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MULTI-TRAITS OF NON-PATHOGENIC FLUORESCENT *PSEUDOMONAS* AND EVALUATION OF THEIR POTENTIEL AS BIOCONTROL AGENTS

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ABSTRACT

In recent years, certain strains of fluorescent pseudomonads called PGPB have drawn attention due to their abilities to promote plant growth. Therefore, in this investigation, we have explored the adhesive properties, the phytostimulator effects and the biocontrol activities of 40 isolates, with the aim to select potential inoculants to improve crop yields. Ten different colony morphotypes were detected on CRA plates. PsTp172 described as "ST" showed the highest adherence ability to abiotic surface (OD_{550} = 2.102). 31 isolates were positive for the plant growth-promoting hormone (IAA) production and 30 stains solubilised tri-calcium phosphate in Pikovskaya's agar. Furthermore, the highest pyoverdine concentration was detected with PsTp172 strain (172.50 µM) under iron starvation conditions. This strain also exhibited a co-resistance against Zn^{2+} and Mn^{2+} and displayed high values of Minimum Inhibitory Concentrations (MIC) for each heavy metal. Additionally, among the tested isolates, eight strains (PsS15, PsTp172, PsS28, PsTp171, PsS31, PsS67, PsS18, PsS39 and PsS93) were found to be efficient antagonists against the 3 pathogenic strains and 6 isolates (PsS15, PsTp156, PsTp172, PsC54, PsTp171 and PsS102) were considered as lactone inhibitors of the 3 tested strains, as shown by their ability to inhibit the cellular communication. The majority of isolates showed various phytobeneficial traits and the most effective strains are *P. putida* (PsTp172 and PsS15) and *P. mosseli* (PsTp171).

Keywords: Fluorescent Pseudomonads, PGPB, Biofertilizer, Biocontrol, Biofilm

1. INTRODUCTION

In diver's environments, beneficial plant-associated bacteria exhibit a significant role in plant growth and health (Wahyudi *et al*., 2011). The success of Plant Growth Promoting Bacteria (PGPB) is due to their efficient colonization of plant roots by forming microcolonies or biofilms which promotes the plant-microbe interaction. These bacterial consortiums have the ability to communicate chemically with one another through *quorum-sensing* (Mishra *et al*., 2010). This signalling mechanism coordinates bacterial communications with plants, comprising antibiotic production, phytohormone excretion, toxin release and Horizontal Gene Transfer (HGT) (Von Bodman *et al*., 2003). Though, it has been portrayed that certain PGPB are able to quench phytopathogens *quorum-sensing* capability by degrading autoinducer signals, thereby blocking expression of virulence genes (Compant *et al*., 2005).

An efficient PGPB should display more than two plant growth promoting activities which are root colonization competency, biofertilizer, phytostimulator and phytopathogen biocontrol activities (Bloemberg and Lugtenberg, 2001). Of the various rhizospheric bacteria, *Pseudomonas* species are the most popular bacteria that combine all these characters; enhance plant growth by direct and indirect mechanisms. The direct promotion involves either supplying plant with growth promoting substances (such as auxins, gibberellins) or helping plant nutrients uptake from environments (Husen *et al*., 2011). The indirect promotion arises when PGPB avoid harmful effects of phytopathogenic microorganisms (bacteria,

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fungi, nematode) by the production of siderophores, HCN, antibiotics, bacteriocins, volatile metabolites (Saharan and Nehra, 2011).

The performance of PGPB is influenced by a wide range of the environmental conditions (soil composition or characteristics: pH, heavy metals contamination, weather conditions, etc.) that may affect the plant growth (Sajani and Muthukkaruppan, 2011). So, application of highly heavy metal tolerant PGPB has been exploited in contaminated soil allowing the vegetables grow under hard conditions (Zhuang *et al*., 2007).

Therefore to exploit the potential of native strains of non-pathogenic fluorescent *Pseudomonas*, the present study attempts to characterize, screen and select non-pathogenic *Pseudomonas* bacteria exhibiting the highest number of traits.

2. MATERIALS AND METHODS

2.1. Growth media and culture conditions

All samples were transfered to the laboratory in sterile stomacher containers, stocked at 4°C and analyzed within 24 h. 10 g or 10 mL of sample were suspended in 90 mL sterile distilled water. 50 µL of the appropriate dilution was spread on King's B (King *et al*., 1954) agar medium. After 48 h of incubation at 25°C, each different colonie showing fluorescent halo was purified (Munsch *et al*., 2000). Each colonie confirmed as putative *Pseudomonas* spp. (gram negative, catalase positive and oxidase positive) was utilized for further characterization.

