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Impact of rhizobacteria on growth and chromium accumulation in *Scirpus lacustris* L. grown under chromium supplementation

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Abstract: *Four chromate tolerant rhizobacterial strains viz., RZB-01, RZB-02, RZB-03 and RZB-04 were isolated from rhizosphere of Scirpus lacustris collected from Cr-contaminated area. These strains characterized at morphological and biochemical levels. The most efficient chromate tolerant strain RZB-03 was inoculated to fresh plant of S. lacustris and grown in 2 μg ml^{-1} and 5 μg ml^{-1} of Cr^{+6} supplemented nutrient solution under controlled laboratory condition. The effects of rhizobacterial inoculation on growth and chromium accumulation in S. lacustris were evaluated. The inoculation of rhizobacteria increased biomass by 59 and 104%, while total chlorophyll content by 1.76 and 15.3% and protein content increased by 23 and 138% under 2 μg ml^{-1} and 5 μg ml^{-1} concentrations of Cr^{+6}, respectively after 14 d as compared to non-inoculated plant. Similarly, the Cr accumulation also increased by 97 and 75% in shoot and 114 and 68% in root of inoculated plants as compared to non inoculated plants at 2 μg ml^{-1} and 5 μg ml^{-1} Cr^{+6} concentrations, respectively, after 14 d. The chromate tolerant rhizobacteria which play an important role in chromium uptake and growth promotion in plant may be useful in development of microbes assisted phytoremediation system for decontamination of chromium polluted sites.*

Key words: *Rhizobacterial strains, Hexavalent chromium (Cr^{+6}), Phytoremediation, Tolerance, Tannery sludge, Rhizosphere*
PDF of full length paper is available online

Introduction

Chromium compounds are being used in a wide variety of commercial processes like tanneries, metal cleaning and processing, electroplating, wood processing and alloy formation. Despite various treatment procedures in use, unregulated disposal of the chromium containing effluent has led to the contamination of soil, sediment, surface and ground water (Szulczewski *et al*., 1997). Health hazards posed by accumulation of toxic metals in the environment and high cost of removal and replacement of metals from polluted soil have prompted efforts to develop phytoremediation strategies. These utilize plants to clean up metal contaminated sites by extracting metals from the soil and concentrating them in the above ground biomass leading to impaired metabolic activities and reduced plant growth (Chaney *et al*., 1997; Smith *et al*., 1998; Kumkum *et al*., 2009). Hence, the alternate way to reduce the toxicity of heavy metals in the plant could be using the rhizospheric microbes to enhance phytoremediation efficiency (Park *et al*., 2005). In this regards, interactions among metals, microbes and plants have attracted attention because of the biotechnological potential of microorganisms for metal removal directly from polluted site or the possible transfer of accumulated metals to higher plants and the diversion of heavy metals towards microbial metabolism and growth (Guo *et al*., 1996; Polti *et al*., 2007). Despite the important role of rhizospheric microorganisms in plant interactions with the soil environment in general and toxic metals in particular, studies focusing on effects of these microorganisms in metal remediation efforts are scanty (Burelle *et al*., 2006). Moreover, the effects on introduced microbial inoculation and/or indigenous communities on phytoremediation practices have been largely ignored.

In this context, present study has been undertaken to isolate chromate tolerant bacterial strains from rhizosphere of *Scirpus lacustris* growing in Cr contaminated area to assess their effects on growth and chromium accumulation in plant under controlled laboratory condition.

