

A comparison between *Pseudomonas aureofaciens* (*chlororaphis*) and *P. fluorescens* in biological control of cotton seedling damping-off disease

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Abstract: Due to the importance of the biological control of plant diseases, testing and introducing new biocontrol-active microorganisms is a major concern among plant pathologists. The causal agent of cotton seedling damping-off disease is *Rhizoctonia solani*. In this regard, we tried to investigate the antagonistic activities of *Pseudomonas aureofaciens* (*chlororaphis*) 30–84 (phenazine producing wild type and non-phenazine producing mutant) strains on *R. solani*, in comparison with some isolates of *P. fluorescens* under both *in vitro* (laboratory) and *in vivo* (greenhouse) conditions. In the laboratory experiment, the inhibitory effects of all the bacteria, on the growth of *R. solani*, were evaluated using the dual culture procedure. Results showed that five isolates of *P. fluorescens* along with both strains of *P. aureofaciens* significantly inhibited the growth of *R. solani*. Effective bacterial antagonists were then evaluated in a greenhouse experiment where cotton seeds were coated with their suspensions and were sown in pasteurised field-soil. The soil had been pre-inoculated with a virulent isolate of *R. solani*. The efficacy of the bacterial antagonists was evaluated by counting the number of surviving seedlings in different treatments, at 15 and 60 days after sowing, for determining pre- and post-emergence damping-off incidence. According to the results of the greenhouse experiment, at both intervals, two isolates of *P. fluorescens* along with both strains of *P. aureofaciens* caused significant increases in the number of healthy seedlings, in comparison with the untreated control, and a commonly used fungicide (carboxin-thiram). The efficacy of phenazine producing a wild type strain of *P. aureofaciens* was higher than its non-phenazine producing mutant, indicating that phenazine plays an important role in the antagonistic activity of *P. aureofaciens*. Effective bacterial antagonists were then studied for their antagonistic mechanisms. The results showed that all four bacteria employed different mechanisms. The bacteria produced siderophore, and volatile metabolites and non-volatile metabolites, in their antagonistic activities. The results of this study suggest that *P. aureofaciens* may be a new biocontrol agent for controlling cotton seedling mortality disease.

Key words: bacterial antagonists, biocontrol, greenhouse, *Rhizoctonia solani*

Introduction

Cotton is considered as an important fibre crop in many countries around the world including Iran. This fibre crop is cultivated in about 20 Iranian provinces (Naraghi *et al.* 2006; Heydari *et al.* 2007). Pests (harmful insects, parasitic weeds, and pathogens) are among the most important yield-reducing factors in cotton cultivation and production. Plant pathogens, particularly soil-born fungi, play very important roles in causing different diseases on cotton fields (Hagedorn *et al.* 1989; Heydari and Misaghi 1998; Naraghi *et al.* 2006; Heydari *et al.* 2007).

Among the soil-born plant pathogens, *Rhizoctonia solani* can attack cotton seeds and seedlings. At various stages of the plant's growth, *R. solani* can cause pre- and post-emergence damping-off or mortality diseases (Rush *et al.* 1994; Heydari *et al.* 2008). Application and the use

of chemical fungicides as seed treatment is the most common strategy for combating and managing this devastating disease (Rush *et al.* 1994; Heydari *et al.* 2008). Continuous application of chemical fungicides for the management of plant diseases on different crops, such as cotton, have become a public concern. The public has scrutinised how much and how often chemical fungicides are applied due to the numerous adverse and harmful impacts on the environment and non-target organisms (Cook 2000; Heydari and Pessarakli 2010). In addition to health and environmental contamination problems, overuse of chemicals in agriculture may lead to the development and appearance of resistant races among plant pathogens. Moreover, the high production costs and problems with registration also need to be considered.