The fluorescence was detected in the liquid iron-poor growth medium (Meyer and Abdallah, 1978), the Casamino Acid (CAA) medium, consisting of (per liter) 5 g of low-iron Bacto Casamino Acid (Difco), 1.54 g of $K_2HPO_4 \cdot 3H_2O$ and 0.25 g of $MgSO_4 \cdot 7H_2O$.

Strains whose designations begin with PsS, PsTp, PsC and PsWw were collected respectively from soil rhizosphere, wastewater treatment plant rhizosphere, compost and wastewater.

The NCBI Accession Numbers for the 16S rDNA gene sequences of 40 isolates determined in this present investigation are listed in **Table 1**.

2.2. Adhesive Properties

2.2.1. Qualitative Detection of Biofilm Formation (Detection of Colony Morphology Variant)

All strains were spread on Congo Red Agar (CRA) containig 0.8 g of Congo red (Sigma) and 36 g of saccharose to 1 l of brain heart infusion agar (Bio-rad). A concentrated aqueous solution of Congo red stain was prepared, autoclaved separately for 15 min at 121°C and added when the agar had cooled to 55°C (Freeman *et al*., 1989). Plates were incubated at 30°C for 48 h. Morphology, pigmentation and mucosity were observed.

2.3. Quantitative Estimation of Biofilm Formation on Polystyrene Surface

All bacteria were cultured overnight in Brain Infusion Broth (BHI-0.25 glucose at 30°C). The culture was diluted 1:20 in fresh BHI plus (0.25%) glucose at 30°C. This suspension (200 µL) was utilized to inoculate sterile 96-well-polystyrene microtiter plates. The plates were incubated at 30°C aerobically for 24h. The cultures were eliminated and the microtiter wells were washed twice with phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) to remove non-adherent cells and were dried in an inverted position. Then, bacteria that not adherate to microtiter plates were stained with 1% Crystal violet for 15 min. The wells were washed once more and the Crystal violet was dissolved in 200 µL of ethanol (95%). An automated PR3 100 TSC (Bio-Rad) was used to measure the absorbance at 550 nm (OD_{550}) (O'Toole *et al*., 1999). Each essay was performed in triplicate. The following values were attributed for biofilm determination: $OD_{550} \leq 0.1$ non biofilm forming; $0.1 \leq OD_{550} \leq 0.5$ weak biofilm forming; $0.5 \leq OD_{550} \leq 1$ medium biofilm forming; $1 \leq OD_{550} \leq 2$ strong biofilm forming; $OD_{550} \geq 2$ very strong biofilm forming.

2.4. Plant Growth Promoter Activity

2.4.1. Indole-3-Acetic Acid (IAA) Production

The production of Indole-3-Acetic Acid (IAA) by the 40 isolates of *Pseudomonas* was assayed following the technique portrayed by Patten and Glick (2002). The auxin amount was determined spectrophotometrically after 72 h of incubation at 535 nm in minimal medium containing (per liter) $MgSO_4 \cdot 7H_2O$, 0.2 g; KH_2PO_4, 6.8 g; $(NH_4)_2SO_4$, 2.0 g; citrate, 2.0 g; ZnO, 0.006 g; H_3BO_3, 0.006 g; $FeCl_3 \cdot 6H_2O$, 0.0024 g; $CaCO_3$, 0.02 g and HCl, 0.13 mL, supplemented with glucose (10 g) and L-tryptophan (100 µg mL^{-1}), using Salkowski's reagent (Gordon and Weber, 1951). In each culture medium, the IAA concentration was established by comparison with a standard curve.

2.5. Phosphate Solubilisation

Onto Pikovskaya's agar medium containing (per liter): 10 g dextrose; 0.5 g yeast extract; 5 g $Ca_3(PO_4)_2$; 0.5 g $(NH_4)_2SO_4$; 0.2 g KCl; 0.1 g $MgSO_4 \cdot 7H_2O$; 0.0001 g

$FeSO_4 \cdot 7H_2O$; 0.0001 g $MnSO_4 \cdot H_2O$ and 15 g agar, all cells were streaked. After 72h of incubation at 28°C, strains inducing clear zone around colonies were noted as positive (Katznelson and Bose, 1959).

2.6. Biocontrol Activity

2.6.1. Pyoverdine Assay

The method was described in details in previous study (Mehri *et al*., 2012). Pyoverdin levels were calculated using the extinction coefficient (ε = 20000 M^{-1} cm^{-1}) following the thechnique of Meyer and Abdallah (1978). Three repeated experiments were envisaged.

