Evaluation of Biological Control Traits in Some Isolates of Fluorescent Pseudomonads and *flavobacterium*

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Abstract

Plant growth promoting rhizobacteria (PGPR) consisting a wide range of beneficial soil bacteria inhabiting rhizosphere of plant. Fluorescent pseudomondas are the most important plant growth promoting rhizosphere bacteria in different crop plants. In this research, twenty-five isolates of fluorescent pseudomonads isolated from forty soil samples of wheat rhizosphere and forty four isolates of *Flavobacterium* selected from the bacterial strains collection were employed. The ability of these isolates for production of chitinase, Salicylic acid, siderophore and hydrogen cyanide and antifungal activity against *Rhizoctonia solani* were determined. Results revealed that all the fluorescent pseudomonads isolates were able to produce siderophore on Chrome Azurol S (CAS) agar plates and siderophore is an important secondary metabolite in plant growth promotion and antifungal activity. In CAS agar method, the range of siderophore production was 0.34-1.21 halo diameter per colony diameter. None of these isolates were capable for chitinase production. Salicylic acid (SA) production by the isolates ranging from 0-10.91 $g.ml^{-1}$ (average 2.48 $g.ml^{-1}$). Bacterial ability for production of hydrogen cyanide had a great variation and based on this ability they were qualitatively classified in to high, relatively high, moderate and no production. Productions of studied metabolites were not detected in *Flavobacterium* isolates. Three isolates (PA24, PA1 and PA18) of fluorescent pseudomonads showed in vitro antifungal activity against *Rhizoctonia solani*. In contrast none of *Flavobacterium* isolates showed growth inhibition against the tested fungus. Results of this study showed that fluorescent pseudomonads which are native to soils of Iran had the

potential to be used for promotion of plant growth and suppression of soil-borne plant pathogens. In contrast, *Flavobacterium* seem to be an inefficient rhizobacterium against plant pathogens because of incapability to production of some important secondary metabolites in plant growth promotion and antifungal activity. There is a need to study their effects on different agricultural crops.

Keywords: Chitinase, *Flavobacterium*, Fluorescent pseudomonads, Hydrogen cyanide, *Rhizoctonia solani*, Salicylic acid and siderophore

1. Introduction

The bacteria that exert some beneficial effects on plant growth are two general types, those that form a symbiotic relationship with the plant and those that are free-living in the soil. Beneficial free-living soil bacteria are usually referred to as plant growth-promoting rhizobacteria or PGPR (Kloepper *et al*., 1989). These bacteria can stimulate plant growth either directly or indirectly (Glick, 1995). PGPRs increase plant growth indirectly either by the suppression of well-known diseases caused by major pathogens or by reducing the deleterious effects of miner pathogens (Whipps, 2001; Someya, 2008). Antibiotic synthesis (Kaaijmakers and Weller, 2001), siderophore production (Alexander and Zuberer, 1991), extra cellular enzymes such as, chitinase, β-1,3glucanase production (Nagarajkumar *et al*., 2004) and Competition for nutrients and space in rhizosphere are some indirect mechanisms used by bacteria (Schippers *et al*., 1990). The direct stimulation of plant development by PGPRs includes either providing the plant with a compound such as, Phytohormones (Glick, 1995), ACC-deaminase enzyme (Glick *et al*., 1994), that is synthesized by the bacteria or simplifying the uptake of certain nutrients such as phosphorus from the soil (Raju and Reddy, 1999). PGPRs include a number of different soil bacteria such as, *Pseudomonas, Azospirillum, Burkholderia, Enterobacter, Azotobacter, Serratia, Alcaligenes, Arthrobacter, Acinetobacter, Flavobacterium* and *Bacillus* species (Glick, 1995; Rodriguez and Fraga, 1999; Sturz and Nowak, 2000; Bloemberg and Lugtenberg, 2001). Bacteria in the genera of fluorescent pseudomonds have captured special attention over the last ten years for their beneficial and deleterious effects on plant development. Mechanisms of biological control of plant pathogens by fluorescent pseudomonads generally involve production of bacterial metabolites such as siderophores, hydrogen cyanide, extra cellular lytic enzymes and induced systemic resistance (O,Sullivan and O,Gara, 1992). Furthermore, *Flavobacterium* is an efficient rhizosphere genus in promotion of plant growth (Asghar *et al*., 2004). Nevertheless, to our knowledge, no report has been published on biological control ability of this genus. This study was carried out to evaluate the potential for production of chitinase, salicylic acid, hydrogen cynanide and siderophore by isolates of fluorescent pseudomonds and *Flavobacterium*. Also antifungal activity of these isolates was investigated against Rhizoctonia solani as a major soil-borne pathogen.

