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Antagonistic Potential of Fluorescent *Pseudomonads* and Control of Crown and Root Rot of Cucumber Caused by *Phytophthora drechsleri*

Akbar Shirzad^{1*}, Vahid Fallahzadeh-Mamaghani² and Maghsoud Pazhouhandeh¹

¹Department of Plant Protection, Azarbaijan University of Tarbiat Moallem, Tabriz, Iran

²Department of Plant Protection, Tehran University, Karaj, Iran

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In this study, 200 isolates of fluorescent pseudomonads were isolated from different fields of East and West Azarbaijan and Ardebil provinces of Iran. These bacterial isolates were screened on the basis of a dual culture assay, the presence of known antibiotic genes, and their ability to successfully colonize roots and to promote plant growth. Twelve isolates exhibited 30% or more inhibition of mycelia growth of *P. drechsleri*. Genes encoding production of the antibiotics 2,4-diacetylphloroglucinol, phenazine-1-carboxylic acid, and pyoluteorin were detected in some strains but none of the strains possessed the coding gene for production of antibiotic pyrrolnitrin. In an *in vitro* test for root colonization, the population density on roots of plants treated with most of the above strains was more than $6 \log_{10}$ CFU g⁻¹ roots, with a maximum of $7.99 \log_{10}$ CFU g⁻¹ roots for strain 58A. Most of the strains promoted significant plant growth in comparison to non-treated controls. In green house studies, the percentage of healthy plants in pots treated with strains 58A and 8B was 90.8% and 88.7%, respectively. The difference between these treatments and treatment with the fungicide metalaxyl was not significant.

Keywords : biocontrol, 2,4-DAPG(2,4-diaetylphloroglucinol), *Phytophthora*, *Pseudomonas*, cucumber

Phytophthora drechsleri, which provokes root and crown rot of cucumber, is one of the most destructive pathogens of cucumber in Iran and other parts of the world (Erwin and Ribeiro, 1996; McGrath, 2001; Ristaino and Johnston, 1999). *Phytophthora capsici* is usually reported as the principal cause of this disease (Babadoost and Islam, 2003), although *P. drechsleri* has been described as a major agent in Iran (Alavi and Strange, 1979). These pathogens cause damping-off, crown and root rot, stem lesions, foliar blight, and fruit rot on cucumber (Kreutzer et al., 1940; McGrath,

2001; Alavi and Strange, 1982). Cucumber varieties resistant to *Phytophthora drechsleri* disease are not available to our knowledge. Consequently, this disease is very difficult to combat and, in particular, no single method is available that can provide adequate control on vegetables (Hwang and Kim, 1995; Ristaino and Johnston, 1999). Moreover, resistance to systemic fungicides develops rapidly in *Phytophthora drechsleri* after their application in the field (Lamour and Hausbeck, 2000; Lamour and Hausbeck, 2001).

Biocontrol of soil-borne phytopathogens by antagonistic rhizobacteria offers a nonpolluting complement and alternative to existing disease management strategies. Plant growth-promoting rhizobacteria (PGPR) can competitively colonize plant roots and stimulate plant growth and/or decrease the incidence of plant diseases. These bacteria can employ a number of different mechanisms to suppress plant diseases, including production of antibiotics (Liu et al. 2007), induction of systemic resistance (Fallahzadeh et al., 2009), and production of powerful siderophores (Buysens et al., 1996). Thus, they can be exploited in various contexts as biologic pesticides to decrease the use of chemical pesticides in agriculture.

