European Journal of Soil Biology 75 (2016) 38–46

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Heavy metal tolerant *Pseudomonas protegens* isolates from agricultural well water in northeastern Algeria with plant growth promoting, insecticidal and antifungal activities

Leila Bensidhoum [a], Elhafid Nabti [a], Nassira Tabli [a], Peter Kupferschmied [b], Angelo Weiss [c], Michael Rothballer [c], Michael Schmid [c], Christoph Keel [b], Anton Hartmann [c, *]

[a] Laboratoire de Maitrise des Energies Renouvelables, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algeria
[b] Université de Lausanne, Département de Microbiologie Fondamentale (DMF), Quartier UNIL-Sorge, Batiment Biophore, CH-1015 Lausanne, Switzerland
[c] Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Research Unit Microbe-Plant Interactions, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany

ARTICLE INFO

Article history:
Received 2 October 2015
Received in revised form
8 April 2016
Accepted 15 April 2016

Handling Editor: C.C. Tebbe

Keywords:
Pseudomonas protegens
Heavy metal tolerance
Plant growth promoting bacteria
Biocontrol and insecticidal activity
Enzymatic activities
Barley

ABSTRACT

The application of plant growth promoting bacteria (PGPB) with biocontrol activities as inoculants of crop plants against phytopathogenic fungi and insect pests provide a biological alternative to the use of agrochemicals. Two *Pseudomonas protegens* strains were isolated from agricultural well water in a heavy metal contaminated area near Bejaia, northeastern Algeria. The isolates S4LiBe and S5LiBe had 16S rRNA gene sequence similarities of 99.4%–99.7% with *P. protegens* CHA0^T and other *P. protegens* strains. The phenotypic profiles tested with BIOLOG-GN2-microplates showed differences in 12 of 95 carbon sources tested, as compared to the type strain *P. protegens* CHA0^T. The isolates S4LiBe and S5LiBe showed plant growth promoting potential which is commonly associated with the production of the phytohormone indole acetic acid and siderophores and the solubilization of insoluble phosphate. In addition, they produce chitinase and other polymer degrading enzymes. As the strains S4LiBe and S5LiBe were isolated from heavy metal polluted well water, they are resistant against several heavy metals (2.0 mM $K_2Cr_2O_7$ and 3.0 mM $CoSO_4$, $HgSO_4$, $CdSO_4$ $8H_2O$ and $PbCl_2$), while the reference strain *P. protegens* CHA0^T was very sensitive to Hg^{2+} and Cd^{2+} and had lower tolerance towards Co^{2+} and Pb^{2+}. The isolates S4LiBe and S5LiBe were active in mycelial growth inhibition assays against *Botrytis cinerea*, *Verticillium dahliae*, *Fusarium graminearum*, *Aspergillus niger* and *Aspergillus flavus* (growth inhibition between 88% and 48%). Furthermore, S4LiBe and S5LiBe showed effective insecticidal activities, when tested in the *Galleria* injection assay and they were tested positive for the insect toxin gene *fitD* alike the reference strain CHA0^T. Finally, inoculation of barley seeds with S5LiBe in non-polluted agricultural soil significantly stimulated the germination rate and growth of seedlings, with increased shoot length (11.96 cm ± 0.59), shoot and root fresh weight (0.10 g ± 0.009, 0.04 g ± 0.006), shoot and root dry weight (0.075 g ± 0.003, 0.03 g ± 0.007) as compared to non-inoculated plants (10.23 cm ± 0.84, 0.06 g ± 0.007, 0.025 g ± 0.006, 0.047 g ± 0.006, and 0.016 g ± 0.004, respectively). In heavy metal contaminated soil, inoculation with strain S5LiBe resulted in similar increase of germination rate and growth parameters of barley like in the non-polluted soil, while *P. protegens* CHA0^T inoculated plants were not stimulated. Thus, the heavy metal tolerant isolates S4LiBe and S5LiBe have a potential as beneficial bacteria for agricultural application even in heavy metal polluted soils, e.g. for the stimulation of biomass crops. The demonstration of successful isolation from agricultural well water may open more ready access for a wide variety of this kind of beneficial bacteria for agricultural application.

* Corresponding author. Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Department for Environmental Sciences, Research Unit Microbe-Plant Interactions, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany.
E-mail address: a.hartmann@helmholtz-muenchen.de (A. Hartmann).

http://dx.doi.org/10.1016/j.ejsobi.2016.04.006
1164-5563/

1. Introduction

Inoculation with plant growth promoting bacteria (PGPB) represents an agro-biotechnology designed to improve growth, yield and health of agricultural crops [53,55]. The use of these beneficial microorganisms appears to be cost-effective, ecosystem friendly and a healthy alternative to the extensive use of chemicals such as fungicides, herbicides and insecticides, which have negative impacts on the environment and human health.

The potential of PGPB to increase crop production involves the solubilization of inorganic phosphate and ferric iron minerals, the increased uptake of mineral nutrients [37], the ability to reduce stress ethylene production in plants [21], N_2 fixation [35], and the production of plant hormones such as auxins, gibberellins and cytokinins [20]. PGPB are able to control pathogens by several mechanisms: competitive root colonization, production of antimicrobial compounds [13], production of hydrolytic enzymes, siderophores, HCN, ammonia, and by inducing systemic resistance of plants towards phytopathogens [12,53]. In addition, insecticidal activities were reported for some PGPB in addition to the existing biocontrol repertoire with perspectives for application against insect crop pests [52]. Recent reports suggest that PGPB also enhance the tolerance of plants towards abiotic stresses such as drought [58,62], chilling injury [3], salinity [25], metal toxicity [16] and elevated temperature stress [1,2].

A high diversity of PGPB have been identified which enhance plant growth by several mechanisms. These PGPB belong for example to the bacterial genera *Pseudomonas*, *Azospirillum*, *Cellulosimicrobium*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* [11,20,24,30,39,40,43].

Among PGPB, *Pseudomonas* spp. have been broadly studied for their roles in plant growth promotion and biological control [18,19,24,28,34,50,63]. Plant growth promoting activities of *Pseudomonas* spp. include production of indole acetic acid (IAA) [18], phosphate solubilization [64], degradation of toxic compounds [42] and production of bioactive metabolites against phytopathogens like the production of siderophores [8] and antibiotics [24,27] and of insect toxins against insect pests [32,45].

Due to industrialization, environmental pollution by heavy metals is a common problem all over the world. However, heavy metal contamination of soils and water may also originate from natural occurring geominerals. Nevertheless, in many sites anthropogenic activities have enriched or deposited huge amounts of heavy metal containing rocks, soil and debris, which are the sources of pollution plumes into the surrounding soil and water bodies. In heavy metal-polluted sites, heavy metal-resistant bacteria have been found, which face the pollution and even have the ability to reduce it by bioremediation activities, partly in combination with plants [42].

In the present investigation, heavy metal contaminated agricultural well water was tested for the presence of plant growth promoting bacteria, because this water was fed from heavy metal polluted soil. The contamination originated from a lead mineral containing rock deposit in some distance, origination from lead mining, which is no longer in operation. The hypothesis was that rhizosphere colonizing PGPB should be part of the bacterial community of the well water. A special emphasis was laid on the *Pseudomonas* group of bacteria, because these have a wide spectrum of plant beneficial activities ranging from antagonistic activities against a wide range of phytopathogenic fungi, insecticidal activity, and phytohormone, siderophore and extracellular enzyme production. The aim was to get PGPB-isolates which could be demonstrated to stimulate plant growth in non-polluted and heavy metal polluted soil.

