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Isolation and characterization of rhizobacteria and their effects on root extracts of *Valeriana officinalis*

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Abstract

Plant growth promoting rhizobacteria (PGPR) are a group of microorganisms which can enhance growth parameters of host plants and can be used as biofertilizers. Valerian (*Valeriana officinalis*) is a perennial herb which is used as a medicinal plant. In the current study 40 colonies of bacteria were isolated from the rhizosphere of valerian by growing in various enriched and selective media including S1 and King B, crystal violet agar and methyl red agar. Furthermore, the ability of bacteria to produce siderophore, indoleacetic acid (IAA), hydrogen cyanide (HCN), lipase and protease were tested *in vitro*. Identification of isolates was performed by using Bergey's manual of systematic bacteriology. Additionally, the effects of seven isolated bacteria (belong to *Pseudomonas* genus) that showed a high potential of siderophore, IAA, HCN, lipase and protease production on the quantity of root extracts were investigated under greenhouse condition. Results showed that the population of *Pseudomonas* was the highest in comparison to other genera in the rhizosphere of plant. Isolated bacteria could mostly produce siderophore, lipase, HCN and protease. Two isolates (belong to *Pseudomonas* genus) significantly increased the amount of valerenic acid in the root extract. The results revealed that PGPR increased shoot length and could also enhance quantity of root extract.

Keywords: Plant growth promoting rhizobacteria, Root extract, Siderophore, Valerenic acid, Valerian.
Abbreviations: CFU- colony forming unit; GLM- general linear model; HCN- hydrogen cyanide; IAA- indoleacetic acid; KB- King et al.'s B medium; PGPR- Plant growth promoting rhizobacteria; SMA- skimmed milk agar; YDC- yeast extract-dextrose-calcium carbonate.

Introduction

Plant growth-promoting rhizobacteria (PGPR) are a group of microorganisms in the rhizosphere that promote plant growth by increasing nutrient availability and may be used as inoculants for biofertilization, phytostimulation and biocontrol and can be classified according to their beneficial effects (Bloemberg and Lugtenberg, 2001; Gwyn, 2006). Some of the rhizobacteria not only benefit from the nutrients secreted by the plant root but also beneficially influence the plant in a direct or indirect way, resulting in a stimulation of its growth. Identification of these mechanisms is a challenge because of i) the diversity of mechanisms by which bacteria can promote plant growth, ii) the difficulty in estimating enhanced growth or improved health, particularly with small increases, iii) the narrow range of abiotic and biotic conditions under which growth promotion may occur. PGPR have been reported to directly enhance plant growth by a variety of mechanisms including fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones (Glick, 1995). Besides, PGPR indirectly enhance plant growth via suppression of phytopathogens using a variety of approaches. The ability to synthesize fungal cell wall-lysing enzymes (protease), or hydrogen cyanide (HCN), which suppress the growth of fungal pathogens; promotes a successful compete with pathogens for nutrients or specific niches on the root surface (Bloemberg and Lugtenberg, 2001; Persello-Cartieaux et al., 2003). A few studies have shown that some non-phytopathogenic bacteria that produce high amounts of indoleacetic acid (IAA) in culture can inhibit or reduce root growth (Barbieri and Galli, 1993; Patten and Glick, 2002). Bacteria belonging to the genera *Azospirillum*, *Pseudomonas*, *Xanthomonas*, and *Rhizobium* as well as *Alcaligenes faecalis*, *Enterobacter cloacae*, *Azetobacter diazotrophicus* and *Bradyrhizobium japonicum* have been shown to produce auxins which stimulate plant growth (Patten and Glick, 1996). Additionally, secretion of siderophore as high-affinity iron chelating compounds and production of chitinases, glucanases, cellulases, lipases and other lytic enzymes by PGPR can affect plant growth (Kloepper et al., 1980; Gwyn, 2006). *Valeriana officinalis* (valerian) is a perennial herb that belongs to family of valerianaceae. During the 200-year period from 1733 to 1936, valerian was one of the six most prescribed medicines in European and American medicine (Hobbs, 1996). The rhizome of valerian contains various compounds including valepotriates, valerenic acid, and volatile oils (Kalyn, 1999). Its extract has been used extensively for treatment of insomnia worldwide (Houghton, 1999). Although the impact of root inoculation with beneficial rhizosphere microorganisms has been explored on some plant quality parameters, to our knowledge this is the first report showing root extract changes in valerian

due to PGPR. In fact, our main purpose was to investigate the effects of plant growth promoting rhizobacteria isolated from rhizosphere of valerian on root extracts of the plant.

