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Metal Accumulation and Growth Response in *Vigna radiata* L. Inoculated with Chromate Tolerant Rhizobacteria and Grown on Tannery Sludge Amended Soil

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Abstract The effects of inoculation of four chromate tolerant rhizobacterial strains previously isolated from rhizosphere of plants from chromium contaminated area in mung plant *Vigna radiata* grown on tannery sludge amended soil were evaluated. An increase of 138%, 88%, 256% and 54.14% in root length, shoot length, biomass and total chlorophyll, respectively was observed after 60 days of treatments by consortium. Similarly, a significant enhancement in Fe, Mn, Zn, Ni, Pb, Cr, Cu and Cd accumulation was observed in consortium inoculated plants as compared to non-inoculated plants. Results showed that rhizobacterial strain helps in ameliorating metal induced phytotoxicity, acquiring higher biomass and metal uptake in the plant may be useful in decontamination of metal from polluted soil.

Keywords Tannery sludge · Rhizobacteria · Metals · Bioaccumulation · Consortium

Heavy metal contamination of soil is one of the world's major environmental problems, posing significant risks to ecosystems; therefore, development of a remediation strategy for metal contaminated soil is necessary for environmental conservation and human health. Phytoremediation, using plants to remove metal pollutants from contaminated soil is being developed as new methods for the remediation of contaminated land (Abou-shanab et al. 2006). This environment friendly, cost effective and plant based technology is expected to have significant economic, aesthetic and technical advantages over traditional engineering techniques (Susarla et al. 2002). Roots proliferation and effective root uptake mechanisms are the key processes in rhizosphere that distinguish metal hyperaccumulators from normal plants, besides role of root exudates and microbial activity are largely unknown (Lombi et al. 2000). Soil microbes can affect trace metal mobility and availability to the plant by producing iron chelators, siderophores, reduce soil pH, solubilize metal-phosphates and influence root parameters, such as root morphology and growth. An increase in root exudation of organic solutes could affect the rate of phytosiderophore symbiotically with root to enhance the potential for metal uptake (Guan et al. 2001).

In the context of above, improving plant–microbes interaction and introduction of beneficial rhizospheric microorganism are vital for increased biomass production and tolerance of plant to heavy metals (Glick 2003). However, effects of single and combined microbial inocula to contaminated soil have been largely ignored. To investigate the role and potential importance of chromate tolerant rhizobacteria in metal uptake and growth of the plant, the present study was undertaken as also to determine whether four different chromate tolerant native strains, isolated from rhizosphere of plants from chromium contaminated site were able to affect growth and metal accumulation in *Vigna radiata* inoculated individually and in combination and grown on chromium contaminated sludge amended soil. Results obtained during this study have been reported in this paper.

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Table 1 Physico-chemical parameters of garden soil and tannery sludge

	Garden soil	Sludge amended soil (1:1)	Tannery sludge
Parameters			
pH	6.80 ± 0.1	7.42 ± 0.01	7.74 ± 0.01
EC (μs cm^{-1})	792 ± 1.21	1,384 ± 0.42	1,735 ± 0.06
Salinity (PPT)	0.48 ± 0.02	1.43 ± 0.08	1.57 ± 0.07
Water holding capacity (%)	62.18 ± 5.30	55.34 ± 4.23	48.62 ± 3.64
Metal (μg g^{-1} dw)			
Cu	1.89	47.1 ± 3.70	62.25 ± 4.5
Ni	Bdl[a]	8.73 ± 1.12	12.5 ± 1.40
Pb	2.13	8.51 ± 7.50	22.36 ± 1.15
Zn	7.12	110.35 ± 10.50	156.36 ± 11.2
Mn	16.02	161.55 ± 14.25	215.73 ± 15.26
Cr	Bdl[a]	23,331.00 ± 2,145.45	32,220 ± 2,350
Cd	Bdl[a]	3.55 ± 0.05	5.90 ± 0.32
Fe	11.05	20,281 ± 1,780.65	35,410 ± 1,800

