

Antifungal Activity and Genetic Diversity of Selected Endophytic Fluorescent Pseudomonads

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Abstract: Fluorescent Pseudomonads have been effectively utilized for controlling major fungal plant diseases because of their antifungal metabolites. In this study, thirty-four bacterial isolates were isolated from root samples of different crops and screened as potential biological control agents against *Rhizoctoniasolani AG 2-2*, *Pythium arrhenomanes* and *Fusarium solani*. On the basis of dual culture assays, twenty isolates were selected for phenotypic characterization, identification and plant growth promoting traits; four isolates were selected for greenhouse studies. The selected *Pseudomonas* strains were characterized by PCR methods using rpoB primer. Eight showed the ability to produce indole acetic acid (IAA) while 12 isolates have no IAA produced, thirteen isolates could solubilize inorganic phosphate and six strains produced hydrogen cyanide. Tested *Pseudomonas* strains had positive response on bean growth and health. Obtained results indicated that the tested antagonistic fluorescent pseudomonads reduced disease severity in beans and disease development under greenhouse pot experiment. However, further study is needed to apply to these results under field conditions.

Keywords: Biological control, antagonists, identification, IAA, inorganic phosphate, solubilizers.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are known to rapidly colonize the rhizosphere and suppress soilborne pathogens at the root surface (Rangarajan *et al.*, 2003). The PGPR can also be beneficial to the plant by stimulating growth (Bloemberg and Lugtenberg, 2001; Moeinzadeh *et al.*, 2010). Among these organisms, fluorescent pseudomonads are considered to be the most promising group of PGPR involved in the biocontrol of plant diseases (Gardner *et al.*, 1984; Moeinzadeh *et al.*, 2010). Fluorescent Pseudomonads can produce supplementary metabolites such as antibiotics (Keel, 1992), phytohormones (Keel, 1992), volatile compound hydrogen cyanide (HCN) (Defago and Haas, 1990), in addition to produce siderophores (Neilands, 1995). Plant growth-promoting ability of these bacteria is as a result of manufacturing of IAA (Patten and Glick, 2002). In the present study, the isolates were identified according to molecular characterizations by rpoB primer. The possible growth-promoting and biocontrol potential of the strains have been investigated by determining the secondary metabolites, *viz* IAA, solubilization of inorganic phosphate and HCN production. Bean growth response to inoculation with the selected four antagonistic *Pseudomonas* strains was also evaluated as well as root colonization and severity disease.

MATERIALS AND METHODS

Isolation of endophytic fluorescent pseudomonads

Root samples of different crops (wheat, bean, clover, tomato and Mango) were collected from the experimental farm, Faculty of Agriculture, Suez Canal University, Ismailia. Roots were removed from the collected plants, thoroughly washed under a running tap water for 5min to remove adhering soil particles and cut into 2-3cm pieces. The root surface was sterilized with

1% chloramines for 15 min (Patriquin *et al.*, 1987), then the roots were rinsed with sterilized distilled water. Fluorescent pseudomonads were obtained by shaking 10 g of roots in 10 ml of King's B (KB) broth for 3hours at 180 rpm and 28°C in a rotary shaker. Serial dilutions of the root suspension (100 µl) were plated onto KB agar (Kings *et al.*, 1954) and incubated for 2 days at 28°C. The colonies were selected based on the presence of fluorescent green under UV light (366 nm).

Screening and Quantification of bacteria for antagonistic activity against phytopathogenic fungi

All selected fluorescent pseudomonad were tested *in vitro* for antagonism towards fungal pathogens; *Rhizoctonia solani AG 2-2*, *Pythium arrhenomanes* and *Fusarium solani*. The phytopathogenic fungi were obtained from a stock reference cultures held in of Laboratory of phytopathology, Faculty of Biotechnology, Ghent University, Belgium. The antagonistic activity of selected fluorescent pseudomonad against these phytopathogenic fungi was tested on PDA plates using a dual culture technique (Skidmore and Dickinson, 1976). Observation on width of inhibition zone and mycelia growth of test pathogen was recorded and the percentage of inhibition of pathogen growth was calculated by using the formula proposed by (Vincent, 1927).

$$\text{Percentage of inhibition} = \frac{C - T}{C} \times 100$$

Where

C: Diameter of pathogen mycelial growth (mm) in control

T: Diameter of pathogen mycelial growth of pathogen in dual plate.

Phenotypic characterization of antagonistic pseudomonads

Based on the percentage of inhibition mycelial growth of tested phytopathogenic fungi, thirty-four

isolates were selected and examined for phenotypic characters *viz* Gram stain, oxidation-fermentation reaction, arginine dihydrolase, cytochrome C oxidase, catalase, fluorescent pigment, citrate utilization and examined for growth at 4 °C and 42 °C according to (Mac Faddin, 1980).

