CHARACTERIZATION OF THE ANTAGONISTIC ACTIVITY OF A NEW INDIGENOUS STRAIN OF PSEUDOMONAS FLUORESCENS ISOLATED FROM ONION RHIZOSPHERE

H. Afsharmanesh, M. Ahmadzadeh, M. Javan-Nikkhah and K. Behboudi

Department of Plant Protection, Faculty of Agricultural Sciences and Engineering, University College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

SUMMARY

Bean damping-off caused by Rhizoctonia solani is one of the most widespread diseases of this plant. Fluorescent pseudomonads able to produce secondary antifungal metabolites can inhibit soil-borne plant pathogens. For this reason the antagonistic activity of Pseudomonas fluorescens UTPF5 against R. solani AG-4 was assessed in bean under in vivo and in vitro conditions. Production of some secondary metabolites and their impact on mycelial growth of R. solani was also studied. The results showed that UTPF5 could inhibit the growth of R. solani both in vitro and in vivo, and suppress the disease by 53.34% and 14.29% by soil drenching and seed treatment, respectively. Population density of UTPF5 decreased from about 10^9 CFU seed^{-1} to 2.07×10^4 CFU g^{-1} root after one month of growth of plants in non-infected soil. Production of HCN, siderophore and protease, and involvement of siderophore, volatile and non-volatile metabolites on growth of the fungus were observed in UTPF5. Occurrence of the phlD gene in UTPF5 was demonstrated using PCR with the primers phl2a and phl2b. The characteristics mentioned above show that P. fluorescens strain UTPF5 is a potential biocontrol agent of R. solani.

Key words: Biological control, Rhizoctonia solani, UTPF5, antifungal metabolites, phlD.

INTRODUCTION

Rhizoctonia solani Kühn anastomosis group (AG)-4 is an economically important soil-borne plant pathogen with a wide host range. It causes damping-off and head rot on many vegetable crops. Bean damping-off caused by R. solani is one of the most widespread diseases of this plant. Chemical control has drawbacks including being costly and hazardous to both the environment and humans. With excess use, there is risk of developing fungicide-resistant strains. Therefore, using biological control methods, for example those using antagonistic bacteria, is very important. Plant growth-promoting rhizobacteria (PGPR) may competitively colonize plant roots, stimulate plant growth and reduce the incidence of plant disease. The PGPR concept has been strengthened by the isolation of many bacterial strains that fulfill at least two of the three above criteria (Haas and Defago, 2005). Among a range of bacteria with this capacity, several strains of fluorescent pseudomonads have been successfully used for biocontrol of soil-borne pathogens (Vidhysaekaran and Muthamilan, 1999). The mechanisms of this control generally involve competition for nutrients, and production of bacterial metabolites such as iron chelating siderophores, antibiotics, volatile metabolites (HCN), extracellular lytic enzymes (proteases) and phytohormones (Gupta et al., 2001; Ashofteh et al., 2009).

Iron competition in pseudomonads has been intensively studied and the role of pyoverdine siderophore, produced by many Pseudomonas species, has been clearly demonstrated in the control of Pythium and Fusarium species, by comparing the effects of purified pyoverdine-minus mutants (Whipp, 2001). In vitro, HCN produced by cultures of Pseudomonas fluorescens on solid medium inhibits fungi such as Thielaviopsis basicola or Gaeumannomyces graminis via its gas phase (Blumer and Haas, 2000).

