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Biocontrol of rhizome soft rot (*Pectobacterium carotovorum)* on valerian by *Pseudomonas* spp. under *in vitro* and greenhouse conditions

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Pectobacterium carotovorum is dreadful causal agent of potato and several agricultural products soft rot and able to damage crops seriously. Recently rhizome soft rot of valerian (*Valeriana officinalis*) has been reported in Iran. Actually, there are no efficient bactericides used to protect valerian against *Pectobacterium*. Biological control could be an interesting approach to manage this disease. In this study, the potential of forty isolates of *Pseudomonas* from Valerian rhizosphere for biological control of *P. carotovorum* were investigated. Sixteen bacterial isolates showed inhibitory effects against *P. carotovorum*. They exhibited antimicrobial activity and prevented the growth of pathogen significantly. Isolates 1 and 3 with 3.43 and 3.83 cm had maximum inhibition zone, respectively. Based on the results of *in vitro* experiments, seven isolates from the *Pseudomonas* genus were selected and studied on valerian growth index under greenhouse condition. Valerian transplants were treated with the isolates. Good prevention of the disease was obtained using the rhizosphere antagonists. The results revealed that two isolates, 1 and 3, were able significantly to increase the fresh and dry weight of root. Detection of *phlD* gene using PCR procedure indicated that these two isolates had *phlD* gene.

Key words: Antagonist, *phlD*, Soft rot, *Pseudomonas*, *Valeriana officinalis*

Introduction

Valeriana officinalis has been used more than 1000 years as a valuable medicinal plant and its roots contain several herbal materials. The roots and rhizomes of *Valeriana* spp. are used in traditional medicine of many cultures (Circosta *et al*., 2007). They contain two main groups of constituents: sesquiterpenes of the volatile oil (valerenic acid and its derivatives, valeranone, valeranal, kessyl esters) and valepotriates (valtrate, didrovaltrate, acevaltrate,

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isovaleroxy hydroxyl valtrate), in addition to other constituents such as flavonoids, triterpenes, lignans, alkaloids (Goppel and Franz, 2004). Valerian essential oils or extracts are used in the formulations of personal care products, cosmetics, aromatherapy, and veterinary practices. *V. officinalis* is one of the most popular herbal supplements for the treatment of anxiety and insomnia (Murphy *et al*., 2010).

Soft rot disease occurring on valerian rhizomes was reported in 2006 from the Medicinal Plants Institute of Karaj, Iran. Plant pathogenic bacteria produce pectolytic and macerating enzymes, most bacteria causing soft rot on many crops belong to certain *Pectobacterium* species and subspecies such as *Pectobacterium carotovorum* (Alippi *et al*., 1997; Catara *et al*., 2001). Because the pathogen invades the inner part of the plants, conventional chemical products, such as copper compounds, may not provide adequate control for the disease. To solve this problem, many scientists have focused on alternative solutions such as biological control in conventional and ecological (organic) agriculture (Aysan *et al*., 2003).

Biological control of soil-borne plant diseases by strains of *Pseudomonas* has been intensively studied (Cook *et al*., 1995). Most promising biocontrol strains produce antimicrobial metabolites that are relevant for their biocontrol activity (Keel and Defago, 1997) and are toxic to a range of different soil microorganisms' *in vitro* (Keel *et al*., 1992; Nowak-Thompson *et al*., 1994). In many biocontrol systems that have been studied, one or more antibiotics play a role in disease suppression. The fact that antibiosis is a common mechanism of biocontrol may be due to a bias in choice of organisms for study (Handelsman and Stabb, 1996).

Pseudomonas spp. is aerobic, gram-negative bacteria, ubiquitous in agricultural soils, and is well adapted to grow in the rhizosphere. Fluorescent pseudomonads are aggressive rhizosphere colonizers and produce a wide range of antimicrobial compounds (Kloepper, 1983). Most known biocontrol agents in the genus *Pseudomonas* produce antifungal metabolites: phenazines, pyrrolnitrin, pyoluteorin, phloroglucinols, cyclic lipopeptides, and hydrogen cyanide (Haas and Defago, 2005). Strains of *P. fluorescens* that produce the polyketide antibiotic 2,4diacetylphloroglucinol (2, 4-DAPG) are some of the most effective PGPR for controlling root and seedling diseases. The 2, 4-DAPG biosynthesis locus contains six genes, *phlA*, *phlB*, *phlC*, *phlD*, *phlE*, and *phlF*, coding for the regulation, synthesis, and putative export of 2, 4-DAPG (Bangera and Thomashow, 1999). The purpose of this study was the detection of fluorescent pseudomonads, which are able to control rhizome soft rot of valerian, under in vitro and greenhouse conditions.

