Available online at www.sciencedirect.com
ScienceDirect
Bioresource Technology 99 (2008) 3491–3498
BIORESOURCE TECHNOLOGY

Effects of inoculation of plant-growth promoting bacteria on Ni uptake by Indian mustard

Mani Rajkumar *, Helena Freitas

Centre for Functional Ecology, Department of Botany, University of Coimbra, Coimbra 3000, Portugal

Received 4 May 2007; received in revised form 27 July 2007; accepted 30 July 2007
Available online 10 September 2007

Abstract

In this study, among a collection of Ni resistant bacterial strains isolated from serpentine soil, two plant growth promoting bacteria (PGPB), Ps29C and Bm4C were selected based on their ability to utilize ACC as the sole N source and promote seedling growth in roll towel assay. The Ni resistant PGPB, Ps29C and Bm4C were characterized as *Pseudomonas* sp. and *Bacillus megaterium*, respectively, on the basis of their 16s rDNA sequences. Assessment of the parameters of plant growth promotion revealed the intrinsic ability of the strains for the production of IAA, siderophore and solubilization of insoluble phosphate. Further, the plant growth promoting activity of Ps29C and Bm4C on the Indian mustard (*Brassica juncea*) were assessed with different concentrations of Ni in soil. Inoculation of Ps29C or Bm4C promoted plant growth and protected the plant from Ni toxicity. However, the maximum growth was observed in the plants inoculated with strain Bm4C. Inoculation with Ps29C or Bm4C had little influence on the accumulation of Ni in root and shoot system, but produced a much larger aboveground biomass. The present observations showed that the strains Ps29C and Bm4C protect the plants against the inhibitory effects of nickel, probably due to the production of IAA, siderophore and solubilization of phosphate. The above results provided a new insight into the phytoremediation of Ni contaminated soil.

Keywords: Nickel; *Brassica juncea*; Siderophore; IAA

1. Introduction

Although Ni is natural component of the soil, industrial operations such as electroplating, steel, alloy, motor vehicles, aircraft paint, chemical, textile, pigment cause phenomenal increase in the extent of nickel (Ni) in the environment. Soil contamination with Ni has become a worldwide problem, leading to losses in agricultural yield and hazardous health effects as they enter the food chain (Guo and Marschner, 1995; Salt et al., 1995). Moreover, the heavy metals cannot be degraded to harmless products and hence persist in the environment indefinitely. In order to remediate the soil contaminated with toxic heavy metals, it should be concentrated and extracted by conventional methods for reuse or for proper disposal. A promising option to achieve this is by phytoremediation, the use of plants to remove, destroy, or sequester hazardous substances from the environment. The success of the phytoremediation process, whereby metals are effectively removed from soil, is dependent on an adequate yield of plants and on the efficient transfer of metals from the roots of the plants into their shoots. Some plants, such as *Thlaspi*, *Urtica*, *Chenopodium*, *Polygonum sachalase* and *Alyssum* have shown the ability to extract, accumulate and tolerate high levels of heavy metals. Such plants are termed hyperaccumulators, but their potential for application in phytoremediation is limited by the fact that they are slow growing and have a small biomass (Mulligan et al., 2001; Puschenreiter et al., 2001). These characteristics are contrary to the ones proposed by Robinson et al. (2000) who suggested that a plant used for phytoremediation should be fast growing, deep rooted, easily propagated and accumulating the target metal. According to Romkens et al.

* Corresponding author. Tel.: +351 239855243; fax: +351 239 855211.
E-mail address: mraaj13@yahoo.com (M. Rajkumar).

0960-8524/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2007.07.046

(2002) it should also have a high biomass production. For these reasons the tobacco plant, *Nicotiana tabacum* is suitable for phytoremediation, in the areas of Latin and South America.

