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Improvement of Ni phytostabilization by inoculation of Ni resistant *Bacillus megaterium* SR28C

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ABSTRACT

The use of metal tolerant plants for the phytostabilization of metal contaminated soil is an area of extensive research and development. In this study the effects of inoculation of Ni-resistant bacterial strains on phytostabilization potential of various plants, including *Brassica juncea*, *Luffa cylindrica* and *Sorghum halepense*, were studied. A Ni-resistant bacterial strain SR28C was isolated from a nickel rich serpentine soil and identified as *Bacillus megaterium* based on the morphological features, biochemical characteristics and partial 16S rDNA sequence analysis. The strain SR28C tolerated concentrations up to 1200 mg Ni L^{-1} on a Luria–Bertani (LB) agar medium. Besides, it showed high degree of resistance to various metals (Cu, Zn, Cd, Pb and Cr) and antibiotics (ampicillin, tetracycline, streptomycin, chloramphenicol, penicillin and kanamycin) tested. In addition, the strain bound considerable amounts of Ni in their resting cells. Besides, the strain exhibited the plant growth promoting traits, such as solubilization of phosphate and production of indole-3-acetic acid (IAA) in modified Pikovskayas medium and LB medium, respectively in the absence and presence of Ni. Considering such potential, the effects of SR28C on the growth and Ni accumulation of *B. juncea*, *L. cylindrica* and *S. halepense*, were assessed with different concentrations of Ni in soil. Inoculation of SR28C stimulated the biomass of the test plants grown in both Ni contaminated and non-contaminated soils. Further, SR28C alleviated the detrimental effects of Ni by reducing its uptake and translocation to the plants. This study suggested that the PGPB inoculant due to its intrinsic abilities of growth promotion and attenuation of the toxic effects of Ni could be exploited for phytostabilization of Ni contaminated site.

1. Introduction

Contamination of soils with toxic heavy metals is becoming a worldwide problem, leading to decreases in soil microbial activity, reduction of crop productivity, and hazardous health effects as they enter the food chain (McGrath et al., 1995; Lim and Schoenung, 2010). Ni is such a heavy metal frequently used on large scale in many different industries, including mineral processing, production of paints and pigments, electroplating, asbestos mining and milling, cement manufacturing, copper sulfate manufacture and steam-electric power plants (Khodadoust et al., 2004; Cecchie and Zanchi, 2005). As heavy metals cannot be biologically degraded, and the metal contaminated soils are more prone to erosion and leaching, the remediation of these soils is gaining considerable momentum and is a challenging task. Although different soil remediation strategies such as physical and chemical methods (e.g., soil excavation followed by coagulation–filtration or ion exchange) have been developed (Khodadoust et al., 2004), these methods are too expensive and disruptive to soil ecological and biological structure (Danh et al., 2009; Wu et al., 2010). Phytoremediation, a biological approach that uses metal tolerant plants to clean up contaminated sites, is a simple, cost effective and self-sustainable alternative to conventional methods (Raskin et al., 1997; Salt et al., 1998; Raskin and Ensley, 2000; Glick, 2010). Particularly, phytostabilization (use of metal tolerant plants to reduce mobility, solubility and/or inactivate toxic heavy metals through *in situ* rhizospheric processes), is currently receiving a great deal of attention (Wu et al., 2011). Although some plants species (e.g., *Jatropha curcas*, *Sesbania virgata*) have the potential to stabilize/inactivate the heavy metals through various rhizopheric reactions, the presence of elevated bioavailable metal concentrations and plant growth limiting factors (e.g., high levels of residual metals, poor nutrients etc.) in polluted soils affect the

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plant growth and its establishment through impairing their ecophysiological metabolism (Wu et al., 2011; Branzini et al., 2012; Pérez-Sanza et al., 2012). In recent years, many different chemical and biological amendments (e.g., limestone, calcinit, urea, calcium carbonate and cow slurry) have been used to improve the plant growth and/or reduce the metal solubility and bioavailability in polluted soils (Epelde et al., 2009; Lee et al., 2011; Wu et al., 2011). Even though some chemical amendments decrease the metal uptake in plants, these amendments (e.g., cyclonic ashes, steel shots, CaO) are toxic to plants and their associated beneficial soil microbes (Mühlbachová and Tlustoš, 2006; Ruttens et al., 2006). Therefore, in recent years metal resistant microbes have been employed because they display a high potential to alter the metal mobility and bioavailability. Moreover, when considering approaches to improve heavy metal phytoremediation, there are several advantages of using biological amendments rather than chemical amendments because they are degradable, less toxic, and they may improve the soil ecological structure and function (Rajkumar et al., 2012).