2. Material and Methods

2.1 Bacteria Isolates

Fluorescent Pseudomonds were isolated from the forty soil samples of wheat rhizosphere collected from different location of Iran with King's medium B (king *et al*.,1954) as described by vidhyasekaran *et al*.(1997). The isolates were identified as species of fluorescent pseudomonads according to Bergey,s Manual of Systematic Bacteriology (Krieg and Holt, 1984). Forty for isolates of *Flavobacterium* were selected from biological research section bacterial collection of soil and water research institute, Tehran, Iran.

2.2 Production of siderophore

Siderophore production by strains was detected as described by Alexander and Zuberer (1991). CAS agar was prepared from four solutions which were sterilized separately before mixing. The Fe-CAS indicator solution (solution 1) was prepared by mixing 10 ml of 1mM $FeCl_3.6H_2O$ [in 10mM HCl] with 50 ml of an aqueous solution of CAS (1.21 $mg.ml^{-1}$). The resulting dark purple mixture was added slowly, with constant stirring, to 40 ml of an aqueous solution of HDTMA (1.82 $mg.ml^{-1}$). This yielded a dark blue solution which was autoclaved, then cooled to 50°C.

The buffer solution (solution 2) was prepared by dissolving 30.24g of PIPES in 750 ml of a salt solution containing 0.3g KH_2PO_4, 0.5g NaCl and 1.0g NH_4Cl. The pH was adjusted to 6.8 with 50% KOH and water was added to bring the volume to 800 ml. The solution was autoclaved after adding 15g of agar, and then cooled to 50°C. Solution 3 contained the following (in 70 ml water): 2g glucose, 2g mannitol, 493 mg $MgSO_4.7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4.H_2O$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4.5H_2O$, 1.2 mg $ZnSO_4.7H_2O$ and 1.0 mg $Na_2MoO_4.2H_2O$. Solution 3 was autoclaved, cooled to 50°C. Then added to the buffer solution along with 30 ml filter-sterilized 10% ($W.V^{-1}$) Casamino acids (solution 4). The indicator solution was added last, with sufficient stirring to mix the ingredients without forming bubbles. Media was poured into sterilized plates after solidification of media a loop full of each strain which was cultured in rich medium (TSB) was placed on the plates (three per plate), and the plates were incubated at 27°C for three days. Change in the dye color from blue

to orange indicated production of siderophore. The ratio of halo diameter per colony diameter was daily evaluated.

2.3 Production of Hydrogen Cyanide

Hydrogen cyanide (HCN) production from glycine was tested by growing the bacteria in 10% tryptic soy agar (TSA) supplemented with glycine (4.4 $g.L^{-1}$) and cyanogenesis was revealed using picric acid and Na_2CO_3 (0.5 and 2%, respectively) impregnated filter paper fixed to the underside of the Petri dish lids. Results were read after 5 days of culture at 28°C. A change in filter paper color from yellow to orange-brown indicated cyanide production (yellow (1): no cyanide production, orange (2): moderate cyanide production, light brown (3): relatively high cyanide production and brown (4): high cyanide production), (Donate-Correa *et al*., 2004).

2.4 Production of Chitinase

A 20 g sample of crab-shell chitin was dissolved in cold concentrated HCl (350 ml) and placed at 4°C for 24 h. The mixture was filtered through glass-wool in to 2 l ethanol at -20°C with rapid stirring. The resulting chitin suspension was centrifuged at 10000 g for 20 min; chitin pellets were washed repeatedly with water until the pH was neutral, and the washed chitin was lyophilized to dryness and stored at -20°C. To prepare chitin plate, chitin was resuspended in water (1 $mg.ml^{-1}$) by passing the suspension over 5 min through a hand-operated cream homogenizer. The chitin suspension was diluted with an equal volume of 1.6% ($w.v^{-1}$) bacto nutrient broth, agar was added to 2% ($w.v^{-1}$), the suspension autoclaved, and 10ml volumes of agar poured into 100 ml Petri plates. After solidification of media a loop full of each strain which was cultured in rich medium (TSB) was placed on the plates (three per plate), and the plates were incubated at 30°C for 96 h. Chitin hydrolysis was indicated by zones of clearing around the colonies (Roberts and Cabib, 1982)

2.5 Production of salicylic acid (SA)

Strains were grown at 28°C for 48 h on a rotary shaker(120 rpm) in 100 ml flasks containing 50 ml succinate medium (succinic acid, 4.0g; K_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4.7H_2O$, 0.2 g; distilled water, 1000ml; pH 7.0). Cells were then collected by centrifugation at 6000g for 5min and 4ml of cell free culture was acidified with 1 N HCl to pH 2.0 and SA was extracted in CHCl3 (2×2 ml). To the pooled $CHCl_3$ phases, 4ml of distilled water and 5 l of 2M $FeCl_3$ were added. The absorbance of the purple iron-SA complex, which was developed in the aqueous phase, was read at 527 nm in a spectrophotometer. A standard curve was prepared with SA dissolved in succinate medium. The quantity of SA in the culture was expressed as gml^{-1} (Meyer *et al*., 1992).