Material and methods

Bacterial isolates

Forty bacterial isolates used in this study were isolated from the rhizosphere of valerian medicinal plant field of Jahad Daneshgahi Research Institute of Karaj (Table 1). *Pectobacterium carotovorum* strain was used in this study obtained from the laboratory of Plant Pathology at the College of Agriculture, University of Tehran, Iran.

In vitro antagonistic activity

For determination of antagonistical activity, the method of Weller and Cook (1993) was used. Sterile paper disks (5mm in diameter) were impregnated with each of the bacteria suspensions 1×10^9 concentration inoculated on NA medium at three replicates. After 48 h incubation at 28 ^oC, bacterial colonies were cleaned with cotton and killed with chloroform vapor. After aeration of Petri dishes, a suspension of strain *Pc* (10^9 cell/ml) was sprayed on this medium and incubated at 28 ^oC for 2 days. Antagonistic activities were evaluated by measuring (as cm) the widths of the clear zones surrounding the spot cultures. Control plates were inoculated only by pathogen.

Detection of phlD gene with gene-specific primers

Four isolates of fluorescent *Pseudomonas* that had the greatest inhibition halo zone were selected. To detect *phl*D gene, the primer *Phl*2a (5'-GAGGACGTCGAAGACCACCA-3') and the reverse primer *Phl*2b (5'-ACCGCAGCATCGTGTATGAG-3) were used. DNA preparation was carried out according to Wang *et al*. (2001). To extract genomic DNA from pure cultures, bacterial cells were grown overnight at 27^oC in King's B. PCR reaction were performed at 20µL mixture including 2 µL PCR (10x) buffer, 2 µL $MgCl_2$, 2 µL dNTPs, 1 µL from each primer, 4 µL lysed cell suspension, 0.3 unit Taq DNA Polymerase. Amplification was performed with a GP001 thermal cycler. The thermo cycler (PCR) program included an initial denaturation at 94^oC for 4 min followed by 35 cycles of 94^oC for 1 min; 60^oC for 1 min; 72^oC for 1 min; and then a final extension at 72^oC for 5 min.

PCR products were separated by electrophoresis on agarose gel (1%) with TAE (1x) buffer at 75 volts for 1hr approximately quantified according to the intensity of bands on the gel and then were stained and examined with GELRED. *P. fluorescenc* CHA0 was used as the positive control and water was selected as negative control. To estimate the length of amplified DNA

fragments, 1kb genomic marker DNA ladder gene was used. All of the materials used in PCR reaction mixtures and primers, were obtained from CinnaGen Inc, Iran.

Volatile metabolites production

This test was performed via modified method of Fiddaman and Rossall (1993). Antagonistic bacterial suspension at 1×10^9 cell/ml were spread uniformly on the NA medium and incubated for 24h at 26^{o}C. Filter papers with 5mm diameter, impregnated with pathogenic bacteria suspension, were placed in three parts the medium. Petri dishes containing antagonistic bacteria were placed on Petri dishes of pathogenic bacteria. The plates were sealed with parafilm to inhibit exit of volatile. Thus, only the volatile metabolites of antagonistic bacteria could have affect on the pathogen. One plate without inoculation of antagonistic bacteria was considered as the control plate. After two days incubation at 26^{o}C, the reduction percent of the pathogen growth was measured. This experiment was conducted in a completely randomized design with three replications.

Greenhouse studies

Greenhouse experiments were conducted in the summer of 2010 in Jahad Daneshgahi Medicinal Plant Research Institute, Karaj, Iran. Six strains of bacteria with the greatest *in vitro* inhibition were tested in a greenhouse on valerian plants to evaluate their control ability bacterial wilt *in planta*. Valerian transplants were planted in 10 cm depth and covered with soil. From 24 h-cultures of each antagonist 1×10^9 cell/ml was prepared by using a spectrophotometer. The suspensions were inoculated on the roots. After 30 min; the 1×10^6 cell/ml suspension of the pathogen inoculated on roots. A randomized complete block design was used with six replications per treatment. Each replicate contained two plants. Control plants were treated with pathogen. Plots were irrigated by overhead sprinklers.

