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

Akbar Shirzad1*, Vahid Falahzadeh-Mamaghani2 and Maghsoud Pazhouhandeh1
1Department of Plant Protection, Azarbaijan University of Tarbiat Moallem, Tabriz, Iran
2Department of Plant Protection, Tehran University, Karaj, Iran
(Received on June 27, 2011; Revised on September 3, 2011; Accepted on December 12, 2011)

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 log10 CFU g−1 roots, with a maximum of 7.99 log10 CFU g−1 roots for strain 58A. Most of the strains promoted significant plant growth in comparison to non-treated controls. In greenhouse 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-diacetylphloroglucinol), 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; 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 (Falahzadeh 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,
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 10^8 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 25 ± 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:

%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 KCI, 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'-TGCCAAAGCCTCGCTCAAC-3') and PCA3b (5'-CGC GITGTTCTCCTGICTCAT-3') for phenazine-1-carboxylic acid (Raaijmakers et al., 1997); B2BF (5'-ACCCCCCGC AGATCGTTTATGAG-3') and B2BR (5'-GGTCGTTTCCTCGTTCAT-3') for 2,4-diacetylphloroglucinol (McSpadden Gardener et al., 2001); PltBr (5'-CGCGGTTATGG AAGATGAAAAAGTC-3') and PltBr (5'-GTGCCGGTATGG AAGATGAAAAAGTC-3') for pyoluteorin and PmrE; and (5'-CCACAAGCCGGCCAGGCAGG-3') and PmrE (5'-GAGAAGAGCGGGTGTGAATGACC-3') for pyrrolnitrin (Mavrodj 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 grow 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 OD_{400} (10^6 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 MgSO_4 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 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 (10^6 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 ml 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
amplified the predicted 629-bp portion of the \textit{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 \textit{phz} locus from the reference strain 2-79 and strains 30B, 36A, 34B, 60B and 8B (A and B), 629-bp portion of \textit{phlD} gene from the reference strain CHA0 and strains 58A, 41A, and 50A (C and D) and 773-bp fragment of \textit{pltB} from the DNA of reference strain CHA0 and strains 8B and 3B (E).

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
Bacterial isolates & Host & Place of collection \\
\hline
8B & Wheat & Maragheh-Hashtrood road (Serajoo) \\
30B & Wheat & Sain defile \\
58A & Wheat & Shahheedj \\
36A & Potato & Ajabshir \\
41A & Cucumber & Maragheh (Mardagh village) \\
34B & Wheat & Sarab \\
50A & Cucumber & Shahstar (Shendabad village) \\
3B & Wheat & Maragheh-Hashtrood road \\
60B & Barley & Azarshahr (Gheshlagh village) \\
63C & Tomato & Azarshahr (Gheshlagh village) \\
23A & Chickpea & Magham \\
69B & Squash & Azarshahr (Gheshlagh village) \\
85C & Tomato & Orumia (Tümtar) \\
23C & Cucumber & Magham \\
82B & Bean & Orumia \\
19C & Cucumber & Magham \\
78B & Potato & Orumia \\
6C & Squash & Basminj \\
26C & Wheat & Duzduzan \\
85B & Cucumber & Orumia \\
41B & Cucumber & Maragheh (mardagh) \\
39B & Wheat & Ajabshir \\
7A & Wheat & Hashtrood-Bostanabad road \\
37B & Agropiron & Maragheh \\
46B & Barley & Marand (Yam) \\
11B & Wheat & Hashtrood-Bostanabad road \\
43B & Wheat & Marand (Yam) \\
29B & Squash & Duzduzan \\
21B & Alfalfa & Bostanabad \\
64B & Onion & Govgan \\
62A & Sunflower & Govgan \\
14A & Alfalfa & Maragheh \\
61B & Alfalfa & Azarshahr (afghan) \\
29A & Barley & Sarab \\
17A & Cucumber & Basmenj \\
59C & Wheat & Shahheedj \\
47A & Wheat & Marand (Yam) \\
\hline
\end{tabular}
\caption{Antagonistic potential fluorescent pseudomonads isolated from different fields}
\end{table}

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.

\textbf{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 log\textsubscript{10} CFU g\textsuperscript{-1} roots (Table 4). The population density on roots of plants treated with 8B and 41A attained 7.76 and 7.55 log\textsubscript{10} CFU g\textsuperscript{-1} root, respectively. Strain 3B, on the other hand, was not an effective colonizer as its cell count was less than 5 log\textsubscript{10} CFU g\textsuperscript{-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
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 \textit{P. drechsleri} in the greenhouse were evaluated for production

### Table 2. Mycelial growth inhibition of \textit{Phytophthora drechsleri} by metabolites produced by selected bacterial isolates

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

\textsuperscript{a} % Mycelial inhibition was calculated as (R – r)/R × 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.