In recent years, several synthetic chelators such as EDTA have been suggested to enhance phytoextraction efficiency in metal contaminated soils (Blaylock et al., 1997; Puschenreiter et al., 2001). However, chelator application in chemically assisted phytoextraction may also have potentially environmental risks. Firstly, some chelators themselves are usually phytotoxic, and increasing metal solubility by them may be also phytotoxic to non-hyperaccumulator plants, thus plant growth may be inhibited, and the chance of success with chemically assisted phytoextraction may be lowered (McGrath and Zhao, 2003). Secondly, chelators cause possible leaching of metal chelates to groundwater, which may have toxic effects on soil microorganisms and soil microfauna, thus affect soil ecosystem stability and function (Romkens et al., 2002). Hence, the alternate ways to enhance phytoextraction efficiency is by using the rhizosphere microbes (Burd et al., 2000). Rhizosphere microbes, with activity and a high surface area-to-volume ratio because of their small size and therefore providing a large contact area may have the potential to act as microbial chelates associated with phytoremediation (Karenlampi et al., 2000). Recently it has been shown that rhizosphere microorganisms may improve the metal solubility and availability by reducing the soil pH or by producing chelators and siderophores. Microbial siderophores prevent iron deficiency of the producing organism and of plants but may also be involved in the uptake of other metals (Hu and Boyer, 1996). The production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, an enzyme that has no function in bacteria but modulates ethylene levels in developing plants (Glick et al., 1998), may further contribute to the heavy metal tolerance of plants. In addition, plant-associated bacteria may produce phytohormones and provide nutrients to the plant (Patten and Glick, 1996).

Thus, the objectives of this study were to isolate and characterize Ni-resistant bacteria from serpentine soils, and to select plant growth promoting bacteria (PGPB) which might be useful to increase plant biomass production and Ni-uptake by plant for improving the efficiency of phytoremediation of Ni-polluted soils.

2. Methods

2.1. Isolation of Ni resistant PGPB

The bacterial strains were isolated from a serpentine site in Bragança, north-east of Portugal, previously described by Freitas et al. (2004). About 1 g of soil samples were serially diluted using 25 mM phosphate buffer and spread over on Luria-Bartani medium (LB) amended with 50 mg of Ni l^{-1} ($NiCl_2$). The plates were incubated at 37 °C for 48 h. From the Ni resistant colonies, different strains were picked and purified on the LB medium containing 50 mg l^{-1} of Ni according to the procedure of Rajkumar et al. (2005). Purified colonies were gradually taken to higher concentration of Ni (50–1000 mg l^{-1}) and the same procedure was continued to isolate Ni resistant strains. In order to isolate the PGPB, the Ni resistant strains were grown on DF salt minimal medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC to provide a nitrogen source at 30 °C for 168 h at 175 rpm. The bacterial growth was measured once in every 24 h by dilution plate method. The diluted culture was plated on to solid DF salt minimal medium with ACC and incubated at 30 °C for 48 h. Further, the ACC utilizing strains were assessed for the plant growth promoting activity by roll towel method (ISTA, 1966). Indian mustard seeds were procured from Centre for Environmental studies, Anna University, Chennai, India. The seeds were inoculated by soaking in a bacterial suspension containing 10^8 cell ml^{-1} for 1 h then placed in wet blotters and incubated in a growth chamber for 20 d. The germination percentage of seeds was recorded and the vigour index was calculated using the formula described by Abdul Baki and Anderson (1973).

2.2. Genetic characterization of Ni resistant PGPB

The bacterial strains were grown in LB broth in presence of 1 mM Ni at 30 °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 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 DNA 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. Influence of PGPB and Ni on Indian mustard growth and Ni uptake

For pot experiments the soil was collected from the Botanical garden, Department of Botany, University of Coimbra, Coimbra, Portugal. The soil was sieved (2 mm)

and sterilized by steaming (100 °C for 1 h on three consecutive days). After sterilization the soil was amended with aqueous solution of $NiCl_2$ to achieve the final concentrations of 100, 200 or 300 mg Ni kg^{-1} and left in a greenhouse for a 2 weeks period (for metal stabilization). Indian mustard seeds were inoculated with PGPB as detailed in earlier section. Seeds soaked in sterile water were used as control. The inoculated and non inoculated seeds were planted in plastic pot (top diameter 120 mm, bottom 100 mm and height 90 mm) containing 300 g of soil. The plants were grown in a glasshouse at 25 °C and a 16/8 day/night regime. After 45 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 length, fresh weight and dry weight of the plants were measured. The accumulation of nickel in root and shoot system was also quantified following the method of Freitas et al. (2004).

2.4. IAA, siderophore production and phosphate solubilization

2.4.1. IAA production

The bacterial strains were inoculated in LB broth supplemented with 500 μg of tryptophan ml^{-1} and incubated at 27 °C for 96 h at 175 rpm. The growth of bacteria was monitored at definite time intervals. The IAA concentration in the culture supernatant was determined according to the method of Bric et al. (1991).