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Biological control using beneficial microorganisms, and their parts and products, has been considered a viable alternative strategy for the replacement of chemical methods (Cook 2000; Heydari and Pessaraki 2010; Naraghi *et al.* 2010). Many biocontrol agents including antagonistic bacteria and fungi have previously been used to control pathogens and diseases they cause on different plants including cotton (Cook 2000; Haas and Défago 2005; Naraghi *et al.* 2006; Shahraki *et al.* 2009; Heydari and Pessaraki 2010; Naraghi *et al.* 2010; Samavat *et al.* 2011; Sadrati *et al.* 2013). The most common antagonistic fungi and bacteria that have been used to control cotton diseases such as seedling damping-off include *Trichoderma harzianum*, *T. hamatum*, *Talaromyces flavus*, and some antagonistic bacteria such as *Pseudomonas*, *Bacillus*, and *Burkholderia* isolates (Sivan *et al.* 1987; Cook 2000; Luzz 2001; Hass and Défago 2005; Naraghi *et al.* 2006; Shahraki *et al.* 2009; Heydari and Pessaraki 2010; Naraghi *et al.* 2010; Ramarathnam *et al.* 2011; El-Hassan *et al.* 2013; Sallam *et al.* 2013).

In previous studies, bacterial antagonists, particularly different species of *Pseudomonas*, have been used to control different diseases on a variety of plants including cotton. The use of bacterial antagonists have produced promising results (Howell and Stipanovic 1980; Weller and Cook 1983; Weller and Cook 1986; Sharifi-Tehrani *et al.* 1998; Sunish Kumar *et al.* 2005; Validov *et al.* 2005; Samavat *et al.* 2008; Ramarathnam *et al.* 2011; Zaim *et al.* 2013). The biocontrol efficacy of rhizobacteria is most likely related to their numerous and different antagonistic mechanisms. These mechanisms include: production of antibiotics, siderophores, cell degradation enzymes, and volatile and non-volatile secondary metabolites. Production of various antibiotics like pyrrolnitrin (Howell and Stipanovic 1980; Hu *et al.* 2005; Prasanna and Reddy 2009), hydrogen cyanide (HCN) (Voisard *et al.* 1989), and 2,4-diacetylphloroglucinal (DAPG) (Shanahan *et al.* 1992; Nowak-Thompson *et al.* 1994; Validov *et al.* 2005) by these beneficial bacteria has been previously reported. In addition to the above-mentioned antibiotics, nitrogen-containing heterocyclic phenazine antibiotics which have wide-spectrum antimicrobial activity, have been proved to be produced by *Pseudomonas* spp. in recent studies (Hu *et al.* 2005; Sunish Kumar *et al.* 2005; Ravindra Naik and Sakthivel 2006).

R. solani is a broad spectrum host plant pathogen. Many phenomena such as mutation, migration and DNA recombination may affect and promote the pathogenicity of *R. solani*. For this reason, testing and introducing new antagonistic bacteria against this pathogen may result in the achievement of more efficient biocontrol strategies for combating this important plant pathogen.

Pseudomonas aureofaciens strain 30–84 is a phenazine producing bacterium that has been capable of biologically controlling *Gaeumannomyces graminis* var. *tritici* as the causal agent of wheat take-all (Pierson and Thomashow 1992; Pierson *et al.* 1994; Wood and Pierson 1996). The ability of this strain to inhibit take-all disease correlates directly with its capacity to produce phenazine antibiotics (Wood and Pierson 1996). Multiple regulators such as quorum sensing genes *phzI* and *phzR* directly control phenazine biosynthesis by 30–84 strain (Pierson *et al.* 1994).

In order to introduce a novel biocontrol agent against *R. solani*, the present study was conducted and executed. Evaluation of the antagonistic activity and mechanisms of *P. aureofaciens* 30–84 (*phzR*⁺ and *phzR*⁻) strains in comparison with some *P. fluorescens* isolates, in suppression of cotton damping-off caused by *R. solani* (AG-4) under both *in vitro* (laboratory) and *in vivo* (greenhouse) conditions, was done.

Materials and Methods

Microorganisms and culture media

The isolate (Co-1) of AG-4 used in this study was obtained from the Microbial Culture Collection, of the Beneficial Microorganisms Research Laboratory, the Iranian Research Institute of Plant Protection. This isolate was previously (2010) isolated from the root and crown of diseased cotton seedlings. The fungus was routinely grown on standard Potato Dextrose Agar (PDA) culture medium (Merck, Germany) and stored in broth containing 15% glycerol at –20°C.

