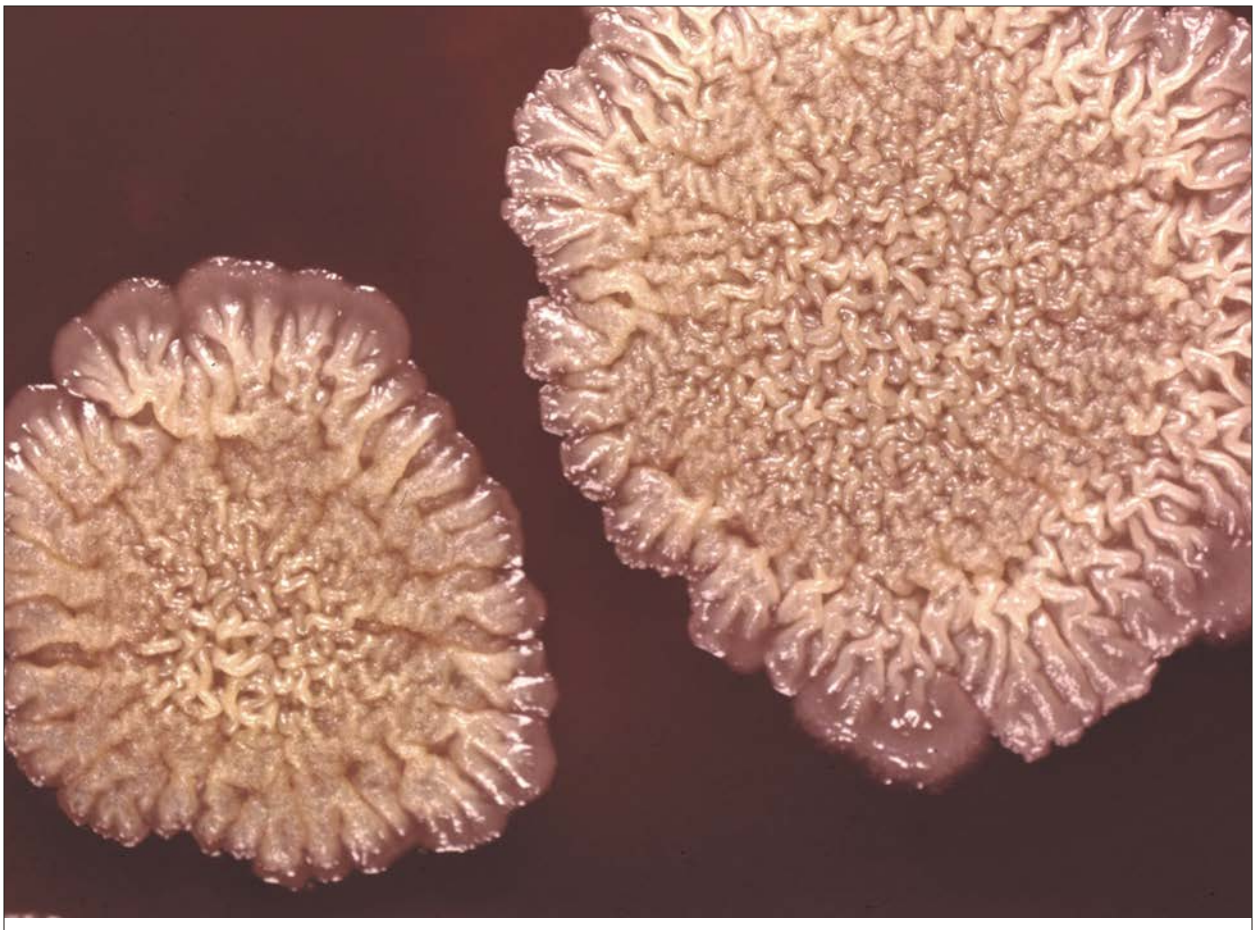

Bacteriology

Journal

ISSN 2153-0211

Academic
Journals Inc.

www.academicjournals.com

Bacteriology Journal 2 (4): 66-78, 2012
ISSN 2153-0211 / DOI: 10.3923/bj.2012.66.78

Bioremediation of Lead by a Plant Growth Promoting *Rhizobium* Species RL9

[1]Parvaze Ahmad Wani and [2]Mohammad Saghir Khan

[1]Department of Biological Sciences, College of Natural and Applied Sciences, Crescent University, Abeokuta, Ogun, Nigeria

[2]Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Corresponding Author: Parvaze Ahmad Wani, Department of Biological Sciences, College of Natural and Applied Sciences, Crescent University, Abeokuta, Ogun, Nigeria Tel: +2348127592298

ABSTRACT

Heavy metal contamination has accelerated due to the rapid industrialization world wide. Accumulation of metals in excess can modify the structure of essential protein or can replace an essential element. *Rhizobium* strain RL9 was isolated from the nodules of lentil grown in metal contaminated soils. The strain tolerated Pb up to a concentration 1600 μg mL^{-1}. The strain RL9 produced Plant Growth Promoting (PGP) substances, it produced a good amount of Indole Acetic Acid (IAA) which was found to produce a maximum amount of 33 μg mL^{-1} of IAA at 100 μg mL^{-1} of tryptophan and the strain RL9 was also positive for siderophore, HCN and ammonia. The PGP activity and Pb tolerance of the strain RL9 was further assessed with increasing concentrations of lead, using lentil as a test crop. The strain RL9 significantly increased growth and nodulation of lentil, compared to plants grown in the absence of bioinoculant but amended with Pb. It also increased chlorophyll, leghaemoglobin, N content, seed protein and seed yield compared to plants grown in absence of bioinoculant but having different concentrations of Pb. The strain RL9 decreased uptake of Pb in lentil compared to plants grown in the absence of bio-inoculant. The intrinsic abilities of nitrogen fixation, growth promotion and the ability to reduce the toxicity of lead by the tested strain could be of practical importance in augmenting the growth and yield of lentil, in lead-polluted soils.

