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Influence of metal resistant-plant growth-promoting bacteria on the growth of *Ricinus communis* in soil contaminated with heavy metals

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Abstract

The metal resistant-plant growth-promoting bacterial (PGPB) strains PsM6 and PjM15 isolated from a serpentine soil were characterized as *Pseudomonas* sp. and *Pseudomonas jessenii*, respectively, on the basis of their morphological, physiological, biochemical characteristics and 16S rDNA sequences. Assessment of plant growth-promoting parameters revealed the intrinsic ability of the strains for the utilization of 1-aminocyclopropane-1-carboxylic acid as the sole N source, solubilization of insoluble phosphate and production of indole-3-acetic acid (IAA). Further, a pot experiment was conducted to elucidate the effects of inoculating metal resistant PGPB on the plant growth and the uptake of Ni, Cu and Zn by *Ricinus communis*. Inoculation of *Pseudomonas* sp. PsM6 or *P. jessenii* PjM15 increased the shoot and root biomass of *R. communis* grown in non-contaminated and contaminated soil. However, the maximum biomass was observed in the plants inoculated with strain PjM15. This effect can be attributed to the solubilization of phosphate and production of IAA. Inoculation of *Pseudomonas* sp. PsM6 and PjM15 did not greatly alter the organ metal concentrations except Zn which concentration was higher in root, stem and leaf of inoculated plants. The results of metal extraction with PGPB strains showed that PsM6 was more efficient at solubilizing Zn than PjM15, and that PjM15 was better at solubilising Ni and Cu than PsM6. Owing to its wide action spectrum, the metal resistant PGPB could serve as an effective metal sequestering and growth-promoting bioinoculant for plants in metal-stressed soil. The present study has provided a new insight into the phytoremediation of metal-contaminated soil.

Keywords: Heavy metals; *Ricinus communis*; PGPB; IAA; Phytoremediation

1. Introduction

The continued industrialization of countries has led to extensive environmental problems. A wide variety of chemicals (e.g. heavy metals, pesticides, chlorinated solvents, etc.) have been detected in different biota such soil, water, and air (Cheng, 2003; Turgut, 2003). Heavy metals pose a critical concern to human health and environmental issues due to their high occurrence as a contaminant, low solubility in biota, and the classification of several heavy metals as carcinogenic and mutagenic (Alloway, 1995; Diels et al., 2002). Moreover, the metals cannot be degraded to harmless products and hence persist in the environment indefinitely. As a result, many different remediation methods have been tried to address the rising number of heavy metal contaminated sites. Most of the traditional methods are either extremely costly (i.e., excavation, solidification and burial) or simply involve the isolation of the contaminated sites. Some methods, such as soil washing, can pose an adverse effect on biological activity, soil structure and fertility, and incur significant engineering costs (Pulford and Watson, 2003). Phytoremediation, the use of hyperaccumulating plants to remove pollutants from the environment or to render them harmless (Raskin et al., 1997), with its lower cost and environmental friendly nature, has received increasing attention in the last decades (Salt et al., 1998). Currently there are a

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number of reports available on metal accumulating plants that are used in removing toxic metals from the soil (Delorme et al., 2001; Whiting et al., 2001; Glick, 2003; Sheng and Xia, 2006). *Ricinus communis* (castor bean) is one of such plant species, which has attracted considerable attention because of its ability to grow in heavily polluted soil together with its capacity for metal ion accumulation (Prasad and Freitas, 2003; Rockwood et al., 2004; Cecchi and Zanchi, 2005). In addition, *R. communis* is an industrial crop with multiple non-food uses and is an excellent rotation and companion crop. The possibilities of easily growing *R. communis* in different climates and using its biomass in biofuel industries can make heavy metal contaminated soils productive, and, although slowly, restore them at the same time. It signifies economic advantage along with a better quality of soil.

The efficiency of phytoaccumulation may not only depend on the plant itself but also on the interaction of the plant roots with microbes and the concentrations of bio-available metals in soil (Wang et al., 1989). Further, the rhizosphere provides a complex and dynamic microenvironment where microorganisms, in association with roots, form unique communities that have considerable potential for detoxification of hazardous waste compounds (De-Souza et al., 1999). Soil microorganisms can resist toxicity by transforming metals into less toxic forms, by immobilising metals on the cell surface or in intracellular polymers, and by precipitation or biomethylation (Silver, 1996). Certain rhizosphere bacteria have exceptional ability to promote the growth of the host plant by various mechanisms, namely fixation of atmospheric nitrogen, utilization of 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole N source, production of siderophores, or production of plant growth regulators (hormones) (Glick et al., 1998, 1999). In addition, many microorganisms in the soil are able to solubilise "unavailable" forms of heavy metal-bearing minerals by excreting organic acids (Abou-Shanab et al., 2003). Therefore, improvement of the interactions between plants and beneficial rhizosphere microbes can enhance biomass production and tolerance of the plants to heavy metals, and are considered to be an important component of phytoremediation technology (Glick, 2003). Although many soil bacteria are tolerant to heavy metals and play important roles in mobilization or immobilization of heavy metals (Gadd, 1990), only a few attempts have been made to study their role in the tolerance to and uptake of heavy metals by the plants.

Thus the aim of this study was to (1) isolate and characterize heavy metal resistant-plant growth-promoting bacteria (PGPB) from serpentine soils, (2) screen the isolates for auxiliary activities including ACC deaminase activity, solubilisation of insoluble phosphate and production of indole-3-acetic acid (IAA) and (3) elucidate the effects of inoculating metal resistant-PGPB on the plant growth and the uptake of Ni, Cu and Zn by *R. communis* in soil.