Molecular method for identification antagonistic fluorescent pseudomonads by rpob primer

In colony polymerase chain reactions (PCR) bacterial cells were directly added to a PCR reaction without extraction of DNA. After touching the colony, place the tip in 20 µL of sterilized milli-q water (in a clean PCR tube) and pipette up and down several times and label the tube with a colony number (Grunenwald, 2000). The PCR were performed using the primers rpob, rpoBf1 CAGTTCATGGACCAGAACAACCCGCT and rpoBr1 CCCATCAACGCACGGTTGGCGTC. The PCR reaction mixture consisted of 1 µL template DNA, 0.25 µL Taq DNA polymerase, 10 µL 5x reaction buffer, 2.5 µL of each primer, 1 µL dNTPs and add H₂O to a final volume of 50 µL. The thermo cycling was programmed at 94 °C for 10 min, was followed by 35 cycles of 1 min denaturation at 94 °C, annealing at 54 °C for 1 min, extension at 72 °C for 1 min, and final extension of 10 minutes at 72 °C. The 10 µL of each extracted DNA sample was firstly mixed with 2.5 µL ethidium bromide and 12.5 µL of each sample was applied to a well on the on 1% agarose gel along with 1 kb ladder as marker in a tray filled with 1xTAE buffer. The PCR results were visualized as bands on the gel during ultraviolet illumination.

All PCR products were purified using the QIAGEN MinElute® PCR Purification Kit. The purified products were directly sequenced; the obtained sequences were aligned using Bioedit programme (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). The BLAST database of the National Center for Biotechnology Information (NCBI) was used to compare resolved sequences of the 20 isolates with rpob sequences data deposited in Gene bank. A phylogenetic tree was constructed by using the software MEGA5 (<http://www.megasoftware.net/>).

Detection of hydrogen cyanide

Production of hydrogen cyanide was detected using the method of (Reddy *et al.*, 2008).

Indole acetic acid (IAA) production

IAA production by the selected fluorescent pseudomonad was detected qualitatively by the modified method as described by (Brick *et al.*, 1991) and quantitatively in the presence of L-tryptophan (L-TRP) by colorimetric method (Sarwar *et al.*, 1992).

Phosphate solubilizing activity

The ability of selected fluorescent pseudomonad to solubilize insoluble inorganic phosphate was estimated using qualitative and quantitative methods. For qualitative method, the bacterial isolates were streaked onto Pikovskaya's agar medium (Pikovskaya, 1948) containing tricalcium phosphate as insoluble inorganic phosphate. After 7 days of incubation at 28 °C, the halo zone around the colony was confirmation of phosphate

solubilization. The ability of fluorescent pseudomonad isolates to solubilize insoluble phosphate was tested quantitatively in liquid culture. Quantitative estimation of phosphate solubilization was carried out according to Jackson (1973).

Pot experiment

The soil used in this experiment was a mixture of sand and soil (Structural; Snebbout, Kaprijke, Belgium) (50/50, w/w). Bean seeds of *Phaseolus vulgaris* cv. *Prelude* (Het Vlaams Zaadhuis, Waarschoot, Belgium) were pre-germinated before sowing. Bean seeds were surface sterilized using 1% sodium hypochlorite solution for 5 min, rinsed thrice in sterile distilled water and about 25 seeds each were placed on wet filter paper in Petri dishes. The seeds were incubated at 28 °C for 3 days prior to the setting up of the experiment. The fungus strains used in this experiment were *R. Solani* AG 2-2, *Pythium arrhenomanes* and *Fusarium solani*. To produce fungus inoculum, sterilized kernels were inoculated with actively growing hyphae from a 3 days old PDA culture of fungus. Flasks were kept in the incubator at 28 °C for two weeks prior to the setting up of the plant experiment. Four bacterial strains were used in this experiment; *Pseudomonas entomophila* SAW7, *Pseudomonas putida* SAW19, *Pseudomonas palleroniana* SAW15 and *Pseudomonas mosselii* SAW1. The bacterial populations were adjusted by adding appropriate amounts of saline solution, to obtain 2800 x 10⁶ CFU per 200 ml. These dilutions served as bacterial inoculums for the plant experiment. Twenty ml diluted bacterial suspension was mixed thoroughly with 2.8 kg of soil for 2 min. Four perforated plastic boxes (22 × 15 × 6 cm) were partially filled with 700 g of soil. Thus, there were four replicates for each treatment was done. Soil mixed with 200 ml saline solution served as positive and negative controls. Ten pre-germinated bean seeds were sown in each box and all plants were incubated in a growth chamber (28 °C, relative humidity of 70%, 16 h photoperiod). The plants were daily watered to maintain soil moisture. Three days after sowing, 40 wheat kernels colonized with fungus were placed in each box at a depth of approximately 2 cm and 3 cm away from the bean seedlings. The positive control was not inoculated with wheat kernels. The negative control was inoculated with wheat kernels and without antagonistic bacteria. The plants were harvested after 30 days from sowing date and some growth parameters were recorded as well disease severity (Fang *et al.*, 2011).

RESULTS AND DISCUSSION

Isolation and screening of antagonistic fluorescent pseudomonads

A total of 200 isolates of fluorescent pseudomonads were isolated from root samples of different crops (wheat, bean, clover, tomato and Mango). The fungal growth inhibition by tested bacterial isolates was assessed by measuring the inhibition of mycelia radial growth in the dual culture plate assay against the three phytopathogenic fungi namely *Rhizoctonia solani* AG 2-2, *Fusarium solani* and *Pythium arrhenomanes* (Table 1).

