

INOCULATION WITH METAL-MOBILIZING PLANT-GROWTH-PROMOTING RHIZOBACTERIUM *Bacillus* sp. SC2b AND ITS ROLE IN RHIZOREMEDIATION

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A plant growth-promoting bacterial (PGPB) strain SC2b was isolated from the rhizosphere of *Sedum plumbizincicola* grown in lead (Pb)/zinc (Zn) mine soils and characterized as *Bacillus* sp. based on (1) morphological and biochemical characteristics and (2) partial 16S ribosomal DNA sequencing analysis. Strain SC2b exhibited high levels of resistance to cadmium (Cd) (300 mg/L), Zn (730 mg/L), and Pb (1400 mg/L). This strain also showed various plant growth-promoting (PGP) features such as utilization of 1-aminocyclopropane-1-carboxylate, solubilization of phosphate, and production of indole-3-acetic acid and siderophore. The strain mobilized high concentration of heavy metals from soils and exhibited different biosorption capacity toward the tested metal ions. Strain SC2b was further assessed for PGP activity by phytagar assay with a model plant *Brassica napus*. Inoculation of SC2b increased the biomass and vigor index of *B. napus*. Considering such potential, a pot experiment was conducted to assess the effects of inoculating the metal-resistant PGPB SC2b on growth and uptake of Cd, Zn and Pb by *S. plumbizincicola* in metal-contaminated agricultural soils. Inoculation with SC2b elevated the shoot and root biomass and leaf chlorophyll content of *S. plumbizincicola*. Similarly, plants inoculated with SC2b demonstrated markedly higher Cd and Zn accumulation in the root and shoot system, indicating that SC2b enhanced Cd and Zn uptake by *S. plumbizincicola* through metal mobilization or plant-microbial mediated changes in chemical or biological soil properties. Data demonstrated that the PGPB *Bacillus* sp. SC2b might serve as a future biofertilizer and an effective metal mobilizing bioinoculant for rhizoremediation of metal polluted soils.

Rapid industrialization, overuse of agrochemicals, and minimal environmental protection over the past three decades resulted in significant environmental problems worldwide (Li et al., 2014). In particular, heavy metal pollution of soils due to intensified exploitation of

mineral resources and emission in smelting process has become a serious concern in many developing countries. Approximately 2×10^7 ha of arable land in China has been contaminated with heavy metals such as arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), and

zinc (Zn) to various degrees, due to local mining and refinery activities (Li, 2005). Accumulation of heavy metals in soils and sediments not only affects soil fertility, crop yield, and quality of agriculture products, but also negatively impacts human and animal health by entering the food chain (Coelho et al., 2012; Dahshan et al., 2013; Figueiredo et al., 2014a, 2014b).

Although traditional physicochemical technologies used for remediation of metal polluted soils have been well developed, these approaches have many problems, including high cost, and adverse effect on soil structure, fertility, and biological activity (Holden, 1989). In recent years, plant-based techniques such as phytoremediation, using metal-tolerant and/or hyperaccumulating plants, have been proposed as an environmentally friendly and low-cost technology for metal stabilization and extraction from polluted soils. However, the major issues hampering the efficiency of these processes are that high biomass-producing plants usually exhibit low metal tolerance and uptake potential, whereas natural hyperaccumulators generally produce low biomass and display slow growth rate (Glick, 2010).

Rhizoremediation is one of the phytoremediation methods and depends upon interactions between plants, microbes, and pollutants, where exudates released by plant roots help stimulate survival and activity of bacteria, which improve soil chemical and physical properties, and enhance nutrient acquisition, metal detoxification, and alleviation of biotic/abiotic stress in plants (Kuiper et al. 2004; Kamaludeen and Ramasamy, 2008). In general, the success of the rhizoremediation process in metal-polluted soils depends upon the type and bioavailability of pollutants, plants, diversity and activity of microbes, and environmental conditions in the rhizosphere (Wenzel, 2009). In this context, metal-mobilizing bacteria may be potential candidates for enhancing rhizoremediation, since these microbes produce various metal mobilizing metabolites, including organic acids, siderophores, or biosurfactants, and thus increase concentrations of bioavailable heavy metals in the rhizosphere, enabling them to be available for plant uptake

(Ma et al., 2011a). Similarly, the effectiveness of rhizoremediation may also be improved by use of plant growth-promoting bacteria (PGPB) as beneficial inoculants, which improve plant nutrient acquisition, reduce metal toxicity, and improve plant health and biomass production under adverse environmental conditions. PGPB exert their beneficial effects on host plants through various mechanisms, such as (1) utilization of 1-aminocyclopropane-1-carboxylate (ACC, the ethylene precursor) produced by plants under stress condition as sole energy source, (2) synthesis of phytohormones including auxins, gibberellins and cytokinins that enhance plant growth and development, and (3) production of different types of siderophores (catechol and hydroxamate) that solubilize and sequester available iron (Fe) from the soil, and mitigate nutrient deficiency by fixation of nitrogen and solubilization of phosphorus and potassium (Ma et al., 2011a). Ahemad and Kibret (2014) demonstrated that plants inoculated with metal-resistant PGPB are usually more tolerant to certain metals than non-inoculated plants. Considering the potential of beneficial bacteria to promote plant growth and heavy metal accumulation in plants, it may be envisaged that increasing the population density of metal-resistant beneficial bacteria in the rhizosphere might alleviate metal stress in plants and elevate bioavailable metal concentration for plant uptake, thus enhancing overall rhizoremediation process in polluted soils.