2. Material and methods

2.1. Isolation of bacteria from heavy metal polluted well water

Plant growth promoting bacteria were isolated from heavy metal polluted well water (receiving effluents from agricultural soil) in the region of Bejaia (northern Algeria). The well water had the following contamination levels (ppm): Cr 65.65 ± 0.22; Cd 8.6 ± 0.23; Hg 3.87 ± 0.12; Co 21.0 ± 0.42; Pb 212.45 ± 0.85; As 2.3 ± 0.1; and Zn 133.80 ± 0.88. The soil in this region is contaminated with heavy metals as follows (ppm): Cr 52.0 ± 0.43; Cd 8.2 ± 0.12; Hg 3.54 ± 0.12; Co 19.19 ± 0.62; Pb 199.51 ± 1.3; As 3.12 ± 0.08; Zn 125.12 ± 0.96. (Laboratory of physico-chemical soil analysis, PROFER-company, Mostaganem-Bejaia, Algeria). Water samples were serially diluted in sterile distilled water and 0.1 ml of each dilution was seeded in triplicates onto nutrient broth (NB) agar plates containing in g/l: trypton 10 g, meat extract 5 g, NaCl 5 g from bioKar diagnostics and agar-agar 18 g from Liofilchem. The agar plates were incubated at 28 °C for 1 week and colonies with different morphologies were selected, re-streaked on nutrient agar medium, and checked for purity.

2.2. Phenotypic and molecular phylogenetic characterization

The isolates were tested by Gram-staining following the standard protocol [5]. The motility test was carried out on mannitol mobility gels according to Guiraud and Galzy [23]. Selected isolates and reference strains were phenotypically characterized for carbon source utilization using the Biolog GN2 MicroPlate™ with 95 different carbon sources according to the manufacturer's instruction. For the phylogenetic characterization, genomic DNA extraction from pure bacterial colonies was carried out using the FastDNA® SPIN kit in conjunction with the FastPrep FP120 instrument (Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. The genomic DNA was further PCR amplified for 16S rDNA gene sequencing using the flanking primer pair 616F (5′AGA GTT TGA TYM TGG CTCAG 3′) and 630R (5′ CAK AAA GGA GGT GAT CC 3′) resulting in the amplification of the whole 16S rRNA-sequence (1500 nucleotides). The amplification program was performed in a thermocycler peqSTAR 96 Universal, with an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 55 °C, 1 min extension at 72 °C, and a final extension step at 72 °C for 10 min. Correct amplification was tested with standard horizontal agarose gel electrophoresis followed by ethidium bromide staining. Amplification products were cloned using the StrataClone PCR cloning system. Clones were then sequenced using the Big Dye Terminator Labeling Kit (Applera, Europe BV) with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, USA). For phylogenetic analyses the obtained 16S rDNA-sequences were aligned with the *Sina Aligner V1.2.11* on the Silva website (www.arb-silva.de) and phylogenetical allocated with the software package ARB [36]. Phylogenetic tree construction was performed by using the Maximum-Likelihood [44].

2.3. Characterization of plant growth promoting properties

2.3.1. Production of indole acetic acid (IAA)

IAA production was measured using the method described by Bric et al. [6]. The isolates were grown in LB liquid medium (containing in g/l: NaCl 4, trypton 10, yeast extract 5) supplemented with 0.5% glucose and 500 μg/ml L-tryptophan using 100 rpm shaking conditions at 30 °C for 72 h. 5 ml of culture was centrifuged at 9000 rpm for 20 min and 2 ml of supernatant was transferred to a fresh tube to which 100 μl of 10 mM orthophosphoric acid and

4 ml of reagent (1 ml of 0, 5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added. The mixture was incubated at room temperature for 25 min and the absorbance of pink color developed was read at 530 nm.

2.3.2. Phosphate solubilization

The isolates were tested for inorganic phosphate solubilization. Freshly grown bacterial culture was inoculated to Pitkovskaya agar containing inorganic phosphate and incubated at 30 °C for 5 days. A clear halo around the bacterial colony indicates solubilization of mineral phosphate [57].

2.3.3. Cellulase activity

Bacteria were inoculated in CMC (Carboxy Methyl Cellulose) agar containing (g/l): Na_2HPO_4 6, KH_2PO_4 3, NaCl 0.5, NH_4Cl 1, yeast extract 3, CMC 7, agar-agar 15 and incubated at 30 °C for 8 days. At the end of the incubation, to visualize the hydrolysis zone, the agar medium was inundated with solution of Congo red (1% w/v) for 20 min. Congo red solution was then poured off, and the plates were further treated with 1 M NaCl [9]. Clear zones around the colony indicated the production of extracellular cellulase.

2.3.4. Esterase activity

The isolates were inoculated in the media which is described by Sierra [60], containing (g/l) peptone 10, NaCl 5, $CaCl_2$ $2H_2O$ 0.1, agar-agar 18, and 1% of sterilized tween 80. After incubation at 30 °C for 48 h a clear halo around the colonies demonstrated esterase activity.

2.3.5. Lipolytic activity

Lipolytic activity was determined as described above for the determination of esterase activity [60]. In this experiment, tween 80 was replaced by tween 20. Clear halos around the colonies indicate lipolytic activity.

2.3.6. Protease activity

Protease activity tests were carried out in skim milk agar plate medium, containing (g/l) casein hydrolysate 10, yeast extract 5, NaCl 4, skim milk powder 20, agar-agar 15. After incubation at 30 °C for 48 h, the presence of a clear zone around colony was the indication of protease production [29].

2.3.7. Urease activity

Urease activity was determined as described by Christensen [14]. The agar-medium contains (g/l) peptone 1, glucose 1, NaCl 5, Na_2HPO_4 1.2, KH_2PO_4 0.8, phenol red 0.012, and agar-agar 15. After autoclaving, 50 ml of urea solution (40%) were added. Urease production was indicated by a bright pink color around colony.

2.3.8. Siderophore production

The experiments were carried out in Chrome Azurol S agar, according to the method of Schwyn and Neilands [59]. CAS agar was prepared from four solutions, which were sterilized separately before mixing: the Fe-CAS indicator solution, buffer solution, nutrient solution and casamino acid solution. After autoclaving and at a temperature of about 50 °C, nutrient solution and casamino acid solution were added to the buffer solution. The indicator solution was added last with sufficient stirring to mix the ingredients. The isolates were streaked on the surface of the blue agar plates, incubated at 30 °C for 3 days, and examined for growth and production of red to orange halos surrounding the colonies, indicating siderophore production.

2.3.9. Chitinase activity

Chitinase activity was determined as described by Kopečný et *al.* [31]. Bacterial isolates were inoculated in minimal salt medium containing 0.8% of colloidal chitin as sole carbon and energy source and the following ingredients (g/l): K_2HPO_4 2.7, KH_2PO_4 0.3, $MgSO_4$ $7H_2O$ 0.7, NaCl 0.5, KCl 0.5, yeast extract 0.13, agar-agar 15. The plates were incubated at 30 °C for 7 days. Clear zone around the colonies indicate extracellular chitinase activity.

2.4. Characterization of biocontrol activities

2.4.1. Antifungal activity

The isolates were assayed for antifungal activities against *Botrytis cinerea*, *Verticillium dahliae* and *Fusarium graminearum* as described by Sagahón et al. [56]. Fungal plugs of 1 cm^2 were inoculated in the center of Luria Bertani (LB) plates and each bacterial isolate was inoculated at a distance of 2.5 cm from the fungal inoculum. Plates without potential bacterial antagonist served as negative control. Three replicates were performed for each confrontation experiment. The plates were then incubated at 25 ± 2 °C for 5 days and verified every day. The percentage of growth inhibition (PGI) of the fungus was recorded and calculated using the formula: PGI (%) = KR-R1/KR-100 where KR corresponds to the distance from the point of inoculation to the colony margin on the control dish (mm). R1 represents the distance (mm) of fungal growth from the point of inoculation to the colony margin on the treated dishes.

2.4.2. Insecticidal activity

As biological insecticidal activity test, the *Galleria mellonella* virulence assay was performed. The injection assays for virulence determination used last-instar larvae of *G. mellonella* (Entomos AG, Grossdietwil, Switzerland) as described before by Péchy-Tarr et al. [45], with 18 larvae per tested bacterial strain.