Materials and Methods

The rooted emergent plant *S. lacustris* was collected from Cr-contaminated area of Unnao (UP) India. The roots of *S. lacustris* were thoroughly washed with tap water for 2 min followed by washing with sterile 0.85% (w/v) saline milli Q water (MQW) (Milli pore Corporation, Bedford, Mass, USA). The roots were then macerated in 0.85% saline MQW with a mortar and pistal. Serial dilutions of the homogenate were plated on nutrient agar (Hi Media Laboratories Pvt. Ltd., India) and incubated at 28±2°C as described earlier (Nautiyal, 1997). These isolates were purified and stored on to nutrient agar slants at 4°C for further tests. The chromate tolerant rhizobacterial strains isolated were designated as RZB-01, RZB-02, RZB-03 and RZB-04 and subjected to morphological and biochemical test by using KBOO3 Hi 25™ Identification kit (Hi Media Laboratories Pvt. Ltd., India). The selected chromate rhizobacterial strains tested also for tolerance to other heavy metals (Luli *et al*., 1983). The fresh overnight peptone water broth cultures of these strains were inoculated (100 µl) aseptically on nutrient agar plates supplemented individually with different concentration ranging from 1 to 1000 µg ml^{-1} of Cr, Cd, Cu and As. Susceptibility for different

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antibiotics of rhizobacterial strains was determined by the disc diffusion method (Bauer *et al*., 1996). The antibiotic impregnated discs were placed on freshly prepared lawns of each isolate on Muller Hinton agar plates and examined for the diameter of inhibition zone.

For experimental studies, the plant buds of *S. lacustris* were detached from mother plant and grown in acid washed sand in 10% Hoagland's solution under laboratory conditions (light / dark cycle 14: 10 hr, temperature 25±2°C, 115 µ mol m^2 s^{-1} illumination provided through day fluorescent tube light) for acclimatization. The pure culture of strain RZB-03 was grown in nutrient broth (Hi Media Laboratories Pvt. Ltd., India) at 28°C and diluted to a final concentration of 10^8 CFU ml^{-1} in sterile distilled water containing 0.025% Tween. Roots of young plant of *S. lacustris* were surface sterilized in 70% ethanol for 2 min and rinsed 10 times in sterile water and then dipped in rhizobacterial suspension of the strain RZB-03 for 4 hr on orbital shaker. The root dipped in sterile water served as non-inoculated control. The 2 and 5 µg ml^{-1} concentrations of Cr^{+6} of were prepared from with 10% Hoagland's solution. The inoculated and non inoculated plants (approximately 2 g fresh wt.) were transferred to glass beaker (250 ml capacity) containing 200 ml of Cr^{+6} (2 and 5 µg ml^{-1} as $K_2Cr_2O_7$) supplemented nutrient solutions. Two sets (comprising of three beakers for each concentration) were placed in random block design plants placed in 10% Hoagland's solution (without Cr) served as control.

One set of each concentration was harvested after 7 and 14 days of the treatment period. Fresh weight of the plants (control and treated) was recorded and root and shoot separated manually. Biomass of the plant was expressed on dry weight basis. The chlorophyll contents extracted in 80% chilled acetone (v/v) and were estimated as per procedure of Arnon (1949). Protein content was estimated following the methods of Lowry *et al*. (1951) using bovine serum albumin as standard. The oven dried plant material was digested in HNO_3: $HClO_4$ (3:1; v/v) and Cr concentration was estimated by atomic absorption spectrophotometer (GBC Σ Avanta). All analysis carried out in triplicates (n = 3). Each treatment was analyzed with six replicates (two experiments each with three replicates) and the standard derivation (SD) was calculated. 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

The morphological and biochemical characteristics of selected bacterial strains are shown in Table 1. All the strains were gram positive, rod-shaped and showed positive response for nitrate reduction, while negative response for ortho-nitrophenyl-β-galactoside, deamination, H_2S production, Voges proskaur's, methyl red, malonate, eseulin hydrolysis, arabinose, xylose, adonitol, rhamnose, cellobiose. Only the strain RZB-01 showed positive response to trehalose and positive response to oxidase, citrate utilization, lysine decorboxylase and negative response to glucose, lactose and urease. The strain RZB-02 showed positive response to lysine decorboxylase, citrate utilization, like RZB-01 and RZB-03, ornithine decorboxylase like RZB-03 and oxidase like RZB-01, whereas negative response to melibiose, indole, saccharose, raffinose, glucose, lactose as reverse to RZB-03, which showed positive response. The strain RZB-04 showed positive response to ortho-nitrophenyl-β-galactoside, indole, urease and negative response to citrate utilization, melibiose, raffinose, trehalose, glucose, lactose and oxidase. The selected strains were designated as RZB-01 RZB-02 RZB-03 and RZB-04 on the basis of their morphological and biochemical characteristics. However, 16-RNA sequencing is required for proper identification. The rhizobacterial strains were tested for their tolerance to different metals *i.e.* chromium, copper, cadmium and arsenic by taking concentration from 1-1000 µg ml^{-1}. These strains showed different minimum inhibitory concentration (MIC) against different metals.