Table (1): Antifungal activity of fluorescent pseudomonad isolates against phytopathogenic fungi used in the study

Isolates	<i>Rhizoctonia solani</i> AG 2-2		<i>Fusarium solani</i>		<i>Pythium arrhenomanes</i>	
	mm [†]	% [‡]	mm [†]	% [‡]	mm [†]	% [‡]
SAW7	17.1 ^l	78.7 ^c	17.1 ^h	78.7 ^c	12.1 ⁿ	84.8 ^a
SAB1	32.1 ^f	60 ⁱ	22.1 ^g	72.5 ^d	22.1 ^k	72.5 ^d
SAW1	24.6 ⁱ	69.3 ^f	14.6 ⁱ	81.8 ^b	24.6 ⁱ	69.3 ^f
SAC1	37.1 ^b	53.7 ^l	17.1 ^h	78.7 ^c	19.6 ^l	75.6 ^c
SAW6	29.6 ^g	63.1 ^h	29.6 ^d	63.1 ^g	27.1 ^h	66 ^g
SAB17	24.6 ⁱ	69.3 ^f	29.6 ^d	63.1 ^g	27.1 ^h	66.2 ^g
SAB7	34.1 ^d	57.5 ^j	80 ^a	0 ^j	80 ^a	0 ^m
SAB15	27.1 ^h	66.2 ^g	24.6 ^f	69.3 ^e	27.1 ^h	66.2 ^g
SAB9	22.1 ^j	72.5 ^e	32.1 ^c	60 ^h	34.6 ^b	56.8 ^l
SAC6	34.1 ^d	57.5 ^j	24.6 ^f	69.3 ^e	24.6 ⁱ	69.3 ^f
SAB11	33.6 ^e	58.1 ^j	32.1 ^c	60 ^h	34.6 ^b	56.8 ^l
SAC8	39.6 ^a	50 ^m	32.1 ^c	60 ^h	32.1 ^d	60 ^j
SAC13	29.4 ^g	63.1 ^h	27.1 ^e	66.2 ^f	80 ^a	0 ^m
SAC14	37.1 ^b	53.7 ^l	32.1 ^c	60 ^h	29.1 ^f	63.7 ^h
SAC14	34 ^d	57.5 ^j	27.1 ^e	66.2 ^f	24.1 ^j	70 ^e
SAB16	29.6 ^g	63.1 ^h	80 ^a	0 ^j	80 ^a	0 ^m
SAB14	27.1 ^h	66.2 ^g	24.6 ^f	69.3 ^e	19.6 ^l	75.6 ^c
SAB12	24.6 ⁱ	69.3 ^f	22.1 ^g	72.5 ^d	22.1 ^k	72.5 ^c
SAB8	22.1 ^j	72.5 ^e	27.1 ^e	66.2 ^f	34.1 ^c	57.5 ^k
SAB10	27.1 ^h	66.2 ^g	22.1 ^g	72.5 ^d	22.1 ^k	72.5 ^c
SAB21	27.1 ^h	66.2 ^g	39.6 ^b	50 ⁱ	29.6 ^e	63.1 ^h
SAW3	27.1 ^h	66.2 ^g	17.1 ^h	78.7 ^c	32.1 ^d	60 ^j
SAW23	24.6 ⁱ	69.3 ^f	32.1 ^c	60 ^h	29.1 ^f	63.7 ^h
SAW24	34.6 ^c	56.8 ^l	17.1 ^h	78.7 ^c	24.6 ⁱ	69.3 ^f
SAW21	19.6 ^k	75.6 ^d	29.6 ^d	63.1 ^g	29.6 ^e	63.1 ⁱ
SAW22	27.1 ^h	66.2 ^g	9.6 ^j	88.1 ^a	32.1 ^d	60 ^j
SAW27	29.6 ^g	63.1 ^h	32.1 ^c	60 ^h	32.1 ^d	60 ^j
SAW19	4.6 ⁿ	94.3 ^a	14.6 ⁱ	81.8 ^b	14.6 ^m	81.8 ^b
SAC29	27.1 ^h	66.2 ^g	17.1 ^h	78.7 ^c	80 ^a	0 ^m
SAW15	24.6 ⁱ	69.3 ^f	14.6 ⁱ	81.8 ^b	27.1 ^h	66.2 ^g
SAC31	14.6 ^m	81.8 ^b	80 ^a	0 ^j	34.6 ^b	56.8 ^l
SAW10	17.1 ^l	78.7 ^c	27.1 ^e	66.2 ^f	27.8 ^g	65.5 ⁱ
SAW9	27.1 ^h	66.2 ^g	39.6 ^b	50 ⁱ	19.6 ^l	75.6 ^c
SAW5	32.1 ^f	60 ⁱ	22.1 ^g	72.5 ^d	27.1 ^h	66.2 ^g

[†]: mycelial growth, [‡]: Percentage of fungal growth inhibition. Values followed by the same letters at the same column are not significantly different by LSD test at 0.05 level.

Thirty-four isolates were showed a broad-spectrum antifungal activity towards tested phytopathogenic fungi. The zone of inhibition of mycelia growth of the

above fungal pathogens ranged from 4.6 to 39.6mm (Table 1). Specifically, the zone of inhibition of mycelia growth of the fungal pathogen, *Rhizoctonia solani* AG

2-2, *Fusarium solani* and *Pythium arrhenomanes*, ranged from 4.6 to 39.6 mm, 9.6 to 39.6 mm, 12.1 to 34.6 mm, respectively (Table 4). These findings are similar to those of Saber *et al.* (2015) who found that the reduction percentages of the mycelial growth of *Rhizoctonia solni* were 45.45% and 32.72% using fluorescent pseudomonads isolate OL2 and AR10, respectively. Toppo and Tiwari (2015) found that four *Pseudomonas* spp. isolates, *Pseudomonas syringae* (PKS10), *Pseudomonas syringae* (PKM11), *Pseudomonas alcaligenes* (PKJ25), *Pseudomonas alcaligenes* (PKB27) in addition to Pmtcc (*Pseudomonas* isolate for standard check) inhibited the hyphal growth of *Rhizoctonia solani* by 47.67, 49.58, 62.60, 53.28 and 55.47%, respectively. The obtained results concerning the effect of fluorescent pseudomonad isolates against *Fusarium solani* are in agreement with those obtained by (Couteaudier and Alabouvette, 1990; Mazzola and Gu, 2002), they found that fluorescent pseudomonads are involved in the natural suppressiveness of some soils to *Fusarium* wilts of various plant species.

