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Interactions of Rhizobia Cultural Filtrates with *Pseudomonas fluorescens* on Bean Damping-off Control

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ABSTRACT

Biotic as well as abiotic factors may influence the biocontrol activity and population density of *Pseudomonas fluorescens*. However, limited studies have been carried out on the effects of extracellular metabolites of other competitor bacteria, especially on the biocontrol efficiency of *P. fluorescens*. A greenhouse experiment was conducted to evaluate the potential of the two *P. fluorescens* isolates UTPF68 & UTPF109 in the biocontrol of bean damping-off caused by *Rhizoctonia solani* (AG-4), when applied individually or in combination with the culture filtrates of five rhizobia isolates (RH3 to RH7). Although all treatments reduced bean damping-off severity in comparison with the untreated control, RH4 + UTPF109 gave the lowest severity of damping-off (0.56 ,<1%). Beside the effect on disease control, seeds treatment with both *P. fluorescens* isolates individually or in combined treatments especially RH4+UTPF109 and RH6+UTPF68 significantly improved bean growth factors such as shoot and root fresh/dry weights. On the other hand, all tested rhizobia and *P. fluorescens* isolates especially, RH4, proved to be siderophore, HCN, IAA, and exopolysaccharide producers. Also, all tested bacteria except RH5 and RH7 produced chitinase. Furthermore, our *in vitro* studies demonstrated that the filtrates of tested rhizobia isolates can effectively increase the population density of both *P. fluorescens* isolates as a biotic factor. Thus, certain rhizobia seem to have a capacity to interact synergistically with *P. fluorescens* isolates having potential biocontrol activity.

Keywords: Extracellular metabolites, biocontrol, Pseudomonas fluorescens, Rhizobium, Rhizoctonia solani

INTRODUCTION

Bean damping-off caused by *Rhizoctonia solani* Kühn. (AG-4) which has great importance among soil-borne plant pathogens, damages a wide range of host plants worldwide (Ghini & Zaroni, 2001). In Iran, it can cause a major constraint to common bean (*Phaseolus vulgaris*) production (Okhovat, 1977). Additionally, bean yeild losses caused by the disease is estimated to be up to 40% (Okhovat, 1977). In spite of the fact that the most common method for disease control is seed treatment with fungicides (Ogoshi, 1996), increased health and environmental concerns with the use of fungicides have urged to find alternative ways such as using antagonistic bacteria and fungi as biological control agents (Cook, 2000).

Hence, introducing beneficial bacteria into the rhizosphere, most notably the use of fluorescent pseudomonads as an important group of plant growth promoting rhizobacteria (PGPR) for the promotion of crop growth, bioremediation, and biocontrol, has been of interest to many microbiologists

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(Haas & Defago, 2005; Luz, 2001; Mello *et al*., 2002). These bacteria are effective against a broad spectrum of soil-borne plant pathogenic fungi such as *R. solani* (Shananhan *et al*., 1992; Sharifi-Tehrani *et al*., 1998; Ahmadzadeh & Sharifi-Tehrani, 2009). Moreover, it has been found that other bacteria especially many species of rhizobia, not only could promote plant growth by fixing atmospheric N2 in the nodules of root legumes, but also could have antagonistic effects on soil-borne plant pathogens (Tu, 1978; Chakraborty and Purkayastha, 1984; Chakraborty and Chakraborty, 1989; Muthamilan and Jeyarajan, 1996; Deshwal *et al*., 2003; Bardin *et al*., 2004). Rhizobia have been reported to inhibit significantly the growth of pathogenic fungi such as *Macrophomina phaseolina*, *Rhizoctonia* spp, *Fusarium* sp. , and *Pythium* spp. in both leguminous and non-leguminous plants (Kibria & Hossain, 2000; Khan, 1998; Hossain & Mohammed, 2002).

Such biocontrol bacteria have different mechanisms or combinations of mechanisms which may be involved in the suppression of different plant diseases; for example, the inhibition of the pathogen by antimicrobial substances (antibiosis) (El-Mehalawy, 2004); production of diverse microbial metabolites such as siderophore, rhizobitoxin (Deshwal *et al*., 2003); competition for nutrients supplied by seeds and roots and colonization sites; induction of plant resistant mechanisms; inactivation of pathogen germination factors present in seed and root exudates and degradation of pathogenicity factors of the pathogen such as toxins; parasitism that may involve production of extracellular cell wall-degrading enzymes, for example, chitinase that can cause pathogen cell walls lysis (El-Mehalawy, 2004), or plant growth enhancement through IAA production (Deshwal *et al*., 2003).