2.4.2. Siderophore production

The production of siderophore was detected in 0.5% casamino acids medium supplemented with different concentration of Fe^{3+} as described by Schwyn and Neilands (1987). The flasks were incubated at 27 °C for 48 h at 175 rpm. To determine the siderophore in liquid culture, 0.5 ml culture supernatant was mixed with 0.5 ml CAS assay solution and the tubes were observed for color change from dark blue to light blue or orange.

2.4.3. Phosphate solubilizing activity

The bacterial strains were grown in modified Pikovskaya's medium (Sundara-Rao and Sinha, 1963) with 0.5% of tricalcium phosphate at 27 °C for 192 h at 175 rpm. The culture supernatants were collected by centrifugation at 10,000 rpm for 15 min. Soluble phosphate in the culture supernatant was estimated according to the method of Fiske and Subbarow (1925).

3. Results and discussion

3.1. Isolation and characterization of Ni resistant PGPB

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, 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 Ni resistant PGPB on the growth of Indian mustard under different concentrations of Ni. During the initial screening, thirty Ni resistant bacterial strains were isolated from the soil samples. In order to isolate the PGPB, the Ni resistant strains were tested for the ability to grow on DF salts minimal medium with ACC. Among the thirty strains tested, Ps29C and Bm4C grew in DF salts minimal medium with ACC as the sole source of nitrogen. However, maximum growth was observed in Ps29C compared with Bm4C. In the absence of ACC, the strains Ps29C and Bm4C showed a limited growth (Fig. 1). 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 ACC utilizing strains (Ps29C and Bm4C) were further assessed for plant growth promoting activity by roll towel method (Table 1). Inoculation of Ps29C and Bm4C showed an increase in the vigour index of Indian mustard. However, Bm4C showed a maximum increase in root length, shoot length and vigour index by 30, 36 and 39%,

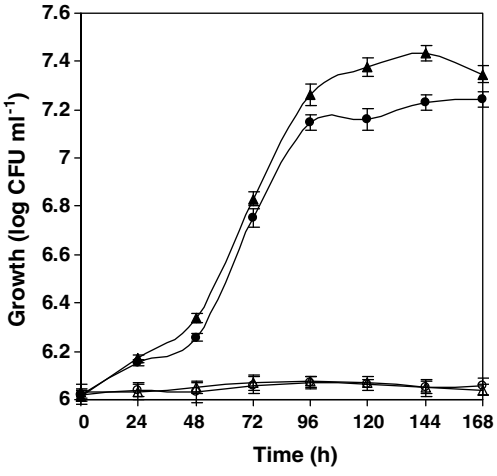

Fig. 1. Growth of PGPB on DF salts minimal medium. *Pseudomonas* sp. Ps29C with ACC (▲), *B. megaterium* Bm4C with ACC (●), *Pseudomonas* sp. Ps29C without ACC (Δ), *B. megaterium* Bm4C without ACC (○). Each value is the mean of triplicates. Error bars represent standard deviation.

Table 1
Influence of PGPB on root length, shoot length and vigour index of Indian mustard

PGPB strain	Root length (cm)	Shoot length (cm)	Vigour index[a]
Control	8.36[b] (±0.61)[c]	7.40 (±0.52)	1406.9
Ps29C	9.56 (±0.0.61)	8.43 (±0.45)	1937.1
Bm4C	10.90 (±1.21)	10.06 (±0.65)	1618.2

[a] Vigour index = germination (%) × seedling length (shoot length + root length).
[b] Values represent average of 3 samples.
[c] Values in parentheses represent standard deviation.

respectively. Similarly, Ps29C increased the root length, shoot length and vigour index by 14, 14 and 15%, respectively. Earlier studies have confirmed the potential of ACC utilizing bacteria to promote the root elongation and growth of canola, lettuce, tomato, Indian mustard, and wheat plants (Glick et al., 1998; Hall et al., 1996). The Ni resistance levels of Ps29C and Bm4C were found to be 550 and 650 mg of Ni l^{-1}, respectively. 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 Ps29C and Bm4C were close to the members of the genus *Pseudomonas* and *Bacillus,* respectively. Partial sequence of 16S rDNA of Ps29C (851 bp) showed 99% homology with the sequence of *Pseudomonas* sp. and Bm4C (808 bp) showed 99% homology with *Bacillus megaterium*. The sequences were deposited at GenBank (*Pseudomonas* sp. Ps29C, accession no. AM709775; *B. megaterium* Bm4C, accession no. AM709774).