Phenotypic characterization

Based on the highly antagonistic potential, twenty isolates of fluorescent pseudomonads were selected for examination of phenotypic tests. The results showed that all tested isolates have positive reactions for fluorescence on KB agar, oxidase, citrate and catalase, while the remained isolates have highly variability for other phenotypic tests such as gelatin hydrolysis, levan production and growth at 4°C. Only one isolate (SAW22) has positive results for levan production test and eight isolates (SAW7, SAB17, SAW5, SAW23, SAW21, SAW22, SAW15 and SAB15) have positive results for hydrolyze of gelatin. Three isolates (SAW5, SAW21 and SAB8) have ability to reduce nitrate and eleven isolates (SAW1, SAB17, SAB8, SAB10, SAW3, SAW23, SAW24, SAW21, SAW9, SAW19 and SAW22) have ability to grow at 4°C. On the other hand, all isolates could not grow at 42°C (Table 2).

Concerning to assimilation of carbohydrates, all strains utilized dextrose but exhibited varying degree of utilization towards other carbon sources such as mannitol, sucrose, fructose, L-arabinose, glycerol and xylose. Four isolates (SAW5, SAW9, SAW3 and SAB17) can utilize mannitol whereas one isolate (SAB15) can utilize sucrose. Five isolates (SAW7, SAW5, SAW21, SAW9 and SAB17) can utilize fructose. All isolates were able to utilize xylose as carbon source except (SAB1 and SAW15). Twelve isolates can be utilizing glycerol as carbon source (SAW7, SAB1, SAW1, SAB17, SAB15, SAB14, SAB12, SAB10, SAW21, SAW15, SAW10 and SAW5). All the isolates were unable to utilize sorbose, sorbitol, maltose and adonitol (Table 2).

Molecular method for identification antagonistic fluorescent pseudomonads by rpob primer.

Phylogenetic tree obtained with rpob sequences determined in this study for the same strains (Fig. 1). On the basis of BLAST analysis of rpob gene (about 1000

bp) the homology of fluorescent pseudomonad species such as *P. palleroniana*, *P. aeruginosa*, *P. putida*, *P. entomophila* and *P. moraviensis* ranged from (96 to 100%). The analysis of sequences showed two strains (SAW7 and SAW5) belonged to *P. entomophila*, five strains (SAW19, SAW22, SAB12, SAW3 and SAB10) belonged to *P. putida*, three strains (SAB15, SAW21 and SAB17) belonged to *P. palleroniana*, two strains (SAC1 and SAW23) belonged to *P. corrugata*, one strain (SAW15) belonged to *P. moraviensis*, one isolate (SAW1) belonged to *Pseudomonas mosselii* and three strains (SAW10, SAB1 and SAB8) belonged to *P. plecoglossicida*.

Phylogenetic analyses of 20 representative fluorescent pseudomonad isolates based on neighbor-joining method with 1000 bootstrap sampling resulted in five major groups Fig. (1). of the 20 isolates, group (I) was formed by three strains of *Pseudomonas palleroniana* (SAB15, SAW21, SAB17) and one strain of *Pseudomonas moraviensis* (SAW15), group (II) was formed by one strain *Pseudomonas anguilliseptica* (SAW24), group (III) was formed by strains, representing the major group, 2 strains of *Pseudomonas entomophila* (SAW7 and SAW5), one strain of *Pseudomonas parafulva* (SAB14), three strains of *Pseudomonas plecoglossicida* (SAW10, SAB1 and SAB8), one strain of *Pseudomonas mosselii* (SAW1) and five strains of *Pseudomonas putida* (SAW19, SAW22, SAB12, SAW3 and SAB10), group (IV) was formed by 1 strain of *Pseudomonas argentinensis* (SAW9) and group (V) was formed by 2 strains of *Pseudomonas corrugate* (SAC1 and SAW23).

Indole acetic acid (IAA) production.

In qualitative method, the results in Table (3) indicate that eight strains (*Pseudomonas entomophila* SAW7, *Pseudomonas mosselii* SAW1, *Pseudomonas parafulva* SAB14, *Pseudomonas corrugate* SAW23, *Pseudomonas anguilliseptica* SAW24, *Pseudomonas putida* SAW19, *Pseudomonas argentinensis* SAW9 and *Pseudomonas entomophila* SAW5) were able to produce IAA. In quantitative determination, IAA production by antagonistic fluorescent pseudomonas isolates ranged from 7.84-19.59 mg l⁻¹. SAW1 exhibit highest IAA production 19.59 mg l⁻¹ (Table 3). On the other hand, the strain SAW23 which was identified as *Pseudomonas corrugate* produced the lowest auxin value (7.84 mg l⁻¹). The differences in the performance of auxin-producing fluorescent pseudomonads may be attributed to the inherent properties of the individual bacteria (Sarwar *et al.*, 1992). Similar results regarding variation in the ability to biosynthesize auxin among fluorescent pseudomonads, many investigators also have reported that auxin production by microbial isolates greatly varied within species and/or strains of the same species (Asghar *et al.*, 2002; Khalid *et al.*, 2004; Ahmed *et al.*, 2005). Additionally, several studies reported that IAA production is widespread by *P. aeruginosa*, *P. chlororaphis*, *P. fluorescens*, *P. putida*, *P. syringae* and *P. stutzeri* (Patten and Glick, 2002; Lucas Garcia *et al.*, 2003; Pedraza *et al.*, 2004; Ayyadurai *et al.*, 2006, 2008; Karnwal, 2009).