To test the presence of the *fitD* toxin gene *fitD* specific primers were applied with DNA extracted from the bacteria. The polymerase chain reaction was performed using the GoTaq DNA Polymerase kit (Promega) according to the manufacturer's instructions and the primer pairs fitD-screen-F: 5′-CCTGCTCAATACCCTGATCG-3′ and fitD-screen-R: 5′-GTGGTTGGCGAAGTACTGCTC-3′ (P. Kupferschmied, unpublished).

2.5. Determination of heavy metal tolerance

Heavy metals incorporated media were used to examine the ability of the isolates to resist heavy metals. Cells of overnight grown cultures were inoculated on nutrient agar plates supplemented with different heavy metals (K_2CrO_7, $HgSO_4$, $CdSO_4$ $8H_2O$, $CoSO_4$, $PbCl_2$), The concentration of each heavy metal solution (0.5, 1, 1.5, 2, 2.5 and 3 mM) was prepared in sterile deionized water and sterilized by autoclaving at 121 °C for 15 min. After incubation for 24–48 h at 30 °C, the plates were examined for cell growth [26].

2.6. Plant growth stimulation tests

2.6.1. Surface sterilization of barley seeds

Barley (*Hordeum vulgare* L.) seeds were surface-sterilized as described by Götz et al. [22]. First, the seeds were treated with 70% ethanol for 1 min and then with 12% acidified hypochlorite for 15 min. The seeds were washed thoroughly in sterile water. Then the seeds were germinated on LB agar plates in the dark at room temperature for 2 days.

2.6.2. Inoculation and growth of barley seedlings

For seed inoculation, S5LiBe was grown in LB medium overnight at 30 °C. The bacterial culture was pelleted by centrifugation and

the supernatant was discarded. Cell pellet was washed twice with 20 ml phosphate buffered saline (PBS, pH 7.2), and suspended in PBS. The optical density of bacterial suspension was 0.1 at 620 nm, corresponding to a cell density of 10^8 cells/ml [17]. Surface sterilized barley seeds were incubated with bacterial suspension for 1 h at room temperature. Control seeds were incubated in sterile distilled water under the same conditions [39]. The seeds were planted in pots filled with unpolluted agricultural soil with following characteristics: pH (7.04), granulometry (clay 17.1%, fine silt 23.0%, coarse silt 7.1%, fine sand 12.4%, coarse sand 28.9%), active limestone 0.38%, conductivity 200 μS/cm, organic carbon 6.38%, exchangeable K_2O 0.11 g/kg, exchangeable CaO 3.99 g/kg, exchangeable MgO 5.24 g/kg.

The experiment consisted of 7 lots and each lot was composed of 7 seeds, inoculated or non-inoculated with bacterial suspension. The seeds were sown at a depth of approximately 1 cm. The experiment was performed under natural dark/light cycles (16 h of light and 8 h of dark) at temperature of 25–35 °C for two weeks [54]. Seed germination was determined by counting germinated seeds at 3 and 7 day after sowing the seeds. Results corresponding to final counts were reported as percentage of germination [29]. Plant growth response parameters were measured after 15 days including shoot length, fresh and dry weight of shoot, fresh and dry weight of root. Fresh weight was determined by measuring the weight of shoots and roots in the fresh state directly after harvest. For dry weight measurements, harvested roots and shoots were dried in a hot oven at 45 °C until stabilization of the weight.

In order to show the ability of the strain S5LiBe to stimulate barley growth in heavy metal contaminated soil, a similarly designed experiment as above (but with 5 lots instead of 7 lots) was carried out using soil with heavy metal contamination (see the composition above). In this experiment, a heavy metal sensitive reference strain *P. protegens* CHA0^T was included as well as a control without any inoculation.

2.7. Statistical analysis

Data obtained for plant growth parameters were subjected to multi-analysis of variance (MANOVA) by the least significant difference (LSD) test at $p \leq 0.05$ with statistical software XLSTAT version 2009.1.02.

3. Results

3.1. Phylogenetic and phenotypic characterization of the isolates

Originally 11 bacterial isolates from the well water were randomly picked and two bacterial strains (S4LiBe and S5LiBe) were selected for further analysis based on their antifungal and enzymatic activities as well as molecular phylogenetic analysis. These isolates gave a Gram-negative staining and were motile. The colonies had a greenish yellow color, resembling *Pseudomonas* spp. bacteria.

For molecular phylogenetic characterization of the isolates, 16S rDNA sequence analysis was performed. PCR-amplified 16S rDNA of the bacterial strains was sequenced and blasted with the NCBI database. Comparative analysis with whole 16S rDNA database sequences suggested that the isolates S4LiBe and S5LiBe were most closely related to *Pseudomonas protegens*. The 16S rDNA sequences of the two isolates were 99.8% similar to each other. S4LiBe showed 99.8% 16S rRNA similarity to *Pseudomonas* sp. AF521651, 99.7% to *P. protegens* PF-5 (sequence AJ417073), 99.6% to *P. protegens* PGNR1 (sequence AJ417071), and 99.5% to *P. protegens* CHA0^T (sequence AJ278812). The 16S rDNA of S5LiBe had 99.4% similarity with *Pseudomonas* sp. AF521651, 99.6% with *Pseudomonas protegens* AJ 417073 and 99.5% with *P. protegens* CHA0^T (sequence AJ278812) (see Table S1). The phylogenetic position of the isolates based on 16S rDNA similarity is also shown in the dendrograms based on maximum likelihood tree calculation (Fig. 1).

The carbon source utilization patterns of the isolates S4LiBe and S5LiBe were assessed using the Biolog GN2 Microplates (Table S2) and compared with the type strain of *P. protegens* CHA0^T and *P. protegens* Pf-5. From 95 carbon sources tested with the isolates S4LiBe and S5LiBe, 89% were identical with the type strain CHA0^T, while 84% were identical with the strain Pf-5. Differences in the utilization pattern between CHA0^T and the isolates were found for m-inositol, D-psicose, sucrose, formic acid, hydroxybutyric acid, itaconic acid, succinamic acid, D-alanine, L-alanine, L-alanylglycine, hydroxy-L-proline and L-threonine (for more information see Table S2).

3.2. Plant growth promotion traits

Table 1 shows the PGP traits of the isolates in comparison with the *P. protegens* type strain CHA0^T. The IAA-production ranged from 3.1 to 4.0 μg ml^{-1} in the presence of 500 μg/ml L-tryptophan. The isolates and the reference strain produced urease, lipase, protease, esterase and cellulase at different levels. However, CHA0^T failed to produce chitinase under the applied test conditions. The abilities to solubilize precipitated phosphate and to produce siderophores were common in S4LiBe, S5LiBe and CHA0^T.

3.3. Heavy metal tolerance

Among the heavy metals, lead and cobalt were less toxic to all isolates. S4LiBe, S5LiBe and CHA0 were able to grow in the presence of K_2CrO_7 at concentrations up to 2 mM. The isolates S4LiBe and S5LiBe showed very high degree of tolerance up to 3 mM $HgSO_4$, $CdSO_4$, and $PbCl_2$. In contrast, CHA0 was very sensitive to $HgSO_4$ and less tolerant towards $CdSO_4$ and $PbCl_2$ (Table 2).

3.4. Biological control activities

3.4.1. Fungal antagonistic activity

Fig. 2 shows the antifungal activity of the isolates against the pathogenic fungi *F. graminearum*, *V. dahliae* and *B. cinerea* as well as *Aspergillus niger* and *Aspergillus flavus* tested in an agar plate confrontation assays. All five fungi were inhibited to different extent by the *P. protegens* isolates S5LiBe and S4LiBe.

