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Alleviation of Phyto-toxic Effects of Chromium by Inoculation of Chromium (VI) Reducing *Pseudomonas aeruginosa* Rb-1 and *Ochrobactrum intermedium* Rb-2

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Abstract

Two indigenous Cr(VI) reducing bacterial strains, *Pseudomonas aeruginosa* Rb-1 and *Ochrobactrum intermedium* Rb-2 were used in this study. Mono as well as mixed cultures of these strains were used to inoculate the seeds of wheat under chromium (III and VI) stressed and chromium free conditions. Inoculated seeds were irrigated with industrial waste water and distilled water. Industrial waste water in combination with Cr(VI) exhibited more adverse effects on the growth as well as biochemical attributes of wheat. Seed germination was severely affected by the application of $CrCl_3$ and K_2CrO_4 irrigated with industrial waste water and distilled water. Bacterial inoculation caused significant enhancement in percentage seed germination when compared with non-inoculated control. Application of chromium salts (III and VI) led to decrease in seedlings length but bacterial inoculation caused improvement in it as compared to respective non-inoculated treatment. Chromium (III and VI) stress in combination with industrial effluent caused significant increase in auxin content of wheat seedlings. Bacteriazation caused significant rise in peroxidase and soluble protein content under chromium (III and VI) stress and unstressed conditions. Maximum augmentation in studied growth and biochemical parameters were observed with the inoculation of bacterial strain *O intermedium* Rb-2. Reduction of chromium (VI) uptake by the inoculated seedlings was most prominent observation. Scanning electron microscopy (SEM) revealed the presence of large number of bacterial cells on the surface of inoculated seedlings. © 2015 Friends Science Publishers

Keywords: Chromium; *Pseudomonas aeruginosa*; *Ochrobactrum intermedium*; Wheat; PGPR

Introduction

Chromium is among one of the most toxic metals detectable in earth crust dueto extensive use of chromium compounds at industrial level. Chromium exists in various oxidation states but trivalent and hexavalent forms are the most dominant forms biologically. In nature, Cr(III) precipitates as minerals that is why it is less mobile and less toxic than Cr(VI). Trivalent forms of chromium are found naturally whereas hexavalent forms are released in to the environment due to anthropogenic activities. These activities lead to increased level of chromium compounds in land as well as in water bodies and ultimately imparting the lethal toxic effects on human beings and soil fertility (Khan *et al.,* 2012). Utilization of industrial waste water for the irrigation of crops is one of a major reason for the significant increase in chromium contamination of soil (Khan *et al.,* 2013). Industrial effluent is mixture of various organic, inorganic components and heavy metals leading to reduce plant growth and accumulation of heavy metals such as Pb, Cd, Cr and Ni (Misra and Tandon, 2009; Kumar *et al.,* 2011). For agronomic plants, Cr is toxic at about 0.5 to 5.0 mg $m L^{-1}$ in nutrient solution and 5000 to 10000 mg kg^{-1} in soil (Hossner *et al.,* 1996). Chromium (VI) compounds are highly toxic to plants and are detrimental to their growth and development. Alterations in germination process, growth of roots, stems and leaves are the toxic effects of chromium on plant resulting in less dry matter production and yield. Physiological processes of plants such as photosynthesis, water relations, total chlorophyll content, rate of transpiration and mineral nutrition are also found to be affected by chromium compounds (Shanker *et al.,* 2005). In wheat, under Cr(VI) stress significant inhibition in the activities of various enzymes such as ascorbate peroxidase, catalase, oxidase and glutathione reductase was also reported. Though, there are numerous physicochemical approaches to reduce the toxic effects of Cr(VI) on plants but biological techniques are more eco-friendly and economical (Khan *et al.,* 2012). One of such biological method is the use of plant growth promoting rhizobacteria (PGPR) to lessen the phytotoxic effects of Cr(VI) (Kang *et al.,* 2012). PGPR are defined as soil bacteria that have ability to colonize the roots

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of plants following inoculation onto seed and they enhance plant growth (Joshi and Bhatt, 2011). These plant growth promoting bacteria have a positive effect on plant growth and development (Castro-Sowinski *et al*., 2007; Filomena *et al.,* 2011). Plant growth promoting bacteria are widely used to promote the growth of hyper accumulating plants for removal of contaminant from the environment (Sabri and Hasnain, 1997; Faisal *et al.,* 2005; Zhuang *et al.,* 2007). Major mechanisms for plant growth promotion include production of phytohormones and siderophores, nitrogen fixation and phosphate solubilization (Vessey, 2003; Ali *et al.,* 2009). Whereas biosorption, bioaccumulation, bioreduction, chromate efflux are certain mechanisms which could be utilized by the plant growth promoting rhizobacteria to lower the lethal effects of Cr(VI) on plants (Khan *et al.,* 2013). Several plant growth promoting rhizobacteria (PGPR) belonging to various genera *Ochrobactrum*, *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Azospirillum*, *Klebsiella*, and *Enterobacter* reported to have significant effects on growth of various plants (Faisal and Hasnain, 2005a; Ali *et al.,* 2009; Egamberdieva, 2010). Wheat (*Triticum aestivum*) is the basic food source for most of the world population including Pakistan. Need of the hour is to develop such wheat varieties which can give good yield and can resist biotic and abiotic stresses so that food requirement of world's growing population can be fulfilled (Datta *et al.,* 2011). Keeping in view the potential of PGPR in the plant growth promotion, this research deals with the assessment of the inoculation effects of both strains (*Pseudomonas aeruginosa* Rb-1 and *Ochrobactrum intermedium* Rb-2) on the growth and biochemical parameters of wheat under chromium(III, VI) stress.

Materials and Methods

Bacterial Strains and Growth Conditions

Pseudomonas aeruginosa Rb-1 (FJ70126) and *Ochrobactrum intermedium* Rb-2 (FJ870125) are gram-negative Cr(VI) reducing bacterial strains previously isolated from tannery effluent. They were obtained from bacterial stock cultures of the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan. Both strains are able to tolerate high levels of K_2CrO_4 i.e. 40 mgL^{-1} on Luria Bertani (LB) agar and 25 mg L^{-1} on Luria Bertani (LB) broth. These strains efficiently reduce significant amount of Cr(VI) (Batool *et al.,* 2012). They were typically cultured on Luria Bertani (LB) agar (pH 7.0) supplemented with 1000 µg mL^{-1} of Cr(VI) at 37°C.