3.2. Influence of PGPB and Ni on Indian mustard growth and Ni uptake

The bacterial inoculated and non-inoculated plants were subjected to three levels of Ni in soil for 45 d responded differently in terms of plant growth. In the absence of Ni, inoculation of *Pseudomonas* sp. Ps29C or *B. megaterium* Bm4C showed an increase in shoot length, fresh and dry weight of plant (Fig. 2). However, maximum plant growth promoting effect was observed in *B. megaterium* Bm4C, which enhances the shoot length, fresh and dry weight by 29, 42 and 28%, respectively, compared with non-inoculated plants. Similarly, *Pseudomonas* sp. Ps29C enhances the shoot length, fresh and dry weight by 23, 22 and 17%, respectively. Growth promotion in plants is due to the presence of PGPB in the soil, a common feature, and has been observed in many plant species (Hall et al., 1996; Burd et al., 2000; Belimov et al., 2005; Wu et al., 2006). The non-inoculated plants exposed to different concentrations of Ni, showed a marked inhibition in the growth. In general, with the increase in concentration of Ni progressive decrease in plant fresh and dry weight was observed. At a concentration of 100 mg Ni kg^{-1} soil, the percent decrease was 6 for fresh weight and 8 for dry weight; for 200 mg Ni, 7 and 10%; and for 300 mg Ni, 17

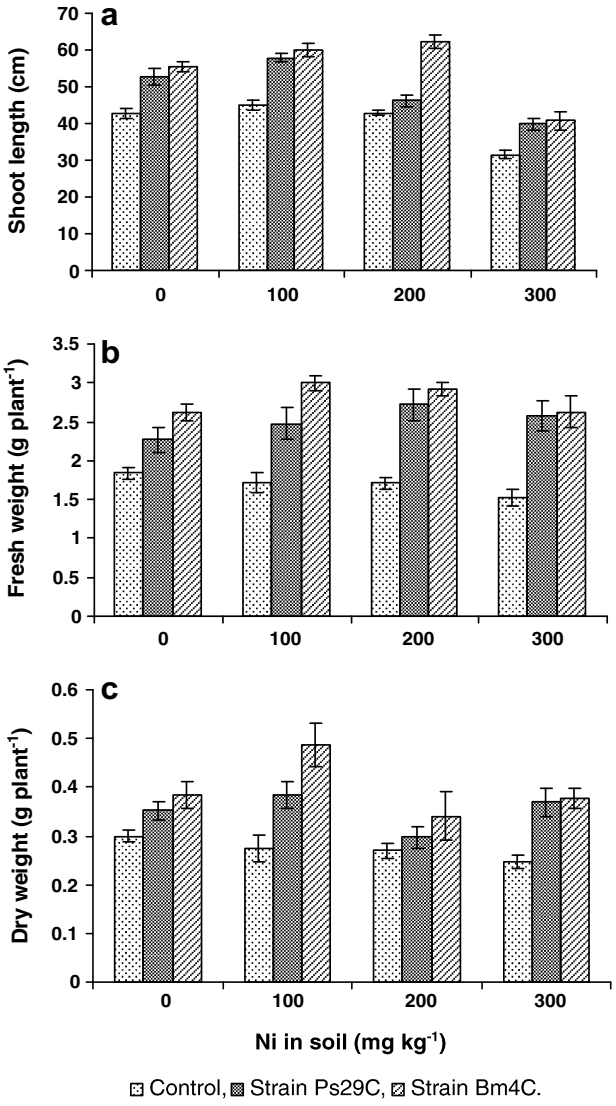

Fig. 2. Influence of PGPB and Ni on shoot length (a), fresh weight (b) and dry weight (c) of Indian mustard. Each value is the mean of triplicates. Error bars represent standard deviation.

and 18%, respectively. Growth reduction at higher concentration may be due to the toxic effects of Ni on plants. Panwar et al. (2002) also reported similar results in *B. juncea* with increasing Ni content of soil (0–80 mg kg^{-1}).

Plants inoculated with *Pseudomonas* sp. Ps29C or *B. megaterium* Bm4C exhibited an increase in shoot length, plant fresh and dry weight in the presence of Ni. At a concentration of 100 mg Ni kg^{-1} soil, the highest effect was found for *B. megaterium* Bm4C, which enhances the shoot length, fresh weight and dry weight by 33, 74 and 81%, respectively. Similarly, at a concentration of 200 mg Ni, the percent increase was 45 for shoot length, 70 for fresh weight and 25 for dry weight; for 300 mg Ni, 31, 73 and 40%, respectively. The results obtained here clearly indicate that inoculation of *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C not only protects Indian mustard from Ni toxicity but also promotes the plant growth. Doelman (1985)

has reported that the efficiency of revegetation and phytoremediation of heavy metal-contaminated sites is closely related to the presence of metal resistant microbial populations in the soil, which likely conferred a better nutritional assimilation and protection effect on plants.

