0.3 (0)
With Ps 8	0.2 (10)	0.5 (20)
With Ps 12	0.5 (0)	0.2 (0)
With Ps 14	0.9 (20)	0.4 (0)
With Ps 17	1.3 (30)	0.9 (10)
With Ps 47	1 (30)	0.4 (10)
With Ps 52	1.2 (20)	0.4 (0)
With Ps 53	0.2 (0)	0.5 (20)
With Ps 65	0.9 (0)	0.3 (0)
With Ps 66	0.9 (20)	0.4 (10)
With Ps 68	0.7 (30)	0.4 (0)

NB: The values in parentheses represent the percentage of plants that had a score ≥2.

Table 5: The effect of isolates on growth of Wheat.

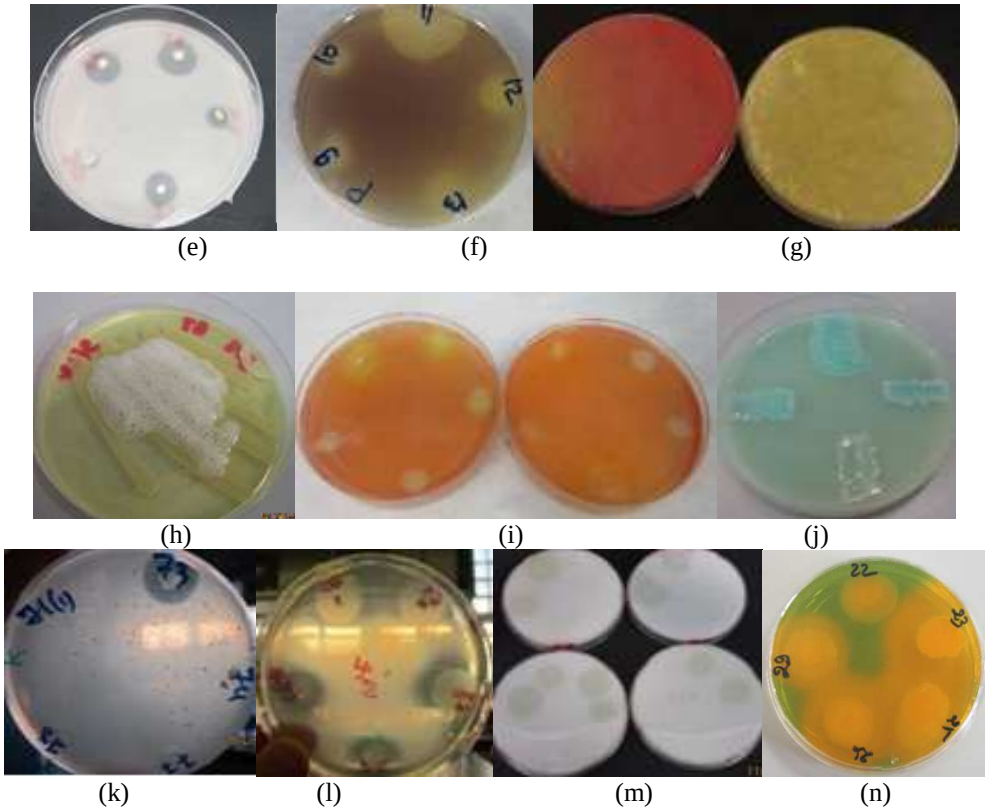
Treatment	Mean length (mm)		Mean fresh weight(cg)		Mean dry weight(mg)	
	Shoot	Root	Shoot	Root	Shoot	Root
Control	28,8±0,78 ^{c}	31±2 ^{c}	31,1±0,73^{d}	14±0,81^{g}	29,6±0,69^{e}	29,8 ±0,42^{d}
Ps 7	28,60±0,51^{d}	28,1±0,73^{e}	23,6±0,69^{g}	23,5±0,52^{a}	19,7±0,48 ^{i}	29,4±0,84 ^{d}
Ps8	32,6±1,17 ^{b}	33,4±0,51^{b}	41,2±0,78^{a}	19,5±0,67^{d}	39,6±0,51^{b}	29,6±0,69 ^{d}
Ps12	33,7±1,15 ^{a}	23,1±0,87^{g}	32,8±0,78^{c}	18,7±0,67^{e}	29,8±0,42 ^{e}	32,3±1,49 ^{c}
Ps14	32,3±0,48^{b}	26,6±0,51^{f}	17±0,66 ^{h}	11,8±0,63^{h}	29,6±0,69^{e}	27,6±1,26 ^{ef}
Ps 17	29,6±0,51^{c}	22,4±0,51^{g}	24,9±0,73^{f}	10,6±0,51^{i}	29, 1±0,87 ^{ef}	27,4±1,07 ^{f}
Ps47	32,9±0,87^{b}	26,9±0,87^{f}	36,5±2,71^{b}	20,9±0,87^{c}	32,3±0,67 ^{d}	28,4±0,84^{e}
Ps52	25,8±1,03^{e}	15,8±1,03^{i}	24,9±0,73^{f}	11,1±0,7 ^{hi}	22,7±0,48 ^{h}	16,6±0,51 ^{h}
Ps53	29,5±0,52^{c}	28±0,81^{e}	36,7±1,01^{b}	14,5±0,52^{fg}	41,7±1,49 ^{a}	41,9±0,99 ^{a}
Ps65	28,9±0,87^{c}	18,7±0,82^{h}	23,2±0,78^{g}	15±0,94^{f}	28,5±1,08 ^{f}	24,8±0,78 ^{g}
Ps66	29,8±0,91^{c}	34,8±1,03^{a}	29,3±0,94^{e}	21,8±1,68^{b}	26,6±0,84^{g}	34,4±1,57 ^{b}
Ps68	29,5±0,52^{c}	29,4±0,69^{d}	31,9±0,87^{cd}	19,5±0,52^{d}	35,1±1,37^{c}	32±0,94 ^{c}
LSD	0,729	0,843	0,999	0,721	0,772	0,900

NT: Control (without bacteria), The data are presented the means ± SD. Different letters indicate statistically significant difference evaluated by LSD.