3.4.2. Insecticidal activity

The *Galleria mellonella* virulence assay of the isolates S4LiBe and S5LiBe as well as the reference strain CHA0^T clearly demonstrated that the isolates were equally effective as the *P. protegens* reference strain (Fig. 3). In both isolates and the CHA0^T reference strain the *fitD* gene for the Fit insecticide toxin could be detected, while in the FitD deficient mutant F113, identified by band at approximately 900 bp, was missing.

3.5. Plant growth promoting effect of isolate S5LiBe

The germination of barley seeds was significantly stimulated at 3 days after inoculation with S5LiBe over non-inoculated controls in unpolluted soil (Fig. 4A). Moreover, S5LiBe significantly increased germination percentage in comparison to the reference strain CHA0 and non-inoculated control seedlings in polluted soil (Fig. 4B).

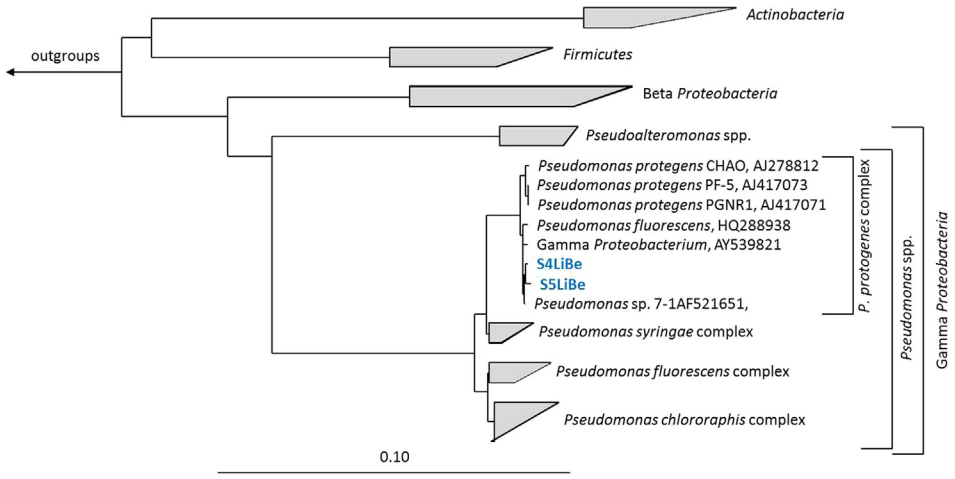

Fig. 1. 16S rRNA gene similarity tree, based on maximum likelihood analysis.

Table 1
Plant growth promoting traits of the isolates S4LiBe, S5LiBe, and *P. protegens* CHA0^T. Zones of clearance or indicator color changes around colonies were measured; "+": diameter between 7 and 10 mm; "++": diameter between 10 and 20 mm; "+++": diameter above 20 mm.

Traits	S4LiBe	S5LiBe	CHA0^T
IAA (μg/ml)	4.0	3.1	3.5
Chitinase	+	++	–
Protease	+	+	++
Esterase	+	+	+
Cellulase	+	+	+
Lipase	+	+	++
Urease	++	+++	+
Phosphate solubilization	++	++	++
Siderophore production	++	+++	++

Table 2
Heavy metal tolerance of the isolates S4LiBe and S5LiBe and *P. protegens* CHA0^T; "+++": High resistance (more than 100 colonies per plate); "++": average resistance (30–50 colonies per plate); "+": resistance (very few colonies per plate); "–": sensitive (complete growth inhibition).

		Isolates		
		S4LiBe	S5LiBe	CHA0^T
K_2CrO_7 (mM)	0.5	+++	+++	+++
	1	+++	+++	++
	1.5	++	++	++
	2	+	+	+
	2.5	–	–	–
	3	–	–	–
$HgSO_4$ (mM)	0.5	+++	+++	–
	1	+++	+++	–
	1.5	++	++	–
	2	++	++	–
	2.5	++	++	–
	3	++	++	–
$CdSO_48H_2O$ (mM)	0.5	+++	+++	++
	1	++	++	+
	1.5	+	++	–
	2	+	+	–
	2.5	+	+	–
	3	+	+	–
$CoSO_4$ (mM)	0.5	+++	+++	+++
	1	+++	+++	+++
	1.5	+++	+++	++
	2	++	++	++
	2.5	+	+	+
	3	+	+	–
$PbCl_2$ (mM)	0.5	+++	+++	+++
	1	+++	+++	+++
	1.5	+++	+++	++
	2	+++	+++	++
	2.5	+++	+++	+
	3	+++	+++	+

Concerning the plant growth parameters measured in unpolluted soil, S5LiBe stimulated significantly the shoot length compared to the control (Fig. 5a). Bacterial inoculation also significantly improved fresh and dry weight (g) of shoots and roots compared to the controls (Fig. 5b and c). In heavy metal polluted soil, S5LiBe improved significantly the following parameters: shoot and root length (Fig. 6a and b); fresh and dry weight (g) of shoot and root (Fig. 6c and d), compared to the heavy metal sensitive reference strain CHA0^T and the non-inoculated control.

4. Discussion

Fluorescent pseudomonads and other rhizobacteria are well known for their abilities to successfully colonize plant roots and to promote plant growth by biological control [24,47] and plant growth promotion activities [46]. In the present study, two *Pseudomonas protegens* isolates from agricultural well water indeed showed a wide variety of different features of plant growth promoting traits. The isolates S4LiBe and S5LiBe are within the *P. protegens* subspecies of the *P. fluorescens* species based on 16S rDNA gene similarity analysis (Fig. 1 and Table S1) and carbon utilization pattern (Table S2). The isolates S4LiBe and S5LiBe are very similar (99.8% 16SrRNA gene similarity) to each other and are very closely related (99.4–99.7%) to the *P. protegens* strains CHA0^T and Pf-5 [48,34].

Biological control of phytopathogens by fluorescent pseudomonads reduces the severity of many plant diseases [53]. In this study, both isolates showed clearly *in vitro* antagonistic potential against several plant pathogenic fungi and *Aspergillus* spp. (Fig. 2). Srinivasan et al. [61] had characterized five members of the genus *Pseudomonas*, *Pseudomonas putida* FC-6B, *P.* sp. FC-7B, *P. putida* FC-8B, *P.* sp. FC-9B and *P.* sp. FC-24B, which showed antifungal activity against *Fusarium oxysporum* and *F.* sp. *lycopersici*. Sagahón et al. [56] reported that *Pseudomonas* spp. 11 inhibited up to 70% of *Stenocarpella maydis* and *Stenocarpella macrospora* and the filtrates

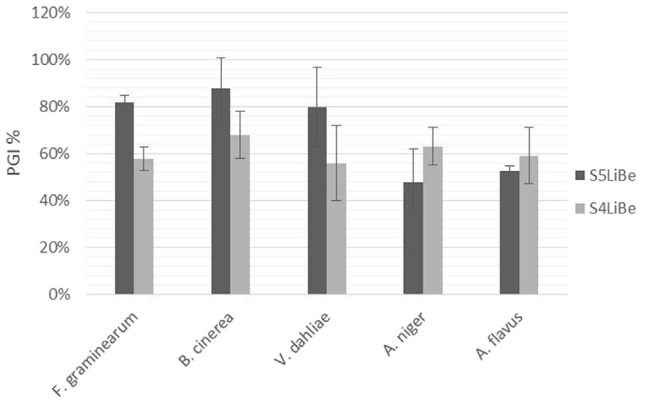

Fig. 2. Percentage Growth Inhibition (PGI %) of plant pathogenic fungi (plate confrontation assays) by the isolates S4LiBe and S5LiBe.