Sedum plumbizincicola (Crassulaceae) is a newly discovered Zn/Cd hyperaccumulator with high potential for rhizoremediation of metal polluted soils (Jiang et al., 2010; Wu et al., 2013). Although several recent investigations addressed rhizosphere and endophytic microbial population of plants and their role on rhizoremediation in metal-polluted soils (Ma et al., 2011b; Rajkumar et al., 2013), the beneficial interactions between *S. plumbizincicola* and PGPB and their possible role on the rhizoremediation of multi-metal-polluted agricultural soils still remain poorly understood. The objectives of this study were to (i) isolate and characterize a beneficial bacterial strain possessing the ability to mobilize metals in soils,

biosorb heavy metals in their cells, and produce various plant growth-promoting (PGP) metabolites, such as indole-3-acetic acid (IAA), ACC deaminase and siderophores, and solubilizing phosphate; and (ii) elucidate the effects of inoculation of isolated metal mobilizing PGPB strain on plant growth and rhizoremediation capacity of *S. plumbizincicola* in multi-metal-polluted agricultural soils.

MATERIALS AND METHODS

Isolation, Identification, and Phylogenetic Analysis

Bacterial strains were isolated from the rhizosphere of *Sedum plumbizincicola* X.H. Guo et S.B. Zhou ex L.H. Wu grown on a Pb/Zn mine area in Zhejiang province, China. To isolate the metal-resistant bacteria, soil samples were serially diluted in sterile deionized water and plated on Luria–Bertani (LB) agar supplemented with 100 mg/L heavy metals $\text{CdSO}_4 \cdot \text{H}_2\text{O}$, PbCl_2 , and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ alone and in combinations. The plates were incubated at 28°C for 48 h. To test the level of resistance to metals, isolated bacterial strains were grown in LB agar medium supplemented with different concentrations of Cd, Pb, and Zn. The bacterial strain resisting highest levels of metals was selected and identified based on morphological, physiological, and biochemical characteristics and the 16S rRNA gene sequencing method. The physiological characteristics such as temperature, pH, and salt tolerance of bacterial strain were examined using standard procedures (Mishra et al., 2009). The genomic DNA was isolated using the QuickExtract bacterial DNA extraction kit and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the conserved eubacterial primers FAM27f (5'-GAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGYTACCTTGTTACGACTT-3'). Each amplification mixture (5 µl) was analyzed by agarose gel (1%, w/v) electrophoresis in TAE buffer (0.04 M Tris acetate, 0.001 MEDTA) containing 1 µg/ml (w/v) ethidium bromide. Partial sequence of the

PCR-amplified 16S rDNA was performed using an ABI 3130XL automatic DNA sequencer (Applied Biosystems, Perkin Elmer) and then compared with similar sequences in the NCBI GenBank database using the BLASTn program. Sequences of the eight most closely related microorganisms and that of *Escherichia coli* were used for comparison to construct a phylogenetic tree. Bacteria used in the construction of the phylogenetic tree with their GenBank accession numbers included *Bacillus megaterium* MB1-42 (KJ843149.1), *B. horikoshii* RB10 (GU232770.2), *B. flexus* NY-1 (EU869200.1), *Bacillaceae bacterium* MSB06 (FJ189761.1), *B. aryabhatai* NN31 (KJ542774.1), *B. weihenstephanensis* MC67 (DQ345791.1), *B. cereus* PR15 (JQ435675.1), *B. thuringiensis* BAB-Bt2 (AM293345.1), and *Escherichia coli* (J01859). The phylogenetic tree was constructed using the neighbor joining method using MEGA version 6.06.

Biochemical Characterization

Resistance to antibiotics including ampicillin, chloramphenicol, kanamycin, streptomycin, and tetracycline of the bacterial strain was analyzed by the disc diffusion method (Rajkumar et al., 2008). The bacterial IAA production in vivo was determined by colorimetric measurement with the optical density (OD) at 530 nm using Salkowski's reagent as described by Patten and Glick (2002). In order to optimize the production of IAA, the bacterial strain was grown in LB broth with different concentration of L-tryptophan (0, 1, 2, 3, 4, or 5 mg/ml) or with L-tryptophan (0.5 mg/ml) at various pH values ranging from 4 to 10. The ACC deaminase activity was measured in bacterial extracts using a modified protocol based on Honma and Shimomura (1978). Cells were collected and washed with 0.1 mol/L Tris-HCl (pH 8.5) and resuspended in 1.5 ml lysate buffer. Cells were lysed on ice by sonication and centrifuged at $4500 \times g$ at 4°C for 15 min. The protein content of the extracts was determined by the method of Bradford (1976). Quantitative estimation of phosphate solubilization was performed by

inoculating 1 ml of bacterial suspension with an OD₆₀₀ of 1.5 in 50 ml modified Pikovskaya's medium (Sundara-Rao and Sinha, 1963) with 0.5% tricalcium phosphate for 5 d. Phosphate content in supernatant was spectrophotometrically estimated by the modified Fiske and Subbarow method (1925) and quantified by comparison with a standard curve derived from known phosphorus concentrations. Siderophore production by the isolate was qualitatively detected by the Chrome Azurol S assay (Schwyn and Neilands, 1987). The bacterial isolate was further quantitatively assayed for the production of catechol and hydroxamate siderophores by inoculating the isolate in casamino acids medium under Fe-limiting conditions based upon method of Arnow (1937) and Atkin et al. (1970), respectively. Bacterial hydrogen cyanide (HCN) production was assayed by the qualitative method of Bakker and Schippers (1987). The bacterial isolate was grown on LB agar supplemented with glycine (4.4 g/L) in petri plates with lids fitted with Whatman number 1 filter paper previously soaked in 0.5% picric acid and 2% sodium carbonate. The changes in the color of the filter paper from orange to red indicated HCN production.