2. Materials and methods

2.1. Isolation of metal resistant-PGPB

The bacterial strains were isolated from a serpentine site in Bragança, north-east of Portugal, previously described by Freitas et al. (2004). For isolation and enumeration of microorganisms, soil samples were serially diluted in sterile distilled water and plated on Luria–Bertani (LB) agar supplemented with 50 mg l^{-1} (ppm) level of heavy metals $NiCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ one metal at a time or as heavy metal mixture. The plates were incubated at 27 °C for 48 h to screen resistant colonies. To check the extent of resistance, the selected bacterial isolates were grown in LB agar media containing different concentrations of Ni, Cu or Zn ranging from 100 to 1000 mg l^{-1} (Rajkumar et al., 2005). In order to isolate the PGPB, the heavy metal resistant strains were grown on DF salts minimal medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC to provide a nitrogen source at 27 °C for 168 h at 175 rpm. The inoculated DF salt minimal medium without ACC was used as a blank. The bacterial growth was monitored as a function of biomass by measuring the optical density at 600 nm against blank.

2.2. Molecular characterization of PGPB

The bacterial strains were grown in LB broth at 27 °C. Cells were harvested after 24 h and processed immediately for DNA isolation using standard procedure (Sambrook et al., 1989). Amplification of 16S rRNA gene sequence was performed by polymerase chain reaction (PCR) with the conserved eubacterial primers pA (5′-AGAGTTTGATCCTGGCTCAG; *Escherichia coli* bases 8–27) and pC5B (5'-TACCTTGTTACGACTT; *E. coli* bases 1507–1492) (Dunbar et al., 1999). Reaction conditions were as described by Branco et al. (2005). Each amplification mixture (5 μl) was analysed by agarose gel (1.5% w/v) electrophoresis in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) containing 1 μg ml^{-1} (w/v) ethidium bromide. For further sequencing reaction, the amplified 16S rDNA was purified from salts and primers using the PCR purification kit (Roche Diagnostics) according to the manufacturer's instructions. Automated sequencing of the purified PCR products was performed using the dRodamina terminator cycle sequencing kit and the ABI 310 DNA Sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Partial 16S rDNA sequences obtained were matched against nucleotide sequences present in GenBank using the BLASTn program (Altschul et al., 1997).

2.3. Characterization of plant growth-promoting features of PGPB

To determine ACC deaminase activity, the PGPB were grown in test tubes containing 10 ml of DF salts minimal

medium. The medium was supplemented with 3 mM ACC. After cultivation for 72 h at 27 °C, the cells were harvested by centrifugation at 9000 rpm for 10 min at room temperature. The ACC deaminase activity in cells was determined by monitoring the amount of α-ketobutylate generated by the enzymatic hydrolysis of ACC as described by Belimov et al. (2005). The protein concentration of cell suspensions was determined by the method of Bradford (1976). IAA production by PGPB was determined according to the method of Bric et al. (1991). Cultures of the isolates were raised in LB broth amended with 500 μg of tryptophan ml^{-1} at 27 °C for 96 h at 200 rpm. Cells were removed by centrifugation at 6000 rpm and the supernatant was assayed for IAA production. The phosphate solubilizing activity of the isolates was analyzed in NBRIP medium (Nautiyal, 1999) amended with tricalcium phosphate. The isolates were grown at 27 °C for 192 h at 200 rpm. The solubilized phosphate in the culture supernatant was quantified as detailed by Fiske and Subbarow (1925).

2.4. Influence of PGPB on R. communis growth and metal uptake

Soil samples were collected from the Botanical garden, Department of Botany, University of Coimbra, Coimbra, Portugal. The soil properties are listed in Table 1. The soil was sieved (2 mm) and sterilized by steaming (100 °C for 1 h on three consecutive days). The soils were artificially contaminated with Ni (275 mg kg^{-1}), Cu (300 mg kg^{-1}) and Zn (400 mg kg^{-1}) and left in a greenhouse for a 3 week period (for metal stabilization). Seeds of *R. communis* were surface sterilized in 2% $Ca(OCl)_2$ (2 h) and rinsed several times with sterile distilled water. The seeds were allowed to germinate in sterilized non-contaminated soil at 25 °C and a 16/8 day/night regime. For inoculation of the seedlings, bacterial cultures were grown for 18 h, cells harvested by centrifugation (6000 rpm, 10 min), washed twice with sterile distilled water, and resuspended in biological saline (0.85% KCl). Fifteen-day-old seedlings were soaked for 2 h in an actively growing bacterial culture (10^9 CFU ml^{-1}) and transplanted in plastic pot (four plants pot^{-1}) containing 300 g of metal contaminated or non-contaminated soil. Each treatment was performed in triplicates. After 35 d the plants were carefully removed from the pots and the root surface was cleaned several times with distilled water. Growth parameters such as shoot dry weight and root dry weight of the plants were measured. The accumulation of total Ni, Cu and Zn in root, stem and leaf system were also analysed using atomic absorption spectrophotometer after nitric acid digestion (Freitas et al., 2004).

Table 1
Selected properties and heavy metals concentrations of the used soil

Parameters	Content
pH	5.95
Organic matter (%)	4.3[a] (±0.3[b])
Metals (HNO_3 extractable) mg kg^{-1} dry soil	
Ni	15.9 (±1.4)
Cu	21.7 (±2.6)
Zn	106.6 (±5.1)

[a] Values represent average of 3 samples except pH.
[b] Values in parentheses represent standard deviation.