Some *Pseudomonas* spp., notably fluorescent pseudomonads, are particularly suitable as agricultural biocontrol agents because they can produce large amounts of secondary metabolites that can protect plants from phytopathogens and stimulate plant growth (Liu et al., 2007). Production of antibiotics such as phenazine-1-carboxylic acid (PCA), pyocyanin, pyrrolnitrin, pyoluteorin, HCN and 2,4-diacetylphloroglucinol (2,4-DAPG) in different species of pseudomonads has been reported (Sunish Kumar et al., 2005). The control of cucumber *Phytophthora* root and crown rot by some isolates of *Trichoderma harzianum* (Nazari, 1991; Khan et al., 2004), and *Bacillus* spp. (Khateri, 2002) has been also demonstrated. The control of soybean phytophthora root rot (Lifshitz et al., 1987), Bean Damping-Off caused by *Rhizoctonia solani* (Ahmadzadeh and Sharifi-Tehrani, 2009), and *Sclerotinia* wilt of sunflower (Moeinzadeh et al., 2010), and of ornamental plants (Yuen and Schorth,

*Corresponding author.

Phone) +98-412-4327500, FAX) +98-412-4327541

E-mail) ashirzad@azaruniv.edu

1986) has been reported with selected isolates of these bacteria. There is, however, very limited knowledge concerning the biological suppression of *Phytophthora* root and crown rot in cucumber by the fluorescent pseudomonads and the goal of this study was to screen for pseudomonad isolates for suitable candidates for biocontrol.

Material and Methods

Field sampling and isolation of fluorescent pseudomonad isolates. In June 2010, a total of 80 soil and plant samples were collected from different fields of the East and West Azarbaijan and Ardebil provinces of Iran. Roots were gently removed from soil and placed in plastic bags before they were transported to the laboratory. Adhering soil was carefully brushed off. Then, 10 gram of roots or soil samples were placed in 100 ml of 0.14 M sodium chloride solution supplemented with 0.01% Tween 20 and the samples were shaken for 30 min at 200 rpm followed by serial dilution of the liquid phase. Then, 100 µl of the first to forth diluents were transferred to modified King's medium B (KMB) agar and KMB⁺⁺⁺ [20 g of proteose peptone per liter, 1.5 g of KH₂PO₄, 1.5 g of MgSO₄·7H₂O, 10 ml of glycerol, and 12 g of agar supplemented with ampicillin (40 µg/ml), chloramphenicol (13 µg/ml), and cycloheximide (200 µg/ml) (Sigma Chemical Co., St. Louis, Mo.)]. These growth conditions have been shown to be selective for fluorescent *Pseudomonas* spp. (Simon and Ridge, 1974). Fluorescent colonies that developed on these plates were isolated.

In vitro antagonistic activity. *In vitro* inhibition of mycelial growth of *P. drechsleri* by non-volatile compounds of the bacterial isolates was tested by using the dual culture technique as described by Ahmed Idris et al. (2007). Three 10 µl drops from a 10⁸ cfu/ml suspension of the bacterial isolate were equidistantly placed on the margins of potato dextrose agar (PDA) plates and incubated at 28 °C for 24 h. A 6 mm agar disc from a fresh culture of *P. drechsleri* was placed at the centre of the PDA plate for each bacterial isolate and incubated at 27 ± 1 °C for seven days. The radii of the fungal colony toward and away from the bacterial colony were measured. The percentage of growth inhibition was calculated using the following formula:

$$\% \text{Inhibition} = \left[\frac{(R - r)}{R} \right] \times 100$$

Where, *r* is the radius of the fungal colony opposite the bacterial colony and, *R* is the maximum radius of the fungal colony distal from the bacterial colony.