Key words: *Rhizobium* RL9, PGP activities, lead tolerance, bioremediation

INTRODUCTION

Considerable amounts of toxic waste that pollute the biosphere is produced by industries which indiscriminately use heavy metals for agricultural and other purposes (Fernandes and Henriques, 1991). Mining, smelting, manufacturing, gas exhaust, energy and fuel production, fertilizer, sewage and pesticide application and municipal waste generation significantly increased the contamination of Heavy Metal (HM) in the environment (Ibekwe *et al*., 1995). It adversely affect about 12% of the world's agricultural land (Moffat, 1999). It is during the beginning of industrial revolution that the heavy metal contamination started increasing dramatically. The application of sewage sludge in agronomic practices is often the most economical means of disposal. It is beneficial because it increases the organic matter content and water holding capacity of soil (Pagliai *et al*., 1981) and can improve the physical, chemical and and biological characteristics of soil and also

provides plant with sufficient nutrients (Katterman and Day, 1989). However, sewage sludges from industrial sources, often contain variable amounts of potentially toxic heavy metals, such as, lead, cadmium, nickel, chromium, copper and zinc (McGrath, 1987). When these sludges are repeatedly applied to agronomic lands, heavy metals accumulate and persist in the top cultivated layer (0-20 cm) (McGrath, 1987). The persistence of these metals in soil adversely affect the agro-ecosystem (Mcllveen and Negusanti, 1994; Broos *et al*., 2004, 2005). The contamination of plants with excess heavy metals can be toxic because these toxic metals can change the structure of essential proteins as well can replace an essential element leading to chlorosis, growth impairment, browning of roots and inactivation of photosystems among others (Gorhe and Paszkowski, 2006). Furthermore, ingestion of contaminated food or drinking water can expose humans and animals to toxic levels of the HM. The accumulation of metals in plant organs to a undesired level show limiting effects on physiological processes such as photosynthesis (Bibi and Hussain, 2005) and also inactivate plant protein (Assche and Clijsters, 1990; Wani *et al*., 2008a), which subsequently severely reduce the crop yields (Moftah, 2000; Wani *et al*., 2008a).

The toxic metal contamination of soil environment therefore, requires an effective and affordable attention. Generally heavy metals can not be degraded biologically to more or less toxic products and thus can still remain in the environment. To circumvent the metal stress, microorganisms of agronomic importance including plant growth promoting rhizobacteria have evolved a number of mechanisms, which they use to tolerate the uptake of heavy metal ions (Nies, 1999). Such mechanisms include (1) the pumping of metal ions exterior to the cell, (2) accumulation and sequestration of the metal ions inside the cell and (3) biotransformation-transformation of toxic metal to less toxic forms (Thacker and Madamwar, 2005) and adsorption/desorption of metals (Mamaril *et al*., 1997). These mechanisms could be constitutive or inducive. Due to these properties, when seed inoculated plant growth promoting rhizobacteria were applied to soil, either treated or amended with metals or the soil which is used for the growth of inoculated plants is already contaminated, has been reported to show a significant reduction in the toxicity of metals and thus has shown an improved over all growth and yield of chickpea (Gupta *et al*., 2004), green gram (*Vigna radiata* L. Wilczek) (Faisal and Hasnain, 2006) and tomato (*Lycopersicon esculentum*), Indian mustard (*Brassica campestris*) and canola (*Brassica rapa*) (Burd *et al*., 2000). Khan *et al*. (2012), also observed that the inoculation of *Paenibacillus lentimorbus* enhance growth of chickpea (*Cicer arietinum*) in chromium amended soil. Saleh and Al-Garni (2006), under field trials observed that the dual inoculation of an arbuscular mycorrhizal fungi and *Rhizobium* increased the growth of cow pea plant under heavy metal stress. In green house experiment Wu *et al*. (2006) observed that the inoculation of *Azotobacter chroococcum* stimulated the growth of *Brassica juncea* under Pb and Zn stress. Further, the nodule bacteria can protect the plants against the toxic effects of lead through adsorption/desorption mechanism (Mamaril *et al*., 1997). In addition, the plant growth promoting rhizobacteria also synthesize plant growth promoting substances (siderophore, indole acetic acid, hydrogen cyanide and ammonia), which augments the crop productivity (Rajkumar *et al*., 2006; Wani *et al*., 2008b). Therefore, the use of PGPR for reduction/detoxification of heavy metals is one of the preferred choices and is considered as cost effective approach in bioremediation technologies. Due to lack of adequate data and conflicting reports on the effect of heavy metals on legumes and nodule bacteria, the present study was therefore, under taken to (1) determine the lead concentration of different soils of Aligarh region and (2) Effect of lead tolerant and plant growth promoting *Rhizobium* species on growth, pigment content, protein and lead uptake by lentil in the presence and absence of lead.

MATERIALS AND METHODS

Concentration of lead in soils: The soil samples for total lead concentrations were collected from the industrial area of Mathura road (S1) and exhibition ground, Aligarh (S2), Uttar Pradesh, India and these had consistent use of industrial sewage water. Soil samples collected from conventional (cultivated) fields of Faculty of Agricultural Sciences (S3), Aligarh Muslim University, Aligarh, were also used for quantitative estimation of lead. For the analysis of lead, oven-dried soil samples were sieved through muslin cloth and ash was prepared at 400-500°C in a muffled furnace overnight. One gram cooled ashed sample was treated with aqua regia [nitric acid and hydrochloric acid (3: 1)]. Digestion was carried out on a hot plate until dense fume evolved and a clear solution was obtained. The clear solution was filtered through Whatman filter paper No. 1 and the volume was made to 100 mL with double distilled water. In the digested sample, the lead was analyzed by the method of McGrath and Cunliffe (1985) using flame atomic absorption spectrophotometer (Model GBC 932B Plus Atomic Absorption Spectrophotometer). All chemicals used in nickel analysis were of analytical grade and solutions used were made in double distilled water.

Evaluation of bacterial strains for lead tolerance: *Rhizobium leguminosarum* strains were isolated from the nodules of lentil plants grown in metal polluted soils of Mathura road using yeast extract mannitol agar. These strains were maintained on the same medium until use. The rhizobial strains were tested for their resistance to lead by agar plate dilution method (Holt *et al*., 1994) using YEM agar medium. The freshly prepared agar plates amended with increasing concentration of lead (0-1600 μg mL^{-1}) were spot inoculated (100 μL) with 10^8 cells mL^{-1}. Plates were incubated at 28±2°C for 3-5 days. The lowest concentration of lead inhibiting the growth of bacteria on nutrient agar plate was defined as a minimum inhibitory concentration. Each experiment was replicated three times.