2.5. Effects of PGPB on the mobility of soil metals

Batch studies on the effects of bacteria on the mobility of soil metals were carried out by using 50-ml scaled polypropylene centrifuge tubes. The sterilized soil was artificially contaminated with Ni, Cu and Zn as detailed in earlier section. Pure culture bacterial strains were grown in LB broth and placed on a shaker at 200 rpm and 27 °C. After 24 h, optical density (600 nm) was measured and adjusted to 1.5; the cultures were centrifuged at 6000 rpm for 10 min, washed in phosphate buffer (pH 7.0) twice, resuspended, washed in sterile water, recentrifuged, and finally resuspended in 5 ml sterile water. Small aliquots of washed bacterial culture (up to 1 ml) were added to the 1 g of soil in the centrifuge tubes. Sterile water was added to soil as an axenic control. All tubes were weighed, wrapped in brown paper and placed on an orbital shaker at 200 rpm at 27 °C. After 1 week, the tubes were again weighed to compensate for evaporation of water. Ten milliliters of sterile water were added to each tube to extract the soil water soluble heavy metals. The soil suspensions were centrifuged at 7000 rpm for 10 min and filtered. The concentrations of Ni, Cu and Zn in the filtrate were determined by atomic absorption spectrophotometer and the mass of tubes and soils was determined immediately.

2.6. Biosorption of metals by PGPB

The biosorption study was carried out as described by Hernandez et al. (1998) with some modifications. Bacteria were grown in 100 ml of LB broth until reaching 1.0 of optical density (600 nm). Cells were then harvested by centrifugation at 6000 rpm for 15 min and the bacterial pellet washed twice with sterile water. The harvested biomass was re-suspended in Eppendorf's tubes containing 1.5 ml of trimetal solution (275 mg Ni l^{-1}, 300 mg Cu l^{-1} and 400 mg Zn l^{-1}). After incubation at room temperature for 10 h, the cells were harvested by centrifugation under the same experimental condition. The amount of residual metal present in the supernatant was measured by atomic absorption spectrophotometer.

3. Results

3.1. Isolation of heavy metal resistant PGPB

Thirty seven colonies were screened from initial (50 mg l^{-1}) level of heavy metal supplemented LB medium. After secondary screening, 7 bacterial strains showing a high degree of metal-resistance were selected for further

studies. In order to isolate the PGPB, the metal resistant strains were tested for the ability to grow on DF salts minimal medium with ACC. Among the seven strains tested, PsM6 and PjM15 grew in DF salts minimal medium with ACC as the sole source of nitrogen. However, maximum growth was observed in PsM6 compared with PjM15. In the absence of ACC, the strains PsM6 and PjM15 showed a limited growth (Fig. 1).

3.2. Molecular characterization of PGPB

On the basis of morphological, physiological, biochemical characteristics (data not shown) and comparative analysis of the sequence with already available database showed that the strains PsM6 and PjM15 were close to the members of the genus *Pseudomonas*. Partial sequence of PsM6 (780 bp) showed 99% homology with the sequence of *Pseudomonas* sp. and PjM15 (923 bp) showed 99% homology with *Pseudomonas jessenii*. The sequences were deposited at GenBank (*Pseudomonas* sp. PsM6, accession no. AM707021; *Pseudomonas jessenii* PjM15, accession no. AM707022).

3.3. Plant growth-promoting features of PGPB

The metal resistant PGPB strains *Pseudomonas* sp. PsM6 and PjM15 were assayed for a number of traits thought to be important for plant growth-promoting activity (Table 2). *Pseudomonas* sp. PsM6 had a high level of ACC deaminase activity, whereas PjM15 exhibited only a low level of activity. Further, *Pseudomonas* sp. PsM6 and PjM15 utilized tryptophane as a precursor for their growth and IAA production. The maximum production of IAA was observed in PjM15 compared with PsM6. Similarly, PsM6 and PjM15 utilized tricalcium phosphate as the sole source of phosphate. The strain PjM15 exhibited higher rate of phosphate solubilization than PsM6.

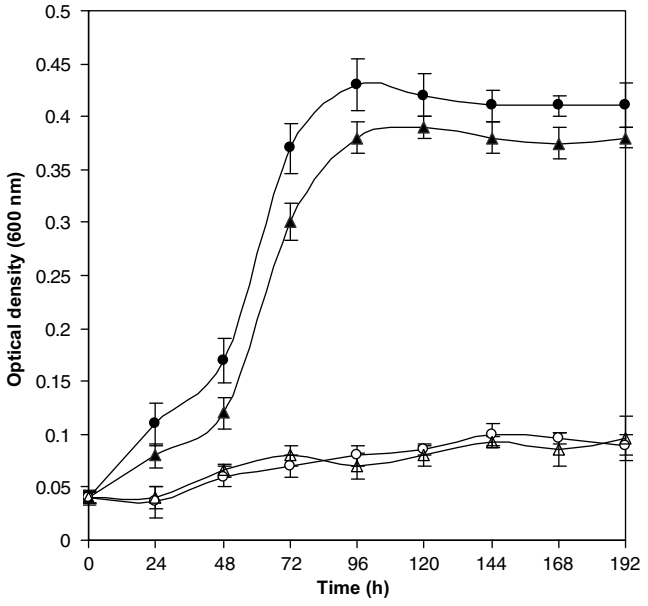

Fig. 1. Growth of PGPB on DF salts minimal medium. *Pseudomonas* sp. PsM6 with ACC (●), *P. jessenii* PjM15 with ACC (▲), *Pseudomonas* sp. PsM6 without ACC (○), *P. jessenii* PjM15 without ACC (△). Each value is the mean of triplicates. Error bars represent standard deviation.