The use of metal resistant bacteria, which are present in the rhizosphere soils, have received much attention as they can affect heavy metal mobility and its uptake by plants through various reactions such as metal biosorption, oxidation/reduction, heavy metal-ligand complexation (Glick, 2010; Ma et al., 2011a; Andreazza et al., 2012; Rajkumar et al., 2012). For example, Chatterjee et al. (2009) reported that the inoculation of Cr^{6+} reducing bacteria *Cellulosimicrobium cellulans* decreased Cr uptake in green chilli plants. Similarly, Vivas et al. (2006) also found that the inoculation of *Trifolium repens* with Zn binding bacteria *Brevibacillus* sp. B–I decreased the concentration of Zn in shoot tissues compared with respective uninoculated control. Moreover, the metal resistant bacteria play a great role in the growth and establishment of plants on the contaminated soils through producing plant growth beneficial metabolites including siderophores, indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase and solubilizing phosphate (P) (Ma et al., 2010, 2011b). Thus, the application of these rhizosphere bacteria is a promising approach for reducing heavy metal toxicity and its accumulation in plants, creating a suitable environment for plant's establishment in heavy metal contaminated soils. Although many studies have demonstrated that the inoculation of plants or soil with plant growth promoting bacteria (PGPB) has promising potential for improving plant growth in metal contaminated soils, only a few metal resistant PGPB have been reported to promote the phytostabilization potential of plants when applied to metal contaminated soils. Moreover, since the microbial inoculums for phytoremediation must be compatible with various plants and soils to be commercially successful, this study investigated the role of Ni-resistant PGPB isolated from serpentine soils on the growth and Ni accumulation of various plant species including *Brassica juncea*, *Luffa cylindrica* and *Sorghum halepense*. These plants were selected based on the factors such as ability to take up large concentrations of heavy metals, adaptation to local climatic conditions, and ease of planting and maintenance (Madejon et al., 2002; Gupta and Sinha, 2006; Singh et al., 2010) and since the potential of these plants with rhizosphere bacteria has rarely been studied for microbial assisted phytoremediation of Ni contaminated soils.

The objectives of this study were to (i) isolate and characterize the serpentine soil bacteria capable of tolerating Ni and other heavy metals, (ii) screen isolates for auxiliary activities including solubilization of P, production of IAA and biosorption of Ni and (iii) study the influence of Ni-resistant bacterium on the growth and Ni accumulation of various plant species under different concentrations of Ni in soil.

2. Materials and methods

2.1. Isolation of Ni-resistant bacterial strain

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 bacteria, soil samples were serially diluted in sterile distilled water and plated on Luria Bertani (LB) agar medium supplemented with 50 mg L^{-1} of Ni as $NiCl_2 \cdot 6H_2O$. The plates were incubated at 27 °C for 48 h. From the Ni-resistant colonies, different strains were picked and purified on LB agar medium containing 50 mg L^{-1} of Ni according to the procedure of Ma et al. (2011b). Purified colonies were gradually taken to higher concentration of Ni (50–1500 mg L^{-1}) and the same procedure was continued to isolate Ni-resistant strains (Ma et al., 2011b).

2.2. Identification of Ni-resistant bacterial strain

The bacterium showing high degree of Ni resistance was selected and identified based on morphological and biochemical features. For further identification, genomic DNA was isolated and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using 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 analyzed by agarose gel (1.5% w/v) electrophoresis in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) containing 1 mg 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. Characterization of Ni-resistant bacterial strain

2.3.1. Heavy metal and antibiotic resistance

For determining the maximal tolerable concentrations (MTCs) of heavy metals (Cu, Pb, Zn, Cd or Cr), the isolate was tested for the ability to grow on LB agar medium with increasing concentrations of the metal ions (ranging from 50 up to 1500 mg L^{-1}). Stock solutions of the metal salts were prepared in double distilled water and sterilized. LB agar plates without metals were used as controls. The experiments were carried out in triplicate. Cultures were incubated at 27 °C for 7 days. The highest metal concentration which allowed bacterial growth was considered as the MTCs (Houdt et al., 2012). The antibiotic resistance of the bacterial strain was determined by the disc diffusion method. The bacterium was grown in LB broth at 27 °C for 24 h and spread on LB agar plates using a sterile swab. Small filter paper discs (6 mm) impregnated with a standard amount of antibiotic were placed on the surface of the LB agar using flame sterilized forceps at the rate of 3 discs per plate. The antibiotic concentrations of the disc used were ampicillin (10 μg), tetracycline (30 μg), streptomycin (30 μg) chloramphenicol (30 μg), penicillin (20 μg) and kanamycin (30 μg), respectively. The diameter of the inhibition zones around the discs was measured. Zones of inhibition of ≥18 mm were considered sensitive, 13–17 mm intermediate and <13 mm resistant (NCCLS, 2002).

2.3.2. Effect of Ni on the growth of Ni-resistant bacterial strain

The culture flask (250 mL) containing 20 mL of LB broth supplemented with different concentrations of Ni (0, 100, 200 and 300 mg L^{-1}) were inoculated with logarithmic-phase bacterial isolate. All the cultures including controls (in triplicate) were incubated at 27 °C for 24 h at 170 rpm. The bacterial growth was monitored at definite time intervals by measuring the optical density at 600 nm.

