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Chromium-resistant bacteria and cyanobacteria: impact on Cr(VI) reduction potential and plant growth

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Abstract Two chromium-resistant bacterial strains, *Bacillus cereus* S-6 and *Ochrobactrum intermedium* CrT-1, and two cyanobacterial strains, *Oscillatoria* sp. and *Synechocystis* sp., were used in this study. At initial chromate concentrations of 300 and 600 μg K_2CrO_4 mL^{-1}, and an inoculum size of 9.6×10^7 cells mL^{-1}, *B. cereus* S-6 completely reduced Cr(VI), while *O. intermedium* CrT-1 reduced Cr(VI) by 98% and 70%, respectively after 96 h. At 100 μg K_2CrO_4 mL^{-1}, *Synechocystis* sp. MK(S) and *Oscillatoria* sp. BJ2 reduced 62.1% and 39.9% of Cr(VI), respectively, at 30°C and pH 8. Application of hexavalent chromate salts adversely affected wheat seedling growth and anatomical characters. However, bacterial inoculation alleviated the toxic effects, as reflected by significant improvements in growth as well as anatomical parameters. Cyanobacterial strains also led to some enhancement of various growth parameters in wheat seedlings.

Keywords *Bacillus cereus* · Chromate reduction · Cyanobacteria · *Ochrobactrum intermedium* · *Triticum aestivum*

Introduction

Increases in material productivity result in the release of substances into the environment that are potentially harmful to living things. Heavy metals found in wastewaters are harmful to the environment and their effects on biological systems are very severe. Efficient and cheap treatments for their removal and for the reuse of spent metals from wastewater need to be developed. Microbe-based technologies can provide an alternative to conventional methods for metal removal [12]. Chromium, which is generated by various industries, occurs in different oxidation states but Cr(III) and Cr(VI) are the most significant. Trivalent chromium occurs naturally in the environment and is an essential nutrient for animals [3]. Hexavalent chromium is a well-known human carcinogen [7]. To avoid the toxic effects of Cr(VI), it is necessary to convert it to Cr(III). Reduction of hexavalent chromium has been observed in many bacterial genera such as *Shewanella* [18] and *Desulfovibrio* [15]. The objective of the present study was to monitor the reduction of toxic Cr(VI) to the less toxic and immobile Cr(III). Microbe-based technology is not limited to waste treatment, and is now becoming important in plant growth improvements, hence the results of plant growth experiments are also reported here.

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Materials and methods

Strains and culture conditions

The two chromium-resistant bacterial strains, *Bacillus cereus* S-6 and *Ochrobactrum intermedium* CrT-1, used in this study were highly resistant to chromate: up to 40 mg K_2CrO_4 mL^{-1} on nutrient agar [27] and up to 10 mg K_2CrO_4 mL^{-1} in acetate-minimal medium [21]. Two cyanobacterial strains, *Oscillatoria* sp. (filamentous) and *Synechocystis* sp. (unicellular), were also able to tolerate up to 200 μg K_2CrO_4 mL^{-1} on BG 11 medium [24].

Cr(VI) reduction experiments

For bacterial hexavalent chromium reduction, two initial K_2CrO_4 concentrations (300 and 600 μg mL^{-1}) and two cell concentrations (2.4×10^7 and 9.6×10^7 cells mL^{-1}) were used. DeLeo and Ehrlich [6] medium (grams per litre: tryptone 10, yeast extract 5, NaCl 5, citric acid 1, Na_2HPO_4 6.9) was used for these experiments. Cultures were grown at 37°C at 150 rpm. Periodically (24, 48, 72

and 96 h), samples were drawn aseptically and analysed for Cr(VI) reduction following the procedure of DeLeo and Ehrlich [6]. To monitor Cr(VI) reduction in cyanobacteria, BG 11 medium at two initial K_2CrO_4 concentrations (100 and 200 mg mL^{-1}) was used. Cultures were harvested after 15- and 30-day incubation periods and Cr(VI) reduction was determined.

