Original Article

Phylogenetic diversity and antagonistic traits of root and rhizosphere pseudomonads of bean from Iran for controlling *Rhizoctonia solani*

Vahid Keshavarz-Tohid [a,b], Parissa Taheri [a,*], Daniel Muller [b], Claire Prigent-Combaret [b], Jordan Vacheron [b], Seyed Mohsen Taghavi [c], Saeed Tarighi [a], Yvan Moënne-Loccoz [b]

[a] *Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran*
[b] *UMR Ecologie Microbienne, CNRS, INRA, VetAgro Sup, UCBL, Université de Lyon, 43 bd du 11 Novembre, F-69622 Villeurbanne, France*
[c] *Department of Plant Protection, Shiraz University, Iran*

Abstract

Fluorescent pseudomonads from bean root and rhizosphere in Iran were investigated for biocontrol of the fungal pathogen *Rhizoctonia solani*. Phylogenetic analysis of concatenated 16S rRNA, *gyrB* and *rpoD* sequences for 33 *Pseudomonas* isolates showed that 15 belonged to four clusters within the 'P. *fluorescens*' group, i.e. one corresponding to *P. thivervalensis*, two others including *P. moraviensis* or *P. baetica*, and the last one without closely-related established species. The 18 other isolates belonged to five clusters within the 'P. *putida*' group, one including *P. mosselii* and *P. entomophila*, another including strains currently described as *P. putida*, and three without closely-related species described. Ten isolates were selected based on in vitro inhibition of *R. solani*. Cellulase activity was identified in three pseudomonads, chitinase activity in two pseudomonads, extracellular protease activity in nine pseudomonads and hydrogen cyanide production in two pseudomonads. Genes coding for production of phenazine, pyoluteorin, pyrrolnitrin and 2,4-diacetylphloroglucinol were not found, whereas the 1-aminocyclopropane-1-carboxylate deamination gene *acdS* was present in three pseudomonads. The antagonistic *acdS*[+] strain VKh13 from the 'P. *putida*' group effectively protected soil-grown bean from *R. solani* AG 4-HGI. Results show that pseudomonads from uncharacterized taxa were readily obtained from Iranian soils and displayed biocontrol potential against *R. solani*.

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) are receiving intense research attention, as they are interesting models for deciphering mechanisms of plant–microbe interactions, and represent an environmentally friendly potential for implementing microbial strategies of crop protection against phytopathogens. The fluorescent *Pseudomonas* spp. have often been considered in this context, and different biocontrol mechanisms are documented in these bacteria [1,2]. Pseudomonads may protect plants via induced systemic resistance (ISR) [3], deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) [4], competition with phytopathogens for organic carbon [5] and iron on the root surface [6], production of extracellular lytic enzymes such as cellulases, chitinases and proteases [7] and antimicrobial secondary metabolites [8]. Several antimicrobial secondary metabolites produced by fluorescent *Pseudomonas* are important for biocontrol effects — for instance, phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin and hydrogen cyanide [8–10].

Fluorescent pseudomonads with biocontrol potential have been isolated in various parts of the world, and many of them can control diverse pathogens. This is the case for strains CHA0 from Switzerland, F113 from Ireland, PITR2 and PILH1 from Italy, Pf29A from France, Pf-5 from Texas, 2.79 and Q2-87 from Washington, PCI2 from Argentina, KD and 2P24 from China [1,11,12], YJR27 from South Korea [13] and Cab57 from Japan [14]. Certain types of biocontrol pseudomonads have been evidenced in different regions of the world, such as *P. protegens* [15], but others display some level of endemicity [16] and have been evidenced locally, for instance, clade TAD10 in western France [17] and new types of 2, 4-diacetylphloroglucinol-producing strains in Switzerland [16]. Most *Pseudomonas* strains have been selected on a reduced range of crop species and/or geographic regions, and endemicity might be more apparent when exploring other ecological situations. Indeed, new types of 2,4-diacetylphloroglucinol-producing pseudomonads have been obtained from finger millet grown in southern India [18].

In this work, we focused on bean agriculture in different regions of Iran, where *Rhizoctonia solani* is the main soil-borne pathogen [19]. Several *Pseudomonas* isolates have been described for biocontrol of *R. solani* [20,21], including in Iran [22]. Here, our hypothesis was that novel types of biocontrol pseudomonads could be isolated under the particular conditions prevailing in Iran, which have not been extensively investigated thus far. These regions encompass a few humid areas in the north of the country, as well as a majority of drier areas in more southern locations (Fig. S1) that might yield novel types of biocontrol agents [18]. To this end, a range of fluorescent pseudomonads were isolated and their taxonomic status investigated. The occurrence of different biocontrol traits effective against various pathogens, including *R. solani*, such as phenazines [10], 2, 4-diacetylphloroglucinol [23], hydrogen cyanide [24] and pyrrolnitrin [25], was investigated.

2. Material and methods

2.1. Isolation of fluorescent pseudomonads from the rhizosphere and root

A total of 409 isolates were obtained in the summer of 2014 from the rhizosphere and roots of healthy bean plants from 15 fields, with 3 fields from each of 5 provinces considered in Iran (Razavi Khorasan, Mazandaran, Golestan, Fars and Markazi, Fig. S1). The bean fields were investigated and plants that grew well and without disease symptoms were selected and collected. These plants were taken out with soil and transferred to the laboratory in plastic bags in a box containing ice.

Rhizosphere samples consisted of 1 g of soil removed aseptically from the roots and shaken 90 min at 5 rpm in Falcon tubes containing 10 mL NaCl 0.8 g/L, whereas root samples consisted of 1 g of washed roots that were surface-sterilized with sodium hypochlorite 10% for 3 min, rinsed twice with sterile distilled water and crushed with mortar and pestle. Isolates from sample extracts and dilutions were obtained at

24–48 h on King's B agar (KBA) [26] and purified on KBA. A total of 409 isolates fluorescent on KBA were kept and 53 selected (43 from rhizosphere and 10 from root) based on the ability to form large colonies on KBA within 24 h, an important trait when considering potential formulation issues if strains are to be developed as commercial biocontrol inoculants.