The metal concentration in plant tissues and total metal removal by shoots are important indicators of whether the addition of a bacterial inoculation affects metal uptake by Indian mustard. Plants growing in metal enriched environments usually take up metals in varying degrees in response to external and internal factors (Reid et al., 1986). In the present study, accumulation of nickel in the root and shoot systems increased with increase in the initial concentration of Ni in soil (Fig. 3). The maximum accumulation of nickel was observed in the plants grown at 300 mg Ni kg^{-1} soil. Inoculation with PGPB did not greatly influence the quantity of accumulation of nickel in root and shoot system. However, inoculation of Bm4C showed a maximum accumulation of Ni in the root and shoot systems. Burd et al. (2000) and Wu et al. (2006) have recorded similar observations upon inoculation with plant growth promoting bacteria under nickel, copper, cadmium, lead and zinc stress. The present observations indicate that the PGPB *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C protect the plants against the inhibitory effects of nickel.

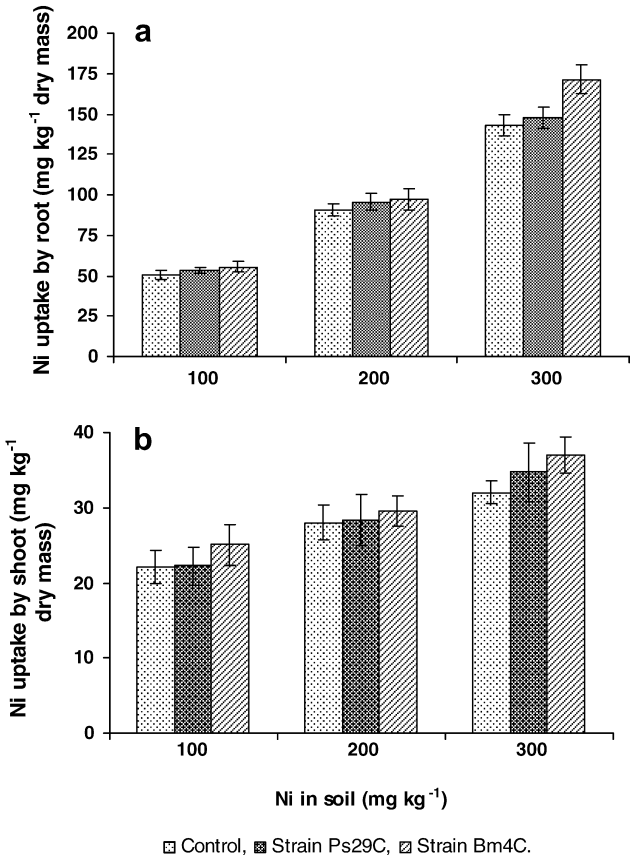

Fig. 3. Ni concentration (mg kg^{-1}) in the root (a) and shoot (b) of Indian mustard. Each value is the mean of triplicates. Error bars represent standard deviation.

3.3. Plant growth promoting features of Ni resistant PGPB

The importance of soil bacteria in metal resistance and their ability to promote the plant growth in metal contaminated environment make them the preferred choice for the bioremediation studies. In addition to ACC utilization, certain metal resistant bacteria could exert their beneficial effects on host plant by several possible mechanisms. The mechanisms include: synthesis of siderophore which can solubilize and sequester iron from the soil (Glick et al., 1999); production of phytohormones which can enhance the growth of plants (Glick et al., 1998); and solubilisation of phosphate (Gupta et al., 2002). In the present study, the improved growth of PGPB inoculated plants under Ni stress has prompted us to assess the plant growth promoting features of PGPB. Assessment of the parameters of plant growth promotion revealed the intrinsic ability of the strains for the production of IAA, siderophore and solubilization of phosphate. IAA production by *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C was estimated in LB medium (Fig. 4). *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C showed the production of IAA indicating the strains utilized L-tryptophane as a precursor for growth and IAA production. The growth and IAA production increased simultaneously and a maximum IAA production (Ps28C – 18; Bm4C – 48 mg l^{-1}) was observed after 84 h of incubation. These results concur with the earlier observations (Garcia de Salamone et al., 2001) indicating induction of IAA production in stationary phase culture probably due to delayed induction of a key enzyme of IAA biosynthesis pathway. The phytohormone IAA production offers great promise for sustaining the increased crop productivity. Most likely, the PGPG producing IAA may indirectly promote metal accumulation by increasing the plant biomass. Previously, bacteria belonging to different genera such as *Pseudomonas, Mycobacterium, Agrobacterium* and