2.7. Heavy Metal Tolerance

Metal Salts were utilized in the following forms: $ZnSO_4 \cdot 7\ H_2O$ (iron content,<10 ppm) and $MnSO_4 \cdot\ H_2O$ (iron content, <0.001%). Stocks of $10mM^1$ $ZnSO_4$ and $MnSO_4$ salts were prepared and sterilized with 0.22 µm filters under aseptic condition. The stock solutions were incorporated to autoclaved CAA media (CAA+Mn and CAA+Zn) at a final concentration of 60 µM for each metal. In the purpose to establish the Maximum Tolerable Concentration (MTC) of metals at which growth was observed, the 40 strains were monitored in CAA medium as a function of increasing concentrations of Mn (II) and Zn (II) from 0.1 to 475 µM in 5 mL CAA medium (Mehri *et al*., 2012).

2.8. In Vitro Antagonism Against Pathogenic and Phytopathogenic *Pseudomonas* Bacteria

Culture supernatant was prepared as follows: A centrifugation at 150 rpm of an overnight culture. Resulting supernatant was neutralized, sterilized by filtering and assayed for the presence of an inhibitor in the broth; following the agar well diffusion assay technique (Barefoot *et al*., 1983). Nutrient agar was first seeded with pathogenic organism (110 µL of overnight culture per 20 mL of agar) in sterile Petri dishes and after solidification, dried for 15 min. Wells were bored in the agar. Aliquots of the supernatant were distributed in holes and plates were incubated 24h at 30°C. Inhibition of growth was determined by an area of inhibition surrounding each agar well.

2.9. *Quorum-Quenching* (QQ) Bioassays

N-Acyl Homoserine Lactone (NAHL) degradation ability of the isolates was detected by cross streaking against Chromobacterium violaceum CV026 as the AHL biosensor (Swift *et al*., 1999). Supernatants from 7 days old cultures of the isolates were adjusted to pH 7 and 15µl of each supernatant blended with pathogenic or phytopathogenic *Pseudomonas* supernatant and dispensed in the wells of a bioassay plate overlaid with *C. violaceum* CV026.

2.10. Pathogens and Phytopathogens Used in Antagonistic and QQ Assays

3 strains of *Pseudomonas* were utilized in this study: *P. aeruginosa* (PsCLHMC1) from clinical origin, *P. Otitidis* (PsWw118) isolated from wastewater (Mehri *et al*., 2011) and *P. savastanoi* from olive knots (Ouzari *et al*., 2008). Access numbers of *Pseudomonas* strains were respectively: HM627572, HM627606 and AM265392.

3. RESULTS

The 40 isolates were screened for functional properties. PGPB traits were represented in **Table 1**.

3.1. Growth Temperature Tolerance

All isolates, except three strains, were able to grow at maximum temperature of 32°C. On the other hand PsS60, PsS91 and PsS73 strains were able to grow at 37°C and 42°C.

3.2. Colony Morphology on CRA Plates

The studied isolates showed ten different colony morphotypes on CRA plates (**Fig. 1**). The morphotype "ST", described in previous study (Kirisits *et al*., 2005), was detected in the strain PsTp172 showing rough, wrinkled and sticky colony (**Fig. 1a**). Another morphotype "SM", previously described by Rainey and Travisano (1998), was found in the strain PsS2 appearing as smooth and regular colony (**Fig. 1b**). Colony morphotype of PsS103 and PsTp169 isolates were light rose or colourless (**Fig. 1c**) suggesting that biofilm formation was affected (Rakhimova *et al*., 2008). Biofilm growth of PsTp155 produced glossy colonies with an inner circle of lysed cells (**Fig. 1d**) (Rakhimova *et al*., 2008). PsS4 and PsS46 showed a particular morphotypes characterized, respectively, by a flat colony having orange pigment in the center of the colony (**Fig. 1e**) and a translucent colony (**Fig. 1f**). The remaining strains exhibited non mucoïd (2 isolates, **Fig. 1g**), mucoïd (19 isolates, **Fig. 1h**), highly mucoïd with the dye Congo red concentrated in colony center (11 isolates, **Fig. 1i**) and stronger mucoïd (one isolate, **Fig.1j**) structures. In fact, the mucosity indicates a weak or a high production of exopolysaccharide (alginate) (Kirisits *et al*., 2005).