Plant growth promoting activities: Indole-3-acetic acid was quantitatively assayed by the method of Gordon and Weber (1951), later modified by Bric *et al.* (1991). For the activity of indole acetic acid bacteria were grown in Luria Bertani (LB) broth (g L^{-1}: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5). Luria Bertani broth (100 mL) supplemented with 0, 20, 40, 60, 80 and 100 μg mL^{-1} of tryptophan was inoculated with 10^8 cells mL^{-1} of 1 mL culture and was incubated for 24 h at 28±2°C with shaking at 125 rpm. After 24 h, five mL of each culture was centrifuged (10,000 rpm) for 15 min and an aliquot of 2 mL supernatant was mixed with 100 μL of orthophosphoric acid and 4 mL of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% per-chloric acid) and incubated at 28±2°C in darkness for 1 h. The absorbance of pink color developed was read at 530 nm. The IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard (Bric *et al*., 1991). The experiments were repeated three times on different time intervals.

The bacterial strains were further assayed for siderophore production on the Chrome Azurol S (CAS) agar medium by the method of Alexander and Zuberer (1991). Chrome Azurol S agar plates were spot inoculated with 100 μL of 10^8 cells mL^{-1} and incubated at 28±2°C for 72-96 h. Development of yellow orange halo around the growth was considered as positive for siderophore production. Each individual experiment was replicated three times. The siderophore produced by the test strains were further quantitatively assayed using Modi medium (K_2HPO_4: 0.05%; $MgSO_4$: 0.04%; NaCl: 0.01%; mannitol: 1%; glutamine: 0.1%; NH_4NO_3: 0.1%). Modi medium was inoculated with 10^8 cells mL^{-1} of bacterial cultures and incubated at 28±2°C for 5 days. Catechol type

phenolates were measured on ethyl acetate extracts of the culture supernatant using a modification of the ferric chloride-ferricyanide reagent of Hathway. Ethyl acetate extracts were prepared by extracting 20 mL of supernatant twice with an equal volume of solvent at pH 2. Hathway's reagent was prepared by adding 1 mL of 0.1 M ferric chloride in 0.1 N HCl to 100 mL of distilled water and to this was then added 1 mL 0.1 M potassium ferricyanide (Reeves *et al*., 1983). For the assay, one volume of the reagent was added to one volume of sample and absorbance was determined at 560 nm for salicylates with sodium salicylate as standard and at 700 nm for dihydroxy phenols with 2,3-dihydroxy benzoic acid as standard. Hydrogen cyanide production by bacterial isolates was detected by the method of Bakker and Schipper (1987). For HCN production, the bacterial strains were grown on an HCN induction medium (30 g tryptic soy broth, 4.4 g glycine, 15 g agar L^{-1}) at 28±2°C for four days. For each bacterial isolate, 100 µL of 10^8 cells mL^{-1} was placed in the centre of the petri plates. A disk of Whatman filter paper No. 1 dipped in 0.5% picric acid and 2% Na_2CO_3 was placed at the lid of the petri plates. Plates were sealed with parafilm. After four days incubation at 28±2°C, an orange brown colour of the paper indicating HCN production was observed. For ammonia production, the rhizobial strains were grown in peptone water (g L^{-1}: peptone 10 g, NaCl 5 g, pH 7) and incubated at 30±2°C for four days. One mL of Nessler reagent was added to each tube and the development of yellow color indicating ammonia production was recorded (Dye, 1962).

Plant growth and lead uptake: The experimental soil was sandy clay loam (organic carbon: 0.4%, Kjeldahl N: 0.75 g kg^{-1}, Olsen P: 16 mg kg^{-1}, pH: 7.2 and WHC: 0.44 mL g^{-1}, Pb: 8.1 µg g^{-1}). Seeds of lentil var. Malka were surface sterilized (70% ethanol, 3 min; 3% sodium hypochlorite, 3 min), rinsed six times with sterile water and shade dried. The sterilized seeds were coated with *Rhizobium* strain RL9, grown in YEM broth, by dipping the seeds in liquid culture medium for two hours using 10% gum Arabic as adhesive to deliver approximately 10^8 cells $seed^{-1}$. The non-coated sterilized seeds were soaked in sterile water served as control. The non-inoculated and inoculated seeds (10 seeds per pot) were sown in clay pots (25 cm high, 22 cm internal diameter) using three kg unsterilized soil with control (without lead) and three treatments each with 97.5, 195 and 390 mg Pb kg^{-1} soil. The normal concentration of Pb (195 mg Pb kg^{-})[1]used in this study was comparable to those found in sewage soil used for lentil production. Six pots used for each treatment were arranged in a complete randomized design. All plants in three pots for each treatment were removed at 90 Days after Seeding (DAS) and were observed for the extent of nodulation and plant growth. Plants uprooted at 90 DAS were oven-dried at 80°C and the dry matter was measured.

The total N content in roots and shoots were measured at 90 DAS by the micro-Kjeldahl method (Iswaran and Marwah, 1980). The total chlorophyll contents in fresh foliage and leghaemoglobin content in fresh nodules grown in metal stressed soil was quantified at 90 DAS by the method of Arnon (1949) and Sadasivam and Manickam (1992), respectively. Lead content at 90 DAS in plant organs (roots and shoots) of lentil was determined by the method of Ouzounidou *et al*. (1992). Data of the measured parameters recorded for two years were pooled together and subjected to Analysis of Variance (ANOVA) for two factor pot culture experiment i.e., inoculation and lead concentration and significant partial difference (LSD) was calculated at 5% probability level.

RESULTS

Concentration of lead in soils: The concentration of lead in polluted soils of Mathura road and exhibition ground, Aligarh and non-polluted soils of Faculty of Agricultural Sciences, AMU, Aligarh, India was determined by atomic absorption spectrophotometer. The concentration of lead

in polluted soils of Mathura Road and Exhibition ground was 195 and 191 mg kg^{-1} soil, respectively (data not shown). In comparison, the heavy metal concentration of the conventional cultivated soils of Faculty of Agricultural Sciences was 8.1 mg kg^{-1} soil (data not shown).

Tolerance of plant growth promoting *Rhizobium* to lead: A total of 50 rhizobial strains were isolated from the nodules of lentil which were grown in the metal contaminated soils of Aligarh region. RL9 strain was specially chosen due to their high tolerance to lead (1400 μg mL^{-1}). The lead tolerant strain RL9 was gram negative and produced circular and white gummy colonies on YEM agar plates. The freshly grown cultures of strain RL9 were positive for catalase, triple sugar iron agar, nitrate, urease and citrate and could hydrolyze gelatin. Strain RL9 gave negative results for starch hydrolysis, indole, methyl red and Voges Proskauer tests.