2.3.3. Biosorption of Ni

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 of optical density (600 nm). Cells were then harvested by centrifugation at 6000 rpm for 10 min and the bacterial pellet washed twice with sterile water. The harvested biomass was resuspended in Eppendorf tubes containing 100, 200 or 300 mg L^{-1} Ni dissolved in sterile Milli-Q water. The samples were incubated at 27 °C for 2, 4 and 6 h, and the cells were harvested again by centrifugation. The amount of residual Ni present in the supernatant was measured by atomic absorption spectrophotometer (AAS).

2.4. Plant growth promoting features of bacterial strain

P solubilizing activity was quantitatively assayed in modified Pikovskayas medium (Sundara-Rao and Sinha, 1963) containing tricalcium phosphate amended with 100, 200 or 300 mg L^{-1} Ni. The isolate was grown at 27 °C for 144 h at 170 rpm. The solubilized P in the culture supernatant was quantified as detailed by Fiske and Subbarow (1925). For IAA analysis, the bacterium was grown for 120 h in LB broth with L-tryptophan (200 mg L^{-1}) in the presence and absence of Ni. The quantitative analysis of IAA was performed as described previously (Bric et al., 1991).

2.5. Influence of Ni-resistant bacterial strain on plant growth and Ni uptake

For pot experiments, the soil was collected from the Botanical garden, Department of Life Sciences, 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 $NiCl_2 \cdot 6H_2O$ to achieve the final concentrations of 100, 200 or 300 mg kg^{-1} Ni and left in a greenhouse for a 2 weeks period (for metal stabilization). Surface-sterilized seeds of *B. juncea*, *L. cylindrica* and *S. halepense* were inoculated by soaking in a bacterial suspension containing 10^8 cell mL^{-1} for 1 h. 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 Ni polluted soils (100, 200 or 300 mg kg^{-1} Ni). A control pot was also maintained without adding Ni. The plants were grown in a glasshouse at 25 °C and a 16/8 day/night regime. After 28 days the plants were carefully removed from the pots and the root surface was cleaned several times with distilled water. Plants fresh weight and dry weight were determined. The accumulation of nickel in root and shoot system was quantified following the method of Freitas et al. (2004).

3. Results and discussion

3.1. Isolation and identification of Ni-resistant bacterial strain

Soil microbes play a major role in the biogeochemical cycling of toxic heavy metals. Bacterial strains isolated from serpentine soils have been frequently shown to tolerate/detoxify increased concentrations of heavy metals and to produce plant growth promoting substance under stress conditions (Abou-Shanab et al., 2006; Barzanti et al., 2007; Mengoni et al., 2010; Turgay et al., 2012). Thus, the inoculation of plants with such bacteria has recently been considered a promising approach for the improvement of heavy metal phytoremediation (Rajkumar et al., 2009; Ma et al., 2010, 2011b). In this study, we isolated Ni-resistant bacterial strain from serpentine soils and assessed its effects as a bioinoculant on Ni phytoremediation potential of various plants. During the initial screening process, we isolated 45 bacterial strains from the serpentine soil. Out of 45 isolates, the strain SR28C was specifically chosen based on its relatively higher growth efficiency and enhanced tolerance up to the concentration of 1200 mg L^{-1} Ni.

On the basis of morphological, physiological, biochemical characteristics (Table 1), comparative analysis of the sequence with already available database and phylogeny based on ClustalW (Fig. 1), the strain SR28C was identified as *Bacillus megaterium*. The strain *B. megaterium* SR28C was gram-positive endospore forming rod-shaped bacterium. The freshly grown culture of strain SR28C was positive for catalase, esculin, casein and gelatin hydrolysis and could produce acid from glucose, mannitol, sorbital and xylose. Strain SR28C gave negative results for starch hydrolysis, oxidase, indole and H_2S production, Voges–Proskauer, ornithine and lysine decarboxylase tests. The sequence (939 bp) was submitted in the NCBI databases under the accession number AM900770.

Table 1
Morphological, physiological and biochemical characteristics of *Bacillus megaterium* SR28C.