Table (2): Phenotypic characteristics of antagonistic fluorescent pseudomonads isolates

Traits	SA W7	SAB 1	SA W1	SAC 1	SAB 17	SAB 15	SAB 14	SAB 12	SAB 8	SAB 10	SA W3	SA W23	SA W24	SA W21	SA W22	SA W19	SA W15	SA W10	SA W9	SA W5	
Fluorescence on KB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	-	-	-	+	+	-	-	-	-	-	+	-	-	+	-	+	-	-	-	+
Levan production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reductase	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4 °C	-	-	+	-	+	-	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-
42 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Assimilation of																					
Mannitol	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+
Sucrose	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+
L-arabinose	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	-	+	+	+	+	-	+	-	-	-	+	-	-	+	+	-	-	+

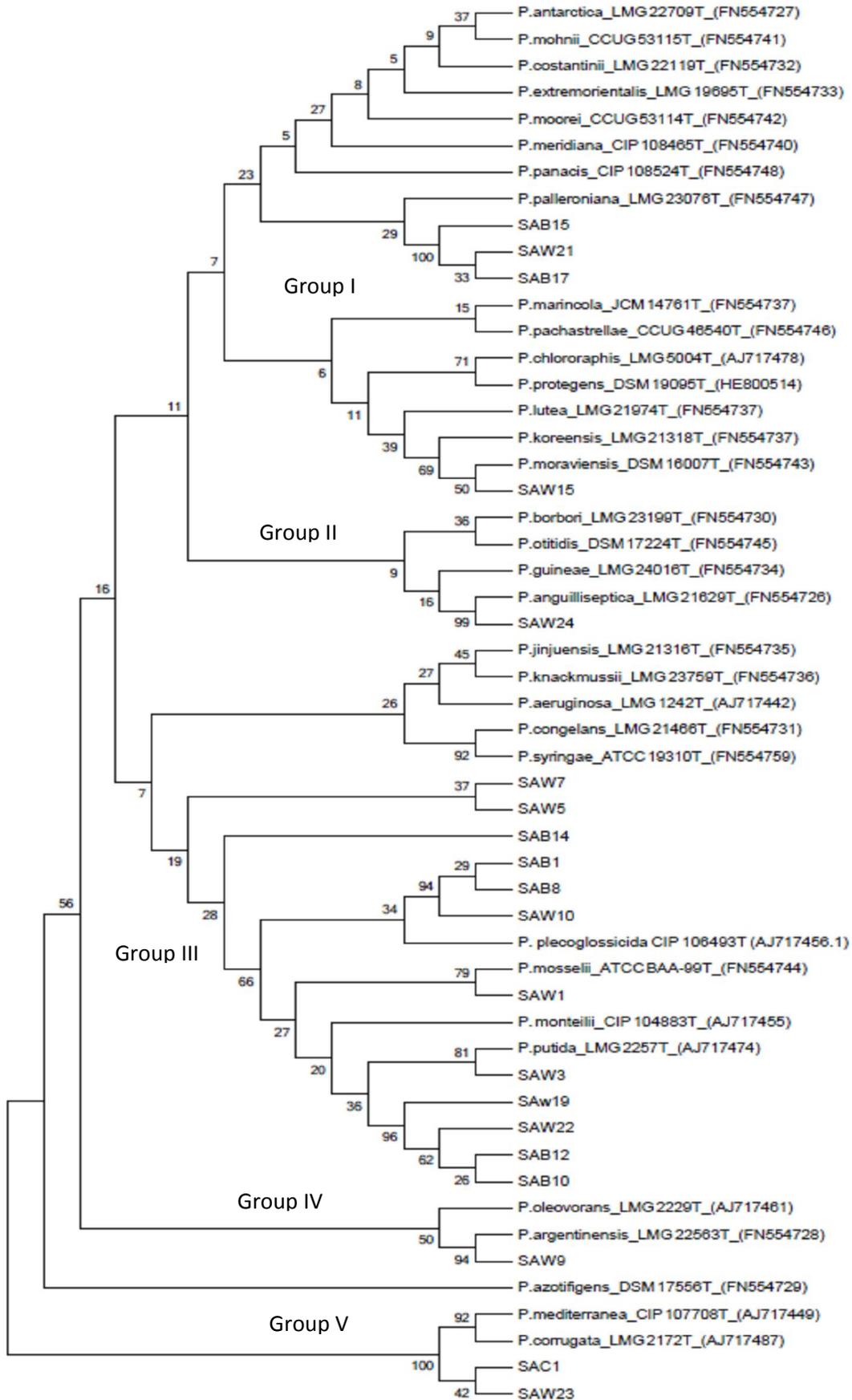


Fig. (1): Phylogenetic tree of 20 antagonistic fluorescent pseudomonad strains based on the *rpoB* sequences.