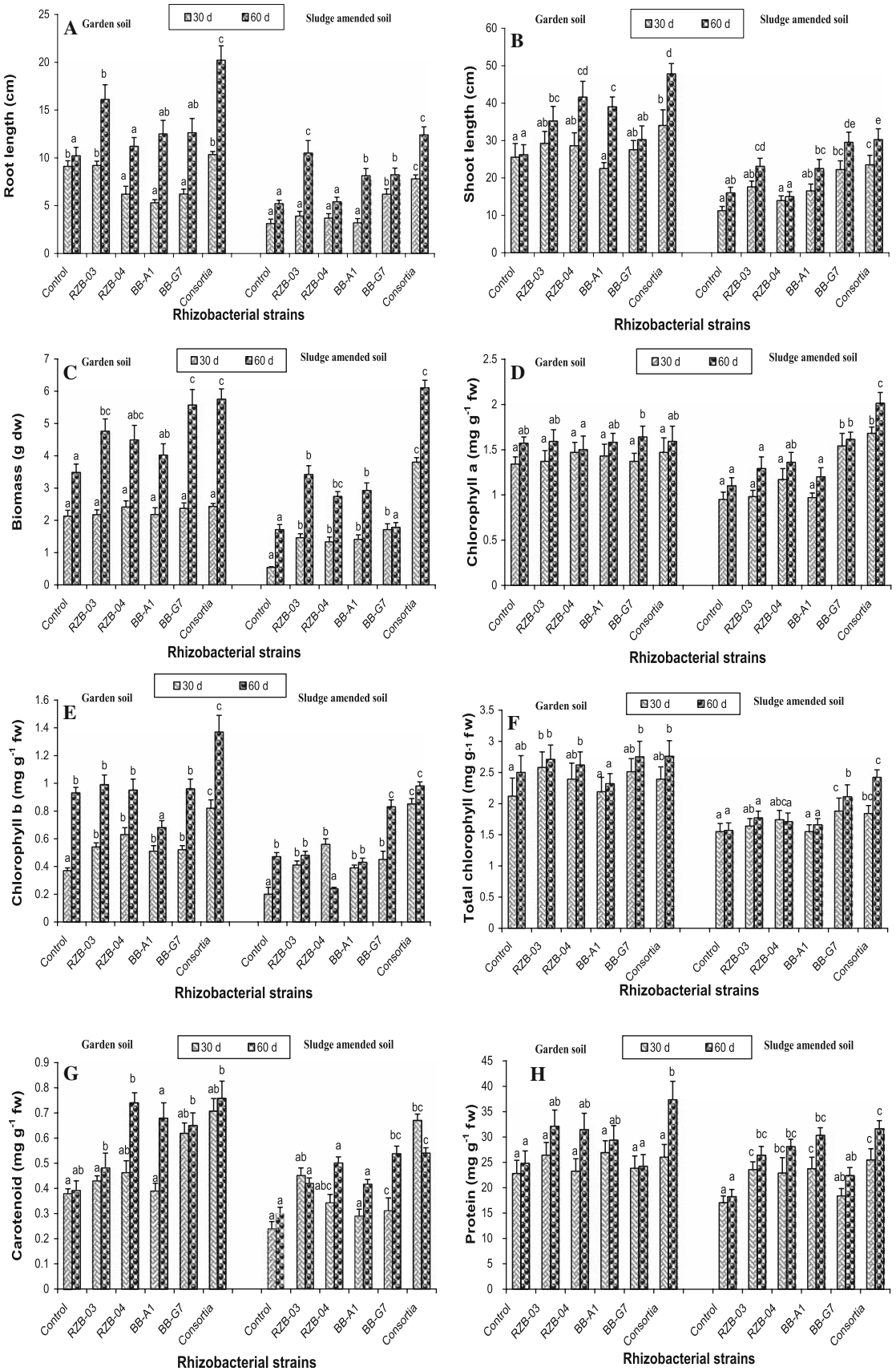
All values are mean ± SD (n = 3)