Results

Isolation and identification of bacteria

Forty bacterial isolates (at least seven different genera) were obtained from the valerian root. Bacterial populations were evaluated by using colony forming unit (CFU) per gram of root that varied between 3×10^3 to 3×10^{10} CFU g^{-1} in four media. The isolates were grouped into *Pseudomonas*, *Klebsiella*, *Xanthomonas*, *Bacillus*, *Erwinia*, *Agrobacterium* and *Arthrobacter* on the basis of cultural, morphological and biochemical characteristics (Figs. 1, 2, 3, 4, 5 and 6 in supplementary file). The maximum and minimum populations were *Pseudomonas* and *Agrobacterium* having 57% and 1% of total population, respectively.

Production of siderophore by isolated bacteria

Up to 98% of isolates were able to produce siderophore (Table 1). The largest diameters of the orange halo around colonies were formed by isolates 1 (belongs to *Pseudomonas* genus) and 35 (belongs to *Bacillus* genus). We selected six isolates which were able to produce the largest siderophore halo, approximately 20 to 40 mm within 1 day of incubation. Maximum amount of pyoverdin production as a fluorescent siderophore in succinate medium was 78.69 µg ml^{-1} by isolate 6 (belongs to *Pseudomonas* genus) followed by isolate 3 (belongs to *Pseudomonas* genus) that produced 31.41 µg ml^{-1} pyoverdin (Table 2).

Production of auxin by isolated bacteria

Auxin (IAA) production was observed in more than 80% of the isolates. As shown in Table 1, isolates 6 and 36 produced 25.96 and 25.70 µg IAA ml^{-1}, respectively, while minimum amount was 2.74 µg ml^{-1}.

Production of hydrogen cyanide (HCN) by isolated bacteria

HCN producing bacteria were categorized in 4 groups with very high, high, medium and low ability. About 38% of the isolates showed high and very high, 33% medium and 20% low ability in producing HCN. Isolate 3 produced the greatest amount of HCN (Table 3).

Production of lipase and protease by isolated bacteria

The results of lipase test revealed that 95% of isolates produced lipase enzyme and 28% of them showed high and very high ability, 23% medium and 45% low ability (Table 3). Up to 78% of the isolates were able to produce protease in skimmed milk agar (SMA) medium. Diameters of halo zone of bacterial colonies on SMA medium were measured after 48 h. The maximum production of this enzyme by isolate 24 (belongs to *Pseudomonas* genus) was 4.17 cm (Table 3). The isolate 39 (belongs to *Bacillus* genus) with 1.30 cm showed a minimum protease production. The isolates used in greenhouse had high ability in HCN, lipase and protease production.

Effect of isolated bacteria on shoot length

The ability of seven isolates 1, 2, 3, 6, 7, 10, 27 and 36 (belong to *Pseudomonas* genus) in promoting the growth of *Valeriana officinalis* was studied under greenhouse condition. The results of greenhouse experiments showed that the highest shoot length (up to 14 cm) was recorded in the presence of isolate 6 and isolate 36 (which belonged to *Pseudomonas* genus) compared to control (6.67 cm) (Table 4).

Effect of isolated bacteria on quantity of root extract and valerenic acid

Evaluation of root extracts of plants in the presence of PGPR showed that isolates 1, 3 and 6 had the greatest impact on the amount of root extract (Table 4). We have also found that the inoculation of some bacteria (e.g. isolates 1 and 3) increased valerenic acid in root extract which were 0.31 and 0.43 mg mL^{-1}, respectively, compared to the control which was 0.30 mg mL^{-1} (Table 5).