Biosorption of Metals

The bacterial isolate was grown in LB medium with shaking at 27°C until the OD₆₀₀ reached 1.5. The cells were centrifuged for 10 min at 4500 × g and the obtained pellet was washed 3 times with deionized sterile water. The harvested biomass was resuspended in Eppendorf microtubes containing 1.5 ml metal solution at a concentration of 50, 100, or 150 mg/L (Cd, Pb, or Zn). After incubation at room temperature for 8 h, cells were harvested by centrifugation at 4500 × g. The residual metal ions in the supernatant were determined by atomic absorption spectrophotometry (AAS) (Varian SpectrAA 220FS, 220Z; Varian, Palo Alto, CA). Total biosorbed metal values were calculated by taking differences between metal contents in supernatant at time zero and at time of sampling.

Biomobilization of Metals

Soil samples were collected from a multi-metal-contaminated agricultural site in Fuyang city, Zhejiang province, China, and sterilized at 100°C by steaming for 1 h on 3 consecutive days. The physicochemical properties of the soil were: pH (1:1 w/v water) 8.1; organic matter 36.3 g/kg; cation exchange capacity 11.4 cmol/kg; total metal concentration 5.9 mg/kg Cd, 1236 mg/kg Zn, 153 mg/kg Pb; and total nutrient concentration 1.7 g/kg N, 1.1 g/kg P, 18.6 g/kg K. A pure culture of the bacterial strain grown in LB broth for 18 h was centrifuged, washed, and recentrifuged at 4500 × g. A 1-ml aliquot of the washed bacterial cells with OD adjusted to 1.5 was added to 1 g soil. Sterile deionized water was used as a control. Samples were weighed and kept at 27°C on an orbital shaker at 120 × g in the dark. Sterile deionized water was added to compensate for daily evaporation. After 10 d, 10 ml sterile deionized water was added to extract metals from the soil. The soil suspensions were centrifuged at 6500 × g for 10 min and filtered. Concentrations of Cd, Pb, and Zn in the filtrate were determined by AAS.

Phytagar Assay

A phytagar assay was used to assess PGP potential of isolated bacterial strain under metal stress condition. *Brassica napus* was used as a model plant because of rapid growth and high biomass production in short duration (Dell'Amico et al., 2008; Zhang et al., 2011). Surface-sterilized seeds of *B. napus* were inoculated with the PGPB and allowed to grow in phytagar medium containing 5 mg/L metal (Cd, Pb, and Zn) according to the modified assay detailed by Ma et al. (2011b). Germination rate, shoot and root length, plant dry weight, and the elongation rate of shoot and root and vigor index were calculated.

Pot Experiment

A pot experiment was performed using the already-described multi-metal-contaminated agricultural soil. Soil was air-dried and passed

through a 2-mm nylon sieve. Seedlings of *S. plumbizincicola* were obtained from a Pb/Zn mine area in Chunan city, Zhejiang province, China. Shoot samples (approximately 5 cm long) were cleaned with deionized water and grown in a half-strength Hoagland's nutrient solution for 1 wk. The roots of the precultured seedlings were disinfected by immersion in 70% (v/v) ethanol for 1 min and in 3% NaClO for 3 min and washed several times with sterile deionized water. For inoculation of seedlings, mutants of PGPB marked with antibiotic resistance were obtained after plating of parental strain onto LB agar containing chloramphenicol (150 mg/L). Cells grown in LB medium for 18 h at 27°C were collected by centrifugation at 4500 × g for 10 min and the pellet was washed twice with biological saline (0.85% KCl). The pellet was re-suspended in saline and the OD₆₀₀ was adjusted to 1.5. The surface-sterilized roots were soaked for 2 h in the bacterial culture or sterile water (controls), and the seedlings were transplanted into plastic pots containing 750 g metal-contaminated soil (6 plants/pot). The seedlings were allowed to grow in a greenhouse at 25°C and a 16:8-h light/dark cycle. Each treatment was performed in five replicates. After 75 d, plants were removed from pots and root surface cleaned several times with deionized water. Plant root and shoot length and fresh and dry weight were measured. The concentrations of chlorophyll (Chl) a, Chl b, and total Chl were determined in the leaves according to the method of Inskeep and Bloom (1985). The concentrations of Cd, Pb, and Zn in the root and shoot system were determined by AAS (Ma et al., 2011b). To examine the colonization of introduced bacterial strain, rhizosphere samples (1 g) of *S. plumbizincicola* were collected and suspended in 10 ml sterile deionized water. Serial dilutions were prepared and spread on LB agar plates containing 150 mg/L chloramphenicol. After incubation for 7 d at 28°C, chloramphenicol-resistant colonies were counted and expressed as colony-forming units (CFU) and compared for colony characteristics (morphology and color), metal resistance, and PGP traits against the parent strains.