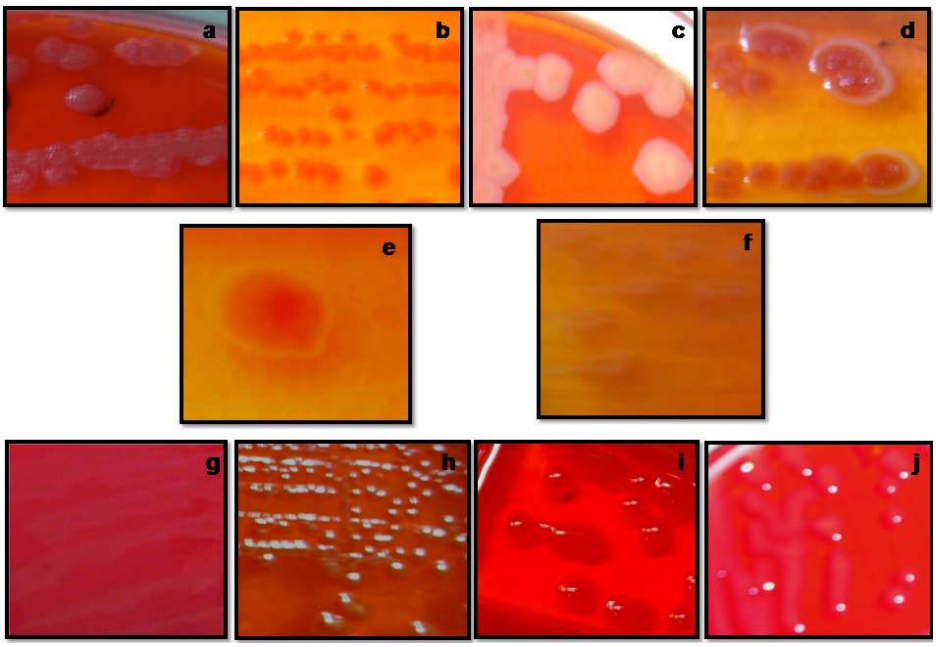

Fig. 1. Colony morphology variants on Congo Red Agar (CRA): (a) rough, wrinkled and sticky "ST"; (b) smooth and regular "SM" (c) light rose or colourless (d) glossy with an inner circle of lysed cells (e) flat with orange pigment in the center (f) translucent (g) non mucoïd (h) mucoïd (i) highly mucoïd with the dye Congo red concentrated in colony center (j) stronger mucoïd

3.3. Biofilm Formation on Polystyrene Surface

About 26 isolates showing mucoid, highly mucoïd and stronger mucoïd colony morphotypes were non-biofilm forming on polystyrene surfaces with an $OD_{550} \leq 0.1$. While, PsTp172 (morphotype "ST") produced a very large amount of biofilm (OD_{550} = 2.102). This type of morphotype was characterized by hyper-adhesion and autoaggregation to abiotic surfaces (Kirisits *et al*., 2005). The PsS67 strain was strongly adhesive to polystyrene with a value of 1,238 at 550 nm. Strains PsTp171 and PsS46 were medium adhesive to the abiotic surface with OD_{550} values respectively of 0.639 and 0.547. On the other hand, Rakhimova *et al*. (2008) explained that isolates with light rose or colourless colony morphotypes, suggesting that biofilm formation was affected. Hence PsTp169 and PsS103 showed optical densities respectively of 0.069 and 0.041. All these data were summarized in **Table 1**.

3.4. IAA production and Phosphorus solubilisation

About 31 strains (76%) of fluorescent *Pseudomonas* produced the IAA hormone in various levels, while 9 strains showed no producing activity. The isolate PsS93 produced the highest level of IAA (7.4 µg mL^{-1}), whereas PsS4 and PsS31 were found the weak producer isolates (respectively 0.11-0.15 µg mL^{-1}). On the other hand, 30 *Pseudomonas* spp. (75%) were capable to solubilise, in Pikovskaya's agar, tri-calcium phosphate by the formation of clear halo zone (**Fig. 2**).

3.5. Pyoverdine Quantification

When the cells grown under iron starvation Conditions (CAA medium) different level of siderophore was detected in culture supernatant. The highest metabolite concentration was obtained with PsTp172 strain (172.50 µM). The least pyoverdine production was detected in PsS73 isolate (2.82 µM).

3.6. Heavy metals Resistance

The 40 isolates were screened for their metal tolerance on CAA media containing different metal concentrations of each Zn^{2+} and Mn^{2+}. The MTC for each metal was determined to select isolates able to grow and resist high level of metal toxicity. Generally, the bacterial isolates showed the maximum resistance against Zn^{2+} (MTC: 100-300 µM) and Mn^{2+} (MTC: 300-475 µM). PsTp172 strain was the most tolerant

bacteria to tested metals. This isolate resisted to Zn^{2+} up to 300 µM and to Mn^{2+} up to 475 µM.

3.7. Antagonism Assay Against Pathogenic and Phytopathogenic *Pseudomonas*

27 isolates inhibited the growth of at least one pathogenic strain. PsS15, PsTp172, PsS28, PsTp171, PsS31, PsS67, PsS18, PsS39 and PsS93 were found to be efficient antagonists against the 3 tested strains (**Fig. 3**).

3.8. *Quorum-Quenching* Activity

The *C. Violaceum* CV026 was used as an indicator of extracellular N-Acyl Homoserine Lactone (NAHL) inhibition of *P. aeruginosa*, *P.otitidis and P. savastanoi*; 18 isolates were considered as lactone inhibitors as shown by their ability to inhibit violacein production of at least one pathogenic strain. PsS15, PsTp156, PsTp172, PsC54, PsTp171 and PsS102 were found to inhibit the cellular communication factor of the 3 tested strains (**Fig. 4**).

