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Characterization of *Pseudomonas fluorescens* strain CV6 isolated from cucumber rhizosphere in Varamin as a potential biocontrol agent

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Abstract

Antagonistic rhizobacteria, more specifically fluorescent pseudomonads and certain species of *Bacillus*, are known to control 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*, causal agent of cucumber root rot, *in vitro* and greenhouse condition. On the basis of dual culture assays, eight isolates were selected for root colonization, PGPR and greenhouse studies. Among these isolates, isolate CV6 exhibited the highest colonization on the roots and significantly promoted plant growth under in vitro condition. In greenhouse studies mortality in pots treated with strain CV6 was very low and percent of healthy plants were 85.71%. Based on biochemical and physiologic tests and 16SrDNA homology, this isolate identified as *Pseudomonas fluorescens*strain CV6. Strain CV6 was shown to have broad spectrum in vitro antibiotic activity against 11 additional plant pathogens. It was able to produce considerable amount of siderophore and acid indole-3-acetic acid (IAA). With the addition of tryptophan from 50 to 500 mg/ml the production of IAA was increased up to 15.3 µg/ml. The bacterium showed positive reactions for HCN, catalase, protease, and phosphatase, and negative for the production of pectinase, lipase and cellulase. Results from this study provide comprehensive information on nutritional requirements and biocontrol mechanisms of strain CV6 that can be used for commercial use and appropriate application of this bacterium.

Keywords: biological control, antagonists, PGPR, colonization, HCN, siderophore

Introduction

The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large active groups of bacteria (Villacieros et al., 2003) known as plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980). Plant growth promoting rhizobacteria are known to rapidly colonize the rhizosphere and suppress soilborne pathogens at the root surface (Rangajaran et al., 2003). These organisms can also be beneficial to the plant by stimulating growth (Bloemberg and Lugtenberg, 2001; Moeinzadeh et al., 2010). Among these organisms, Fluorescent *Pseudomonads* are considered to be the most promising group of plant growth-promoting rhizobacteria involved in biocontrol of plant diseases (Gardner et al., 1984; Moeinzadeh et al., 2010). They produce secondary metabolites such as antibiotics (Keel et al., 1992), phytohormones (Keel et al., 1992), volatile compound Hydrogen cyanide (HCN) (Defago and Haas 1990), and siderophores (Neiland, 1995). Plant growth-promoting ability of these bacteria is mainly because of the production of indole-3-acetic acid (IAA; Patten and Glick 2002), siderophores (Schippers et al., 1987) and antibiotics (Colyer and Mount 1984). Production of antibiotics such as phenazine-1-carboxylic acid (PCA), pyocyanin, 2-acetamidophenol, pyrrolnitrin, pyoluteorin, Phenazine-1-Carboxylic Acid, 2,4-diacetylphloro-glucinol, viscosinamide and tensin in different species of pseudomonads has been reported (Sunish Kumar et al., 2005). Production of siderophores has also been linked to the disease-suppressing ability of certain fluorescent *Pseudomonas* species (Loper and Buyer, 1991). The control of phytophthora root rot of soybean (Lifshitz et al., 1987), tobacco black root rot (Keel et al., 1989), fungal diseases of orange, lemon citrus roots (Gardner et al., 1984), and ornamental plants (Yuen and Schorth, 1986) has been demonstrated with fluorescent *Pseudomonads*. In the present study, a soil isolate CV6, identified according to chemotaxonomic characterizations as well as 16S rDNA gene sequence analysis. The possible growth-promoting and biocontrol potential of the afore-mentioned strain has been investigated by determining the secondary metabolites, viz. IAA, siderophore, and HCN production.

Material and methods

Microbial cultures and media

Fluorescent pseudomonad strains, *Pseudomonas fluorescens* PF5, *P. fluorescens* CHA0, *P. fluorescens* 2–79, and *P. aeruginosa* 7NSK2, and plant pathogenic fungal strains, were obtained from the collection of microbial culture (Department of Plant Protection, University of Tehran, Karaj, Iran). Stock cultures of bacteria were prepared for storage at -80°C in 1.5 ml vials by mixing equal volumes of 50 % glycerol and 24-h culture broth [from single colony inoculum, 25ml LB medium, 100ml flask, 130 rpm]. Fungal strains after growth on slants of potato dextrose agar (PDA) were maintained under liquid paraffin.