2.6 Dual culture inhibition assays

Tests were performed in King's medium B agar. Bacterial antagonists were spotted at the sides of the Petri dish (10 µl of an overnight culture in succinate medium from a single colony) and preincubated at 27ºC for 2 day. *R.solani* isolates was then inoculated at center of the bacterial growth in three replicate plates and incubated at 27°C. Inhibition of fungal growth was assessed 3 day later by measuring the size of the inhibition zone (in mm).

2.7 Data analysis

Data were analyzed for significance by analysis of variance, followed by Duncan multiple range test (α= 0.01), with SAS software (SAS institute, Cary, NC). Normal distribution and homogeneity of variances were checked beforehand. Completely randomized design with four replications was used in all experiments.

3. Results and Discussion

In this study 25 isolates of fluorescent pseudomonds (PA1-PA25) were isolated from the wheat rhizosphere collected from different locations of Iran. Production of siderophore, Salicylic acid, chitinase and cyanide hydrogen were evaluated in these isolates and Forty for isolates of *Flavobacterium*. The results are shown in table (1, 2). Many *Pseudomonas fluorescens* strains are known to secrete fluorescent, yellow-green, water-soluble siderophores under iron-limiting conditions (O, Sullivan and O, Gara, 1992). Belimov *et al*. (2005) reported that AY197010, AY197006 and AY197009 had the ability to produce siderophores. In this study the results of siderophore production of the fluorescent pseudomonads isolates showed that all of the isolates were able to produce siderophores in form of an orange to dark yellow halo surrounding their colonies in CAS- agar medium. This halo had a clear edge for most of the bacteria. The assay of siderophore production was down by measuring the ratio of halo diameter to colony diameter. Proportion of halo diameter to the colony diameter (the average of three day) of the isolates are between 0.34- 1.21 (table 2). Analysis of variance and means comparison showed that there was a significant difference among the isolates. Production of siderophore in isolate P24 was significantly more than others. Rasouli *et al*. (2005) reported that 201 indigenous *pseudomonas* spp. Isolated from Iranian soils were sidrophore producers in CAS-agar medium.

Salicylic acid produced by PGPR in the rhizosphere can play an important role in Induced Systemic Resistance

(ISR). Salicylic acid production by WCS374, WCS4172 (Leeman *et al*., 1996), CHA0 Strains of *P.fluorescens* (Maurhofer *et al*., 1994) and also 7NSK2 Strain of *P.aeruginosa* (Demeyer and Hofte, 1997) has been reported. NagaraJkumar *et al*. (2004) reported that 14 strains of *Pseudomonas* could produce salicylic acid and its amount varied from 2 $mg.ml^{-1}$ to 16 $mg.ml^{-1}$. In our study 11 isolates of studied fluorescent pseudomonads produced different amounts of SA from 0.31 to 10.91 $mgml^{-1}$ in PA23 and PA22 respectively (table 1). Analysis of variance and means comparison showed that there was significant difference among the isolates (table 1). Production of SA by isolate PA22 was significantly more than the others.

A wide range of bacteria have been reported to have chitinase enzyme. The studies done by different researchers showed that *P. fluorescens* could produce this enzyme (Nagarajikuma *et al*., 2004; Ajit *et al*., 2006; Saikiar *et al*., 2005). Lack of this ability has also been reported for some bacteria. O'Brien *et al*. (1987) reported that the *P.putida* and *P.aerugiuosa* in their study did not produce chitinase. In other study Cattelan *et al*. (1999) showed that GW2103 and LC1118 isolates of *Flavobacterium indologenes* did not produce chitinase. The results of our study also showed that all of the tested isolates of fluorescent Pseudomonds could not produce chitinase.

Hydrogen cyanide production by some bacteria has been reported (Lork, 1984). Researchers showed that some strains of *P.fluorescens*, *P.aeroginosa* and *Chromobacterium violaceum* can produce hydrogen cyanide (Siddiqui *et al*., 2003). Anton *et al*. (1998) introduced some Rhizobial strains as hydrogen cyanide producer. Fluorescent pseudomonads isolates of our study were able to produce of hydrogen cyanide in different ranges. production of hydrogen cyanide by isolates PA25, PA18, PA14, PA11 were at high level(4), by PA2, PA5, PA8, PA19 at relatively high level(3), by PA10, PA20, PA23 at moderate level(2), and other isolates did not produce hydrogen cyanide (1) (Table 1).