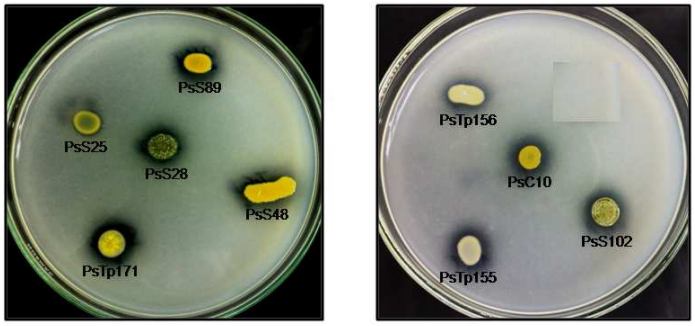

Fig. 2. Phosphate solubilisation halo produced by *Pseudomonas* isolates

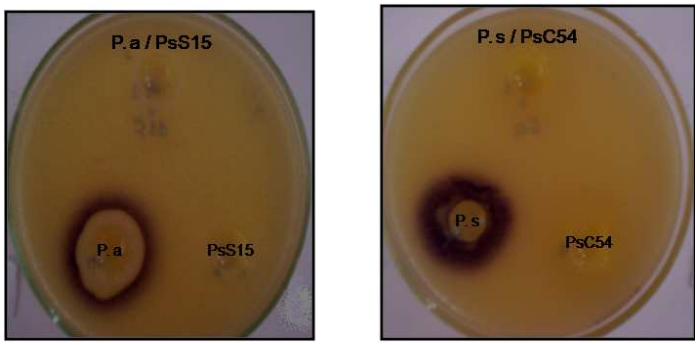

Fig. 3. Antagonistic effect of *Pseudomonas* isolates on pathogenic *Pseudomonas* (*P. savastanoi* and *P. otitidis*)

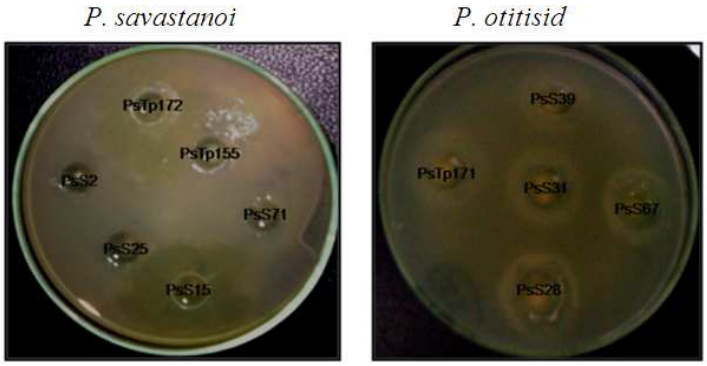

Fig. 4. NAHL inhibition activity (QQ) of PsS15 and PsC54 via *P. aeruginosa* (P. a) and *P. savastanoi* (P. s)

Table 1. List of the forty fluorescent *Pseudomonas* isolates and their PGPB traits and biocontrol activities