In order to show all above mentioned suppressing mechanisms, high ability of biocontrol agents in root colonization and threshold populations are typically required. However, colonization, traits, genes contributing to rhizosphere competence, and the mechanisms of pathogen suppression by them may be influenced by a number of biotic and abiotic factors (Weller, 2007). These factors may be limiting or intensifying for the success of PGPR in the rhizosphere (Cavigelli *et al*., 1995). Staley & Brauer (2006) showed that because of such limiting factors, PGPR introduced into the plant rhizosphere, often grew slowly and typically declined in number. Therefore, such beneficial bacteria were unable to show their efficiency completely. In addition, to enhance their biocontrol efficiency, the mixture of two or more biocontrol microorganisms have occasionally been applied and subsequently their efficiency could be influenced by their interaction. Incompatibility of the co-inoculants caused by the competition and antagonism against each other, may sometimes arise and thus inhibit each other as well as the target pathogens (Leeman *et al*., 1996). Therefore,, an important prerequisite for the successful development of strain mixtures appears to be the compatibility of the co-inoculated microorganisms (Baker, 1990; De Boer *et al*., 1997). Accordingly, by choosing compatible microorganisms with diverse mechanisms for biocontrol, it may be possible to improve biocontrol potential in the rhizosphere.

Because of the importance of bean damping-off caused by *R. solani* (AG-4) in Iran, and also the possibility that *Pseudomonas* and *Rhizobium* isolates as its biological control agents may affect each other, this study was commenced with the purpose of identifying the effect of the extracellular metabolites of some *Rhizobium* spp. isolates on both the growth and biocontrol efficiency of some *P. fluorescens* isolates as biocontrol agents of *R. solani*.

MATERIALS AND METHODS

Source of microorganisms and culture conditions

Bacterial isolates were assessed as potential biocontrol agents of *Rhizoctonia*

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solani (AG-4) during previous studies (Samavat *et al*., 2008). Rhizobia isolates, *Rhizobium etli* (RH5) and *R. leguminosarum* (RH3, RH4, RH6, RH7), were obtained from Department of Soil Biology, Soil and Water Research Institute, Karaj. *Pseudomonas fluorescens* isolates (UTPF68 & UTPF109) and the fungal isolate of *R. solani* (AG-4) were obtained from the Department of Plant Protection, University of Tehran.

The bacteria were stored in 0.1 M magnesium sulfate ($MgSO_4.7H_2O$) solution at room temperature. The isolates were cultivated in nutrient broth (Merck, Germany) and stored in broth containing 15% glycerol at −20 ºC for short-term preservation. For the preparation of the bacteria, a starter culture was grown on nutrient broth in tubes and was incubated for 48 h at 25 ºC in darkness.

The fungus was routinely grown on standard potato dextrose agar (Merck, Germany) and stored in broth containing 15% glycerol at −20 ºC. Bacterial isolates used in this study are shown in Table 1.

Preparation of rhizobia culture filtrates

During the experiment, the cultures of rhizobia were stored on yeast extract mannitol agar (YEMA; Vincent, 1970) slants in screw cap tubes at 4°C. Cell-free culture filtrates of *Rhizobium* spp. isolates were prepared for studying the effects of their extracellular metabolites on *P. fluorescens*, using 24 h cultures from TS agar plates (Tryptic soy broth agar, 3 g/l, Technical agar, 10 g/l), suspended in 0.01M $MgSO_4$ and diluted to a cell density of 10^5 cfu/ml. About 1ml of cell suspension was transferred to Erlenmeyer flasks (250 ml) containing 50 ml of yeast extract mannitol broth (YEMB). The cultures were grown on a rotary shaker, at 120 rpm, in darkness at 22°C. After 72 h of growth the cell densities of the cultures were between 4×10^8 and 2×10^{10} cfu/ml. All cultures were centrifuged 5000 *g* for 15 min at 5°C, and the supernatants were stored in 1ml portions at −80°C. Just before use, portions of the supernatants were thawed at 4°C and filtered through a sterile 0.2 µm syringe filter (Berggren *et al*., 2001).