Phosphate solubilizing activity

Qualitative determination of phosphate-solubilizing capacity of the tested fluorescent pseudomonads strains show that thirteen of the tested twenty strains (65.0%) were able to solubilize of $\text{Ca}_3(\text{PO}_4)_2$ (Table 3). These strains are *Pseudomonas plecoglossicida* SAB1, *Pseudomonas corrugata* SAC1, *Pseudomonas palleroniana* SAB17, *Pseudomonas palleroniana* SAB15, *Pseudomonas corrugata* SAW23, *Pseudomonas palleroniana* SAW21, *Pseudomonas putida* SAW22, *Pseudomonas moraviensis* SAW15, *Pseudomonas putida* SAB12, *Pseudomonas plecoglossicida* SAB8, *Pseudomonas plecoglossicida* SAB10, *Pseudomonas putida* SAW3, and *Pseudomonas plecoglossicida* SAW10. The quantitative estimation of phosphate solubilization showed diverse levels of phosphate solubilizing activity. After an incubation period of 7 days, the amounts of P solubilized from $\text{Ca}_3(\text{PO}_4)_2$ by the tested strains ranged from 12.75 to 131.11 mg P l^{-1} and the pH values of the cultures were reduced from initial value of 7.1 to values varied between 4.20 and 5.87. Strain SAB12 was the most efficient strain in solubilizing of phosphate and in decreasing pH value (Table 3). On the other hand, the strain SAW23 was the lowest efficient strain in solubilizing of phosphate (12.57 mg P l^{-1}). The decrease in pH values clearly indicates the production of organic acids, which is considered to be the mechanism responsible for P solubilization (Rodriguez *et al.*, 2004). Similar results were confirmed by previous works by (Kpombrekou and Tabatabai, 1994; Nautiyal *et al.*, 2000). In addition to organic acids, other mechanisms such as inorganic acids, chelating agents and mineralization of organic phosphorus may be also responsible for solubilization of phosphate. Previous studies reported that several species of fluorescent pseudomonads such as *P. fluorescens* NJ-101 and EM85 (Bano and Musarrat, 2004; Dey *et al.*, 2004), *P. aeruginosa* (Bano and Musarrat, 2004), *Pseudomonas* sp. (Leinhos, 1994), *P. chlororaphis*, *P. savastanoi*, *P. pickettii* (Cattelan *et al.*, 1999), *P. lutea* OK2 (Peix *et al.*, 2003), *P. rhizophaeae* LMG 21640, *P. graminis* DSM 11363 (Peix *et al.*, 2003), *P. striata* (Gaind and Gaur, 2002) and *P. corrugata* (Pandey and Palni, 1998) have been reported as efficient phosphate solubilizers.

Production of hydrogen cyanide (HCN)

Hydrogen cyanide production was observed after 96 h for strains, the HCN formed a brownish red compound with sodium picrate. In the present study, seven strains produced HCN (*Pseudomonas entomophila* SAW7, *Pseudomonas mosselii* SAW1, *Pseudomonas corrugata* SAC1, *Pseudomonas moraviensis* SAW15, *Pseudomonas palleroniana* SAB17, *Pseudomonas parafulva* SAB14, and *Pseudomonas palleroniana* SAW21) (Table 3). HCN production has been evidenced in several *Pseudomonas* species (*P. fluorescens*, *P. aeruginosa*, *P. chlororaphis*, and *P. aureofaciens*), (Haas and Defago, 2005).

Cultivation experiment

Plant response to inoculation with the selected four antagonistic *Pseudomonas* strains was evaluated by measuring some growth parameters after 30 days from

sowing date. These parameters include plant height, fresh and dry weight (Table 4). The results in Table (4) show that all tested strains significantly increased plant height ranging from 65.47% with the strain SAW15 to 112.39% with SAW19 strain when compared to the negative control (diseased treatment). However, the differences between these four strains were not statistically significant (Table 4). Bean plants treated with four *Pseudomonas* strains (SAW1, SAW15, SAW19 and SAW7) without any infection of fungus showed plant growth promoting effect of these bacteria for all tested growth parameters. Moreover, the results showed that the plant treated with tested phytopathogenic fungi (the diseased treatment) significantly decreased the plant height compared to the plant treated with tested *Pseudomonas* strains. Obtained results indicated that the highest value of plant height (68 cm) was pronounced with SAW19 strain, which identified as *P. putida* in soil infested with *R. solani* AG 2-2.