Strains	Closest16S r DNA speice (assession Number)	Growth at37 -42°C	Biofilm form ation (OD_{550})	Morphotypes[a]	IAA produ ction (µg mL)	Phos phate solubiliz ationa	Antagonistic ctivity[b]			NAHL inhibition (QQ)[c]			Sidero phore produc tion (µM)	MTC (µM of Zn)	MTC (µM of Mn)
							p.a	p.o	p.s	p.a	p.o	p.s			
PsS2	*P. Putida* (HM627611)	-	0, 266	smooth morph :"SM"	0,27	++	-	-	-	-	-	-	35,33	100	350
PsS4	Pseubom on sp (HM627612)	-	0, 135	Flat, or pigment incolony center	0,11	+	-	-	-	-	-	-	14,90	150	350
PsS15	*P. Putida* (HM627595)	-	0, 021	Mucoid	1,50	+	+	+	+	+	+	+	32,83	200	400
PsS46	*P. Putida* (HM627613)	-	0,547	Flat translucent	2,52	+	-	-	+	-	-	+	98,18	250	450
PsWw124	*P. Putida* (HM 627628)	-	0,007	Mucoid	1,25	+	-	+	+	-	-	+	18,17	150	300
PsTp169	*P. Putida* (HM 627607)	-	0,040	Mucoid	1,35	+	+	-	+	+	+	+	13,87	200	300
PsTp 172	*P. Putida* (HM 627615)	-	0,041	Flat, light ro colourless	-	+	+	+	-	+	+	+	106,17	250	450
PsTp 172	*P. Putida* (HM 627615)	-	2,102	Rough, wrinkled, sticky: "ST"	0,27	+	+	+	+	+	+	+	172,50	300	475
PsS 28	*P. Putida* (HM 627615)	-	0,176	Mucoid	-	++	+	+	+	+	+	-	85,33	200	400
PsC 54	*P. Putida* (HM 627615)	-	0,103	Mucoid	1,45	+	-	+	+	+	+	+	49,62	200	350
PsS 103	*P. Putida* (HM 627615)	-	0,069	Flat, light ro colourless	1,02	+	+	-	-	+	-	-	80,80	200	300
PsS 48	*P. Putida* (HM 627615)	-	0,032	Mucoid	0,78	++	-	-	-	-	+	-	31,50	150	350
PsS 79	*P. Putida* (HM 627615)	-	0,042	Mucoid	-	+	-	-	-	+	-	-	31,50	150	300
PsS 71	*P. Putida* (HM 627615)	-	0,095	Mucoid	1,41	++	-	-	-	-	-	-	85,38	250	350
PsS 11	P.m. onteilii (HM 627615)	-	0,426	Mucoid	0,82	+	+	-	-	+	+	+	65,90	200	400
PsTp 139	*P. Putida* (HM 627615)	-	0,007	Mucoid	1,11	++	-	-	-	-	-	-	52,50	200	350
PsTp 153	*P. Putida* (HM 627615)	-	0,003	Mucoid	1,21	+	-	-	-	-	-	-	12,10	100	300
PsTp 155	P.m. onteilii (HM 627615)	-	0,405	Shiny autolysis (inner circle of Lysed cell)	0,91	++	-	-	-	-	-	-	17,22	150	300
PsTp 154	P.m. onteilii (HM 627615)	-	0,450	Mucoid	0,98	+	-	-	-	-	-	-	11,55	100	300
PsC 10	*P. Putida* (HM 627615)	-	0,156	Mucoid	0,62	++	+	+	-		b		21,33	150	400
PsC 75	*P. Putida* (HM 627615)	-	0,008	Mucoid	-	+	+	+	-	+	+	-	17,67	100	300
PsT p171	P.mosseli (HM627603)	-	0,639	flat,non m ucoid	-	+++	+	+	+	+	+	+	82,35	200	425
PsS31	P.Putida (HM 627624)	-	0,017	mucoid	0, 15	++	+	+	+		b		21,23	100	300
PsS102	(HM 627601)	-	0,081	mucoid	-	++	+	+	+	+	+	+	34,76	100	400

Table 1. Continue

Strains	Closest16S r DNA speice (assession Number)	Growth at 37 -42°C	Biofilm formation (OD_{550})	Morphotypes[a]	IAA production (µg mL)	Phosphate solubilizationa	Antagonistic ctivity[b] p.a	p.o	p.s	NAHL inhibition (QQ)[c] p.a	p.o	p.s	Siderophore production (µM)	MTC (µM of Zn)	MTC (µM of Mn)
PsS67	P.Putida (HM 627602)	-	1, 238	flat,non mucoid mucoid	- -	+	+	-	+		b		8567	250	425
PsS18	P.Putida (HM 627510)	-	0,001	stronger mucoid (moist and bubbly)	0, 82	+	+	+	+		b		3567	150	350
PsWw128	P.fluorescens (HM 627582)	-	0,053	mucoid	4, 83	++	+	+	+	-	-	-	24,55	100	300
PsS89	P.fluorescens (HM 627583)	-	0,139	Highly mucoid dye Congo red in the center of colony	1, 53	++	-	-	-	-	-	+	77,67	200	425
PsS60	P.fluorescens (HM 627584)	+	0,031	Highly mucoid dye Congo red in the center of colony	1, 30	-	-	-	-		b		5,17	250	425
PsS29	P.fluorescens (HM 627585)		0,010	Highly mucoid dye Congo red in the center of colony	1, 18	-	+	+	-		b		26,28	200	475
PsS39	P.fluorescens (HM 627586)	-	0,063	Highly mucoid dye Congo red in the center of colony	0, 03	-	-	-	+		b		9,93	200	450
PsS25	P.fluorescens (HM 627587)	-	0,000	Highly mucoid dye Congo red in the center of colony	4, 49	+/-	+	+	+		b		7,37	150	350
PsS49	P.fluorescens (HM 627588)	-	0,003	Highly mucoid dye Congo red in the center of colony	7, 4	-	-	-	-		b		9,07	250	475
PsS93	P.fluorescens (HM 627589)	-	0,007	Highly mucoid dye Congo red in the center of colony	4, 53	-	-	-	+		b		9,85	250	450
PsS90	P.fluorescens (HM 627590)	-	0,004	Highly mucoid dye Congo red in the center of colony	3, 94	-	+	+	+		b		7,15	100	350
PsS91	P.fluorescens (HM 627591)	+	0,004	Highly mucoid dye Congo red in the center of colony	2, 32	-	-	-	-		b		4,12	150	400
PsS23	P.fluorescens (HM 627592)	-	0,020	Highly mucoid dye Congo red in the center of colony	2, 24	-	+	-	+		b		5,32	100	300
PsS26	P.fluorescens (HM 627593)	-	0,089	Highly mucoid dye Congo red in the center of colony	2, 83	-	+	-	+		b		13,62	200	425
PsS73	P.fluorescens (HM 627594)	+	0,005	Highly mucoid dye Congo red in the center of colony		-	+	+	-		b		2,82	250	475