[a] Below detection limit

Materials and Methods

The roots of *Scirpus lacustris* and *Prosopis julifolia* were collected from chromium contaminated area near common effluent treatment plant (CETP), located at Unnao, Uttar Pradesh (India) for isolation of chromate resistant rhizobacteria. The roots of plants were washed thoroughly with tap water for 2 min followed by washing with sterile 0.85% (w/v) saline milli Q water (MQW) and macerated in 0.85% saline MQW with a mortar and pistil. Serial dilutions of the homogenate were plated on nutrient agar supplemented with different chromate concentrations (100–1,000 μg mL^{-1}) as $K_2Cr_2O_7$ (Hi Media Laboratories Pvt. Ltd, India) and incubated at 28 ± 2°C as described earlier (Nautiyal 1997). The four more efficient chromate tolerant rhizobacterial strains were designated as RZB-03, RZB-04, BB-A1 and BB-G7. These chromate tolerant rhizobacterial isolates were grown overnight in 500 mL Erlenmeyer flasks containing 250 mL of sterilized nutrient broth and incubated at 28°C in a incubation shaker at 150 rev min^{-1} for 24 h. Bacterial cells were harvested by centrifugation (12,000*g*, 20°C, and 10 min) and the pellets were then washed twice with sterile distilled water. Bacterial suspension in distilled water were adjusted to an absorbance at 600 nm of 0.5 (equivalent to approximately 1.5×10^8 cfu mL^{-1}) of each isolate separately and in combination and added to 500 g sterilized talc powder and incubated for 24 h at 28°C. The seeds were surface sterilized by shaking in 70% ethanol for 5 min followed by shaking in 1% mercuric chloride for 1 min and then mixed with incubated talc powder containing culture of rhizobacterial isolates. The seeds mixed with only sterilized talc powder without any rhizobacterial strains served as control. The sludge sample used in this study was also collected from drying beds of Common Effluent Treatment plant (CETP), located at Unnao, Uttar Pradesh (India). The sludge was air dried crushed and passed through a 4 mm sieve to disaggregate and then mixed with uncontaminated garden soil collected from National Botanical Research Institute, Lucknow (India) in equal proportion (1:1, w/w) in large container and dried at room temperature. The physico-chemical analysis of soil and their amendments with tannery sludge were carried out by following the methods of Kalra and Maynard (1991). The inoculated and uninoculated seeds of *V. radiata* with rhizobacterial strains were sown in sterilized earthen pots filled with 1 kg air dried sludge amended garden soil (1:1, w/w). Two sets of each combination were taken in triplicate, one for sludge amended garden soil and other for garden soil. Sludge amended soil in pots was kept moist by adding 350 mL double distilled water (DDW) in each pots on alternate days. The plants were grown for 2 months in a glass house at 25°C in a randomized block design and tap watered routinely as required.

Plants were harvested after 30 and 60 days of growth. The whole plants of *V. radiata* were uprooted from the pots and washed repeatedly with deionized water, blotted dry then roots and shoots were separated manually. Fresh weight of the plants (control and treated) was recorded and root and shoot length were measured after each harvesting with the help of a meter scale. Biomass was estimated on dry weight basis (g dw) after oven dried at 105°C till a constant weight is obtained. Chlorophyll and carotenoid


A
Garden soil
30 d
60 d
Sludge amended soil
Root length (cm)
B
Shoot length (cm)
C
Biomass (g dw)
D
Chlorophyll a (mg g-1 fw)
E
Chlorophyll b (mg g-1 fw)
F
Total chlorophyll (mg g-1 fw)
G
Carotenoid (mg g-1 fw)
H
Protein (mg g-1 fw)
Control
RZB-03
RZB-04
BB-A1
BB-G7
Consortia
Rhizobacterial strains