Discussion

Pseudomonas species were the dominant isolated bacteria in the rhizosphere of valerian as a medicinal plant. Tamilarasi et al. (2008) isolated various bacteria from rhizosphere of 50 medicinal plants which among the isolated bacteria the dominant species was *Bacillus* followed by *Pseudomonas, Enterobacter, Corynebacterium, Micrococcus and Serratia*. The main reason of microbial specificity towards the various medicinal plants could be due to exchange of plant metabolites (Ramesh et al., 2012). Plant growth promoting rhizobacteria exhibits beneficial effects on host plants. Production of phytohormones (e.g ethylene) by PGPR may affect plant growth and development, and can improve fruit quality (Lucas-Garcia et al., 2004; Mena-Violante and Olalde-Portugal, 2007). Asghar et al. (2002) showed that PGPR strains produced 24.6 g ml^{-1} of auxins in the presence of the precursor L-tryptophan in the medium. Lopper and Schroth (1989) showed that twelve of 14 rhizobacteria strains produced 5 to 10 µg ml^{-1} of auxin, while in the present study, 80% of isolates were able to produce auxin considering that the maximum amount of auxin was 25.96 µg ml^{-1}. In our study, the frequency of isolated bacteria with capability of auxin and siderophore production differed strongly from that observed by Antoun et al. (1998) who found that 58% of the isolates were IAA producers and 83% siderophore producer.
Previous researches have showed that the introduction of PGPR for greenhouse tomato stimulated plant growth and could also significantly increase the fruit quality (Van Peer and Schippers, 1989; Mena-Violante and Olalde-Portugal, 2007). However, according to our knowledge, our study is the first which shows that PGPR increase marketable valerian root extract. The effect of PGPR on auxin production and shoot length revealed that there was a positive correlation between the amount of auxin production and shoot length where the isolates 6 and 36 with high ability of auxin production exerted the highest shoot length. Ahmadzadeh et al. (2006) reported that among the 47 strains, the highest halo diameters of siderophore and protease were 1 and 1.5 cm, respectively, whereas in the current study the related data were 2.03 cm and 4.17 cm, respectively indicating a high potential of isolated bacteria from the rhizosphere of valerian. The results showed that 100%, 77.5% and 90% of isolates were capable to produce siderophore, protease and HCN, respectively. Characterization of PGPR associated with *Cicer arietinum* revealed that *Pseudomonas* and *Bacillus* species had a high potential to produce siderophores (Joseph et al., 2007). Regarding the results of production of IAA, siderophore, protease and HCN, very distinct behaviors were

Table 1. Production of siderophore in CAS medium and auxin concentration in the isolates.