Characteristics	*Bacillus megaterium* SR28C
Gram staining	+
Cell shape	Rod
Colony morphology	Round, smooth and white
Spore	+
Motile	+
Growth at/on	
5 °C	−
40 °C	+
6% NaCl	+
Oxidase	−
Catalase	+
Indole production	−
H_2S production	−
Voges–Proskauer test	−
Utilization of	
Arabinose	+
Mannitol	+
Maltose	+
Glucose	+
Citrate	+
Butyrate	+
Lactate	+
Acid production from	
Glucose	+
Mannitol	+
Xylose	+
Sorbitol	+
Ornithine decarboxylase	−
Lysine decarboxylase	−
Nitrate reduction	+
Hydrolysis of	
Casein	+
Starch	−
Gelatin	+
Esculin	+

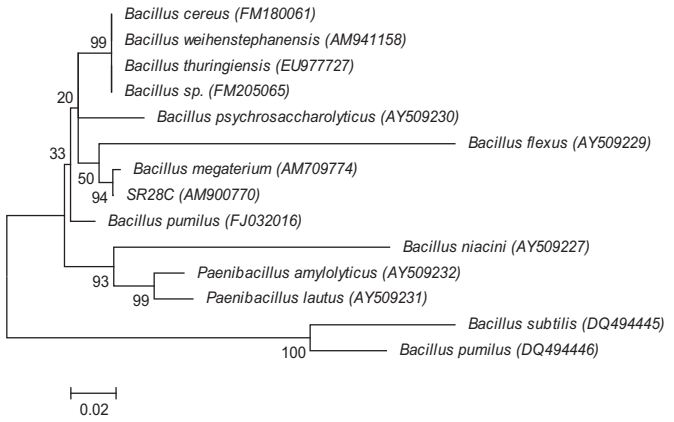

Fig. 1. Phylogenetic tree showing the relationship of partial 16S rDNA gene sequences from Ni-resistant PGPB strain SR28C with other related sequences from Ni-resistant bacteria in the NCBI database (accession numbers are given in parentheses). The tree was clustered with the neighbor-joining method using MEGA 5.05 package. Bootstrap values based on 1000 replications are listed as percentages at the nodes. The scale bar indicates 0.02 substitutions per nucleotide position.

3.2. Characterization of Ni-resistant Bacillus megaterium SR28C

3.2.1. Metal tolerance and antibiotic resistance

The strain *B. megaterium* SR28C was found to exhibit multiple heavy metal and antibiotic resistance characteristics (Table 2). The strain SR28C showed resistance against 1200 mg L^{-1} of Ni, 450 mg L^{-1} of Cu, 500 mg L^{-1} of Zn, 100 mg L^{-1} of Cd, 1200 mg L^{-1} of Pb and 300 mg L^{-1} of Cr. Among the heavy metals, Ni and Pb were less toxic, whereas Cd and Cr were highly toxic to strain SR28C with the order of resistance is Ni = Pb > Zn > Cu > Cr > Cd. In comparison to previous studies on the metal tolerance of *Bacillus* strains (Hassen et al., 1998; Yilmaz, 2003), this soil bacterium exhibited more tolerance. It has been suggested that under conditions of various stresses including compounds acting as antimicrobials and signal molecules, in addition to metal tolerance, the antibiotic resistance in microorganisms help them to adapt adverse environmental conditions (Hibbing et al., 2010; Piotrowska-Seget et al., 2012). Hence, *B. megaterium* SR28C was tested for the ability to grow in various antibiotic-supplemented media, and the strain showed resistance to ampicillin, tetracycline, streptomycin, chloramphenicol, penicillin and kanamycin (Table 2). The results indicate that the high degree of antibiotic resistance might be associated with heavy metal tolerance (Rosen, 1996; Hassen et al., 1998). Previously, Wright et al. (2006) also found that the bacteria isolated from metal polluted environment were able to resist various antibiotics. The present observation indicates that in order to tolerate and reduce the toxic effects of pollutants in serpentine soils, the isolate *B. megaterium* SR28C might have developed distinguishable properties of antibiotic resistance and tolerance to various heavy metals.

3.2.2. Effects of Ni on the growth of B. megaterium SR28C

Although the beneficial bacteria possess several traits to promote plant growth and mobilize/immobilize heavy metals, the metal tolerance is an important factor for PGPB-assisted phytoremediation because the survival of inoculated bacterium under metal stress is highly dependent on its metal tolerance level. Hence, the effect of different concentrations of Ni on the growth of SR28C was tested in liquid medium (Fig. 2). *B. megaterium* SR28C could tolerate high levels of Ni; however, there was a general decrease in the bacterial growth with increasing Ni concentration. This could be explained by the toxicity effects of heavy metals, which cause alterations in the metabolic and physiological traits of bacteria (Kamika and Momba, 2013). During the initial 12 h, the maximum growth was observed in the control followed by that exposed to a concentration of 100 mg L^{-1} Ni. Further, the higher concentrations of Ni (200 and 300 mg L^{-1} Ni) initially inhibited the growth rate of *B. megaterium* SR28C. However, after few hours SR28C recovered its ability to grow in a Ni-polluted medium. A similar finding was reported elsewhere (Kamika and Momba, 2013). The growth response of SR28C under Ni stress clearly indicates its potential to tolerate higher concentration of Ni.

Table 2
Heavy metal tolerance and antibiotic resistance of *Bacillus megaterium* SR28C.