2.2. Molecular identification of fluorescent pseudomonads

Genus identification of 53 KBA isolates was carried out by sequencing16S rRNA gene *rrs*, after amplification of 1492 bp using primers PA and PH [27], whereas taxonomic identification within the *Pseudomonas* genus was carried out by sequencing genes *rpoD* and *gyrB*, after amplification of 810 bp of *rpoD* using primers 70F and 70R [28] and 747 bp of *gyrB* using primers UP-1 and UP-2R [29] (Table S1). The 50-μl PCR reaction included 5 μl buffer (10×), 1.5 μl MgCl$_2$ (50 mM), 5 μl dNTP (2 mM), 2.5 μl of each primer (10 μM), 0.2 μl Taq polymerase, 1.85 μl DMSO, 30.9 μl water and 0.55 μl DNA. PCR was performed with 5 min initial denaturation at 94 °C, followed by 30 cycles with (i) 1 min at 94 °C, (ii) 45 s annealing at 55 °C (for *rrs*) or 30 s annealing at 63 °C (for *rpoD*) or 67 °C (for *gyrB*), (iii) 1 min extension at 72 °C, and a final extension 5 min at 72 °C. Sequencing was carried out on both strands by Biofidal (Vaulx-en-Velin, France), using primers PA/PH (for *rrs*), 70Fs/ 70Rs (for *rpoD*) and UP-1S/UP-2Sr (for *gyrB*) (Table S1). Accession numbers for *rrs*, *rpoD* and *gyrB* are LT718456-88, LT718489-521 and LT719095-127, respectively.

Strand sequences were merged using http://mobyle.pasteur. fr and assessed with BLASTn (NCBI GenBank database). For *rrs*, *rpoD* and *gyrB*, the sequences were aligned with the corresponding sequences of *Pseudomonas* type strains (obtained from NCBI GenBank database) using SeaView version 4 (Sequence Alignment and Phylogenetic Tree Building) and cut with Jalview. The aligned sequences were concatenated with SeaView (giving 2310 bp per strain). A maximum-likelihood phylogenetic tree was obtained using PhyML.

2.3. Standard biochemical tests

The 53 *Pseudomonas* isolates were subjected to standard biochemical tests for *Pseudomonas* identification based on the LOPAT approach (L, levan production; O, oxidase production; P, pectinase activity; A, arginine dihydrolase production; T, tobacco hypersensitivity). Tests were done as described by Schaad et al. [30]. The biochemical tests were repeated three times.

2.4. Antagonistic assay in vitro against pathogenic fungi

The 53 *Pseudomonas* isolates were tested for in vitro growth inhibition of *R. solani* AG 4-HGI and AG 2-2 on potato dextrose agar (PDA) and Waksman agar (WA) [31]. The plates (3 per isolate) were incubated at 25 °C for five days and the width of inhibition zones (IZ) measured. The inhibition percentage was calculated as (IZ/distance between bacteria and

plug of fungi) $\times$ 100. Sterile water was used as control and inhibition percentages were compared. The experiments were repeated three times.

2.5. Mycelium observation by microscope

Mycelium formation of *Rhizoctonia* against antagonistic *Pseudomonas* isolates was investigated based on the method described by D'aes et al. [71]. In this assay, 10 μL overnight antagonistic pseudomonads in liquid KB were pipetted on one site of the slides covered with water agar 1.5%. A 5 mm plug of 3-day-old *R. solani* AG 4-HGI and AG 2-2 was placed in the center of each slide. The slides were placed in Petri dishes with a wet filter paper and incubated in an incubator at 25 °C. After 48 h, mycelium formation was observed by an Olympus microscope and digital pictures were taken by a Dino capture 2.0 camera. The assay was repeated three times.

2.6. Biochemical screening for enzymatic activities, hydrogen cyanide and siderophore production

The 10 *Pseudomonas* isolates giving the best results in fungal inhibition tests were assessed for cellulase (halo after 2 days of growth on CMC agar containing 0.5 g carboxymethylcellulose (CMC), 0.1 g K_2HPO_4, 0.05 g $MgSO_4$, 0.1 g KCl, 0.05 g yeast extract and 0.1 g $NaNO_3$ in 100 mL distilled water, followed by 15 min treatment with 1% (w/v) Congo red) [32], chitinase (halo after 5 days of growth on semi-minimal medium containing K_2HPO_4, 0.7; KH_2PO_4, 0.3; $MgSO_4 \cdot 5H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4$, 0.001; $MnCl_2$, 0.001 g/L, supplemented with 0.2% colloidal chitin followed by treatment with 1% (w/v) Congo red for 15 min and washing with 1 M NaCl) [33] and extracellular protease activities (halo after 5 days of growth on skim milk agar containing 1.5% skim milk (w/v), 0.05% yeast extract (w/v), 0.4% blood agar base (w/v) and 1.4% agar (w/v)) [34]. HCN production by KBA-grown bacteria was assessed based on an orange/brown color [35] of indicator filter paper soaked with 0.05% picric acid (in 2% sodium carbonate solution) and placed in the lid of parafilm-sealed plates. Production of siderophores was investigated using CAS-agar medium (Chrome Azurol S 60.5 mg/50 mL, HDTMA 72.9 mg/40 mL, which was mixed with $FeCl_3 \cdot 6H_2O$ in 10 mM HCl). The components were mixed as an indicator and color changed to dark blue. The indicator autoclaved after cooling to 50 °C added to PIPES, salt and casamino acid solutions, as described by Schwyn and Neilands [72]. The final volume was adjusted to 1000 mL. The yellow to orange color showed siderophore production.

2.7. PCR screening of genes for production of antimicrobials and ACC deamination

Genomic DNA of the 10 *Pseudomonas* isolates was extracted using the NucleoSpin kit (Macherey−Nagel, Hoerd, France). Genes for production of pyrrolnitrin (*prnD*), 2,4-diacetylphloroglucinol (*phlD*), pyoluteorin (*pltC*), phenazines (*phz*) and ACC deaminase activity (*acdS*) were

investigated by PCR using 50 mM $MgCl_2$, 5% DMSO, 10 mM of each primer (Table S1), 2 mM dNTP, 1 U of Taq polymerase (Invitrogen, Cergy-Pontoise, France) and 50 ng of DNA in 10$\times$ PCR buffer. All amplifications (Eppendorf thermocycler, Germany) were performed for 5 min at 94 °C, followed by 30 cycles with 1 min denaturation at 95 °C, 40 s primer annealing at 68, 64, 67, 56 and 57 °C (for *prnD*, *phlD*, *pltC*, *phz* and *acdS*, respectively), 1 min extension at 72 °C, and a final extension for 5 min at 72 °C. The PCR products were checked for size in 1% agarose gels and sequenced by Biofidal (accession numbers LT795007 to LT795009 for *acdS*). *P. protegens* CHA0 was used as positive control for *phlD*, *pltC* and *prnD* genes, *P. kilonensis* F113 for *acdS* and *P. chlororaphis* CFBP 2132 for *phz*.