Regarding the dry weight of the bean, data presented in Table (4) indicated that fresh and dry weights of bean plants were highly affected by inoculation with different *Pseudomonas* strains. Generally, the highest plant fresh weight (23.3 g pot^{-1}) was observed when plant inoculated with SAW15 strain, which identified as *Pseudomonas palleroniana*, and infected with *F. solni*. Similarity, the highest plant dry weight (10.3 g pot^{-1}) was observed when plant inoculated with SAW15 and SAW19 strains, which identified as *Pseudomonas palleroniana* and *Pseudomonas putida*, when soil infested with *R. solani* AG 2-2 and *F. solni*, respectively. Additionally, inoculation with the *Pseudomonas* strains without any infection with phytopathogenic fungi led to an improvement in the plant fresh and dry weights when compared to diseased treatment. Based on the results of this study, the application of biocontrol agents of fluorescent pseudomonads significantly promoted plant vigor and health. These findings were primarily due to the positive effects of the tested *Pseudomonas* strains on plant growth that are always correlated with noteworthy changes in root morphology such as increased lateral root length and root hair number and length. In this regard, some plant growth-promoting bacteria *Pseudomonas* sp. strain promote root development and alter root architecture by the production of phytohormones such as IAA, cytokinins, and gibberellins (Bloemberg and Lugtenberg 2001; Persello-Cartieaux *et al.*, 2003). Plant-growth promotion for some rhizobacteria has been found to be related with the solubilization and increased uptake of phosphate (Gyaneshwar *et al.*, 2002). These findings are in agreement with the results in this study because of the tested three strains (SAW1, SAW19 and SAW7) have ability to produce IAA and one strain has induced to solubilize insoluble inorganic phosphate (Table 4). Obtained results also are similar to those of Saber, Fekria *et al.* (2015), who found that the inoculation of tomato seedlings with *Pseudomonas* strains led to support plant growth and increased of NPK uptake. Previous studies have shown that rhizobacteria had positive response on plant growth and plant health (Naureen Zakira, 2009; Erdogan and Benlioglu, 2010).

Table (3): Plant growth promoting activities of antagonistic fluorescent pseudomonads

strains	P-Solubilization			IAA [†] production (mg l ⁻¹)		HCN [§]
	On solid medium	pH	mg P l ⁻¹	On liquid medium	With L-TRP ^{‡‡}	
<i>Pseudomonas entomophila</i> SAW7	-	-	-	+	13.06	+
<i>Pseudomonas plecoglossicida</i> SAB1	+	5.87	12.75	-	-	-
<i>Pseudomonas mosselii</i> SAW1	-	-	-	+	19.59	+
<i>Pseudomonas corrugata</i> SAC1	+	5.66	70.60	-	-	+
<i>Pseudomonas palleroniana</i> SAB17	+	4.68	105.02	-	-	+
<i>Pseudomonas palleroniana</i> SAB15	+	5.55	87.62	-	-	-
<i>Pseudomonas parafulva</i> SAB14	-	-	-	+	16.98	+
<i>Pseudomonas putida</i> SAB12	+	4.2	131.11	-	-	-
<i>Pseudomonas plecoglossicida</i> SAB8	+	4.55	119.52	-	-	-
<i>Pseudomonas putida</i> SAB10	+	4.34	127.58	-	-	-
<i>Pseudomonas putida</i> SAW3	+	5.22	82.32	-	-	-
<i>Pseudomonas corrugata</i> SAW23	+	5.30	79.68	+	7.84	-
<i>Pseudomonas anguilliseptica</i> SAW24	-	-	-	+	10.45	-
<i>Pseudomonas palleroniana</i> SAW21	+	4.90	100.73	-	-	+
<i>Pseudomonas putida</i> SAW22	+	4.65	102.24	-	-	-
<i>Pseudomonas putida</i> SAW19	-	-	-	+	15.67	-
<i>Pseudomonas moraviensis</i> SAW15	+	5.44	89.63	-	-	+
<i>Pseudomonas plecoglossicida</i> SAW10	+	5.17	96.44	-	-	-
<i>Pseudomonas argentinensis</i> SAW9	-	-	-	+	11.75	-
<i>Pseudomonas entomophila</i> SAW5	-	-	-	+	14.36	-

[†]IAA: Indole acetic acid, [‡]L-TRP: L-tryptophan, [§]HCN: Hydrogen cyanide

Table (4): Effect of *Pseudomonas* strains on plant growth of bean plants with three different phytopathogenic fungi

Strains	Plant height (cm)			Plant fresh weight (g)			Plant dry weight (g)		
	<i>R. solani</i> AG 2-2	<i>F. solani</i>	<i>P. arrhenomanes</i>	<i>R. solani</i> AG 2-2	<i>F. solani</i>	<i>P. arrhenomanes</i>	<i>R. solani</i> AG 2-2	<i>F. solani</i>	<i>P. arrhenomanes</i>
<i>Pseudomonas mosselii</i> SAW1	54 ^c	66 ^a	55.33 ^b	18.3 ^b	22 ^a	19.8 ^a	8.3 ^a	10.1 ^a	8.9 ^a
<i>Pseudomonas moraviensis</i> SAW15	53 ^c	67 ^a	55.6 ^b	17 ^b	23.3 ^a	19.4 ^a	7.2 ^{ab}	10.3 ^a	8.7 ^a
<i>Pseudomonas putida</i> SAW19	68.0 ^{3a}	65.03 ^{ab}	53.16 ^b	23 ^a	23 ^a	18.5 ^a	10.3 ^a	10.1 ^a	7.5 ^{ab}
<i>Pseudomonas entomophila</i> SAW7	60 ^b	62 ^b	60 ^a	20 ^{ab}	22 ^a	17 ^a	9 ^a	9.5 ^a	8.9 ^a
Diseased	32.0 ^{3d}	35.03 ^d	34.66 ^c	12 ^c	12.2 ^c	11.8 ^b	4.3 ^b	4.5 ^b	4.3 ^b
Healthy Control	52 ^c	52 ^c	52 ^b	16.8 ^b	16.8 ^b	16.8 ^a	7 ^{ab}	7 ^{ab}	7 ^{ab}

Values followed by the same letters at the same column are not significantly different by LSD test at 0.05 level.