Metal tolerance		Antibiotic resistance		
Metals	Maximal tolerable concentrations (mg L^{-1})	Antibiotics	Concentration (μg)	Diameter of inhibition zone (mm)
Nickel	1200	Ampicillin	10	NZ (R)
Copper	450	Tetracycline	30	12 (R)
Zinc	500	Streptomycin	20	7 (R)
Cadmium	100	Chloramphenicol	30	8 (R)
Lead	1200	Penicillin	20	7 (R)
Chromium	300	Kanamycin	30	12 (R)

Note: NZ – no zone; R – resistant.

3.2.3. Biosorption of Ni by B. megaterium SR28C

To analyze the biosorption of nickel, *B. megaterium* SR28C was exposed to different concentrations of nickel. The strain SR28C was capable of removing significant concentrations of Ni within 2 h of incubation. For instance, the biosorption of Ni increases from 9.38 to 26.62 mg g^{-1} of cell (dry wt.) at a concentration ranging from 100 to 300 mg L^{-1} after 2 h of incubation (Fig. 3). Further, the data revealed that biosorption increases as the initial metal concentration in the reaction mixture increases. However, no significant difference was observed in the biosorption of Ni on increasing the incubation time. Our study suggested that metal concentration had specific equilibrium, after which there was no significant effect on biosorption by increasing the time of incubation. These results were in accordance with those of Watanabe et al. (2003). They reported that by increasing the concentration of Ni from 5 to 20 mg L^{-1}, the biosorption also increases by *Rhodovulum* sp. and *Rhodobacter sphaeroides* and found that for each concentration, the

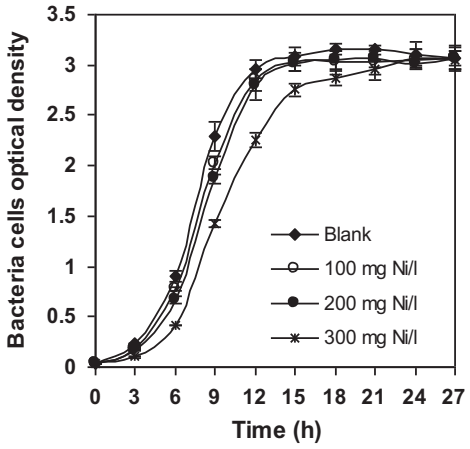

Fig. 2. Growth pattern of *B. megaterium* SR28C on LB medium supplemented with different concentrations of Ni. The optical density of bacteria cells was measured at 600 nm. Each value is the mean of triplicates. Error bars represent standard deviation.

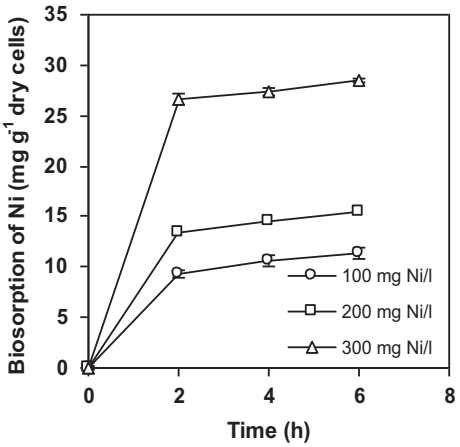

Fig. 3. Biosorption of Ni by *B. megaterium* SR28C cells. Each value is the mean of triplicates. Error bars represent standard deviation.

maximum biosorption occurs in 2 h and after which there is a slight increase in biosorption. Similarly, the plant associated bacteria, *Pseudomonas putida*, *Stenotrophomonas maltophilia* and *Acinetobacter* sp. (Andreazza et al., 2012), *Methylobacterium oryzae* strain CBMB20 and *Burkholderia* sp. (Madhaiyan et al., 2007) isolated from metal polluted environment have also been reported to absorb various heavy metals including Cu, Ni, and Cd. In yet another study Wei et al. (2011) recently found that the *P. putida* X4 producing extracellular polymeric substances was able to absorb large amounts of Cd and reported that carboxyl and phosphate groups of polymeric substances were responsible for Cd binding on bacterial cells.

