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Role of Plant Growth Promoting Rhizobacteria in Accumulation of Heavy Metal in Metal Contaminated Soil

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Abstract

Heavy metal pollution by industrial, agricultural and municipal wastewater is a frequent phenomenon. Bioremediation is a biological process for cleaning up of pollutants from the environment. In the present study, *B. megaterium* and *P. aeruginosa* isolated from a weed *Suaeda nudiflora* at chemical polluted site from Ankleshwar, Gujarat was used for bioremediation. These isolates were characterized for their ability of metal assimilation like phosphorus and iron, and on the basis of their best assimilation ability, the isolates were selected for heavy metal tolerance like zinc and lead. *B. megaterium* and *P. aeruginosa* with highest heavy metal tolerance up to 200 mgl^{-1} were inoculated in maize plants by test tube inoculation method and analyzed for the growth promotion efficacy in heavy metal polluted soil. The plant inoculated with isolates showed 20% higher plant height, 20% longer root length and 5% greater dry weight as compared to non-inoculated control. Further analyses of leaves were carried out for the accumulation of heavy metal in maize. Results of the study showed positive growth response of maize on heavy metal polluted soil in green house condition and also accumulate a significant amount of zinc (587 mg kg−1) and lead (227 mg kg−1) in plants. The study concluded that the metal mobilizing PGPR could be used as an effective inoculant for improving the phytoremediation in multi-metal polluted soil, as well as for the reclamation of heavy metal polluted soil.

Keywords bioremediation, heavy metal, maize, metal mobilizing rhizobacteria

Introduction

Nowadays, environmental pollution is a major concern worldwide due to intensive anthropogenic activities and extensive industrialization, which releases pollutant having various types of organic, inorganic as well as heavy metal contaminants. Among major primary inorganic contaminants, heavy metals are the one, which accumulates in the environment and contaminate the food chain [1] due to their non-biodegradable nature.

Different industrial and agricultural activities like usage of agrochemicals, waste disposal and deposition of urban sewage sludge add huge amounts of heavy metals to the soil and endanger human food safety [2]. Various chemical, physical and biological techniques have been developed for the pollutant eradication from the ecosystem. In comparison to physico-chemical remediation technologies, which has detrimental effects on soil characteristics, phytoremediation is a convenient and economic technique for removal of heavy metal form polluted soil [3].

The realization of this technique depends on the ability of plants to survive under heavy metal stress with high biomass production. The phytoremediation efficiency can be enhanced by increasing the heavy metal solubility and mobilization in the soil [4] and enhanced plant biomass by the help of plant growth promoting bacteria. This can be accomplished by emerging the relationship of hyper accumulator plants with plant growth promoting rhizobacteria (PGPR) like *Bacillus* and *Pseudomonas* having the ability for plant growth in adverse conditions. The plant growth promoting rhizobacteria (PGPR) justify exceptional consideration as it can directly increase the phytoremediation procedure by altering the metal bio-accessibility through the production of phytohormones, changing pH, discharge of chelators etc. [5], and hence, it deserves special attention among all other rhizospheric microorganisms. Major role of *B. megaterium* and *P. aeruginosa* secretion in phytoremediation of heavy metals has been reported by Bakiyaraj et al. [6]. Direct mechanisms include synthesis of siderophores, production of phytohormones, solubilization of phosphorus and biological nitrogen fixation; while secondary mechanisms comprise averting phytopathogens from impeding plant growth and development. The metal-chelating mediators called siderophores, have an important role in the acquirement of several heavy metals which are also produced by rhizobacteria. Due to their small size, it has resulted in a large contact area and high surface area-to-volume ratio, having the ability to act as microbial chelates that are associated with phytoremediation. Aim of this study is the isolation of rhizobacteria from metal contaminated soil and in-vitro inoculation of these isolates in maize plants to estimate their plant growth promoting potential. The inoculated plants were also analyzed for accumulation of heavy metals.

Methodology

Isolation of bacteria

Bacterial strains were isolated from rhizosphere soil and roots of plant, *Suaeda nudiflora* wild mosque from a chemical industrial site at Ankleshwar. For isolation of rhizosphere bacteria, cluster of soil present on the root surface was used by suspending it in sterile 1% saline solution. For the isolation of endorhizosphere bacteria, the surface sterilized roots were macerated in sterile 1% saline solution using a sterile mortar and pestle. The suspension obtained was serially diluted and inoculated in 5 ml of semi-solid nitrogen-free medium (NFb) with 0.05 g yeast extract 100 ml^{-1} in vials for 48 hrs. at 30 °C. One loop of pellicle-forming culture was transferred on fresh semisolid NFb medium and subsurface pellicle forming cultures were streaked on the solid NFb media supplement with NH_4Cl [7].