Plant Germination and Growth Experiments

In order to check the plant growth promotion ability of the chromium (VI) reducing bacterial strains, plant growth experiments were carried out with economically important cash crop (*Triticum aestivum*) in axenic conditions. Both of these strains were found compatible for each other as these strains were isolated from the same sample (tannery effluent) collectively. Mono and mixed cultures of bacteria were used to inoculate the seeds. In axenic conditions, experiments were performed in two sets. One set was irrigated with distilled water whereas the other set was supplied with industrial effluent collected from drain of industrial area of KalashahKaku (Latitude: 31° 37' 18 N, Longitude: 74° 9' 59 E), near Lahore. This drain was receiving waste water from different industries. The pH and temperature of industrial effluent were 5.7 and 27°C, respectively. Other metals were also determined (Cr(VI) 350 µg mL^{-1}; Fe 101 µg mL^{-1}; Cu 75 µg mL^{-1}; Zn 8 µg mL^{-1}; Ni 114 µg mL^{-1}; Co 4 µg mL^{-1}). Seeds were germinated and seedlings were grown under respective trivalent (350 µg g^{-1} $CrCl_3$) and hexavalent chromium (350 µg g^{-1} K_2CrO_4) stress.

Inoculum Preparation

For preparation of bacterial suspensions, bacterial strains were grown in L-broth (g L^{-1}: Tryptone: 10, yeast extract: 5, NaCl: 5) overnight at 37°C with 150 rpm. Bacterial cells from cultures were harvested aseptically, washed and re-suspended in sterilized distilled water. To ensure the equal cell population of each bacterial strain in the suspension, cell density (10^7–10^8 CFU mL^{-1}) was maintained by keeping the optical density of cultures (OD=0.5) at 600 nm. For the preparation of mixed cultures of bacteria, equal quantity of suspension of each bacterial culture (after adjusting the cell density) was taken and mixed.

Surface Sterilization of Seeds

Certified seeds of *Triticum aestivum* var. Inqlab-91 were obtained from Pakistan Agriculture Research Council – PARC. The surface of healthy seeds was sterilized by washing with 0.1% $HgCI_2$ for 5 min, with continuous shaking followed by repeated washing with sterilized water, so that all traces of $HgCI_2$ were removed from the seeds.

Pot Experiments under Axenic Conditions

Plant growth experiments were conducted in plastics pots (12 cm length, 13 cm width), each pot containing 300 g of air dried soil. It was a sandy clay loam textured soil and had pH of 7.2, EC 4.4 dS m^{-1}, organic content 0.60%. This air dried soil was artificially polluted with trivalent (350 µg g^{-1} $CrCl_3$) and hexavalent chromium (350 µg g^{-1} K_2CrO_4) along with untreated control. Chromium salt solutions was homogenously mixed with air dried soil and kept for 2 weeks to become stable. Under aseptic conditions, surface sterilized seeds were dipped in respective bacterial suspension for fifteen minutes. For control, the seeds were soaked in sterilized water and in the effluent for the same time period. Ten inoculated seeds were sown with uniform distance in each pot aseptically. Experiment was performed with four replicates for each treatment. Pots were placed in completely randomized design in plant growth chambers at 25°C

temperature, 50% humidity, 16 h photoperiod with light intensity of 180- 200 µ E m^2 S^{-1}. After germination, 10 mL of Hoagland's solution (Jensen and Bassham, 1966) was added to supplement the nutrient requirements of the seedlings just once. Seedlings were grown for two weeks. Observations were made daily and general appearance of seedlings was noticed.

Harvest

After two weeks, seedlings were removed from pots washed and excess of moisture was removed by gently pressing the seedlings between blotting paper and different growth parameters (seed germination, root length, shoot length, number of roots) were observed. For biochemical analysis of seedlings, peroxidase activity, auxin estimation and soluble protein content were noticed.

Estimation of Peroxidase Activity

Weighed and frozen plant material was crushed in phosphate buffer (0.1 M, pH 7.0) in a cold pestle and mortar in the ratio of 1:4 (w/v, one gram of plant material, 4 mL of phosphate buffer). The samples were centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant was used for estimation of peroxidase enzyme. David and Murry (1965) method was used for the quantitative estimation of peroxidases.

Extraction and Estimation of Auxin

Mahadevan (1984) method was followed for the extraction of auxin content of seedlings. Auxin content was determined by using Salkowski colorimetric technique (Glickmann and Dessaux, 1995). Auxin content was estimated by measuring the absorbance at 535 nm with Beckman D-2 spectrophotometer. Optical densities of various concentrations of indole-3-acetic acid (IAA) (standard) were also measured to construct a standard curve. From the standard curve the actual amount of auxin was measured and calculated as µg g^{-1} fresh weight of plant.

Soluble Protein Extraction for Protein Estimation

Extraction of soluble proteins was accomplished by following Bhatti *et al.* (1993) whereas for soluble protein analysis method of Lowry *et al.* (1951) was adopted. Amount of soluble proteins was calculated from standard curve obtained by using Bovine serum albumin (BSA) as standard at wavelength of 750 nm on Beckman D-2 spectrophotometer.

Estimation of Chromium Uptake

Chromium uptake by wheat seedlings was determined. Acid digestion of the plant material was done for the estimation of total chromium accumulated (both trivalent and hexavalent). For this, any trivalent chromium present was oxidized into hexavalent chromium with $KMnO_4$ and then was determined spectrophotometrically at 540 nm using diphenylcarbazide as the complexion agent (Rand *et al.,* 1976).