Isolation and screening of fluorescent bacteria

In April 2009, a total of 50 cucumber plants were collected from fields and greenhouses of Varamin, Tehran province of 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 placed in 9 ml of 0.1 M phosphate buffer (pH 7) supplemented with 0.025% Tween 20 and vortexed for 5 min. Then, 100 µl of first to forth diluents transferred to Gould's S1 medium (CA) plates and incubated at 27 °C for 2 days. Single colonies that fluoresced under UV light (366 nm) were selected and further cultured to establish pure cultures. The in vitro inhibition of mycelial growth of *Ph. drechsleri* by isolates was tested by using the dual culture technique as described by Ahmed Idris et al., (2007). Volatile compounds produced by bacteria were detected in a split plate experiment (Kraus and loper 1992). The isolates, which showed antagonistic activity in the dual culture and split plate assays, were tested for their ability to promotion of plant growth and colonize the roots by using the methods describe by Ahmadzadeh and Shrifi-Tehrani (2009). The ability of isolates to suppress *Phytophthora* damping-off of cucumber in greenhouse 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, 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 topped up with 30 g of the potting mix that had been blended with white bean seed inoculum of *Ph. drechsleri* and drenched with 20 ml of bacterial suspensions or a solution of 1 g L^{-1} Ridomil MZ-72 WP. Then pots were watered and arranged in a randomized complete block design and incubated in mentioned condition. The treatments in this experiment were: Pots inoculation with *Ph. drechsleri* and bacteria, inoculation with *Ph. drechsleri* and mix of bacterial strains, inoculation with *Ph. drechsleri* and Ridomil, inoculation only with *Ph. drechsleri* but no bacteria (CI) and control without inoculation (C). There were four pots for each treatment. After 2 and 3 weeks, percentage of the healthy plants was determined. After last assessment of disease severity, plants were removed from pots and fresh and dry weight parameters of them were measured.

Identification of strain CV6, on the basis of biochemical and physiological characterization

Strain CV6 was identified on the basis of tests for cytochrome oxidase (Kovacs, 1956), arginine dihydrolase (Thornley, 1960), gelatin liquefaction (Dye, 1968), production of diffusible non-fluorescent pigment (pyocyanin) on King's A medium (King et al., 1954), tobacco HR (Klement, 1963), nitrate reduction, growth at 4 and 41°C, levan production, (Shaad et al., 2001) and the ability to growth on carbon sources such as L-arabinose, D-galactose, trehalose, meso-inositol, sorbitol, L-tartrate was tested with the basal medium of Ayers et al., (1919).

16S rDNA gene sequencing

Genomic DNA of CV6 was extracted and purified according to the method described by Wang et al., (2001). 16S rDNA was amplified from CV6 genomic DNA with the primers PS16f 5´TGGCTCAGATTGAACGCTGGCGG-3´and PS16r5´-GATCCAGCCGCAGGTTCCCCT AC -3´. PCR amplification was carried out in 20 µl reaction mixtures containing 4 µl of lysed bacterial suspension, 1× PCR bovine serum albumin, 5% dimethyl sulfoxide, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.40 µM of each primer and 1.4 U of Taq DNA polymerase Amplifications were performed with a Corpet research g001 cycler. The initial denaturation (2 min at 94 °C) was followed by 30 PCR cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s) and a final extension at 72 °C for 10 min. The amplified DNA was purified using polymerase chain reaction (PCR) purification kit (Promega, Madison, WI, USA), diluted to 200 ng $\mu 1^{-1}$, and was sequenced at Microsynth Inc. (Balgach, Switzerland).

Assessment of antiphytopathogenic activity against several phytopathogens

The antagonistic ability of *P. fluorescens* CV6 was checked against 12 phytopathogenic fungi, according the method described by Ahmed Idris et al., (2007) Three 10 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 ºC for 24 h. A 6 mm agar disc from fresh cultures of *Ph. drechsleri* was placed at the centre of the PDA plate for each bacterial isolate and incubated at 27 ± 1 ºC 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:

$$\% \ Inhibition = \left[\frac{(R - r)}{R} \right] \times 100$$

Where, r is the radius of the fungal colony opposite the bacterial colony and, R is the maximum radius of the fungal colony away from the bacterial colony. There were three replicate in this assay.

Detection and production of siderophore

The siderophore production was determined by Chrome Azurol S (CAS) assay (Alexander and Zuberer, 1991). Culture of test strain CV6 was grown in M9 minimal medium (Sambrook et al., 1989) at 27°C to a density of 10^8 CFU/ml. Cells in late log phase were removed by centrifugation at 3000 rpm, and the filtrate was tested for siderophore on CAS agar plates. Also, the quantitative estimation was performed according to the method of Chambers et al., (1996). Specific tests were carried out for quatification of pyoverdine according to the method of Meyer and Abdallah (1978). There were three replicate in this assay.