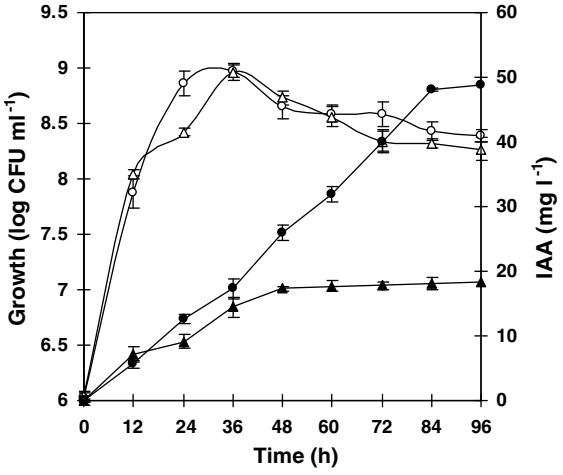

Fig. 4. Growth and IAA production. Growth of *Pseudomonas* sp. Ps29C (Δ), Growth of *B. megaterium* Bm4C (○), IAA production by *Pseudomonas* sp. Ps29C (▲), IAA production by *B. megaterium* Bm4C (●). Each value is the mean of triplicates. Error bars represent standard deviation.

Arthrobacter are reported to produce IAA and aid in plant growth (Dell'Amico et al., 2005).

Siderophore production was determined in casamino acids medium with different concentrations of Fe^{3+}. Growth of *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C increased with increase in Fe^{3+} concentrations in the medium (Table 2). In both strains, the colony forming unit (CFU) ml^{-1} increased when 1.0 μM of Fe^{3+} was added. However, there were only slight differences in CFU with iron concentrations from 10 to 50 μM. Under iron deficient conditions, *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C showed the production of siderophore. However, addition of 10 and 25 μM of Fe^{3+} repressed the production of siderophore in Ps29C and Bm4C, respectively. Siderophores may be important for the mobilization of the heavy metal in the rhizosphere. They show high affinity for ferric iron but also form complexes with bivalent heavy metal ions (Evers et al., 1989) that can be assimilated by the plant. Furthermore, heavy metals have been shown to stimulate the production of bacterial siderophores (van der Lelie et al., 1999). In general, the reduction of plant growth in Ni contaminated soil is often associated with iron deficiency and reduced uptake of some other essential element (Mishra and Kar, 1974). In the present study, siderophore produced by *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C might have helped Indian mustard root proliferation and enhanced the uptake of soil minerals such as iron.

Quantitative estimation of phosphorous solubilization was carried out in modified Pikovskaya's medium for 192 h (Fig. 5). *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C utilized tricalcium phosphate as the sole source of phosphate. However, there was no significant difference of solubilisation of phosphate between *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C. Growth of *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C increased linearly up to 96 h incubation and thereafter the stationary phase was reached. The maximum solubilization by Ps29C and Bm4C was achieved after 144 h of incubation (Ps29C – 101; Bm4C – 109 mg P l^{-1}). Further incubation up to 192 h, did not improve the extent of solubilization. At the end of each day, the final pH was determined to find out whether solubilization of phosphate was accompanied by the production of acid in the growth medium. The

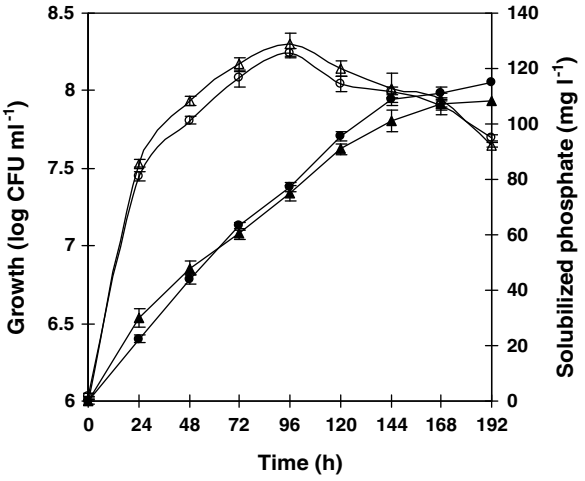

Fig. 5. Growth and phosphate solubilization. Growth of *Pseudomonas* sp. Ps29C (△), Growth of *B. megaterium* Bm4C (○), Phosphate solubilization by *Pseudomonas* sp. Ps29C (▲), Phosphate solubilization by *B. megaterium* Bm4C (●). Each value is the mean of triplicates. Error bars represent standard deviation.