Screening for known antibiotic coding genes. The

isolates which showed antagonistic activity in the dual culture assays were tested for the presence of genes encoding certain antibiotics. Colonies growing on King B agar (KB) medium were suspended in lysis solution [50 mM KCl, 0.1% Tween 20, 10 mM Tris-HCl (pH 8.3)] in 250 µl tubes to an optical density at 600 nm (OD₆₀₀) of 0.15 to 0.45. Then suspensions were centrifuged at 5000 rpm for 1 min and incubated for 10 min at 99 °C. The heat-lysed bacterial suspensions were frozen (−20 °C) during 30 min. After thawing, 4 µl volumes of supernatant were carefully taken and used for PCR (McSpadden Gardener et al., 2000; Wang et al., 2001). The initial denaturation (2 min at 94 °C) was followed by 34 PCR cycles (94 °C for 30 s, 62 °C for 30 s and 72 °C for 60 s) and a final extension at 72 °C for 10 min. Primer pairs specific for the selected antibiotic-encoding genes were as follows: PCA2a (5'-TGCCAAGCCTCGCTCCAAC-3') and PCA3b (5'-CGCGTTGTTCTCGTTCAT-3') for phenazine-1-carboxylic acid (Raaijmakers et al., 1997); B2BF (5'-ACCCACCGCAGCATCGTTTATGAGC-3') BPR4 (5'-CCGCCGGTATGG AAGATGAAAAAGTC-3') for 2,4-diacetylphloroglucinol (McSpadden Gardener et al., 2001); PltBf (5'-CGGAGCATGGACCCCCAGC-3') and PltBr (5'-GTGCCCGGATA TTGGTCTTGACCGAG-3') for pyoluteorin and Prn cf; and (5'-CCACAAGCCCGGCCAGGAGC-3') and Prn cr (5'-GAGAAGAGCGGGTCGATGAAGCC-3') for pyrrolnitrin (Mavrodi et al., 2001).

Identification and characterization of antagonistic bacteria. Isolates were identified on the basis of tests for cytochrome oxidase (Kovacs, 1956), arginine dihydrolase (Thornley, 1960), gelatin liquefaction (Dye, 1968), tobacco HR (Klement, 1963), nitrate reduction, growth at 4 and 41 °C, levan production, (Shaad et al., 2001) and the ability to growth on carbon sources such as L-arabinose, D-galactose, trehalose, meso-inositol, sorbitol, and L-tartrate in the basal medium of Ayers et al. (1919).

In vitro root colonization. Isolates displaying high antagonistic activity in the dual culture assays and that carried antibiotic-encoding genes were tested for their ability to colonize cucumber roots *in vitro* using the method described by Ahmed Idris et al. (2007). Cucumber seeds (Soltan cultivar) were surface-sterilized with 5% sodium hypochlorite for 3 min, then with 70% ethanol for 1 min before rinsing three times in sterile distilled water. For each treatment, 15 seeds were transferred to a sterile moist chamber, i.e. a disc of filter paper placed in a 90 mm diameter plastic Petri dish and moistened with sterile distilled water. For inoculation, antagonistic isolates were grown on Nutrient Agar (NA) plates for 30 h at 26 °C. Bacteria were harvested by scraping cells from the agar,

suspending them in 0.1 M phosphate buffer (pH 7), and washing the suspensions twice by centrifugation (10 min at 6,000 rpm). Washed pellets were suspended in 1% methylcellulose solution and the optical density of these suspensions adjusted to 0.1 at OD₆₀₀ (10⁷ cell/ml). A 100 µl aliquot of each inoculum was added to the seeds in the moist chambers and the plates were incubated at 27°C for a week in the dark for root development. 1% methylcellulose solution was added to control seeds. After seven days, one gram of roots (from different parts of roots) from each treatment was aseptically excised, one seed per treatment, and transferred to 0.1 M MgSO₄ solution, shaken and serially diluted. From each dilution, a 0.1 ml aliquot was plated on KMB⁺⁺⁺ and the plates were incubated at 27°C for colony counts. The number of bacteria colonizing the root was calculated as colony-forming units/g root (CFU/g root).

In vitro plant growth promotion. Surface sterilized cucumber seeds were pregerminated in moist chambers at 27°C for 36 h. Successful sterilization of seeds was tested by placing germinated seeds on nutrient agar. The bacterial inoculum was prepared as described above. Then, two pregerminated cucumber seeds were placed in glass tubes (20 cm height and 1.5 cm diameter) filled with 0.8% sterile water agar (Merck) and the seeds were inoculated with 100 µl of bacterial suspension (containing 10⁶ cells ml⁻¹). Two weeks after incubation (27°C, 16h/8h brightness/darkness) in a phytochamber, fresh and dry weight of foliage and roots were measured to determine the effects of bacterial treatment on plant growth. Each isolate was tested in four replicates.