2.8. Greenhouse assay for protection of the bean from R. solani

Seeds of the bean (*Phaseolus vulgaris* cv. Naaz) were surface-sterilized in 1.2% sodium hypochlorite for 4 min and rinsed twice with sterile distilled water. The seeds were then placed in 6 cm $\times$ 10 cm pots containing 50% perlite and 50% peat. At 2 weeks, the seedlings were inoculated with *Pseudomonas* sp. VKh13 (which had the best antagonistic effect against *R. solani* in vitro) or the reference biocontrol strain *P. protegens* CHA0, by unearthing the roots and dipping them in the corresponding bacterial suspension (OD_{600} = 1) for 20 min, whereas in the control, the same procedure was followed, but with sterile distilled water, as described [36]. Then, the seedlings were transplanted in 18 cm $\times$ 20 cm pots containing 50% clay and 50% sand and, where necessary, 0.3 g barley seeds colonized with *R. solani* AG 4-HGI (obtained from the fungal collection of Ferdowsi University of Mashhad, isolated from tomato) were placed near the seedlings. Three pots with 4 seedlings per pot were used in each treatment. Disease severity was scored 10 days later using the scale of Wen et al. [37]: 0 = no necrotic lesion; 1 = root necrosis up to 2.5 mm in length; 2 = necrosis 2.5−5.0 mm in length; 3 = necrosis longer than 5.0 mm; 4 = lesions covering the crown and shoots; and 5 = seedling damping-off. Then, a disease index (DI) was calculated as described by Taheri and Tarighi [62], where DI = $[(1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5)/5N] \times 100$, with n_1 the number of plants with score 1, n_2 the number of plants with score 2, etc., n_5 the number of plants with score 5 and N the total number of plants used in the experiment. After checking the symptoms, the biomass of inoculated bean plants was investigated by determining dry weight of root and shoot compared to control. The roots and shoots were separated and placed in paper bags. The paper bags were placed in an oven with 70 °C temperature. After 48 h, the dry weight was determined. The greenhouse assay was repeated three times.

2.9. Bean leaf assay

To assess potential induced resistance, additional plants (24 plants) from the above experiment were used, and apical

leaves (all were completely healthy) were sampled at 6, 12 and 24 h after seedling inoculation (as above). First, antioxidant enzyme activity and the defense enzyme phenylalanine ammonia lyase (PAL) were investigated in triplicate at each sampling. Total protein was extracted using 0.4 g of leaves, as described [64]. Peroxidase (POX) activity was determined spectrophotometrically following the method of Lin and Kao [65] and expressed as μmol/min/mg protein of oxidized guaiacol substrate. Catalase (CAT) activity was assayed using the method of Aebi [66] and expressed as μmol/min/mg protein of H_2O_2 reduction. Ascorbate peroxidase (APX) activity was measured as described by Nakano and Asada [67] and expressed as μmol/min/mg protein of ascorbate oxidation. PAL activity was measured based on McCallum and Walker's method [68], starting from 1 g of leaves, and expressed as enzymatic units (U)/mg protein.

Second, 2×2 cm square samples were cut from the apical leaves collected at 24 h (3 leaf squares per treatment, using 3 distinct plants) and each leaf square was inoculated with a 5 mm mycelial plug of *R. solani* AG 4-HGI (in the three treatments where *R. solani* had been added) or a 5 mm agar plug (in the non-inoculated control), essentially as described [36]. Each leaf square was put on a glass slide placed above wet filter paper inside a Petri dish. The Petri dishes were sealed and placed in an incubator at 25 °C and 12 h light/12 h dark. The disease index was assessed 72 h after leaf inoculation, using the following scale [36]: $0 =$ no infection; $1 = 1{-}25\%$; $2 = 26{-}50\%$; $3 = 51{-}75\%$; $4 = 76{-}100\%$ infected leaf surface. Then DI was calculated using $DI = [(1n_1 + 2n_2 + 3n_3 + 4n_4)/4N] \times 100$, with n_1 the number of plants with score 1, etc., n_4 the number of plants with score 4 and N the total number of plants used in the experiment. The leaf square assay was done three times.

2.10. Statistical analysis

All data were assessed by one-way ANOVA and Duncan tests ($P < 0.05$). SPSS 16 software (SPSS, Chicago, IL) was used.

3. Results

3.1. Taxonomic identification of Pseudomonas strains

Analysis of 16S rRNA gene sequences indicated that 36 of the 53 strains belonged to the *Pseudomonas* genus, and the other strains corresponded to *Stenotrophomonas* spp. or *Enterobacter* spp. Three strains (from the Markazi province) corresponded to *Pseudomonas aeruginosa*. The 33 non-*aeruginosa*, *Pseudomonas* strains were kept.

The LOPAT tests for phenotypic identification placed all 33 non-*aeruginosa Pseudomonas* isolates in the V b group, to which *P. fluorescens* belongs. This was based on levan production, oxidase activity, no pectinase activity, arginine dihydrolase production and avirulence on tobacco.

Analysis of concatenated *rrs*, *rpoD* and *gyrB* sequences showed that 15 isolates belonged to the 'P. fluorescens' group and 18 isolates to the 'P. putida' group (Fig. 1). The 15 isolates from the 'P. fluorescens' group fell into four clusters hereafter named Pf, i.e. (i) cluster Pf1 (which includes *P. thivervalensis*) for strain VF5, (ii) cluster Pf2 (including *P. moraviensis*) for strains VM-1, VM5, VM7, VF3, VF11 and VKh3, (iii) cluster Pf3 (more distantly related to *P. moraviensis* and including the fish pathogen *P. baetica*) for strain VF15, and (iv) cluster Pf4 (distantly related to *P. vancouverensis* and *P. jessenii*) for strains VF1, VF2, VF7 to VF10, and VF14 (Fig. 1). The 18 isolates from the 'P. putida' group fell into five clusters, i.e. (i) cluster Pp1 (including *P. mosselii*, *P. entomophila*, and *P. soli*) for strains VF13 and VF16, (ii) cluster Pp2 (without closely-related species) for strains VM16 and VKh13, (iii) cluster Pp3 (without closely-related species) for strains VKh2, VKh4, VKh9, VKh10 and VKh17, (iv) cluster Pp4 (without closely-related species) for strains VM2, VM3, VM6, VM11, VM13, VM14 and (v) cluster Pp5 (which includes well-known strains *P. putida* BIRD-1 and KT2440, but is distantly related to type strain *P. putida* ATCC12633ᵀ) for strains VKh7, VKh14 and VM-10 (Fig. 1).