Germination experiments

Wheat (*Triticum aestivum* var. Inqlab-97) seeds were procured from NARC, Islamabad, Pakistan. Two chromate salts (trivalent chromium $CrCl_3$ and hexavalent K_2CrO_4; 300 μg mL^{-1}) were used. Surfaced-sterilised seeds were inoculated with bacterial cultures. Inoculum was prepared from overnight cultures suspended in 10 ml sterilised distilled water and adjusted to an absorbance of 1.2 at 600 nm for both strains to ensure equal numbers of bacteria for each inoculation. Uninoculated seeds were used as a control treatment. The experimental design was as described by Afrasayab et al. [1]. After 10 days, seedlings were harvested and different growth parameters were measured. For biochemical analysis, the activity of acid phosphatase was measured following the method of Iqbal and Rafique [11]. For the determination of chromium content, seedlings were dried at 80°C for 24 h and digested according to Humphries [10]. Estimation of chromium content was determined by the method of Rand et al. [23]. In case of cyanobacteria, 25-day incubated cultures (cells suspended in sterile water and OD adjusted as above) were used to inoculate surface-sterile seeds, which were subsequently grown as described above. Growth parameters only are reported; further work is in progress. For each experiment, four replicates of 25 seeds were performed.

Anatomical observations

Some of the germinated seedlings were processed for microtomy [28]; 1 cm samples of roots and leaves were embedded in Paraffin wax and transversely sectioned (10–12 μm). The effects of bacterial or chromate application on the internal anatomy of seedlings of *T. aestivum* were observed under a compound microscope.

Statistical analysis

Standard errors of the means and least squares difference (LSD) were calculated following Steel and Torrie [29].

Results

Cr(VI) reduction

At an initial Cr(VI) concentration of 300 μg mL^{-1}, *B. cereus* S-6 was able to reduce all the chromate within 72 h, while at 600 μg mL^{-1}, complete reduction was accomplished after 96 h. Strain *O. intermedium* CrT-1 was able to reduce 98% and 70% of Cr(VI) at initial Cr(VI) concentrations of 300 and 600 μg mL^{-1}, respectively, after a 96-h incubation period (Fig. 1a). The rate of reduction was much faster during the first 24 h, but with the passage of time it decelerated. Controls showed no change in the Cr(VI) concentration from the initial to the final stage of the experiment [i.e. no evidence of spontaneous Cr(VI) reduction]. No major difference in the reduction capability of either strain was observed at the two initial inoculum sizes used, although small differences were observed at high initial Cr(VI) concentration (600 μg mL^{-1}). In cyanobacteria, the Cr(VI) reduction potential of both *Synechocystis* sp. MK(S) (62.14%) and *Oscillatoria* sp. BJ2 (39.99%) strains was maximal at 30°C and pH 8 after 30 days of incubation (Fig. 1b). In both strains, there was a gradual decrease in reduction potential upon increasing the concentration and time period (data not shown).

Plant growth experiments

Application of either chromate salt caused a significant reduction in seedling length (Table 1). A significant decrease (159.2%) in seedling length was observed at 300 μg mL^{-1} K_2CrO_4; trivalent chromium was less toxic, with a decrease in seedling length of ~18.9% compared to the control. Bacterial inoculation caused enhancement of seedling length under either $CrCl_3$ or K_2CrO_4 treatment. Maximum enhancement (49%) was observed with strain *B. cereus* S-6 at 300 μg K_2CrO_4 mL^{-1} compared to the corresponding non-inoculated control. The activity of acid phosphatase increases under metal stress. Increases in acid phosphatase activity of wheat seedlings of 230% and 49% were observed upon application of 300 μg mL^{-1} of $CrCl_3$ and K_2CrO_4, respectively, as compared to controls. Inoculation of strains *B. cereus* S-6 and *O. intermedium* CrT-1 caused increments of 55.6% and 18% in the activity of acid phosphatase as compared to the corresponding non-inoculated controls (Table 1). Under chromium stress, the auxin content increased as compared to controls. In the absence of chromium, all bacterial inoculations caused an increase in auxin content of wheat seedlings as compared to the respective non-inoculated controls, with the maximum increment (63%) being observed in seedlings inoculated with *B. cereus* S-6 (Table 1). Both strains also caused some enhancement in auxin content even under chromium stress when compared with non-inoculated controls. As can be seen in Table 1, uptake of hexavalent chromium was much higher as compared to trivalent chromium. Bacterial strains significantly hindered the uptake of chromate, especially in the case of hexavalent chromium.