[a]The different colony morphotypes after 48 h incubation at 30°C in Congo red agar (**Fig. 1.**),
[b]Lactone inhibition activity was tested only with not lactone producers strains (PsC10, PsS31, PsS67, PsS18, PsS60, PsS29, PsS39, PsS25, PsS49, PsS93, PsS90, PsS91, PsS23, PsS26 and PsS73 are lactone producers strains).
IAA: Indole-3-Acetic Acid, NAHL: N-*acyl* homoserine lactone, QQ: Quorum-Quenching, P. a: *Pseudomonas aeruginosa*, P. o: *Pseudomonas otitidis*, P. s: *Pseudomonas savastanoi*, MTC: Maximum Tolerable Concentration, Zn: Zinc, Mn: Manganese.

4. DISCUSSION

Among PGPB, fluorescent pseudomonads have proved a boon in sustainable agriculture for use as biofertilizer (plant growth promoting capacity), biocontrol agent (antagonistic activity) and in bioremediation (degradation of pollutants) (Husen *et al*., 2011). A total of 40 isolates recovered from diver's origin were subsequently screened for plant growth promoting characteristics and biological control of plant deleterious microbes.

IAA, a member of phytohormone group, acts as important signal molecule in the regulation of plant growth (Kumar *et al*., 2012). In our study most of the *Pseudomonas* isolates were positive for IAA production (76%). Among them, *P. fluorescens* strains isolated from soil rhizosphere are found to be good producers of the phytohormone (PsS93, PsS89, PsS49, PsS90). Saharan and Nehra (2011) study revealed that isolates of the *Pseudomonas putida-fluorescens* group, which produce auxin, are able to induce statistically important yield increases up to 144% in field tests.

In the soil, most of phosphorus is present in the insoluble form and cannot be used by plants (Pradhan and Sukla, 2006). The study of Rodriguez and Fraga (1999) revealed that the availability of phosphor in soil was increased by strains of Bacillus, *Pseudomonas* and *Rhizobium*. In our experiments, all *P. putida* isolates are able to produce the phosphatase enzyme. Villegas and Fortin (2002) selected *P. putida* strains as effective agents able to solubilise soil phosphate. The aptitude of various strains to transform the insoluble phosphate suggested their application in agriculture (Wahyudi *et al*., 2011).

Apart the PGPB biofertilizer actions, it is prominent to note that the conformation of adherent microbial population affects significantly the duration and intensity of the plant-microbe interactions (Danhorn and Fuqua, 2007). In general, there should be a correlation between the colony morphotype and the capacity of adhesion to different surfaces. Therefore, PsTp172 strain with rough, wrinkled and Sticky (ST) colony morphotype showed a higher adherence ability (OD_{550} = 2.102). "ST" variant is so called because of the hyperadherence to abiotic surfaces and the strong autoaggregative phenotype in liquid culture (Kirisits *et al*., 2005). On the other hand, some isolated strains showed hypermucoid colony (PsWw128, PsS60, PsS29). In this context, Bianciotto *et al*. (2001) described that the hypermucoid mutant of *P. fluorescens* CHA0 demonstrated an important adherence_to roots and to the mycelium of arbuscular mycorrhizal fungi. Exopolysaccharide production (alginate) contributes to surface colonising of plants (Danhorn and Fuqua, 2007). Study of Stoodley *et al*. (2002) portrayed that structure and adherent nature of cells in biofilms modulate the biocontrol activities and antimicrobial tolerance. *P. putida* and *P. fluorescens* bacteria are able to coat roots of plant by forming a biofilm, which may save roots against phytopathogens (Espinosa-Urgel *et al*., 2002; Walker *et al*., 2004; Ude *et al*., 2006). Additionally, it has been shown that besides biocontrol activities, biofilmed *Pseudomonas* spp. inocula possess N_2-fixing properties and nutrient uptake (Seneviratne *et al*., 2010).