PGPB strains reduced the pH of growth medium from 7.5 to 3.5–4.5 after 48 h of incubation (data not shown). The decrease in pH clearly indicates the production of acids, which is considered to be responsible for P solubilization (Bano and Mussarat, 2003). Earlier studies have demonstrated that the elevated levels of Ni 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 with the concomitant reduction in pH of the medium. Presumably, the increased accumulation of Ni in Indian mustard plants in presence of Bm4C could be due to more Ni uptake under acidic soil conditions, which develops as a result of activity of phosphate solubilization in soil. Halstead et al. (1969) have also reported that the process of solubilizing inorganic phosphates facilitates the uptake of the metals from soil.

4. Conclusion

In conclusion, the results indicate that inoculation with PGPB may facilitate plant growth and thus increase phyto-

Table 2
Growth and Chrome Azural S reaction (CAS) in PGPB grown in 0.5% casamino acids medium

PGPB strain	Parameters	Fe (μM)				
		0	1	10	25	50
Ps29C	CAS assay	+	+	+	–	–
	Growth (log CFU ml^{-1})	7.66[a] (±0.05)[b]	8.10 (±0.03)	8.23 (±0.02)	8.30 (±0.02)	8.32 (±0.03)
Bm4C	CAS assay	+	+	+	+	–
	Growth (log CFU ml^{-1})	7.19 (±0.03)	8.11 (±0.04)	8.27 (±0.03)	8.29 (±0.02)	8.33 (±0.03)

+ = Indicates the production of siderophores.
[a] Values represent average of 3 samples.
[b] Values in parentheses represent standard deviation.

remediation efficiency. Enhancing metal accumulation in high yielding crop plants without diminishing their yield is fundamental to successful phytoremediation (Blaylock et al., 1997). Compared with control treatments, inoculation with *B. megaterium* Bm4C did not greatly influence the quantity of accumulation of Ni in plant tissues, but achieved a much larger aboveground biomass harvest, thus resulting in a much higher metal removal. The increase in plant growth caused by *B. megaterium* Bm4C may be attributed to the maximum production of IAA and solubilisation of phosphate. Earlier studies have also confirmed the potential of soil microorganisms to assist plant establishment on contaminated soils through mediating nutrient mineralization and uptake by plants (Gupta et al., 2002), production of plant growth hormones and siderophores (Patten and Glick, 1996; Glick et al., 1998, 1999) and utilization of ACC as a sole N source (Glick et al., 1998; Burd et al., 2000). These beneficial effects caused by inoculation with PGPB, together with the suggested interrelationship between microbial heavy metal resistance and plant growth promoting efficiency, indicates that inoculation with microbes might have some potential to improve phytoextraction efficiency in metal contaminated soils. Further work aims to assess the suitability of PGPB, *B. megaterium* Bm4C for efficient bioremediation of heavy metals in natural ecosystem.

Acknowledgement

M. Rajkumar thanks the Portuguese Foundation for Science and Technology (FCT) for awarding a Post-doctoral research grant (SFRH/BPD/21309/2005).

References

Abdul Baki, A.A., Anderson, J.D., 1973. Vigour determination in soya bean seed by multiple criteria. Crop Sci. 13, 630–633.

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.

Bano, N., Mussarat, J., 2003. Isolation and characterization of phorate degrading soil bacteria of environmental and agronomic significance. Lett. Appl. Micribiol. 36, 349–353.

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* L.Czern.). Soil Biol. Biochem. 37, 241–250.

Blaylock, J.M., Salt, D.E., Dushenkov, S., Zakharova, O., Gussman, C., Kapulnik, Y., Ensley, B.D., Raskin, I., 1997. Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. Environ. Sci. Technol. 31, 860–865.

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. Microbiol. 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.

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.

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. Microbiol. 65, 1662–1669.