The cyanobacterial strains *Oscillatoria* sp. and *Synechocystis* sp. both caused a significant improvement in seedling length (Table 2). With *Oscillatoria* sp. an

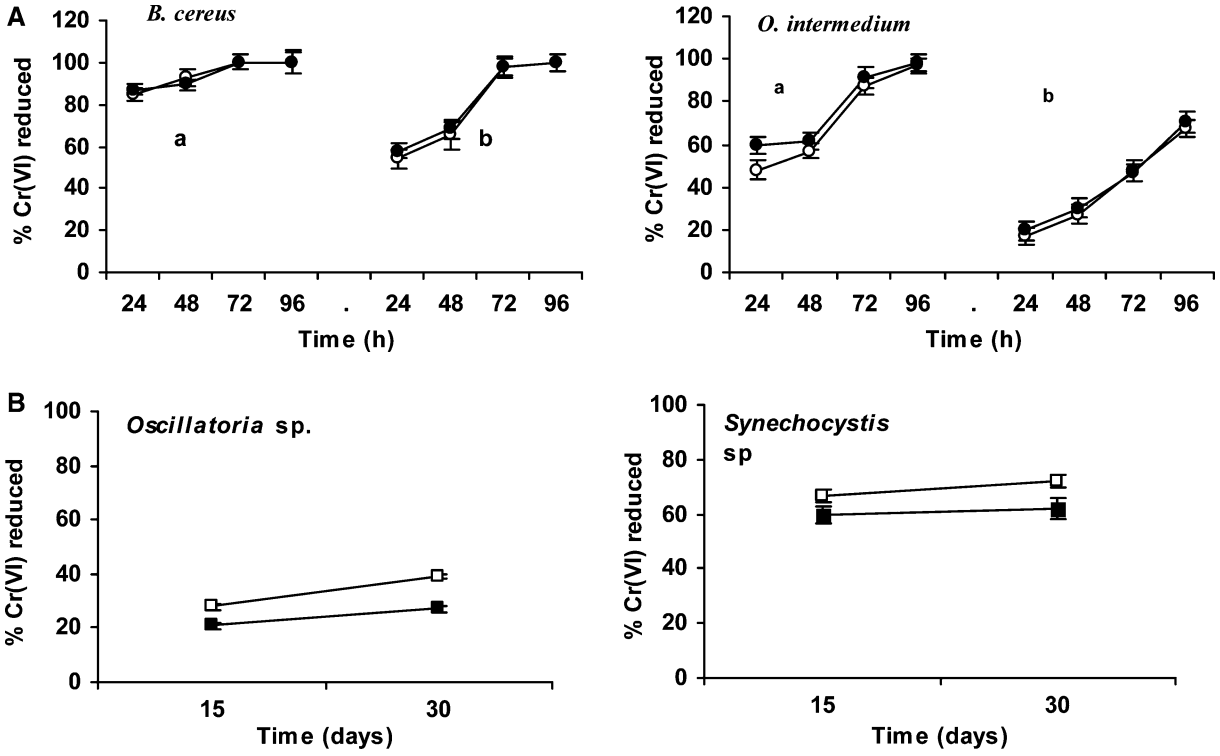

Fig. 1 **a** Reduction of K_2CrO_4 by *Bacillus cereus* S-6 and *Ochrobactrum intermedium* CrT-1 at two levels of chromate. Reduction was monitored after 24, 48, 72 and 98 h of growth incubation. Initial inoculation: *Open circles* 2.4×10^7 cells mL^{-1}, *filled circles* 9.6×10^7 cells ml^{-1}. *a* Cr(VI) 300 μg mL^{-1}, *b* Cr(VI) 600 μg mL^{-1}. **b** Reduction of K_2CrO_4 by *Oscillatoria* sp. and *Synechocystis* sp. at two levels of chromate. Reduction was monitored after 15 and 30 days of growth incubation. Initial chromate: *Open circles* 100 μg mL^{-1} Cr(VI), *filled squares* 200 μg ml^{-1}Cr(VI)

increase of almost 18% was observed when compared to the non-inoculated control. Under hexavalent stress, the number of roots increased (30.1%) as compared to the control (Table 2). The number of leaves remained constant, both under chromium stress and cyanobacterial inoculation.

Anatomical aspects

Trivalent chromium stress (300 μg mL^{-1}) caused some contraction in the diameter of the internal region of roots in comparison to controls (Table 3). The stimulating effect of Cr(VI) salt was more obvious in the cortical regions. However, bacterial inoculation led to expansion of the diameter of the cortical region compared to the corresponding non-inoculated control (Table 3). *B. cereus* S-6 inoculation resulted in an increase in the cortex region of almost 22% compared to the non-inoculated control. Hexavalent chromium resulted in disintegration of root cells/tissues, while bacterial inoculation improved cell diameter by alleviating the toxic effect of Cr(VI). The same