Screening of Rhizobacteria for Biological Control of Cucumber Root and Crown Rot Caused by *Phytophthora drechsleri*

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Antagonistic rhizobacteria, more specifically fluorescent pseudomonads and certain species of *Bacillus*, are known as biocontrol agents of fungal root diseases of agronomic crops. In this study, 144 bacteria were isolated from cucumber rhizosphere and screened as potential biological control agents against *Phytophthora drechsleri*, the causal agent of cucumber root rot, *in vitro* condition. Non-volatile compounds of 23 isolates showed noticeable inhibition zone (> 30%) against *P. drechsleri*, whereas volatile compounds of 7 isolates could prevent more than 30% of the mycelial growth of the fungus. All promising isolates, except of *Pseudomonas fluorescens* V69, promoted significantly plant growth under *in vitro* condition. *P. fluorescens* CV69 and V11 exhibited the highest colonization on the root. Results of the greenhouse studies showed that a reduction in disease incidence by use of some strains, and particularly use of strains CV6 and V11 as a soil treatment, exhibited a reduction in disease incidence so that suppressed disease by 85.71 and 69.39% respectively. *Pseudomonas fluorescens* CV6 significantly suppressed disease in comparison to Ridomil fungicide. The use of mixture bacterial strains in the soil inoculated by the fungus resulting in falling down the most of the plants which didn't show significant difference with infected control soils without bacteria.

Keywords : biological control, cucumber, *Phytophthora drechsleri*, rhizobacteria, root rot

Phytophthora blight, root and crown rot of cucumber is one of the most destructive pathogens of cucumber in entire the world (Erwin and Ribeiro, 1996; McGrath, 2001; Ristaino and Johnston, 1999). *Phytophthora capsici* Leonian usually reported as major agent of this disease (Babadoost and Islam, 2003) whereas *P. drechsleri* reported as a major agent of that in Iran (Alavi and Strange, 1979). On cucumber, these pathogens causes damping-off, crown and root rot, stem lesions, foliar blight, and fruit rot (Alavi and Strange,

1982; Kreutzer et al., 1940; McGrath, 2001). This pathogen infects susceptible hosts throughout the growing season and survives as dormant oospores during the winter (Lamour, and Hausbeck, 2001). Cucumber varieties resistant to *Phytophthora* disease are not available to our knowledge. Some practices such as planting on raised beds, management of field moisture, straw mulching, planting tolerant cultivars, and application of some fungicides may reduce severity of *Phytophthora* diseases on vegetables (Hwang and Kim, 1995). So, this disease is very difficult to control and no single method is available (Hwang and Kim, 1995; Ristaino and Johnston, 1999). Moreover, resistance to the systemic fungicides developed rapidly in *Phytophthora* after its application in the field (Lamour, and Hausbeck, 2000; Lamour and Hausbeck, 2001). Recently, soilborne diseases have been controlled by means of certain beneficial bacteria that are indigenous to the rhizosphere of plants (Thomshaw, 1996) and known as plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980). Bacterial isolates of *Actinomycetes*, *Streptomyces* spp., *Flavobacterium* sp., *Pseudomonas fluorescens* and *Bacillus* spp. have been reported to control of *Phytophthora* disease of plants (Alexander, 1999; Kun et al. 2002; 1984; Osburn et al., 1995; Tracey et al., 2004; Sid Ahmed et al., 2003). PGPR are known to rapidly colonize the rhizosphere (Rangajaran et al., 2003), produce secondary metabolites such as antibiotics and phytohormones (Keel et al., 1992), and can also be beneficial to the plant by stimulating growth of plants (Bloemberg and Lugtenberg, 2001).

Currently there is very limited knowledge regarding the biological suppression of *Phytophthora* root and crown rot in cucumber by application of PGPR. The aim of this study is therefore to isolate bacteria from the cucumber rhizosphere and screen them against *P. drechsleri*, one of the major causes of root and crown rot in Iran. This study may contribute to the introduction of PGPR systems in biological control of soil-born phytopathogenic fungi in cucumber from Iran.

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Material and Methods

Pathogen isolate and preparation of inoculum. *Phytophthora drechsleri* strain CP4, isolated from cucumber, Pishva region, Varamin, was received from Iranian Research Institute of Plant Protection, Evin, Tehran, and was stored on agar in sterile distilled water (SDW) in the dark at 5–7°C. For inoculums preparation, 100 g of white bean seed add to 250 ml of water in a 500 ml Erlenmeyer flask and then autoclaved for 20 min at 121°C in two successive days. The flask was inoculated with a mycelial plug from a 7-day-old culture of *P. drechsleri*, incubated 3 weeks at 26°C, and shaken daily to break up particles. This inoculum ground in a mill and used for greenhouse studies.