Production of antibiotics 2,4-diacetylphloroglucinol (Phl or 2,4-DAPG), pyoluteorin (Plt), pyrrolnitrin (Pn) and phenazine-1-carboxylic acid (PCA) in several strains of fluorescent pseudomonads has been recognized as a major factor in suppression of root formation (Dwivedi and Johri, 2003). 2,4-DAPG is a polyketide that displays antifungal as well as antibacterial, antiviral, antihelminthic, antinematode and phytotoxic properties in vitro (Wang et al., 2001). The use of Phl-minus derivatives of P. fluorescens CHA0, F113 and Q2-87 in soil microcosms has shown the importance of Phl in disease suppression by the corresponding wild-type strains (Wang et al., 2001). Genes required for the synthesis of 2,4-DAPG have been cloned and sequenced. Five complete open reading frames (ORFs) and one partial ORF,...
within the 6.8 kb segment of DNA, are responsible for biosynthesis of DAPG (Dwivedi and Johri, 2003). The biosynthetic locus includes phlA, phlC, phlB, and phlD (Bangera and Thomasow, 1999). phlD is responsible for the production of monoacetylphloroglucinol (MAPG), and phlA, phlC, phlB are necessary to convert MAPG to 2,4-DAPG. The first step required to investigate the genetic diversity of these bacteria is to detect the genes involved in synthesising this antibiotic. Probes and primers specific for sequences in phlD have been used in combination with colony hybridization and PCR to quantify the population size of 2,4-DAPG-producers in the rhizosphere environment (Raaijmakers et al. 1997; Raaijmakers and Weller, 1998; Picard et al., 2000).

The objectives of the research presented here were to evaluate the antagonistic activity of P. fluorescens UTPF5 against R. solani AG-4 on bean in vitro and in vivo, and to study the mechanisms of this antagonism. Also, because Phl is important in the biocontrol of a variety of root and seedling pathogens, the occurrence of phlD gene in strain UTPF5 was assessed by a PCR-based assay using the specific primers phl2a and phl2b.

MATERIALS AND METHODS

Source of microorganisms. Two bacterial strains, P. fluorescens strain UTPF5 (formerly named P-5) a biocontrol agent isolated during previous studies from the rhizosphere of onion in Iran (Ahmadzadeh et al., 2006) and P. fluorescens strain CHA0 obtained from the Swiss Federal Institute of Technology, were used in this study. Rhizoctonia solani AG-4 isolated from root and crown of bean obtained from the Mycology laboratory at the Department of Plant Protection, University of Tehran, with proven pathogenicity on bean was used as a test fungus.

Fungal inhibition assay. In a plate procedure, strains UTPF5 and CHA0 were spotted 0.5 cm from the edge of a PDA plate (four spots per plate). After 4 days of incubation at 25°C, one 5 mm disc of 48 h old R. solani mycelium was placed in the centre of the plate. Plates were incubated at 25°C and examined for evidence of fungal growth inhibition. The distance between the edges of the bacterial colony and the fungal mycelium were measured after three days (Hagedorn et al., 1989).

Production of antifungal metabolites. Evaluation of siderophore production was semi-quantitative, using Chrome Azurol S (CAS) medium as described by Schwyn and Neillands (1987). The diameter of an orange halo around colonies after incubation at 27°C for 48 to 72 h indicated relative level of siderophore production. Production of HCN was assessed on King’s B medium containing 4.4 g·L⁻¹ of glycinine with indicator paper [Whatman filter paper, soaked in 0.5% (w/v) picric acid and 2% (w/v) sodium carbonate] and plates were incubated at 27°C for 48 to 72 h. Presence of HCN caused the indicator paper to turn from yellow to cream, light brown, dark brown and brick-red (Alstrom and Burns, 1989). Extracellular protease activity was determined in skim milk agar (SMA) according to Maurhofer et al. (1995). Clear halos, indicating activity, were measured after 3 days incubation at 27°C.

Involvement of metabolites produced by UTPF5 against R. solani. Inhibition of mycelial growth of R. solani by volatile metabolites was studied as described by Kraus and Loper (1990). One hundred µl of UTPF5 and CHA0 suspensions (10⁸ CFU ml⁻¹) were placed on plates containing KB amended with 4.4 g·L⁻¹ glycinine. Incoculated plates were incubated at 27°C for 24 h. Subsequently, one 5 mm disc of 48 h old R. solani mycelium was placed in the centre of another KB plate. The plates were placed face to face but without contact between the pathogen and the bacterial suspension and were sealed. Plates were incubated at 27°C for three days, growth of the fungus was measured and compared to controls substituted with sterilized distilled water. Percentage inhibition was calculated using the following formula (Sivan et al., 1987):

\[
\% \text{ inhibition} = \frac{1-(\text{fungal growth/control growth})}{1} \times 100
\]

Inhibition of the fungus by non-volatile metabolites was studied using the method of Kraus and Loper (1990). Bacterial suspensions (10⁷ CFU ml⁻¹) were grown on KB medium supplemented with 1000 µM FeCl₃ + 6H₂O at 27°C for three days. The bacteria were then wiped off using chloroform. One hour later a 5 mm disc of 48 h old mycelium from R. solani (grown on PDA) was placed in the centre of the plates and growth of the fungus was measured and compared to the control substituted with sterile distilled water. Percent of inhibition was calculated. A complete randomized design was used with three treatments and three replicates for the assessment of volatile and non-volatile metabolites.