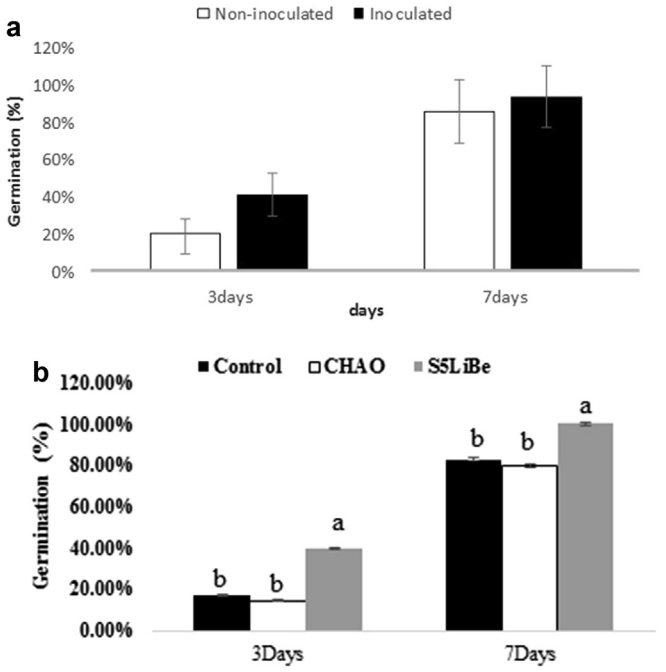

Fig. 4. Germination percentage of non-inoculated, S5LiBe-, and CHA0-inoculated barley seeds after 3 and 7 days in A) un-polluted soil and B) heavy metal polluted soil. Different letters indicate significant differences obtained by the Fischer LSD test ($p \leq 0.05$).

obtained in logarithmic growth phase from the *P. fluorescens* 16 inhibited 54% of the growth of *Stenocarpella maydis*. In addition to siderophore and chitinase production, the observed biological control activity of the isolates is probably also due to the production of several antibiotic compounds, like 2,4-diacetylphloroglucinol and pyoluteorin, as is well known for fluorescent pseudomonads [24,48]. Currently, genomic analysis of the two isolates is in progress, which will demonstrate which biosynthetic gene clusters for antibiotic production and other antagonistic activities are present in the two well water isolates from Algeria. Like the reference strain *P. protegens* CHA0^T and related strains [45,51], the isolates S4LiBe and S5LiBe also harbor insecticidal activities, as could be demonstrated in the positive *Galleria mellonella* injection assay and the presence of the *fitD* gene (Fig. 3). Since the infection and growth within insect larvae constitutes a second growth cycle of these bacteria – apart from plant roots – it may be hypothesized, that these types of bacteria were enriched in the well water also harboring insect larvae. Therefore, the isolation of PGPB with insecticidal activities from agricultural well water may be a quite straight forward approach, which may be useful in future isolation attempts.

S5LiBe-inoculated plants significantly increased germination rate, shoot length, fresh and dry biomass of barley comparing with non-inoculated after two weeks. A plant growth promoting strain of *Pseudomonas* spp. has been already described by Rosas et al. [50], who reported that the strain *Pseudomonas aurantiaca* SR1, when applied on maize and wheat seeds showed a significant plant growth promoting effect. The beneficial effect of *Pseudomonas* was also confirmed by Egamberdieva et al. [17] who tested the co-inoculation of *Pseudomonas* spp. with *Rhizobium* on the growth of fodder galega (*Galega orientalis* Lam.). Co-inoculation of plants showed increased shoot and root dry matter compared to the inoculation with *Rhizobium galega* HAMBI 540 alone. The isolates S4LiBe and S5LiBe as well as the reference strain CHA0 had IAA production activity in the presence of L-tryptophan in the range of 3–4 μg/ml, which is in the range of other PGPBs. Naik and Sakthivel [41] suggested that the plant growth promoting ability of

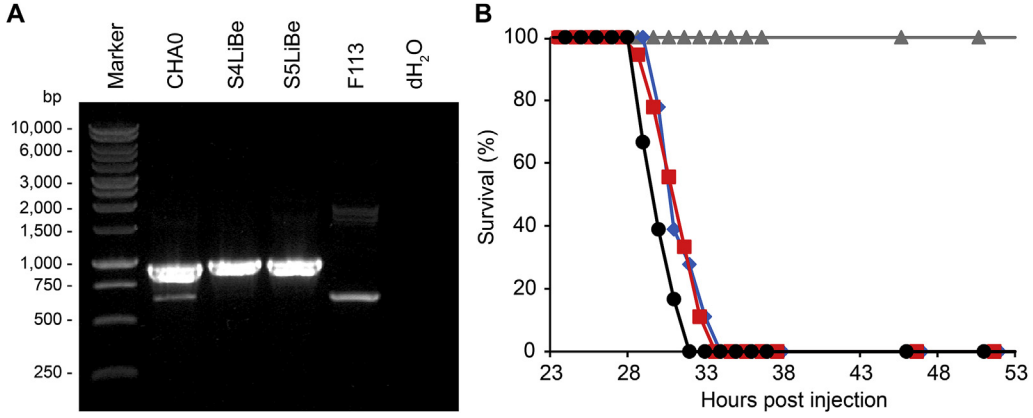

Fig. 3. Insecticidal activity of the isolates S4LiBe and S5LiBe compared to the reference strain CHA0^T. A) PCR-analysis of the *fitD* gene: In lane F113, a *fitD* deletion mutant of CHA0 was applied; dH_2O: blank control. B) *Galleria mellonella* virulence assay, according to Péchy-Tarr et al. [41]; circles: *P. protegens* CHA0^T (positive control); diamonds: *Pseudomonas* sp. S4LiBe; squares: *Pseudomonas* sp. S5LiBe; triangles: 0.9% NaCl solution (negative control).

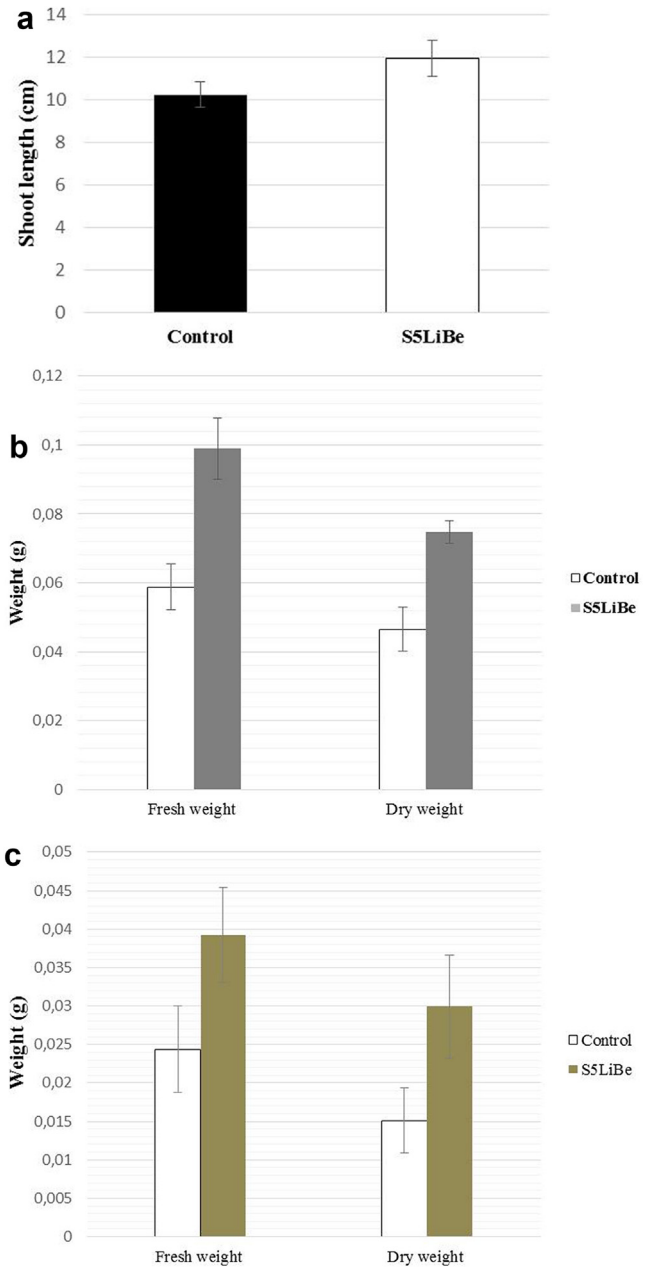

Fig. 5. Stimulation of growth of barley seedlings in unpolluted soil due to inoculation with strain S5BiLe after 15 days of growth as compared to non-inoculated control plants. Different letters indicate significant differences obtained by the Fischer LSD test ($p \leq 0.05$). A) Effect on shoot length, B) Effect on fresh and dry weight of shoots, C) Effect on fresh and dry weight of roots.