Identification of the bacteria

Identification of the bacteria was done by PCR amplification of 16s rDNA using primers 16S F: 5'AGAGTTTGATCCTGGCTCAG3' and 16S R: 5'AGGTTACCTTGTTACGACTT3' followed by sequencing (Bangalore, GeNei). The sequences obtained were compared with the sequence from the nucleotide database. The sequences were aligned with the CLUSTAL-W program and evolutionary distances were generated. Alignment gaps and ambiguous bases were not taken into consideration for comparison. Phylogenetic trees were constructed using the neighbor-joining method and the maximum likelihood method in PHYLIP package [8].

Characterization of bacteria as metal mobilizing plant growth promoting bacteria

Isolated bacteria were analyzed for its metal mobilizing ability by solubilizing rock phosphate and potassium. The isolates were analyzed for their ability to solubilize insoluble K by culturing serially diluted (up to 106) enriched samples on Aleksandrov agar medium constituting 1g glucose, 0.05M $MgSO_4.7H_2O$, 0.0005M $FeCl_3$, 0.01M $CaCO_3$, 0.2M $CaPO_4$ and 0.5M potassium aluminium silicate, agar 3% pH 6.5 [9] and incubated at 37 ºC for 1 week. Colonies exhibiting clear zone on the 104, 105, and 106 dilutions plates were selected as potential potassium solubilizer. Based on the zone activity of different isolates further screening was carried by using Khandeparkar's selection ratio.
Ratio = D/d =Diameter of zone of clearance /Diameter of growth

Similarly, isolates were further analyzed for their ability to solubilize insoluble P using modified Pikovaskaya's agar medium according to Park et al. [10] method.

Heavy metal resistance levels

Bacterial isolates having best rock P and K solubilizing ability were selected to analyze its heavy metal resistant ability, the selected bacterial strains were grown in LB agar media containing different concentrations of Zn or Pb ranging from 100 to 1200 mgl^{-1}. Cultures were incubated at 27 ºC for 7days. The highest concentration of metal supporting growth was defined as the maximum resistance level. Moreover, the growth pattern of isolated bacterial strain in metal contaminated liquid medium was also determined. Briefly 250ml culture flask containing 20 ml LB broth supplemented with heavy metals at the concentration of 200 mgl^{-1} (Zn or Pb) were inoculated with logarithmic-phase bacterial isolates. All the cultures including controls (in triplicate) were incubated at 27 ºC for 36 hrs at 200 rpm. Bacterial growth was estimated once in every four hrs. by measuring the OD at 600 nm.

Inoculation of maize with selected isolates

Seeds of the maize variety, Pioneer 30 V92 were surface sterilized with 0.2 % $HgCl_2$ solution for 5 min and 70 % ethanol for 10 min. The sterilized seeds were soaked in sterile distilled water on a rotary shaker. To check the contamination, soaked seeds were transferred on tryptone glucose yeast extract agar medium and incubated in dark at 30 °C. Seeds devoid of any contamination were used for inoculation experiments [11]. The germinated seedlings were transferred in 400 µl Hoagland's nutrient medium, 400 µl micronutrients and 1% agar in 40 ml distilled water in presence of bacterial inoculums of isolated bacteria at a concentration of 6 x 108 cfu ml^{-1} culture tubes. The inoculated plants were transferred in growth chamber and incubated at 27 °C in a 12 hrs light – dark cycle and transferred to the pots for salinity treatment [12]. Plants were maintained at 150mM salinity by adding NaCl solution (150mM NaCl kg-1 soil) to the pots in greenhouse at 20 to 25 °C with a relative humidity of 70 to 80%.

Measurement of plant length and dry weight

Plants from each treatment after 35 days of seed sowing, were collected carefully with plant root. Shoot and root length were measured and dried in an oven at 80°C for 72 hrs to determine dry weight.