To investigate the possible involvement of siderophores in reducing fungal growth, the method of Rachid and Ahmed (2005) was used. One hundred µl of the bacterial suspensions (10⁸ CFU ml⁻¹) were transferred to KB plates with or without addition of 1000 µM FeCl₃ + 6H₂O. Then, four mycelial discs of 48 h old R. solani were placed on the plates. Growth of the fungus was measured and compared to the control substituted with sterile distilled water and the percentage inhibition calculated. Placement of the strains and the two media was done in a complete randomized design with two factorial agents and three treatments with three replicates. The first factor included UTPF5 and CHA0. The second included the media KB and KB amended with FeCl₃.
PCR experiments. Bacterial templates were prepared as described by Wang et al. (2001). Two bacterial colonies on KB medium after 48 h incubation at 27°C were transferred to 100 µl of lysis solution (50 mM KCl, 10 mM Tris-HCl at pH 8.3 with 0.1% (v/v) Tween 20). The resulting suspension was centrifuged at 1200 g for 1 min and incubated for 10 min at 99°C in a GP001 thermal cycler (Corbett Research, Australia) The heat-lysed bacterial suspension was frozen at -20ºC for 30 min. After thawing, 4 µl of the supernatant was used for PCR.

PCR amplification of phlD was performed using the forward primer phl2a (5’-GAG GAC GTC GAA GAC CAC CA -3’) and reverse primer phl2b (5’- ACC GCA GCA TCG TGT ATG AG -3’), which were developed from the phlD sequence of P. fluorescens Q2-87 (Raaijmakers et al., 1997). PCR was done in 20 µl reaction mixtures containing 4 µl of lyzed bacterial suspension, 10x PCR buffer, 2 mM MgCl2, 20 mg ml-1 bovine serum albumin (BSA), 5% (v/v) dimethyl sulfoxide, 0.4 mM dNTPs, 0.4 pmol of each primer and 1.5 U of Smart-Taq DNA polymerase, using a palm GP001 thermal cycler (Corbett Research, Australia). The cycling program was as described by Wang et al. (2001), but with modification of the annealing temperature. The program included an initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 65°C for 30 sec, 72ºC for 60 sec, and then a final extension at 72°C for 10 min.

All materials used in PCRs except for the primers were obtained from CinnaGen (Iran). The primers were synthesized by MWG Biotech (Germany). The products were electrophoresed in a 1% (w/v) agarose gel with 1x TBE buffer at 80V at room temperature, stained with ethidium bromide, and photographed under UV light by Gel-Documentation (IMAGO, B & L systems). The 1 kbp DNA ladder (MBI Fermentas, Germany) was used as molecular size marker. In each experiment, strain CHA0 and lysis buffer were used as positive and negative controls, respectively.

Greenhouse experiments. Preparation of fungal and bacterial inoculum. The R. solani inoculum was prepared by wetting 200 g millet seeds with 100 ml water, twice autoclaving at 15 psi for 30 min, adding ten 5 mm mycelial discs from a 4-day-old culture of R. solani on PDA, and incubating at 25°C for 2 weeks in darkness. Then 2 g of the infected seeds were mixed with 1 kg of sterilized soil in pots. Cells of strains UTPF5 and CHA0 were grown on KB agar and incubated at 25°C for 48 h (King et al., 1994). Bacteria were harvested by scraping cells from the agar, and suspending them in 9 ml sterile water. Solutions were serially diluted and bacterial concentration was determined. The bacterial suspensions were diluted in KB medium to about 10⁹ CFU ml⁻¹. These suspensions were used for seed coating and soil drenching.