3.2. Pseudomonas status in relation to strain geographic origin

The 33 non-*aeruginosa Pseudomonas* isolates were obtained mostly from rhizosphere (14 for the 'P. fluorescens' group and 15 for the 'P. putida' group) and a few from root samples (1 for the 'P. fluorescens' group and 3 for the 'P. putida' group) (Table 1). When considering their distribution amongst the *Pseudomonas* clusters, 1 isolate from the 'P. fluorescens' group and 8 isolates from the 'P. putida' group originated from Khorasan Razavi province, 3 isolates from the Pf2 cluster, 8 from Pp2, Pp4 and Pp5 clusters originated from Mazandaran province, 11 isolates from Pf1, Pf2, Pf3 and Pf4 clusters and 2 isolates from Pp1 originated from the Fars province, whereas no isolate originated from Golestan or Markazi provinces.

3.3. Antagonistic ability of Pseudomonas isolates in vitro

Among the 33 *Pseudomonas* isolates, 10 isolates could inhibit in vitro growth of the two important bean-pathogenic fungi *R. solani* AG 4-HGI and AG 2-2 (Table 2), i.e. isolates VF5, VM-1, VF15, VF14, VF13, VF16, VM16, VKh13, VKh7, VKh14 (belonging to Pf1—4 clusters and Pp1, 2 and 5 clusters). No isolate with an antagonistic effect in vitro was obtained from the two clusters Pp3 and Pp4 from the 'P. putida' group. Statistically, the inhibition percentage of VF5, VF14-16, VKh13 and VM16 isolates were more than CHA0 for *R. solani* AG 4-HGI in PDA. Statistically, isolates VF5, VF14, VF13, VF16, VM16 and VKh13 made larger inhibition zones against *R. solani* AG 2-2 compared to the CHA0 isolate in PDA. Although, this effect in WA somewhat changed, VF15

Fig. 1. Phylogenetic tree based on concatenated *rrs*, *gyrB* and *rpoD* sequences of 33 *Pseudomonas* isolates collected from the rhizosphere and root of bean plants from different provinces of Iran, along with type strains from related *Pseudomonas* species and reference biocontrol strains. Within the 'P. putida' group, a black circle is used to indicate the large clade containing *P. putida* type strain ATCC12633[T] and in which the name *P. putida* is traditionally used, even for strains distant from the type strain. Cluster Pp4 contains two strains (S16 and HB-3267) previously assigned to Yonezuka's cluster V and cluster Pp5 six strains (L846, B6-2, ND6, F1, KT2440 and BIRD-1) assigned to Yonezuka's cluster I [41].

Table 1
Taxonomy and origin of the 33 *Pseudomonas* isolates characterized.

Name of isolates	Sample of origin	Geographic region[a]
'P. fluorescens' group		
Cluster Pf1		
VF5	Rhizosphere	Dehno Qalandari village, field DN1
Cluster Pf2		
VM-1, VM5, VM7	Rhizosphere	Behshahr city, field B3
VF3, VF11	Rhizosphere	Dehno Qalandari village, field DN1
VKh3	Root	Neyshaboor city, field N1
Cluster Pf3		
VF15	Rhizosphere	Dehno Qalandari village, field DN1
Cluster Pf4		
VF1, VF2, VF7 to VF10, VF14	Rhizosphere	Dehno Qalandari village, field DN1
'P. putida' group		
Cluster Pp1		
VF13, VF16	Rhizosphere	Dehno Qalandari village, field DN1
Cluster Pp2		
VM16	Root	Behshahr city, field B3
VKh13	Rhizosphere	Neyshaboor city, field N1
Cluster Pp3		
VKh2, VKh4, VKh9, VKh10	Rhizosphere	Neyshaboor city, field N1
VKh17	Root	Neyshaboor city, field N1
Cluster Pp4		
VM2, VM3, VM6, VM11, VM13, VM14	Rhizosphere	Behshahr city, field B3
Cluster Pp5		
VKh14	Rhizosphere	Neyshaboor city, field N1
VKh7	Root	Neyshaboor city, field N1
VM-10	Rhizosphere	Behshahr city, field B3

[a] Dehno Qalandari village is in the Fars province (average temperature 15.8 °C, average rainfall 400 mm/year), Behshahr city in the Mazandaran province (average temperature 18.2 °C, average rainfall 700 mm/year), and Neyshaboor city in the Razavi Khorasan province (average temperature 14.8 °C, average rainfall 240 mm/year) (http://www.irimo.ir/eng/wd/720-Products-Services.html).

Table 2
Biocontrol traits identified in 10 antagonistic *Pseudomonas* isolates and growth inhibition percentage (IP) of bean pathogens in vitro.

Isolate	Biocontrol traits						IP of *R. solani* AG 4-HGI		IP of *R. solani* AG 2-2	
	Cellulase	Chitinase	Protease	HCN	*acdS*	Siderophore	On PDA	On WA	On PDA	On WA
'P. fluorescens' group										
Cluster Pf1										
VF5	+		+			+	23.3 e[a]	22.2 d	31.0 de	37.7 c
Cluster Pf2										
VM-1			+			+	6.6 b	10.0 b	13.3 b	15.5 b
Cluster Pf3										
VF15			+	+		+	18.8 cde	11.0 b	18.8 bc	21.0 b
Cluster Pf4										
VF14			+			+	22.2 de	17.7 cd	24.4 cd	20 b
'P. putida' group										
Cluster Pp1										
VF13		+	+			+	15.5 cd	12.2 bc	23.3 cd	23.3 b
VF16			+			+	20.0 de	12.2 bc	24.4 cd	15.5 b
Cluster Pp2										
VM16		+	+	+		+	50.6 f	45.5 e	55.5 e	42.2 c
VKh13			+	+		+	53.3 f	45.5 e	48.6 e	35.5 c
Cluster Pp5										
VKh7	+		+			+	15.5 cd	10.0 b	21.0 bc	14.4 b
VKh14	+		+	+		+	7.7 b	8.8 b	25.5 bc	21.0 b
Reference biocontrol strain										
CHA0		+	+				16.6 cd	15.0 bc	20.0 bc	21.0 b