The impact of rhizobia culture filtrates on Pseudomonas growth

Cell-free filtrates from *Rhizobium* spp. cell cultures were prepared as already described. *P. fluorescens* cell suspensions of isolates UTPF68 and UTPF109 were cultured in Erlenmeyer flasks (250 ml) containing 50 ml King's B medium (Merck, Germany) on a rotary shaker in darkness for 72 h and thereafter suspended in 0.02 M $MgSO_4$ to OD 0.1 at 600 nm (10^7 cfu/ ml). Sterile microtitre plates, NunclonTM, 96 wells, were used for the experiments. About 40 µl of *P. fluorescens* cell suspension, 80µl of King's B medium and 80 µl of rhizobia cell-free filtrate were added into each well, with 4 replicates. For controls, 80 µl of King's B medium, 80 µl of YEMB (yeast extract mannitol broth) medium, and 40 µl of *P. fluorescens* cell suspension were used. Thereafter, the plates were sealed and incubated for five days at 22°C on a shaker

Table1. Bacterial isolates used in this study.

Isolates	Host plant	Geographical origin
R. leguminosarum RH3	*Phaseolus vulgaris*	Iran_Tehran
R. leguminosarum RH4	*P.vulgaris*	Iran_Tehran
R. etli RH5	*P.vulgaris*	Iran_Zanjan
R. leguminosarum RH6	*P.vulgaris*	Iran_Tehran
R. leguminosarum RH7	*P.vulgaris*	Iran_Tehran
P. fluorescens UTPF68	*Brassica napus*	Iran_Mazandaran
*P. fluorescens*UTPF109	*Rosmarinus afficinalis*	Iran_Semnan

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in darkness. The growth of *P. fluorescens* cells was checked regularly by measuring the optical density at 600 nm on a multi-scan spectrophotometer during five days (Berggren *et al*., 2001).

In vitro tests of antimicrobial activity

The antimicrobial activity of the wild rhizobia isolates (RH3 to RH7) and *P. fluorescens* isolates (UTPF68 & UTPF109) were studied under *in vitro* conditions. Siderophore production was tested according to Deshwal *et al*. (2003), their ability to produce hydrocyanic acid (HCN) was checked as described by Bakker and Schippers (1987), and chitinase production was checked according to Chernin *et al.* (1955). Moreover, the production of phytohormones such as indole acetic acid (IAA) as well as exopolysaccharides was determined according to De Britto Alvareg *et al*. (1995) and Hebbar *et al*. (1992), respectively.

Greenhouse assay

A loamy soil with the pH of 7.6 having 0.6% organic C, 0.05% N, 12% CCE, and 9, 180, 3 and 0.8 mg/Kg available P, K, Fe and Zn, respectively was used in all greenhouse experiments. The soil was passed through a 3-mm sieve, air-dried, and stored in plastic bags at 4ºC. Fungi-free soil was obtained by treating the soil with live steam (121 ºC) for 30 min (Tarpero-Casas *et al*., 1990).

Greenhouse studies were carried out with Goli cultivar of common bean, obtained from the Department of Agronomy and Plant Breeding, University of Tehran. Bean seeds were surface-sterilized by washing with 96% ethanol for 30s and 2.5% sodium hypochlorite for 5 min, and then rinsed four times with sterile, distilled water. The seeds were put on water agar (WA) (Technical agar, 15 g/l) to germinate in the dark condition at 26ºC in the incubator. After 48h, seedlings were treated with *P. fluorescens* isolates (UTPF68 or UTPF109) cell suspensions cultured in cell free culture filtrates from *Rhizobium* spp. isolates (RH3 to RH7) by the method of Weller and Cook (1983) with some modifications. A lawn of *P. fluorescens* (UTPF68 or UTPF109) was grown for 48 h on King's B agar in a Petri dish and then scraped from the surface of the medium and suspended in 1.0% methylcellulose. The *Pseudomonas*-methylcellulose suspension was equally mixed with rhizobia cell free filtrate; and added to seedlings. Then, the seedlings were shaked on an orbital shaker at 120 rpm for 1 to 3 h under a stream of filtered air. This method resulted in $1–5 \times 10^8$ colonyforming units (cfu) $seed^{-1}$ at planting. Control treatments consisted of non-treated seedlings which were only coated with 1.0% methylcellulose. Then three seedlings were transplanted into each pot containing 300 gram sterile soil which was infected with 200 cfu of *R. solani* (AG-4) per gram of soil. The plants were grown in a greenhouse under natural light supplemented with artificial light (80 $\mu Ms^{-1}m^{-2}$; 16-h day, 8-h night). The day time temperature ranged from 22 to 27 ^oC, and at night the temperature was 19 ºC. All plants were harvested 21 days after planting. Plant growth measurements included disease severity, and root and shoot fresh/dry weights. The severity of *Rhizoctonia* root rot was evaluated on the scale of 0 to 6 in which 0 = no lesion evident, 1 =<10% roots with a single typical brown sunken lesion, 2 = >10% roots each having a few lesions, 3 =<20% roots each with one or more lesions, 4 = >20% roots with brown sunken lesions within 1 cm from the seed, 5 = postemergence bean damping-off, bean seedlings shorter than 5 cm, and 6 = almost no roots with stunting or death of seedling (Kim *et al*., 1997).