Table 2
Some key traits of PGPB strains *Pseudomonas* sp. PsM6 and *P. jessenii* PjM15

Parameters	Strain	
	Pseudomonas sp. PsM6	*P. jessenii* PjM15
ACC deaminase, α-ketobutyrate mg^{-1} protein h^{-1} (nmol)	66.52[a] (±7.05[b])	34.23 (±4.67)
Phosphate solubilization (mg l^{-1})	73.11 (±3.26)	88.67 (±4.46)
IAA synthesis (mg l^{-1})	17.74 (±2.06)	39.88 (±3.68)
Metal tolerance level (mg l^{-1})		
Ni	800	900
Cu	750	600
Zn	700	700

[a] Values represent average of 3 samples except the metal tolerance levels.
[b] Values in parentheses represent standard deviation.

3.4. Influence of PGPB on the growth of R. communis

In control soil, inoculation of *Pseudomonas* sp. PsM6 and PjM15 showed an increase in shoot and root dry weight of plant (Fig. 2). However, maximum plant growth-promoting effect was observed in PjM15, which enhances shoot and root dry weight by 14% and 19%, respectively, compared with non-inoculated plants. Similarly, PsM6 enhances the shoot dry weight and root dry weight by 7% and 12%, respectively. The non-inoculated plants grown in metal contaminated soil showed a decrease of 7% and 3% in shoot and root dry weight, respectively. In metal contaminated soils, plants inoculated with *Pseudomonas* sp. PsM6 and PjM15 exhibited an increase in shoot and root dry weight. However, the highest plant growth-promoting effect was found for PjM15, which enhances shoot and root dry weight by 20% and 25%, respectively. Similarly, PsM6 enhances shoot and root dry weight by 15% and 18%, respectively.

3.5. Metal accumulation in R. communis tissues

The metal concentrations in the root, stem and leaf tissues of *R. communis* grown in artificially metal contaminated soil are given in Table 3. In general, inoculation of *Pseudomonas* sp. PsM6 and PjM15 did not greatly alter the concentration of metal except Zn in plant tissues. The concentration of Zn in root tissues was increased after inoculation with PsM6 and PjM15. However, this effect was higher in PjM15 than PsM6. Further it can be observed that inoculation enhanced the translocation of Zn from root to shoot. Compared with the control, the

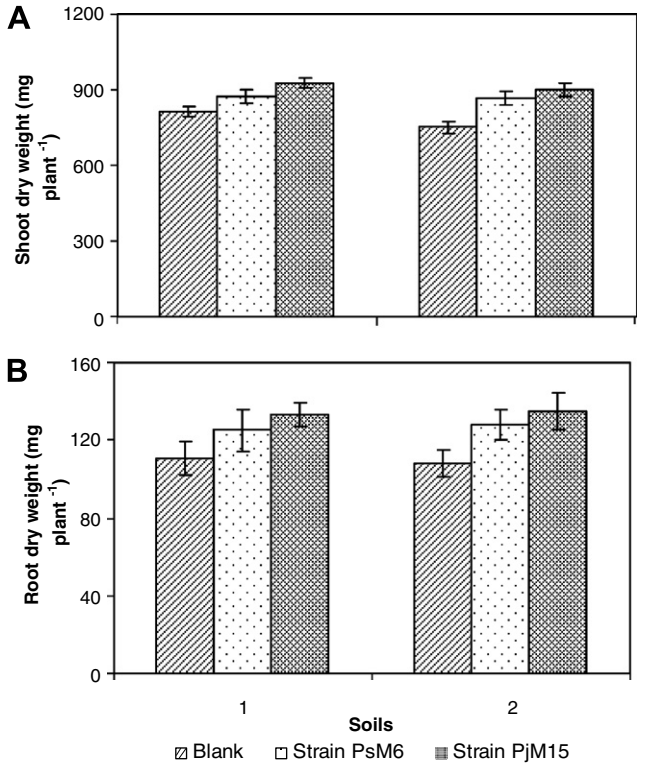

Fig. 2. Effects of inoculation with PGPB strains on shoot (A) and root (B) dry weights of *R. communis*. (1) Non-contaminated soil; (2) metal-contaminated soil. Each value is the mean of triplicates. Error bars represent standard deviation.

Table 3
Ni, Cu and Zn concentrations in *R. communis* tissues

	Root	Stem	Leaf
Nickel concentration (mg kg^{-1})			
Blank	185.0[a] (±9.8[b])	35.0 (±4.0)	53.0 (±5.2)
Pseudomonas sp. PsM6	166.6 (±10.0)	41.3 (±3.0)	56.1 (±6.6)
P. jessenii PjM15	203.0 (±7.2)	44.0 (±6.0)	62.3 (±4.0)
Copper concentration (mg kg^{-1})			
Blank	147.3 (±7.5)	12.5 (±2.2)	10.4 (±1.9)
Pseudomonas sp. PsM6	157.0 (±9.1)	15.9 (±2.3)	9.6 (±1.1)
P. jessenii PjM15	175.6 (±7.1)	14.0 (±2.3)	13.9 (±1.4)
Zinc concentration (mg kg^{-1})			
Blank	375.3 (±9.6)	105.0 (±6.0)	161.6 (±4.5)
Pseudomonas sp. PsM6	391.0 (±10.8)	138.3 (±11.0)	185.0 (±7.5)
P. jessenii PjM15	407.3 (±8.6)	121.6 (±12.2)	179.3 (±4.5)

[a] Values represent average of 3 samples.
[b] Values in parentheses represent standard deviation.

inoculation of *Pseudomonas* sp. PsM6 and PjM15 increased the concentrations of Zn in stem and leaf tissues. However, the highest effect was observed in PsM6, which increases the Zn concentration in the stem and leaf tissues by 32% and 15%, respectively. Similarly, PjM15 increases the Zn concentrations in stem and leaf tissues by 16% and 11%, respectively. By contrast, inoculation of PsM6 and PjM15 did not greatly alter the concentrations of Ni and Cu in root, leaf and stem tissues.