None of the 10 isolates displayed *phlD*, *pltC*, *prnD* or *phzC-phzD* genes.
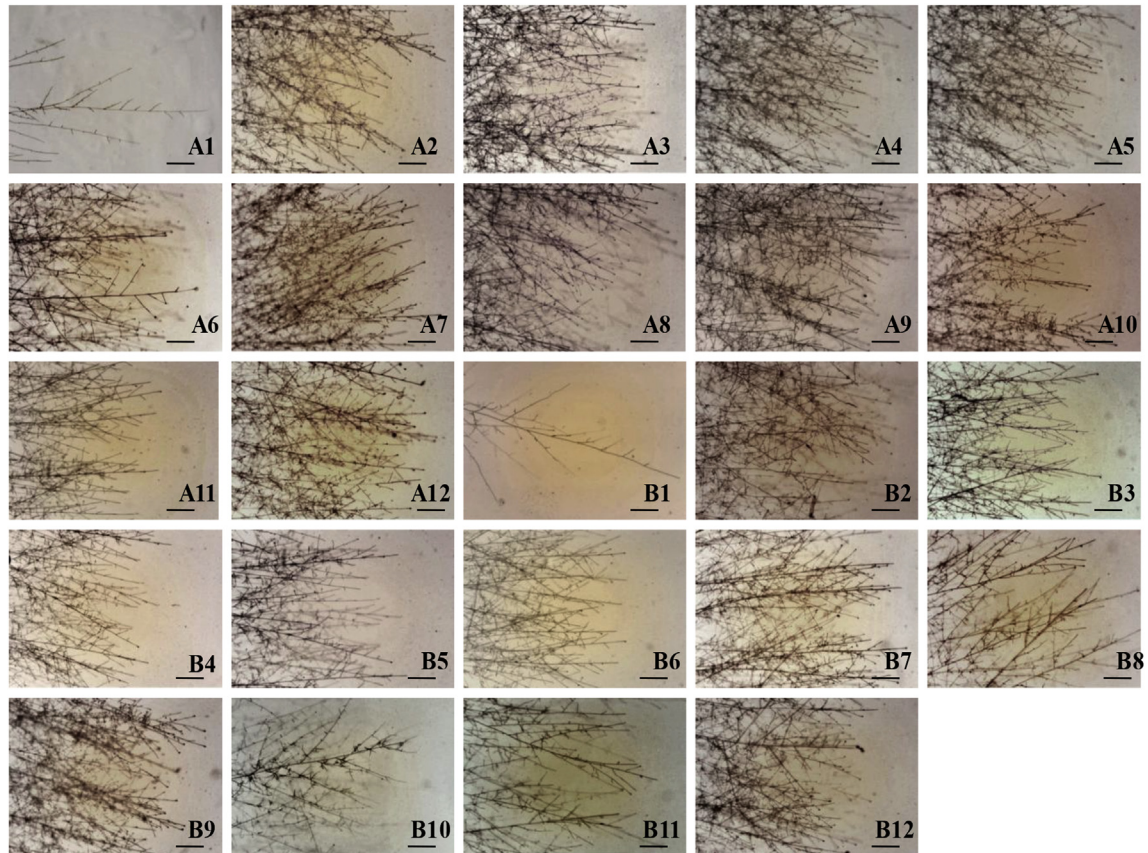[a] Values with different letters are significantly different (ANOVA and Duncan test; $P < 0.05$).

Fig. 2. Microscopic analysis of the mycelial growth pattern of *R. solani* AG 4-HGI (A1−A12) and AG 2-2 (B1−B12) on water agar covered glass slides in dual culture with sterile water (image A1 and B1) and antagonistic *Pseudomonas*. Images A2−A12 showed *R. solani* AG 4-HGI mycelium formation against CHA0, VF5, VF13, VF14, VF15, VF16, VKh7, VKh13, VKh14, VM-1 and VM16, respectively. Images B2−B12 showed *R. solani* AG 2-2 mycelium formation against CHA0, VF5, VF13, VF14, VF15, VF16, VKh7, VKh13, VKh14, VM-1 and VM16, respectively. Scale bar = 200 μm. The assay was repeated three times with similar results.

for AG 4-HGI and VF13−16 for AG 2-2 had less or the same effects compared to CHA0, respectively.

3.4. Microscopic observation of mycelium

The mycelium formation of *Rhizoctonia* in dual culture with antagonistic pseudomonads was investigated by microscopic analysis on glass slides covered by water agar. The observation showed that the mycelium growth type changed, especially at the edge of the inhibition zone (Fig. 2.). The mycelium of both anastomosis groups of *R. solani* dual-cultured with antagonistic *Pseudomonas* isolates were compressed, shorter and more branched than controls. This type of mycelial growth was observed in all dual cultures of *R. solani* AG 2-2 and AG 4-HGI against the antagonistic isolates.

3.5. Biocontrol traits in Pseudomonas isolates

Based on the use of PCR tools, none of the 10 *Pseudomonas* isolates selected displayed *phlD*, *pltC*, *prnD* or *phzC-phzD* genes involved in synthesis of prominent antimicrobial secondary metabolites important for disease control by fluorescent pseudomonads (Fig. S2). ACC deaminase gene *acdS* was detected in strains VM16, VKh13 (cluster Pp2) and

VKh14 (cluster Pp5), and HCN production in strains VM-1 (cluster Pf2) and VF15 (cluster Pf3) (Table 2 and Fig. S2). Cellulase activity was found in strains VF5 (cluster Pf1), VKh7 and VKh14 (cluster Pp5), and chitinase activity in strains VF13 (cluster Pp1) and VM16 (cluster Pp2). All strains were siderophore- and extracellular protease activity-positive except VM-1, that could not produce extracellular protease (Fig. 5).

3.6. Biocontrol activity in planta of Pseudomonas sp. VKh13

Non-inoculated bean seedlings did not exhibit disease symptoms, whereas plants exposed to *R. solani* AG 4-HGI displayed extensive necrosis, and several plants were damped off (Fig. 3A). Fewer lesions were observed when the reference biocontrol strain *P. protegens* CHA0 had been added, as indicated by a significant reduction in the disease index (Table 3). Investigating the dry weight of root and shoot samples confirmed disease index results. Statistically, shoot and root dry weight of control was more than *Rs*, *Rs* + CHA0 and *Rs* + VKh13 treatments. The *Rs* + VKh13 treatment was placed in second position. *Rs* + CHA0 and *Rs* treatments were placed in lower positions in terms of dry weight.