Plant growth promoting activities: The strain RL9 showed plant growth-promoting activities like IAA, siderophore, HCN and ammonia under *in vitro* conditions. The lead tolerant strain RL9 demonstrated the production of substantial amounts of Indole Acetic Acid (IAA) during 24 h growth in Luria Bertani broth supplemented with 20, 60 and 100 μg mL^{-1} of tryptophan. Generally, the amounts of IAA produced by this strain increased consistently with increase in tryptophan concentration in LB broth. A maximum amount of IAA (33 μg mL^{-1}) was observed in the LB broth having 100 μg mL^{-1} of tryptophan, which was followed by 15.2 and 6.4 μg mL^{-1} of IAA at 60 and 20 μg tryptophan mL^{-1}, respectively (Table 1). On CAS agar plates, lead tolerant *Rhizobium* showed a positive siderophore activity, as indicated by the development of orange colour zone (12 mm) after four days of growth (Table 1). The quantitative estimation of phenolate type of siderophore was performed in Modi medium after five days of incubation. The ethyl acetate extraction from culture supernatant yielded 15 mg L^{-1} of salicylate and 18.3 mg L^{-1} of 2,3-dihydroxy benzoic acid, respectively. Furthermore, the strain was positive for both HCN and ammonia (Table 1).

Influence of lead tolerant *Rhizobium* RL9 species on lentil grown in lead amended soil
Plant growth and nodulation of lentil plant: The lead tolerant *Rhizobium* strain RL9 was used to assess the effect RL9 strain on lentil plants sown in soils treated with three concentrations of lead. Lentil plants grown in lead amended soil showed variable growth and nodulation

Table 1: Plant growth promoting activities of *Rhizobium* species RL9

PGP activities	Values
Siderophores	
CAS agar (mm)	12±2
SA (mg L^{-1})	15±2
2,3 DBA (mg L^{-1})	18.3±3
IAA (μg mL^{-1})	
20 T	6.4±0.9
60 T	15.2±1.5
100 T	33±3.0
HCN	+
Ammonia	+

T: Tryptophan, +: Positive, -: Negative for the strain, CAS: Chrome Azurol S agar, SA: Salicylic acid, DBA: Dihydroxy benzoic acid, IAA: Indole acetic acid, HCN: Hydrogen cyanide

(Table 2). Generally, length, total dry weight and nodulation at 90 DAS, decreased progressively with increase in the concentration of lead. Lead at 390 mg kg^{-1} soil had the greatest phytotoxic effect and significantly ($p \leq 0.05$) decreased the length of roots and shoots by 33 and 39%, nodule numbers and nodule dry weights by 42 and 33% and total dry weight at 90 DAS by 22%, at 390 mg kg^{-1} of lead compared to control plants (Table 2). In contrast, plants inoculated with strain RL9 increased the measured parameters, even in the presence of different concentrations of lead (Table 2). Rhizobial strain RL9 when used with 195 mg Pb kg^{-1} had the highest stimulatory effect and increased the root length, shoots length, nodule numbers, nodule dry weight and total dry weight by 67, 87, 100, 138 and 172% at 90 DAS, compared to un-inoculated but amended with 195 mg Pb kg^{-1} soil (Table 2). Furthermore, the growth and nodulation also increased even at 580 mg Pb kg^{-1} soil in the presence of bio-inoculant. The two way ANOVA revealed that the individual effects of inoculation and lead and their interaction (inoculation×Pb) was significant ($p \leq 0.05$) for the measured parameters at 90 DAS.

Chlorophyll, leghaemoglobin and N content: Chlorophyll, leghaemoglobin and N content in roots and shoots at 90 DAS decreased consistently with increase in the concentration of nickel (Table 3) without the inoculation of strain RL9. Lead at 390 mg kg^{-1} had the greatest effect on the photosynthetic pigments of lentil plants and decreased the chlorophyll, leghaemoglobin and root N and shoot N by 7, 44, 9 and 5%, respectively compared to un-inoculated control (Table 3). On the other hand *Rhizobium* RL9 species with 195 mg Pb kg^{-1}, increased the chlorophyll in fresh foliage, leghaemoglobin content in fresh nodules, N content in roots and N content in shoots by 221, 100, 11 and 7%, respectively compared to un-inoculated but having 195 mg Pb kg^{-1} soil. Furthermore, chlorophyll, leghaemoglobin content, N content in roots and shoots also increased even at 390 mg Pb kg^{-1} soil in the presence of *Rhizobium* species RL9 (Table 3). Two factor ANOVA revealed that the individual effects of inoculation and lead and their interaction (inoculation×lead) were significant ($p \leq 0.05$) for the measured parameters except the interaction on root N and shoot content at 90 DAS.

Table 2: Effect of inoculation with *Rhizobium* sp. RL9 on biological characteristics of lentil subjected to different soil lead concentrations

		Length/plant (cm)		Dry weight (mg $plant^{-1}$)		Nodulation		
Treatment	Pb (mg kg^{-1} soil)	Root	Shoot	Root	Shoot	No. $plant^{-1}$	Dry weight (mg $plant^{-1}$)	Total dry weight (mg $plant^{-1}$)
Uninoculated	Control	21^{c}	18^{c}	44^{c}	123^{c}	12^{e}	13^{c}	180^{d}
	97.5	21^{c}	18^{c}	40^{b}	118^{c}	10^{c}	8^{b}	166^{c}
	195	18^{b}	15^{b}	38^{b}	110^{b}	8^{b}	8^{b}	156^{b}
	390	14^{a}	11^{a}	33^{a}	102^{a}	7^{a}	6^{a}	141^{a}
Inoculated	Control	24^{d}	21^{d}	60^{d}	125^{d}	13^{f}	15^{d}	200^{e}
	97.5	29^{g}	26^{e}	101^{d}	205^{a}	14^{g}	16^{d}	322^{f}
	195	30^{g}	28^{e}	135^{e}	270^{e}	16^{h}	19^{e}	424^{g}
	390	20^{c}	20^{c}	47^{c}	128^{d}	11^{d}	13^{c}	188^{d}
LSD		1.4	2.0	3.7	6.6	0.7	1.1	8.4
F-value								
Inoculation (df = 1)		233*	486*	1953*	457*	505*	502*	1909*
Metals (df = 3)		54.1*	62.4*	370*	116*	71.7*	44.0*	398.6*
Interaction (df = 3)		15.1*	30.2*	349*	120*	57.7*	29.1*	406.5*

*Significantly different from control at $p \leq 0.05$, Each value is a mean of three replicates where each replicate constituted three plants/pot, Mean values followed by different letters in the same column are different at $p \leq 0.05$ according to Tukey test

Table 3: Effect of inoculation with *Rhizobium* sp. RL9 on the biological and chemical characteristics of lentil plants subjected to different soil lead concentrations