Electron Microscopy

For this, firstly, seeds of wheat were surface sterilized with 0.1% $HgCl_2$ and then aseptically transferred to petridishes containing Whatman filter paper No. 1. The filter paper was moistened with autoclaved distilled water. Plates were kept in the dark for three days. The germination process was followed daily. After germination, plates were incubated under light (16:8 h day: night, 200 µE m^2 S^{-1}) for four days. Roots of seven day-old seedlings of wheat were suspended in 50 mL tubes containing 25 mL of Hoagland's nutrient solution supplemented with 0 and 350 µg $mL^{-1}$of hexavalent chromium. The amount of Cr(VI) was adjusted to a level that will stress the plant, but allow it to grow. One mL of bacterial inoculum was added to suspended seedling roots in Hoagland's nutrient solution. Incubation of bacteria and wheat seedlings was carried out at 25°C (16:8 day: night, 200 µE m^2 S^{-1}). After 7 days of incubation, the seedlings were harvested and roots were excised.

Plant roots with bacterial colonization were cut into small pieces of 5-10 mm. Samples were prepared for scanning electron microscopy as described by Lounatmaa (1985) and observed using field emission scanning (JEOL JSM-6335F) electron microscope operated at 60 kV.

Statistical Analysis

Data was statistically analyzed using SPSS personal (version 16, SPSS Inc, Chicago). Analysis of variance (ANOVA) was performed and means were separated using Duncan's multiple range test ($P \leq 0.05$).

Results

Effect on Growth Parameters of *Triticum aestivum* var Inqlab-91

Seed germination: Generally, bacterial inoculation significantly augmented the germination of seeds of wheat in both the watering conditions. Treatment with two different chromium salts i.e., $CrCl_3$ and K_2CrO_4 severely affected the germination of *T. aestivum* over control under both watering conditions but the effects of K_2CrO_4 were more adverse. Impact of Cr toxicity was more pronounced with industrial effluent than distilled water. Application of $CrCl_3$ caused 18.87 and 29.58% (with water and industrial effluent, respectively) reduction in seed germination, while K_2CrO_4 treatment caused 32.97 and 36.62% (with water and industrial effluent, respectively) reduction in this parameter when compared with control (0 µg g^{-1} of Cr) treatment (Table 1). Single as well as combined inoculation of Rb-1 and Rb-2 lessen the adverse effects of chromium (III and VI) salts and significantly improved the germination of wheat

Table 1: Effect of bacterial inoculation on seed germination, shoot length and root length of *T. aestivum* var Inqlab-91 seedlings at 0 and 350 µg g^{-1} of $CrCl_3$ and K_2CrO_4 concentrations under both watering conditions (distilled water and industrial effluent)

Parameter/Treatment			Control/ Cr stress	Bacterial strains		
				Rb-1	Rb-2	Rb-1+Rb-2
Seed germination (%age)	Distilled water	Control	88.75±0.37 (a)	97.25±0.30 (a)	99.62±0.19(a)	96.00±0.31(a)
		Cr(III)	72.00±1.60 (a)	87.5±3.60 (ab)	93.75±1.80(b)	76.87±2.72 (a)
		Cr(VI)	65.01±1.69 (a)	81.25±1.80 (b)	92.75±1.80(c)	68.75±1.80 (a)
	Industrial effluent	0(Cr)	72.5±2.10 (b)	87.75±0.99 (a)	93.75±1.80(b)	90.00±3.06 (b)
		Cr(III)	62.5±3.61 (a)	71.87±2.99(a)	74.37±2.57	67.51±3.57 (a)
		Cr(VI)	56.25±4.03 (a)	67.52±1.53 (a)	73.75±2.52(b)	70.25±1.50(ab)
Shoot length (cm)	Distilled water	Control	15.11±0.53(a)	16.56±0.29(a)	16.98±0.14(a)	15.9±0.34(a)
		Cr(III)	9.01±0.19(a)	17.35±0.40(c)	18.41±0.23(d)	12.41±0.09(b)
		Cr(VI)	7.75±0.15(a)	17.78±0.46(c)	18.15±0.37(d)	12.71±0.43(b)
	Industrial effluent	0(Cr)	13.71±0.35(a)	13.95±0.08(b)	14.52±0.25(c)	18.7±0.34(d)
		Cr(III)	9.66±0.39(a)	14.09±0.72(c)	16.63±0.41(d)	12.83±0.65(b)
		Cr(VI)	7.41±0.36(a)	13.61±0.39(c)	14.29±0.14(d)	8.58±0.25(b)
Root length (cm)	Distilled water	Control	4.13±0.09(a)	5.16±0.16(c)	6.41±0.19(d)	5.04±0.15(b)
		Cr(III)	3.75±0.12(a)	6.00±0.14(c)	7.19±0.22(d)	5.68±0.20(b)
		Cr(VI)	3.3±0.12(a)	5.45±0.12(b	6.46±0.23(c)	5.39±0.09(b)
	Industrial effluent	0(Cr)	4.05±0.07(a)	4.49±0.09(c)	6.49±0.22(d)	4.15±0.07(b)
		Cr(III)	3.96±0.07(a)	4.24±0.21(b)	5.76±0.17(d)	5.01±0.13(c)
		Cr(VI)	3.35±0.08(a)	4.49±0.12(c)	5.81±0.23(d)	4.06±0.11(b)
Number of roots	Distilled water	Control	4.75±0.09(a)	4.50±0.09(ab)	5.50±0.16(b)	5.25±0.06(ab)
		Cr(III)	6.00±0.09(a)	6.25±0.11(a)	6.25±0.09(a)	6.50±0.12(a)
		Cr(VI)	7.25±0.17(a)	7.50±0.18(a)	7.35±0.23(a)	7.25±0.18(a)
	Industrial effluent	0(Cr)	5.00±0.13(ab)	5.37±0.09(a)	5.25±0.13(ab)	5.5±0.09(b)
		Cr(III)	6.05±0.09(a)	6.25±0.11(a)	6.87±0.12(a)	6.13±0.17(a)
		Cr(VI)	6.5±0.16(a)	7.125±0.16(a)	7.75±0.19(a)	6.75±0.19(a)