3.3. Plant growth promoting features of B. megaterium SR28C

Although the beneficial bacteria possess several traits to produce various plant growth promoting metabolites, their ability to express such traits under stress is an important factor for PGPB-assisted phytoremediation because the activity of inoculated bacteria is necessary to produce beneficial substances in metal contaminated soils. Hence, we compared the levels of ACC deaminase activity, siderophores production, P solubilization and IAA production by the strain SR28C in the absence and presence of Ni. The strain SR28C showed negative for siderophores production and ACC deaminase activity in the absence or the presence of Ni (data not shown). The strain SR28C solubilized a substantial amount of P indicating that SR28C utilized tricalcium phosphate as the sole source of P (Fig. 4A). The maximum solubilization of P was achieved after 144 h of incubation. Further, the presence of Ni in NBRIP medium did not affect the ability of SR28C to solubilize the P. However, at a concentration of 300 mg L^{-1} Ni, a noticeable decrease in P solubilization by SR28C (11%) was observed. Wani et al. (2007a) have also recorded similar observations in *Bacillus* spp. under chromium stress. Another important trait of PGPB is the production of IAA, which may directly affect the growth of plants. It is known that the IAA released by the PGPB enhances root growth directly by stimulating elongation of the plant cell or affecting cell division (Malhotra and Srivastava, 2009; Davies, 2010). In the present investigation, SR28C also produced a substantial amount of IAA after 96 h of incubation both in the absence and presence of Ni (Fig. 4B). The production of IAA by SR28C indicated that the tested strain utilized L-tryptophan as a precursor for growth and IAA production. Further, IAA synthesis by SR28C was not affected by the application of Ni. At a concentration of 100 mg L^{-1} Ni, the percent decrease of IAA production was 2; for 200, 5%; and for 300, 11%.

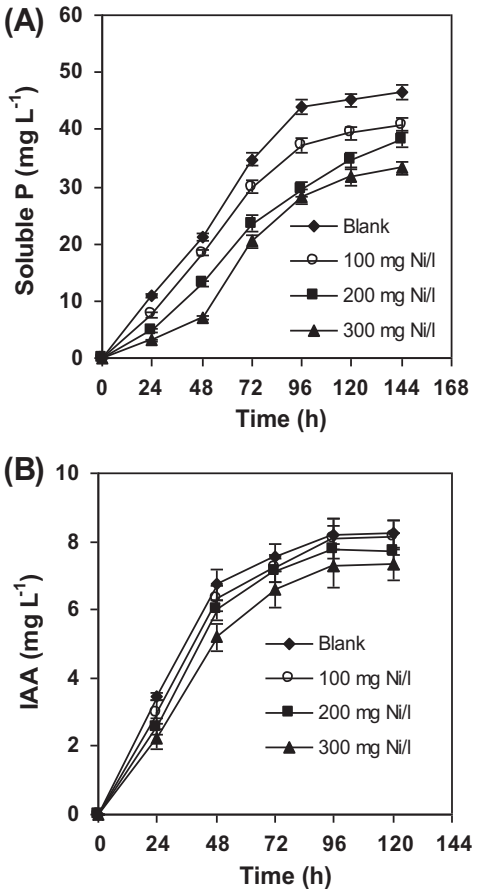

Fig. 4. P solubilization (A) and IAA production (B) by *B. megaterium*SR28C. Each value is the mean of triplicates. Error bars represent standard deviation.

Similar evidence of IAA production by *Bacillus weihenstephanensis* (Rajkumar et al., 2008), *Bacillus* sp. (Wani et al., 2007a) and *Bradyrhizobium* sp. (Wani et al., 2007b) under heavy metal stress is reported. In other study, *Pseudomonas fluorescens*, *Pseudomonas tolaasii* and *Mycobacterium* sp. has also been reported to produce IAA and promote the growth of *Brassica napus* under Cd stress (Dell'Amico et al., 2008).

3.4. Influence of B. megaterium SR28C on plant growth and Ni uptake

The solubilization of phosphate and production of IAA in the presence of high concentrations of Ni indicate that the strain SR28C has an exceptional ability to produce plant growth promoting substance in metal polluted soils and thereby promote the plant growth. Considering such potential, the plant growth promoting efficiency of SR28C was tested on various plant species under different concentrations of Ni in soil (Table 3). Generally, in each plant the strain SR28C treatment resulted in a significant increase in plant fresh and dry weight. Among the plants investigated, *L. cylindrica* inoculated with SR28C showed a maximum increase in both fresh weight (91%) and dry weight (124%) compared with non-inoculated control. The growth stimulation by PGPB was observed by several authors (Madhaiyan et al., 2007; Chatterjee et al., 2009; Malhotra and Srivastava, 2009; Li and Ramakrishna, 2011; Park et al., 2011; Turgay et al., 2012), which might be due to the production of plant growth promoting metabolites including the production of siderophores, IAA, ACC deaminase enzyme and solubilization of P. The non-inoculated plants exposed to different

Table 3
Influence of *B. megaterium* SR28C and Ni on fresh weight and dry weight of *B. juncea*, *L. cylindrica* and *S. halepense*.