Treatment	Dose rate of Pb (mg kg^{-1} soil)	Chlorophyll content (mg g^{-1})	Leghaemoglobin content (mmol g^{-1} FM)	Nitrogen content (mg g^{-1}) Root	Shoot
Uninoculated	Control	0.28[a]	0.09[b]	13.7[b]	41.0[a]
	97.5	0.29[a]	0.08[b]	13.1[a]	40.1[a]
	195	0.28[a]	0.07[a]	12.9[a]	39.7[a]
	390	0.26[a]	0.05[a]	12.5[a]	38.8[a]
Inoculated	Control	0.30[a]	0.10[b]	14.2[b]	42.0[b]
	97.5	0.80[b]	0.13[c]	14.2[b]	42.1[b]
	195	0.90[c]	0.14[c]	14.3[b]	42.4[b]
	390	0.60[b]	0.10[b]	13.7[b]	39.4[a]
LSD	0.27	0.023	1.15	1.8	
F-value					
Inoculation (df = 1)		256.9*	212.6*	83.9*	21.1*
Pb (df = 3)		33.2*	21.0*	9.3*	9.9*
Interaction (df = 3)		31.7*	16.6*	3.1	1.9

*Significantly different from control at $p \leq 0.05$, Mean values followed by different letters in the same column are different at $p \leq 0.05$ according to Tukey test, FM: Fresh matter

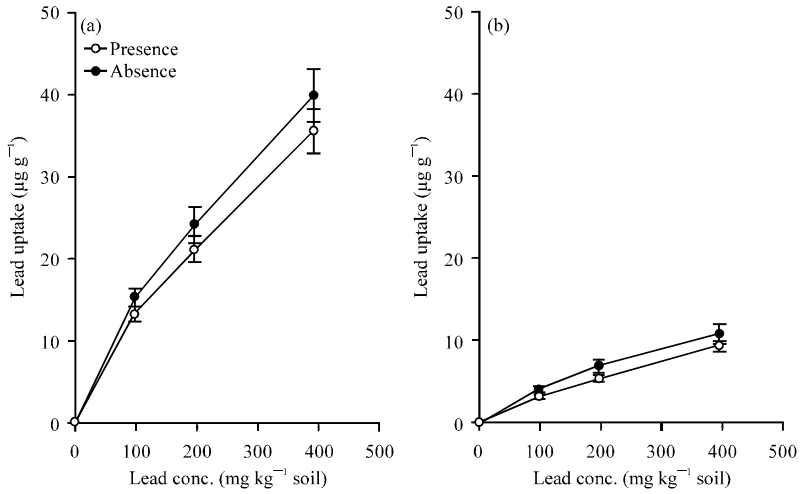

Fig. 1: Lead accumulation in (a) Roots and (b) Shoots at 90 days after seeding lentil in the absence and presence of bioinoculant strain *Rhizobium* RL9 with 97.5, 195 and 390 mg Pb kg^{-1} soil. Values are Mean±SD of three replicates

Lead uptake: The uptake of lead by plant organs (roots and shoots) at 90 DAS was maximum at 390 mg kg^{-1} of lead (Fig. 1) both in the presence and absence of bio-inoculant. Moreover, the accumulation of lead in roots and shoots were less in the presence of bio-inoculant RL9 compared to the un-inoculated but lead amended plants. Generally, roots accumulated more concentrations of lead compared to those observed for shoots, under both inoculated and metal stressed condition.

DISCUSSION

Deposition of metal into soil over a long period of time results in high concentration of metal in the soil which adversely affects the microflora of the soil (Matsuda *et al.*, 2002). Heavy metals in

general show adverse effect on the soil microbial flora by blocking their functional groups or these metals modify the biological molecules in particular their active sites. But these metals when present in low concentrations are important for the microbes as they supply the microorganisms with the essential co-factors for metalloproteins and enzymes (Nies, 1999). The metal-microbe interaction in natural environment is complex and is influenced by pH or organic matter content (Saeki *et al*., 2002). The ability to grow at concentration of metals is however, found in many plant growth promoting rhizobacteria including symbiotic nitrogen fixing bacteria (Lakzian *et al*., 2002) and may be the result of intrinsic or induced mechanism (Giller *et al*., 1998). There are reports that have shown a high level tolerance to heavy metals by rhizobia (Wani and Khan, 2010). Conflicting reports are, however, also available in the literature on the tolerance level of rhizobia, which could possibly be due to the variation in the tolerance level of rhizobia and growth conditions employed (Rajkumar *et al*., 2005). For instance, *Rhizobium leguminosarum* isolated from metal contaminated soil tolerated 92.9 μM of zinc (Delorme *et al*., 2003) while *Rhizobium* species isolated from nodules of *Trifolium repens* tolerated 300 mg kg^{-1} nickel and showed an effective symbiosis with its legume host, when grown in nickel amended soils (Smith and Giller, 1992). Similarly Luo *et al*. (2011), isolated *Serratia* sp. LRE07 from cadmium hyper-accumulator *Solanum nigrum* L. was resistant to the toxic effects of heavy metals.

In the present study, *Rhizobium* isolated from lentil nodules, displayed a high resistance towards lead (1400 μg mL^{-1}). The lead tolerant strain RL9 was characterized by physiological and biochemical methods. In other studies, the gram-negative bacteria have also shown resistance to cadmium and zinc salts. For instance, cadmium and zinc tolerance by Protobacteria, Actinobacteria and Bacteroidetes were resistant to Zn and Cadmium (Kuffner *et al*., 2010).

In the present study *Rhizobium* strain RL9 was positive for plant growth promoting activities and produced substantial amount of IAA, siderophore, HCN and ammonia. Bacterial strains produce ammonia; this ammonia plays a signalling role when the plant growth promoting bacteria and plants interact with each other (Becker *et al*., 2002). Moreover, the ammonia released by the bacterial strain is known to increase the glutamine synthetase activity (Chitra *et al*., 2002). In addition, ammonium transporters found in several plant growth promoting rhizobacteria are thought to be involved in the reabsorption of NH_4^+ released as a consequence of NH_3 diffusion through the bacterial membrane (Van Dommelen *et al*., 1997). Similarly phytohormone production (Wani and Khan, 2010; Rajkumar *et al*., 2006; Ahmad *et al*., 2008) and IAA by rhizobia is reported (Antoun *et al*., 1998). Siderophores is also known to bind to the available form of iron Fe^{3+} in the rhizosphere thus making it unavailable to the phytopathogens and consequently protects the plant health. In other study, the heavy metal resistant *Bacillus* species are also known to produce considerable amount of plant growth promoting substances (Wani *et al*., 2007).