Highlighted areas indicate control treatments. Mean of 32 values ± standard error of the mean. In each row, figures followed by different letter (s) in parenthesis indicate significant difference by Duncan's multiple range test (P<0.05)

seeds under both the watering conditions. Under 350 µg g^{-1} of $CrCl_3$, Rb-1 stimulated germination 21.5 and 14.9% (with water and industrial effluent, respectively), whereas Rb-2 inoculation lead to 29.3 and 18.9% stimulation in seed germination with water and industrial effluent respectively, when compared to non-inoculated treatment. Effects of hexavalent chromium on the germination of wheat seeds were more pronounced as compared to trivalent chromium. At 350 µg g^{-1} of Cr(VI) treatment, Rb-2 showed significant enhancement (44.2 and 31.1% with water and industrial effluent, respectively) in percentage seed germination of *T. aestivum,* while Rb-1 showed 24.9 and 20% (with water and industrial effluent, respectively) increase in seed germination over non inoculated treatment. Mixed culture inoculation of Rb-1 and Rb-2, nevertheless enhanced (5.8–24.9%) germination over non inoculated treatment but germination parameter was hindered with combined inoculation when compared with single inoculation of two strains (Table 1).

Shoot length: Plant growth parameters were severely affected by the application of chromium (III and VI) salts but effect of Cr(VI) was more pronounced. Effects of Cr(VI) salt along with industrial effluent were more drastic and caused 50.96% reduction in shoot length when compared with control. Application of $CrCl_3$ resulted in comparatively less reduction in shoot length (18.88% and 29.58% with water and industrial effluent, respectively) than Cr(VI) salt (Table 1). Bacterial inoculation reduced the adverse effects of chromium salts by increasing the shoot length of wheat seedlings. Inoculation with Rb-1 as well as Rb-2 stimulated the shoot length (9.6% and 11.55%, respectively) of *T. aestivum* when seedlings were provided with distilled water over control. With industrial effluent, bacterization of Rb-1 revealed 1.12% reduction whereas Rb-2 resulted in marginal increase (5.63%) in shoot length of wheat seedling as compared to non-inoculated treatment. Inoculation with Rb-2 resulted in maximum stimulation in shoot length. With water, Rb-2 inoculation revealed 104.5% and 134.2% increases in shoot length under Cr(III) and Cr(IV) stress, respectively when compared with non- inoculated treatment. Among seedlings provided with industrial effluent, Rb-1 inoculation caused 45.9 and 83.7% enhancement in the shoot length under Cr(III) and Cr(IV) stress, respectively when compared with non-inoculated treatment. Mixed culture inoculation of Rb-1 and Rb-2 caused 5.2, 26.7 and 64% (with water) stimulation in shoot length under Cr free, Cr(III) and Cr(VI) treatment respectively over respective non inoculated treatments, while in case of wheat seedlings provided with industrial effluent, mixed culture inoculation resulted in 36.55, 32.8 and 15.8% increases in this parameter under Cr free, Cr(III) and Cr(VI) treatment, respectively when compared with non-inoculated treatments (Table 1). Shoot length was better than control with bacterial inoculation Rb-1 (all water conditions), Rb-2 (all water treatments, Cr(III) industrial effluent treatment) and mixed culture (Cr free industrial effluent).

Root length: Watering with industrial effluent along with

stress of hexavalent chromium proved to be more toxic towards the root length of wheat seedlings and caused 18.31% reduction when compared with control. Generally, root length was found to be stimulated with bacterial inoculation. Maximum increase in root length (74.1%) of wheat seedlings was attained by inoculation of Rb-2 with water under Cr(III) stress over un-inoculated treatment (Table 1). Application of both chromium (III and VI) salts adversely affected the root growth and caused 18.87 and 29.2% (with water and industrial effluent, respectively) reduction in root length of *T. aestivum* seedlings under Cr(III) and 32.97 and 36.62% (with water and industrial effluent, respectively) reduction under Cr(VI) stress when compared with control treatment. Inoculation with Rb-1 caused 24.9, 45.3 and 45.8% increases in root length of seedlings under Cr free, $CrCl_3$ and K_2CrO_4 stresses, respectively, as compared to non-inoculated treatments when seedlings were provided with distilled water. Whereas Rb-2 inoculation, caused 55.2, 74.1 and 43.7% (with water) stimulation in root length under Cr free, Cr(III) and Cr(VI) stresses, respectively, over un-inoculated treatments. Combined inoculation of Rb-1 and Rb-2 lead to 22, 37.5 and 43.7% (with distilled water) increases in root length under Cr free, Cr(III) and Cr(IV) stresses, respectively, over non-inoculated treatments. With industrial effluent, maximum increase in root length (73.4%) was observed under Cr(VI) stress when Rb-2 was given as inoculum to seeds of *T. aestivum.* Increases with Rb-2 inoculation were 60.2 and 45.4% under Cr free and Cr(III) stresses, respectively when compared with non-inoculated treatment. Inoculation of Rb-1 caused 10.8, 7.1 and 34% (with industrial effluent) enhancement in root length under Cr free, Cr(III) and Cr(IV) treatments, respectively, in comparison with un-inoculated treatments. Combined inoculation of Rb-1 and Rb-2*,* revealed enhancement in the root length of wheat seedling 26.5 and 21.2% (with industrial effluent) under Cr(III) and Cr(IV) stresses, respectively, when compared with non-inoculated treatments (Table 1). With all bacterial inoculations (except mixed culture at Cr(VI) and industrial effluent) root growth was better than control.