Statistical Analysis

Data were analyzed using Student's *t*-test ($p < .05$). Correlations were determined by linear regression analysis by the least square method ($p < .05$). All the analyses were performed using SPSS 10.0.

RESULTS

Characterization of the Bacterial Strain

In total, 45 metal-resistant bacterial strains were initially isolated from the rhizosphere of *S. plumbizincicola* and strain SC2b was specifically selected due to its high resistance to Cd (300 mg/L), Pb (1400 mg/L), and Zn (750 mg/L). Strain SC2b was a gram-positive, endospore-forming, rod-shaped bacterium. The isolate was able to grow at a wide temperature range from 4 to 37°C; however, maximal growth occurred at 26°C. The strain also showed ability to grow over a wide range of pH (5–9) and tolerated an NaCl concentration of up to 5%. Although it was susceptible to tetracycline (30 µg/ml), kanamycin (30 µg/ml), and streptomycin (20 µg/ml), this strain exhibited resistance to high concentrations of ampicillin (500 µg/ml), penicillin (300 µg/ml), and chloramphenicol (150 µg/ml). Based on the morphological, physiobiochemical characteristics (data not shown) and 16S rRNA gene sequencing analysis, evidence indicated that strain SC2b was related to the *Bacillus* genus. The highest sequence similarity (100%) and phylogeny based on ClustalW alignments indicated that SC2b is a strain of *Bacillus* sp. (Figure 1). The sequence obtained (1426 bp) was submitted in the NCBI databases under the accession number JX512223.1.

Plant Growth-Promoting Features

Bacillus sp. SC2b showed positive for HCN production and was able to solubilize a significant amount of phosphate (56.6 mg/L) and produce IAA up to 64.8 mg/L after 5 d of incubation (Table 1). Data also demonstrated ACC deaminase activity at 25 µmαKB/mg/hr and displayed positive siderophore activity, as

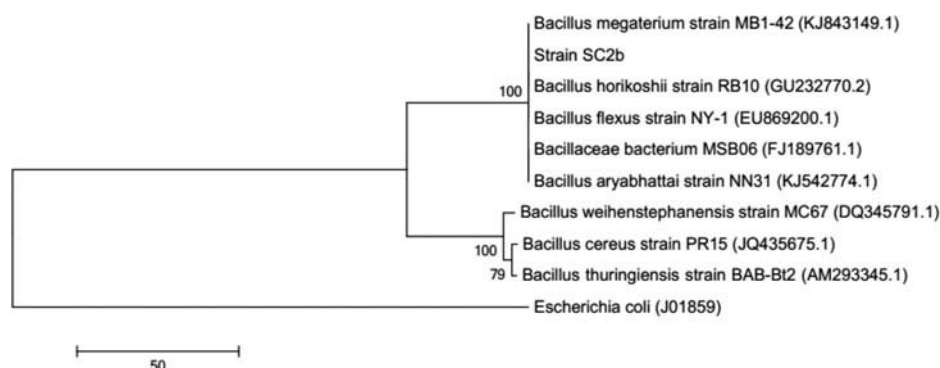


FIGURE 1. Phylogenetic tree showing the relationship of partial 16S rDNA gene sequences from metal mobilizing *Bacillus* sp. SC2b with other related sequences from *Bacillus*. *Escherichia coli* was used as the out-group. The value on each branch is the percentage of bootstrap replications supporting the branch.

TABLE 1. Biochemical Characteristics of *Bacillus* sp. SC2b

Characteristics	Parameter	Unit	<i>Bacillus</i> sp. SC2b
Metal resistance	Cd	mg/L	300
	Zn	mg/L	750
	Pb	mg/L	1400
Antibiotic resistance	Ampicillin	mm	4 (R)
	Tetracycline	mm	17 (S)
	Streptomycin	mm	18 (S)
	Chloramphenicol	mm	15 (I)
	Kanamycin	mm	17 (S)
Plant growth-promoting feature	ACC deaminase production	$\mu\text{mol KB/mg/h}$	25 ± 3.6
	P solubilization	mg/L	56.6 ± 4.3
	IAA production	mg/L	64.8 ± 2.0
	HCN production		+
	Siderophore		
	CAS	cm	1.2 ± 0.2
	Catechol	mg/L	198.3 ± 18.0
	Hydroxamate	mg/L	13.2 ± 0.2

Note. R, resistant (<10 mm); I, intermediate (10–15 mm); S, susceptible (>15 mm); ACC, 1-aminocyclopropane-1-carboxylate; α -KB, α -ketobutyrate; P, phosphate; IAA, indole-3-acetic acid; HCN, hydrogen cyanide; CAS, chrome azurol S; +, positive.

indicated by development of orange-colored zone on CAS agar plates after 5 d of growth. Under Fe-limiting conditions, strain SC2b produced 198.3 and 13.2 mg/L catechol and hydroxamate siderophore, respectively. Further, the effects of L-tryptophan concentration and pH on bacterial growth and IAA production by strain SC2b were also determined. As shown in Figure 2A, SC2b produced the highest amount of IAA when cultured in LB broth amended with 2 mg/ml L-tryptophan, whereas at higher L-tryptophan concentrations (3, 4, and 5 mg/ml) an adverse effect on IAA production was observed. Strain SC2b was also able to grow in the medium over a wide range of initial pH ranging from 4 to 10; however, the highest

synthesis of IAA by SC2b was obtained when cultivated in acidic media at pH 6 (Figure 2B). Significant correlations were obtained between bacterial growth and IAA production at different L-tryptophan concentrations (Figure 2A) and pH (Figure 2B).