Heavy metal accumulation in leaves of maize

Plant leaf samples were washed thoroughly with deionized water to remove surface dust and soil, dried at 80°C until completely dry, weighed, and ground to <0.5 mm size. Plant subsamples (0.5 g) of finely ground tissue were digested with concentrated HNO_3 (16 $moll^{-1}$) and $HClO_4$ (12 $moll^{-1}$) in 5:1 ratio (v/v). Metal concentrations in plants were determined by flame atomic absorption spectrometry (FAAS, PerkinElmer 3030). Two measurements of heavy metals were performed for each sample.

Statistical analysis

Data were analyzed by one way ANOVA (analysis of variance). All treatments were replicated 5 times, with 15 plants per experiment. Differences were considered to be significant at the $P<0.05$ level. Obtained means were compared by Fisher's protected LSD.

Results

In the present study, fifteen bacteria were isolated from the rhizosphere of *Suaeda nudiflora* plant. The rhizospheric soil sample was tested in a SICART (Sophisticated Instrumentation Centre for Applied Research and Testing laboratory) by water sample extraction method. The physio-chemical properties of the soil areas were as follows: pH 6.58, electrical conductivity 1480 μScm^{-1}, salinity 8.6%, nitrate 112.5mg kg^{-1}, chloride 128 mg kg^{-1}, sulphate 155 mg kg^{-1}, ammonia nitrogen 23.3 mg kg^{-1}, CEC:3 cmol, organic carbon: 5500 mg kg^{-1}, zinc 0.992 g kg^{-1} and lead 14.2 g kg^{-1}. Among all isolated bacteria, two isolates were selected on the basis of mineral mobilization for further experiments, in which one was Gram positive

endospore forming, approx. 2.6 X1.2µm size and non-capsulated bacteria, motile by perichate flagella. Another was Gram negative short rods shape, motile having polar flagella and non-sporulating. The bacterial isolates were molecular biologically identified by the isolation of total genomic DNA and amplified by 16S rDNA specific primers. PCR amplicons of 16S rDNA of about 1500 bp were obtained for both the isolates as discrete bands in agarose gel as shown in figure 1.

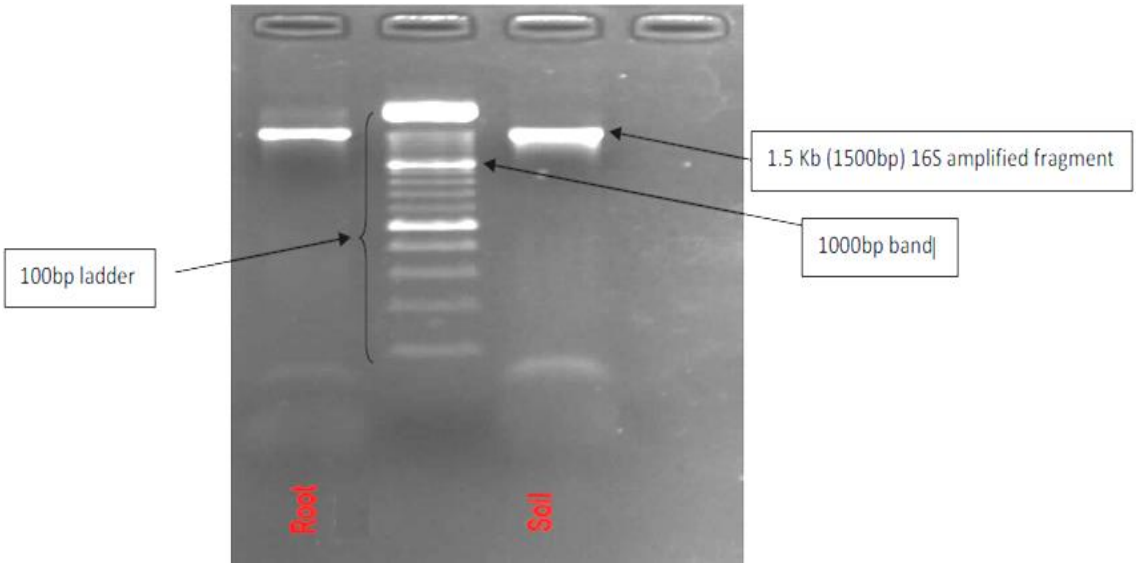

Figure 1. Agarose gel showing the amplified 16S rDNA of isolates. 1st lane is of 16S rDNA of *Pseudomonas aeruginosa* 2nd lane is 100bp marker, and 3rd lane is of 16S rDNA of *Bacillus megaterium* having molecular weight of about 1500 bp each

The phylogenetic trees were constructed using BLAST software by the comparison of the 16S rDNA sequence of isolates and related genera from a database using the neighbor-joining (NJ) algorithm and maximum likelihood (ML) method. Nucleotides homology and phylogenetic analysis of the root isolate was identified as *Pseudomonas aeruginosa* (GenBank Accession Number: JQ790515) and the soil isolate was identified as *Bacillus megaterium* (GeneBank Accession Number: JQ790514).