Measurement of plant growth parameters

Four months after inoculation, plants were removed from the soil and washed. Fresh and dry weights of roots were measured. The averages of treatments were compared using the least significant difference (LSD).

Statistical analysis

All data were analyzed by SAS 9.1 software (SAS institute, USA) and GLM procedure. Least significant difference values were used at $P \leq 0.01$. All experiments were conducted in three replicates.

Results

A total of 40 rhizospheric bacteria were screened against *Pc* strain, 16 strains had inhibitory effects that ranged from an average of 1.20 -3.83 cm radius of inhibition zone. The antagonistic effects of the candidates on the pathogen are shown in Table 1. Six isolates with inhibition zones of 1.86- 3.83 cm radius were selected and used for further study. All of the six isolates were fluorescent under UV light when cultured on KB medium, and were identified as fluorescent pseudomonads based on the Bergey's Manual.

Table 1. In vitro inhibition (cm) of growth of Pc strain by antagonistic bacterial isolates. NA media incubated at 28 ^{o}C for 48 hr. Comparison of inhibition halo diameter of isolates and amount of reduction diameter of pathogen colony by volatile metabolites of isolates

Isolates	Genus	Average diameter of inhibition halo zone (cm)	Average diameter of pathogen colony in volatile metabolites test
1	*Pseudomonas*	3.43333 b	0.66667 ghi
2	*Pseudomonas*	1.33333 gh	0.76667 defg
3	*Pseudomonas*	3.83333 a	0.63333 hi
4	*Pseudomonas*	1.96667 e	0.60000 i
5	*Erwinia*	0.00000 i	0.90000 abc
6	*Pseudomonas*	2.06667 e	0.63333 hi
7	*Pseudomonas*	2.46667 d	0.76667 defg
8	*Pseudomonas*	0.00000 i	1.00000 a
9	*Pseudomonas*	2.80000 c	0.70000 fghi
10	*Pseudomonas*	2.30000 d	0.73333 efgh
11	*Pseudomonas*	1.20000 h	0.60000 i
12	*Erwinia*	0.00000 i	1.00000 a
13	*Bacillus*	0.00000 i	0.80000 cdef
14	*Xanthomonas*	0.00000 i	0.83333 bcde
15	*Xanthomonas*	0.00000 i	0.73333 efgh
16	*Pseudomonas*	0.00000 i	0.70000 fghi
17	*Xanthomonas*	0.00000 i	1.00000 a
18	unknown	0.00000 i	0.90000 abc
19	*Bacillus*	0.00000 i	0.90000 abc
20	*Pseudomonas*	0.00000 i	0.86667 bcd

21	*Bacillus*	0.00000 i	0.86667 bcd
22	*Bacillus*	0.00000 i	1.00000 a
23	*Xanthomonas*	0.00000 i	1.00000 a
24	*Pseudomonas*	0.00000 i	0.90000 abc
25	*Pseudomonas*	1.23333 gh	0.93333 ab
26	*Pseudomonas*	2.00000 e	0.80000 cdef
27	*Pseudomonas*	2.06667 e	0.76667 defg
28	*Pseudomonas*	1.43333 fg	0.86667 bcd
29	*Agrobacterium*	1.56667 f	0.76667 defg
30	unknown	2.00000 e	0.63333 hi
31	*Erwinia*	0.00000 i	0.90000 abc
32	*Pseudomonas*	0.00000 i	0.83333 bcde
33	*Bacillus*	0.00000 i	0.80000 cdef
34	*Pseudomonas*	0.00000 i	0.90000 abc
35	*Bacillus*	0.00000 i	0.90000 abc
36	*Pseudomonas*	1.86667 e	0.63333 hi
37	*Pseudomonas*	0.00000 i	0.80000 cdef
38	*Bacillus*	0.00000 i	0.73333 efgh
39	*Bacillus*	0.00000 i	0.80000 cdef
40	*Pseudomonas*	0.00000 i	0.83333 bcde
control		0.00000 i	1.00000 a

Means in the same column followed by the same letter did not differ significantly at $P \leq 0.01$; Control plates were inoculated only by pathogen.