Metal Biosorption and Mobilization

As shown in Figure 3, metal-resistant *Bacillus* sp. SC2b was capable of absorbing significant amounts of Cd, Pb, and Zn. Maximal biosorption by *Bacillus* sp. SC2b was achieved after 8 h of incubation. Further incubation up to 10 h did not enhance the extent of biosorption (data not shown). The highest

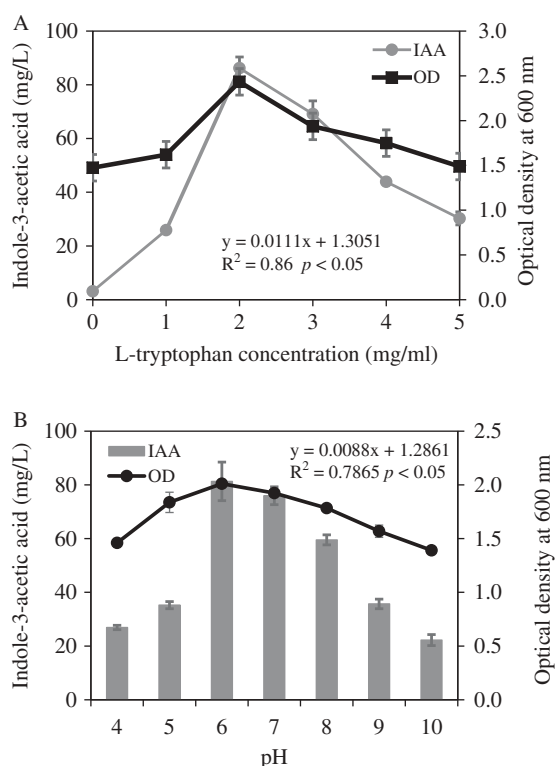


FIGURE 2. Impact of L-tryptophan concentration (A) and pH (B) on the growth of *Bacillus* sp. SC2b and its indole-3-acetic acid (IAA) production. Bars represent standard deviations of triplicates. Correlations between bacterial growth and IAA production at different L-tryptophan concentrations (A) and pH (B) were determined by linear regression analysis ($p < .05$).

amount of biosorption of heavy metals was observed with Zn, while the lowest was seen with Pb. At 50, 100, and 150 mg/L initial concentrations, the biosorption of Cd after 8 h of incubation was 3.8, 4.3, and 5.8 mg/g dry mass (Figure 3A); Pb was 1.4, 4.2, and 3.1 mg/g dry mass (Figure 3B); and Zn was 5.9, 7, and 10.8 mg/g dry mass, respectively (Figure 3C). A batch assay was conducted to analyze metal mobilization potential of strain SC2b in multi-metal-contaminated agricultural soil. Inoculation of strain SC2b for 10 d increased concentrations of water-extractable Cd, Pb, and Zn in soil by 13.9-, 4.5-, and 3.8-fold, respectively, compared with non-inoculated control soil (Figure 4).

Effects of Inoculation of SC2b on the Growth of *Brassica napus*

The PGP effects of *Bacillus* sp. SC2b were initially evaluated in the phytagar assay. It was

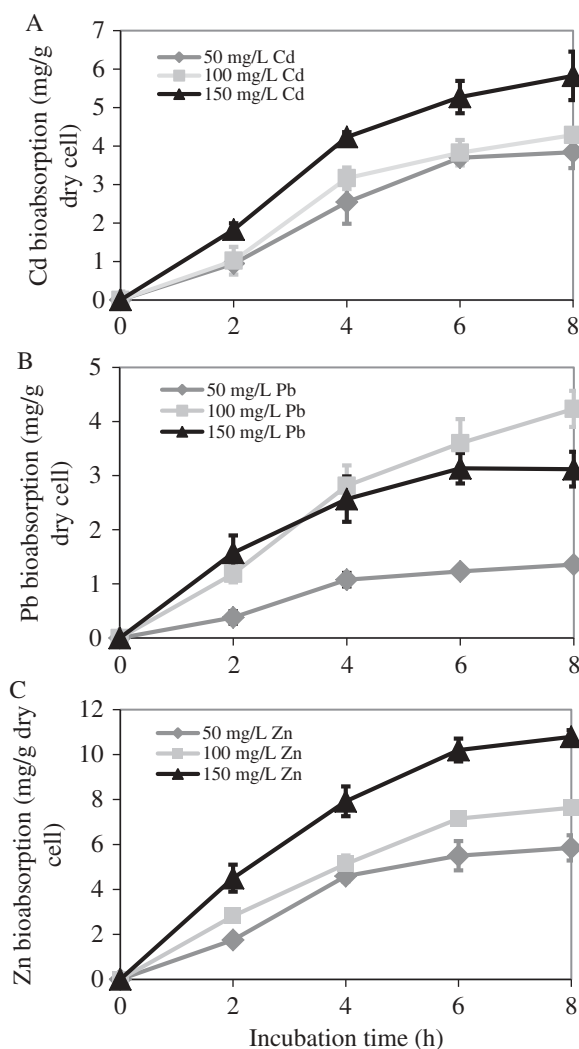


FIGURE 3. Biosorption of Cd (A), Pb (B) and Zn (C) on *Bacillus* sp. SC2b cells. Bars represent standard deviations of triplicates.

found that inoculated *B. napus* exhibited 17% higher seed germination rate, 26% higher plant dry weight, 51% higher vigor index, and 116 and 139% shoot and root elongation rates, respectively, compared with non-inoculated control treatments (Table 2).