3.6. Effects of PGPB on the mobility of soil metals

The concentrations of water soluble Ni, Cu and Zn in soil were examined to assess the relative efficiency of *Pseudomonas* sp. PsM6 and PjM15 in enhancing metal solubilisation from the soil. Compared with control treatment, inoculation of *Pseudomonas* sp. PsM6 and PjM15 for 7 d increased the concentrations of soluble heavy metals in soil (Fig. 3). The inoculation of *Pseudomonas* sp. PsM6 increased the concentrations of soluble Ni, Cu and Zn in soil, which were 2-, 2.7- and 7.4-folds higher than those in the control soil, respectively. Similarly, PjM15 increased the concentrations of soluble Ni, Cu and Zn in soil by 2.4-, 3.2- and 4.93-folds, respectively.

3.7. Biosorption of metals by PGPB

The data expressing the capabilities of *Pseudomonas* sp. PsM6 and PjM15 to uptake Ni, Cu and Zn are given in Table 4. It is clearly evident that the isolates exhibited different biosorption capacity towards the tested metal ions. The maximum biosorption by *Pseudomonas* sp. PsM6 and PjM15 was achieved after 6 h of incubation. Further incubation up to 10 h did not improve the extent of biosorption (data not shown). The maximum biosorption capacity for Cu and Zn was observed in the case of PjM15, and the highest quantity of Ni was adsorbed by PsM6.

4. Discussion

Bacteria present in serpentine soil and their interaction with hyperaccumulating plants have attracted the attention of several investigators (Mengoni et al., 2001; Pal et al., 2005) due to biotechnological applications for bioremediation. The serpentine areas are considered as an interesting model for the evolution of metal resistant microorganisms,

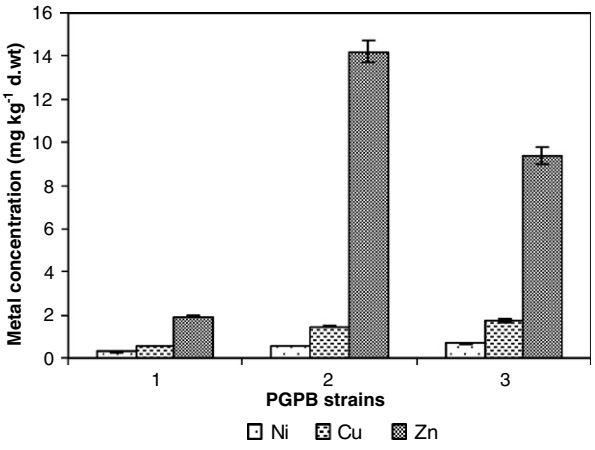

Fig. 3. Effects of inoculation with PGPB strains on the solubilization of Ni, Cu and Zn in soil. (1) Blank; (2) *Pseudomonas* sp. PsM6; (3) *P. jessenii* PjM15. Each value is the mean of triplicates. Error bars represent standard deviation.

Table 4
Biosorption of Ni, Cu and Zn by *Pseudomonas* sp. PsM6 and *P. jessenii* PjM15 at different time intervals

Metal	Biosorption of metal ion (mg g^{-1} dry cell)					
	Pseudomonas sp. PsM6			*P. jessenii* PjM15		
	2 h	4 h	6 h	2 h	4 h	6 h
Ni	0.78[a] (±0.01[b])	1.87 (±0.08)	2.79 (±0.05)	0.19 (±0.02)	1.19 (±0.04)	1.36 (±0.07)
Cu	4.32 (±0.11)	5.47 (±0.08)	5.52 (±0.16)	8.08 (±0.05)	9.19 (±0.23)	10.22 (±0.31)
Zn	1.64 (±0.08)	2.68 (±0.15)	3.66 (±0.09)	1.36 (±0.08)	3.84 (±0.11)	4.39 (±0.09)

[a] Values represent average of 3 samples.
[b] Values in parentheses represent standard deviation.

completely different from that of artificially contaminated soils. In recent years, such newer strains and new genetic determinants for heavy metal-resistance could be exploited in bioremediation practices. In this investigation, the bacterial strains were isolated from serpentine soils with an objective to assess the effects of metal resistant PGPB on the plant growth and the uptake of Ni, Cu and Zn by *R. communis*. Among the 7 metal resistant strains tested, *Pseudomonas* sp. PsM6 and *P. jessenii* PjM15 grew in DF salts minimal medium with ACC as the sole source of nitrogen. Bacterial strains utilizing ACC as a sole source of nitrogen possess ACC deaminase which hydrolyses ACC and enhance the elongation of plant roots (Glick et al., 1998). Certain heavy metal resistant bacterial strains potentially hydrolyse ACC and promote the plant growth. Nickel resistant *Kluyvera ascorbata* isolated from soil contaminated with Ni and other heavy metals has been shown to promote plant growth (Burd et al., 2000). Similarly, Belimov et al. (2005) isolated cadmium-resistant *Variovorax paradoxus* from the rhizosphere of *Brassica juncea* for promoting plant growth. The PGPB strains *Pseudomonas* sp. PsM6 and PjM15 exhibited a higher metal tolerance when cultivated under increasing metal levels in the growth medium (Table 2). This high tolerance to heavy metals could be attributed to the fact that the bacteria were isolated from a serpentine soil containing high levels of metals (Freitas et al., 2004). It is known that microorganisms isolated from natural environments contaminated with heavy metals often exhibit tolerance to multiple pollutants because they have adapted to such environments (Pal et al., 2005).