Field sampling and isolation of bacteria. In April 2009, a total of 50 cucumber plants were collected from fields and greenhouses of Varamin, Tehran province, Iran. Roots gently removed from soil and placed in plastic bags before they were transported to the laboratory. Adhering soil was carefully brushed off the plant roots, followed by gentle washing of the roots in sterile water. One gram of roots was added in 9 ml of 0.1 M phosphate buffer (pH 7.0) supplemented with 0.025% Tween 20 and serially diluted. Then the tubes were vortexed for 5 min. Then, 100 µl of dilutions 10^{-1} to 10^{-4} was plated on Nutrient Agar (NA) or Gould's S1 medium 18 g of agar, 10 g of sucrose, 10 ml of glycerol, 5.0 g of Casamino Acids (Difco), 1.0 g of NaHCO₃, 1.0 g of MgSO₄·7H₂O, 2.3 g of K₂HPO₄, 1.2 g of sodium lauroyl sarcosine (SLS), and 20 mg of trimethoprim. NA medium was used as a common medium to isolation of both gram positive and gram negative bacteria. S1 medium is a selective medium for isolation of fluorescent pseudomonad's (Gould et al., 1985).

In vitro antagonistic activity. 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 20 µl drops from the 10^8 cfu/ml suspension 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 fresh cultures of *P. drechsleri* was placed at the centre of the PDA plate for each bacterial isolate and incubated at $27 \pm 1^\circ\text{C}$ for seven days. The radii of the fungal colony towards and away from the bacterial colony were measured. The percentage growth inhibition was calculated using the following formula:

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

Where, r is the radius of the fungal colony opposite the

bacterial colony and, R is the maximum radius of the fungal colony away from the bacterial colony (control without bacterial colony).

Volatile compounds produced by bacteria were detected in a split plate experiment (Kraus and Ioper, 1992). Bacteria were grown for 3 days on tryptic soy agar (TSA; Difco) in one side of a split plate before inoculation of the PDA in the other side with a 6-mm-diameter plug of PDA culture of *P. drechsleri*. Radial growth of *P. drechsleri* on the fungal side was measured after 5 day of incubation at 27°C and the percentage of growth inhibition was calculated. Promising isolates were identified as an above technique.

Identification and characterization of antagonistic bacteria. The bacteriological characteristics (Gram staining, growth on media, catalase reactions and hypersensitivity reactions on tobacco) were carried out as described by Schaad et al. (2001).

The determination of physiological characteristics of antagonistic bacteria was performed as described by Schaad et al. (2001).

Plant growth promotion and root colonization. Isolates which showed antagonistic activity in the dual culture assays, were tested for their ability to promotion of plant growth and root colonization. Surface sterilized (2.5% NaOCl, 5 min) cucumber seeds of Soltan cultivar were pregerminated in moist chambers at 22°C for 36 h. Sterility of seeds was proved by a print on nutrient agar. Bacteria were grown on NB for 16 h and then centrifuged and resuspend in physiological salt solution (0.85%). At the next stage, standard 24-well microplates were filled with 1 ml of water agar (1.2%, pH 6.8). In each well, one seed was placed on the agar surface and inoculated with 20 µl of bacterial suspension (10^5 cfu per ml physiological salt solution (0.85%). Inoculated microplates were transferred to a phytochamber for two weeks (22/16°C, 16/8 h day/night, and artificial light) and the length and dry weight of stems and roots were measured to determine the effects of bacterial treatments on plant growth. Moreover one gram of root from each treatment was aseptically excised and placed in 25-ml flasks containing 5 ml of sterile 0.85% NaCl, and vigorously shaken at 300 rpm for 30 min. The resulting suspensions were serially diluted and plated on NA, then incubated at 28°C for colony counts. The level of root colonizing-acteria was calculated as colony forming units/g root (cfu/g root).