Effects of Inoculation of SC2b on the Growth and Metal Uptake of *Sedum plumbizincicola*

The influence of inoculation of strain SC2b on *S. plumbizincicola* growth and metal (Cd, Pb, and Zn) uptake was determined in a pot experiment. Plants inoculated with strain SC2b exhibited a 46 and 42% increase in plant fresh

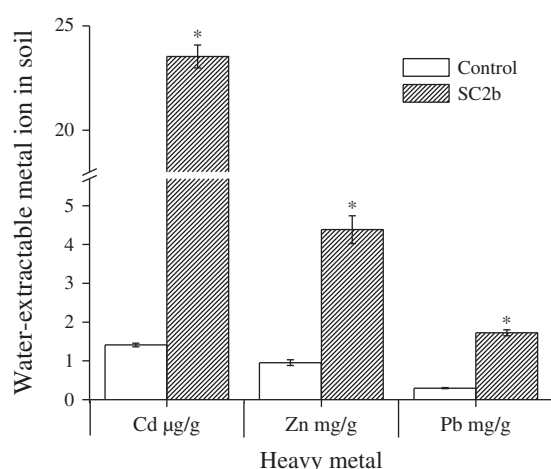


FIGURE 4. Effect of inoculation with *Bacillus* sp. SC2b on the mobilization of Cd, Pb and Zn in soil. Bars represent standard deviations of triplicates. An asterisk denotes a value significantly greater than the corresponding control value according to Student's *t*-test ($p < .05$).

and dry weight, respectively, compared with non-inoculated control (Table 2). In addition, SC2b inoculation elevated Chl a, Chl b, and total Chl content in leaves by 46, 80, and 58%, respectively. Inoculation of SC2b also increased concentrations of Cd and Zn in *S. plumbizincicola* by 15 and 13%, respectively, over the

non-inoculated controls (Figure 5A and 5C). In contrast, inoculation of SC2b decreased concentrations of Pb in the plant tissues (Figure 5B). The strain SC2b showed high level of colonization on rhizosphere of *S. plumbizincicola* (6.8×10^5 CFU/g) grown in multi-metal-contaminated agricultural soil (Table 2).

DISCUSSION

The natural ability of plants in removal of heavy metals from polluted soils may be integrated and improved by metal-resistant PGPB, which are naturally present in metal-polluted rhizosphere soils where they play important roles in plant growth and stress tolerance (Ma et al., 2011a; Ahemad and Kibret, 2014). In addition to improving plant growth, PGPB are themselves involved in metal immobilization, mobilization, or transformation through various mechanisms including physical sequestration, exclusion, complexation, and detoxification (Rajkumar et al., 2010). PGPB inoculation enhances plant growth, as well as tolerance toward various heavy metals including Cd (Dell'Amico et al., 2008), nickel (Ni) (Ma et al.,

TABLE 2. Plant Growth-Promoting Effects of *Bacillus* sp. SC2b in the Microcosm Assays

Phytagar assay	Non-inoculated control	<i>Bacillus</i> sp. SC2b
Percent germination	72 ± 4 b	89 ± 2 a
Shoot length (cm)	4.5 ± 0.3 b	5.2 ± 0.2 a
Root length (cm)	1.8 ± 0.3 b	2.5 ± 0.2 a
Shoot elongation rate (%) ^a	—	115.6 ± 35
Root elongation rate (%) ^b	—	138.9 ± 24
Vigor index ^c	454 ± 36 b	685 ± 42 a
Plant dry weight (g)	2.3 ± 0.1 b	2.9 ± 0.1 a
Pot experiment	Non-inoculated control	<i>Bacillus</i> sp. SC2b
Shoot fresh weight (g)	47.9 ± 6.4 b	68.9 ± 7.2 a
Root fresh weight (g)	0.7 ± 0.0 b	2.2 ± 0.6 a
Shoot dry biomass (g)	4.4 ± 0.7 b	6.2 ± 0.5 a
Root dry biomass (mg)	154.8 ± 16.2 b	278.4 ± 18.9 a
Chlorophyll a (mg/g fw)	1.3 ± 0.2 b	1.9 ± 0.3 a
Chlorophyll b (mg/g fw)	0.5 ± 0.0 b	0.9 ± 0.1 a
Total chlorophyll (mg/g fw)	1.9 ± 0.3 b	3.0 ± 0.4 a
Bacterial colonization (10 ⁵ CFU/g)	nd	6.8 ± 0.1

Note. Values are means ± standard deviations of three samples. Data of rows indexed by the same letter are not significantly different between treatments according to Student's *t*-test ($p < 0.05$). fw, fresh weight; CFU, colony-forming units; nd, not detected.

^aShoot elongation ratio (%) = mean shoot length of tested plant/mean shoot length of control × 100%.

^bRoot elongation ratio (%) = mean root length of tested plant/mean root length of control × 100%.

^cVigor index = germination (%) × seedling length (shoot length + root length).