Seed coating. After surface sterilizing of bean seeds (Phaseolus vulgaris cv. Naz) with 5% sodium hypochlorite for 2 min, they were soaked in the bacterial suspensions containing 0.5% carboxy methyl cellulose (CMC) and shaken for 3 h. For control treatment, the bacterial suspensions were replaced by 0.5% CMC. The treated seeds were allowed to dry on filter paper in a laminar flow cabinet. Four seeds were sown in each pot filled with soil infested with the fungal inoculum. The untreated control seeds were planted in infested soil. A negative control using pathogen-free soil was included in each test. Population densities of the applied bacteria on seeds were about 10⁸ CFU ml⁻¹.

Soil drenching. Four surface-sterilized bean seeds were sown in each pot filled with soil infested with the fungal inoculum. Then 50 ml of bacterial suspensions (10¹⁰ CFU per g of soil) containing 0.5% CMC were added to each pot. In non-infested (no pathogen) and infested (with pathogen) controls, 50 ml of 0.5% CMC without any bacteria were added to each pot. In both methods, Benomyl fungicide (1/1000 dilution) was used as a chemical control for comparison with the biocontrol experiments. Four replicates were applied for each of five treatments in a complete randomized design. Each replicate consisted of four seeds per pot. After 2 weeks of growth, reduction percentages of bean damping-off were assessed by counting the number of healthy plants. We measured plant growth by determining fresh and dry weights of roots and aerial parts after 30 days of growth. Dry weight was determined after placing plant material for 3-5 days at 70°C (Bakker et al., 2002).

Root colonization. Bean seeds surface-sterilized with 5% sodium hypochlorite for 2 min were placed in UTPF5 and CHA0 suspensions (10⁶ CFU ml⁻¹) containing 0.5% carboxy methyl cellulose (CMC), on a rotary shaker for 2-3 h at 60 rpm. The seeds were then air-dried in a laminar flow cabinet and sown in pots of sterilized soil. A complete randomized design was used with two treatments and four replicates. Each replicate consisted of four seeds per pot. After 4 weeks, plants were harvested, and excess soil was removed from the roots, then 1g of root sample with tightly adhering soil was shaken vigorously for 30 sec in test tubes containing 9 ml sterile water. Root samples were prepared from four selected plants and numbers of the introduced bacteria grown on the selected roots of both strains (UTPF5 and CHA0) were determined based on a plate assay as follows: tenfold dilutions were prepared from the suspensions of UTPF5 and CHA0. Hundred µl of each suspension were separately plated on Pseudomonas selective medium S1 [per litre: 10 g saccharose; 5 g casamino acids; 1 g NaHCO₃; 10 ml 87% glycerin; 1 g MgSO₄.7H₂O; 2.3 g K₂HPO₄; 1.2 g sodium lauryl sarcosine (SLS); 17 g BactoTM agar; 20 mg trimethoprim
added after autoclaving]. The plates were incubated for 2 days at 25°C and CFUs counted.

**Statistical analysis.** All data were analyzed by ANOVA using SAS (V6.12). Duncan’s Multiple Range Test was used to determine differences between treatments at 5% significance level.

**RESULTS**

Fungal inhibition plate assay and production of secondary metabolites. In the *in vitro* fungal inhibition assay strain UTPF5 showed an inhibition zone of 15 mm, and strain CHA0 10 mm. Siderophore production, as determined by colour change of Chrome Azurol S (CAS) in agar medium, was observed in both strains UTPF5 and CHA0. The average diameter of the orange zone around UTPF5 and CHA0 colonies was 8 and 10 mm, respectively. UTPF5 and CHA0 displayed proteolytic activity in SMA medium, so that the average diameters of haloes around UTPF5 and CHA0 colonies were 7 and 11 mm, respectively. Both strains produced HCN on KB medium supplemented with glycine and caused the indicator paper to turn from yellow to brick-red.