Pseudomonas sp. PUP6 could be based on the production of phytohormone IAA, siderophores and phosphate-solubilizing enzymes. Wahyudiet al. [63] reported that the capability to increase plant growth parameters in germination seed bioassays was highly related to the IAA level produced by *Pseudomonas* spp. applied. The growth promoting effect of S5LiBe can also be due to another mechanism, which is linked to its phosphate-solubilizing activity. Phosphorus (phosphate) is one of the major essential macronutrients for biological growth and development and the release of insoluble forms of phosphorus increase soil phosphorus accessibility. Rodriguez and Fraga [49] reported that *Pseudomonas* and other Phosphate Solubilizing Bacteria (PSB) are capable to increase the availability of phosphate in soil. Siderophore production is another important characteristic of efficient plant growth promoting which was observed with the isolates as well as the applied reference strain CHA0. Siderophores are widely studied as one biocontrol mechanism against plant pathogenic microbes, but they also may support iron nutrition of plants. Siderophores can stimulate plant growth indirectly by the inhibition of phytopathogenic microorganisms competing for the growth limiting mostly insoluble ferric iron resource in soils [17,33,54].

Furthermore, the isolates S4LiBe and S5LiBe produce several exo-cellular enzymes (protease, cellulose, chitinase and urease) which are potentially relevant for soil fertility. The degradation of protein by microbial peptidases is important in N-cycling in soils by making organically bound nitrogen accessible for plants [4]. Chitinases are well known to lyse the fungal cell wall [10] and thus could effectively contribute to control plant-pathogenic fungi. As reported by Egamberdieva et al. [17], cellulose production of bacteria can enhance nodule formation. Co-inoculation of the cellulose-producing strain *Pseudomonas trivialis* 3Re27 with *Rhizobium galegae* HAMBI 540 significantly increased nodulation and nitrogen content of fodder Galega, whereas cellulose-negative *Pseudomonas extremorientalis* TSAU20 showed no significant stimulation.

Since heavy metals cannot be biologically degraded to harmless products and hence persist in the environment indefinitely, heavy metal contamination of agriculture soil is a significant environmental problem and has several disadvantages on human health and agriculture [65]. The selection of metal-tolerant plant growth promoting microorganisms can be advantageous to speed up the recolonization of the plant rhizosphere in the polluted soil [15]. Both isolates in this study were characterized by considerable tolerance to rather high levels (up to 3 mM) of heavy metals Co, Pb, Hg and Cd and up to 2 mM to chromium (Table 2). Dell' Amico et al. [16] showed that inoculation with cadmium-resistant strains *Pseudomonas tolassi* and *Pseudomonas fluorescens* enabled *Brassica napus* to grow under cadmium stress because of the production of indole acetic acid (IAA), siderophores and ACC (1-aminocyclopropane-1-carboxylate) deaminase, which protected the plants against cadmium stress. Since the *P. protegens* reference strain CHA0^T (like related PGPB) is rather sensitive to these heavy metals (Table 2), the heavy metal tolerance of S4LiBe and S5LiBe can be regarded as unique among this group of bacteria. Probably, the vicinity of the heavy metal deposit had resulted in soil contamination which fostered the distribution of heavy metal tolerance. It is well known, that metal resistance transposons or plasmids are shared between Gram-negative bacteria [38]. Further detailed investigation of the actual contamination in the area of isolation and the well water as well as about the genetic basis in the isolated bacteria are necessary.

In conclusion, the isolates S5LiBe and S4LiBe revealed plant growth promoting potentials, as shown by the presence of many key features of plant growth promotion and by the stimulation of germination and growth barley seedlings upon inoculation. Furthermore, the estimation of the antagonistic effect against pathogenic fungi and also insecticidal activity add further valuable activities for possible plant beneficial bacterial inoculants. To find out most efficient biological control agents against plant diseases under given application conditions, an increasing number of isolates is still to be investigated [7]. The observed heavy metal tolerance of the new isolates towards mercury, cadmium, cobalt and lead may enable the newly isolated *P. protegens* strains as superior candidates for inoculation of biomass crops in heavy metal contaminated soils.

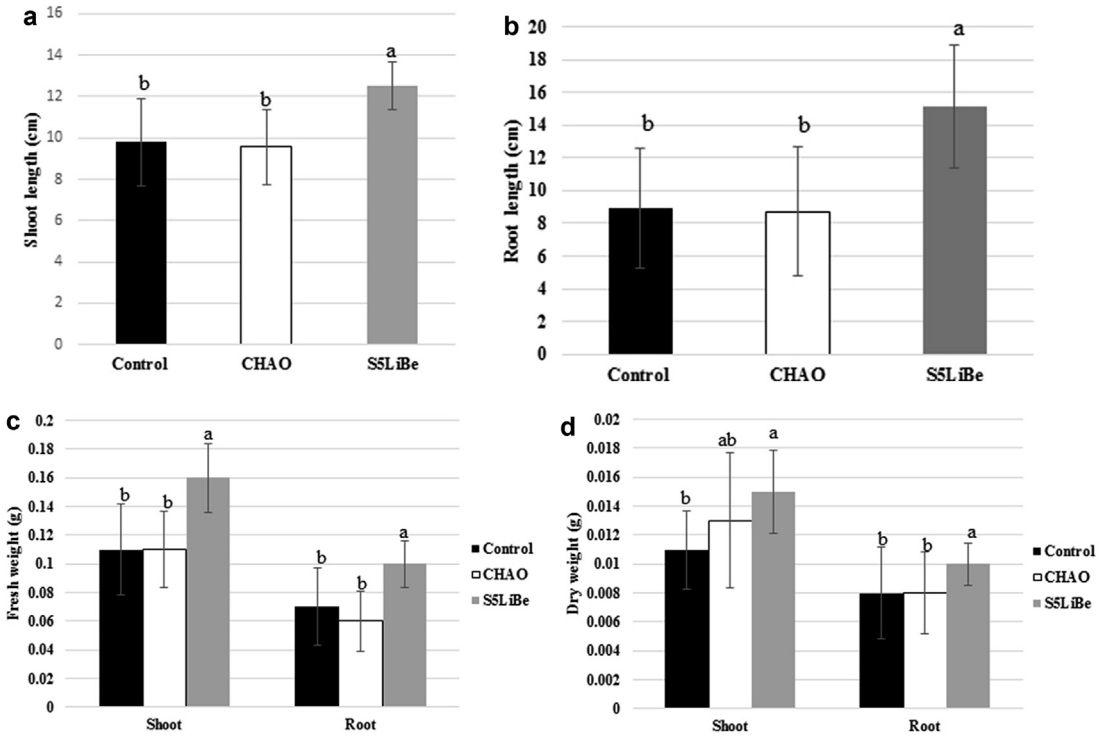

Fig. 6. Stimulation of growth of barley seedlings in heavy metal polluted soil due to inoculation with strain S5BiLe or CHA0 after 15 days of growth in comparison with non-inoculated (control) seedlings. Different letters indicate significant differences obtained by the Fischer LSD test ($p \leq 0.05$). A) Effect on shoot length, B) Effect on root growth, C) Effect on fresh weight of shoots and roots, D) Effect on dry weight of shoots and roots.