As shown in the Table 2 the strain RZB-01 has maximum MIC, 800 µg ml^{-1} for arsenic and cadmium while, minimum 400 µg ml^{-1} for chromium and copper. The strain RZB-02 showed maximum tolerance to cadmium up to 600 µg ml^{-1} and minimum for chromium and arsenic up to 400 µg ml^{-1}, whereas, the strain RZB-03 showed maximum MIC value for chromium, cadmium and arsenic up to 600 µg ml^{-1} and minimum for copper up to 400 µg ml^{-1}.

The strain RZB-04 showed maximum tolerance for cadmium up to 800 µg ml^{-1} and minimum for copper and chromium up to 500 µg ml^{-1}. The rhizobacterial strain's antibiotic susceptibility profile has been depicted in Table 3. The strain RZB-01 showed sensitivity (S) to ampicillin, vankomycin, nalidixic acid, norflaxin, ceffepime, tetracycline, while resistance (R) to cefaclor and no inhibition (NI) for chloramphenicol, streptomycin, kanamycin. The strain RZB-02 exhibited intermediate (I) response to ampicillin, vankomycin, and S for chloramphenicol, nalidixic acid, norfflaxin, ceffepime, tetracycline, cefaclor while NI for streptomycin and kanamycin. However, strain RZB-03 showed S to chloramphenicol, nalidixic acid, norflaxacin, ceffepime, tetracycline and I to ampicillin, vancomycin and R to streptomycin, NI for kanamycin. The strain RZB-04 showed S to chloramphenicol, nalidixic acid, norflaxacin, tetracycline, cefaclor and I for ampicillin, vancomycin, ceffepime and NI to streptomycin and kanamycin.

The plant of S. *lacustris* was found suitable for growing in Cr supplemented nutrient solution. However, as the concentration of Cr in the medium increased, the biomass of *S. lacustris* decreased (Fig. 1A). It is interesting to note that a considerable increase by 59 and 104% were found in biomass after inoculation with RZB-03 as compared with non-inoculated plants at 2 and 5 µg ml^{-1} Cr^{+6} concentration. Similarly, an increase of 23 and 138% in protein content was found in inoculated plants with RZB-03 as compared with non-inoculated plants at 2 and 5 µg ml^{-1} Cr^{+6} concentration after 14 d of exposure, however, higher concentration of Cr had toxic effect on the protein content (Fig. 1B). A decrease in protein content in presence of chromium may be due to the breakdown of soluble protein or due to the increased activity of protease or other catabolic