Antagonistic bacteria used against *R. solani* were also obtained from the above-mentioned source. These bacteria were isolated from the rhizosphere of different host plants in 2011 and 2012. Bacteria were stored in a 0.1 M magnesium sulfate (MgSO₄ × 7H₂O) solution at room temperature. The isolates were cultured in nutrient broth and stored in broth containing 15% glycerol at –20°C for short-term preservation. For the preparation of the bacterial suspension, a starter culture was grown in nutrient broth in tubes, and was incubated for 48 h at 25°C. Table 1 shows a list of bacterial isolates used in this study and their characteristics.

In vitro study of the antagonistic activity of bacterial isolates

To test the effects of bacterial isolates on the growth of *R. solani* under *in vitro* conditions, a dual culture experiment with 8 treatments and 4 replications was carried out in laboratory conditions. Each bacterial isolate was streaked in the center (as diameter) of a Petri plate containing PDA medium. After 48 h, two 5-mm mycelial plugs of a 3-day-old *R. solani* culture were placed at the opposite sides of the Petri plate. The mycelial growth of the fungus toward the bacterial streak was measured when the fungal colony in the control plate completely encircled the Petri plate area. Inhibition percent in each Petri plate was calculated using the following formula:

$$\text{Inhibition} = [1 - (\text{fungal colony diameter in the treatment Petri} / \text{fungal colony diameter in the control Petri})] \times 100.$$

Greenhouse study

Preparation of bacterial suspension

For the preparation of bacterial suspension, from the 3-day-old culture of each bacterial isolate grown on King's B

medium (KB), several loopfulls were transferred into test tubes containing sterilised distilled water. Bacterial cell density was adjusted to 10^9 CFU/ml at 620 nm, using a spectrophotometer. The prepared suspensions were used immediately or stored at 4°C for a short period of time.

Effects of bacterial antagonists on cotton seedling damping-off in greenhouse conditions

The biocontrol potential of bacterial antagonists against *R. solani*, the casual agent of cotton seedling damping-off, was evaluated in a completely randomised greenhouse experiment. Twelve cotton seeds (cv. Varamin) were separately coated with the suspension of bacterial antagonists and were sown in plastic pots containing 3 kg of pasteurised field soil. The soil was pre-inoculated with *R. solani* inoculums. There were 10 treatments each, with 4 replications. Pots were placed in a greenhouse with a 12 h photo period, and were watered as needed.

The efficacy of the bacterial antagonists on pre- and post-emergence damping-off diseases of cotton seedlings were determined by counting the number of surviving seedlings after 15 days (for pre-emergence diseases) and 60 days (for post-emergence disease). The obtained data collected from different treatments were then statistically analysed and were compared to evaluate the efficacy of the bacterial antagonists.

Study of the mechanisms of the effective antagonistic bacteria

The production of siderophore

The production of siderophore by effective bacterial isolates was studied according to the procedure of Shah-raki *et al.* (2009), using *Geotrichum candidum* fungus. The growth of the fungus is highly dependent on the presence of iron as described below.

Each bacterial isolate was streaked at the center of a Petri plate containing KB culture medium amended with 0, 25 and 100 μM FeCl_3 in a completely randomised experiment of 5 treatments each with 3 replications. Petri plates were incubated at 25°C for 48 h. After the incubation period, two 5-mm plugs of a 3-day-old *G. candidum* culture grown on PDA were placed at both of the opposite sides of the bacterial line. The mycelial growth of the fungus toward the bacterial streak was measured when the fungal colony in the control plates completely covered the Petri plate area.