Inoculation of metal resistant PGPB increased the growth of *R. communis* plants and it seemed it was effective in protecting plants from growth inhibition caused by heavy metals. Previously, Doelman (1985) has reported that the efficiency of revegetation and phytoremediation of heavy metal-contaminated sites is closely related to the presence of higher proportions of metal resistant microbial populations in the soil, which likely conferred a better nutritional assimilation and protection effect on plants. In general the metal resistant rhizosphere bacteria have exceptional ability to protect the host plants from metal toxicity by several possible mechanisms. The best-known mechanism is the utilization ACC by rhizosphere bacteria. A number of PGPB, which stimulate the growth of different plant species (Burd et al., 2000; Belimov et al., 2002; Rajkumar et al., 2006), contain the enzyme ACC deaminase, which hydrolyses ACC (the immediate precursor of the plant hormone ethylene). Some of the plant ACC is exuded from roots or seeds and cleaved by ACC deaminase to NH_3 and α-ketobutyrate (Penrose and Glick, 2001). The bacteria utilize the NH_3 evolved from ACC as a source of N and thereby decrease ACC within the plant with the concomitant reduction of plant ethylene (Grichko and Glick, 2001). In the present study, in addition to ACC deaminase activity, *Pseudomonas* sp. PsM6 and PjM15 exhibited the solubulization of phosphate and production of IAA (Table 2). In general the elevated levels of heavy metals in soil interfere with uptake of nutrients such as P and lead to plant growth retardation (Halstead et al., 1969). This deficiency can be compensated by the phosphate-solubilizing ability of PGPB strains (Gupta et al., 2002; Zaidi et al., 2006). Further, the IAA produced by PGPB promotes root growth by directly stimulating plant cell elongation or cell division (Glick et al., 1998). A low level of IAA produced by rhizosphere bacteria promotes primary root elongation whereas a high level of IAA stimulates lateral and adventitious root formation but inhibit primary root growth (Xie et al., 1996). Thus PGPB can facilitate plant growth by altering the plant hormonal balance. The phytohormone IAA production offers great promise for sustaining the increased crop productivity. The metal resistant bacteria belonging to different genera such as *Pseudomonas, Mycobacterium, Agrobacterium* and *Arthrobacter* were found to have plant growth-promoting features that can potentially promote plants growth and reduce stress symptoms in plants (Dell'Amico et al., 2005; Rajkumar et al., 2005). The maximum plant growth promotion by *P. jessenii* PjM15 observed in the present study can be attributed to the solubilisation of phosphate and production of IAA.

The process of metal uptake and accumulation by different plants depends on the concentration of available metals in soils, solubility sequences and the plant species (Gupta and Sinha, 2006). The rhizosphere microbes can affect trace metal mobility and availability to the plant, they can produce siderophores for ensuring iron availability, reduce soil pH, and or solubilize phosphates (Smith and Read, 1997; Sheng and Xia, 2006; Zaidi et al., 2006). In the present study, inoculation with *Pseudomonas* sp. PsM6 or *P. jessenii* PjM15 did not greatly influence the quantity of accumu-

lation of metals in root and shoot system. However, on average, only in the case of Zn, was the metal concentration higher in stem and leaves of inoculated plants. Similar observations have also reported by Whiting et al. (2001), who found that the addition of a mixed inoculum of *Microbacterium saperdae*, *Pseudomonas monteilii*, and *Enterobacter cancerogenes* to surface sterilized seeds of *Thalaspi caerulescens* sown in autoclaved soil increased the Zn concentration in shoots 2-fold compared with non-inoculated controls; the total accumulation of Zn was enhanced 4-fold. The increased accumulation of Zn in presence of *Pseudomonas* sp. PsM6 or *P. jessenii* PjM15 might be due to more Zn uptake under acidic soil conditions, which develops as a result of activity of phosphate solubilization in soil. Effects of pH on the solubility and speciation of metals are well documented (Gadd, 2001; Gadd and Sayer, 2000). Sheng and Xia (2006) reported that the addition of Cd-resistant bacterial strains to *Brassica napus* grown in metal contaminated soil significantly increased the plant uptake of Cd when compared with the non-inoculated controls as a result of pH reduction. Similarly, Delorme et al. (2001) hypothesized that soil acidification in the rhizosphere of *T. caerulescens* facilitates metal ion uptake by increasing metal ion mobility around the roots. Our results indicate that bacteria facilitated the release of Zn from the non-soluble phases in the soil, thus enhancing the availability of Zn to *R. communis*. This result is in good agreement with the value shown in Fig. 3 concerning bacterial metal solubilisation in the soils. The higher water soluble Zn induced by *Pseudomonas* sp. PsM6 inoculation resulted in a correspondingly higher Zn accumulation in both the shoots and roots of *R. communis* suggesting that the bioavailability of Zn was increased by through bacterial metabolic activities or their interactions with the plants.