Acknowledgments

We kindly appreciate the exceptional help of Mr. Abbaci Hocine and Mr. Bensaid Karim (University of Bejaia, Algeria). Many thanks go to PROFERT-Company (Bejaia, Algeria) for the great help in heavy metal composition analysis in soil and water samples.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejsobi.2016.04.006.

References

[1] Sk Z. Ali, V. Sandhya, M. Grover, N. Kishore, V.R. Lingab, B. Venkateswarlu, *Pseudomonas* sp. strain AKM-P6 enhances tolerance of sorghum seedlings to elevated temperatures, Biol. Fertil. Soils 46 (2009) 45–55.
[2] Sk Z. Ali, V. Sandhya, M. Grover, V.R. Lingab, V. Bandi, Effect of inoculation with a thermotolerant plant growth promoting *Pseudomonas putida* strain AKMP7 on growth of wheat (*Triticum* spp.) under heat stress, J. Plant Interact. 6 (2011) 239–246.
[3] E.A. Barka, J. Nowak, C. Clement, Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth promoting rhizobacterium; *Burkholderia phytofirmans* strain PsJN, Appl. Environ. Microbiol. 72 (2006) 7246–7252.
[4] H.J. Bach, J.C. Munch, Identification of bacterial sources of soil peptidase, Biol. Fertil. Soil 31 (2000) 219–224.
[5] H.J. Benson, Microbiological Applications: a Laboratory Manual in General Microbiology, The McGraw-Hill Companies, 2001, p. 478.
[6] J.M. Bric, R.M. Bostock, S.E. Silverstone, Rapid *in situ* assay for indole acetic acid production by bacteria immobilization on a nitrocellulose membrane, Appl. Environ. Microbiol. 57 (1991) 535–538.
[7] M. Bardin, S. Ajouz, M. Comby, M. Lopez-Ferber, B. Graillot, M. Siegwart, P.C. Nicot, Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? Front. Plant Sci. 6 (2015) http://dx.doi.org/10.3389/fpls.2015.00566.
[8] H. Budzikiewicz, Siderophores of fluorescent pseudomonads, Z. Naturforsch. 52 (1997) 713–720.
[9] J.H. Carder, Detection and quantification of cellulase by congo red staining of substrates in a cup-plate diffusion assay, Anal. Biochem. 153 (1986) 75–79.
[10] L. Chet, *Trichoderma* – application, mode of action, and potential as a biocontrol agent of soil borne plant pathogenic fungi, in: I. Chet (Ed.), Innovative Approaches to Plant Disease Control, John Wiley and Sons, New York, 1987, pp. 137–160.
[11] P.S. Chowdhury, K. Dietel, M. Rändler, H. Junge, R. Borriss, A. Hartmann, R. Grosch, Effects of *Bacillus amyloliquefaciens* FZB42 on lettuce growth and its impact on rhizosphere bacterial community under *Rhizoctonia solani* pathogen pressure in pot and field experiments, Plos One 8 (2013) e68818.
[12] P.S. Chowdhury, J. Uhl, R. Grosch, S. Alqueres, S. Pittrof, S. Dietel, M. Schmid, R. Borriss, P. Schmitt-Kopplin, A. Hartmann, Cyclic lipopeptides of *Bacillus amyloliquefaciens* FZB42 subsp. *plantarum* colonizing the lettuce rhizosphere enhance plant defense responses towards the bottom rot pathogen *Rhizoctonia solani*, Mol. Plant Microbe Interact. 28 (2015) 984–995.
[13] S. Compant, B. Duffy, J. Nowak, C. Clement, E.A. Barka, Use of plant growth promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects, Appl. Environ. Microbiol. 71 (2005) 4951–4959.
[14] W.B. Christensen, Urea decomposition as a means of differentiating proteus and paracolon cultures from each other and from salmonella and shigella types, J. Bacteriol. 52 (1946) 461–466.
[15] M.S. Deepthi, T. Reena, M.S. Deepu, *In vitro* study on the effect of heavy metals on PGPR microbes from two different soils and their growth efficiency on *Oryza sativa* (L.), J. Biopestic. 7 (2014) 64–72.
[16] E. Dell' Amico, L. Cavalca, V. Andreoni, Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistance rhizobacteria, Soil Biol. Biochem. 40 (2008) 74–84.
[17] D. Egamberdieva, G. Berg, K. Lindström, L.A. Räsänen, Co-inoculation of *Pseudomonas spp.* with *Rhizobium* improves growth and symbiotic performance of fodder galega (*Galega orientalis* Lam.), Eur. J. Soil Biol. 46 (2010) 269–272.
[18] A. Fröhlich, K. Buddrus-Schiemann, J. Durner, A. Hartmann, U. von Rad, Response of barley to root colonization by *Pseudomonas* sp. DSMZ 13134 under laboratory, greenhouse, and field conditions, J. Plant Interact. 7 (2012) 1–9.
[19] Q. Fu, C. Liu, N. Ding, Y. Lin, B. Guo, Ameliorative effects of inoculation with the plant growth-promoting rhizobacterium *Pseudomonas* sp. DW1 on growth of eggplant (*Solanum melongena* L.) seedlings under salt stress, Agric. Water Manag. 97 (2010) 1994–2000.
[20] B.R. Glick, The enhancement of plant growth by free-living bacteria, Can. J. Microbiol. 41 (1995) 109–117.
[21] B.R. Glick, D.M. Penrose, J. Li, A model for lowering of plant ethylene concentration by plant growth-promoting bacteria, J. Theor. Biol. 190 (1998) 63–68.