Siderophores such as pyoverdins are iron chelators molecule. Privated from iron, telluric harmful flora slows its growth and its density is decreased in the rhizosphere. This feature can enhance fluorescent *Pseudomonas* (PGPR) in the process of colonization and competition for the substrate better than other microbes in the rhizosphere (Lemanceau, 1992). A significant pyoverdin production was detected with isolates PsTp172, PsTp169, PsS46, PsTp171 (>80 µM). Loper and Buyer (1991) explain that the pyoverdine concentration produced by *Pseudomonas* spp. in the rhizosphere, is important to influence the microbial interactions. Many authors found that mutants, who lost the synthesizing siderophore ability (Sid^-), are not able to determine the beneficial effects recorded with the wild strain (Becker and Cook, 1988). Unfortunately, the involvement of pyoverdin in animals and humans virulence is very well studied in pathogenic isolates (*P. aeruginosa*), but it is unlikely exposed in saprophytic strains (mainly *P. fluorescens*) grown at or above 37°C (Donnarumma *et al*., 2010). These siderophores acquire iron from lactoferrin and transferrin and regulate the production of virulence factors (exotoxin A and endoprotease) (Takase *et al*., 2000).

Besides iron, several siderophores can bind other essential metals (Mg, K) serving as micronutrients (Shinozaki-Tajiri *et al*., 2004). Furthermore, microorganisms producing siderophores are able to bind a variety of toxic metals such as Al, Pb, Cd (Mureseanu *et al*., 2003). While essential metals have important biological role, at high levels they can alter cell membranes, disturb cellular functions, damage the DNA structure and can reduce crop yields (Teitzel *et al*., 2006). *Pseudomonas* strains are widely utilized in bioremediation processes as they are able to survive and adapt in extreme environments. PsTp172 isolate is the most tolerant bacteria to tested metals. This metal resistance could be maintained by active efflux, uptake reduction, sequestration, detoxification and binding

proteins synthesis (Zhuang *et al*., 2007). In previous investigation, it has been demonstrated that PsTp171 strain was able to bind Zn and to tolerate high concentration of the metal. The result shows that pyoverdins may have the capacity to complex zinc instead of iron. This strain might be able to sequester one of essential element and make it unattainable to phytopathogens (competition phenomenon) (Mehri *et al*., 2012).

In addition to these traits, PGPB have to be competent in the rhizospheric soil (Sajani and Muthukkaruppan, 2011). So, growth inhibition of pathogenic and phytopathogenic bacteria by antimicrobials released mainly by PsS15, PsTp172, PsS28, PsTp171, PsS31, PsS67, PsS18, PsS39 and PsS93 reflect their rhizospheric competitiveness that can be beneficially combined with plant protection. On the other hand, the discovery of *QS* enzymes did not only offered a promising means to control bacterial infections, but also provided new challenges to study their roles in host organisms and their potential impacts on environments (Dong and Zhang, 2005). Molina *et al*. (2003) showed that *QS* signal of Erwinia carotovora and *Agrobacterium tumefaciens* can be disturbed by lactonase of *P. fluorescens* which significantly reduce respectively potato soft rot and crown gall of tomato. Our results revealed that PsS15, PsTp156, PsTp172, PsC54, PsTp171 and PsS102 isolates are capable to disturb and inhibit the *QS* signal of the 3 pathogenic and phytopathogenic bacteria. Thus, the *quorum-quenching* mechanism has emerged as novel biocontrol strategy that could substitute pesticides.

5. CONCLUSION

On the basis of biofertilizer, plant growth promoter and biocontrol capacities we propose three strains (PsTp172, PsTp171 and PsS15) as potential bioinoculants for crop plants to increase seedling emergence, vigor and yield. Selected isolates are metal tolerant and could be used in polluted soils. Further siderophore production and antagonistic activity, which helps in establishing and resisting against deleterious microorganisms, reflect their rhizospheric competitiveness and plant protection that can be beneficially combined with PGPR traits. Additionally, in vitro production of hyper-adherent and/or hyper-mucoïd PGPB inocula could be utilized to increase the crop yields by improving N2 fixation, nutrient uptake and to protect plant against soil pathogens. Moreover, the concept of biological control of plant diseases, using non-pathogenic bacteria as bio-pesticide could substitute chemical fertilizers and pesticides, since they cause an accumulation of toxic compounds in soil. Thus, the use of microorganisms as biological control agent (antimicrobial secretion or NAHL degradation) is a promising strategy to ensure effective plant protection in agricultural ecosystems.

Therefore, most effective *Pseudomonas* isolates could well be commercialized but we should not overlook the involvement of some species of *P. fluorescens*, able to grow at or above 37°C that can behave as opportunistic agent in human pathogenicity (endotoxin release). Inoculation of these species (PsS60, PsS73 and PsS91) in raw consumption plants (carrots, cabbage) can cause severe effects in patients with weakened immune systems (Chapalain *et al*., 2008).

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