The results obtained here indicate that inoculation of metal resistant PGPB seemed to be very effective in protecting plants from growth inhibition caused by metals, and this was strongly supported by the root and shoot biomass data. The increase in plant growth caused by PGPB may be attributed to the maximum production of IAA and solubilisation of phosphate. In addition, the PGPG exhibited a high degree of metal biosorption potential (Table 4). Bacteria have a high surface area to volume ratio (Beveridge, 1988) and, as a strictly physical cellular interface, should have a high capacity for sorbing metals from solutions (Mullen et al., 1989). Several investigations have shown that relatively large quantities of metallic cations are complexed by fungi (Zhou, 1999) and bacteria (Samuelson et al., 2000). With this intrinsic characteristic, the PGPB may also contribute in reducing the phytotoxic effects of the metals by sharing the metal load due to its demonstrated ability of biosorption and bioaccumulation (Zaidi and Musarrat, 2004). To the best of our knowledge, this is the first report elucidating the role of metal resistant serpentine isolates in heavy metal accumulation by *R. communis* with concurrent reduction of metal phytotoxicity and promotion of plant growth.

5. Conclusions

The accumulation and distribution of metals in the plant tissue are important aspects to evaluate the role of plants in remediation of contaminated sites (Salt et al., 1998). The results of the present study revealed that inoculation of metal resistant PGPB *Pseudomonas* sp. PsM6 and *P. jessenii* PjM15 increases the efficiency of phytoextraction directly by enhancing the metal accumulation in plant tissues (especially Zn) and indirectly by promoting the shoot and root biomass of *R. communis*. The use of these metal resistant PGPB can be considered as a biotechnological tool of great economical and ecological relevance. As the technology of metal 'phytomining' matures and is commercially developed, even small increases in metal uptake can have very significant impacts on profitability. Thus, suitable modification of the roots/rhizosphere system of heavy metal phytoaccumulators with beneficial microflora could promote metal bioavailability and phytoextraction. Further research will be aimed to assess the suitability of metal resistant PGPB for efficient bioremediation of heavy metals in natural ecosystem.

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References

Abou-Shanab, R.A., Angle, J.S., Delorme, T.A., Chaney, R.L., van Berkum, P., Moawad, H., Ghanem, K., Ghozlan, H.A., 2003. Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. New Phytol. 158, 219–224.

Alloway, B.J., 1995. Heavy Metal in Soils, second ed. Chapman & Hall, London.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Belimov, A.A., Hontzeas, N., Safronova, V.I., Demchinskaya, S.V., Piluzza, G., Bullitta, S., Glick, B.R., 2005. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* LCzern.). Soil Biol. Biochem. 37, 241–250.

Belimov, A.A., Safronova, V.I., Mimura, T., 2002. Response of spring rape to inoculation with plant growth-promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate deaminase depends on nutrient status of the plant. Can. J. Microbiol. 48, 189–199.

Beveridge, T.J., 1988. The bacterial surface: general considerations towards design and function. Can. J. Microbiol. 34, 363–372.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.

Branco, R., Chung, A.P., Veríssimo, A., Morais, P.V., 2005. Impact of chromium-contaminated wastewaters on the microbial community of a river. FEMS Microbiol. Ecol. 54, 35–46.

Bric, J.M., Bostock, R.M., Silversone, S.E., 1991. Rapid in situ assay for indole acetic acid production by bacteria immobilization

on a nitrocellulose membrane. Appl. Environ. Microb. 57, 535–538.

Burd, G.I., Dixon, D.G., Glick, B.R., 2000. Plant growth promoting bacteria that decreases heavy metal toxicity in plants. Can. J. Microbiol. 46, 237–245.

Cecchi, C.G.S., Zanchi, C., 2005. Phytoremediation of soil polluted by nickel using agricultural crops. Environ. Manage. 36, 675–681.

Cheng, S., 2003. Heavy metal pollution in China: origin, pattern and control. Environ. Sci. Pollut. Res. 10, 192–198.

Dell'Amico, E., Cavalca, L., Andreoni, V., 2005. Analysis of rhizobacterial communities in perennial *Graminaceae* from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. FEMS Microbiol. Ecol. 52, 153–162.

Delorme, T.A., Gagliardi, J.V., Angle, J.S., Chaney, R.L., 2001. Influence of the zinc hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. and the nonmetal accumulator *Trifolium pratense* L. on soil microbial population. Can. J. Microbiol. 67, 190–197.

De-Souza, M.P., Huang, C.P.A., Chee, N., Terry, N., 1999. Rhizosphere bacteria enhance that accumulation of selenium and mercury in wetland plants. Planta 209, 259–263.

Diels, L., van der Lelie, N., Bastiaens, L., 2002. New development in treatment of heavy metal contaminated soils. Rev. Environ. Sci. Biotechnol. 1, 75–82.

Doelman, P., 1985. Resistance of soil microbial communities to heavy metals. In: Jensen, V., Kjoelles, A., Soerensen, L.H. (Eds.), Microbial Communities in Soil. Elsevier, London, pp. 369–384.

Dunbar, J., Takala, S., Barns, S.M., Davis, J.A., Kuske, C.R., 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microb. 65, 1662–1669.

Dworkin, M., Foster, J., 1958. Experiments with some microorganisms which utilize ethane and hydrogen. J. Bacteriol. 75, 592–601.

Fiske, C.H., Subbarow, Y., 1925. A colorimetric determination of phosphorus. J. Biol. Chem. 66, 375–400.

Freitas, H., Prasad, M.N.V., Pratas, J., 2004. Analysis of serpentinophytes from north–east of Portugal for trace metal accumulation-relevance to the management of mine environment. Chemosphere 54, 1625–1642.

Gadd, G.M., 1990. Heavy metal accumulation by bacteria and other microorganisms. Experientia 46, 834–840.

Gadd, G.M., 2001. Metal transformations. In: Gadd, G.M. (Ed.), Fungi in Bioremediation. Cambridge University Press, Cambridge, UK, pp. 359–382.