Assay for indole acetic acid (IAA) production

Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986) at different concentrations of tryptophan (0, 50, 150, 300, 400 and 500 mg/ml). Bacterial cultures were grown for 48 h in nutrient broth (peptone, 5 g; yeast extract, 1.5 g; beef extract, 1.5 g; and NaCl, 5 g; each per liter).

Table 1. Mycelial growth inhibition of *Phytophthora drechsleri* by volatile and non-volatile metabolites produced by bacterial isolates

Bacterial isolates	% mycelial inhibition*		Bacterial isolates	%mycelial inhibition	
	non-volatile	Volatile		non-volatile	volatile
CV3	55.56a**	21.66ef	V10	31.1ikjl	15.33ij
CV6	45.56b	32.33b	V42	30kjlm	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 × 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 roots colonization of cucumber plants by rhizobacterial strains

	PGPR				In vitro root colonization
Bacterial isolate	Stem length	Root length	Foliage weight	Root weight	log cfu/g roots
CV3	9.46bc	7.5b	3.28bc	2.6cd	5.83
CV6	12.23a	9.4a	4.76a	3.87ab	8.03
V11	9.26c	7b	3.95ab	3.58abc	7.76
V14	12.96a	9.5a	4.83a	4.39a	5.1
V69	6.56d	5.66	2.6cd	1.53e	4.75
V28	12.53a	7.93b	4.05ab	3.02bc	6.86
V16	11.76	9.26a	4ab	3.58abc	6.98
V64	10.46bc	7.56b	4.35a	3.02bc	4.33
C	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.

Table 3. Pearson correlations between antagonistic parameters of bacteria and disease incidence.

Antagonistic parameters	disease incidence
Non-volatile compounds	579*
Volatile compounds	316n
Root colonization	721**

**, Correlation is significant at the 0.01 level. *, Correlation is significant at the 0.05 level. n, Correlation is nonsignificant

Table 4. Biochemical and physiologic characteristics of *P. fluorescence* CV6

Test	Reaction
Dilfusible non-fluorescent pigment	-
Non-dilfusible, non-fluorescent Pigment	-
Levan	+
Oxidase	+
Aginine dihydrolase	+
Pectolytic Activity	-
Tobacco HR	-
Growth @ 41°C	-
Growth @ 4C	+
Nitrate to N_2	+
Gelatin liquefaction	+
Growth on:	
L-arabinose	+
D-galactose	+
Trehalose	+
Saccharate	+
Butyrate	-
Valerate	-
Azalate	+
Sorbitol	+
Meso-inositol	+
Adonitol	-
Propylene glycol	-
Ethanol	-
Geraniol	-
Testosterone	-
Phenyl acetate	-
Butylamine	-
Nicotinate	-
Trigonelline	+
Lecithinase	+

Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink color indicates IAA production. Optical density was taken at 530 nm with a spectrophotometer (T70+ UV/VIS, PG instruments). Concentration of IAA produced by cultures was measured with the help of standard graph of IAA (Hi-media) obtained in the range of 10–100 mg/ml. There were three replicates in this experiment.

Assays for hydrolytic activity and detection of HCN

Cellulase and pectinase production by the strain was determined as described by Cattelan et al., (1999). M9 medium agar (Miller, 1974) amended with 10 g of cellulose and 1.2 g of yeast extract per liter of distilled water was used to test the cellulase activity, in which a clear halo after 8 days of incubation of the colonies at 28°C was considered as positive for cellulase production. For determining the pectinase activity, 10 g pectin plus 1.2 g yeast extract was amended in M9 medium agar and the plates were flooded with 2 M HCl after 2 days of incubation at 28°C. Clear halos around the colonies were considered as positive for pectinase production. Lipase medium was used to check the lipase production, which was indicated by clear zones surrounding the colonies (Smibert and Krieg 1994). Phosphatase activity was determined by development of a clear zone in Pikovskaya's agar medium after 2–5 days incubation of assay plates at 28°C (Pikovskaya 1948). Proteolytic activity was determined by using skimmed milk agar (pancreatic digest of casein 5 g, yeast extract 2.5 g, glucose 1 g, 7% skim milk solution 100 ml, agar 15 g dissolved in 1 l distilled water) (Sunish Kumar et al. 2005). After 2 days incubation at 28°C, a clear zone around the cells indicated positive proteolytic activity (Smibert and Krieg 1994). Production of HCN by CV6 on KB media with different concentrations of iron was observed according to the method of Lorck (1948). There were three replicates in all assays.