was observed with stomata, where hexavalent chromium severely damages the guard cells and trichomes. Inoculation of bacterial strains under Cr(VI) stress results in an improvement in the morphology of guard cells and epidermal appendages as compared to the corresponding control treatments (Fig. 2).

Discussion

The present work focussed on the reduction of toxic Cr(VI) to the less toxic Cr(III) by two bacterial strains, *O. intermedium* CrT-1 and *B. cereus* S-6, and two cyanobacterial strains, *Oscillatoria* sp. and *Synechocystis* sp., which showed high-level resistance to chromate. The chromate resistance level of these strains is very high in minimal medium relative to strains reported by other workers. Chromium-resistant bacteria isolated from tannery effluents could resist up to 250 μg mL^{-1} hexavalent chromium in the medium [4]. Megharaj et al. [17] observed that strains isolated from chromium-contaminated soil could grow at concentrations of Cr(VI) up to 100 μg mL^{-1} in minimal medium. The strains reported here could grow at 10 mg K_2CrO_4 mL^{-1} (2.68 mg Cr mL^{-1}) in acetate-minimal medium. Strains *O. intermedium* CrT-1 and *B. cereus* S-6 both have the capacity to reduce high amounts of Cr(VI) into Cr(III). In both strains the rate of Cr(VI) reduction was rapid during the first 24 h, declining thereafter. The initial K_2CrO_4 concentrations used in this experiment were much higher than in others studies reported in the literature.

Table 1 Effect of inoculation of chromium-resistant bacteria on seedling length, auxin content, acid phosphatase, and chromium content of *Triticum aestivum* var. Inqlab-97 seedlings following application of $CrCl_3$ and K_2CrO_4 at concentrations of 0 and 300 μg mL^{-1} (means of four replicates). *LSD* Least squares difference