Table (5): Effect of *Pseudomonas* strains on Bacterial population of bean with three different phytopathogenic fungi

Strains	Bacterial population (log CFU g ⁻¹ fresh root)			
	<i>R. solani</i> AG 2-2	<i>Fusarium solani</i>	<i>Pythium arrhenomanes</i>	control
SAW1	4.5 ^b	5.3 ^a	5.1 ^a	3.0 ^c
SAW15	4.7 ^b	5.7 ^a	4.8 ^b	3.7 ^c
SAW19	5.6 ^b	5.5 ^a	4.3 ^b	3.5 ^c
SAW7	5.2 ^a	4.9 ^a	5.3 ^a	4.0 ^b

Values followed by the same letters at the same row are not significantly different by LSD test at 0.05 level.

Table (6): Effect of *Pseudomonas* strains on disease severity of bean with three different phytopathogenic fungi

Isolates	disease severity		
	<i>R. solani</i> AG 2-2	<i>Fusarium solani</i>	<i>Pythium arrhenomanes</i>
SAW1	0.15 ^b	0.0 ^c	0.0 ^d
SAW15	0.1 ^b	0.0 ^c	0.25 ^b
SAW19	0.0 ^c	0.0 ^c	0.2 ^c
SAW7	0.0 ^b	0.1 ^b	0.0 ^d
Healthy control	0.0 ^c	0.0 ^c	0.0 ^d
Diseased	2.2 ^a	3.5 ^a	2.33 ^a

Values followed by the same letters at the same column are not significantly different by LSD test at 0.05 level.

The role of *Pseudomonas* spp. in promoting root and shoot growth of different crops has been demonstrated by Kamal *et al.* (2008).

Bacterial root colonization

The obtained results showed that all the tested *Pseudomonas* strains had capacity for root colonization when compared with the control treatment (Table 5). The population densities of *Pseudomonas* spp. are ranged from 4.5 to 5.7 log CFU g⁻¹ fresh root. The highest population of bacteria (5.7 log CFU g⁻¹ root) was observed when bean plant inoculated with SAW15 strain (*Pseudomonas palleroniana*) and infected with *F. solni*. Plant root colonization by bacteria can be considered as an enrichment of the best adapted microorganisms to a particular ecological niche. Colonization of roots by inoculated bacteria is an important step in the interaction between beneficial bacteria and the host plant. Seed colonization is the first step in root colonization. Microorganisms established on the germinating seed can multiply and colonize the root as it emerges and grows through soil. Thus colonization of the imbibing seed may predispose future colonization of the root. Previous studies have shown that the fluorescent pseudomonads are aggressive colonizers of the rhizosphere of various crop plants (Weller *et al.*, 2002; Bhattacharyya and Jha, 2012).

Disease severity index

The pathogenicity of the tested *Pseudomonas* strains was estimated in the current study by a disease severity index. The index was expressed according to a 4-point disease index scale based on the progressive wilt symptoms. The absence of symptoms was rated zero. Disease severity was significantly but variously affected by the tested four *Pseudomonas* strains (Table 6). The obtained results indicated that the plants that had inoculated with only fungus had the highest disease index. The average disease severity index values are 2.2, 3.5 and 2.33 for *R. solani* AG 2-2, *F. solani* and *Pythium arrhenomanes*, respectively. The disease index was significantly lower when plants inoculated with SAW1 or SAW15 strains and infected with *R. solani* AG 2-2 (Table 6). The disease index of plants receiving SAW19 or SAW7 strains plus *R. solani* was equal or similar to that in non-treated control and the index value is zero (that is absence of wilt symptoms). The same trend was observed in the plants that inoculated with SAW1 or SAW15 or SAW19 plus *F. solani* and the plants inoculated SAW1 or SAW7 plus *Pythium arrhenomanes*. A significantly lower disease severity, as compared to treatment with *F. solani* or *Pythium arrhenomanes*, was also observed in plants treated with SAW7 and plants treated with SAW15 and SAW19 strains, respectively. This indicated that the tested antagonistic fluorescent pseudomonads reduced disease severity in beans and disease development. These results were confirmed the result obtained *in vitro* antagonistic activity that the colonization of roots by pathogenic fungi was suppressed by with antagonistic strains (Table 6). In general, the results showed that the strains which have ability to colonize the root of plant were more effective in suppressing pathogenic fungi on bean plants.

It seems that performance of rhizobacteria was effective in disease development. This delay may be due to either preliminary induced systemic resistance (Van loon *et al.*, 1998) or preliminary colonization which suppress pathogen onset directly via different mechanisms such as production of antibiotics and volatile compound competition for space or nutrition (Mafia *et al.*, 2009).

CONCLUSION

The present study clearly indicates the potential of fluorescent pseudomonads as biocontrol agents and fulfills diverse beneficial interaction in plants leading to promising solution for sustainable and environment friendly agriculture. Thirty-four isolates were showed a broad-spectrum antifungal activity against phytopathogenic fungi, *Rhizoctonia solani* AG 2-2, *Fusarium solani* and *Pythium arrhenomanes*. The phenotypic characterization of twenty isolates confirmed their relation to fluorescent pseudomonads. The gene sequencing of these bacteria revealed that all isolates belonged different species and strains of *pseudomonas*. The current study indicated that the *Pseudomonas* strains have ability to colonize the root of bean and more effective in suppressing pathogenic fungi on plants under greenhouse pot experiment as well as promoted the plant growth.

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