Results

Isolation and screening of fluorescent bacteria

According to the table 1 (isolates with no inhibition were not included), among of the 144 bacterial isolates, 23 isolates exhibited more than 30% inhibition of mycelia growth of *Ph. drechsleri* by non-volatile inhibitors. The maximum inhibition achieved by any isolate was 55.56% (CV3). Control plates not treated with the bacterial isolates were completely covered by the phytopathogens showing no inhibition (Table1). In tests for detection of volatile metabolites by isolates, eight isolates exhibited a more than 25% of mycelia growth of *Ph. drechsleri*. The maximum inhibition achieved by any isolate was 39.33% (V69). Isolates CV3, CV6, V11, V14, V69, V28, V16 and V64 were amongst the most effective isolates against *Ph. drechsleri* and selected for greenhouse studies. Isolates V16 and V64 produced noticeable amount of the volatile metabolites which inhibited mycelial growth by more than 30% (Table 1). The effect of bacterial isolates on the growth of seedlings was monitored by measuring of length and weight of the root and stem (Table 2). All strains except V69 promoted plant growth significantly compared to the untreated 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 to enhance the foliar weight of cucumber seedling. However, both strains, CV6 and V14, exhibited the greatest effect to enhance root weight of seedling. In vitro root study of the colonization, 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 (Table 2). Strains V69 and V64 were not effective colonizers as their cell count was less than 5 $\log_{10}$ CFU g^{-1} root. Statistical analysis exhibited that there is a significant correlation between root colonization talent of strains and their growth promoting ability, as in most cases the strains with a higher efficacy of root colonization resulted in the more growth of plants (Table 3).

In greenhouse studies, mortality in pots treated with strain CV6 and V11 was very low and three weeks after treatment of pots with pathogen, percent of healthy plants in these treatments was 85.71 and 69.39%, respectively (Figure 1). Percent of healthy plants in pots treated with CV6 significantly was more than those treated with Ridomil. Moreover, there was no significant difference between fresh and dry weight of plants treated with isolate CV6 and non-infected control plants (Data not shown).

Table 5. Broad-spectrum antifungal activity of *Pseudomonas fluorescence* CV6 against phytopathogenic fungi

Pathogen	Host	Disease	% mycelial inhibition
Rhizoctonia solani AG4	Bean	Bean damping-Off	55.51
Magnaporthe grisea	Rice	Sheath blight	85.82
Sclerotinia sclerotiorum	Sunflower	Sclerotinia wilt	75.87
Macrophomina phaseolina	Cotton	Charcoal rot	65.48
Botrytis cinera	Tomato	Grey mildew	57.53
Colletotrichum gloeosporioides	Citrus	Anthracnose	79.43
Alternaria solani	Tomato	Early blight	15.32
Bipolaris sorokiniana	Barley	Crown rot	68.09
F. oxysporum f. sp. *lycopersici*	Tomato	Fusarium wilt	10.02
F. oxysporum f. sp. *vasinfectum*	Cotton	Fusarium wilt	45.32
Monilinia fructicola	Peach	Brown rot	76.34

Table 6. Detection and quantification of siderophore produced by *Pseudomonas fluorescence* CV6

strains	CAS plate assay	CAS activity (OD at 630 nm)
PF 5	+	0.169d
CHA0	+	0.179cd
2–79	+	0.194bc
7NSK2	+	0.310a
CV6	+	0.202b

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

Table 7. Hydrogen cyanide production by *Pseudomonas fluorescence* CV6 and reference strains on KB media with glycin and different concentrations of iron

	Different concentrations of iron				
Bacterial strains	0 µM	25 µM	50 µM	100 µM	200 µM
CV6	+	+	++	++	++
CHA0	+	+	++	+++	++++
7NSK2	-	-	-	-	-
Control	-	-	-	-	-

Identification and characterization of strain CV6

The result of biochemical and physiologic testing is listed in Table 4. Strain CV6 showed positive reactions of oxidase, gelatinase, nitrate reductase, arginine dihydrolase and levan production but it was negative for tobacco HR and growth at 41 °C. Primers, PS16f and PS16r amplified a complete DNA fragment of 1.49 kb 16S rDNA when the total genomic DNA of CV6 was used as template in PCR. The 16S rRNA gene sequence of this strain exhibited 99.1 % similarity to that of *P. fluorescens.*

Antiphytopathogenic activity of CV6 against several phytopathogens

P. fluorescens CV6 was shown to have broad spectrum in vitro antibiotic activity against 11 additional plant pathogens (Table 5). *Fusarium oxysporum* f. sp. *lycopersici* and *Alternaria solani* were the only pathogens tested that were not inhibited.