Involvement of metabolites in reducing growth of *R. solani*. UTPF5 inhibited mycelial growth of *R. solani* by production of volatile metabolites such as HCN on KB medium containing glycine, and diffusible metabolites on KB medium containing FeCl₃. UTPF5 growth inhibition of *R. solani* by volatile and non-volatile metabolites was 80% and 87%, respectively while it was 55% and 73% for CHA0, respectively (Fig. 1). The percentage of growth inhibition by volatile and non-volatile metabolites was not significantly different between UTPF5 and CHA0.

*R. solani* mycelial growth on KB medium in the presence or absence of 1000 µM FeCl₃ was inhibited both by UTPF5 and CHA0. By adding FeCl₃ to KB medium, the percentage of growth inhibition increased by 16% with CHA0, but was reduced by 8% with UTPF5. As shown in Table 1, the strongest growth inhibition was observed with UTPF5 on KB medium without FeCl₃ as compared to the medium amended with FeCl₃ (p=0.05). Therefore inhibition of *R. solani* depended on both FeCl₃ and the isolate used, so that FeCl₃ had a positive impact on CHA0 but negative impact on UTPF5 in activity against *R. solani*.

**Detection of phlD by gene-specific primers.** The results of the PCR analysis using primers phl2a and phl2b showed that a DNA fragment approximately 745 bp in size, as predicted from the known phlD sequence, was

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### Table 1. Interaction between bacteria and medium on inhibition of mycelial growth of *R. solani.*

<table>
<thead>
<tr>
<th>Strain and medium</th>
<th>Growth inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTPF5+KB</td>
<td>88 a</td>
</tr>
<tr>
<td>UTPF5+(KB+Fe)</td>
<td>80 b</td>
</tr>
<tr>
<td>CHA0+(KB+Fe)</td>
<td>37 c</td>
</tr>
<tr>
<td>CHA0+KB</td>
<td>21 d</td>
</tr>
<tr>
<td>KB+ Control</td>
<td>0 e</td>
</tr>
<tr>
<td>(KB+Fe) + Control</td>
<td>0 e</td>
</tr>
</tbody>
</table>

Each number is the mean of three replicates. Mean values followed by the same letter are not significantly different according to Duncan’s test at 5% significance level.

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![Fig. 1. Growth inhibition (%) of *R. solani* by volatile and non-volatile metabolites produced by *Pseudomonas fluorescens* strains UTPF5 and CHA0. Mean values followed by the same letter are not significantly difference according to Duncan’s test at 5% significance level.](image1)

![Fig. 2. PCR amplification of the *phlD* gene. Lane M, 1-kb ladder; lane 1, CHA0; lane 2, UTPF5; C, negative control (lysis buffer).](image2)
amplified in both UTPF5 and CHA0, which was used as a positive standard, known from previous work (Raaijmakers et al., 1997; Wang et al., 2001) to have the phlD gene (Fig. 2).

**Antifungal activity on R. solani in greenhouse.** After two weeks of plant growth, both strains reduced damping-off in soil inoculated with *R. solani* as compared to the inoculated, but not bacterially treated control. Disease percentage reductions in UTPF5 for soil drenching and seed coating were 33.34% and 14.29%, respectively, while CHA0 reduced disease by 60% and 35.72% (Fig. 3). Bean damping-off percentage reductions by Benomyl in soil drenching and seed coating were 33.34% and 50%, respectively. Four weeks after the treatment, the average plant fresh and dry weights after both drenching and seed coating were higher in the CHA0 treatment than with UTPF5 (Tables 2 and 3).

**Root colonization ability of UTPF5.** After four weeks of plant growth, the numbers of UTPF5 and CHA0 bacteria on bean roots were determined. These were $2.07 \times 10^4$ (UTPF5) and $2.39 \times 10^4$ (CHA0) CFU g$^{-1}$ root. A considerable reduction ($10^5$ times) in population sizes of both bacteria occurred from the original $10^9$ CFU ml$^{-1}$ (primary population on seeds) to $10^4$ CFU g$^{-1}$ root after one month.

**DISCUSSION**

Antagonistic root-associated bacteria are important for the control of soil-borne pathogens (Weller, 1988; Sørensen, 1997). In recent years, fluorescent pseudomonads have drawn attention worldwide because of their production of secondary metabolites such as siderophores, antibiotics like 2, 4-diacetylphloroglucinol, volatile compounds (HCN), enzymes and phytohormones.