Preparation of pathogen inoculum and greenhouse evaluation of selected isolates. *P. drechsleri* was obtained from the Iranian Research Institute of Plant Protection, Evin, Tehran. For inoculum preparation, 100 g of white bean seed were added to 200 ml of water in a 500-ml Erlenmeyer flask and then autoclaved for 20 min at 121°C on two successive days. The flask was inoculated with a mycelial plug from an 8 day-old culture of *P. drechsleri*, incubated two weeks at 25°C with daily shaking to break up particles. This inoculum was air dried, ground in a mill and used for greenhouse studies.

The ability of bacterial isolates to suppress *Phytophthora drechsleri* damping-off of cucumber was examined on *Cucumis sativus* L. Super Dominus cultivar. Seven surface-sterilized seeds were seeded in polyethylene pots (8 cm in diameter) filled with a 1:1:2 mixture of peat, sand and field soil. The pots were transferred to the greenhouse (24–28°C, 16/8 h day/night) and watered at three-day intervals. Ten days after seeding, the pots were topped up with 30 g of the

potting mix that had been blended with 1 g of white bean seed inoculum of *P. drechsleri* and drenched with 20 ml of bacterial suspension (10⁸ cell⁻¹) or a solution of 1 g L⁻¹ Ridomil MZ-72 WP (8% metalaxyl + 64% mancozeb). Then the pots were watered, arranged in a randomized complete block design and incubated as described above. The various treatments in this experiment were as follows: inoculation with *P. drechsleri* and different strains of the bacteria; inoculation with *P. drechsleri* and Ridomil; inoculation with *P. drechsleri* but no bacteria (CI); and controls without inoculation (C). After 3 weeks, the percentage of healthy plants was determined and plants were removed from pots for measuring fresh weight of foliage and roots. There were four pots for each treatment and seven plants for each pot.

Quantification of 2,4-DAPG production. Production of DAPG was quantified by using an analytical high performance liquid chromatography assay (HPLC) as described by (Duffy and Defago, 1997). The rhizobacterial isolates were grown in 15 ml of yeast-malt extract (YME) medium (yeast extract, 3 g; malt extract, 3 g; Bacto Peptone, 5 g; glucose, 10 g) for 72 h at 27°C. Liquid cultures of 15 ml were acidified to pH 2 with 200 µl of 5 N HCl and extracted with 15 ml of ethyl acetate for 30 min with vigorous shaking. Phase separation was accelerated by 10 min of centrifugation at 6000 rpm. The organic phase was transferred to a round-bottom glass flask and flash-evaporated, and the residue was dissolved in 1 ml of HPLC-grade methanol. Compounds were separated on a 250 mm × 4.6 mm ID, 5-µm particle, Perfectsil Target ODS-3 column (MZ-Analysentechnik, Germany) with a ODS-3 precolumn (10 × 4.0 mm I.D., 5-µm), which was maintained at ambient temperature. The isocratic mobile phase consisted of acetonitrile-phosphate buffer (pH 5.0) in the ratio of 50:50 v/v, flowing through the column at a constant flow rate of 1 ml min⁻¹. The eluent was monitored using UV detection.

Results

Isolation of fluorescent pseudomonads and in vitro antagonistic activity. Out of the about approximately 200 isolates tested, 12 isolates exhibited more than 30% inhibition of mycelia growth of *P. drechsleri* (Table 2 and 1). The maximum inhibition achieved by any isolate was 71.42% (Isolate 8B). Control plates which had not been treated with a bacterial isolate were completely covered by the phytopathogen (Table 2).