Detection and production of siderophore and IAA

The strain CV6 also showed the production of siderophore on CAS agar plates. Appearance of a reddish-brown zone surrounding the colony on CAS agar plates clearly suggests siderophore production by this strain. Strains *P. fluorescens* PF5, *P. fluorescens* CHA0, *P. fluorescens* 2–79, and *P. aeruginosa* 7NSK2 were used as reference strains. CAS activity by CV6 was significantly more than those of all the reference strains except 7NSK2 (Table 6). Specific tests were carried out for quantification of pyoverdine. Pyoverdine production by strain CV6 was significantly higher than those of CHA0 and PF5 but not those of other reference strains (Figure 2).

Strain CV6 was tested for the quantitative estimation of IAA in the presence of different concentrations of tryptophan (Figure 3). With no addition of tryptophan, production of IAA was not observed. With the addition of tryptophan from 50 to 500 mg/ml the production of IAA was increased up to 15.3 µg/ml.

Assays for hydrolytic activity and detection of HCN

A remarkable change in color from yellow to reddish-brown with strain CV6 compared with the control indicates HCN production. The data revealed a decreased level of HCN production under low iron conditions (Table 7). Production of HCN by strain CHA0 was drastically dependent on iron concentration in the medium. Strain 7NSK2 was not able to produce HCN. Strain CV6 showed positive reactions for catalase, protease, and phosphatase, and negative for the production of pectinase, lipase and cellulase.

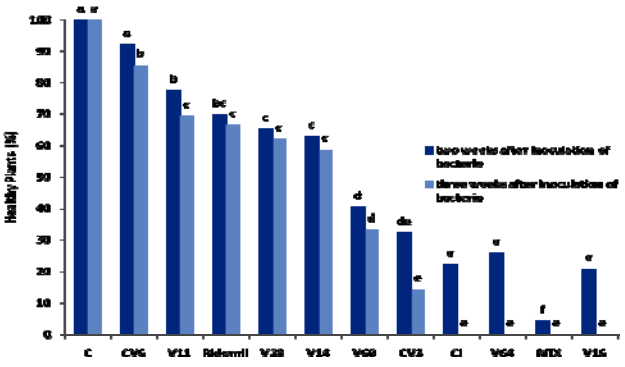

Fig 1. Suppression of *Phytophthora* root rot by selected strains in greenhouse. Isolates was applied 10 days after sowing and after 2 and 3 weeks, percent of healthy plants was determined. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test

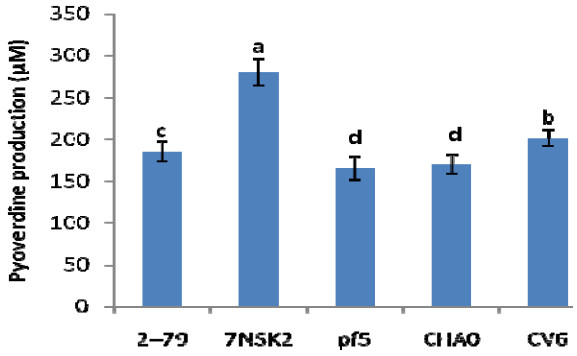

Fig 2. Pyoverdine production by CV6 and reference strains under low iron conditions in succinate medium. All values were significantly different at 1%, as determined by variance analysis followed by Duncan's test. Bars indicate standard error.

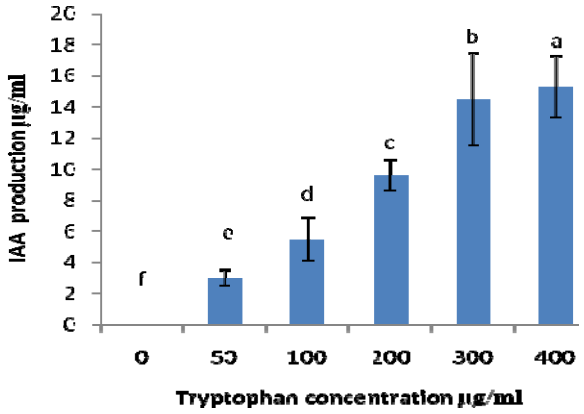

Fig 3. Auxin production by CV6 in TSB medium with different concentrations of tryptophan. All values were significantly different at 1%, as determined by variance analysis followed by Duncan's test. Bars indicate standard error.