Our work demonstrates the ability of *P. fluorescens* UTPF5 to control the fungus *R. solani*. Dual culture studies showed that this strain inhibited fungal growth on agar more than strain CHA0. The inhibitory zone suggests the presence of fungistatic metabolites secreted by the bacteria. Strain UTPF5 produced HCN, siderophores and protease, as did CHA0.

**Pseudomonas** spp. are known to produce volatile compounds. One such metabolite is HCN (Castric and Castric, 1983), although other volatiles such as aldehydes are also produced by fluorescent pseudomonads. Considering that we used KB containing glycine (an

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Table 2. Effect of bacterial strains CHA0 and UTPF5 and Benomyl (applied by soil drenching) on bean plant growth in soil infected with *R. solani*.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fresh weight aerial parts (g)</th>
<th>Dry weight aerial parts (g)</th>
<th>Fresh weight roots (g)</th>
<th>Dry weight roots (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHA0</td>
<td>3.625 ab</td>
<td>0.452 ab</td>
<td>1.077 ab</td>
<td>0.325 ab</td>
</tr>
<tr>
<td>UTPF5</td>
<td>2.01 bc</td>
<td>0.332 bc</td>
<td>0.507 b</td>
<td>0.152 abc</td>
</tr>
<tr>
<td>Benomyl</td>
<td>1.832 bc</td>
<td>0.255 bc</td>
<td>0.43 b</td>
<td>0.147 bc</td>
</tr>
<tr>
<td>Infected control</td>
<td>1.247 c</td>
<td>0.127 c</td>
<td>0.327 b</td>
<td>0.042 c</td>
</tr>
<tr>
<td>Non-infected control</td>
<td>5.17 c</td>
<td>0.67 a</td>
<td>1.51 a</td>
<td>0.357 a</td>
</tr>
</tbody>
</table>

Each number is the mean of four replicates. Mean values followed by the same letter are not significantly different according to Duncan’s test at 5% significance level.

Table 3. Effect of bacterial strains and Benomyl on growth factors of bean plant in seed coating method.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fresh weight aerial parts (g)</th>
<th>Dry weight aerial parts (g)</th>
<th>Fresh weight roots (g)</th>
<th>Dry weight roots (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHA0</td>
<td>2.345 bc</td>
<td>0.197 bc</td>
<td>0.89 bc</td>
<td>0.237 bc</td>
</tr>
<tr>
<td>UTPF5</td>
<td>1.672 c</td>
<td>0.232 bc</td>
<td>0.43 bc</td>
<td>0.19 bc</td>
</tr>
<tr>
<td>Benomyl</td>
<td>2.91 bc</td>
<td>0.445 ab</td>
<td>0.532 bc</td>
<td>0.187 bc</td>
</tr>
<tr>
<td>Infected control</td>
<td>0.875 c</td>
<td>0.13 c</td>
<td>0.235 c</td>
<td>0.095 c</td>
</tr>
<tr>
<td>Non-infected control</td>
<td>5.92 a</td>
<td>0.61 a</td>
<td>2.05 a</td>
<td>0.77 a</td>
</tr>
</tbody>
</table>

Each number is the mean of four replicates. Mean values followed by the same letter are not significantly different according to Duncan’s test at 5% significance level.
HCN precursor), we conclude that the inhibition of mycelial growth of \textit{R. solani} is attributable to HCN.

Siderophores are low molecular weight compounds with high affinity for \( \text{Fe}^{3+} \) (Nielands, 1981), which are produced under limiting concentrations of iron. These compounds are able to transport this element inside the cell for metabolic functions (Press \textit{et al.}, 2001) and microorganisms that produce siderophores show competitive advantage over those that do not. Thus competition for iron increases in conditions where this element is limiting, but this condition is reverted when iron is added to culture medium (Elad and Baker, 1985). In our study, involvement of siderophores in reducing mycelial growth of \textit{R. solani} was observed in strain UTPF5. In this strain, percentage inhibition against the fungus on KB medium increased by 8% compared to when KB was amended with \( \text{FeCl}_3 \). Considering the increased siderophore production under limiting iron concentration, we suggest role of siderophores in inhibition of fungal growth.