Treatment	*B. juncea* (mg plant^{-1})		*L. cylindrica* (mg plant^{-1})		*S. halepense* (mg plant^{-1})	
	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight
Blank	623.0[a] (±14.5[b]) d	53.3 (±4.7) b	2041.0 (±246.9) d	162.2 (±33.5) d	2845.0 (±70.6) c	227.8 (±3.5) bc
Blank + SR28C	867.6 (±16.5) a	77.3 (±5.5) a	3907.0 (±323.2) a	364.2 (±64.1) a	4233.2 (±243.2) a	344.6 (±4.6) a
100 mg Ni kg^{-1} soil	601.6 (±12.5) d	49.6 (±3.0) bc	1322.3 (±138.0) f	122.8 (±13.0) e	2123.1 (±58.2) d	204.4 (±5.2) c
100 mg Ni kg^{-1} soil + SR28C	825.0 (±11.5) b	80.2 (±4.0) a	2784.2 (±138.0) c	203.4 (±39.9) c	3177.4 (±10.5) b	242.5(±11.5) b
200 mg Ni kg^{-1} soil	477.0 (±9.5) f	51.6 (±4.0) b	1693.9 (±50.1) e	104.9 (±5.7) e	1585.4 (±141.0) e	155.9 (±13.2) d
200 mg Ni kg^{-1} soil + SR28C	753.6 (±14.0) c	76.3 (±3.3) a	3183.9 (±237.8) b	238.1 (±9.8) b	2699.3 (±104.8) c	204.1 (±5.5) bc
300 mg Ni kg^{-1} soil	380.5 (±11.3) g	35.0 (±3.6) d	677.7 (±39.6) g	51.9 (±7.1) f	1257.0 (±39.9) f	121.5 (±8.4) e
300 mg Ni kg^{-1} soil + SR28C	524.0 (±12.4) e	55.3 (±3.3) b	1504.8 (±132.8) ef	112.4 (±8.7) e	2078.5 (±112.1) d	119.1 (±8.3) e

[a] Values represent average of three samples.
[b] Values in parentheses represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

concentrations of Ni showed a marked inhibition in the growth. In general, with the increase in the concentration of Ni progressive decrease in plant fresh and dry weight was observed as reported by others (Wani et al., 2007b; Zaidi et al., 2006). In contrast, plants inoculated with SR28C exhibited an increase in plant fresh and dry weight in the presence of different concentrations of Ni. Among the three plants, *L. cylindrica* inoculated with SR28C showed a maximum increase in fresh and dry weight compared with respective Ni treated control plants. For instance, the strain SR28C increased the fresh weight and dry weight of *L. cylindrica* by 122% and 117%, respectively, even at 300 mg Ni kg^{-1} soil, compared to non-inoculated but amended with the same dose of Ni. Increase in plant growth caused by *B. megaterium* SR28C may be attributed to the solubilization of P and production of IAA. In our study high P solubilization was observed in the tested strain (Fig. 4A), which is in good agreement with the higher fresh and dry weight of the tested plants inoculated with this strain. Recent studies also demonstrated that rhizosphere/seed inoculation with beneficial bacteria helps plants to alleviate heavy metal stress through enhancing the plant nutrient acquisition (Rajkumar et al., 2008; Ma et al., 2010; Li and Wong, 2012). For instance, experiments with Cd/Zn hyperaccumulating plant *Sedum alfredii* revealed that inoculation with *Burkholderia cepacia* reduced Cd and Zn toxicity in plants through increasing available P in soil and P uptake in plants (Li and Wong, 2012). Likewise, the increased plant growth and P uptake have also been reported on the inoculations of *Pseudomonas trivialis* BIHB 745, *P. trivialis* BIHB 747, *Pseudomonas* sp. BIHB 756 and *Pseudomonas poae* BIHB 808 in maize (Vyas and Gulati, 2009), *B. megaterium* var. *phosphaticum* in sugarcane (Sundara et al., 2002), *Pantoea* sp. J49 in peanut (Taurian et al., 2010) and *Pseudomonas* sp. in wheat (Babana and Antoun, 2006). The bacterial strains producing IAA may also improve the plant growth and nutrient acquisition under metal stress condition through stimulating cell elongation, cell division and root initiation. In agreement with our data, Ganesan (2008) found that the inoculation of P solubilizing and IAA producing *Pseudomonas aeruginosa* MKRh3 resulted in significant increase of biomass in black gram plants. Some other metal resistant bacteria such as, *Pseudomonas* sp., *Pantoea* sp. and *Enterobacter* sp., were also found to have plant growth promoting characters including P solubilization and IAA production that can improve plant growth and reduce metal stress symptoms in plants (Li and Ramakrishna, 2011; Park et al., 2011).