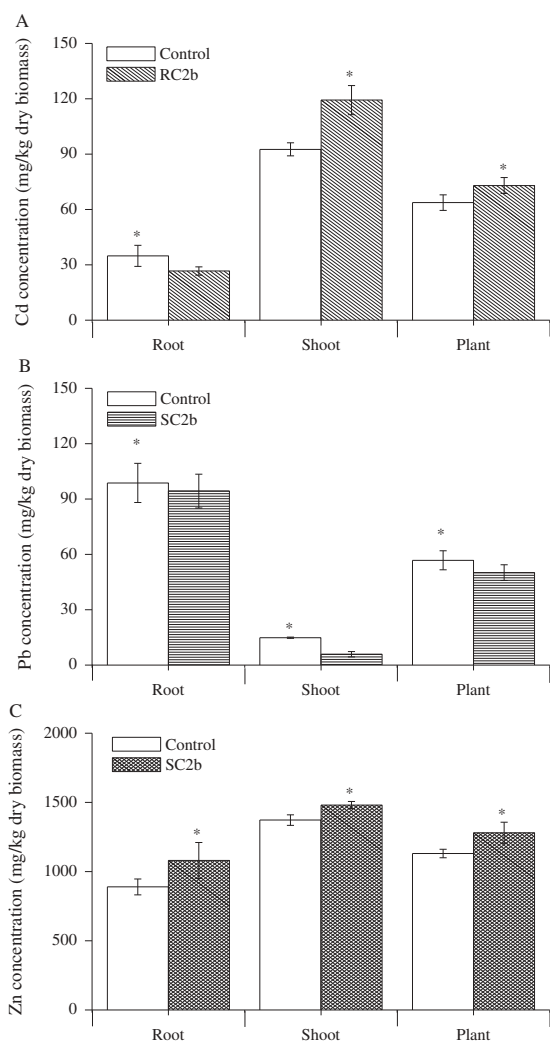


FIGURE 5. Effect of *Bacillus* sp. SC2b on the uptake of Cd (A), Pb (B), and Zn (C) by *Sedum plumbizincicola*. Bars represent standard deviations of triplicates. An asterisk denotes a value significantly greater than the corresponding control value according to Student's *t*-test ($p < .05$).

2011b), and copper (Cu) (Zhang et al., 2011). In some cases, increased growth and tolerance against heavy metal stress observed in PGPB inoculated plants have been explained by plant beneficial traits of PGPB and/or reduced metal uptake by plants (Rajkumar et al., 2013). However, different PGPB–hyperaccumulator associations might provide different responses, and therefore, further research is required to understand whether PGPB-inoculated plants and specific strains favor rhizoremediation of pollutants and through which mechanisms.

Although heavy metals exert inhibitory effects on microorganisms by displacing essential element ions or hindering functional groups, bacterial strains isolated from different habitats may exhibit different degrees of metal resistance, and those from metal-polluted soils are usually more resistant (Ma et al., 2011a). Thus, bacterial strains isolated from metal-polluted natural soils may be exploited for heavy metal bioremediation. Several bacterial strains isolated from metal-polluted soils tolerant to Cd, Pb, and Zn were previously reported (Becerra-Castro et al., 2012). Our results showed that *Bacillus* sp. SC2b was tolerant to high concentrations of Cd, Pb, and Zn. This bacterial strain was isolated from a multi-metal-polluted agricultural soil, where it may have evolved a strong resistance to heavy metals.

Various studies confirmed that metal-resistant bacteria possessing plant beneficial traits increase plant growth, nutrient uptake, metal tolerance, and/or rhizoremediation process in polluted soils (Rajkumar et al., 2008; Ma et al., 2011b; Zhang et al., 2011). In this study, strain SC2b possessed multiple PGP traits such as production of IAA, production of siderophore, utilization of ACC, and solubilization of phosphate. Among the PGP traits, bacterial synthesis of IAA was found to help plants in production of longer roots for nutrient uptake under metal stress conditions (Golubev et al., 2011). Thus, the intent was to optimize production of IAA by supplementing *Bacillus* sp. SC2b with different levels of L-tryptophan and growing the bacterial strain under various pH conditions. As a precursor of IAA, the addition of L-tryptophan to bacterial cultures generally enhanced IAA biosynthesis (Costacurta and Venderleyden, 1995). In our study, IAA was produced in negligible quantities in L-tryptophan-free medium. Addition of 2 mg/ml L-tryptophan of culture media resulted in an increase in production of IAA by this isolate, whereas higher concentrations of L-tryptophan (3, 4, and 5 mg/ml) showed negative effects on IAA production (Figure 2A). The results indicate that bacterial IAA production was modulated by adding L-tryptophan, and excessive L-tryptophan may result in the synthesis of

IAA-degrading/metabolizing enzymes such as IAA oxidase and peroxidase, and polyphenol oxidase (Datta and Basu, 2000). The regression analysis of bacterial growth and IAA production in medium indicated that conversion of L-tryptophan into IAA is closely dependent on growth and activity of the bacterial strain. The pH of the medium influenced the growth of *Bacillus* sp. SC2b and consequently the production of IAA, as reported by Acuña et al. (2011) in studies with other *Bacillus* spp.