Both the isolates showed phosphate solubilizing activity on Pikovaskaya's agar medium. The phosphate solubilizing activity by *P. aeruginosa* was higher as compared to that of *B. megaterium* as observed by a clear zone around the inoculated strain, after 3 days. Both bacteria prefer neutral pH for their growth. Thus, to estimate the effect of pH on K solubilization, selected bacterial cultures were grown under several pH conditions as shown in Table 1.

The potassium released and titratable acidity of *B. pumilus* was increased by 8.3 times and 2.2 times respectively, while potassium released and titratable acidity of *P. pseudoalcaligenes* was increased by 11.2 times and 3.8 times, respectively after 1 week of inoculation in the medium.

The growth rate of isolates in the presence of heavy metals was also determined (Fig. 2a and 2b). The growth pattern of isolates during initial 24hrs showed maximum growth in control followed by Zn, although a slight decrease in the overall growth of *P. aeruginosa* and *B. megaterium* was evident in the presence of heavy metals during initial 12hrs. Among the heavy metals, Zn was less toxic than Pb to both the isolates.

In present study, maize plants inoculated with *B. megaterium* and *P. aeruginosa*, showed significantly higher plant height, root length and dry weight. Plant inoculated with *B. megaterium* showed 20% high plant height, 15% longer root length and 11% enhanced dry weight as compared to non-

Table 1. Titratable acidity, organic acid concentration and pH during solubilisation of potassium over incubation period of 1 week by the isolates (n=5)

	***P. pseudoalcaligenes* (Mean±S.D)**	
Week	**pH**	**Potassium solubilization**
0	7.0±0.01	345.6±0.10
1	4.8±0.05	213.4±0.21
	***B. pumilus* (Mean±S.D)**	
0	7.0±0.05	276.3±0.11
1	5.7±0.01	189.2±0.32

inoculated control (Table-2); while *P. aeruginosa* treated plants showed 30% more dry weight, 25% higher plant height, 46% longer root length as compared to non-inoculated control. Plants inoculated with