Isolates	Genus	Auxin concentration (µg ml $^{-1}$)	Siderophore production (halo diameter cm)
1	*Pseudomonas*	23.17 bcd[†]	2.03 a
2	*Pseudomonas*	23.33 abc	1.47 fg
3	*Pseudomonas*	24.22 ab	1.87 abcd
4	*Pseudomonas*	7.44 ijklm	0.97 kj
5	*Erwinia*	4.43 mn	0.53 l
6	*Pseudomonas*	25.95 a	2.00 ab
7	*Pseudomonas*	18.66 ef	1.83 abcd
8	*Pseudomonas*	2.74 n	1.40 hg
9	*Pseudomonas*	20.49 cde	2.00 ab
10	*Pseudomonas*	20.28 cde	1.96 abc
11	*Pseudomonas*	21.64 bcde	1.40 hg
12	*Erwinia*	5.82 klmn	1.13 ij
13	*Bacillus*	6.92 jklm	1.06 kj
14	*Xanthomonas*	2.74 n	0.36 l
15	*Xanthomonas*	2.74 n	1.13 ij
16	*Pseudomonas*	7.60 ijkl	2.00 ab
17	*Xanthomonas*	2.74 n	0.36 l
18	unknown	4.47 lmn	0.46 l
19	*Bacillus*	4.83 lmn	1.16 hij
20	*Pseudomonas*	6.49 jklm	1.46 fg
21	*Bacillus*	5.22 klmn	1.00 kj
22	*Bacillus*	4.62 mnl	0.86 k
23	*Xanthomonas*	2.74 n	1.90 abcd
24	*Pseudomonas*	9.14 hij	1.36 gih
25	*Pseudomonas*	2.74 n	0.00 l
26	*Pseudomonas*	8.15 ijk	1.36 ghi
27	*Pseudomonas*	15.5809 gf	1.83 abcd
28	*Pseudomonas*	11.3818 h	1.13 ij
29	*Agrobacterium*	16.0187 fg	1.40 hg
30	unknown	2.74 n	1.70 def
31	*Erwinia*	10.421 hi	1.50 efg
32	*Pseudomonas*	21.78bcde	1.76 bcd
33	*Bacillus*	7.19 jklm	1.13 ij
34	*Pseudomonas*	6.01 jklm	1.10 kj
35	*Bacillus*	20.19 de	2.03 a
36	*Pseudomonas*	25.69 a	1.86 abcd
37	*Pseudomonas*	15.22 g	1.43 g
38	*Bacillus*	2.74 n	1.03 kj
39	*Bacillus*	15.37 g	1.73 cde
40	*Pseudomonas*	21.07 cde	1.86 abcd

[†] Similar letters in each column represent insignificant difference in LSD test at *(P<0.01)*. Data are means of 3 replications.

observed among different isolates. Extracellular protease, lipase and HCN can contribute to the ability of bacteria to suppress fungal diseases. Meanwhile, production of these components by many of isolates demonstrated potential of valerian PGPR for biological control. Some of *Pseudomonas* species as a group of PGPR can involved in controlling of plant diseases (Ahmadzadeh and Sharifi-Tehrani, 2009). Limited supply of iron and other metals due to chelation by siderophores could act as a biocontrol agent of some plant disease in natural habitats (Joseph et al., 2007; Loper and Henkels, 1997). The results of laboratory and greenhouse experiments indicated that PGPR with ability of IAA and siderophore production could improve plant growth. The shoot length of seedlings inoculated with isolate 6 was more than twice of the non-inoculated control. The population of *Pseudomonas* dominated in valerian rhizosphere and other rhizobacteria of this medicinal plant showed a high capability to be used as a biofertilizer. The maximum increase in quantity of root extract and valerenic acid were observed in response to inoculation with isolates 1 and 3 (belong to *Pseudomonas* genus) compared to non-inoculated controls (Tables 4 and 5). Overall, the results of the present study suggested that *Pseudomonas* species had a great potential to increase the root extracts and shoot length of valerian which are important factors for commercial production of this medicinal plant. The use of PGPR of valerian as a biofertilizer could be an efficient approach to replace chemical fertilizers and pesticides for sustainable valerian cultivation. Further investigation, including efficiency test under field condition with various plants is needed to clarify the role of PGPR and their beneficial effects on plant growth and development.

Materials and methods

Isolation and identification of bacteria from valerian rhizosphere

Samples of mature rhizome of *Valeriana officinalis* were collected from field of Medicinal Plant Research Institute of Karaj, Iran. Adhering soil was carefully brushed off from the roots and consequently the roots were washed with tap water.

Table 2. Pyoverdine production by selected bacterial isolates.

Isolates	Concentration (mol L^{-1})
6 (*Pseudomonas*)	78.69 a†
3 (*Pseudomonas*)	31.41 b
1 (*Pseudomonas*)	17.71 c
27(*Pseudomonas*)	25.36 cb
36(*Pseudomonas*)	17.71 c

† Similar letters in each column represent insignificant difference in LSD test at *(P<0.01)*. Data are means of 3 replications.