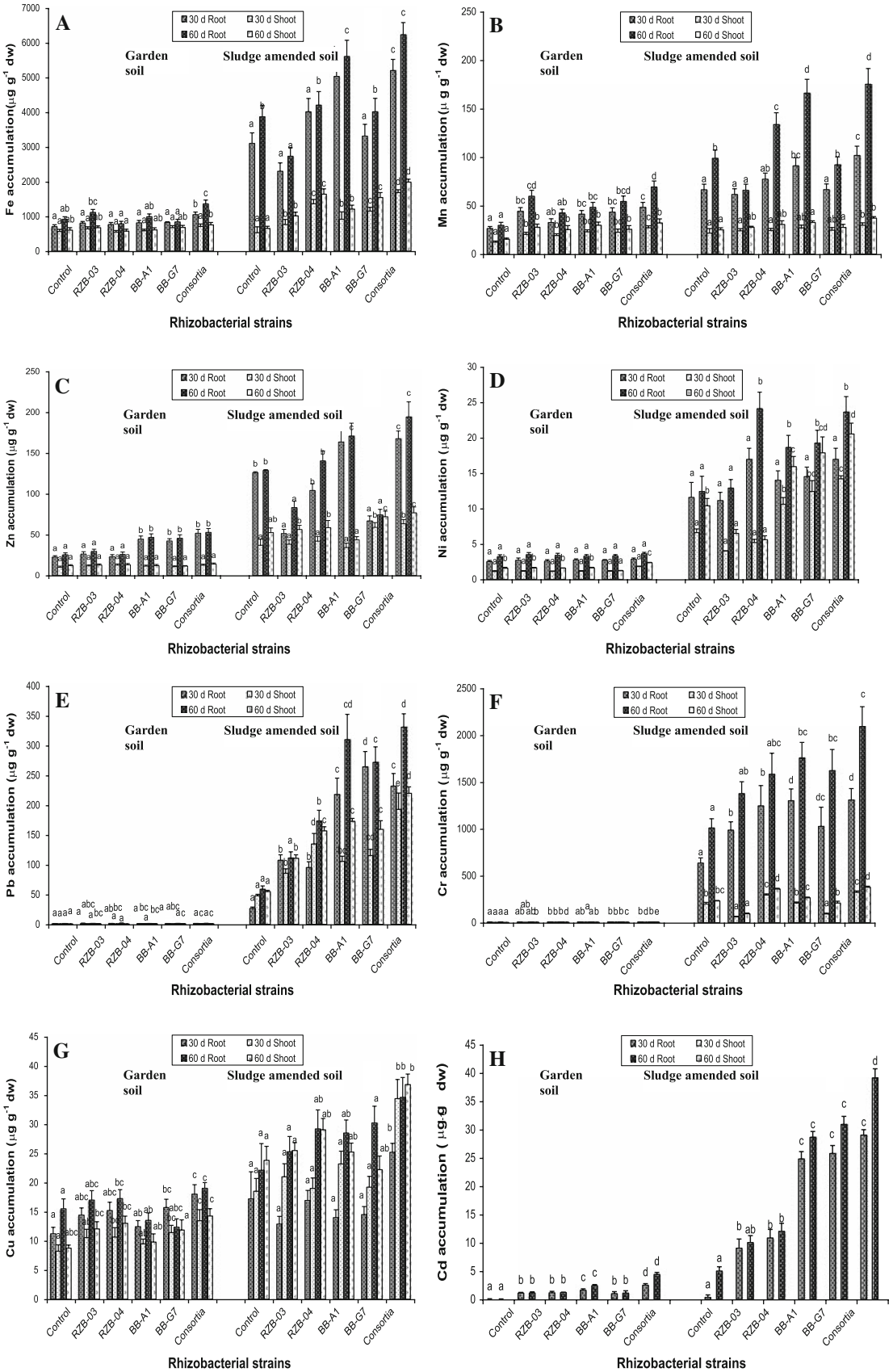
◀ **Fig. 1** Effect of inoculation of different rhizobacterial strains and their consortium on **a** root length (cm), **b** shoot length (cm), **c** biomass (g dw), **d** chlorophyll *a* (mg g^{-1} fw), **e** chlorophyll *b* (mg g^{-1} fw), **f** total chlorophyll (mg g^{-1} fw), **g** carotenoid (mg g^{-1} fw) and **h** protein (mg g^{-1} fw) of *V. radiata* grown on garden soil and tannery sludge amended soil (1:1 w/w) at different treatment durations. All values are mean ± SD (n = 3). ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different value (DMRT, $p < 0.05$)

contents were calculated using the formula given by Arnon (1949) and Duxbury and Yentsch (1956), respectively. The protein content was estimated following the method of Lowry et al. (1951) in the shoot using bovine serum albumin as standard. The root, shoot and soil samples were oven dried to a constant weight separately and digested in a mixture of HNO_3:$HClO_4$ (3:1 ratio). The volume was made 10 mL by demineralized water. The metal concentrations (Fe, Mn, Zn, Ni, Pb Cr, Cu and Cd) were estimated by GBC Avanta Σ Atomic Absorption Spectrophotometer. The standard reference material of metals (E-Merck, Germany) was used for the calibration and quality assurance for each analytical batch. Analytical data quality of metals was ensured through repeated analysis (n = 3) of EPA quality control samples (Lot TMA 989) and the results were found to be within ±2.71% of certified values. Recoveries of metals from the plant tissues were found to be more than 98.5% as determined by digesting three samples each from an untreated plant with known amount of metals. The blanks were run in triplicate to check the precision of the method with each set of samples. The detection limit of Fe, Mn, Zn, Ni, Pb Cr, Cu and Cd were 0.02, 0.02, 0.005, 0.02, 0.06, 0.012, 0.001 and 0.013 ppm, respectively. All experiments were setup in triplicate following randomized block design. To confirm the variability of data and validity of results, all data were subjected to analysis of variance (ANOVA) and for group wise comparison of means Duncan's multiple range test (DMRT) was applied to see the significant level (Gomez and Gomez 1984).