The PGP effects of strain SC2b were further evaluated on *B. napus* using the phytagar assay. Inoculation of strain SC2b exhibited a significant increase in germination rate, length and elongation rate of shoot and root, vigor index, and dry weight of plants. Previous studies demonstrated the potential of rhizosphere bacteria, which possessed various PGP traits to promote the root elongation and growth of *Brassica juncea* and *B. napus* (Sheng and Xia, 2006; Ma et al., 2009). The enhanced growth response of plants induced by SC2b inoculation showed the ability of organisms to survive on the root and exhibit beneficial effects on the host plant growth. Considering such potential, a pot experiment was performed with an objective to assess usefulness of SC2b and *S. plumbizincicola* on rhizoremediation of metal-polluted agricultural soils. Strain SC2b significantly elevated fresh and dry weight of *S. plumbizincicola* in metal-polluted soils, indicating that plants adapt to metal stress in soils more effectively with the help of rhizosphere bacteria.

The survival and colonization of bacteria in the rhizosphere are important aspects to evaluate the role of the inoculated PGPB in microbe-assisted phytoremediation of contaminated sites (Compant et al., 2010). Although the success of the microbe-assisted rhizoremediation process depends upon heavy metal uptake by plants, survival and activity of metal-resistant PGPB markedly influence the level of metal uptake by plants through metal mobilization or immobilization process (Ma et al., 2011a, 2015). The results of the present study also showed a high level of colonization by strain SC2b, indicating the ability of this strain to survive and develop in the metal-polluted

rhizosphere of *S. plumbizincicola*. Further, the ability of SC2b in alleviating metal stress in *S. plumbizincicola* was also demonstrated by a significant rise in Chl a, Chl b, and total Chl content in inoculated plants. The enhanced germination rate, biomass, and related physiological parameters of plants produced by strain SC2b may be attributed to its PGP features, such as solubilization of phosphate and production of IAA, ACC deaminase, and siderophores (Ma et al., 2011a; Ahemad and Kibret, 2014). Our results corroborate those of Dell'Amico et al. (2005), suggesting that the siderophore-producing bacteria facilitate Fe uptake in plants through formation of mobile Fe–siderophore complexes, thus favoring both chloroplast development and Chl biosynthesis (Rajkumar et al., 2010). In addition, strain SC2b exhibited a high degree of metal biosorption potential (Figure 3). The capacity of bacterial adsorption of metals is generally dependent upon ionic radius of each metal (Karakagh et al., 2012). Data demonstrated that Zn (0.88 Å) having a smaller ionic radius may be more rapidly complexed by bacteria compared to Cd (0.97 Å) and Pb (1.2 Å). Ma et al. (2011a) reported that PGPB inoculation decreased metal toxicity through biosorption and bioaccumulation mechanisms, thus exerting a protective effect on host plants against heavy metal toxicity and leading to higher plant growth and yields. Nevertheless, the mechanism underlying bacterial biosorption in protecting effect on plant against metal stress is poorly understood.

Accumulation of Cd, Pb, and Zn in roots and shoots of *S. plumbizincicola* with or without PGPB inoculation was determined in the pot experiment. In general, the shoot system accumulated significantly more Cd and Zn than the root system, irrespective of inoculation treatment (Figure 5). This might be attributed to the effective translocation of heavy metals from the root to the shoot system (Jiang et al., 2010). As shown in Figure 5 (A and C), inoculation of strain SC2b significantly increased concentrations of Cd and Zn in plant system by 15 and 13%, respectively. These findings are in agreement with Rajkumar et al.

(2008), who found that PGPB *Bacillus weihenstephanensis* SM3 inoculated onto *Helianthus annuus* enhanced Zn accumulation in root and shoot tissues by 22 and 35% respectively, compared with non-inoculated plants. The elevated concentrations of Cd and Zn in SC2b inoculated *S. plumbizincicola* corresponds to the effect of the bacterial strain on metal mobilization in soil (Figure 4). Sessitsch et al. (2013) found that the presence of metal-resistant bacteria induced acidification of rhizosphere soils of plants by producing organic acids or siderophores, which enhance metal bioavailability around the root zone and thus facilitate plant metal uptake. In our study, inoculation with the metal-resistant bacterial strain SC2b significantly increased soil water-extractable metals Cd, Pb, and Zn concentrations (Figure 4), in accordance with the previous finding of Rajkumar et al. (2008). This was probably attributed to acidification and chelation reactions in soil, which were initiated by solubilization of phosphate and production of catechol and hydroxamate siderophores. Further studies are needed to assess fate of metals in soil solution, as they leach from polluted agricultural soils and contribute to groundwater contamination.

Bacterial inoculation decreased Pb accumulation in root and shoot systems by 26 and 46%, respectively. Similar observations were also reported by Rajkumar et al. (2013), who found that inoculation of metal-resistant *B. megaterium* on surface-sterilized seeds of *B. juncea*, *Luffa cylindrica*, and *Sorghum halepense* significantly reduced concentration of Ni in roots and shoots compared with non-inoculated plants. This situation was probably due to direct dilution of Ni concentration by increased plant biomass.

Data demonstrated that inoculation of *Bacillus* sp. SC2b not only promoted biomass of plants, but also enhanced uptake of Cd and Zn in plant tissues, especially in shoots. These beneficial effects produced by inoculation with *Bacillus* sp. SC2b, together with metal biosorption and biomobilization potential, indicate that metal-resistant PGPB possess

potential to improve rhizoremediation efficiency of metal-contaminated soils.

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