Since the plant associated microbes play pivotal roles in altering metal mobility/solubility in the rhizosphere and in enhancing the overall phytoremediation potential of plants, we assessed whether inoculation with *B. megaterium* SR28C influence the uptake of nickel by plants. The uptake of Ni by *B. juncea*, *L. cylindrica* and *S. halepense* increased with increase in the initial concentration of Ni in soil. Among the three plants studied, *S. halepense* showed a maximum accumulation of Ni in both root and shoot tissues (Fig. 5). However, when SR28C was inoculated with plants, a significant decrease in the uptake of Ni was observed in all the plants investigated, in comparison with respective non-inoculated plants. For instance, SR28C reduced the concentration of Ni in roots and shoots of *B. juncea* by 52% and 15%, respectively, when plants were grown in soil amended with 300 mg kg^{-1} Ni compared to non-inoculated plants. The decreased accumulation of Ni in the presence of *B. megaterium* SR28C might be due to bacterial biosorption of metals. Previously, experiments with tomato plants also revealed that the inoculation with *M. oryzae* CBMB20 reduced translocation of Ni and Cd from roots to shoots compared with the controls. This

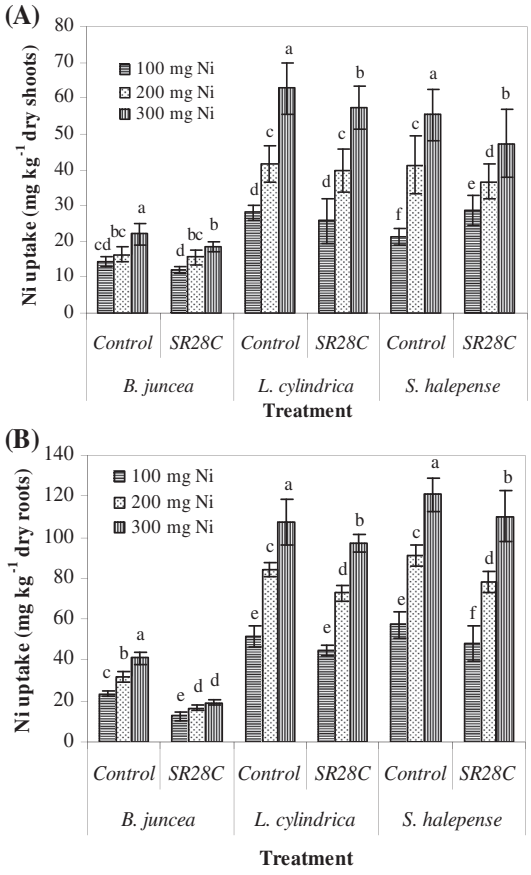

Fig. 5. Ni concentrations in shoots (A) and roots (B) of *B. juncea*, *L. cylindrica* and *S. halepense*. Each value is the mean of triplicates. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

effect was attributed to the increased metal biosorption by *M. oryzae* CBMB20 (Madhaiyan et al., 2007). These effects of inoculation were also reported by Sinha and Mukherjee (2008), who found that the inoculation pumpkin plants with *P. aeruginosa* KUCd1 decreased the concentration of Cd in roots by 59.2% and in shoots by 47.4% compared with respective non-inoculated control. Further, they reported that Cd biosorption or immobilization by bacteria accounted for decreased concentration of Cd in plants. The decreased metal accumulation in all plants tested in the presence *B. megaterium* SR28C indicates that inoculation of this strain seemed to be effective in improving Ni phytostabilization through reducing Ni accumulation in both root and shoot tissues. Though the observation indicates that *B. megaterium* SR28C reduced the metal uptake in plants with this metal binding feature, several authors have pointed out that microbial biosorption was not solely responsible for the reduced metal accumulation and/or translocation in plants (Vivas et al., 2003; Babu and Reddy, 2011). Recently, Park et al. (2011) investigated the immobilization of Pb in soils by inoculating two phosphate solubilizing bacteria (*Pantoea* sp. and *Enterobacter* sp.) and found that these isolates were able to immobilize Pb as a carbonated fluoropyromorphite-like mineral in soils through the release of P from insoluble P compounds. Since the other metal mobilizing or immobilizing metabolites (e.g., extracellular metabolites) complexing metal or forming insoluble precipitates (Glick, 2010; Ma et al., 2011a; Park et al., 2011; Wei et al., 2011; Rajkumar et al., 2012) produced by rhizosphere microbes could also alter metal accumulation in plants, further work including the analysis of the nature of metal immobilizing metabolites released by the *B. megaterium* SR28C and its role in Ni immobilization in soil will be carried out in order to elucidate the complete mechanisms.

4. Conclusions

We conclude that Ni-resistant PGPB, *B. megaterium* SR28C, exhibited resistance to high concentration of Ni and protected the plants against the inhibitory effects of Ni through producing IAA, solubilizing the phosphate and reducing Ni accumulation in plant tissues. Therefore successful inoculation of this bacterial strain SR28C may be potentially useful for Ni phytostabilization and for possible control of Ni entry into food chain. To the best of our knowledge, this is the first research report elucidating the potential of Ni-resistant serpentine isolate in Ni phytostabilization by various plant species with concurrent promotion of plant growth. Though our results open new perspectives for the phytostabilization technology for metal polluted soils and since in this study, *B. juncea*, *L. cylindrica* and *S. halepense* have been used as model plant species under greenhouse controlled conditions, further investigations are needed to verify the beneficial effects of this strain on Ni phytostabilization under long term field conditions.

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