The method of in vitro (test chloroform vapor) indicated that the strains differed in inhibiting the pathogen. Isolates 1 and 3 exhibited significantly greater inhibition zones than the other isolates (Figure 2).

The results of the PCR analysis with primers phl2a and phl2b indicated that a DNA fragment approximately 745 bp in size was obtained in three strains 1, 3 and 6 (Figure 1). For comparative purposes, strain CHA0 was used as a positive control for the detection of *phlD* gene. Correlation analysis was used to identify whether there was a link statistically between inhibition of bacteria growth on plates and production of the antibacterial metabolites by the antagonistic isolates or the existence of *phlD* gene in these bacteria. The results revealed that collectively there was obvious link between them. In strains such as 1, 3 and 6 a correlation observed between the presence of *phlD* genes and antibacterial activities.

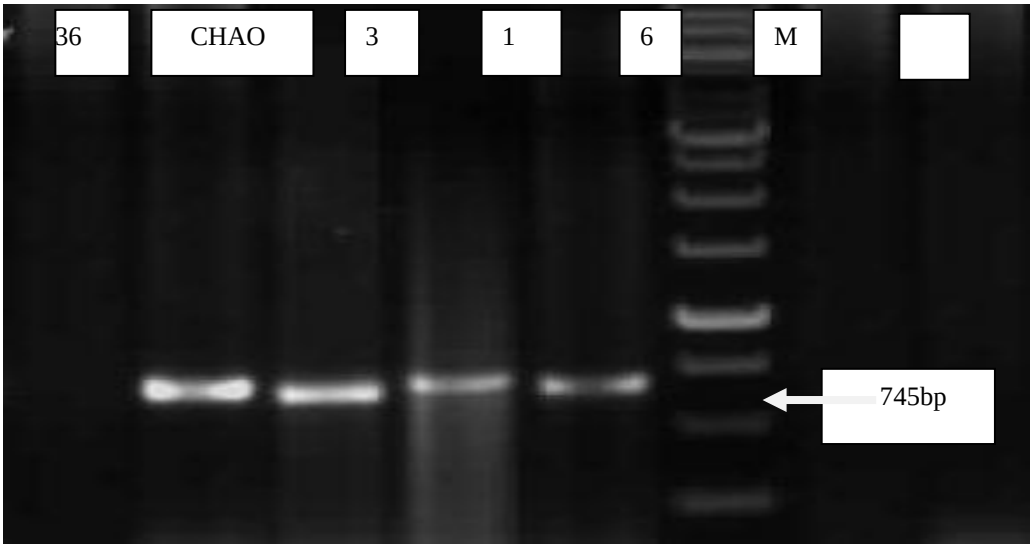

Fig. 1. PCR amplification of the *phlD* gene for fluorescent *Pseuodomonas*. Lanes M, 1-kb ladder; C, negative control (lysis buffer); CHAO, positive control.

The result of volatile metabolites on NA medium against *Pc* showed that 87.5% of isolates decreased the pathogen colony diameter. Based on average comparison, the highest reduction of colony diameter of pathogen was 40% and caused by isolates 4 and 11.

The in-planta efficacy of selected antagonists for the control of Pc wilt in valerian plants was evaluated under greenhouse conditions. The antagonistic isolates monitored by measuring plant biomass (fresh and dry weight) showed variation among plants treated with antagonists and the untreated control. There were significant differences among treatments for both dry and fresh weight of valerian plants. The effects of the six antagonists 1, 3, 6, 10, 27 and 36 on the transplants can be seen in Table 2.

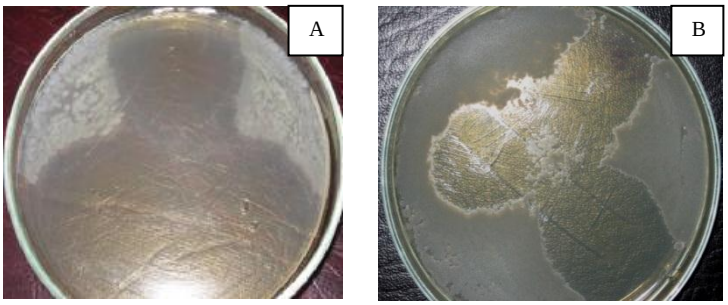

Fig. 2. Inhibition halo is caused by isolates 1 (A) and 3 (B) against *Pc*.