Statistical analysis

The green-house experiments were designed as a randomized complete block with six replications, and repeated in two independent trials. Means comparison were

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conducted using an ANOVA protected least significant difference (LSD) (P<0.01) test. Standard deviations for each treatment were calculated using the SPSS software. For the statistical treatment, nonlinear regression analysis was used by fitting logistic curves to the data. Estimated asymptotes were compared by Student's t-test (P <0.05).

RESULTS

The impact of rhizobia culture filtrates on Pseudomonas growth

Results revealed that among rhizobia isolates, only cell-free culture filtrates of RH3, RH6, and RH7 could significantly increase *P. fluorescens* isolate UTPF109 growth in comparison with the control (P<0.01) (Figure 1); besides, none of them showed antagonistic effects on UTPF109.

As shown in Figure 2, a significant increase in *P. fluorescens* isolate UTPF68 growth was found with the application of each cell-free culture filtrate of *Rhizobium* isolates. This effect was especially significant (P< 0.01) when combined with the filtrate of RH6.

In vitro tests for antimicrobial activity

Results in Table 2 show that siderophore, HCN, IAA, and exopolysaccharides were produced by all tested rhizobia and *P. fluorescens* isolates but at different degrees; *Rhizobium* isolate RH4 produced more siderophore, HCN, IAA, and exopolysaccharides than other isolates. Chitinase was also produced in all tested bacteria isolates with different amounts except *Rhizobium* spp. isolates RH5 and RH7.

Greenhouse experiment

According to the results, the extracellular metabolites produced by *Rhizobium* spp. were able to influence *Pseudomonas* isolates (UTPF68, UTPF109) ability to improve the growth indices of common bean infected with *R. solani* (AG-4). In this case, their effects might be synergetic or antagonistic. As shown in Table 3, the extracellular metabolites of *Rhizobium* isolates RH7 and

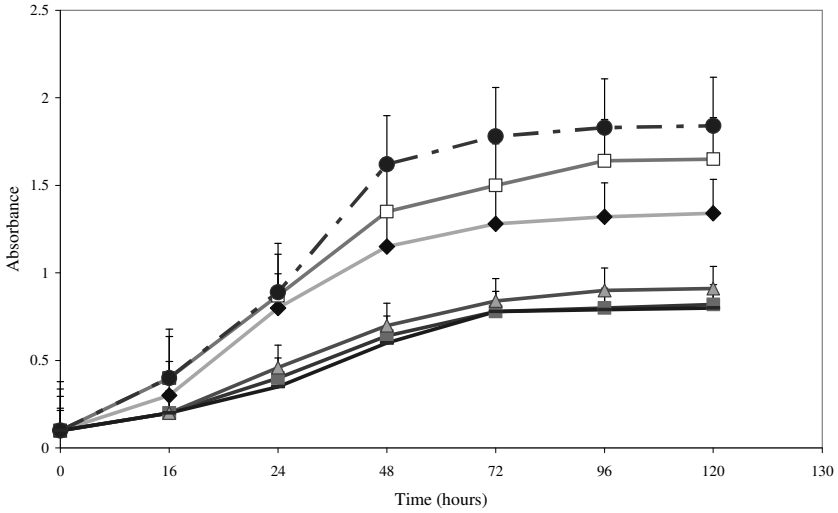

Figure 1. The effect of cell-free culture filtrates of *Rhizobium* spp. isolates RH3 (♦), RH4 (■), RH5 (▲), RH6 (□), RH7 (●), and control (-) on growth of *Pseudomonas fluorescence* isolate UTPF109 in King's B medium. Number of replicates = 4. ** = significant results refer to asymptotes compared with non-treatment, P= <0.05. Error bars are +standard error.