Screening for known antibiotic coding genes. Antagonistic isolates were screened for the presence of known antibiotic coding genes (Fig. 1). Primers B2BF and BPR4

Table 1. Antagonistic petential flourescent pseudomonads isolated from different fileds

Bacterial isolates	Host	Place of collection
8B	Wheat	Maragheh-Hashtroud road (Serajoo)
30B	Wheat	Sain defile
58A	Wheat	Shaheendedj
36A	Potato	Ajabshir
41A	Cucumber	Maragheh (Mardagh village)
34B	Wheat	Sarab
50A	Cucumber	Shabstar (Shendabad village)
3B	Wheat	Maragheh-Hashtroud road
60B	Barley	Azarshahr (Gheshlagh village)
63C	Tomato	Azarshahr (Gheshlagh village)
23A	Chickpea	Mamaghan
69B	Squash	Azarshahr (Gheshlagh village)
85C	Tomato	Orumia (Tümtar)
23C	Cucumber	Mamaghan
82B	Bean	Orumia
19C	Cucumber	Mamaghan
78B	Potato	Orumia
6C	Squash	Basminj
26C	Wheat	Duzdizan
85B	Cucumber	Orumia
41B	Cucumber	Maragheh (mardagh)
39B	Wheat	Ajabshir
7A	Wheat	Hashtroud-Bostanabad road
37B	Agropiron	Maragheh
46B	Barley	Marand (Yam)
11B	Wheat	Hashtroud-Bostanabad road
43B	Wheat	Marand (Yam)
29B	Squash	Duzdizan
21B	Alfalfa	Bostanabad
64B	Onion	Govgan
62A	Sunflower	Govgan
14A	Alfalfa	Maragheh
61B	Alfalfa	Azarshahr (afgham)
29A	Barley	Sarab
17A	Cucumber	Basmenj
59C	Wheat	Shaheendedj
47A	Wheat	Marand (Yam)

amplified the predicted 629-bp portion of the *phlD* gene from the reference strain CHA0 and strains 58A, 41A, and 50A (McSpadden Gardener et al., 2001). Primers PCA2a and PCA3b amplified the predicted 1150-bp fragment of *phz* locus from the reference strain 2-79 and strains 30B, 36A, 34B, 60B and 8B (Raaijmakers et al., 1997). Primers PltBf2 and PltBr amplified the predicted 773-bp fragment of *pltB* (Mavrodi et al., 2001) from the DNA of reference strain CHA0 and strains 8B and 3B. Primers PrnCf and

Fig. 1. Polymerase chain reaction (PCR) tests for screening of genes involved in antibiotic production. Amplification of 1150-bp fragment of *phz* locus from the reference strain 2-79 and strains 30B, 36A, 34B, 60B and 8B (A and B), 629-bp portion of *phlD* gene from the reference strain CHA0 and strains 58A, 41A, and 50A (C and D) and 773-bp fragment of *pltB* from the DNA of reference strain CHA0 and strains 8B and 3B (E).

PrnC_r amplified the predicted 719-bp fragment of *prnC* from the reference strain CHA0 but no PCR product was amplified from any of the newly isolated bacterial strains.

Identification and characterization of antagonistic bacteria. Results of physiological and biochemical tests for all selected strains for oxidase and arginine dihydrolase was positive. Results for pectinase production, growth at 41 °C and tobacco hypersensitivity for all strains was negative. With the exception of strain 58A, all strains were able to grow at 4 °C. Results of physiological and biochemical tests listed in Table 3.

In vitro root colonization and promotion of plant growth. Selected strains were tested for their ability to promote plant growth and colonize cucumber roots. Significant differences in the ability of the different strains to colonize roots were noted (Table 4). The highest population density was achieved by strain 58A, whose population attained 7.99 log₁₀ CFU g⁻¹ roots (Table 4). The population density on roots of plants treated with 8B and 41A attained 7.76 and 7.55 log₁₀ CFU g⁻¹ root, respectively. Strain 3B, on the other hand, was not an effective colonizer as its cell count was less than 5 log₁₀ CFU g⁻¹ root.