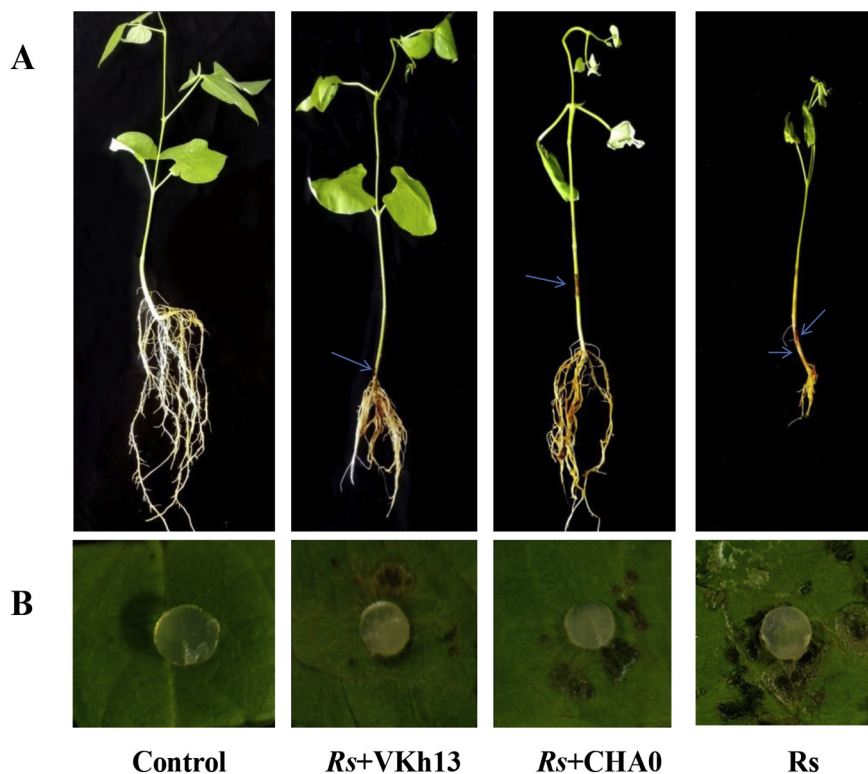**Control**   *Rs*+VKh13   *Rs*+CHA0   **Rs**

Fig. 3. Effect of *Pseudomonas* sp. VKh13 (from cluster Pp 2) on bean plants exposed to *R. solani* AG 4-HGI under non-sterile greenhouse conditions. Treatments included a non-inoculated control (indicated as Control), inoculation with *R. solani* alone (*Rs*), inoculation with *Rs* and the reference biocontrol strain *P. protegens* CHA0 (*Rs* + CHA0), and inoculation with *Rs* and with *Pseudomonas* sp. VKh13 (*Rs* + VKh13). **A.** Whole plants at 10 days after inoculation. Arrows show lesions in the stem of bean plants that developed following infection by *Rs*. **B.** Bean leaf bioassay at 72 h after leaf inoculation. The assay was repeated three times with similar results.

Table 3
Disease index of *R. solani* AG 4-HGI (*Rs*), *Rs* and CHA0 (*Rs* + CHA0) as reference antagonistic *Pseudomonas* and *Rs* and VKh13 (*Rs* + VKh13) as best antagonistic isolate from this research (cluster Pp2) in greenhouse and on leaf square.

Treatments	Disease index of whole bean plant	Disease index of leaf square	Root dry weight (mg)	Shoot dry weight (mg)	Total root and shoot dry weight (mg)
Control	0% a[a]	0% a	131 a	269 a	411 a
Rs	68.3% c	66.6% c	44 c	148 c	213 c
Rs + CHA0	40.0% b	25.0% b	57 bc	177 bc	232 bc
Rs + VKh13	19.9% ab	16.7% ab	77 b	228 b	272 b

Effect of *Rs*, *Rs* + CHA0 and *Rs* + VKh13 on root, shoot and total of root and shoot dry weight.
[a] Values with different letters are significantly different (ANOVA and Duncan test; $P < 0.05$).

3.7. Physiological responses of the bean to Pseudomonas sp. VKh13

The leaf square bioassay showed that disease did not develop in non-inoculated leaf squares taken from non-inoculated plants, whereas strong disease was found upon leaf inoculation with *R. solani* AG 4-HGI (Fig. 3B and Table 3). However, disease severity of leaf samples was significantly lower for plants whose roots had been inoculated with the ISR-inducing reference strain *P. protegens* CHA0 or the *Pseudomonas* isolate VKh13 (belonging to cluster Pp2).

As expected, the necrotrophic pathogen *R. solani* AG 4-HGI enhanced POX, APX, CAT and PAL activities, at least at 6 and 12 h (Fig. 4). This was also the case with the ISR-inducing reference strain *P. protegens* CHA0. The VKh13 treatment also showed significantly increased activities of all four enzymes compared with the non-inoculated control. Enzymatic activities at 6 h in the VKh13 treatment were even higher than in the CHA0 treatment for all four enzymes POX, APX, CAT and PAL.