The production of volatile metabolites

The production of volatile metabolites by bacterial antagonists was studied through the inhibition of the mycelial growth of *R. solani* as follows. First, 200 μl of each bacterial suspension (10^9 CFU/ml) was transferred to Petri plates containing Nutrient Agar (NA) culture medium. Then the suspension in the medium was incubated at 25°C for 24 h. Simultaneously, a 5-mm plug of *R. solani* mycelium was placed in the centre of a plate containing PDA culture medium. The lids of both plates were removed. Bacterial

plates were placed on the top of fungal plates with open lids, and were sealed and incubated at 25°C. The design of the experiment was completely randomised and there were 5 treatments each with 4 replications. When *R. solani* in the control plates grew fully and covered the area of the plates, the experiment was stopped. Production of volatile metabolites by bacterial antagonists was investigated by measuring *R. solani* growth in different treatments and comparing them with those of the control.

Production of non-volatile metabolites

Bacterial antagonists' possible production of non-volatile metabolites was investigated through the inhibition of *R. solani* mycelial growth. For this purpose, 200 μl of each bacterial suspension (10^9 CFU/ml) was transferred to the Petri plates containing NA medium and incubated at 25°C for 3 days. The bacterial colonies were then wiped off the plate using 40% formaldehyde. A five-mm mycelia plug of *R. solani* was placed in the centre of the plates and the plates were placed in the incubator at 25°C. Bacterial antagonists' production of non-volatile metabolites was investigated by measuring *R. solani* growth in different treatments and comparing them with those of the controls (without bacterial antagonists). This experiment was conducted in a completely randomised design with 5 treatments each with 4 replications.

Statistical analysis

Data obtained in different experiments were first subjected to analysis of variance (ANOVA) using Co-Stat statistical software (Cohort, CA, USA). The mean comparison was then performed using Duncan's multiple range test.

Results

Results of different parts of the study are presented in tables 1–5. In table 1 there is a list of all bacterial isolates and their characteristics. Table 1 shows, that five isolates of *P. fluorescens* and two strains of *P. aureofaciens* (*phzR*+ wild type and *phzR*- mutant) were used in this study. Also shown are that *P. fluorescens* isolates were previously isolated from diseased cotton and sugar beet plants in different provinces of Iran. *P. aureofaciens* (*phzR*+ wild type and *phzR*- mutant) were obtained from the Department of Plant Sciences, University of Arizona, USA.

Table 2 shows the laboratory experiment results of the antagonistic activities of bacterial isolates against *R. solani*. According to table 2, all seven bacterial isolates, at different rates, significantly inhibited the growth of *R. solani* (compared to the control). The most effective isolates in this experiment were *Pa* (*Wt*), *Pf*-5, *Pf*-4, *Pf*-2, *Pf*-1, *Pf*-3 and *Pa* (*Mu*), respectively (Table 2).

The results of the biocontrol effects of bacterial antagonists on pre- and post-emergence damping-off diseases of cotton seedlings are presented in table 3. As table 3 indicates, 15 days after sowing, three bacterial antagonists including *Pa* (*Wt*), *Pf*-5, *Pf*-4 along with fungicide, increased the number of emerged seedlings (compared to

Table 1. Characteristics of antagonistic bacterial isolates used in the study

Isolate code	Isolate identity	Isolation host	Isolation location
<i>Pf-1</i>	<i>P. fluorescens</i>	sugar beet	Karaj
<i>Pf-2</i>	<i>P. fluorescens</i>	sugar beet	Kermanshah
<i>Pf-3</i>	<i>P. fluorescens</i>	sugar beet	Kermanshah
<i>Pf-4</i>	<i>P. fluorescens</i>	cotton	Karkandeh
<i>Pf-5</i>	<i>P. fluorescens</i>	cotton	Karkandeh
<i>Pa (Wt)</i>	<i>P. aureofaciens</i> (<i>phzR+</i>)	–	University of Arizona, USA
<i>Pa (Mu)</i>	<i>P. aureofaciens</i> (<i>phzR-</i>)	–	University of Arizona, USA

Table 2. Effects of bacterial antagonists on *R. solani* (Co-1) growth in the laboratory experiment