Strain	Seedling length (cm)			Auxin content (μg g^{-1})			Acid phosphatase (units g^{-1})			Chromium content (mg g^{-1} dry weight)		
	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4
Control	25.4 ± 0.98	20.6 ± 1.30	9.8 ± 0.57	0.73 ± 0.041	1.67 ± 0.038	2.68 ± 0.037	72 ± 3.45	105 ± 6.34	238 ± 5.34	0.0 ± 0	0.368 ± 0.021	2.85 ± 0.10
Bacillus cereus	35.9 ± 0.89	30.6 ± 0.98	14.4 ± 0.75	1.18 ± 0.057	2.20 ± 0.047	3.40 ± 0.078	112 ± 5.48	139 ± 4.57	283 ± 4.56	0.0 ± 0	0.387 ± 0.014	1.42 ± 0.067
Ochrobactrum intermedium	30.8 ± 1.20	24.6 ± 1.02	12.6 ± 0.48	0.93 ± 0.034	1.92 ± 0.054	3.17 ± 0.067	85 ± 4.59	109 ± 5.21	258 ± 0.61	0.0 ± 0	0.360 ± 0.024	2.48 ± 0.045
LSD at 0.05	4.40			0.21			10.6			34.4		

Table 2 Effect of inoculation of chromium-resistant cyanobacteria on seedling length, number of roots and number of leaves of *T. aestivum* var. Inqlab-97 seedlings at 0 and 150 μg mL^{-1} $CrCl_3$ and K_2CrO_4 (means of four replicates)

Strain	Seedling length (cm)			Number of roots			Number of leaves		
	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4
Control	37.5 ± 2.14	32.2 ± 1.87	27.4 ± 3.54	3.75 ± 0.14	3.75 ± 0.15	4.8 ± 0.34	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Oscillatoria sp.	44.2 ± 2.34	38.5 ± 2.14	38.5 ± 1.89	4.88 ± 0.15	4.27 ± 0.47	5.0 ± 0.45	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Synechocystis sp.	39.9 ± 1.98	36.7 ± 2.01	35.7 ± 2.54	4.83 ± 0.21	4.24 ± 0.64	4.67 ± 0.47	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LSD at 0.05	4.44			0.82			0.00		

Table 3 Effect of inoculation of chromium-resistant bacteria on the internal anatomy of root and leaves of *T. aestivum* var. Inqlab-97 plants (15 days after germination) at 0 and 300 μg mL^{-1} $CrCl_3$ and K_2CrO_4 (means of four replicates)

	Diameter of cortex (μm)			Diameter of xylem (μm)			Diameter of epidermis (μm)		
Root									
Strain	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4
Control	247 ± 6.97	240 ± 5.97	252 ± 11.2	28.2 ± 1.54	27.4 ± 1.60	29.6 ± 2.4	12.8 ± 0.89	13.2 ± 1.20	14.3 ± 1.07
B. cereus	300 ± 7.54	293 ± 7.89	317 ± 7.68	40.8 ± 2.54	39.2 ± 1.47	41.7 ± 1.8	16.8 ± 1.02	15.8 ± 0.58	15.9 ± 0.67
O. intermedium	285 ± 5.98	279 ± 9.78	279 ± 9.57	35.2 ± 1.68	33.2 ± 1.38	34.6 ± 3.0	15.6 ± 0.79	15.9 ± 1.08	15.9 ± 0.57
LSD at 0.05	17.6			1.39			1.64		
	Diameter of epidermis (μm)			Diameter of xylem vessel (μm)			Diameter of phloem (μm)		
Leaf									
Strains	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4
Control	8.0 ± 0.87	7.0 ± 0.37	8.5 ± 0.47	10.0 ± 0.58	10.0 ± 0.87	12.0 ± 1.0	13.0 ± 0.98	12.0 ± 1.32	10.5 ± 0.57
B. cereus	9.4 ± 0.67	8.0 ± 0.58	9.30 ± 0.64	11.5 ± 0.47	10.5 ± 0.67	13.0 ± 1.3	13.8 ± 1.54	12.6 ± 1.24	11.5 ± 0.68
O. intermedium	9.5 ± 0.75	8.1 ± 1.0	9.0 ± 0.87	11.0 ± 0.34	11.0 ± 0.87	12.5 ± 0.87	13.5 ± 0.98	12.5 ± 1.0	11.0 ± 0.97
LSD at 0.05	0.67			0.86			0.29		