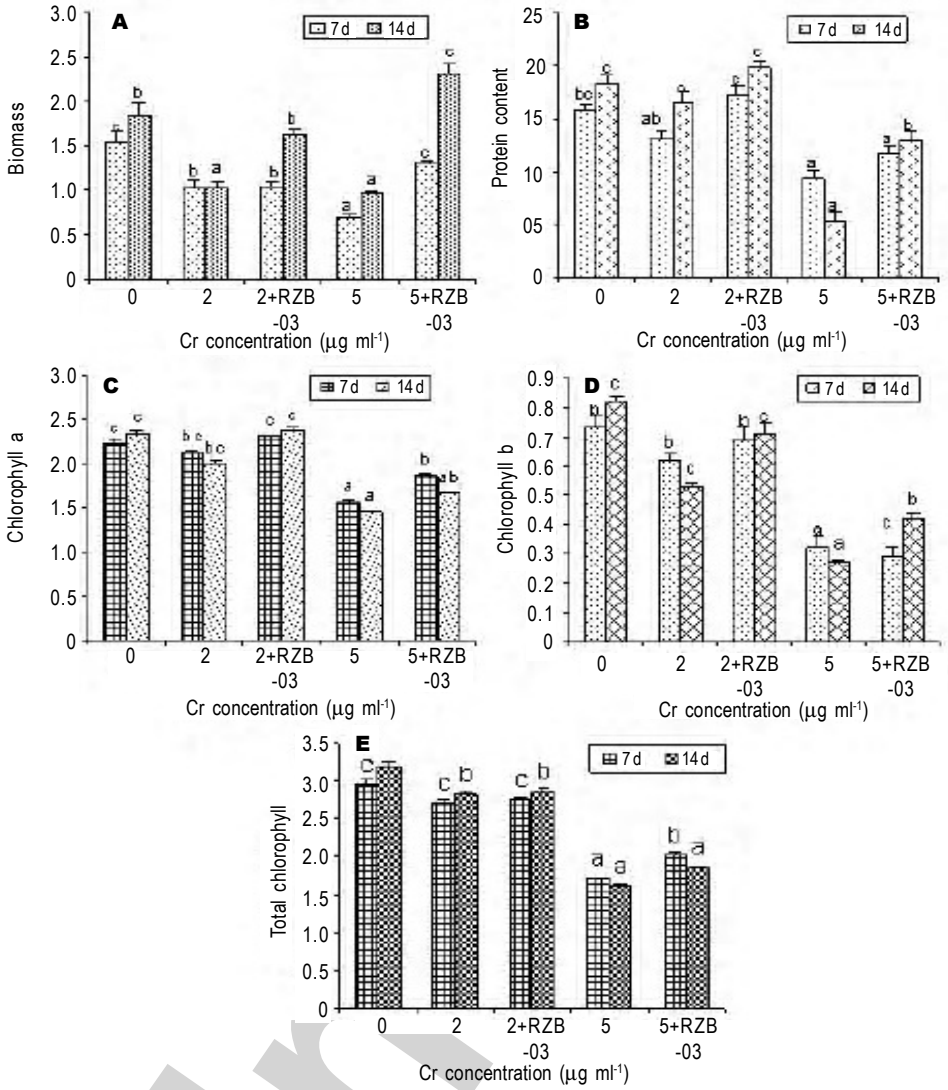

Fig. 1: Effect of rhizobacterial inoculation (RZB-03) in *S. lacustris* grown under different concentration of Cr at different treatment durations: (A) Biomass (g dry wt); (B) Protein content (mg g^{-1} fresh wt); (C) Chlorophyll a (mg g^{-1} fresh wt); (D) Chlorophyll b (mg g^{-1} fresh wt); (E) Total Chlorophyll (mg g^{-1} fresh wt). All values are mean of three replicate ± S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different value (DMRT, $p<0.05$)

enzymes, which were activated and destroyed the proteins. The decline of protein content under heavy metal stress in aquatic plants have been reported (Mazhoudi *et al*., 1997; Chaoui *et al*., 1997). Inoculation with rhizobacterial (RZB-03) most likely mitigated the toxicity of the metals and thus increased biomass and protein content in presence of Cr. Similar observation have been reported earlier (Wu *et al*., 2006; Reed *et al*., 2005; Jiang *et al*., 2008; Zaidi *et al*., 2006). Inoculation of RZB-03 significantly increased the photosynthetic pigments in *S. lacustris* after 7 and 14 d of treatments (Fig. 1 C-E) (DMRT, $p<0.05$). Chlorophyll a was found to increase by 17 and 14.38% in inoculated plants compared to non-inoculated plants at 2 and 5 µg ml^{-1} Cr^{+6} concentration after 14 d of exposure. However, a decrease of 14.46 and 37.81% was found at 2 and 5 µg ml^{-1} Cr^{+6} in non- inoculated plants which may be due to Cr^{+6} toxicity in plant. The chlorophyll b, was increased by 33 and 67% in inoculated plants as compared to non-inoculated plants at 2 and 5 µg ml^{-1} Cr^{+6} concentration after 14 d of exposure, while total chlorophyll found increased by 1.76 and 15.43% in inoculated plant as compared to non inoculated plant at 2 and 5 µg ml^{-1} Cr^{+6} concentration after 14 d of exposure. The photosynthetic pigment degradation has routinely been observed as a response of plants exposed to various metals (Rai *et al*., 1995; Vajpayee *et al*., 2000).