Preparation of pathogen inoculum and greenhouse studies. The ability of isolates to suppress cucumber *Phytophthora* damping-off was examined on *Cucumis sativus* L. Soltan cultivar. Seven surface sterilized (2.5% NaOCl, 3

min) seeds of Soltan cultivar seeded in polyethylene pots (8 cm in diameter) filled with a 1:1:2 mixture of peat, sand and soil from Varamin region, Iran. Then, the pots were transferred to the greenhouse (16 h of light and 25–27°C) watered at a three-day interval and kept for 10 days. Ten days after seeding, pots were inoculated with 30 g of the potting mix that had been blended with white bean seed inoculum of *P. drechsleri* and drenched with 20 ml of bacterial suspensions (10^6 cells ml⁻¹) or a solution of 1 g L⁻¹ Ridomil MZ-72 (Metalaxyl + Mancozeb = 8 + 64% WP). Then the pots watered and arranged in a randomized complete block design and incubated in condition mentioned above. The treatments in this experiment were: Pots inoculation with *P. drechsleri* and each of bacterial strains inoculation with *P. drechsleri* and mix of bacterial strains, inoculation with *P. drechsleri* and Ridomil, inoculation only with *P. drechsleri* but no bacteria (CI) and control without microbial inoculation (C).

After 2 and 3 weeks, percentage of the healthy plants was determined. After last assessment of disease severity, fresh and dry weight parameters of the plants were measured.

Statistical analysis. *In vitro* and greenhouse experiments

were repeated three times in a completely randomized design and randomized complete block design, respectively. The individual plant in the pot was the experimental unit. Statistical analyses were carried out using the software package SAS (V. 9.1). A mean comparison was performed using the Duncan's multiple range test. Correlations between volatile and non-volatile antifungal activities and root colonization ability with disease suppression were done by using the software SPSS (V. 10.1).

Results

Isolation and screening of antagonistic isolates. According to the Table 1, among of the 144 bacterial isolates, 23 isolates exhibited a more than 30% inhibition of mycelial growth of *P. drechsleri* by non-volatile inhibitors. The maximum inhibition achieved by isolate CV3 (55.56%). Inhibition of mycelial growth by non-volatile compounds of isolates CV6, V11, V14, V69, V127 and CV12 determined by 45.56, 43.33, 43.33, 42.53, 42.3 and 42.23 percent, respectively. Furthermore, volatile metabolites of seven isolates CV6, V16, V28, V81, V28, and V64 inhibited the mycelial growth of *P. drechsleri* considerably (Table

Table 1. Inhibition of *Phytophthora drechsleri* mycelial growth on potato dextrose agar by rhizobacterial isolates

Bacterial isolates	Mycelial inhibition (%)*		Bacterial isolates	Mycelial inhibition (%)	
	Non-volatile	Volatile		Non-volatile	Volatile
CV3	55.56*a**	21.66ef	V10	31.1ikjl	15.33ij
CV6	45.56b	32.33b	V42	30kjl	22.06ef
V11	43.33bc	22.66de	V41	29.8klmn	4q
V14	43.33bc	13jkl	V81	29.1klmn	38a
V69	42.53bc	39.3a	V38	29 klmn	17hi
V127	42.3bc	12klm	V2	27.76lmno	18ghi
CV12	42.23bc	22ef	V17	27.6lmno	27c
V28	41cd	33.33b	V79	26.66mnop	13.66jkl
V51	40.7cd	11lmn	V49	25.56nop	17hi
V30	40.3cd	6pq	V3	24.43opq	11.8klm
CV8	40cd	8.66no	V55	24.47opq	17.1hi
V27	38.9cde	13.3jkl	V1	23.33pq	17.03hi
V13	37.76efd	18.5gh	V37	21.19qr	23.06de
V26	36.66efdg	22ef	V62	21.16qr	15.36ij
V12	36.60efdgf	10mno	V21	21.14qr	19.83fg
V46	35.56efgh	9no	V61	21.1qr	13.86jk
V54	34.43fghi	23.1de	V66	18.97r	24.78dc
V71	34.43fghi	13.66jkl	V47	18.9r	8op
V15	15hi	17.9h	V64	18r	34.33b
V25	32.23hijk	27c	C	0s	0r
V16	31.8ikjl	37.66a			

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

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

Table 2. *In vitro* growth promotion and root colonization of cucumber plants by rhizobacterial strains

Bacterial isolate	PGPR			
	Stem length	Root length	Foliage weight	Root weight
<i>B. subtilis</i> V14	12.96a	9.5a	4.83a	4.39a
<i>P. aeruginosa</i> CV3	9.46bc*	7.5b	3.28bc	2.6cd
<i>P. fluorescens</i> CV6	12.23a	9.4a	4.76a	3.87ab
<i>P. fluorescens</i> V11	9.26c	7b	3.95ab	3.58abc
<i>P. fluorescens</i> V16	11.76	9.26a	4ab	3.58abc
<i>P. fluorescens</i> V69	6.56d	5.66	2.6cd	1.53e
<i>P. putida</i> V28	12.53a	7.93b	4.05ab	3.02bc
<i>P. putida</i> V64	10.46bc	7.56b	4.35a	3.02bc
Control	7.03d	5.56c	1.86d	1.7de

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

1), so that the maximum inhibition achieved by any isolate was 39.33% (V69).