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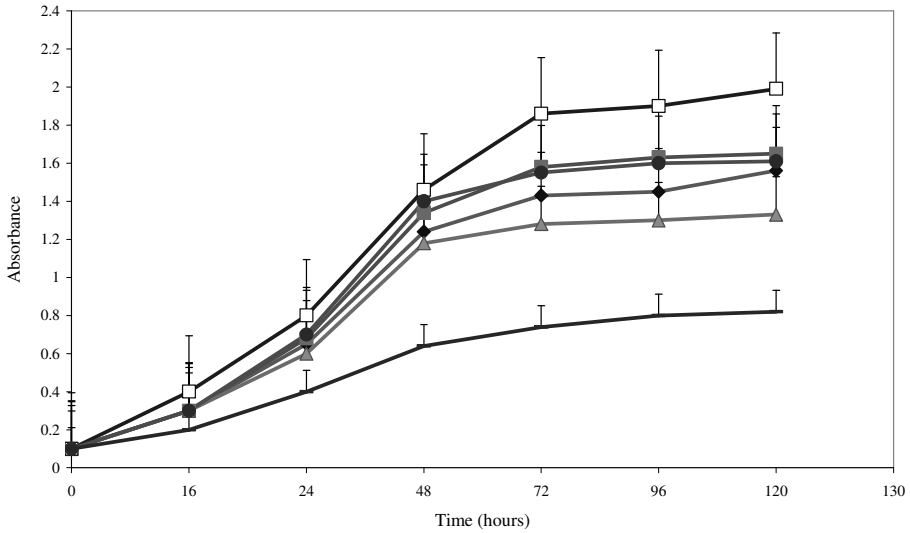

Figure 2. The effect of cell-free culture filtrates of *Rhizobium* spp. isolates RH3 (♦), RH4 (■), RH5 (▲), RH6 (□), RH7 (●), and control (-) on growth of *Pseudomonas fluorescence* isolate UTPF68 in King's B medium. Number of replicates = 4. ** = significant results refer to asymptotes compared with non-treatment, P= <0.05. Error bars are + standard error.

RH5 had antagonistic effects on *P. fluorescence* isolates UTPF109 and UTPF68 ability to promote infected bean growth, respectively. However, the effects of *Rhizobium* isolates RH4 and RH6 filtrates on *P. fluorescence* isolates UTPF109 and UTPF68 promoting ability were synergetic, respectively. No significant difference in root dry weight of infected bean could be detected.

Our results showed that *Rhizobium* spp. extracellular metabolites could affect not only *P. fluorescence* isolates ability to improve the mentioned bean growth factors, but also their ability to control bean damping-off caused by *R. solani*. Although seed treatment with both *P. fluorescens* isolates (UTPF109 & UTPF68) individually or in combined treatments could not completely inhibit bean damping-off incidence, disease severity was noticeably reduced (Figure 3). This effect was more pronounced (P<0.01) in RH4 filtrate+UTPF109.

Compared with UTPF109, only co-inoculation with RH4 filtrate showed a significant increase in the reduction of bean damping-off severity.

Seeds inoculation with the combination of *P. fluorescens* UTPF68 and *Rhizobium* RH6

Table 2. The qualitative assessment of siderophore, HCN, IAA, exopolysaccarides and chitinase production by *Rhizobium* spp. isolates (RH3 to RH7) and *P. fluorescens* isolates (UTPF68 & UTPF109) (n=3).

Isolates	Siderophore	HCN	IAA	Exopolysaccarides	Chitinase
RH3	+++	+++	+++	++++	+++
RH4	++++	++++	++++	++++	++
RH5	++	+	++	++	_
RH6	++	++++	+++	+++	+++
RH7	+	+++	++	+	_
UTPF68	+	++++	+	++	+
UTPF109	++	+	+++	+++	++

HCN: Hydrocyanic acid, IAA: Indole acetic acid, (++++): Very high, (+++): , (++): Moderate, (+): Low, (-): Negative.

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Table 3. The effect of five rhizobia filterates (RH3 to RH7) on *Pseudomonas* isolates (UTPF68, UTPF109) ability to improve shoot and root fresh/dry weights of infected common bean with *Rhizoctonia solani* in two independent greenhouse trials (n=6). (P<0.01).