Most of the strains tested promoted significant plant growth in comparison to non-treated control. Strains 58A and 8B were most effective in promoting fresh and dry weight of foliage and roots (Table 4). Growth parameters of

Table 2. Mycelial growth inhibition of *Phytophthora drechsleri* by metabolites produced by selected bacterial isolates

Bacterial isolate	% mycelial inhibition ^a	Bacterial isolate	%mycelial inhibition
8B	71.42a ^b	85B	20.24jklm
30B	71.42a	41B	17.85klmn
58A	66.66ab	39B	16.66lmno
36A	65.47ab	7A	15.47lmnop
41A	61.90bc	37B	15.47lmnop
34B	55.95cd	46B	13.96lmnopq
50A	50.00de	11B	13.10lmnopq
3B	42.85ef	43B	11.90mnopq
60B	36.90fg	29B	10.71nopq
63C	35.71fgh	21B	10.71nopq
23A	34.52gh	64B	8.33opq
69B	33.93gh	62A	7.14pq
85C	28.57ghij	14A	7.34pq
23C	28.57ghij	61B	4.86q
82B	27.38hij	29A	4.66q
19C	27.38hij	17A	4.55q
78B	25.00ijk	59C	4.34q
6C	21.43jkl	47A	4.10q
26C	21.43jkl		

^a% Mycelial inhibition was calculated as $(R - r)/R \times 100$, where R is mycelial growth away from the bacterial colony (the maximum growth of the fungal mycelia) and r is mycelial growth towards the bacteria.

^bValues followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

plants treated with 3B were not significantly different from the control whereas strain 34B had a negative effect on

cucumber plants as both fresh and dry weights were significantly lower than for the control.

Greenhouse evaluation of selected isolates. Selected antagonistic strains were evaluated in a greenhouse trial and compared with plants grown in the presence of a fungicide (Ridomil) and in non-treated soil. Two weeks after inoculation of pots with the pathogen and the different bacterial strains, some plants started to display significant disease symptoms such as wilting and a watery rot on stem near the soil line, and some plants collapsed, but differences in mortality between the different treatments was not significant. As further time passed, additional plants collapsed and three weeks after inoculation of pathogen, all the plants in pots treated with isolates 3B, 34B and the infected control had collapsed (Table 5). Disease severity in pots treated with the other strains was lower, with the percentage of healthy plants in pots treated with strains 58A and 8B attaining 90.8% and 88.7%, respectively. The difference in plant mortality between the plants treated with isolates 58A and 8B and the metalaxyl-treated plants was not significant. Moreover, the fresh and dry weights of plants treated with these strains and those treated with fungicide was not significantly different from untreated control plants. Another strain that performed well was 30B, where the percentage of healthy plants in pots treated with this strain was 78.28%.

Quantification of 2,4-DAPG production. The five strains displaying the best antagonistic activity against *P. drechsleri* in the greenhouse were evaluated for production

Table 3. Physiological and biochemical tests for identification of selected isolates

Tests	34B	3B	36A	60B	50A	41A	30B	8B	58A
Levan	+	+	–	+	–	–	–	+	–
Oxidase	+	+	+	+	+	+	+	+	+
Pectolytic activity	–	–	–	–	–	–	–	–	–
Arginin dihydrolase	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	–	+	+	+	–	+
Gelatin liquefaction	+	+	–	+	+	+	+	+	–
Tobacco HR	–	–	–	–	–	–	–	–	–
Growth @ 41 °C	–	–	–	–	–	–	–	–	–
Growth @ 4 °C	+	+	+	+	+	+	+	+	–
Growth on:									
D-galactose	+	+	+	–	+	+	+	+	–
Trehalose	+	+	+	+	+	+	+	+	+
Meso-inositol	+	+	+	+	+	–	–	+	–
Sorbitol	+	–	+	–	+	+	+	+	+
L-arabinose	+	–	+	–	+	+	+	–	+
L-tartrate	–	–	–	+	+	–	–	–	+

Table 4. *In vitro* growth promotion and roots colonization of cucumber plants by rhizobacterial strains