4. Discussion

This work was carried out under the assumption that isolation of *Pseudomonas* from a bean grown in soil from Iran, especially from arid regions, could yield new kinds of pseudomonads in comparison with those described in the literature, which originate mainly from other types of soil conditions and
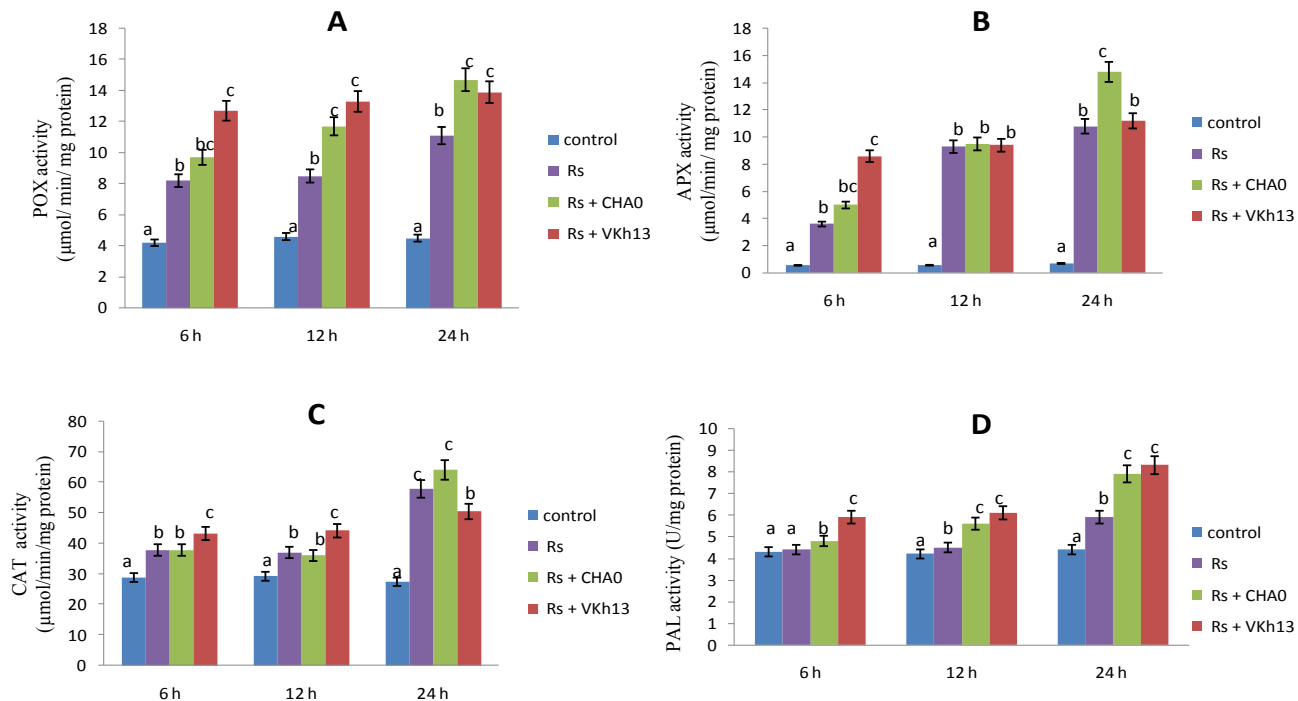
Fig. 4. Effect of *Pseudomonas* sp. VKh13 (from cluster Pp 2) on the activity of (**A**) peroxidase (POX), (**B**) ascorbate peroxidase (APX), (**C**) catalase (CAT) and (**D**) phenylalanine ammonia lyase (PAL) in bean leaves. Treatments included a non-inoculated control (indicated as Control), inoculation with *R. solani* (*Rs*) alone, inoculation with *Rs* and the reference biocontrol strain *P. protegens* CHA0 (*Rs* + CHA0), and inoculation with *Rs* and *Pseudomonas* sp. VKh13 (*Rs* + VKh13). Leaves were studied 6, 12 and 24 h after inoculation of bean roots. Data are shown as means ± standard deviations; n = 3. At each sampling, values with different letters are significantly different (ANOVA and Duncan test; $P < 0.05$). The experiments were repeated three times with similar results.

climates. Indeed, new types of strains were evidenced, since one of four clusters (i.e. Pf4) within the 'P. fluorescens' group and three of five clusters (i.e. Pp2, Pp3 and Pp4) within the 'P. putida' group that they belonged to were comprised of strains without described closely related species. The Fars province (with moderate temperatures and semi-arid conditions) yielded isolates mostly from the Pf clusters (11 of 13 isolates, with 7 of them from Pf4), whereas both the Mazandaran province (with warm temperatures and humid conditions) and the Razavi Khorasan province (with cool temperatures and arid conditions) yielded isolates mostly from the Pp clusters, i.e. 8 of 11 isolates (with 6 of them from Pp4) and 8 of 9 isolates (with 5 of them from Pp3), respectively. Thus, isolate patterns differed between provinces but, contrary to expectations, they did not clearly coincide with broad temperature or aridity contrasts within the country.

One conclusion from phylogenetic analysis is that the five clusters defined that did not contain any species described are likely to correspond to new species within the 'P. fluorescens' or 'P. putida' groups, and this deserves further research attention. Previously, this type of molecular approach had identified the possibility that P. "fluorescens" CHA0 and related ARDRA-1 strains should be described as a separate species [11,38], which was subsequently carried out and yielded *P. protegens* [15]. Another conclusion from the phylogeny of this work is that some of the nomenclatures currently in use are incorrect in both the 'P. fluorescens' and 'P. putida' groups, and the affiliation of several misnamed

P. fluorescens and *P. putida* strains needs to be changed, as mentioned previously [39,40]. Here we portrayed them as *Pseudomonas* sp., except that we kept the name *P. putida* traditionally used for many strains in the large clade (Fig. 1) containing the *P. putida* type strain (and clusters Pp2 to Pp5), but it is obvious that many pseudomonads there are distant from the *P. putida* type strain and the true boundaries of this species should be clarified [41].

A total of 12 of the 33 isolates obtained belonged to *Pseudomonas* clusters already known to include plant-beneficial bacteria, e.g. *P. thivervalensis* strains PITR2 (from Italy) and C10-190 (from Switzerland) in cluster Pf1 and the species *P. moraviensis* (which contains biocontrol strains isolated in Tunisia and Switzerland) [11,42,43] in cluster Pf2 of the 'P. fluorescens' group, as well as the species *P. mosselii* (which includes biocontrol strain FP13 from India) [44], in cluster Pp1, and *P. putida* KT2440 [45] plus many other *P. putida* strains from various countries in cluster Pp5 of the 'P. putida' group. Therefore, these four clusters display a rather cosmopolitan biogeography, as representatives from Iran were also found.

Microscopic observation of mycelium showed that all antagonistic *Pseudomonas* isolates lead to change in the growth pattern of mycelium. The fungal mycelia in dual culture with the antagonistic bacteria were dense and more branched compared to the controls.