The uptake of Cr following exposure of Cr of *S. lacustris* was studied with and without inoculation of rhizobacteria (RZB-03) after 7 and 14 d (Fig. 2A,B), which showed a differential accumulation of Cr in plant tissues influenced by inoculation of rhizobacterial strain. Maximum accumulation of Cr was recorded 164.70 and 154.22 µg g^{-1} dry wt in shoot and root in inoculated plants as compared to non-inoculated 93.86 and 91.56 µg g^{-1} dry wt., respectively, exposed to 5 µg ml^{-1} Cr after 14 d of treatment (DMRT, $p<0.05$). The inoculation of rhizobacterial strain RZB-03 increased Cr accumulation by 97

Table - 1: Morphological and biochemical characteristics of different bacterial strains isolated from rhizosphere of *S. lacustris*

Test	RZB-01	RZB-02	RZB-03	RZB-04
Gram reaction	+	+	+	+
Shape	Rod single	Rods in pairs	Rods in pairs	Rods in pairs
Colony colour	White	Pink	Bright Yellow	Orange
Ortho-nitrophenyl-â-galactoside	-	-	-	+
Lysine decorboxylase	+	+	+	-
Ornithine decorboxylase	-	+	+	-
Urease	-	-	-	+
Deamination	-	-	-	-
Nitrate reduction	+	+	+	+
H_2S production	-	-	-	-
Citrate utilization	+	+	+	-
Voges proskauer's	-	-	-	-
Methyl red	-	-	-	-
Indole	-	-	+	+
Malonate	-	-	-	-
Eseulin hydrolysis	-	-	-	-
Arabinose	-	-	-	-
Xylose	-	-	-	-
Adonitol	-	-	-	-
Rhamnose	-	-	-	-
Cellobiose	-	-	-	-
Melibiose	-	-	+	-
Saccharose	-	-	+	+
Raffinose	-	-	+	-
Trehalose	+	-	-	-
Glucose	-	-	+	-
Lactose	-	-	+	-
Oxidase	+	+	-	-

+ = Positive, - = Negative

Table - 2: Minimum Inhibitory concentration (MIC) value (μg ml^{-1}) for different metals of rhizobacterial strains isolated from *S. lacustris*

Test	RZB-01	RZB-02	RZB-03	RZB-04
Chromium	400	400	600	500
Cadmium	800	600	600	800
Copper	400	500	400	500
Arsenic	800	400	600	700

Table - 3: Antibiotic profile of different bacterial strains isolated from rhizosphere of S. *lacustris*

Antibiotic	Disc content	Diameter (mm) of the inhibition zone			
		RZB-01	RZB-02	RZB-03	RZB-04
Ampicillin	10 μg	18 (S)	11(I)	12(I)	16(I)
Chloramphenicol	30 μg	0 (NI)	30(S)	30(S)	30(S)
Streptomycin	10 μg	0 (NI)	0(NI)	6(R)	0(NI)
Vancomycin	30 μg	18 (S)	11(I)	16(I)	16(I)
Nalidixic acid	30 μg	27 (S)	29(S)	28(S)	28(S)
Norflaxacin	10 μg	25 (S)	25(S)	22(S)	26(S)
Ceffepime	30 μg	18 (S)	18(S)	18(S)	16(I)
Kanamycin	30 μg	0 (NI)	0 (NI)	0(NI)	0(NI)
Tetracyclin	30 μg	18 (S)	20(S)	28(S)	21(S)
Cefaclor	30 μg	11 (R)	20(S)	20(S)	22(S)