Identification and characterization of antagonistic bacteria. According to morphology, physiological and biochemical characteristics of promising isolates, isolates CV6, V11, V16 and V69 identified as *Pseudomonas fluorescens*. Isolates V28 and V64 got best matches with *P. putida*. Isolates CV3 and V14 were closely match to *P. aeruginosa* and *Bacillus subtilis*, respectively (data not shown).

Plant growth promotion and root colonization in phyto-chamber. The effect of bacterial isolates on the growth of cucumber seedlings was monitored by measuring of length and dry weight of the roots and stems (Table 2). All strains except of *P. fluorescens* V69 promoted significantly plant growth in comparison to nontreated control. Strains CV6, V14 and V28 caused to increase the length of stem and root, considerably. In addition, Strains CV6, V14 and V64 showed the most effect on enhancing the foliar weight of cucumber seedling. However, both strains, CV6 and V14, exhibited the greatest effect to enhance root weight of seedling.

Study of root colonization *in vitro* demonstrated that some of the strains have more ability to root colonization than others. The population density on roots of plants treated with CV6 and V11 reached to 8.03 and 7.76 $\log_{10}$ CFU g^{-1} root respectively (Fig. 1). Strains V69 and V64 were not effective root- colonizers as their cell count was less than 5 $\log_{10}$ CFU g^{-1} root. It seems there is a correlation between root colonization talent of selected strains and their growth promoting ability, as in the most cases the strains with a highefficacy of root colonization resulted in increasing growth of plants (Table 2, Fig. 1).

Fig. 1. Colonization levels of eight bacterial biocontrol strains on the roots of the cucumber cultivar Soltan (in $\log_{10}$ CFU/g of root mass). **Fig. 2.** Suppression of *Phytophthora* root rot by selected strains in greenhouse. Isolates was applied as soil and seed treatments, percent of healthy plants was determined after 3 weeks. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

Greenhouse experiment. According to the results (Fig. 2), the use of bacteria as a soil treatment was more effective to suppress the disease than those as a seed treatment. The percentage of healthy plants in the presence of strains V14, V28 and V69 as soil treatments and *Phytophthora* inoculums was significantly more than those of infected control plants without bacterial inoculums. Mortality in pots treated with strain CV6 and V11 was very low and the percentage of healthy plants was 85.71 and 69.39%, respectively. Disease suppression by strain CV6 significantly was more than that Ridomil, whereas some strains such as V16 and V64 were not able to reduce the disease. Moreover, there was no significant difference between fresh and dry weight of plants treated with strain CV6 and non-infected control

plants (Data not shown). However, the application of mixture of bacterial strains as soil and seed treatments on pots inoculated with the pathogen resulting in falling down the most of the plants, so that only 4.83 percent of plants showed healthy condition (Fig. 2). In other hand, by using mixture of bacterial strains, numbers of healthy plants were significantly lower than those of infected plants without bacteria.

Discussion

In current study we isolated and screened the rhizobacteria from cucumber plants with antagonistic activity against *P. drechsleri*, the causal agent of cucumber root rot. The approach provided an opportunity to select effective biocontrol strains capable of antagonizing soil-borne pathogens in the same environment where they will be used commercially (Landa et al., 1997). Moreover, isolation of bacteria from rhizosphere of the target crop is essential for successful identification of potential biocontrol agents (Williams and Asher, 1996). Use of Gould's S1 and NA media culture for isolation of bacteria, provided a very efficient tool for screening of fluorescent pseudomonads. The selectivity of Gould's S1 medium is based on an iron limitation, and high glycerol and sucrose contents. It is also based on other compounds such as sodium lauroyl sarcosine (which inhibits the growth of Gram-positive bacteria) and trimethoprim (an antibiotic limiting the growth of non-fluorescent *Pseudomonas*). This medium allowed a high recovery of fluorescent *Pseudomonas* phenotypes from soil samples (Gould et al., 1985).