\textsuperscript{b} Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan’s test.

### 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


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* associated 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 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.
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 phiID 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., 2000) 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 $10^7–10^8$ 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.

### Acknowledgments

This research has been supported by Azarbaijan University of Tarbiat Moallem (Research Project Number: 217/S/324). We thank Habib Razmi and Hasan Heidari for help with the HPLC analysis and Richards Kenneth for critical review of the manuscript prior to submission.

### References

Ahmadzadeh, M. and Sharifi-Tehrani, A. 2009. Evaluation of fluorescent pseudomonads for plant growth promotion, antifungal activity against Rhizoctonia solani on common bean, and biocontrol potential. *Biolog. Control* 48:101–107.

Ahmed Idris, H., Labuschagne, N. and Korsten, L. 2007. Screening rhizobacteria for biological control of Fusarium root and...
crown rot of sorghum in Ethiopia. *Biol. Control* 40:97–106.

Alavi, A. and Strange, R. N. 1979. A baiting for isolating *Phytophthora drechsleri*, causal agent of crown rot of *Cucumis* species in Iran. *Plant Dis. Rep.* 63:1084–1086.

Alavi, A. and Strange, R. N. 1982. The relative susceptibility of some cucurbits to an Iranian isolate of *Phytophthora drechsleri*. *Plant Path.* 31:221–227.

Ayers, S. H., Rupp, P. and Johnson, W. T. 1919. A study of the alkali forming bacteria in milk. *U. S. Dept. Agric. Bull.* 782 pp.

Babadoost, M. and Islam, S. Z. 2003. Fungicide seed treatment effects on seedling damping-off of pumpkin caused by *Phytophthora capsici*. *Plant Dis.* 87:63–68.

Bull, C. T., Weller, D. M. and Thomashow, L. S. 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis var. tritici* by *Pseudomonas fluorescens* strain 2–79. *Phytopathology* 81:954–959.

Buyens, S., Heangens, K., Poppe, J. and Hofte, M. 1996. Involvement of pyochelin and pyoverdin in suppression of *Pythium*-induced damping-off of tomato by *Pseudomonas aeruginosa* 7NSK2. *Appl. Environ. Microbiol.* 62:865–871.

de Souza, J. T., Arnold, C., Deulov, C., Lemanceau, P., Gianinazzi-Pearson, V. and Raaijmakers, J. M. 2003. Effect of 2,4-diacyl-3,5-dihydroxy-HQ on *Pythium*: cellular responses and variation in sensitivity among propagules and species. *Phytopathology* 93:966–975.

Dowling, D. N. and O’Gara, F. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *Trends Biotechnol.* 12:133–144.

Dye, D. W. 1968. A taxonomic study of the genus *Erwinia*. I. the “amylovora” group. *N.Z. J. Sci.* 11:590–607.

Duffy, B. K. and Défago, G. 1997. Zinc improves biocontrol of *Fusarium* crown and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. *Phytopathology* 87:1250–1257.

Erwin, D. C. and Ribeiro, O. K. 1996. *Phytophthora capsici*. Pages 262–268 in: *Phytophthora Diseases World wide*. American Phytopathological Society, St. Paul, MN.

Expert, J. M. and Digat, B. 1995. Biocontrol of *Sclerotinia* wilt of sunflower by *Pseudomonas fluorescens* and *Pseudomonas putida* strains. *Can J. Microbiol.* 41:685–691.

Fallahzadeh-Mamaghani, V., Ahmadzadeh, M. and Sharifi, R. 2009. Screening systemic resistance-inducing fluorescent pseudomonads for control of bacterial blight of cotton caused by *Xanthomonas campestris* pv. *malvacearum*. *J. Plant Pathol.* 91:663–670.

Haas, D. and Keel, C. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annu. Rev. Phytopathol.* 41:117–153.

Hagedorn, C., Gould, W. D. and Bardinelli, T. R. 1998. Rhizobacteria of cotton and their repression of seedling disease pathogens. *Appl. Environ. Microbiol.* 55:2793–2797.

Handelsman, J. and Stabb, E. V. 1996. Biocontrol of soil-borne plant pathogens. *Plant Cell* 8:1855–1869.

Howell, C. R. and Stipanovic R. D. 1980. Suppression of *Pythium ultimum* induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology* 70:712–715.

Hu, H. B., Xu, Y. Q., Cheng, F., Zhang, X. H. and Hur, B. 2005. Isolation and characterization of a new *Pseudomonas* strain produced both phenazine-1-carboxylic acid and pyoluteorin. *J. Microbiol. Biotech.* 15:86–90.

Hwang, B. K. and Kim, C. H. 1995. *Phytophthora* blight of pepper and its control in Korea. *Plant Dis.* 79:221–227.