Investigation of siderophore, HCN, SA and chitinase production in *Flavobacterium* isolates exhibited that none of these isolates had ability to produce of these metabolites.

Antifungal activity:

Strains PA24, PA1 and PA18 with 35, 19 and 13 mm had larger inhibition zone than other strains (table 1). The PA24 and PA1 strains showed significant siderophore production, this may suggest that their antagonistic effects on *R. solani* may be attributed mainly to the production of this metabolite. These results were similar to Ahmad *et al*. (2007) and Leong-Xian *et al*. (2005). As it expected none of *Flavobacterium* isolates exhibit antifungal activity against *R. solani* that it maybe due to inability of this isolates in production antifungal Metabolites.

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Table 1. Chitinase, salicylic acid, hydrogen cynanide production and fungal growth inhibition by isolates of fluorescent pseudomonads

isolates	Hydrogen cyanide❋	Chitinase enzyme	Salicylic acid	fungal growth inhibition
			(µg.mL)	(mm)
PA1	1	–	0 L	19b
PA2	3	–	0 L	0 h
PA3	1	–	3.22 E	0 h
PA4	1	–	0 L	0 h
PA5	3	–	*	0 h
PA6	1	–	1.64 H	3g
PA7	1	–	1.37 I	0 h
PA8	3	–	*	0 h
PA9	1	–	0 L	0 h
PA10	2	–	0.84 J	4f
PA11	4	–	0 L	0 h
PA12	1	–	0 L	3g
PA13	1	–	*	0 h
PA14	4	–	9.05 B	4f
PA15	1	–	4.02 D	6d
PA16	1	–	*	0 h
PA17	1	–	0 L	4f
PA18	4	–	8.52 C	13c
PA19	3	–	*	0 h
PA20	2	–	2.69 F	0 h
PA21	1	–	2.16 G	5e
PA22	1	–	10.91 A	0 h
PA23	2	–	0.31 K	6d
PA24	1	–	*	35a
PA25	4	–	*	0 h
Range	1–4	–	0-10.91	0-35
Average		–	2.49	

Values followed by the same letters are not significantly different at p= 0.01 Duncan multiple range test

× No growth -No enzyme production

❋HCN scale:	1	2	3	4
Indicator color:	yellow	orange	light brown	brown
HCN production:	–	moderate	relatively high	high

Values followed by the same letters are not significantly different

Table 2. Siderophore production by isolates of fluorescent pseudomonads in CAS agar medium

Isolates	First day	Second day	Third day	Average of three days
	Ratio of halo diameter to colony diameter	Ratio of halo diameter to colony diameter	Ratio of halo diameter to colony diameter	Ratio of halo diameter to colony diameter
PA1	1.05BCD	1.00B	0.88BC	0.97BC
PA2	0.48HI	0.53GH	0.40HIJ	0.47HIJK
PA3	1.18AB	0.93BCD	0.79BCD	0.96BC
PA4	0.34I	0.32H	0.36IJ	0.34K
PA5	0.88CDEF	0.56GH	0.48GHIJ	0.64FGHIJ
PA6	1.31A	0.99BC	0.88BC	1.06AB
PA7	0.68FGH	0.50GH	0.48GHIJ	0.56GHIJ
PA8	0.87CDEF	0.72CDEFG	0.59DEFGH	0.73DEFG
PA9	0.67FGH	0.33H	0.40HIJ	0.46IJK
PA10	0.89CDEF	0.57FGH	0.70BCDEFG	0.72DEFG
PA11	0.78EF	0.66DEFG	0.59DEFGH	0.68EFG
PA12	1.10BC	0.86BCDE	0.71BCDEF	0.89BCDE
PA13	0.99BCDE	0.85BCDE	0.83BC	0.89BCDE
PA14	1.14AB	0.87BCDE	0.75BCDE	0.92BCD
PA15	0.81EF	0.88BCDE	0.66CDEFG	0.78CDEF
PA16	0.82CEF	0.57FGH	0.61DEFGH	0.67FGHI
PA17	0.55GHI	0.51GH	0.32J	0.46JK
PA18	0.96BCDE	0.75BCDEFG	0.55EFGHI	0.76DEFG
PA19	0.81EF	0.71DEFG	0.51FGHIJ	0.68FGH
PA20	0.89CDEF	0.64EFG	0.61DEFGH	0.71DEFG
PA21	1.13AB	0.85BCDE	0.90B	0.96BC
PA22	0.86DEF	0.65EFG	0.56EFGHI	0.69EFG
PA23	0.52HI	0.72CDEFG	0.51FGHIJ	0.58FGHIJ
PA24	1.14AB	1.26A	1.23A	1.21A
PA25	0.75EFG	0.84BCDEF	0.55EFGHI	0.71DEFG

Values followed by the same letters are not significantly different at p=0.01 Duncan multiple range test

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