Treatment code	Treatment description	<i>R. solani</i> colony diameter [mm]	Percent inhibition
The control	<i>R. solani</i> alone	*75.00 a	–
<i>Pf-1</i>	<i>P. fluorescens</i>	32.50 b	57
<i>Pf-2</i>	<i>P. fluorescens</i>	32.17 b	58
<i>Pf-3</i>	<i>P. fluorescens</i>	34.33 b	54
<i>Pf-4</i>	<i>P. fluorescens</i>	25.00 c	67
<i>Pf-5</i>	<i>P. fluorescens</i>	24.17 c	68
<i>Pa (Wt)</i>	<i>P. aureofaciens</i> (<i>phzR+</i>)	21.00 c	72
<i>Pa (Mu)</i>	<i>P. aureofaciens</i> (<i>phzR-</i>)	32.75 b	51

*each figure is the average of four replicates; figures in the columns marked with the same letter(s) are not statistically different ($p > 0.05$)

Table 3. Effect of bacterial antagonists on cotton seedling damping-off disease caused by *R. solani* (Co-1) 15 and 60 days after sowing, under greenhouse conditions

Treatment code	Treatment description	No. of healthy seedlings 15 days after sowing	Pre-emergence disease [%]	No. of healthy seedlings 60 days after sowing	Post-emergence disease [%]
Cont-	untreated seed without <i>R. solani</i>	*12.00 a	0	*12 a	0
Cont+	<i>R. solani</i> + untreated seeds	3.75 e	69	2.50 e	17
Fungic.	seeds treated with Carboxin-thiram	7.25 bcd	34	7.00 bcd	4
<i>Pf-1</i>	seeds treated with <i>Pf-1</i> bacterium	5.00 de	55	4.00 de	10
<i>Pf-2</i>	seeds treated with <i>Pf-2</i> bacterium	4.75 de	57	3.75 de	21
<i>Pf-3</i>	seeds treated with <i>Pf-3</i> bacterium	5.50 cde	50	4.25 de	23
<i>Pf-4</i>	seeds treated with <i>Pf-4</i> bacterium	8.00 bc	27	8.00 bc	0
<i>Pf-5</i>	seeds treated with <i>Pf-5</i> bacterium	8.50 b	23	8.00 bc	6
<i>Pa (Wt)</i>	seeds treated with <i>Pa (Wt)</i> bacterium	8.25 b	25	8.25 bc	0
<i>Pa (Mu)</i>	seeds treated with <i>Pa (Mu)</i> bacterium	5.25 cde	53	5.00 d	5

*each figure is the average of four replicates; figures in the columns marked with the same letter(s) are not statistically different ($p > 0.05$)

the inoculated control) and significantly reduced the incidence of pre-emergence damping-off incidence (Table 3). At the final interval (60 days after sowing), four bacterial antagonists including *Pa (Wt)*, *Pf-5*, *Pf-4* and *Pa (Mu)* were capable of increasing the number of survived seed-

lings significantly and decreased the incidence of post-emergence damping-off significantly (Table 3). These four effective bacterial isolates were selected and were further studied for their antagonistic mechanisms in the laboratory. A description is in the following sections.

Study on the antagonistic mechanisms of bacterial isolates

The production of siderophore

The results of siderophore production are presented in table 4. As this table shows, in the absence of FeCl_3 , *G. candidum* did not grow at all in the presence or absence of bacterial antagonists because iron is an essential element for its growth. According to the table 4, when the culture media was amended with 25 μmol of FeCl_3 in the control plates, *G. candidum* grew fully and covered the whole area of the plate. In the plates treated with bacterial suspension, though, the growth of *G. candidum* was significantly reduced due to iron deprivation (chelate) by siderophore. Antagonistic bacteria produce siderophore. As table 4 shows, when the concentration of FeCl_3 was increased to 100 μmol , in the culture medium, the growth of *G. candidum* in the presence of bacteria showed some increase in comparison with 25 μmol , but still was significantly reduced by bacterial antagonists and the production of siderophore. In both concentrations of FeCl_3 , all bacterial isolates caused various but significant growth inhibition of *G. candidum*. These results indicate that bacteria's production of siderophore was one of the antagonistic mechanisms (Table 4).