Gadd, G.M., Sayer, J.A., 2000. Fungal transformations of metals and metalloids. In: Lovley, D.R. (Ed.), Environmental Microbe–Metal Interactions. American Society for Microbiology, Washington, DC, pp. 237–256.

Glick, B.R., 2003. Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol. Adv. 21, 383–393.

Glick, B.R., Patten, C.L., Holguin, G., Penrose, G.M., 1999. Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria. Imperial College Press, London.

Glick, B.R., Penrose, D.M., Li, J., 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190, 63–68.

Grichko, V.P., Glick, B.R., 2001. Amelioration of flooding stress by ACC deaminase-containing plant growth-promoting bacteria. Plant Physiol. Biochem. 39, 11–17.

Gupta, A.K., Sinha, S., 2006. Chemical fractionation and heavy metals accumulation in the plants of *Sesamum indicum* (L.) var. T55 grown on soil amended with tannery sludge: selection of single extractants. Chemosphere 64, 161–173.

Gupta, A., Meyer, J.M., Goel, R., 2002. Development of heavy metal resistant mutants of phosphate solubilizing *Pseudomonas* sp. NBRI4014 and their characterization. Curr. Microbiol. 45, 323–327.

Halstead, R.L., Finn, B.J., MacLean, A.J., 1969. Extractibility of nickel added to soils and its concentration in plants. Can. J. Soil Sci. 49, 335–342.

Hernandez, A., Mellado, R.P., Martinez, J.L., 1998. Metal accumulation and vanadium-induced multidrug resistance by environmental isolates of *Escherichia hermannii* and *Enterobacter cloacae*. Appl. Environ. Microb. 64, 4317–4320.

Mengoni, A., Barzanti, R., Gonneli, C., Gabbrielli, R., Bazzicalupo, M., 2001. Characterization of nickel-resistant bacteria isolated from serpentine soil. Environ. Microbiol. 3, 691–708.

Mullen, M.D., Wolf, D.C., Ferris, F.G., Beveridge, T.J., Flemming, C.A., Bailey, G.W., 1989. Bacterial sorption of heavy metals. Appl. Environ. Microb. 55, 3143–3149.

Nautiyal, C.S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol. Lett. 170, 265–270.

Pal, A., Dutta, S., Mukherjee, P.K., Paul, A.K., 2005. Occurrence of heavy metal-resistance in microflora from serpentine soil of Andaman. J. Basic Microbiol. 45, 207–218.

Penrose, D.M., Glick, B.R., 2001. Levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in exudates and extracts of canola seeds treated with plant growth-promoting bacteria. Can. J. Microbiol. 47, 368–372.

Prasad, M.N.V., Freitas, H., 2003. Metal hyperaccumulation in plants – biodiversity prospecting for phytoremediation technology. Electron. J. Biotechn. 6, 285–321.

Pulford, I.D., Watson, C., 2003. Phytoremediation of heavy metal contaminated land by trees – a review. Environ. Int. 29, 529–540.

Rajkumar, M., Nagendran, R., Lee, K.J., Lee, W.H., 2005. Characterization of a novel Cr^{6+} reducing *Pseudomonas* sp. with plant growth–promoting potential. Curr. Microbiol. 50, 266–271.

Rajkumar, M., Nagendran, R., Lee, K.J., Lee, W.H., Kim, S.Z., 2006. Influence of plant growth promoting bacteria and Cr^{6+} on the growth of Indian mustard. Chemosphere 62, 741–748.

Raskin, I., Smith, R.D., Salt, D.E., 1997. Phytoremediation of metals: using plants to remove pollutants from the environment. Curr. Opin. Biotechnol. 8, 221–226.

Rockwood, D.L., Naidu, C.V., Carter, D.R., Rahmani, M., Spriggs, T.A., Lin, C., Alker, G.R., Isebrands, J.G., Segrest, S.A., 2004. Short-rotation woody crops and phytoremediation: opportunities for agroforestry?. Agrofores. Sys. 61 51–63.

Salt, D.E., Smith, R.D., Raskin, I., 1998. Phytoremediation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 13, 468–474.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning; A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Samuelson, P., Wernérus, H., Svedberg, M., Stahl, S., 2000. Staphylococcal surface display of metal-binding polyhistidyl peptides. Appl. Environ. Microb. 66, 1243–1248.

Sheng, X.F., Xia, J.J., 2006. Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. Chemosphere 64, 1036–1042.

Silver, S., 1996. Bacterial resistance to toxic metal ions – a review. Gene 179, 9–19.

Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis. Academic Press, London.

Turgut, C., 2003. The contamination with organochlorine pesticides and heavy metals in surface water in Kucuk Menderes River in Turkey, 2000–2002. Environ. Int. 29, 29–32.

Wang, P.C., Mori, T., Komori, K., Sasatsu, M., Toda, K., Ohtake, H., 1989. Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. Appl. Environ. Microb. 55, 1665–1669.

Whiting, S.N., de Souza, M.P., Terry, N., 2001. Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environ. Sci. Technol. 15, 3144–3150.

Xie, H., Pasternak, J.J., Glick, B.R., 1996. Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR 12-2 that overproduce indoleacetic acid. Curr. Microbiol. 32, 67–71.

Zaidi, S., Musarrat, J., 2004. Characterisation and nickel sorption kinetics of a new metal hyper-accumulator *Bacillus* sp.. J. Environ. Sci. Health A 39, 681–691.

Zaidi, S., Usmani, S., Singh, B.R., Musarrat, J., 2006. Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. Chemosphere 64, 991–997.

Zhou, J.L., 1999. Zn biosorption by *Rhizopus arrhizus* and other fungi. Appl. Microbiol. Biot. 51, 686–693.