Evers, A., Hancock, R.D., Martell, A.E., Motekaitis, R.J., 1989. Metal ion recognition in ligands with negatively charged oxygen donor groups. Complexation of Fe(III), Ga(III), In(III), Al(III) and other highly charged metal ions. Inorg. Chem. 28, 2189–2195.

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.

Garcia de Salamone, I.E., Hynes, R.K., Nelson, L.N., 2001. Cytokinin production by plant growth promoting rhizobacteria and selected mutants. Can. J. Microbiol. 47, 103–113.

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.

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.

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.

Guo, Y., Marschner, H., 1995. Uptake, distribution, and binding of cadmium and nickel in different plant species. J. Plant Nutr. 18, 2691–2706.

Hall, J.A., Peirson, D., Ghosh, S., Glick, B.R., 1996. Root elongation in various agronomic crops by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Israel J. Plant Sci. 44, 37–42.

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.

Hu, X., Boyer, G.L., 1996. Siderophore-mediated aluminum uptake by *Bacillus megaterium* ATCC 19213. Appl. Environ. Microbiol. 62, 4044–4048.

ISTA, 1966. International rules for seed testing. Proc. Int. Seed Test Assoc. 31, 1–152.

Karenlampi, S., Schat, H., Vangronsveld, J., Verkleij, J.A.C., van der Lelie, D., Mergeay, M., Tervahauta, A.I., 2000. Genetic engineering in the improvement of plants for phytoremediation of metal polluted soils. Environ. Pollut. 107, 225–231.

McGrath, S.P., Zhao, F.J., 2003. Phytoextraction of metals and metalloids from contaminated soils. Curr. Opin. Biotechnol. 14, 277–282.

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.

Mishra, D., Kar, M., 1974. Nickel in plant growth and metabolism. Bot. Rev. 40, 395–452.

Mulligan, C.N., Yong, R.N., Gibbs, B.F., 2001. Remediation technologies for metal-contaminated soils and groundwater: an evaluation. Eng. Geol. 60, 193–207.

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.

Panwar, B.S., Ahmad, K.S., Mittal, S.B., 2002. Phytoremediation of Nickel-contaminated soils by *Brasssica* species. Environ. Dev. Sustain. 4, 1–6.

Patten, C.L., Glick, R.B., 1996. Bacterial biosynthesis of indole-3-acetic acid. Can. J. Microbiol. 42, 207–220.

Puschenreiter, M., Stoger, G., Lombi, E., Horak, O., Wenzel, W.W., 2001. Phytoextraction of heavy metal contaminated soils with *Thlaspi goesingense* and *Amaranthus hybridus*: rhizosphere manipulation using EDTA and ammonium sulfate. J. Plant Nutr. Soil Sci. 164, 615–621.

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.

Reid, C.P.P., Szaniszlo, P.J., Crowley, D.E., 1986. Siderophore involvement in plant iron nutrition. In: Swinburne, T.R. (Ed.), Iron Siderophores and Plant Diseases. Plenum Press, New York, NY, pp. 29–42.

Robinson, B.H., Millis, T.M., Petit, D., Fung, L.E., Green, S.R., Clothier, B.E., 2000. Natural and induced cadmium accumulation in poplar and willow: implications for phytoremediation. Plant Soil 227, 301–306.

Romkens, P., Bouwman, L., Japenga, J., Draaisma, C., 2002. Potentials and drawbacks of chelate-enhanced phytoremediation of soils. Environ. Pollut. 116, 109–121.

Salt, D.E., Blaylock, M., Kumar, N.P.B.A., Dusenkov, V., Ensley, B.D., Chet, I., Raskin, I., 1995. Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnology 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.

Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for detection and determination of siderophores. Anal. Biochem. 160, 47–56.

Sundara-Rao, W.V.B., Sinha, M.K., 1963. Phosphate dissolving microorganisms in the soil and rhizosphere. Indian J. Agric. Sci. 33, 272–278.

van der Lelie, D., Corbisier, P., Diels, L., Gilis, A., Lodewyckx, C., Mergeay, M., Taghavi, S., Spelmans, N., Vangronsveld, J., 1999. The role of bacteria in the phytoremediation of heavy metals. In: Terry, N., Banuelos, G. (Eds.), Phytoremediation of Contaminated Soil and Water. Lewis Publishers, London, United Kingdom, pp. 265–281.

Wu, S.C., Cheung, K.C., Luo, Y.M., Wong, M.H., 2006. Effects of inoculation of plant growth-promoting rhizobacteria on metal uptake by *Brassica juncea*. Environ. Pollut. 140, 124–135.