Production of volatile metabolites

Table 5 shows the results of the bacterial antagonistic mechanism of the production of volatile metabolites. As this table shows, the quality and the quantity of the bacteria's production of volatile metabolites have been evaluated based on their growth inhibition of *R. solani*. According to table 5, none of the bacterial isolates' production

of volatile metabolites significantly inhibited the growth of *R. solani* (compared to the control). The indication is, that the bacterial production of volatile metabolites did not play an important role in the antagonistic activity of the bacterial isolates.

The production of non-volatile metabolites

Results of the production of non-volatile metabolites are also presented in table 5. According to these results, all bacterial isolates, except *Pa* (*Mu*), reduced the growth of *R. solani* significantly. This means that bacterial production of non-volatile metabolites played an important role in the antagonistic activities of these bacteria. In this experiment, phenazine producing bacterial strain of *P. aureofaciens*, showed the highest efficacy in the inhibition of *R. solani* growth. The indication is, that production of this antibiotic is a major antagonistic mechanism of this bacterium (Table 5).

Discussion

Results of the present study indicate that it may be possible to control and reduce cotton seedling damping-off disease, which is a serious disease of this crop, by the application of *P. aureofaciens* 30–84.

In our *in vitro* experiment where wild type phenazine producing (*phzR+*) and mutant non-phenazine producing (*phzR-*) strains of *P. aureofaciens* were used against *R. solani*, along with some *P. fluorescens* isolates, all antagonistic bacteria, at different rates, inhibited the growth of pathogenic fungus. Yet, inhibition rate caused by the wild type strain of *P. aureofaciens* was much higher. So, it appears that the phenazine antibiotic plays an important role in the antagonistic activity of this bacterium. Indeed, this activity

Table 4. Evaluation of the selected bacterial antagonists on *G. candidum* growth in the production mechanism of siderophore, at different concentrations of FeCl_3

Treatment code	<i>G. candidum</i> colony diameter [mm] at no FeCl_3	<i>G. candidum</i> colony diameter [mm] at 25 μmol FeCl_3	<i>G. candidum</i> colony diameter [mm] at 100 μmol FeCl_3
The control	0	*75.00 a	*75.00 a
<i>Pf-4</i>	0	29.50 b	30.00 b
<i>Pf-5</i>	0	24.00 c	28.70 cd
<i>Pa</i> (<i>Wt</i>)	0	18.33 d	27.00 d
<i>Pa</i> (<i>Mu</i>)	0	25.17 c	29.33 bc

*each figure is the average of four replicates; figures in the columns marked with the same letter(s) are not statistically different ($p > 0.05$)

Table 5. Evaluation of the selected bacterial antagonists on *R. solani* (Co-1) growth in the production mechanism of volatile and non-volatile metabolites

Isolate code	<i>R. solani</i> colony diameter [mm] in the production of volatile metabolites	<i>R. solani</i> colony diameter [mm] in the production of non-volatile metabolites
The control	*75.00 a	*75.00 a
<i>Pf-4</i>	58.00 ab	28.00 d
<i>Pf-5</i>	70.00 a	34.00 c
<i>Pa</i> (<i>Wt</i>)	68.00 a	24.00 d
<i>Pa</i> (<i>Mu</i>)	63.00 ab	69.00 a

*each figure is the average of four replicates; figures in the columns marked with the same letter(s) are not statistically different ($p > 0.05$)

has also been reported in previous studies (Pierson and Thomashow 1992; Pierson *et al.* 1994; Ravindra Naik and Sakthivel 2006). Results of the laboratory experiments in the efficacy of *P. fluorescens* isolates, also showed that in addition to phenazine production, other mechanisms are involved in the antagonisms of bacteria against *R. solani*. Such mechanisms may include the production of siderophore, and other non-volatile and volatile metabolites that have previously been demonstrated by other researchers (Shahraki *et al.* 2009; Athukorala *et al.* 2010).