Bacterial isolate	PGPR				<i>In vitro</i> root colonization
	Foliage fresh weight	Root fresh weight	Foliage dry weight	Root dry weight	Log CFU g ⁻¹ roots
<i>P. fluorescens</i> 58A	2.520a ^z	1.683a	0.420a	0.286ab	7.99a
<i>P. fluorescens</i> 8B	2.401a	1.863a	0.406a	0.311a	7.76ab
<i>P. fluorescens</i> 30B	2.223b	1.754a	0.379b	0.292ab	7.26cd
<i>P. fluorescens</i> 41A	2.085bc	1.333b	0.322cd	0.230bc	7.55bc
<i>P. fluorescens</i> 50A	1.973c	1.102bc	0.300de	0.284cd	6.51ef
<i>P. fluorescens</i> 60B	1.966c	1.186b	0.295e	0.178cd	6.10f
<i>P. fluorescens</i> 36A	1.944c	1.193b	0.335c	0.199cd	6.91de
<i>P. fluorescens</i> 3B	1.167d	0.811d	0.158g	0.153d	4.60h
<i>P. fluorescens</i> 34B	0.853e	0.530e	0.135f	0.139e	5.67g
Control	1.183d	0.876cd	0.213f	0.146d	

^zMeans with the same letters were not significantly different at 5%, according to the Duncan's tests using the GLM procedure.

Table 5. Suppression of *Phytophthora drechsleri* root and crown rot by selected strains in greenhouse. Isolates was applied 10 days after sowing and the percentage of healthy plants was determined after 3 weeks

	Foliage fresh weight	Roots fresh weight	Percent of healthy plants
<i>P. fluorescens</i> 58A	20.113a	13.408a	90.810b
<i>P. fluorescens</i> 8B	19.763a	14.297a	88.714b
Ridomil	18.093ab	14.062a	84.714bc
<i>P. fluorescens</i> 30B	16.676b	9.927b	78.288c
<i>P. fluorescens</i> 41A	12.180c	8.120c	49.710d
<i>P. fluorescens</i> 50A	4.866d	2.703e	35.280e
<i>P. fluorescens</i> 60B	5.970d	3.739d	35.553e
<i>P. fluorescens</i> 36A	10.676c	6.536c	43.723e
<i>P. fluorescens</i> 3B	0e	0e	0f
<i>P. fluorescens</i> 34B	0e	0e	0f
Control	0e	0e	0f
Mock	19.940a	14.31a	100a

*Means with the same letters were not significantly different at 5%, according to the Duncan's tests using the GLM procedure.

of 2,4-DAPG. The highest level of antibiotic production (4.33 µg/ml) was observed in strain 58A. This amount of antibiotic was more than that observed in reference strain UTPF68 (4 µg/ml). Strain 41A produced only very low quantities of DAPG but we still consider it likely that it is a DAPG-producing strain in view of the PCR results. No DAPG antibiotic was detected in broth cultures of strains 30B and 8B and in the control.

Discussion

The aim of this study was to screen for fluorescent pseudomonads that are innocuous to cucumber plants and display antagonistic activity against the fungus *P. drechsleri* associ-

ated with cucumber damping-off. The bacterial isolates used were initially isolated from different fields of west and east Azarbaijan and Ardebil provinces of Iran. The use of modified King's medium B in the primary screen is based on an iron limitation and the presence of antibiotics (Simon and Ridge, 1974) and it proved to be a very efficient tool as about 98% of colonies that developed on this medium were fluorescent pseudomonads. An inhibition zone assay on PDA was then employed to screen for strains with high antagonistic potential. This assay had been used as a basic procedure in numerous screens for antagonistic agents (Ahmed Idris et al., 2007; Tjamos, 2004; Weller, 1985; Hagedorn et al., 1989; Ahmadzadeh and Sharifi-Tehrani, 2009). However, in some works there was no clear correlation between the results of this assay and *in situ* biological control results (Expert and Digat, 1995; Lindow, 1988; Reddy et al., 1993). There are several possible explanations for such a lack of correlation. For example, it is possible that in some strains one or more genes directing production

Fig. 2. Production of DAPG by selected isolates in YEM medium. All values were significantly different at 5%, as determined by variance analysis followed by Duncan's test. Bars indicate standard error.