Plant-microbe interactions have been proposed to increase the bioremediation capacity of plants (Zhuang *et al*., 2007; Khan *et al*., 2009; Faisal and Hasnain, 2006). Legumes in association with different species of rhizobia and other PGPR strains are receiving greater attention for their application used in the remediation of metal contaminated soils (Isk *et al*., 2007). Moreover, the phytohormone is reported to reduce the effect of high concentration of certain metals (e.g., Cd) on the growth of non-inoculated soybean plants (Ghorbanli *et al*., 1999). Inoculation of *Rhizobium* strain RL9 augmented the growth and nodulation of legume when the bacterial strain was applied as seed inoculant in lead amended soil. This study consolidated the fact that the selected strains possesses metal reducing/tolerance ability and PGP activity which in turn might have provided the protection to legume plants against the inhibitory effects of each metal (Faisal and Hasnain, 2006; Zaidi *et al*., 2006). The IAA produced by the rhizobial strains promotes root growth directly by

stimulating plant cell elongation or cell division (Minamisawa and Fukai, 1991). Similar evidence of increase in plant growth in metal amended soil has been reported (Faisal and Hasnain, 2005; Pajuelo *et al.*, 2007). In other study, Burd *et al.* (2000), observed an increase in the growth of tomato, canola and mustard, when these plants were grown in the presence of *Kluyvera ascorbata* in Ni, Pb and Zn amended soil. However, reports on the effect of metals on *Rhizobium*-legume symbiosis are conflicting. For example, Chaudrie *et al.* (2000), observed a significant reduction in nodulation, when field grown pea was grown in soil amended with Zn and Cu. While Ibekwe *et al.* (1995) reported a considerable increase in nodulation of alfalfa (*Medicago sativa* L.), white clover (*Trifolium repens* L.) and red clover (*Trifolium pratense* L.) when grown in metal amended soil.

Chlorophyll, leghaemoglobin and N content at 90 DAS decreased consistently with increase in the concentration of nickel without the inoculation of strain RL9. In comparison the strain RL9 increased the measured parameters significantly compared to uninoculated plants. Similar increase in chlorophyll content of lentil plants following inoculation of siderophore producing and lead and cadmium resistant *Pseudomonas putida* KNP9 under metal stressed conditions has also been reported (Tripathi *et al.*, 2005). Furthermore, the nodules on the root system of legume plant raised in soil amended with lead, had considerably a lower concentration of leghaemoglobin. On the contrary, the plants grown in the presence of bio-inoculant increased the leghaemoglobin content under the influence of lead. Comparable observations on the effect of cadmium, nickel, copper and zinc on soybean nodules has been reported (Vesper and Weidensaul, 1978).

In contrast, plants grown in the presence of bio-inoculant considerably increased the N content under the influence of lead. Similar increase in N availability to the crops under different concentrations of lead has also been reported (Ibekwe *et al.*, 1995). The plants grown in the soil amended with lead decreased the N content which possibly could be due to the toxic effects of lead on the proliferation of roots and to shoots (Ibekwe *et al.*, 1996). The reduction in roots and shoots in turn might have the suppressive effect on dry matter production and consequently the N content (Bisessar *et al.*, 1983).

The uptake of lead by the roots and shoots of lentil plant used in this study increased with increase in the concentration of lead both in the presence and absence of lead. The inoculated strain in general, decreased the concentration of nickel in roots and shoots considerably when plants were grown in soil treated with nickel, compared to non-inoculated plants. Moreover it was also observed that the roots accumulated more concentrations of lead compared to shoots, under both inoculated and metal stressed soil. Furthermore, it was interesting to note that the bio-inoculant strain used in this study reduced the concentration of metals in the plant organs of inoculated legumes, grown in soil treated with different concentrations of lead. The decrease in lead concentration after rhizobial inoculation suggested the role of lead tolerant strain used in this experiment in the removal of lead through an adsorption/desorption mechanism (Mamaril *et al.*, 1997). Moreover, this study suggested that the ability of the bio-inoculant to protect legume against the inhibitory effects of high concentration of metal could be related to the bio-inoculant strain providing the legume plant with the sufficient PGP substances including iron (Burd *et al.*, 2000). Similarly Faisal and Hasnain, 2005) reported a lesser accumulation of chromium in sunflower (*Helianthus annuus*) inoculated with *Ochrobacterum intermedium* while in another study, *Ochrobacterum intermedium* and *Bacillus cereus* significantly decreased the chromium toxicity and concomitantly increased the growth of green gram plants under chromium stress (Faisal and Hasnain, 2006). The greater uptake of lead by the roots of legume plant could be due to the poor translocation of lead from roots to shoots (Zayed *et al.*, 1998; Rajkumar *et al.*, 2006).

Implication: Heavy metals after release from various sources may enter into soil, vegetation and water depending on their density. The metals disrupt cellular functions, damage the DNA structure and finally result in cell death. The use of bioinoculants result in the detoxification of these heavy metals which are toxic and increase the production of the legume crops to legume growers when they use these legumes in metal contaminated soils.

CONCLUSIONS

This study concluded that the inoculation of *Rhizobium* strain RL9 not only protected the lentil plants against the lead toxicity but also reduced the uptake of lead in lentil plants. The increased growth of lentil plants in the presence of bioinoculant could be due to the effect of phytohormones produced by the *Rhizobium* strain. Due to multifarious properties expressed by this strain, RL9 could therefore be used as bioinoculant to increase the performance of lentil crop in soils contaminated with lead.

REFERENCES

Ahmad, F., I. Ahmad and M.S. Khan, 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res., 168: 173-181.

Alexander, D.B. and D.A. Zuberer, 1991. Use of chrome azurol S reagents to evaluate siderophore production by *Rhizosphere* bacteria. Biol. Fertil. Soils, 12: 39-45.

Antoun, H., C.J. Beachamp, N. Goussard, R. Chabot and R. Lalande, 1998. Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: Effects on radishes (*Rhaphanus sativus* L.). Plant Soil, 204: 56-67.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplats, polyphenol oxidase in *Beta vulgaris*. Plant Physiol., 25: 1-15.

Assche, F.V. and H. Clijsters, 1990. Effects of metals on enzyme activity in plants. Plant Cell Environ., 13: 195-206.