Results and Discussion

Out of 64 rhizobacterial isolates, isolated from rhizosphere of plants *Scirpus lacustris* and *Prosopis julifolia,* only four strains viz., RZB-03, RZB-04 and BB-A1, BB-G7 were found tolerate to high Cr^{+6} concentration (600, 500 and 400, 800 μg mL^{-1}, respectively). Physico-chemical properties of tannery sludge amended soil (1:1, w/w) and garden soil used in the experiment have been shown in Table 1. Addition of tannery sludge in the garden soil increased pH and EC. In garden soil, the levels of Cr, Ni, Cd and Pb were below detection limits (BDL). However, the data showed high concentration of metals in the tannery sludge amended soil. Chromium, was found maximum i.e. 23,331.00 ± 2,145.45 μg g^{-1} dw followed by Fe, i.e. 20,281 ± 1,780.65 μg g^{-1} dw in the tannery sludge. The plant of *Vigna radiata* have been able to grow in tannery sludge amended soil, however, the growth was diminished as compared to plants grown in garden soil. The inoculation of rhizobacterial strains significantly enhanced growth of *V. radiata* and provided tolerance to grow in tannery sludge amended soil. It was interesting to note that there was maximum increase i.e. 138% in root length, 88% in shoot length and 256% in biomass of plant inoculated with consortium grown in tannery sludge amended soil after 60 days of treatment as compared to un-inoculated plant. (Fig. 1a–c). Similarly, root elongation in *B. napus* has also been shown to be stimulated by un-identified rhizobacteria (Belimov et al. 2005). The plants grown on tannery sludge amended soil showed reduction in biomass as compared to plants grown on garden soil and minimum biomass 0.54 g dw was recorded in non-inoculated plant in tannery sludge amended soil in contrast to 2.12 g dw in garden soil (Fig. 1c). The inoculation of plant with the rhizobacterial isolates resulted in increased biomass. Similar trend was observed by Wu et al. (2006) in which increasing rate of plant inoculation was found 71.9% and 22.6% in plants without tailings amendments and 40% in tailings added soil. The PGPR was found to modify the plant growth and development by increasing nutrient and water uptake and, therefore, the plant biomass (Belimov et al. 2004). The other mechanisms or regulators driven by PGPR, such as siderophores, specific enzymes, organic acids involved in phosphorus solubilization, fixation of atmospheric N_2 also contributed to this effect (O' Sullivan and O' Gara 1992; Pattern and Glick 1996).

A decrease in chlorophyll content was observed in plants grown on the tannery sludge amended soil as compared to garden soil (Fig. 1d–f). However, chlorophyll *a*, *b* and total increased by 83%, 47.3% and 54.14%, respectively in consortium inoculated plant grown in tannery sludge amended soil after 60 days of treatment. Metals have a potential to alter the rate of photosynthesis by disturbing the structure of chloroplast leading to the changes in the fatty acid composition, inhibiting photosynthetic pigments and enzymes of Calvin cycle (Vazquez et al. 1987). Similar effects of rhizobacterial inoculation was observed on carotenoid content which showed maximum increase of 93.84% in garden soil followed by 179% in tannery sludge amended soil after 60 days of treatment in consortium inoculated plants, respectively (Fig. 1g). The increase in the carotenoid level may be due to the ability of the plant to counteract the toxic effect of free radicals generated under metal stress. Carotenoid serves as an accessory pigment for photosynthesis and protects the plants from photooxidation, replace peroxidation and


A
Fe accumulation(μg g-1 dw)
B
Mn accumulation (μ g g-1 dw)
C
Zn accumulation (μg g-1 dw)
D
Ni accumulation (μg g-1 dw)
E
Pb accumulation (μg g-1 dw)
F
Cr accumulation (μg g-1 dw)
G
Cu accumulation (μg g-1 dw)
H
Cd accumulation (μg g dw)
30 d Root
30 d Shoot
60 d Root
60 d Shoot
Garden soil
Sludge amended soil
Control
RZB-03
RZB-04
BB-A1
BB-G7
Consortia
Rhizobacterial strains