Keel, C., Schneider, U., Mauhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D. and Défago, G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHAO: importance of the bacterial secondary metabolite 2,4-diacyl-3,5-dihydroxy-HQ. *Mol. Plant-Microbe Interact.* 5:4–13.

Khan, J., Ooka, J. J., Miller, S. A., Madden, L. V. and Hoitink, H. A. J. 2004. Systemic resistance induced by *Trichoderma hamatum* 382 in cucumber against *Phytophthora* crown rot and leaf blight. *Plant Dis.* 88:280–286.

Khateri, H. 2002. Studying the effect of some antagonistic bacteria against *Phytophthora drechsleri* the causal agent of cucumber root and crown rot. MSc thesis. University of Tehran, Karaj.

King, E. O., Ward, M. K. and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *Lab. Clin. Med.* 44:301–307.

Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. *Nature* 199:299–300.

Kovacs, N. 1956. Identification of *Pseudomonas pyocyana* by the oxidative reaction. *Nature* 178, p. 703.

Kreutzer, W. A., Bodine, E. W. and Durrell, L. W. 1940. Cucumber diseases and rot of tomato fruit caused by *Phytophthora capsici*. *Phytopathology* 30:972–976.

Lamour, K. H. and Hausbeck, M. K. 2000. Mefenoxam insensitivity and the sexual stage of *Phytophthora capsici* in Michigan cucurbit fields. *Phytopathology* 90:396–400.

Lamour, K. H. and Hausbeck, M. K. 2001. Investigating the spatiotemporal genetic structure of *Phytophthora capsici* in Michigan. *Phytopathology* 91:973–980.

Lee, J. Y., Moon, S. S. and Hwang, B. K. 2003. Isolation and in vitro and in vivo activity against *Phytophthora capsici* and *Colletotrichum orbiculare* of phenazine-1-carboxylic acid from *Pseudomonas aeruginosa* strain GC-B26. *Pest Manag. Sci.* 59:872–882.

Lifshitz, R., Kloepper, J. W., Kozlowski, M., Simonson, C., Carlson, J., Tipping, E. N. and Zaleska, I. 1987. Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. *Can. J. Microbiol.* 8:102–106.

Lindow, S. E. 1988. Lack of correlation of in vitro antibiotic with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. *Phytopathology* 78:444–450.

Liu, H., He, Y., Jiang, H., Peng, H., Huang, X., Zhang, X., Thomashow, L. S. and Xu, Y. 2007. Characterization of a Pheno-zine-Producing Strain *Pseudomonas chlororaphis* GP72 with broad-spectrum antifungal activity from green pepper rhizo-
Antagonism of Pseudomonads against *Phytophthora drechsleri*

Simon, A. and Ridge, E. H. 1974. The use of ampicillin in a simplified selective medium for the isolation of fluorescent pseudomonads. *J. Appl. Bacteriol.* 37:459–460.

Sunish-Kumar, R., Ayyadurai, N., Pandiaraja, P., Reddy, A. V., Venkateswarlu, Y., Prakash, O. and Sakhivel, N. 2005. Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broad-spectrum antifungal activity and biofertilizing traits. *J. Appl. Microbiol.* 98:145–154.

Thomashow, L. S. and Weller, D. M. 1995. Current concepts in the use of introduced bacteria for biological disease control, p. 187–235. In G. Stacey and N. Keen (ed.), Plant-microbe interactions, vol. 1. Chapman & Hall, New York, N.Y.

Thornley, M. I. 1960. The differentiation of Pseudomonas from other Gram negative bacteria on the basis of arginine metabolism. *Appl. Bacteriol.* 1:37–52.

Tjamos, E. C., Tsitsigiannis, D. I., Tjamos, S. E., Antoniou, P. P. and Katinakis, P. 2004. Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahliae* of solanaceous hosts. *Eur. J. Plant Pathol.* 110:35–44.

Turner, J. M. and Messenger, A. J. 1986. Occurrence, biochemistry and physiology of phenazine pigment production. *Adv. Microb. Physiol.* 27:211–275.

Wang, C., Ramette, A., Punjasamamwong, P., Zala, M., Natsch, A., Moenne-Loccoz, Y. and Defago, G. 2001. Cosmopolitan distribution of phlD-containing dicotyledonous crop-associated biocontrol pseudomonads of worldwide origin. *FEMS Microbiol. Ecology* 37:105–116.

Weller, D. M., Zhang, B. X. and Cook, R. J. 1985. Application of a rapid screening test for selection of bacteria suppressive to take-all of wheat. *Plant Dis.* 69:710–713.

Yuen, G. Y. and Schroth, M. N. 1986. Interaction of *Pseudomonads fluorescens* strains E6 with ornamental plants and its effect on the composition of root colonization microflora. *Phytopathology* 76:176–179.