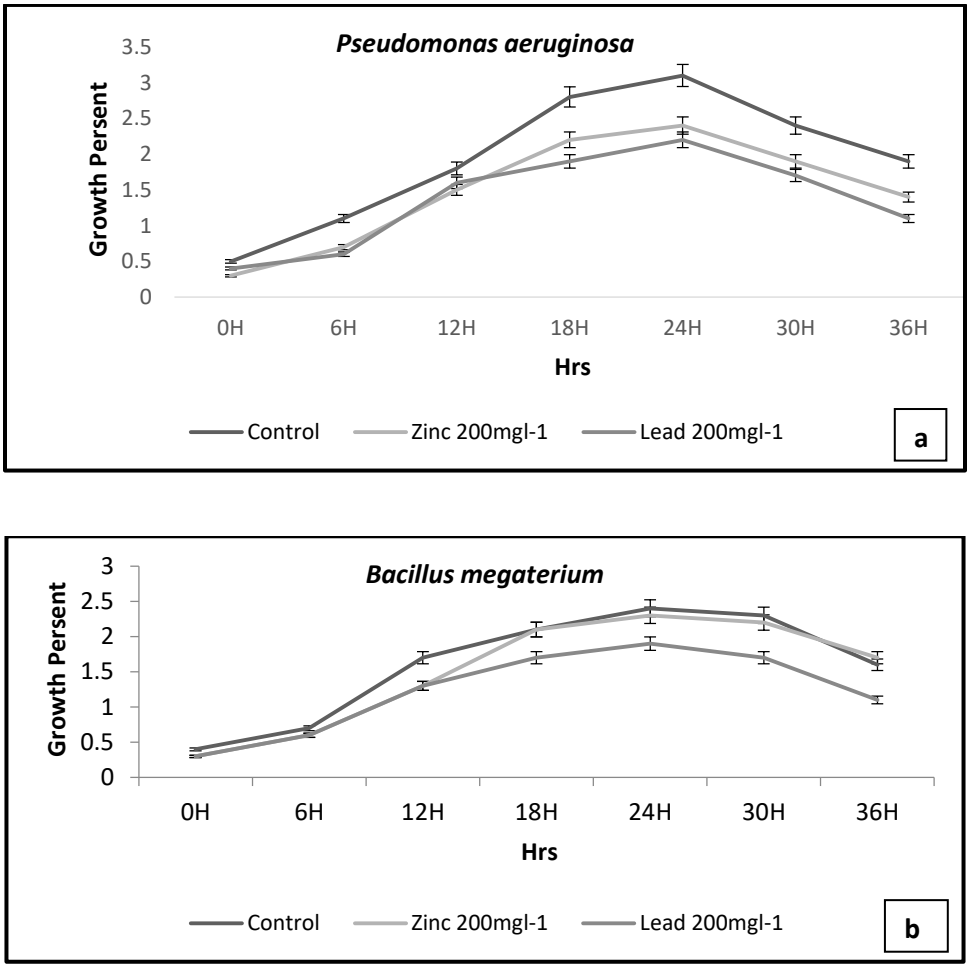

Figure 2. Growth of (a) *P. aeruginosa* and (b) *B. megaterium* on 200 mgl^{-1} of heavy metal Zn, Pb at different time interval on suitable medium (n=3)

both *B. megaterium* and *P. aeruginosa* showed 20% higher plant height, 20% longer root length and 5% greater dry weight as compared to non-inoculated control.

The accumulation of heavy metal in the leaves of inoculated plant was analyzed to known the in-vivo efficiency of isolates. The concentrations of Pb and Zn showed significant difference in inoculated and non-inoculated plants leaves (Table -3). Accumulation of Zn varied between 42 to 587 mg kg^{-1} in maize and inoculation with PGPR helped the plant to survive in presence of 10-12 times higher Zn concentration.

Table 2. Effect of PGPR strains on maize plant growth promoting activity (n=4)

Values are mean of three replications Means within columns sharing the same letters are not significantly different (p≤ 0.05; LSD test)

Treatment	Germination (%)	Shoot length (cm)	Root length (cm)	Plant height (cm)	Shoot weight (g)	Root weight (g)	Total Biomass Fresh wt.(g)	Dry wt.(g)
Control	66±0.11d	14±0.1d	15±2.1d	29±1.2d	0.9±1d	2±1.2d	1±0.1d	0.2±3d
Control+ *B. megaterium*	81±0.12abc	22±0.16bc	22±1.3abc	44±2.2bc	1.8±5bc	4±2.1bc	2±1.2c	0.4±6c
Control+ *P. aeruginosa*	83±0.21ab	24±1.1ab	22±3.4ab	49±1.6b	2.9±2b	4±3.4ab	3±2.1ab	0.5±4b
Control+ *B. megaterium*+ *P. aeruginosa*	86±0.31a	28±0.34a	24±1.2a	52±3.1a	2.9±3a	5±0.2a	3±0.2a	0.6±7a

Values are mean of three replications. Means within columns sharing the same letters are not significantly different (p≤ 0.05; LSD test)

Table 3. Concentrations of Zn, and Pb, in leaves of maize inoculated with the isolates from the contaminated field (mean ± SD, n=4)

Plant	Treatments	Zn	Pb
Maize	**Control**	**42±2.2cd**	**12±4.5cd**
	Control + *B. megaterium*	425±4.2c	144±2.1c
	Control + *P. aeruginosa*	542±32ab	213±11ab
	Control + *B. megaterium* + *P. aeruginosa*	587±42a	227±37a

Values are mean of three replications. Means within columns sharing the same letters are not significantly different (p≤ 0.05; LSD test)

Similarly, accumulation of heavy metal Pb also enhanced in plant shoot in the presence of PGPR, it varied from 12-227 mg kg-1. Inoculation with both the isolates showed highest accumulation of heavy metal in leaves of maize plants. In the present study, both the isolates helped the maize plant to accumulate considerable amount of Zn and Pb in their leaves.