Results of the greenhouse experiments of the present study, in the use of bacterial antagonists for controlling cotton seedlings damping-off disease, were similar to those results of the laboratory in the effects of *P. auerofaciens* strains. Like the laboratory experiment, the wild type strain of *P. auerofaciens* showed higher effectiveness than the mutant strain in controlling both pre- and post-emergence damping-off disease, confirming the importance of the phenazine antibiotic (Hu *et al.* 2005; Ravindra Naik and Sakthivel 2006). In the greenhouse experiment, surprisingly only two isolates of *P. fluorescens* performed effectively in controlling cotton seedling damping-off disease. The other three isolates did not show effectiveness in controlling and suppressing the damping-off disease. The differences in the antagonism among different isolates of *P. fluorescens* could be due to several factors, including their genetics, their antagonistic mechanisms, and isolation hosts which have previously been reported (Cook 2000; Shahraki *et al.* 2009; Heydari and Pessaraki 2010).

Another important point which can be obtained from our greenhouse results is the trend of disease in two phases of pre- and post-emergence. As the results show, the number of dead seedlings 15 days after sowing was much higher than those of between 15 and 60 days. So, it seems that pre-emergence damping-off was the major form of the disease occurrence. Similar results in the damping-off disease trend have been observed in previous studies (Heydari *et al.* 2008; Shahraki *et al.* 2009).

Since several bacterial isolates performed effectively in controlling cotton seedling damping-off in the greenhouse, further *in vitro* experiments were conducted to investigate the antagonistic mechanisms of the bacterial isolates. Such antagonistic mechanisms included the isolates' production of siderophore, volatile and non-volatile metabolites. According to the results of this section, all effective bacterial isolates produced siderophore, which was reported for the *P. fluorescens* bacteria in the previous studies (Prasanna and Reddy 2009; Shahraki *et al.* 2009).

In the experiment concerning the production of volatile metabolites, the results showed that none of the bacteria could significantly inhibit the growth of *R. solani*, indicating that volatile metabolites did not have a significant role in bacterial antagonistic activities. However, some *Pseudomonas* bacteria have produced volatile metabolites as has previously been observed (Shahraki *et al.* 2009; Athukorala *et al.* 2010). Finally, in the experiment concerning the bacterial production of non-volatile metabolites, the results indicated that all four bacterial isolates produced these compounds which may include phenazine and other antibiotics. The important point obtained from this experiment is that the effectiveness of

the non-phenazine producing mutant of *P. auerofaciens* in the greenhouse could be due to the production of other non-volatile metabolites such as pyrrolnitrin, and 2,4-diacetylphloroglucinal (DAPG). The same justification applies to the effective isolates of *P. fluorescens*. As in our study, non-volatile metabolites including phenazines, pyrrolnitrin and other antibiotics that were produced by several species of *Pseudomonas* bacteria including *P. fluorescens* and *P. auerofaciens*, have been demonstrated in previous studies (Howell and Stipanovic 1980; Nowak-Thompson *et al.* 1994; Hu *et al.* 2005; Sunish Kumar *et al.* 2005; Validov *et al.* 2005; Ravindra Naik and Sakthivel 2006; Athukorala *et al.* 2010; Ramarathnam *et al.* 2011).

The overall results of this study showed that two isolates of *P. fluorescens*, along with both strains of *P. auerofaciens* employing different mechanisms, performed effectively in controlling and combating *R. solani*, the causal agent of cotton seedling damping-off disease both in the laboratory and greenhouse conditions. The results also indicated that although the phenazine antibiotic plays an important role in the antagonistic activity of *P. auerofaciens*, other secondary metabolites are also involved in the antagonism of this bacterium.

Results of this study in introducing *P. auerofaciens* 30–84 as a novel and effective biocontrol agent against *R. solani* after being examined and verified in an open field experiment, may have practical application in the biological control of seedling damping-off. This is a devastating disease of cotton in the field. The introduction of *P. auerofaciens* 30–84 as a biocontrol agent against *R. solani* can result in an increased cotton yield, decreased chemical fungicide application, and protection of the agricultural environment and biological resources.

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