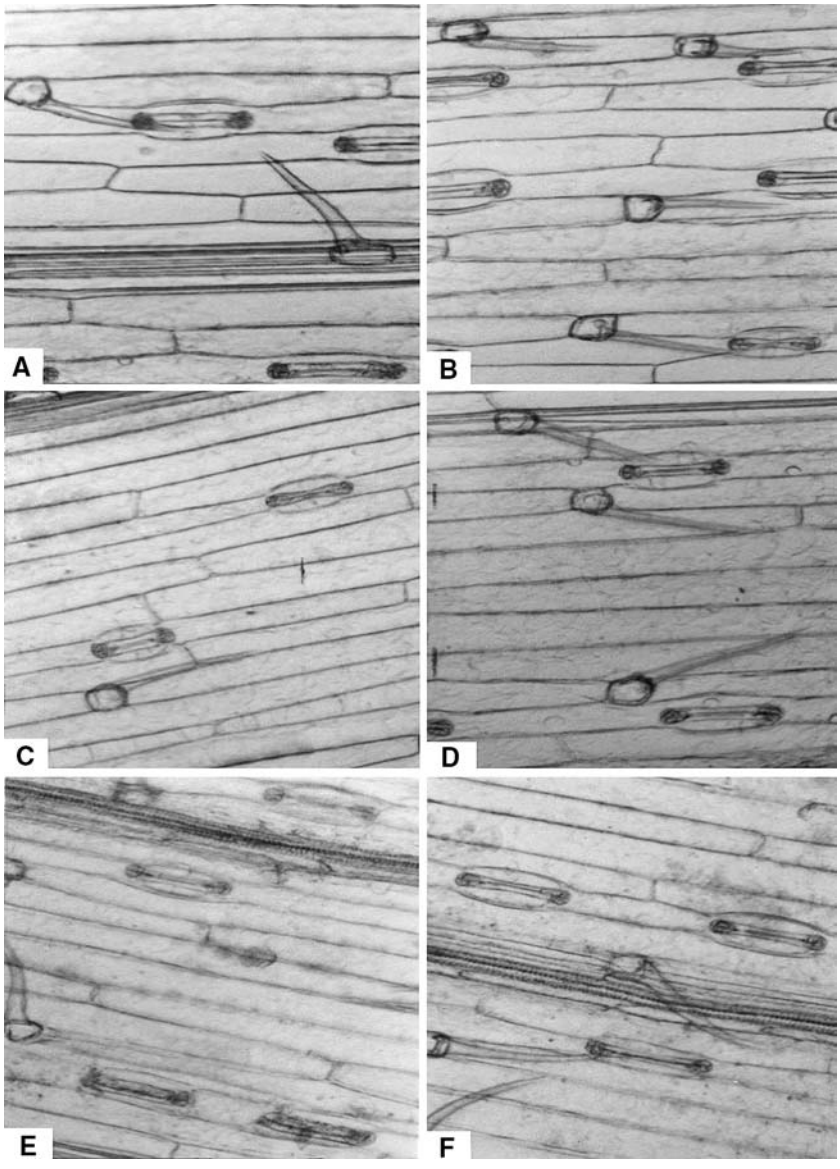

Fig. 2a–f Effect of bacterial strains and chromate salts on stomatal morphology of seedlings of *Triticum aestivum* var. Inqlab-97. **a** Control at 0 μg mL^{-1}, **b** inoculated with *B. cereus* S-6 at 0 μg mL^{-1}, **c** without inoculation at 300 μg mL^{-1} $CrCl_3$, **d** inoculated with *B. cereus* S-6 at 300 μg mL^{-1} $CrCl_3$; **e** without inoculation at 300 μg mL^{-1} K_2CrO_4; **f** inoculated with *B. cereus* S-6 at 300 μg mL^{-1} K_2CrO_4

Middleton et al. [18] observed that *Shewanella oneidensis* MR-1 could grow and reduce Cr(VI) when supplied with 100 μM Cr(VI) (5.2 μg mL^{-1} Cr) but was inhibited at a concentration of 150 μM.