[22] M.C.M. Götz, N. Gomes, A. Dratwinski, R. Costa, G. Berg, R. Peixoto, L.M. Mendoça-Hagler, K. Smalla, Survival of gfp-tagged antagonistic bacteria in the rhizosphere of tomato plants and their effects on the indigenous bacterial community, FEMS Microbiol. Ecol. 56 (2006) 207–218.
[23] J.P. Guiraud, P. Galzy, Analyse microbiologique dans les industries alimentaires, Editions Usine nouvelle, in: Collection genie alimentaire, Paris, 1980, p. 236.
[24] D. Haas, G. Défago, Biological control of soil-borne pathogens by fluorescent Pseudomonads, Nat. Rev. Microbiol. 3 (2005) 307–319.
[25] H.S. Han, K.D. Lee, Plant growth promoting rhizobacteria effect on antioxidant status, photosynthesis, mineral uptake and growth of lettuce under soil salinity, Res. J. Agric. Biol. Sci. 1 (2005) 210–215.
[26] M. Hookoom, D. Puchooa, Isolation and identification of heavy metals tolerant bacteria from industrial and agricultural areas in Mauritius, Curr. Res. Microbiol. Biotechnol. 1 (2013) 119–123.
[27] D.W. James Jr., N.I. Gutterson, Multiple antibiotics produced by *Pseudomonas fluorescens* HV37a and their differential regulation by glucose, Appl. Environ. Microbiol. 52 (1986) 1183–1189.
[28] V. Katiyar, R. Goel, Solubilization of inorganic phosphate and plant growth promotion by cold tolerant mutants of *Pseudomonas fluorescens*, Microbiol. Res. 158 (2003) 163–168.
[29] T. Kavitha, R. Nelson, S.J. Jesi, Screening of rhizobacteria for plant growth promoting traits and antifungal activity against charcoal rot pathogen *Macrophomina phaseolina*, Int. J. Pharm. Bio Sci. 4 (2013) 177–186.
[30] J.W. Kloepper, R. Lifshitz, R.M. Zablotowicz, Free-living bacterial inocula for enhancing crop productivity, Trends Biotechnol. 7 (1989) 39–43.
[31] J. Kopečný, B. Hodrová, C.S. Stewart, The isolation and characterization of a rumen chitinolytic bacterium, Lett. Appl. Microbiol. 23 (1996) 195–198.
[32] P. Kupferschmied, M. Maurhofer, C. Keel, Promise for plant pest control: root-associated pseudomonads with insecticidal activities, Front. Plant Sci. 4 (2013), http://dx.doi.org/10.3389/fpls.2013.00287.
[33] E. Kurek, E. Ozimek, P. Sobiczewski, A. Słomka, J.J. Scisełа, Effect of *Pseudomonas luteola* on mobilization of phosphorus and growth of young apple trees (Ligol)-pot experiment, Sci. Hortic. 164 (2013) 270–276.
[34] L.E. Loper, K.A. Hassan, D.V. Mavrodi, E.W. Davis, C.K. Lim, et al., Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions, Plos Genet. 8 (2012) e1002784.
[35] M. Lucy, E. Reed, B.R. Glick, Applications of free living plant growth promoting rhizobacteria, Antonnie Leeuwenhoek 86 (2004) 1–25.
[36] W. Ludwig, O. Strunk, R. Westram, L. Richter, H. Meier, et al., ARB: a software environment for sequence data, Nucl. Acid. Res. 32 (2004) 1363–1371.
[37] O. Martinez-Viveros, M.A. Jorquera, D.E. Crowley, G. Gajardo, M.L. Mora, Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria, J. Soil Sci. Plant Nutr. 10 (2010) 293–319.
[38] S. Mindlin, G. Kholodii, Z. Gorlenko, S. Minakhina, L. Minakhin, et al., Mercury resistance transposons of Gram-negative environmental bacteria and their classification, Res. Microbiol. 152 (2001) 811–822.
[39] E. Nabti, L. Bensidhoum, N. Tabli, D. Dahel, A. Weiss, M. Rothballer, M. Schmid, A. Hartmann, Growth stimulation of barley and biocontrol effect on plant pathogenic fungi by a *Cellulosimicrobium* strain isolated from salt-affected rhizosphere soil in northwestern Algeria, Eur. J. Soil Biol. 61 (2014) 20–26.
[40] E. Nabti, M. Sahnoune, M. Ghoul, D. Fischer, A. Hofmann, M. Rothballer, M. Schmid, A. Hartmann, Restoration of growth of durum wheat (*Triticum durum* var. waha) under saline conditions due to inoculation with the rhizosphere bacterium *Azospirillum brasilense* NH and extracts of the marine alga *Ulva lactuca*, J. Plant Growth Regul. 29 (2010) 6–22.
[41] P.R. Naik, N. Sakthivel, Functional characterization of a novel hydrocarbonoclastic *Pseudomonas* sp. strain PUP6 with plant-growth-promoting traits and antifungal potential, Res. Microbiol. 157 (2006) 538–546.
[42] D.H. Nies, Microbial heavy-metal resistance, Appl. Microbiol. Biotechnol. 51 (1999) 730–750.
[43] Y. Okon, C.A. Labandera-Gonzalez, Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation, Soil Biol. Biochem. 26 (1994) 1591–1601.
[44] G.J. Olsen, H. Matsuda, R. Hagstrom, R. Overbeek, Fast DNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood, Comput. Appl. Biosci. 10 (1994) 41–48.
[45] M. Péchy-Tarr, D.J. Bruck, M. Maurhofer, E. Fischer, C. Vogne, M.D. Henkels, K.M. Donahue, J. Grunder, J.E. Loper, C. Keel, Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*, Environ. Microbiol. 10 (2008) 2368–2386.
[46] J.M. Raaijmakers, T.C. Paulitz, C. Steinberg, C. Alabouvette, Y. Moenne-Loccoz, The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms, Plant Soil 321 (2009) 341–361.
[47] S. Ramesh, N. Mathivanan, Screening of marine actinomycetes isolated from the Bay of Bengal, India, for antimicrobial activity and industrial enzymes, World J. Microbiol. Biotechnol. 25 (2009) 2103–2111.
[48] A. Ramette, M. Frapolli, M. Fischer-Le Saux, C. Gruffaz, J.M. Meyer, et al., *Pseudomonas protegens* sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin, Syst. Appl. Microbiol. 34 (2011) 180–188.
[49] H. Rodríguez, R. Fraga, Phosphate solubilizing bacteria and their role in plant growth promotion, Biotechnol. Adv. 17 (1999) 319–339.
[50] S.B. Rosas, G. Avanzini, E. Carlier, C. Pasluosta, N. Pastor, M. Rovera, Root colonization and growth promotion of wheat and maize by *Pseudomonas aurantiaca* SR1, Soil Biol. Biochem. 41 (2009) 1802–1806.
[51] B. Ruffner, M. Péchy-Tarr, F. Ryffel, P. Hoegger, C. Obrist, A. Rindlisbacher, C. Keel, M. Maurhofer, Oral insecticidal activity of plant-associated pseudomonads, Environ. Microbiol. 15 (2013) 751–763.
[52] L. Ruiu, Insect pathogenic bacteria in integrated pest management, Insects 6 (2015) 352–367.
[53] P.R. Ryan, Y. Dessaux, L.S. Thomashow, D.M. Weller, Rhizosphere engineering and management for sustainable agriculture, Plant Soil 321 (2009) 363–383.
[54] D. Sachin, Effect of *Azotobacter chroococcum* (PGPR) on the growth of bamboo (*Bambusa bamboo*) and maize (*Zea mays*) plants, Biofrontiers 1 (2009) 1–24.
[55] B.S. Saharan, V. Nehra, Plant growth promoting rhizobacteria: a critical review, Life Sci. Med. Res. 21 (2011) 1–30.
[56] I.P. Sagahón, M.A.A. Reyes, H.V.S. Rojas, A.A. Cuenca, A.T. Jurado, I.O.C. Álvarez, Y.M. Flores, Isolation of bacteria with antifungal activity against the phytopathogenic fungi *Stenocarpella maydis* and *Stenocarpella macrospora*, Int. J. Mol. Sci. 12 (2011) 5522–5537.
[57] A. Sagervanshi, P. Kumari, A. Nagee, A. Kumar, Isolation and characterization of phosphate solubilizing bacteria from an agricultural soil, Int. J. Life Sci. Pharma Res. 2 (2012) 256–266.
[58] V. Sandhya, Sk Z. Ali, G. Minakshi, R. Gopal, B. Venkateswarlu, Alleviation of drought stress effects in sunflower seedlings by the exopolysaccharides producing *Pseudomonas putida* strain GAP-P45, Biol. Fertil. Soils 46 (2009) 17–26.
[59] B. Schwyn, J.B. Neilands, Universal chemical assay for the detection and determination of siderophores, Anal. Biochem. 160 (1986) 47–56.
[60] G.A. Sierra, A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates, Antonie Leeuwenhoek 28 (1957) 15–22.
[61] K. Srinivasan, G. Gilardi, A. Garibaldi, M.L. Gullino, Bacterial antagonists from used rockwool soilless substrates suppress *Fusarium* wilt of tomato, J. Plant Pathol. 91 (2009) 147–154.
[62] S. Timmusk, E.G.H. Wagner, The plant growth promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: a possible connection between biotic and abiotic stress responses, Mol. Plant Microbe Interact. 12 (1999) 951–959.
[63] A.T. Wahyudi, R.I. Astuti, A.S. Gianto, Screening of *Pseudomonas* sp. isolated from rhizosphere of soybean plant as plant growth promoter and biocontrol agent, Am. J. Agric. Biol. Sci. 6 (2011) 134–141.
[64] S.C. Wu, Z.H. Cao, Z.G. Li, K.C. Cheung, M.H. Wonga, Effects of biofertilizer containing N-fixer, P- and K-solubilizers and AM fungi on maize growth: a greenhouse trial, Geoderma 125 (2005) 155–166.
[65] Y.F. Zhang, L.Y. He, Z.J. Chen, Q.Y. Wang, M. Qian, X.F. Sheng, Characterization of ACC deaminase-producing endophytic bacteria isolated from copper-tolerant plants and their potential in promoting the growth and copper accumulation of *Brassica napus*, Chemosphere 83 (2011) 57–62.