Number of roots: Application of chromium (III and VI) salts as well as industrial effluent increased the number of roots when compared with control. The enhancement in root parameter was more pronounced with distilled water treatments at Cr(III) and with industrial effluent at Cr(VI) (Table 1). Bacterial inoculation caused significant increase in number of roots under Cr stress conditions in comparison to non-inoculated treatments. Number of roots was relatively more with Cr(III and VI) treatments as well as bacterial inoculations (except Rb-1 in Cr free treatment) than that of control. Inoculation with Rb-1 resulted in marginal increases (5.5 and 7.6% with water and industrial effluent, respectively) in this parameter over respective un-inoculated treatments. Under Cr(III and VI) stress, inoculation of Rb-1 to wheat seedlings leads to marginal improvement in the number of roots. Increments in number of roots of wheat seedlings were 3.4 and 4.2% under Cr(III) and Cr(VI) stresses, respectively when seedlings were provided with water. While seedlings provided with effluent exhibited 3.3 and 9.7% augmentation under Cr(III) and Cr(VI) stresses, respectively as compared to non-inoculated treatments. Rb-2 inoculation augmented the number of roots under Cr free as well as Cr stress conditions. The increases were 22.2 and 5% under Cr free, 4.2 and 13.7% under Cr(III) stress, 1.4 and 19.2% under Cr(VI) stress with water and industrial effluent respectively over un-inoculated treatments. Combined inoculation of Rb-1 and Rb-2 either enhanced (1.3-16.6%) or had no effect on number of roots under Cr free as well as Cr stress conditions when provided with distilled water as well as industrial effluent. The increases were 16.6 and 8.3% (with water) under Cr free and Cr(III) stress respectively, whereas combined inoculation along with industrial effluent resulted in 10, 1.3 and 3.8% increases in number of roots of wheat seedlings under Cr free, Cr(III) and Cr(VI) stresses respectively, when compared with inoculum free treatments (Table 1).

Effect on Biochemical Parameters of *Triticum aestivum* var Inqlab-91 (Wheat)

Peroxidase content: Chromium (III and VI) treated seedlings of *T. aestivum* showed enhancement in the peroxidase content in comparison with control. Bacterial inoculation enhanced the peroxidase content of wheat seedlings when compared to non-inoculated treatment in chromium free as well as chromium supplemented conditions. Seedlings of *T. aestivum* provided with industrial effluent under Cr(VI) stress had higher peroxidase content and 47.49% increase in peroxidase content was observed when compared to control. Trivalent chromium caused 16.8 and 26.9% (with water and industrial effluent, respectively) increases in the peroxidase content when compared with control. Bacterial inoculation resulted in significant increases in the peroxidase content of wheat seedlings which were provided with effluent as compared to seedlings supplied with distilled water. Wheat seedlings inoculated with *P. aeruginosa* Rb-1 showed increased (37.4–74.9%) peroxidase content. Inoculation of Rb-1 caused 74.9 and 61.3% (with water and industrial effluent, respectively) increases in peroxidase content under Cr free conditions, whereas 43.4 and 42.3% (with water and industrial effluent, respectively) increases in peroxidase content under Cr(III) stress. Under Cr(VI) stress, the inoculation of *P. aeruginosa* Rb-1 caused 61.5 and 37.4% (with water and industrial effluent, respectively) stimulation in this parameter over non-inoculated treatment. Inoculation with Rb-2 under Cr free conditions showed significant increase in peroxidase content (91.8 and 51.5% with water and with industrial effluent, respectively) when compared to inoculum free treatments. Rb-2 inoculation under trivalent chromium stress, caused 64.5 and 30.1% increases in peroxidase content (with water and industrial effluent, respectively), whereas under

Table 2: Effect of bacterial inoculation on auxin, soluble protein and chromium content of *T. aestivum* var Inqlab-91 seedlings at 0 and 350 µgg^{-1} of $CrCl_3$ and K_2CrO_4 concentrations under both watering conditions (distilled water and industrial effluent)

Parameter/Treatment			Control/Cr stress	Bacterial strains		
				Rb-1	Rb-2	Rb-1+Rb-2
Peroxidase content (µgg^{-1})	Distilled water	Control	16.74±0.11(a)	29.29±0.07(c)	33.11±0.19(d)	23.2±0.10(b)
		Cr(III)	19.55±0.05(a)	28.03±0.09(c)	32.16±1.39(d)	25.23±0.60(b)
		Cr(VI)	22.82±0.05(a)	36.85±0.37(d)	38.04±0.33(c)	30.82±1.24(b)
	Industrial effluent	0(Cr)	13.75±0.03(a)	20.83±0.77(d)	22.18±0.41(c)	18.66±0.50(b)
		Cr(III)	17.46±0.02(a)	22.71±1.45(d)	24.84±0.35(c)	19.2±0.04(b)
		Cr(VI)	20.28±0.11(a)	27.87±0.07(c)	35.98±0.58(d)	23.02±0.52(b)
Auxin content (µgg^{-1})	Distilled water	Control	0.67±0.02(a)	1.15±0.04(b)	1.65±0.05(c)	1.02±0.03(b)
		Cr(III)	1.51±0.05(a)	1.99±0.06(b)	2.47±0.08(c)	1.77±0.06(ab)
		Cr(VI)	2.37±0.08(a)	3.11±0.10(b)	3.42±0.11(c)	2.74±0.09(ab)
	Industrial effluent	0(Cr)	0.54±0.02(a)	1.11±0.04(b)	1.43±0.05(c)	1.15±0.03(b)
		Cr(III)	1.49±0.04(a)	2.08±0.03(b)	2.20±0.06(b)	1.74±0.04(a)
		Cr(VI)	2.03±0.04(a)	2.63±0.05(c)	2.45±0.05(bc)	2.25±0.05(b)
Soluble protein content (µgg^{-1})	Distilled water	Control	320.69±0.77(a)	394±0.95(c)	456.5±1.10(d)	373.42±0.90(b)
		Cr(III)	414.27±0.99(a)	456.95±1.10(c)	481.01±1.15(d)	433.15±1.04(b)
		Cr(VI)	571.32±1.37(a)	634.03±1.52(c)	602.56±1.45(d)	585.87±1.41(b)
	Industrial effluent	0(Cr)	306.28±0.73(a)	342.6±0.82(c)	408±0.98(d)	322.28±0.77(b)
		Cr(III)	405.19±0.97(a)	433.73±1.04(c)	466.2±1.12(d)	424.62±1.02(b)
		Cr(VI)	534.08±1.28(a)	575.65±1.38(c)	617.14±1.48(d)	551.54±1.32(b)
Chromium content (mg kg^{-1})	Distilled water	Control	0.00±0.00(a)	0.00±0.00(a)	0.00±0.00(a)	0.00±0.00(a)
		Cr(III)	730±0.02(d)	360±0.01(b)	180±0.04(a)	430±0.03(c)
		Cr(VI)	1210±0.03(d)	220±0.04(b)	130±0.06(a)	290±0.02(c)
	Industrial effluent	0(Cr)	100±0.08(d)	70±0.01(b)	20±0.04(a)	70±0.08(c)
		Cr(III)	850±0.08(d)	480±0.05(b)	210±0.00(a)	680±0.01(c)
		Cr(VI)	1310±0.03(d)	830±0.03(c)	170±0.02(a)	720±0.09(b)