The involvement of non-volatile metabolites, such as antibiotics, was observed with both UTPF5 and CHA0 on KB amended with \( \text{FeCl}_3 \) (Fig. 1). As siderophore production is abolished in this medium (Elad and Baker, 1985), siderophores could not be involved in growth inhibition of \textit{R. solani} by both CHA0 and UTPF5. On the other hand inhibition of the fungus in these experiments could not have depended on HCN, as it would diffuse out of the plates immediately. Therefore inhibition of mycelial growth in KB medium amended with \( \text{FeCl}_3 \) may be attributable to antibiotics.

For UTPF5, growth inhibition by volatile and non-volatile metabolites appeared stronger than for CHA0, but this was not significant at the 5% level.

There are numerous reports of antibiotic production by fluorescent pseudomonads \textit{in vitro} that may be effective under field condition as well. These include 2,4-diacylphloroglucinol (Phl), pyoluteorin (Plt), pyrrolnitrin (Pn), phenazine-1-carboxylic acid (PCA), butyrolactones and viscosinamide (Keel and Defago, 1997). Antibiotic-producing PGPR have been studied intensively during the last decade, and special attention has been given to 2,4-DAPG-producing \textit{Pseudomonas} spp. because of their ability to control a wide variety of soil-borne plant pathogens (Duffy and Defago, 1997; Sharifi-Tehrani \textit{et al.}, 1998). Probes and primers for sequences within \textit{phlD} have been used to monitor the population dynamics of 2,4-DAPG producers in take-all decline disease-suppressive and conducive soils (Raaijmakers \textit{et al.}, 1997; Raaijmakers and Weller, 1998) and in the rhizosphere of maize (Picard \textit{et al.}, 2000). In this report, we used PCR to detect 2,4-DAPG-producing \textit{Pseudomonas} populations based on amplification of \textit{phlD} gene sequence. Occurrence of the \textit{phlD} gene was demonstrated by detecting a 745 bp DNA fragment in \textit{P. fluorescens} UTPF5, as well as the standard strain CHA0. These results concur with those of Wang \textit{et al.} (2001).

Many root-associated bacteria have a direct positive influence on plant growth and can also stimulate plant health indirectly (Höflich \textit{et al.}, 1994). The results of our study showed that UTPF5 and CHA0 were more effective as a soil drench than as a seed coating. CHA0 controlled damping-off better than UTPF5 under greenhouse conditions. Correlation analyses did not reveal any significant link between inhibition of fungal growth on plates and protection in plants. A lack of correlation between data obtained \textit{in vitro} and \textit{in vivo} seems to be the rule when dealing with biocontrol of soil-borne diseases (Kloepper, 1991). Our result corresponded to findings of Sharifi-Tehrani \textit{et al.} (1998), Hagedorn \textit{et al.} (1989) and Ellis \textit{et al.} (2000).

Populations of UTPF5 and CHA0 decreased from about 10^6 CFU seed^{-1} to 2.07×10^4 and 2.39×10^4 CFU g^{-1} root respectively, one month after growth of plants in non-infected soil. Vincent \textit{et al.} (1991) showed that pseudomonads are able to colonize wheat roots at a density of 10^4 to 10^6 CFU g^{-1} root. Berg \textit{et al.} (2001) indicated that \textit{Pseudomonas putida} BE2 colonized inoculated roots of strawberry at densities of 5.4×10^4 CFU g^{-1} root.

We conclude that UTPF5 can inhibit the growth of \textit{R. solani} both \textit{in vitro} and \textit{in vivo}, and can colonize bean roots at densities 10^4 CFU g^{-1} root. Involvement of an iron siderophore, and volatile and non-volatile metabolites in inhibiting mycelial growth of \textit{R. solani} was also shown. Detection of the \textit{phlD} gene shows the potential of this strain to produce 2,4-DAPG, which is able to suppress a variety of soil-borne plant pathogens. We conclude that strain UTPF5 is in several ways a suitable candidate for application under field conditions to control bean damping-off caused by \textit{R. solani}.

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Probiotic activity of \textit{P. fluorescens}