The 'P. fluorescens' group has been extensively studied for the ability to produce emblematic biocontrol antimicrobials
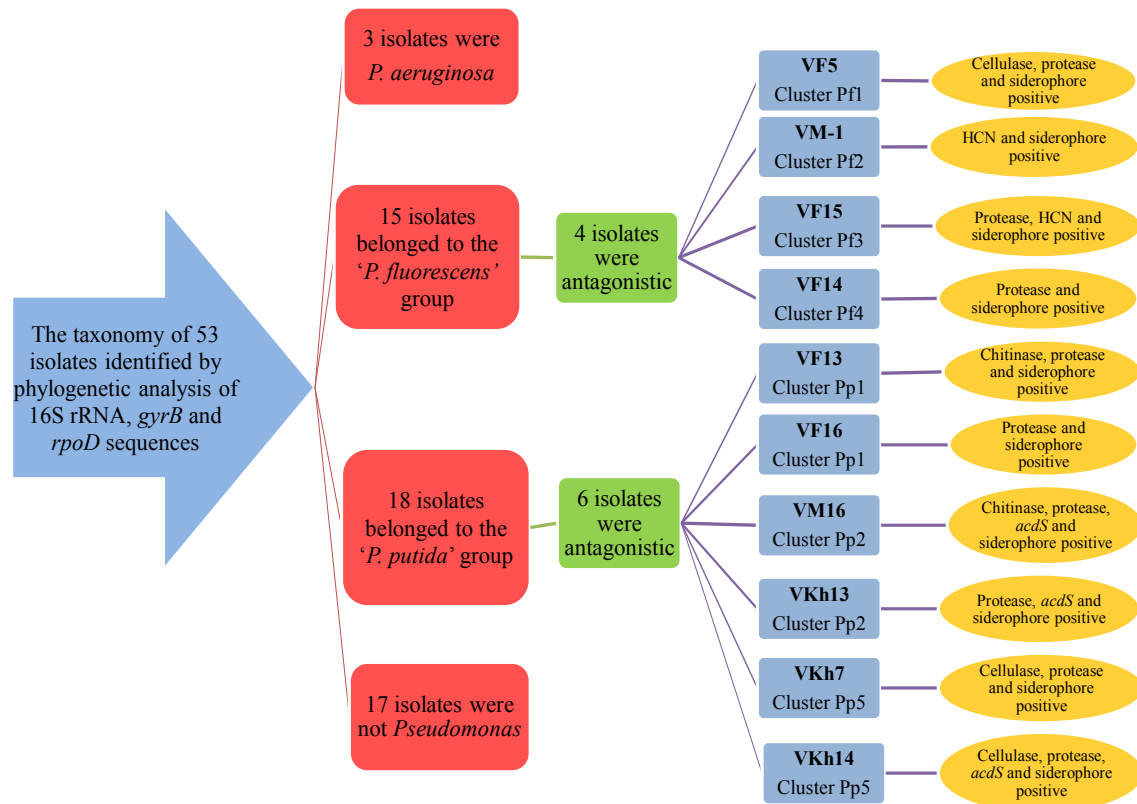
Fig. 5. The schematic picture of identification of isolates collected from bean plants in Iran by *rrs* sequencing and blasting. Taxonomic position recognition of non-*aeruginosa* isolates by multilocus sequence analysis (MLSA) of *rrs*, *rpoD* and *gyrB*. Detecting antagonistic isolates and screening genes for antibiotics pyrrolnitrin (*prnD*), 2,4-diacetylphloroglucinol (*phlD*), pyoluteorin (*pltC*), phenazines (*phz*) and for ACC deaminase activity (*acdS*) by PCR. Screening of biocontrol traits of antagonistic isolates for enzymatic activities, hydrogen cyanide and siderophore production.

such as 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin and phenazines [46−48]. However, these secondary metabolites were not evidenced in current isolates, even in Pf1 strain VF5 close to antagonistic *P. thivervalensis* strains [16] and that inhibits *R. solani* (and the phytopathogen *Macrophomina phaseolina*; data not shown) in vitro. This was unexpected, as fluorescent pseudomonads producing 2,4-diacetylphloroglucinol were obtained from a bean grown in Iranian soil from Karaj [22], in the Alborz province located next to other provinces studied in this work. Only HCN production was found in certain Pf strains (i.e. Pf2 strain VM-1 and Pf3 strain VF15), but other types of secondary metabolites (cyclic lipopeptides etc) [49] and biocontrol-relevant functions (e.g. type III secretion) [2] have not been investigated thus far. Among the six Pf2 isolates, only strain VM-1 inhibited *R. solani* in vitro, which is compatible with the moderate ability of the Pf2 species *P. moraviensis* to control *R. solani* [42].

Within the 'P. putida' group, pseudomonads from three clusters (Pp1, Pp2 and Pp5) inhibited *R. solani* on plates. Cluster Pp1 harbors species not typically associated with plant-beneficial effects, but that nevertheless contain biocontrol strains, i.e. *P. mosselii* [44,50] and *P. entomophila* [51,52], in which production of siderophores, lytic enzymes, cyclic lipopeptides and/or HCN is documented. Here, the Pp1 strain VF13 produced chitinase and protease but the other strain

inhibitory to *R. solani* (VF16) did not produce both. *P. entomophila* can colonize the rhizosphere [53], and isolates affiliated with *P. entomophila* have already been obtained from Iranian soils [54].

In contrast to Pp1, cluster Pp5 gathers emblematic strains well studied for their plant-beneficial properties (and their potential for bioremediation or bioproductions), such as *P. putida* KT2440 and BIRD-1 [55,56]. Here, the two Pp5 isolates VKh7 and VKh14 presented antagonistic effects upon fungi. Both VKh7 and VKh14 are cellulase-positive (which may be important for *Rhizoctonia* control) [7], whereas VKh14 is also ACC deaminase-positive, a trait interfering with ethylene metabolism in plants and potentially plant health [57].

Cluster Pp2 (similarly to clusters Pp3 and Pp4 branching nearby) did not contain any described species of the 'P. putida' group (Fig. 1). Two isolates (VM16 and VKh13) from this cluster controlled *R. solani* AG 4-HGI and AG 2-2 (as well as the bean pathogen *M. phaseolina*) very efficiently on plates. Other Iranian pseudomonads, which belonged to the 'P. fluorescens' group, could also benefit a bean exposed to *R. solani* AG 4 [22]. Here, both Pp2 isolates display ACC deaminase activity, an important trait for beneficial plant−microbe interactions [4,58] that is rather widespread in pseudomonads [59,60]. Strain VM16 is also chitinase-positive, which may contribute to *Rhizoctonia* control [7]. However, a more

thorough investigation of phytoprotection mechanisms is needed in light of the promising biocontrol potential of strain VKh13 and other Pp2 pseudomonads.

This investigation showed that *Pseudomonas* sp. VKh13 (from cluster Pp2) can control *R. solani* AG 4-HGI better than reference strain *P. protegens* CHA0 under non-sterile greenhouse conditions (Table 1). Previous leaf bioassays results pointed to induced resistance triggered in bean plants by *P. protegens* CHA0 [36], a finding that was also made with strain VKh13 in the current work. The dry weight of root and shoot confirmed disease index results. Consistent with this, *Pseudomonas* sp. VKh13 enhanced the activity of PAL and antioxidant enzymes POX, APX and CAT. These enzymatic responses are expected in the case of ISR, as indicated by previous inoculation studies with *P. protegens* CHA0 on bean (for all four enzymes) [36] or tomato (for POX) [69] and *Pseudomonas aureofaciens/chlororaphis* 63-28 on soybean (for CAT, APX and PAL) [70].

In conclusion, this study shows that novel types of pseudomonads with biocontrol potential can be readily obtained from Iranian soils. Many of them belong to taxa thus far uncharacterized and for which almost nothing is known about their phytoprotection mechanisms.

Conflict of interest

There is no conflict of interest to declare.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2017.08.002.

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