Table 6. Pearson correlations between antagonistic parameters of bacteria with disease suppression and PGPR activity of antagonistic bacteria

Antagonistic parameters	Percent of healthy plants	PGPR
% mycelial inhibition	0.729 ^a	0.596 ^c
Root colonization	0.915 ^b	0.874 ^b

^a Correlation is significant at the 0.01 level.^b Correlation is significant at the 0.05 level.^c Correlation is nonsignificant

of antibiotic(s) or some other metabolite with pathogen-inhibitory properties is turned off in *in vitro* conditions, leading to loss of antagonistic activity. Our results with strain 41A may be an example of such a situation since our PCR analysis indicated that the strain contains the *phlD* gene directing DAPG synthesis, although production of the antibiotic by this strain was negligible in medium YEM (Fig. 2). Similar observations have been reported by Mavrodi et al. (2001) who showed that DAPG was not detectable by HPLC in strains like CHA0 and pf5 although these strains possess the gene directing production of this antibiotic. Nevertheless, our correlation analysis did show that there was a significant relation between *P. drechsleri* inhibition activity on PDA plates and disease suppression in the greenhouse (Table 6).

Another approach for selection of potentially antagonistic bacteria in this study was by testing for the presence of genes directing antibiotic production in the different antagonistic strains. We performed our PCR-based analysis for genes directing synthesis of four known antibiotics produced by fluorescent pseudomonads. Out of 35 strains we studied, 9 possessed such genes. Among the antibiotics produced by pseudomonads, 2,4-DAPG has received particular attention because it is synthesized by a wide range of pseudomonads used for the biological control of root diseases (Dowling and O’Gara, 1994; Keel et al., 1992). It is a phenolic compound with broad-spectrum antifungal, antibacterial, antihelminthic, and phytotoxic activity (Dowling and O’Gara, 1994; Keel et al., 1992; Thomashow and Weller, 1995). This antibiotic causes membrane damage to *Pythium* spp. and is particularly inhibitory to zoospores of this Oomycete (de Souza et al., 2003). An anti-oomycete activity has also been demonstrated for PCA (Lee et al., 2003) and pyoluteorin (Howell and Stipanovic, 1980). In one of strains we tested (strain 8B) genes encoding production of both PCA and Pyoluteorin were detected (Fig. 1). Production of two different types of antibiotics, PCA via the shikimic acid pathway (Turner and Messenger, 1986) and Plt via the polyketide pathway (Handelsman and Stabb, 1996) is rare and first reported by Hu et al. (2005). Strain 8B exhibited strong inhibitory activity against *P. drechsleri*

in *in vitro* conditions (Table 2) and it is possible that production of these two antibiotics had a synergistic effect. However, in greenhouse condition the performance of strain 8B was similar to strains 58A and 30B.

Statistical analyses indicate that there was a significant correlation between the disease-suppressive ability of strains in the greenhouse and PGPR activity with the degree of root colonization by the bacterial inoculants (Table 6). Successful root colonization by a biocontrol agent is important for two reasons: first, it is a prerequisite for successful expression of other biocontrol mechanisms, and, second, it can act as a biocontrol mechanism in its own right, since the biocontrol agent might occupy ecological niches on roots that might otherwise be colonized by pathogens (Haas and Defago, 2005). The crucial colonization level that must be reached has been estimated at 10^5 – 10^6 CFU g⁻¹ of root in the case of *Pseudomonas* spp., which protect plants from *Gaeumannomyces graminis* var. *tritici* or *Pythium* spp. Most of our selected strains were efficient colonizers of roots and CFU counts for strains 58A, 8B, 30B and 41A were more than 10^7 CFU g⁻¹ root.

Overall, the findings of this study show that some of the fluorescent pseudomonad strains that we have isolated have strong antifungal activity against *P. drechsleri*. Furthermore, most of the selected strains promoted cucumber plant growth. Our results suggest that production of antibiotics, notably DAPG and a combination of PCA and Plt, may have an important role in disease suppression by these strains. Moreover, it appears that the ability to successfully colonize roots and to promote plant growth is important factors in performance of these strains. Nevertheless, in order to determine if one or more of these promising strains can be developed into commercial inoculants, their efficiency as biocontrol agents must first be confirmed under field conditions.

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