Bakker, A.W. and B. Schippers, 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* SPP-mediated plant growth-stimulation. Soil Biol. Biochem., 19: 451-457.

Becker, D., R. Stanke, I. Fendrik, W.B. Frommer, J. Vanderleyden, W.M. Kaiser and R. Hedrich, 2002. Expression of the NH_4^+-transporter gene *LEAMT1;2* is induced in tomato roots upon association with N_2-fixing bacteria. Planta, 215: 424-429.

Bibi, M. and M. Hussain, 2005. Effect of copper and lead on photosynthesis and plant pigments in black gram [*Vigna mungo* (L.) Hepper]. Bull. Environ. Contam. Toxicol., 74: 1126-1133.

Bisessar, S., R.J. Rinne and J.W. Potter, 1983. Effect of heavy metals and *Meloidogyne hapla* on celery grown on organic soil near nickel refinery. Plant Dis., 67: 11-14.

Bric, J.M., R.M. Bostock and S.E. Silverstone, 1991. Rapid *In situ* assay for indoleacetic acid production by bacteria immobilized on nitrocellulose membrane. Applied Environ. Microbiol., 57: 535-538.

Broos, K., M. Uyttebroek, J. Mertens and E. Smolders, 2004. A survey of symbiotic nitrogen fixation by white clover grown on metal contaminated soils. Soil Biol. Biochem., 36: 633-640.

Broos, K., H. Beyens and E. Smolders, 2005. Survival of rhizobia in soil is sensitive to elevated zinc in the absence of the host plant. Soil Biol. Biochem., 37: 573-579.

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

Chaudrie, A.M., C.M.G. Allain, V.L.B. Jefferson, F.A. Nicholson, P. Chambers and M. Baud Grath, 2000. A study of the impact of Zn and Cu on two rhizobial species in soils of along-term field experiment. Plant Soil, 221: 167-179.

Chitra, R.S., V.C. Sumitra and D.S. Yash, 2002. Effect of different nitrogen sources and plant growth regulators on glutamine synthetase and glutamate synthase activities of radish cotyledons. Bulg. J. Plant Physiol., 28: 46-56.

Delorme, T.A., J.V. Gagliardi, J.S. Angle, P.B. van Berkum and R.L. Chaney, 2003. Phenotypic and genetic diversity of rhizobia isolated from nodules of clover grown in a zinc and cadmium contaminated soil. Soil Sci. Soc. Am. J., 67: 1746-1754.

Dye, D.W., 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. N. Z. J. Sci., 5: 393-416.

Faisal, M. and S. Hasnain, 2005. Bacterial Cr(VI) reduction concurrently improves sunflower (*Helianthus annuus* L.) growth. Biotechnol. Lett., 27: 943-947.

Faisal, M. and S. Hasnain, 2006. Growth stimulatory effect of *Ochrobactrum intermedium* and *Bacillus cereus* on *Vigna radiata* plants. Lett. Applied Microbiol., 43: 461-466.

Fernandes, J.C. and F.S. Henriques, 1991. Biochemical, physiological and structural effects of excess copper in plants. Bot. Rev., 57: 246-273.

Ghorbanli, M., S.H. Hadade-Kaveh and M.F. Sepehr, 1999. Effects of cadmium and gibberellin on growth and photosynthesis of *Glycin max* L.. Photosynthetica, 37: 627-631.

Giller, K.E., E. Witter and S.P. McGrath, 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. Soil Biol. Biochem., 30: 1389-1414.

Gordon, S.A. and R.P. Weber, 1951. The calorimetric estimation of IAA. Plant Physiol., 26: 192-195.

Gorhe, V. and U. Paszkowski, 2006. Contribution of the arbscular mycorrhizal symbiosis to heavy metal phytoremediation. Planta, 223: 1115-1122.

Gupta, D.K., U.N. Rai, S. Sinha, R.D. Tripathi, B.D. Nautiyal, P. Rai and M. Inouhe, 2004. Role of *Rhizobium* (CA-1) inoculation in increasing growth and metal accumulation in *Cicer arietinum* L. growing under fly-ash stress condition. Bull. Environ. Contam. Toxicol., 73: 424-431.

Holt, J.G., N.R. Kreig, P.H.A. Sneath, J.T. Staley and S.T. Williams, 1994. Bergeys Manual of Determinative Bacteriology. 9th Edn., Williams and Wilkins, New York, USA.

Ibekwe, A.M., J.S. Angle, R.L. Chaney and P. Van Berkum, 1995. Sewage sludge and heavy metal effects on nodulation and nitrogen fixation of legumes. J. Environ. Qual., 24: 1199-1204.

Ibekwe, A.M., J.S. Angle, R.L. Chaney and P. van Berkum, 1996. Zinc and cadmium toxicity to alfalfa and its microsymbiont. J. Environ. Qual., 25: 1032-1040.

Isk, K., H.A. Kayali, N. Sahin, E.O. Gundogdu and L. Tarhan, 2007. Antioxidant response of a novel *Streptomyces* sp. M3004 isolated from legume rhizosphere to H_2O_2 and paraquat. Process Biochem., 42: 235-243.

Iswaran, V. and T.S. Marwah, 1980. A modified rapid Kjeldahl method for determination of total nitrogen in agricultural and biological materials. Geobios, 7: 281-282.

Katterman, F.R.H. and A.D. Day, 1989. Plant growth factors in sewage sludge. Biocycle, 3: 64-65.

Khan, M.S., A. Zaidi, P.A. Wani and M. Oves, 2009. Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environ. Chem. Lett., 7: 1-19.

Khan, N., A. Mishra, P.S. Chauhan, Y.K. Sharma and C.S. Nautiyal, 2012. *Penibacillus lentimorbus* enhances growth of chickpea (*Cicer arietinum* L.) in chromium-amended soil. Ant. Van Leeuwen, 101: 453-459.

Kuffner, M., S. De Maria, M. Puschenreiter, K. Fallmann and G. Wieshammer *et al*., 2010. Culturable bacteria from Zn and Cd accumulating *Salix caprea* with different effects on plant growth and heavy metal availability. J. Applied Microbiol., 108: 1471-1484.

Lakzian, A., P. Murphy, A. Turner, J.L. Beynon and K.E. Giller, 2002. *Rhizobium leguminosarum* bv. *viciae* populations in soils with increasing heavy metal contamination: Abundance, plasmid profiles, diversity and metal tolerance. Soil Biol. Biochem., 34: 519-529.