Table 2. Effect of selected antagonists on valerian root dry weight as compared to control under greenhouse conditions

Isolates	**Average root dry weight per plant (g)**
1	6.3000 bc
2	5.1133 bcd
3	9.0233 a
6	4.1000 cde
10	4.1700 cde
27	6.4700 bc
36	6.6300 ab
Control	2.6567 e

Significant differences among some of the treated valerians after four months were observed. Isolates 1, 3, 6 and 36 had the highest effect on promotion of fresh weight of valerian roots, whereas isolate 27 had the least effect and has no significant difference with the control (Table 3).

Table 3. Effect of antagonistic bacterial isolates on valerian root fresh weight; Control plants were treated with pathogen.

Isolates	Average root fresh weight per plant (g)
1	11.862 bc
2	6.520 bcd
3	14.007 a
6	10.242 cde
10	5.468 cde
27	7.645 bc
36	8.167 ab
Control	4.147 e

Values within the same column followed by the same letter do not differ significantly (a = 0.05)

Discussion

The soft rot bacterium (*P. carotovorum*) is a pathogen of many plant species, affecting crops in subtropical and temperate areas worldwide (Catara *et al*., 2001). Several studies have shown that the effect of PGPR on plant growth can be associated with the inhibition of deleterious microorganisms (Kloepper and Schroth, 1981). Biological control of plant diseases has no harmful effects upon nature. More attention to non-chemical control procedures is the necessary.

In the present research, antagonistic ability of forty bacteria isolated from valerian rhizosphere against *Pc* was investigated. The results on NA medium

showed that 40 percent of isolates had the ability to produce an inhibition halo against the *Pc* and reduction of pathogen colony diameter. The maximum diameter of inhibition zone was 3.83 and 3.43 cm, belonging to the isolates 1 and 3, respectively. Minimum diameter of inhibition zone was 1.20 cm belonging to isolate 11. The antibacterial activity of each isolate under *in vitro* condition is shown in Table 1.

According to Cook *et al*. (1995), antibiotic-producing PGPR have been studied widely during the last decade, and special attention has been paid to 2, 4-DAPG-producing *Pseudomonas* spp. because of their ability to control a wide variety of soil-borne plant pathogens. In other words, antibiotic production has recently been recognized as an important feature in the biological control of plant diseases by fluorescent pseudomonads. The presence of *phl* genes can be used as a suitable marker for the screening and selection of bacteria with potential biocontrol activity. In this study, we used PCR analysis to detect 2, 4-DAPG-producing *Pseudomonas* isolates based on the amplification of *phlD* gene sequences. Occurrence of the *phlD* gene was demonstrated through the detection of a 745 bp DNA fragment in three strains 1, 3 and 6 as well as the control strain CHA0, using a PCR assay with primers phl2a and phl2b. Overall, correlation analyses reveal significant links between inhibition of bacterial growth on plates and production of the antibacterial metabolites by the fluorescent *Pseudomonas* spp. or the existence of *phlD* genes in these bacteria.

Hydrogen cyanide produced by *P. aeruginosa* had the characteristics of a secondary metabolite which played an important role in biocontrol (Castric, 1975). Fluorescent pseudomonads are aggressive rhizosphere colonizers and produce a wide range of antimicrobial compounds (Kloepper, 1983). The result of volatile metabolites on NA medium against *Pc* strains revealed significant links between inhibition of bacteria growth on plates and production of the volatile extracellular metabolites by the fluorescent *Pseudomonas* spp.

Our results suggested that isolate number 3 is a beneficial antagonist which can reduce soft rot disease caused by *P. carotovorum* on valerian in greenhouse experiments and may be a useful element for the disease management in valerian cultivated region (Figure 3).

In as much as roots containing soft rot pathogen are hazardous in valerian planting, preinoculation of antagonist seedlings may provide a convenient strategy for the production of *Valeriana officinalis* plantlets resistant to soft rot on a large scale.

In conclusion, the results of this study showed that biological control is an efficient procedure for control of soft rot and fluorescent pseudomonads are suitable agents for controlling this disease.

Fig. 3. Differentiation between growths of treated plants with isolate 3 and infected control; A, treatment with pathogen; B, treatment with antagonist (Isolate 3) and pathogen.

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