Discussion

Heavy metals cannot be ended biologically, but are only converted from one oxidation state to another [13], so remediation of heavy metal contaminated soils is extremely difficult. Although there are several procedures for remediation such as excavation, landfill, thermal treatment, acid leaching and electro

reclamation, but these are not successfully practiced due to their high cost, low efficiency, large destruction of soil structure, soil fertility, soil properties, site conditions, and so on.

Potential for phytoremediation depends upon the interactions among soil, heavy metals, bacteria, and plants. The roots of plants interact with a large number of different microorganisms that are major determinants of the extent of phytoremediation. Different bacterial genera are vital components of soils, involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production. Largely, PGPR endorse plant growth directly either by enabling resource procurement or moderating plant hormone levels, or secondarily by diminishing the inhibitory effects of several pathogens on plant growth in the forms of biocontrol agents [14]. Bacterial genera such as *Bacillus*, *Pseudomonas* and *Brevibacillus* are well known to promote growth and yield in different non-leguminous plants [15].

Phosphorus and potassium are the major nutrients required by the plants for proper growth and development. Most of the phosphorus and potassium in soil is present in the insoluble form and cannot be utilized by plants [16]. It was found that P and K solubilization was maximum when bacterial strains were grown in a medium with acidic pH as both the isolates were able to solubilize these minerals with the production of organic acid. PGPR precipitate these minerals with secretion organic acid and enhance minerals availability in maize plants. It represents a possible mechanism of plant growth promotion by PGPR [17].

The microorganisms isolated from metal contaminated natural environment can be constitutively or adaptively resistant to increasing metal concentrations by physical sequestration, exclusion, complexion, and detoxification can be adapted by strain to resist high metal concentrations [18]. Detailed information on the behavior of microbial strains were analyzed in metal contaminated liquid medium. In the present study, the growth of isolates in the presence of heavy metals indicates its resistance or adaptability for these metals. Among the heavy metals, Zn was less toxic as more growth of both the isolates was observed in it than Pb. Similar results were also reported for other metal resistant rhizobacteria e.g. *Bacillus thuringiensis* OSM29, *Agrobacterium tumefaciens* LMG196 by Oves et al. [19]. Bacterial strains isolated from heavy metal polluted soils have adapted to multiple heavy metal stress by developing various mechanisms. It has straight impact on metal solubility of altering heavy metal speciation in the rhizosphere. The study showed that in the rhizosphere of maize plant roots, speciation of Zn and Pb changed significantly due to the presence of PGPR and resulted in increased host plant tolerance against excessive heavy metals in soil.

Plant growth in agricultural soil is influenced by several environmental factors. While using physical and chemical approaches to manage the soil environment to improve crop yield is frequent, application of microbial products for this purpose is less common. Beneficial microorganisms can be a significant component for management practices to achieve the yield. In the present study, maize plants inoculated with *B. megaterium* and *P. aeruginosa*, showed significantly higher plant height, root length and dry weight.

Effects of heavy metals on plants include growth inhibition, structure damage, decreased physiological and biochemical activities, as well as function of plants. The effect and bioavailability of heavy metals depend on many factors, such as environmental conditions, pH, and types of element, organic substances and plant species [20]. However, there are integrated mechanisms to protect the plants against heavy metals injury such as combining heavy metals with proteins and expression of detoxifying enzyme. In the present study, accumulation of Zn and Pb has been reported in the leaves of maize plant inoculated with isolates and both the isolates helped the maize plant to accumulate considerable amount of Zn and Pb in their leaves. There are two aspects on the interaction of plants and heavy metals. On one hand, heavy metals show negative effects on plants, and other hand, plants have their own resistance mechanisms against toxic effects of heavy metal and for detoxifying the heavy metal pollution [20]. PGPR provide additional support in the survival of plant in presence of heavy metals.

The effect of these isolated rhizobacteria in phytoremediation of contaminated soil, regardless of the precise effects used by the bacterium in plant growth and protection is a good strategy for green and clean environment [21]. However, phytoremediation is a slow process, so removal of heavy metal from the polluted soil is done either by increasing the amount of plant biomass or by increasing the metal-

accumulating ability of the plants. Plants inoculated with plant growth-promoting rhizobacteria have increased plant biomass and helps the plants to develop tolerance in heavily contaminated soil, where the metal content exceeds the limit of plant tolerance. Thereby, re-vegetating, remediating and stabilizing metal-polluted soils and the amount of heavy metal accumulated in the grains are subjects of future studies.

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