S = Sensitive, I = Intermediate, R = Resistant, NI = No inhibition

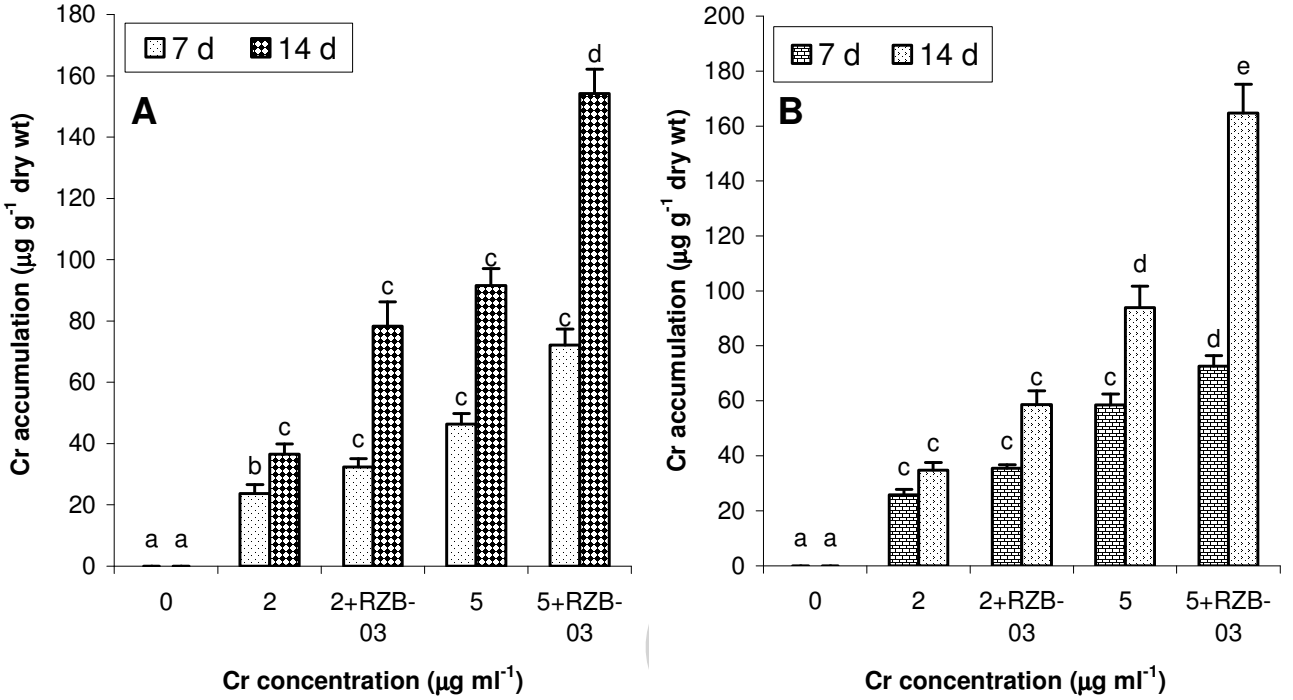

Fig. 2: Effect of rhizobacterial inoculation (RZB-03) on chromium accumulation (µg g^{-1} dry wt) in *S. lacustris* grown under different concentration of Cr at different treatment durations: (A) Root and (B)- Shoot. All values are mean of three replicate ± S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different value (DMRT, $p<0.05$)

and 75% in shoot, however, this increase was 114 and 68.43% in root in inoculated plant as compared to non inoculated plant at 2 and 5 µg ml^{-1} Cr^{+6} concentration after 14 d of treatment.

Inoculation of rhizobacteria increased concentration of Zn (Whiting *et al*., 2001), Ni (Abou-shanab *et al*., 2003) and Se (De-souza *et al*., 1999) in *T. caerulensces, A. murale* and *B. juncea*, respectively. Similarly, Rai *et al*. (2004) reported that inoculation of plant with a fly ash tolerant *Rhizobium* strain conferred tolerance in *Prosopis juliflora* under fly ash stress conditions with more translocation of metals to the above ground parts. The inoculation of rhizobacteria greatly influenced the chromium concentration in plant tissue and achieved a larger biomass harvest, thus resulting in a faster removal of chromium as compared to non-inoculated plant. Thus, plants with such rhizobacterial strain could be used to remove chromium from contaminated site more efficiently.

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