In this study, the strains with high ability of root colonization were more effective in suppressing of *Phytophthora* root rot on cucumber plants (Figs. 1 and 2). In the most cases, strains with a higher level of root colonization caused to higher growth of plants. Growth promotion of plants can increase their resistance to disease. Moreover, in many cases, variable performance of introduced rhizobacteria has been attributed to insufficient root colonization, a process whereby the bacteria inoculated onto seed attach to the root surface, and colonize the developing root system (Weller, 1988).

It is known that *in vitro* assays have certain limitations in that the biocontrol efficiencies may not be equally expressed under gnotobiotic (axenic) and *in vivo* conditions (Inam-ul-Haq et al., 2003). However, the *in vitro* assays conducted in our study (pathogen inhibition ability) were used to screen and select potential biocontrol agents and subsequently test their ability to suppress *Phytophthora* root rot under greenhouse conditions. Correlation analyses indicate that disease incidence is significantly correlated with the inhibition activity by non-volatile compounds and

Table 3. Pearson correlations between inhibition activity, root colonisation and disease suppression by beneficial bacteria

		Disease suppression	
		Seed treatment	Soil treatment
Inhibition zone	Volatile	−0.543**	−0.316 ^{ns}
	Non-volatile	0.406*	0.424*
Root colonization of plants		0.533**	0.262 ^{ns}
Disease suppression by seed treatment		1**	0.892**

* and** mean $P < 0.05$ and $P < 0.01$, respectively

^{ns} means non significant difference

the degree of root colonization by the bacterial inoculants (Table 3). Our results suggest that these variations in colonization levels may be affect the beneficial activity of the bacteria.

Among the bacterial genera used in biocontrol, *Pseudomonas* and *Bacillus* species are the most widely studied (Haas and Defago, 2005; Raaijmakers et al., 2002; Whipps, 2001). The mechanisms by which they protect plants against pathogens are diverse and include competition, antibiosis, degradation of fungal pathogenicity factors, and induced systemic resistance (Fallahzadeh et al. 2009; Haas and Deffago, 2005). Correlation analyses in this study indicate that disease incidence is significantly correlated with the inhibition activity by non-volatile compounds and the degree of root colonization by the bacterial inoculants (Table 3). Our results suggest that these variations in colonization levels may be affect the beneficial activity of the bacteria. Recent studies have shown that biosurfactant-producing *Pseudomonads* are very effective in controlling disease caused by Oomycete pathogens, including *Pythium* and *Phytophthora* species (De Jonghe, 2006; Stanghellini et al., 1996; Stanghellini and Miller, 1997; Stanghellini et al., 2000; Nielsen et al., 2006). Biosurfactants, including rhamnolipids and cyclic lipopeptides (CLPs), disrupt the membrane integrity of *Phytophthora* zoospores leading to complete elimination of infectious zoospore population within minutes (de Bruinjn et al., 2007; De Jonghe, 2006; Stanghellini and Miller, 1997; de Souza et al., 2003). Only limited attempts have been made to control *Phytophthora* infections using *Bacillus* species as a biological agent. One of our promising strains identified as *B. subtilis* v14 that significantly suppressed the disease in greenhouse studies, but it was not as effective as *Pseudomonas* strains. Lee et al. (2007) screened 41 isolates of *Bacillus* from rhizosphere of Red pepper that two of them exhibited more than 70% of ability to reduce the severity of *Phytophthora* blight on Red pepper.

Introduction of PGPR strains to phyllosphere, spermo-

sphere or rhizosphere may be moderately effective or sometimes totally ineffective under field conditions to control plant diseases (Duffy et al., 1996). Inefficacy of the strains under field conditions may be due to the variation in climatic conditions that suppress growth and survival of biocontrol agents (Guetsky et al., 2001). In addition both pathogen and biocontrol agents does not have similar ecological niche for their growth and survival. Hence the efficacy of biocontrol agents could be improved through the usage of compatible mixed inoculum of biocontrol agents which could have a consistent performance under diverse environmental conditions (Guetsky et al., 2001; Janisiewicz, 1996). For this propose we used a condition of bacterial isolates as a treatments for assessing their interaction on damping off disease. In the contrary, our study showed that the combination mixture of our biocontrol strains had a negative effect on disease incidence. It seems that interaction of isolates resulted in production of some deleterious metabolites that have a negative effect on plants. In summary, the results of this investigation are very promising for the selection of beneficial bacteria for organic and sustainable cropping systems.

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