◀ **Fig. 2** Effect of inoculation of different rhizobacterial strains and their consortium on **a** Fe, **b** Mn, **c** Zn, **d** Ni, **e** Pb, **f** Cr, **g** Cu and **h** Cd accumulation (μg g^{-1} dw) in root and shoot of *V. radiata* grown on garden soil and tannery sludge amended soil (1:1) at different treatment durations. All values are mean ± SD (n = 3). ANOVA significant at $p < 0.01$. Different letters indicate significantly different value (DMRT, $p < 0.05$)

collapse of membrane chloroplasts (Knox and Dodge 1985). The maximum increase in protein content was observed by 73.62% and 50.68% in plants inoculated with consortium in tannery sludge amended soil and garden soil after 60 days of treatment, respectively (Fig. 1h). Chlorophyll *a*, *b* and total content increased in rhizobacterial inoculated plants *V. radiata* as compared to non-inoculated plants grown in metal contaminated sludge amended garden soil, however, a decrease in chlorophyll content was observed at higher treatment duration. A decrease in protein content in the presence of chromium or other metals may be due to the breakdown of soluble protein or due to the increased activity of protease or other catabolic enzymes, which were activated and destroyed the proteins. Similar trend in decline of protein content under heavy metal stress in a terrestrial plant soybean (*Glycine max* L.) have been observed (Ganesh et al. 2008). Tripathi et al. (2005) reported a facilitating growth in mung plants inoculated with siderophore-producing bacteria and grown in the presence of $CdCl_2$.

The plants of *V. radiata* inoculated with rhizobacterial isolates singly and in combination (consortium) showed a jubilant growth in garden soil, however, the growth was diminished in tannery sludge amended soil. But, it is interesting to note that plant inoculated with different rhizobacterial strains showed better growth as compared to non -inoculated plants. Data further, revealed that inoculation of rhizobacteria enhanced metal accumulation which was more pronounced in root than shoot (Fig. 2). Different rhizobacterial isolates affect distinctly and up to different extent, however, the strains in combination (consortium) yielded better performance in enhancing metal accumulation in both tannery sludge amended soil and garden soil. The accumulation of metals was found in order Fe > Cr > Zn > Mn > Pb > Cd > Cu > Ni in the root of the *V. radiata* grown in tannery sludge amended soil. However, translocation of metals from root to shoot was varied for each metal and rhizobacterial strain inoculated. In case of metal Cu and Pb which showed nearly equal concentrations in both root and shoot while other metals (Fe, Mn, Zn, Cr and Ni) seems to be localized in roots of the plant. Similar to this finding Banerjee et al. (2008) reported maximum accumulation of Cr in the root with respect to the total chromium accumulation by *V. radiata* towards Cr(III) and Cr(VI) to have an insight on the migration and bio-magnification of Cr while other parts e.g. cotyledons, shoot and leaves, showed negligible accumulation. The maximum increase in Fe, Mn, Zn, Ni, Pb, Cr and Cu accumulation were observed by 60.9%, 77.4%, 51.1%, 90.16%, 46%, 107.12% and 54.42% in the root and 148%, 48.41%, 42.28%, 97.4%, 300%, 62.5% and 56.56% in shoot of the plants inoculated with consortium and grown in tannery sludge amended soil, respectively, after 60 days of treatment duration. In contrast to other metals, Cd accumulation in shoot of *V. radiata* was found below detection limit (<0.001 μg g^{-1}), however, root of consortium inoculated plant accumulated significant amount of Cd i.e. 39.1 μg g^{-1} dw and 4.4 μg g^{-1} dw showing an increase of 680%, and 48.79% grown in sludge amended soil and garden soil, respectively after 60 days of treatment duration. Similarly, increase in both the rate of metal accumulated by plants and biomass by inoculation of plant growth promoting rhizobacteria were reported earlier (Carlot et al. 2002). Similarly, Rai et al. (2004) reported inoculation of plant with a fly ash tolerant *Rhizobium* strain (PJ-1) conferred tolerance for the plant to under fly ash stress conditions with more translocation of metals to the above ground parts. Our findings revealed that addition of mixed inoculum of rhizobacterial strains RZB-03. RZB-04, BB-A1 and BB-G7 in *V. radiata* increased metal accumulation and plant showed comparatively better growth response. However, a combined application of biosurfactants and biodegradable chelating agents with bioinoculating PGPR should be considered in order to develop more environmentally sound treatment protocol (Gregorio et al. 2006). Therefore, it may be concluded that application of native chromate tolerant growth promoting rhizobacteria for inoculating plants for amelioration of metal contaminated site is one of the most important approach in the development of enhanced microbe assisted phytoremediation system for metals. Rhizobacterial isolate behaves differently in promotion of growth and metal accumulation and it seems possible that the synergistic effect of these isolates will be more useful.

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