In the present study, seedling length of *T. aestivum* was severely affected upon application of 300 μg mL^{-1} $CrCl_3$ and K_2CrO_4. The effects of hexavalent chromium were very severe when compared to controls. Many workers have also reported the adverse effects of chromium salt on seedling length [8, 25]. Inoculation of seeds with bacterial strains significantly enhanced seedling length when compared with non-inoculated controls. Nichols et al. [20] also observed that different growth parameters decreased in *Salvinia minima* under Cr(VI) stress. According to some reports, growth stimulatory bacteria release some chemotaxis chemicals to root exudates, which helps plants grow better. Rhizospheric bacteria, such as *Pseudomonas* spp., *Azospirillum* spp., and *Agrobacterium* spp., increase plant growth and nutrient uptake in maize, wheat and legumes [9]. The chromium content of *T. aestivum* seedlings increased upon hexavalent chromium stress as compared to trivalent chromium. Hexavalent chromium is considered the most hazardous form of chromium to animals and plants due to its solubility, mobility, and toxicity, as well as its carcinogenic and mutagenic properties. Bioavailability of Cr(VI) is largely a function of its ability to cross biological membranes, its powerful oxidizing capabilities [14], and its interference with electron transport in respiration and photosynthesis [13]. Cr(VI) uptake by *S. minima* (mg Cr g^{-1} dry weight) was reported to increase as the Cr(VI) concentration in the growth medium increased [20]. In the present study, upon application of 300 μg mL^{-1} Cr(VI), most bacterial inoculations resulted in a decrease in chromate uptake in *T. aestivum* seedlings as compared to the corresponding non-inoculated controls.

Organic substances, produced either endogenously or applied exogenously, that are capable of regulating plant growth are named plant growth regulators. They regulate growth by affecting physiochemical and morphological processes at very low concentration [2]. Several bacterial strains are capable of producing auxin, gibberellins, ethylene or abscisic acid [30]. Auxin appears to be a master hormone, exercising regulatory action over many other plant hormones. In the present study, all the bacterial strains used caused an increase in auxin content in inoculated seedlings compared with non-inoculated controls. High auxin activity has also been observed by many workers in chromium-treated seedlings [5, 8]. Enhancement of auxin content upon bacterial inoculation was also reported by Hasnain and co-workers [1]. Mutaftchier et al. [19] describe the action of growth hormones and explained that auxin improves plant growth through different mechanisms by combining with oligosaccharides, proteins, cell wall fragments and other biological components. Enzyme studies in *T. aestivum* seedlings revealed that the activity of acid phosphatase was affected in chromium stress. The activity of acid phosphatase is related to metal accumulation by the cell [16]. In the present study, a significant increase in acid phosphatase activity was observed under 300 μg mL^{-1} K_2CrO_4 as compared to controls. Most bacterial inoculation resulted in an increase in acid phosphatase activity as compared to non-inoculated controls. Increased acid phosphatase activity upon bacterial strain inoculation was also reported by Preneta et al. [22]. As reported by Saleh and Belisle [26], bacterial strains, in addition to stimulating the acid phosphatase activity of the inoculated plants, may secrete acid phosphatase. Hexavalent chromium caused disintegration of guard cells but bacterial inoculation prevented the damage caused by the chromate salt. These results represent new findings that have not previously been reported in the literature. Hence, from the above discussion, it is concluded that, along with many others factors (phytohormones production, nitrogen fixation, phosphorous solubilisation, etc.) chromium-resistant bacterial strains perk up *T. aestivum* plant growth by reducing the toxicity of hexavalent chromium Cr(VI), which is reduced by the bacterial strains into the less toxic and less mobile trivalent chromium Cr(III).

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