One gram of roots was placed in tubes containing sterile physiologic water (0.85% NaCl in distilled water) supplemeted with 0.025% Tween 20 and vortexed for 5 min followed by serial dilution procedure. Then, 100 µl of dilutions were spread on four media plates in triplicate. The media included S1 and King B for fluorescent pseudomonads, crystal violet agar (Merck, Germany) for gram negative bacteria, and methyl red agar (Merck, Germany) for gram positive bacteria. The plates were incubated at 28°C for 48 h, and then bacterial populations were determined as colony forming unit (CFU). Identification of isolates was performed by using Bergey's manual of systematic bacteriology as one of the most comprehensive works in the field of bacterial taxonomy which has been extensively used (Sr and Rao, 2012; Fotou et al., 2011; Jyothi et al., 2010; Arora et al., 2000) considering the following tests: gram reaction, endospores production, oxidase, aerobic and anaerobic growth, aerial mycelium, catalase, fluorescent on King et al.'s B (KB) medium, mucoid growth on yeast extract-dextrose-calcium carbonate (YDC) medium at 30°C, growth on DIM agar (Merck, Germany), yellow or orange colonies on YDC and growth at 40°C. Although molecular techniques are new powerful approaches for microbial identification which mostly considered for that purpose nowadays, Bergey's manual of systematic bacteriology as one of the most comprehensive works in the field of bacterial taxonomy has been also used (Sr and Rao, 2012; Fotou et al., 2011; Jyothi et al., 2010; Arora et al., 2000). Furthermore, most of the selected media in the current study have been proposed for isolation of different PGPR (Bashan et al., 1993).

The characterization of plant growth promoting rhizobacteria

Siderophore production

Siderophore production was measured as described by Alexander and Zuberer (1991). Briefly, 10 µL of the bacterial suspension in triplicate was inoculated on chrome azurol S (CAS) medium (Merck, Germany) and the plates were incubated at 28°C for 24 h. Change of color from blue to orange indicated production of siderophore and the diameter of the orange halo around colonies were measured. Siderophore production was assessed by measuring a ratio of halo diameter to colony diameter on CAS medium (Sung et al., 2001). Furthermore, 6 bacterial isolates of *Pseudomonas* were evaluated in producing pyoverdin (a fluorescent siderophore) according to the method described by Meyer and Abdullah (1978).

Auxin production

Production of auxin by PGPR strains was assayed based on the method described by Patten and Glick (1996). Briefly, each bacterium was cultured in nutrient broth medium and incubated at 28°C for 48 h in a shaker incubator. Then 50 µL of each bacterial suspension were transferred to nutrient broth containing 50 µg mL^{-1} L-tryptophane. After 48 h, the suspensions were centrifuged at 10000 rpm for 10 min. Consequently, 1 mL of supernatant was mixed with 4 mL Salkowski reagent (2 mL 0.5 mol L^{-1} $FeCl_3$ + 98 mL 35% $HClO_4$). After 20 min, the samples that turned red were considered as positive and the absorbance of the mixture was measured at 535 nm with a spectrophotometer.

Hydrogen cyanide (HCN) production

HCN production was determined by color change of filter paper (Alstrom and Burns, 1989). 100 µL of bacteria suspension was inoculated on nutrient agar medium (Merck, Germany) contained 4.4 g L^{-1} glycine. Filter papers were soaked in a reagent solution (sodium carbonate 2% and picric acid 0.5%) and placed in the upper lid of Petri dishes. To prevent volatilization, the plates were sealed with parafilm and incubated at 28 °C for 4 days. One plate without inoculation of bacterium was considered as control. If HCN was produced, yellow filter papers changed to cream, light brown, dark brown and eventually turn into reddish-brown. To compare the bacterial isolates in HCN production semi-quantitatively, isolated bacteria that showed yellow, cream, light brown and dark brown (reddish- brown) colors received 0, 1, 2, 3 and 4 scores, respectively. The scores including 0, 1, 2, 3 and 4 represented no ability and low, medium, high and very high ability of HCN production, respectively.