Highlighted areas indicate control treatments. Mean of 32 values ± standard error of the mean. In each row, figures followed by different letter (s) in parenthesis indicate significant difference by Duncan's multiple range test (P<0.05)

hexavalent chromium stress, bacterization with Rb-2 caused 66.7 and 77.4% (with water and industrial effluent, respectively) increases in peroxidase content of wheat seedling when compared with non-inoculated treatment (Table 2).

T. aestivum seedlings which were inoculated with mixed culture of Rb-1 and Rb-2 showed higher peroxidase content as compared to control. The increments in peroxidase content by mixed culture were 8.6 and 35.8% (with water and industrial effluent, respectively) under Cr free conditions, 29.1 and 9.7% (with distilled water and industrial effluent, respectively) under Cr(III) stress, 35 and 13.5% (with water and industrial effluent, respectively) after Cr(VI) treatment, when compared with non-inoculated treatment (Table 2).

Auxin content: Considerable increase in the auxin content of wheat seedlings was observed with bacterial inoculation as compared to non-inoculated treatment (Table 2). Cr (III and VI) stress resulted in more auxin content in *T. aestivum* seedlings as compared to control. The increases in auxin content were 125.37 and 175.93% (with water and industrial effluent, respectively) under Cr(III) stress and 253.73 and 275.93% (with water and industrial effluent, respectively) under Cr(VI) stress when compared to control (Table 2). Under Cr free conditions, wheat seedlings inoculated with Rb-2 manifested marked 146.3 and 164.8% (with water and industrial effluent, respectively) increases in auxin content as compared to non-inoculated treatment. At 350 µg g^{-1} of $CrCl_3$, inoculation with Rb-1 led to 31.8 and 39.6% (with water and industrial effluent, respectively) increases in auxin content while Rb-2 inoculation caused 63.6 and 47.6% (with water and industrial effluent, respectively) increment in auxin content of wheat seedlings as compared to inoculum free treatment. Mixed culture inoculation of Rb-1 and Rb-2 resulted in significant 17.2 and 16.7% (with water and industrial effluent, respectively) increments in auxin content of wheat seedlings when compared to un-inoculated treatments. Under 350 µg g^{-1} of K_2CrO_4 stress, significant increases in auxin content were observed with Rb-2 inoculation (43.4 and 29.5% with water and industrial effluent, respectively). Whereas Rb-1 inoculation exhibited 31.2 and 20.7% increases in auxin content with water and industrial effluent, respectively. Mixed culture inoculation of Rb-1 and Rb-2 revealed 15.6 and 10.8% increment in auxin content with water and industrial effluent, respectively as compared to non-inoculated treatments (Table 2).

Protein content: Generally, increment in protein content was observed in all the inoculated seedlings but Rb-2 inoculation exhibited significant enhancement in protein content (42.3 and 33.2% with water and industrial effluent, respectively) in comparison to non-inoculated treatments. Cr(III) and Cr(VI) stresses caused increase in the protein content of wheat seedling as compared to control. At 350 µg g^{-1} of $CrCl_3$, inoculation of wheat seedling (mono and mixed culture) considerably enhanced the protein content under both the watering conditions when compared with non-

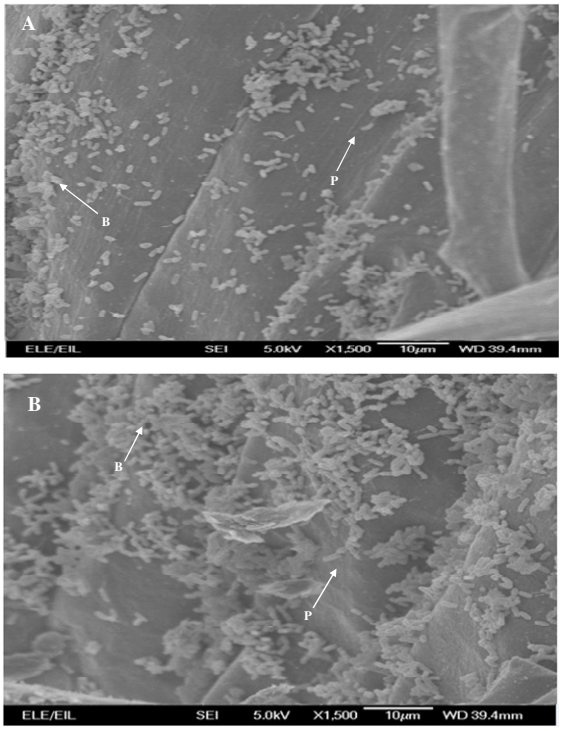

Fig. 1: Scanning electron microscopy of wheat root sample exhibiting surface distribution of bacteria after co-incubation with **(A)** *P. aeruginosa* Rb-1 **(B)** *O. intermedium* Rb-2 (B, bacteria; P, plant root surface)

inoculated treatments (Table 2). Bacterial inoculation (single as well as mixed culture inoculation) caused increase in protein content of *T. aestivum* seedlings at 350 µg g^{-1} of K_2CrO_4 stress. Rb-1 inoculation showed 10.9 and 15.5% (with water and industrial effluent, respectively) increases, while Rb-2 inoculation exhibited 5.46 and 20.7% (with water and industrial effluent, respectively) increases in protein content of *T. aestivum* seedlings in comparison with non-inoculated treatment. Mixed culture inoculation (Rb-1 and Rb-2) caused 2.5 and 3.3% (with water and industrial effluent, respectively) increases in protein content when compared to non-inoculated treatments (Table 2).