Table 6: Identification of isolates based on 16s rDNA partial sequence analysis.

Isolate	Identified as	%similarity	Accession number	Organism, strain
Ps7	*Pseudomonas fluorescens*	98%	KR267325	*P.fluorescens* strain DmBR 2
Ps8	*Pseudomonas fluorescens*	98%	KR267326	*P.fluorescens* strain NITDPY
Ps12	*Pseudomonassp*	98%	KR267327	*Pseudomonas sp* EP_S_49
Ps26	*Pseudomonas fluorescens*	99%	KR267328	*P.fluorescens* strain dqe01
Ps34	*Pseudomonas fluorescens*	98%	KR267330	*P fluorescens* strain NITDPY
Ps43	*Pseudomonas fluorescens*	94%	KR267331	*P.geniculata* strain MD 05
Ps47	*Pseudomonas fluorescens*	98%	KR267332	*P fluorescens* A506
Ps65	*Pseudomonas fluorescens*	89%	KR267333	*P fluorescens* strain B-Exp9
Ps66	*Pseudomonas fluorescens*	98%	KR267334	*P fluorescens*strainDmBR 2

(a) (b) (c) (d)



(e) (f) (g)

(h) (i) (j)

(k) (l) (m) (n)

Fig. 1: Growth promoting properties and extracellular enzyme activity of isolates.

(a) Picture of the isolates under an UV lamp, showing fluorescent colonies, (**b**)+(**c**) Screening for antagonistic activity against respectively *Fusarium culmorum* et *Fusarium pseudograminearum*, (**d**) Microscopic photos showing Gram negative bacteria,(**e**) Clearing zone around the colonies indicating the ability of this isolates to solubilize phosphate in Pikowskaya agar medium, (**f**) Pectinase activity, (**g**) HCN production, (**h**) Catalase test result, (**i**) Cellulose activity, (**j**) Lipase activity, (**k**) Chitinase activity, (**l**) Gelatinase hydrolisis, (**m**) Protease activity, (**n**) Siderophore production.

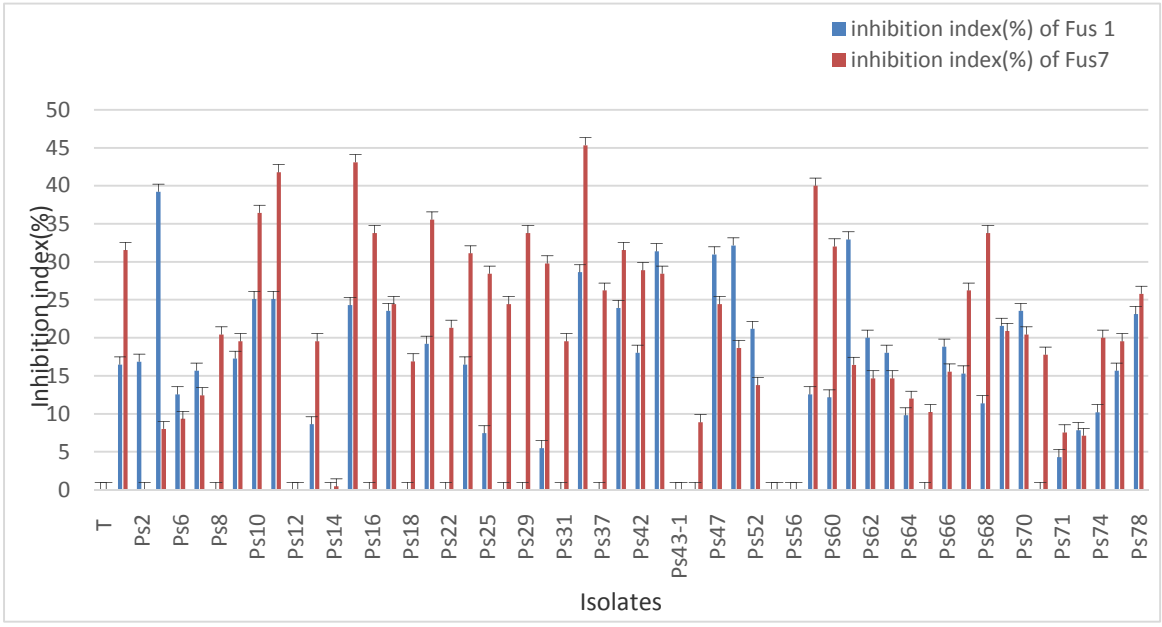


Fig. 2: Invitro inhibition of *Fusarium culmorum* (Fus1) *and Fusarium pseudograminearum* (Fus7). Results shown as means ± SD.

Conclusion:

This study concludes that strains of *Pseudomonas fluorescens*, isolated from wheat rhizospheric soil from the region of Constantine(Algeria), showed variation in their plant promoting characteristics production. Such as siderophore, IAA production, solubilization of phosphate, ammonia and extracellular cellulase, pectinase,protease, lipase, chitinase and HCN, that can contribute to the ability of these isolates to suppress fungal diseases. Based on the positive results of the antagonistic effect of selected strains, it is interesting to use the PGPR *Pseudomonas fluorescens* as inoculants biofertilizers to replace chemical fertilizers and pesticides for Wheat.

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