Lipase production

For determination of lipase enzyme the following medium was used (Omidvari, 2008): Peptone 10 g, calcium chloride 0.1 g, sodium chloride 5 g, Agar 15 g, distilled water 1 Liter, 10 mL sterile Tween 20. All of bacteria were streaked on this medium and incubated at 27 °C for 48 h. Depositions around the bacterial colonies indicted activity of lipase enzyme (Supplementary file). The scores including 0, 1, 2, 3 and 4 represented no ability and low, medium, high and very high ability of lipase production, respectively.

Protease production

Determination of protease enzyme carried out according to method of Marhofer et al. (1995). The bacteria were spotted on plates of SMA medium containing 15 g skim milk, 0.5 g yeast extract, 9.13 g agar and 1 L distilled water) and incubated at 27°C for 48 h. The diameters of colorless halo zone around the bacterial colonies were measured to determine the ability of protease production.

Greenhouse experiments

Greenhouse experiments were carried out in the certified greenhouse of Medicinal Plants Research Institute of Karaj, Iran where valerian transplants were obtained from there. In order to study the effects of eight bacterial isolates (belong to *Pseudomonas* genus) on plant growth, bacterial suspensions were adjusted to 10^9 CFU mL^{-1} by a spectrophotometer. Then the same valerian transplants were collected and their roots were soaked in a 10-ml of bacterial suspension for 10 to 15 min and the transplants were sown in plastic pots. Pots were watered by using the overhead sprinklers. After four months, the shoot length, root dry weight and weight of root extract were determined.

Table 3. Protease, HCN and lipase production of the isolates.

NO. of isolates	Genus	Protease (halo diameter cm)	HCN‡	Lipase£
1	*Pseudomonas*	2.47 cde†	3.00 abc	3.00 a
2	*Pseudomonas*	2.00 ghi	2.00 cdef	3.00 a
3	*Pseudomonas*	2.93 b	4.00 a	2.66 a
4	*Pseudomonas*	2.37 cdefg	1.00 fg	3.00 a
5	*Erwinia*	0.00 m	1.00 fg	1.00 c
6	*Pseudomonas*	2.70 bcd	3.67 ab	1.00 c
7	*Pseudomonas*	2.03 fghi	2.00 cdef	3.00 a
8	*Pseudomonas*	2.27 efgh	1.00 fg	3.00 a
9	*Pseudomonas*	2.27 efgh	3.67 ab	2.66 a
10	*Pseudomonas*	2.30 defgh	3.66 ab	3.00 a
11	*Pseudomonas*	2.46 cde	2.00 cdef	1.00 c
12	*Erwinia*	0.00 m	1.00 fg	1.00 c
13	*Bacillus*	1.93 hi	3.66 ab	3.00 a
14	*Xanthomonas*	1.40 jk	2.00 cdef	3.00 a
15	*Xanthomonas*	2.40 cdefg	3.66 ab	2.66 a
16	*Pseudomonas*	2.66 bcde	2.00 cdef	3.00 a
17	*Xanthomonas*	0.00 m	1.00 fg	1.00 c
18	unknown	0.23 m	0.00 g	1.00 c
19	*Bacillus*	2.90 b	0.00 g	3.00 a
20	*Pseudomonas*	2.03 fghi	2.00 cdef	3.00 a
21	*Bacillus*	0.00 l	2.00 cdef	2.66 a
22	*Bacillus*	0.00 l	1.00 fg	3.00 a
23	*Xanthomonas*	0.00 l	1.00 fg	0.00 d
24	*Pseudomonas*	4.16 a	1.00 fg	1.00 c
25	*Pseudomonas*	2.70 bcd	0.00 g	0.00 d
26	*Pseudomonas*	0.00 m	2.00 cdef	3.00 a
27	*Pseudomonas*	1.93 hi	2.00 cdef	2.66 a
28	*Pseudomonas*	1.73 ij	0.00 g	3.00 a
29	*Agrobacterium*	2.33 cdefgh	3.00 abc	1.00 c
30	unknown	2.73 bc	3.00 abc	1.00 c
31	*Erwinia*	1.73 ij	1.66 def	3.00 a
32	*Pseudomonas*	2.43 cdef	2.33 cde	3.00 a
33	*Bacillus*	2.46 cde	1.33 ef	2.66 a
34	*Pseudomonas*	2.00 ghi	1.66 def	3.00 a
35	*Bacillus*	2.93 b	1.00 fg	1.00 c
36	*Pseudomonas*	2.36 cdefg	3.00 abc	1.00 c
37	*Pseudomonas*	0.00 m	2.00 cdef	3.00 a
38	*Bacillus*	2.70 bcd	3.66 ab	3.00 a
39	*Bacillus*	2.03 fghi	3.00 abc	2.66 a
40	*Pseudomonas*	2.26 efgh	2.66 bcd	3.00 a