Treatment	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
RH3	3.10± 0.10 DE	0.87± 0.06 GH	0.30± 0.008 DEF	0.077± 0.002 ABC
RH4	3.50± 0.19 BC	0.64± 0.05 I	0.35± 0.004 CDE	0.050± 0.005 BC
RH5	3.52± 0.31 B	1.12± 0.09 BCD	0.39± 0.006 ABC	0.10± 0.003ABC
RH6	2.94± 0.22 EF	0.95± 0.04 EFG	0.25± 0.011 FGH	0.083± 0.006 ABC
RH7	3.11± 0.14 DE	1.03± 0.08 CDEF	0.30± 0.010DEF	0.080± 0.008 ABC
UTPF68	2.60 ± 0.17 FG	0.95 ± 0.04 EFG	0.23 ± 0.008 FGH	0.083 ± 0.007 ABC
UTPF109	3.05 ± 0.11 E	0.90 ± 0.05 FG	0.29 ± 0.016 DEFG	0.076 ± 0.005 ABC
RH3+UTPF68	2.84 ± 0.20 EF	0.96 ± 0.04 EFG	0.28 ± 0.013 EFG	0.063 ± 0.007 ABC
RH3+UTPF109	3.15 ± 0.10 CDE	1.03 ± 0.06 DEF	0.31 ± 0.015 DEF	0.057 ± 0.004 ABC
RH4+UTPF68	3.43 ± 0.19 BCD	1.08 ± 0.02 CDE	0.33 ± 0.015 CDE	0.083 ± 0.009 ABC
RH4+UTPF109	4.48 ± 0.25 A	1.27 ± 0.07 AB	0.44 ± 0.016 A	0.113 ± 0.007 AB
RH5+UTPF68	2.13 ± 0.07 H	0.74 ± 0.08 HI	0.20 ± 0.012 H	0.043 ± 0.004 BC
RH5+UTPF109	3.67± 0.12 B	0.89 ± 0.05 FG	0.36 ± 0.014 BCD	0.060 ± 0.006 ABC
RH6+UTPF68	4.35 ± 0.22 A	1.19 ± 0.07 ABC	0.43 ± 0.017 AB	0.080 ± 0.007 ABC
RH6+UTPF109	2.97 ± 0.06 E	0.96 ± 0.09 EFG	0.29 ± 0.013 DEFG	0.067 ± 0.005 ABC
RH7+UTPF68	3.42 ± 0.11 BCD	1.04 ± 0.03 CDEF	0.34 ± 0.015 CDE	0.080 ± 0.006 ABC
RH7+UTPF109	2.30 ± 0.09 GH	0.65 ± 0.06 I	0.22 ± 0.011 GH	0.047 ± 0.003 BC
Control	4.22 ± 0.28 A	1.31 ± 0.10 A	0.41 ± 0.018 ABC	0.137 ± 0.009 A
Infected control	0.33 ± 0.04 I	0.09 ± 0.00 J	0.031 ± 0.006 I	0.017 ± 0.001 C

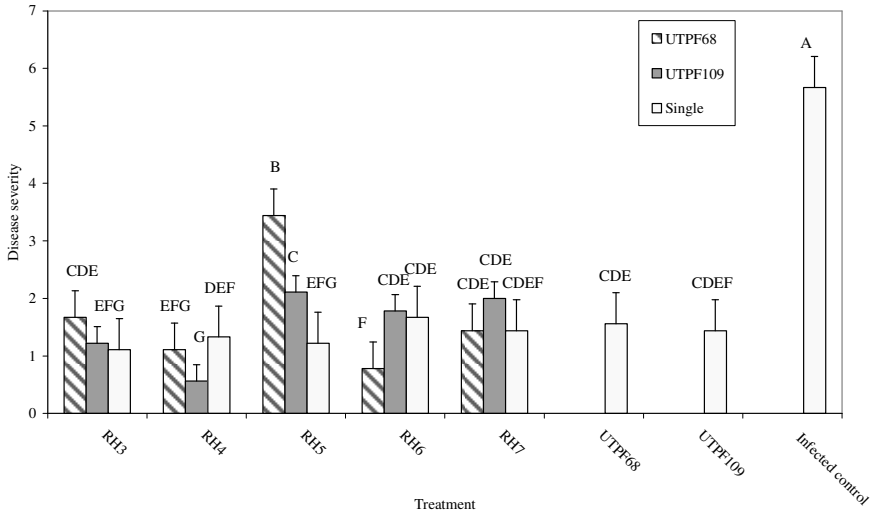

Figure 3. The effect of five rhizobia filterates (RH3 to RH7) on *Pseudomonas* isolates (UTPF68, UTPF109) ability to decrease severity of bean damping-off caused by *Rhizoctonia solani* (P<0.01) in two independent greenhouse trials. Error bars are + standard error. The number of replicates = 6.

filtrate resulted in a significant increase in the control of the disease in comparison with UTPF68.