Chromium content (mg Cr kg $^{-1}$ dry weight): Generally bacterial inoculation caused reduction in chromium uptake by wheat seedlings. Amount of Cr taken up by seedlings grown under Cr(VI) stress was more in comparison with the seedlings grown under Cr(III) stress (Table 2). The amount was found to be 730 mg kg^{-1} (with distilled water) and 850 mg kg^{-1} (with industrial effluent) under Cr(III) stress and 1210 mg kg^{-1} (with distilled water) and 1310 mg kg^{-1} (with industrial effluent) under Cr(VI) stress. Monoculture as well as mixed culture inoculation exhibited significant decreases in the chromium content taken up by the seedlings under Cr(III) and Cr(VI) stress, when provided with water and industrial effluent as compared to non-inoculated treatments (Table 2). Maximum decrease in chromium uptake by the seedlings was caused by inoculation of *O. intermedium* Rb-2. The amount of Cr taken up were 180 mg kg^{-1} and 210 mg kg^{-1} (with water and industrial effluent, respectively) under Cr(III) and 130 mg kg^{-1} and 170 mg kg^{-1} (with water and industrial effluent, respectively) under Cr(VI) stress when compared with non-inoculated treatments (Table 2).

Electron Microscopy

SEM revealed the even distribution of Rb-1 throughout the root surface of inoculated wheat seedling. It preferred to attach in the form of clusters on the root surface rather than singly (Fig. 1A). Rb-2 exhibited significant colonization potential with wheat roots and found to be associated in the form of group of two or three cells to root surface (Fig. 1B).

Discussion

Chromium is a toxic heavy metal causing severe damage to all living organisms as Cr(VI). In current study, Cr(VI) resistant bacterial strains were used for pot experiment. These bacterial strains were used as inoculum for wheat seeds, which were sown in Cr(III) and Cr(VI) contaminated soil for screening of their plant growth promoting and chromium removal abilities. Bacterization with chromate reducing Rb-1 and Rb-2 (single and mixed culture inoculation) caused improvement in the growth and biochemical attributes of *T. aestivum* under chromium stress in both watering conditions. Inoculated seeds were germinated under stress of two different chromium salts (350 µg $g^{-1}$of $CrCl_3$ and K_2CrO_4) axenically. In plant microbe interaction experiment, seedlings were provided with distilled water and industrial effluent.

Treatment with both salts of chromium ($CrCl_3$ and K_2CrO_4) had adverse effects on the seed germination as well as on growth parameters of *T. aestivum* under both watering conditions. Hexavalent salt had more adverse effects than trivalent salt. Adverse effects of chromium salts on the seed germination have been reported by many workers (Sabri and Hasnain, 1997; Faisal *et al.,* 2005). Similarly, reduction in seed germination of the weed *Echinochloa colona* was 25% with 200 mol L^{-1} Cr (Nagajyoti *et al.,* 2010). Previously, Faisal and Hasnain (2005b) and Fozia *et al.* (2008) reported the reduced seed germination of sunflower due to toxic effects of chromium salt. The reduced germination of seeds could be related to less activity of amylase and successive transport of sugars to the embryo axes and increased protease activity under chromium stress (Nagajyoti *et al.,* 2010). Bacterization of wheat seedlings under both watering conditions significantly enhanced the seed germination (up to 99%) under Cr free as well as Cr stress conditions. Improvement in seed germination is associated with ability of bacteria to produce auxin and solublize phosphate (Sharma *et al*., 2007). Enhancement in percentage germination of bacterial inoculated seeds has been reported by many workers (Afrasyab *et al.,* 2001; Faisal and Hasnain,

2005b; He *et al.,* 2008; Cassán *et al.,* 2009). Inoculation with Rb-2 showed maximum stimulation in seed germination in studied crop under both watering conditions after Cr free, Cr(III) and Cr(VI) treatment as compared to non-inoculated treatment as well as control.

Chromium stress adversely affected the growth attributes of *T. aestivum*. Plant growth parameters were severely affected with Cr salts when compared with Cr-free treatments under both watering conditions. Bacterial inoculations alleviate the toxic effects of chromium salts on length parameters of wheat seedlings under both watering conditions as compared to non-inoculated treatments. Many bacterial species are found to promote the plant growth under heavy metal stress conditions (Sabri and Hasnain, 1997). Rb-1 and Rb-2 stimulated the root and shoot length under both watering conditions in single as well as mixed culture inoculation as compared to non-inoculated treatment. One of the pronounced effects of heavy metals in crops is the reduction of root growth (Fozia *et al.,* 2008). Chromium compounds cause inhibition of root cell division/elongation or extension of the cell cycle, enhancement of growth of root hairs and increment of relative fraction of pith and cortical tissue layers (Chen *et al.,* 2010). Plant growth promoting rhizobacteria stimulates plant growth by producing phytohormones, escalating the availability and uptake of nutrients, decreasing heavy metal toxicity and antagonizing plant pathogens (Gholami *et al.,* 2009). Chromate salts are highly soluble and readily taken up by plant (Nath *et al.,* 2008). Number of roots per seedling was increased under Cr(VI) stress in both watering conditions.The roots were darker in color and their texture become hard and stiff due to binding of Cr(VI) on the cell wall of roots. Increment in number of roots and root hairs under stress conditions is one of the methods adapted by plants to cope up with stress environment (Kahle, 1993; Suseela *et al.,* 2002).

Seedlings of *T. aestivum* grown under Cr(VI) stress had shown more chromium accumulation under both watering conditions. Plants growing in metal enriched environments usually take up metals in varying amounts in response to external and internal factors (Rajkumar and Freitas, 2008). Henriques (2010) reported that Cr(VI) is considered more toxic to plants due to its strong oxidizing potential and high mobility. Chromium, primarily Cr(VI), brings on intense disturbances in various aspects of plant metabolism and physiology, mainly in the plant water, mineral nutrient balance, mitochondrial and photosynthetic electron transport chains and respiration as well as photosynthesis. Bacterial single as well as mixed culture inoculation caused reduction in amount of chromium uptake in *T. aestivum* under both watering conditions as compared to their non-inoculated treatments. In fact, chromium reducing bacteria have the ability to reduce Cr(VI) into Cr(III) in soil adjacent to the root so making it unavailable to plants for absorption. Previously, Faisal and Hasnain (2005b) also found that rhizobacteria reduced most of the toxic Cr(VI) into less toxic Cr(III) supplied to sunflower seedlings.