† Similar letters in each column represent insignificant difference in LSD test at *(P<0.01)*. Data are means of 3 replications. ‡ 0: No ability, 1: low ability, 2: medium ability, 3: high ability, 4: very high ability of HCN production , £ 0: No ability, 1: low ability, 2: medium ability, 3: high ability, 4: very high ability of lipase production

Table 4. Effect of rhizobacterial strains on shoot length and percentage of root extract

Isolates	Root extract (%)	Shoot length (cm)
1 (*Pseudomonas*)	30.09 a†	12.50 ab
2 (*Pseudomonas*)	21.61 bc	8.67 bc
3 (*Pseudomonas*)	29.97 a	11.83 ab
6 (*Pseudomonas*)	29.84 a	14.17 a
7 (*Pseudomonas*)	21.30 bc	11.17 abc
10 (*Pseudomonas*)	20.57 bc	10.50 abc
27 (*Pseudomonas*)	25.80 ab	11.50 abc
36 (*Pseudomonas*)	18.47 c	13.83 a
Control (No Inoculation)	16.26 c	6.67 c

† Similar letters in each column represent insignificant difference in LSD test at *(P<0.01)*. Data are means of 3 replications.

Table 5. Amounts of valerenic acid in treated roots with isolates 3, 1 and control (non-inoculated isolate).

No. Of Isolates	Total valerinic acid (mg mL $^{-1}$)
3	0.43 a†
1	0.31 b
Control (No Inoculation)	0.30 c

† Similar letters in each column represent insignificant difference in LSD test at *(P<0.01)*. Data are means of 3 replications.

Evaluation of shoot length

Four months after inoculation, the roots and shoots were separated and rinsed with tap water until all soil particles removed. Then, lengths of shoots of each treatment were measured.

Extraction of root

The roots were dried at laboratory for 48 h. Then 1.3 g of grinded root was mixed with ethanol 70% and placed in the device of ultrasonic cleaner for 15 min. After 48 h, the mixture was placed in the ultrasonic for 15 min again and passed through Whatman filter papers. The solvent was evaporated using a rotary evaporator and after that dry weights of essential oils were determined.

Quantification of valerenic acid

Two treatments of roots having greatest essential oil and dry weight were selected and the valerenic acids of them were evaluated. 1.25 g of each sample, treating with bacterial isolates was weighted and mixed with 25 ml of ethanol 70%. The mixtures were placed in ultrasonic cleaner for 2 h. After centrifuge of samples at 10000 rpm, the supernatants were passed through a 0.45-µm filter and injected to a high pressure liquid chromatography (HPLC). To prepare the mobile phase, solution A (5 ml of phosphoric acid) was added to distilled water to reach 100 ml. The mixture of solution A and solution B (methanol) with ratio of 27:73 was used as the mobile phase. HPLC specifications were as follows: Model KNAVER, Column: C18 10 µm 125 A - 3.9×300 nm, detector: UV 225 nm, 20µL of injection was done manually.

Statistical analysis

All data were analyzed by SAS 9.1 software (SAS institute, USA) using general linear model (GLM) procedure. Comparison of mean data was carried out using least significant difference (LSD) test and data with $P<0.01$ were considered significant. All experiments were conducted with three replicates.

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