DISCUSSION

The application of useful microorganisms mixtures for increasing crop production is currently under practice in agriculture. The mixtures of biocontrol agents may better adapt to the environmental changes, protect against a broader range of pathogens, increase the genetic diversity of biocontrol systems that persist longer in the rhizosphere, utilize a wider array of biocontrol mechanisms (Pierson & Weller, 1994), enhance the efficacy and

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reliability of control (Duffy & Weller, 1995), and allow the combination of various mechanisms of biocontrol without the need for genetic engineering (Janisiewicz, 1988).

Certain isolates of fluorescent pseudomonads are important biological components of agricultural soils that are suppressive to diseases caused by pathogenic fungi on crop plants. The population density and biocontrol abilities of such isolates may be affected by other beneficial bacteria such as rhizobia.

Our *in vitro* results revealed that all of the bacterial isolates (*Rhizobium* or *Pseudomonas*) produce siderophore and HCN. These results agreed with those of many other investigators (Carrillo & Del Rosario, 1992; Arora *et al*., 2001; Compant *et al*., 2005; Flaishman *et al*., 1996; Antoun *et al*., 1998). Arora *et al*, (2001) and Patten & Glick (2002) proved that the production of IAA is common in rhizobia and pseudomonads, respectively. Their findings were agreed with the data obtained in the current study. Also others reported that both rhizobia and *Pseudomonas* isolates produce exopolysaccharides and this result was in agreement with many other investigators who proved that an extra cellular polysaccharide (EPS) was produced by a *Rhizobium* sp. isolated from the root nodules of *Vigna mungo* (L.) (Mandal *et al*., 2007). Furthermore, the results obtained showed that most of *Rhizobium* and *Pseudomonas* isolates produced chitinase; this is in agreement with data obtained by many other workers (Chernin *et al*., 1955). They reported that many species of bacteria, *Streptomyces*, Actinomyces, fungi, and plants produced chitinolytic enzymes.

Data obtained from the greenhouse trial showed that there were heavy attacks on the untreated control plants by the end of the experiment, all of which noticeably showed the symptoms of root rot and damping-off caused by *R. solani*. The treatment of common bean seeds with individual *P. fluorescens* isolates generally reduced the disease severity. However, the combined treatments of rhizobia cultural filtrates and *P. fluorescens* isolates reduced root rot and damping-off severity of infected plants more than *P. fluorescens* single treatments in comparison with untreated control, particularly the combined treatment RH4+UTPF109. These results were in the same way with those of Esteve de Jensen *et al*, (2002), who found that the application of *Bacillus subtilis* with *Rhizobium* is a promising approach for the improvement of bean root rot control. Furthermore, Dileep Kumar *et al*, (2001) proved that seed treatment with *P. fluorescens* isolates alone and together with a rhizobial isolate reduced the number of infected peas grown in *Fusarium oxysporum* infected soils. Moreover, El-Batanony *et al*, (2007) found that the cultural filtrates of *Rhizobium leguminosarum* showed potential synergetic activity with arbuscular mycorrhizal (AM) fungi in the biocontrol of *R. solani*, *Fusarium solani***,** and *F.oxysporum* of faba bean.

Although the main mechanisms behind such protection against root diseases are not clearly defined**,** there are several hypotheses for such mechanisms**;** one of them may be the possibility of producing chitinolytic enzymes which was also detected in the tested rhizobial isolates. It is well known that chitin is the major structural component of most fungal cell walls**,** and that many species of bacteria, *Streptomycetes*, actinomycetes, fungi**,** and plants produce chitinolytic enzymes (Chernin *et al*., 1955). Another hypothesis may be the possibility of inducing systemic resistance by the metabolic products found in the cultural filtrates of the tested rhizobia e.g. exopolysaccharides. These findings were in agreement with Abdelaziz *et al*, (1996), who found that the rhizobia induced plant defence mechanisms against faba bean root rot. Moreover, many authors suggested that the heat-stable surface structures of *R. etli* G12 which consist mainly of EPS and Lipopolysaccharides (LPS) are invoved in the induction of induced systemic resistance as inducing factors (Van Peer and Schippers, 1992; Mandal *et al*., 2007).