Discussion

Over the last decades, many studies have reported on natural activity of some fungi and bacteria against pathogens, and this is considered as a very appealing alternative to the use of chemical fungicides (Gerhardson 2002; Welbaum et al. 2004). The rhizosphere microorganisms, especially fluorescent pseudomonads, have exceptional ability to promote the growth of host plant by various mechanisms (O'Sullivan and O'Gara 1992). Also these bacteria have various mechanisms to suppress plant diseases including production of antibiotics, efficient root colonization and production of powerful siderophores (O'Sullivan and O'Gara 1992; Hass and Defago 2005). In our study, 144 bacterial strains were isolated from soil samples collected from agricultural fields of Varamin regione of Iran. All isolates subjected to several screening procedures for selecting the best strain for effective biocontrol of phytopathogens. Of all the strains tested, isolates CV3, CV6, V11, V14, V69, V28, V16 and V64 were amongst the most effective isolates against *Ph. drechsleri* and selected for further investigations. However, the main problem of biological control is its low consistency and reliability under field conditions due to a high variability in efficacy (Ojiambo, 2006). The complex *in situ* condition in the natural rhizosphere influences survival, growth and production of secondary metabolites by the biocontrol strains. One of the important measures in developing reliable biocontrol systems is to identify the biocontrol mechanisms and nutritional and physiological requirements of biocontrol agents to provide these optimum conditions in plant cultivation systems. Detailed characterization of a biocontrol agent is an essential step towards achieving this goal, and towards improving the level and reliability of its biocontrol activity. For example nutritional composition of root exudates in different plants is variable (Lugtenberg and Dekkers, 1999) and knowing the nutritional requirements of biocontrol agents will help us to introduce them to appropriate pathosystems. Moreover knowing the mechanisms of a biocontrol agent allow us to improve its efficacy. In the percent study, the ability of these strains for promotion of plant growth and efficient root colonization was investigated *in vitro*. Strains V14 and CV6 were able to significantly enhance all measured plant growth parameters. The strains with higher ability of root colonization were more effective in suppressing *Phytophthora* root rot on cucumber plants (Table 2 and Figure 1). In all most cases, strains with a higher efficacy of root colonization caused to higher growth of plants. In several works, 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). On the other hand, the results of greenhouse studies revealed that, strains with higher ability of root colonization were more effective in suppressing *Phytophthora* root rot on cucumber plants. On the basis of these in vitro and in situ screening procedures, we selected strain CV6 for further investigations and characterization. This strain was identified as *P. fluorescens* using the biochemical and physiological tests as well as 16S rDNA sequence analysis. This procedure in identification is more accurate than the traditional methods (Weisburg, 1991; Guo et al., 2007). Strain CV6 revealed a broad spectrum antifungal activity against different phythopathogens and it can be used as an effective biological control candidate against devastating fungal pathogens that attack various plant crops. Results from this

study exhibited that strain CV6 is partly powerful producer of IAA. There was an increase in the level of IAA with the increasing concentration of tryptophan (50–400 mg/ ml) similarly to the results reported by Barazani and Friedman (2000). IAA is responsible for division, enlargement and differentiation of plant cells and tissues; it plays a major role in xylem- and root formation (Davies, 1995). Auxin biosynthesis is also widespread among soil- and plant-associated bacteria. Moreover, its production is a determinant trait both for plant growth promoting rhizobacteria (PGPR) and plant pathogens (Patten and Glick, 1996, 2002). Another important trait of PGPR, that may indirectly influence the plant growth, is the production of siderophores. Siderophores chelates iron and other metals contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats (Hofte et al., 1992; Loper and Henkels, 1997). Siderophores may directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria. Results from the qualitative and quantitative estimations of siderophore by CV6 showed that, it's a powerful producer of siderophores. Strain CV6 showed positive reactions for catalase, protease, phosphatase and HCN. Solubilization of phosphorous because of phosphatase were found to significantly increase crop yield (Brown 1974) and bacterial strains showing catalase activity must be highly resistant to environmental, mechanical, and chemical stresses (Joseph et al., 2007). Production of HCN by pseudomonads is associated with biological control of the black root rot of tobacco, but other workers observed that it can have a detrimental effect on plant growth (O'Sullivan and O'Gara, 1992).

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