Table 3: P values of multifactorial analysis (ANOVA) of different factors used

INTERACTIONS	Growth parameters	Biochemical parameters
Watering conditions	0.000	0.000
Inoculation	0.000	0.000
Cr- levels	0.000	0.000
Watering conditions X Inoculation	0.803	0.000
Watering conditions X Cr- levels	0.716	0.000
Inoculation X Cr- levels	0.000	0.000
Watering conditions X Inoculation X Cr- levels	0.075	0.000

P values < 0.05 represent significant difference/interaction

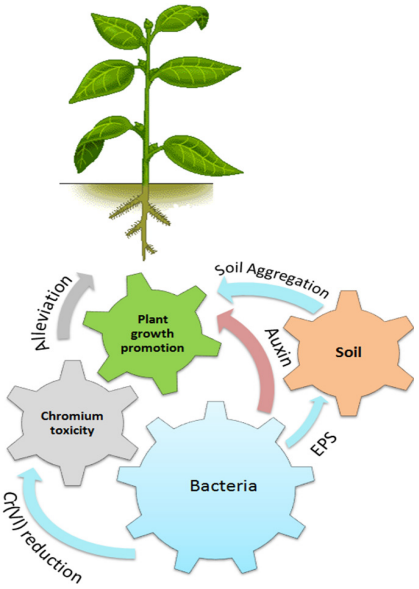

Fig. 2: Interaction between the bacteria, chromium and plants

Plant growth promoting rhizobacteria can improve plant growth and development either indirectly by lessen the adverse effects of heavy metals or directly by production of the plant growth regulatory substances called phytohormones (Wani *et al.,* 2007). Phytohormones regulate plant growth by modifying physiological and morphological processes at very low concentrations (Babalola, 2010). Many bacteria are able to produce phytohormones including auxin, cytokinins, gibberellins and ethylene. Auxin is regarded as major hormone involving in regulation of plant development including organogenesis, cell expansion, division and differentiation (Saharan and Nehra, 2011). The highest concentration of auxin is reported in growing tips of plants and in young and healthy root nodules (Mandal *et al.,* 2007). Auxin content was significantly enhanced in *T. aestivum* seedlings under chromium (III and VI) stress under both watering conditions when compared to control. Faisal *et al.* (2005) also reported increased auxin activity under chromium stress. Bacterial strains stimulated the auxin content in both the watering conditions under chromium stress as well as Cr free conditions. Stimulation of plant growth due to augmentation of auxin content as a result of

bacterial inoculation has been reported earlier (Çakmakçı *et al.,* 2007). Peroxidases are intracellular enzymes, which catalyze the peroxidase dependent oxidation of variety of organic and inorganic substances. Bacterial inoculation caused stimulation in activity of peroxidase (Munir *et al.,* 2003). Peroxidase activity of the *T. aestivum* found to be increased under chromium stress with both the watering conditions when compared to non-inoculated treatments as well as control but the impact of industrial effluent under Cr(VI) stress was more pronounced. Stress conditions cause enhancement in activities of various enzymes. Peroxidase activity is reported to be enhanced with increasing chromium stress conditions in wheat plants. Increased peroxidase activity can bring about variation in cell wall, which may be related to a decline in plant growth rate (Ganesh *et al.,* 2008).

Protein content was significantly increased in *T. aestivum* and under chromium (III and VI) stress in both watering conditions as compared to non-inoculated treatments as well as control. Heavy metals stress resulted in the production of new proteins and inhibition of cellular proteins synthesis. The proteins produced as a response of heavy metal stress might be involve in synthesis of membrane stabilization protein (Misra and Tandon, 2009). Increased protein content in seedlingsof *T. aestivum* under Cr(VI) stress has been reported earlier by Faisal *et al.* (2005). Efficient plant root colonization by the plant growth promoting rhizobacteria is one of the key factors in plant growth promotion. The qualitative and quantitative difference in production of root exudates by the crop determines the root colonization potential of the bacteria (Hassen and Labuschagne, 2010).

Significant impact of watering conditions, inoculation and Cr-levels on growth as well as on biochemical parameters of wheat was observed. Multifactorial analysis of different factors revealed significant interaction as shown in Table 3. On the whole, watering with industrial effluent had more drastic effects and caused significant reduction in some growth attributes and marked increases in biochemical parameters of the studied crop. Bacterial inoculation (mono and mixed culture) caused enhancements in these parameters by reducing toxicity of Cr (III and VI) salts and yielding better than the non-inoculated treatments. As industrial effluent contains variety of heavy metals and nutrients in excessive amount and thus having variable effects on growth and development of plants (Khan *et al.,* 2011). Monoculture of Rb-1 and Rb-2 showed more competence in elevating the toxic effects of Cr(III and VI) on wheat seedlings than the mixed culture inoculation. This may be due to the possibility that when the bacterial strains are grown together they produced certain type of metabolites that interact with each other and have negative impact on growth of plant. Curtailed effect of mixed culture inoculation on plant growth has previously reported by Afrasyab *et al.* (2001) and Batool and Hasnain (2005). It is obvious from these results that Rb-2 performed more efficiently as compared to control with wheat provided either with distilled water or with the effluent. As plant growth promoting bacteria have potential to attract a variety of microbes through the production of range of root exudates thus favoring the microbial growth and lessening the phytotoxic effects of chromium (Gautam and Pandey, 2008; Medhi *et al.,* 2011).

Conclusion

The findings of the present study revealed that both bacterial strains Rb-1 and Rb-2 capable of surviving in chromium (III and VI) contaminated environment could be very useful in improving growth of wheat along with lessening the toxicity of chromate by various direct and indirect mechanisms (Batool *et al.,* 2012; Fig. 2). Further studies are needed to explore the possible mechanism for plant growth promotion under stress conditions.

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