On the other hand, our *in vitro* studies demonstrated that the cultural filtrates of tested rhizobial isolates were able to effectively increase the growth of both *P. fluorescens* isolates (UTPF68 & UTPF109). This may be

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another probable synergetic mechanism of rhizobia for intensifying the biocontrol ability of such beneficial *P. fluorescens* isolates against bean damping-off caused by *R. solani*. It is well known that *P. fluorescens* as biocontrol bacteria must be present on the roots in sufficient numbers to have a beneficial effect on the plant. The crucial colonization level that must be reached has been estimated at 10^5–10^6 CFU (colony-forming units) g^{-1} of root in the case of *Pseudomonas* spp., which protects plants from soil-born plant pathogens (Haas & Défago, 2005).

Besides the effect on disease control, seed treatments with both *P. fluorescens* isolates as individual or combined treatments with the culture filtrates of tested rhizobia also improved plant growth in the current study, as shown by significant increase in plant growth indices. Dileep Kumar *et al*, (2001) showed that seed treatment with fluorescent *Pseudomonas* strains alone and together with a rhizobial isolate significantly reduced the number of infected peas with wilt symptoms and promoted the growth of pea plants grown in *F. oxysporum* infested soils. The increase in growth indices in combined treatments with the cultural filtrates of the tested rhizobia may be due not only to the reduction in disease severity but also to the IAA (Al-Kahal *et al*., 2003), exopolysaccharides (EPS) (Gonzalez *et al*., 1996)**,** or siderophore (Dileep Kumar *et al*., 2001) which was found in rhizobial culture filtrates in our investigation.

In conclusion, our results showed the potential use of combinations of pseudomonads and rhizobia native to Iranian soils in improving plant growth and/or suppressing damping-off disease in bean plants. Furthermore, the biocontrol efficiency and population density of *P. fluorescens* isolates may be affected by biotic factors such as extracellular metabolites of rhizobial isolates in interaction with each other.

Further research is also necessary to discover the mechanisms of action and the efficacy of combined application of these useful bacteria at field level.

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برهمکنش های عصاره ریزوبیوم ها با *Pseudomonas fluorescens* در کنترل مرگ گیاهچه لوبیا

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چکیده

فاکتورهای زنده همچون فاکتورهای غیر زنده ممکن است تراکم جمعیت و کارایی بیوکنترل باکتری های *Pseudomonas fluorescens* را تحت تاثیر قرار دهند. اما مطالعات کمی در این رابطه، به‌خصوص در مورد اثرات متابولیت‌های خارج سلولی دیگر باکتری‌های رقابت کننده که در منطقه ریزوسفر آزاد می‌شوند بر روی کارآمدی بیوکنترل*P. fluorescens* صورت گرفته است. آزمایش گلخانه ای به منظور ارزیابی پتانسیل آنتاگونیستی سویه های UTPF68وUTPF109 باکتری *P.fluorescens* در کاربرد انفرادی و یا توأم با عصاره های پنج جدایه ریزوبیومی (RH3-RH7) در کنترل مرگ گیاهچه لوبیا ناشی از قارچ *Rhizoctonia solani* (AG-4) صورت گرفت. تمامی تیمارها در قیاس با تیمار شاهد شدت مرگ گیاهچه را کاهش دادند. از این نظر UTPF109 + RH4 مؤثر تر از سایر تیمارها بود و کمترین شدت مرگ گیاهچه (۰/۵۶) را نشان داد. علاوه بر تأثیر بر کنترل بیماری، تیمار بذور با سویه های *P.fluorescens* به طور انفرادی و یا توأم منجر به بهبود رشد لوبیا شد، به‌خصوص تیمارهای توأم RH4+UTPF109 وRH6+UTPF68 میزان وزن تر و خشک ریشه و اندامهای هوایی را به طرز چشمگیری افزایش دادند. از سوی دیگر اثبات شد که ریزوبیوم‌ها و سویه‌های *P.fluorescens* به کار رفته، به‌خصوص RH4، تولید کننده سیدروفور، HCN، IAAو پلی ساکاریدهای خارج سلولی هستند. باکتری‌های بررسی شده به استثنای RH5 وRH7 تولیدکننده آنزیم کیتیناز بودند. علاوه بر این، مطالعات صورت گرفته تحت شرایط درون شیشه نشان می دهند که عصاره‌های جدایه-های ریزوبیومی بررسی شده به عنوان یک فاکتور زنده می توانند به طور مؤثری تراکم جمعیت هر دو سویه *P.fluorescens* را افزایش دهند. بنابراین به نظر می‌رسد که ریزوبیوم‌های به‌خصوصی از قابلیت برهمکنش سینرژیستی مؤثر با سویه های کارآمد بیوکنترل *P.fluorescens* برخوردار هستند.

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