Luo, S., Y. Wan, X. Xiao, H. Guo and L. Chen *et al*., 2011. Isolation and characterization of endophytic bacterium LRE07 from cadmium hyperaccumulator *Solanum nigrum* L. and its potential for remediation. Applied Microbiol. Biotechnol., 89: 1637-1644.

Mamaril, J.C., E.T. Paner and B.M. Alpante, 1997. Biosorption and desorption studies of chromium (iii) by free and immobilized *Rhizobium* (BJVr 12) cell biomass. Biodegradation, 8: 275-285.

Matsuda, A., F.M.S. Moreira and J.O. Siqueira, 2002. Tolerance of rhizobai from different origins to zinc, copper and cadmium. Pesq. Agro. Bras., 37: 343-355.

McGrath, S.P. and C.H. Cunliffe, 1985. A simplified method for the extraction of metals Fe, Zn, Cu, Ni, Cd, Pb, Cr and Mn from soils and sewage sludge. J. Sci. Food Agri., 36: 794-798.

McGrath, S.P., 1987. Long Term Studies of Metal Transfers Following Application of Sewage Sludge. In: Pollutant Transport and Fate in Ecosystems, Coughtrey, P.J., M.H. Martin and M.H. Unsworth (Eds.). Blackwell Scientific, Publication, UK., pp: 301-307.

Mcllveen, W.D. and J.J. Negusanti, 1994. Nickel in the terrestrial environment. Sci. Total Environ., 148: 109-138.

Minamisawa, K. and K. Fukai, 1991. Production of indole-3-acetic acid by *Bradyrhizobium japonicum*: A correlation with genotype grouping and rhizobitoxine production. Plant Cell Physiol., 32: 1-9.

Moffat, A.S., 1999. Engineering plants to cope with metals. Science, 285: 369-370.

Moftah, A.E., 2000. Physiological response of lead polluted tomato and egg plant to the antioxidant ethylene diurea. Menufiya Agri. Res., 25: 933-955.

Nies, D.H., 1999. Microbial heavy metal resistance. Applied Microbiol. Biotechnol., 51: 730-750.

Ouzounidou, G., E.P. Eleftheriou and S. Karatagfis, 1992. Ecophysiological and ultrastructural effects of copper in *Thlaspi ochroleucum* (Cruciferae). Can. J. Bot., 70: 947-957.

Pagliai, M., G. Guidi, M. La Marca, M. Giachetti and G. Lucamante, 1981. Effect of sewage sludges and composts on soil porosity and aggregation. J. Environ. Qual., 10: 556-561.

Pajuelo, E., I.D. Rodriguez-Llorente, M. Dary and A.J. Palomares, 2007. Toxic effects of arsenic on *Sinorhizobium-Medicago sativa* symbiotic interaction. Environ. Pollut., 154: 203-211.

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

Rajkumar, M., R. Nagendran., J.L. Kui., H.L. Wang. and Z.K. Sung, 2006. Influence of plant growth promoting bacteria and Cr^{6+} on the growth of Indian mustard. Chemosphere, 62: 741-748.

Reeves, M.W., L. Pine, J.B. Neilands and A. Balows, 1983. Absence of siderophore activity in *Legionella* species grown in iron-deficient media. J. Bacteriol., 154: 324-329.

Sadasivam, S. and A. Manickam, 1992. Biochemical Methods for Agricultural Sciences. Wiley Eastern Ltd., New Delhi, India, ISBN: 8122403883.

Saeki, K., T. Kunito, H. Oyaizu and S. Matsumoto, 2002. Relationships between bacterial tolerance levels and forms of copper and zinc in soils. J. Environ. Qual., 31: 1570-1575.

Saleh, M. and S. Al-Garni, 2006. Increased heavy metal tolerance of cowpea plants by dual inoculation of an arbuscular mycorrhizal fungi and nitrogen-fixer *Rhizobium bacterium*. Aferican J. Biotechnol., 5: 133-142.

Smith, S.R. and K.E. Giller, 1992. Effective *Rhizobium leguminosarum biovar trifolii* present in five soils contaminated with heavy metals from long term application of sewage sludge or metal mine spoil. Soil Biol. Biochem., 24: 781-788.

Thacker, U. and D. Madamwar, 2005. Reduction of toxic chromium and partial localization of chromium reductase activity in bacterial isolate DM1. World J. Microbiol. Biotechnol., 21: 891-899.

Tripathi, M., H.P. Munot, Y. Shouch, J.M. Meyer and R. Goel, 2005. Isolation and functional characterization of siderophore-producing lead-and cadmium-resistant *Pseudomonas putida* KNP9. Curr. Microbiol., 5: 233-237.

Van Dommelen, A., E. Van Bastelaere, V. Keijers and J. Vanderleyden, 1997. Genetics of *Azospirillum brasilense* with respect to ammonium transport, sugar uptake and chemotaxis. Plant Soil, 194: 155-160.

Vesper, S.J. and T.C. Weidensaul, 1978. Effects of cadmium, nickel, copper and zinc on nitrogen fixation by soybeans. Water Air Soil Pollut., 9: 413-422.

Wani, P.A. and M.S. Khan, 2010. *Bacillus* species enhance growth parameters of chickpea (*Cicer arietinum* L.) in chromium stressed soils. Food Chem. Toxicol., 48: 3262-3267.

Wani, P.A., M.S. Khan and A. Zaidi, 2007. Effect of metal tolerant plant growth promoting *Bradyrhizobium* sp. (vigna) on growth, symbiosis, seed yield and metal uptake by greengram plants. Chemosphere, 70: 36-45.

Wani, P.A., M.S. Khan and A. Zaidi, 2008a. Effect of heavy metal toxicity on growth, symbiosis, seed yield and metal uptake in pea grown in metal amended soil. Bull. Environ. Contam. Toxicol., 81: 152-158.

Wani, P.A., M.S. Khan and A. Zaidi, 2008b. Chromium reducing and plant growth promoting *Mesorhizobium* improves chickpea growth in chromium amended soil. Biotechnol. Lett., 30: 159-163.

Wu, C.H., T.K. Wood, A. Mulchandani and W. Chen, 2006. Engineering plant-microbe symbiosis for rhizoremediation of heavy metals. Applied Environ. Microbiol., 72: 1129-1134.

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

Zayed, A., C.M. Lytle, J.H. Qian and N. Terry, 1998. Chromium accumulation, translocation and chemical speciation in vegetables crops. Plantrum, 206: 293-299.

Zhuang, X., J. Chen, H. Shim and Z. Bai, 2007. New advances in plant growth-promoting rhizobacteria for bioremediation. Environ. Int., 33: 406-413.