Growth improvements of Sunflower seedlings by Cr(VI)-resistant bacteria

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Abstract
In the present study, three chromium resistant bacterial strains (CrT-1, CrT-2, CrT-3) which could resist very high concentration of K_2CrO_4 (up to 40 mg ml^{-1} on nutrient agar plates and 10 mg ml^{-1} in acetate-minimal medium) were used to inoculate the sunflower seeds both as control and under chromium stress. Cr(VI) caused severe reduction in different growth parameters (seedling length, fresh weight, dry weight g^{-1} fresh weight) as compared to control, while bacterial inoculations improved different growth parameters both as control and under chromate stress when compared with non-inoculated respective controls. With respect to biochemical parameters, acid phosphatase and auxin content showed marked increment with bacterial inoculation both in chromium stress and unstressed condition. Uptake of chromium in inoculated plants decreased significantly as compared to non-inoculated control. Cr (VI) application also severely damages different plant cells/tissues but bacterial inoculation not only improves the growth and yields parameters but also prevent cell damages caused by the Cr (VI) salt.
Keywords: *Cr(VI); Ochrobactrum intermedium; Helianthus annuus; PGPR.*

INTRODUCTION

One of the most common polluting metals is chromium, arising from discharged effluents from leather tanning, electroplating, alloy preparation (Ozdemir *et al.*, 2005). Because of its widespread use and adverse impact on the environment, chromium is currently receiving increased interest from various national and international organizations (Valko *et al*., 2005). Besides its essentiality for normal glucose utilization, at high concentration it is problematic for both fauna and flora (Proctor *et al.*, 2002). It mostly occurs as Cr (III) and Cr (VI) in nature. Cr (VI) is more mobile and easily taken up by the plants and subsequently enters into the food chain (Amezcua-Allieri *et al*., 2005). Hence there must be some strategy, which limits its mobility and permeability. Several bacterial strains are now known that reduced Cr (VI) in to Cr (III) which in turn is less toxic (Faisal and Hasnain, 2004). Microbes in the rhizosphere interact with plants thereby affecting plant growth, enhancing mineral and water uptake, providing antibiotics to inhibit soil pathogens and producing plant growth regulators (Morgan *et al.*, 2005). The purpose of this study was to check the role chromium resistant bacteria in plant growth improvements through the reduction of toxic Cr (VI) to less permeable Cr (III).

MATERIALS AND METHODS

Bacterial strains and culture conditions: Three chromium resistant bacterial strains (CrT-1, CrT-2 and CrT-3) were used to inoculate the growth of *Helianthus annuus* in the laboratory experiments. These strains (CrT-1, CrT-2 and CrT-3) were isolated from wastewater of Tanneries, Dingarh, Pakistan (Faisal and Hasnain, 1999). Initially strains were isolated on nutrient agar plates supplemented with 1 mg ml^{-1} of K_2CrO_4. Colonies obtained were picked, purified and were taken to higher level of K_2CrO_4 both in nutrient agar as well as in acetate minimal medium

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(Pattanapipitpaisal *et al.*, 2001). Strains were normally stored at 4°C on nutrient agar plates amended with 40 mg ml^{-1} of K_2CrO_4. To confirm taxonomic identity of the strain, 16S rRNA gene sequencing analysis was carried out. DNA was extracted and a part of the 16S rRNA gene (500 bp) was amplified and the amplicon sequenced using fluorescent di-deoxy terminator cycle sequencing chemistry (Mills *et al.*, 2003). The extension product was then separated on an ABI PRISM® (automated DNA sequencer) and the data was compared to the MicroSeq® databases (ACCUGENIX™ Newark DE 19702).

Germination experiments: Certified seeds of sunflower (*Helianthus annuus* var SF-187) were obtained from National Agriculture Research Center, Islamabad, Pakistan. Seeds were surface sterilized in 5% sodium hypochlorite solution for 5 minutes and then thoroughly washed with sterilized glass-distilled water thrice. Two different chromate salts were used (trivalent chromium $CrCl_3$ and hexavalent K_2CrO_4, 300 μg ml^{-1}). For seeds inoculation freshly prepared overnight cultures were suspended in a 10 ml sterilized glass distilled water and optical density was adjusted to 1.2 at 600 nm for all the strains to ensure equal number of cells for each inoculation. Un-inoculated seeds were used as control treatment. Both inoculated and un-inoculated (control) seeds (20 seeds per plate) were spread uniformly on the filter papers in sterilized petri dishes. Seeds were kept in dark for germination. After germination, seedlings were provided with nutrient solution (Hewitt, 1963) supplemented with respective chromium salts ($CrCl_3$ and K_2CrO_4, 300 μg ml^{-1}) and were shifted to light with a 10 hours photoperiod. After 10 days, seedlings were harvested and different growth parameters (seedling length, fresh weight of seedlings, dry weight g^{-1} fresh weight of seedlings) were measured. For biochemical analysis, the activity of acid phosphatase was measured following Iqbal and Rafique (1986). Mahadevan method was followed for the extraction of auxin content of seedlings (Mahadevan, 1984). For the determination of chromium content *Helianthus annuus* seedlings were thoroughly rinsed with distilled water, placed in between filter papers to remove excess water and dried at 80°C for 24h. Dried material was crushed into small pieces and chromium analysis in the tissues was carried out by acid digestion method following Humphries (1975). Estimation of chromium content was determined following Rand *et al*. (1979).

Anatomical study: Some seedlings were processed for microtomy (Sanderson, 1994). For this 1 cm samples of root and shoot were fixed in paraffin wax and transversely sectioned (10-12 μm). Any changes posed by the bacterial strains and chromate application on the internal anatomy of seedlings were observed under microscope.

Chromate reduction: To check whether bacterial strains as well as *Helianthus annuus* seedlings or both were involved in the reduction of toxic hexavalent chromium into less bioavailable trivalent chromium, separate experiments were conducted. Seedlings were grown both as control and inoculated in the presence of 300 μg ml^{-1} of K_2CrO_4. After ten days, seedlings were harvested and the amount of Cr(VI) reduced was determined both in the remaining nutrient solution (initially supplemented with 300 μg ml^{-1} of K_2CrO_4) as well as in seedlings present in Petri dishes.

Statistical analysis: Standard errors of the means and LSD were calculated following Steel and Torrie (1981).

RESULTS

Bacterial strains: Three chromium resistant bacterial strains (CrT-1, CrT-2 and CrT-3) which could resist very high concentration of K_2CrO_4 (up to 40 $mgml^{-1}$ on nutrient agar plates and 10 mg ml^{-1} in acetate-minimal medium) were used to stimulate the growth of *Helianthus annuus* in the laboratory experiments. On the basis of 16S rRNA gene sequencing analysis (500 bp 16S rRNA), the isolate CrT-1 is identified as *Ochrobactrum intermedium* CrT-1 (Fig. 5).

Plant growth experiments: Seed germination was affected drastically under hexavalent chromium Cr(VI) stress while bacterial inoculation promoted seed germination over respective non-inoculated control (Table 1). Seedling length was severely affected by the application of chromate salt especially with the Cr(VI). About 3.9 and 66% reduction in seedling length was observed at 300 μg ml^{-1} of $CrCl_3$ and K_2CrO_4, respectively, over chromium free control. Bacterial inoculations stimulated seedlings length as compared to non-inoculated control. At 0 μg ml^{-1}, CrT-1 caused maximum stimulation where 27% increases were recorded as compared to its respective non-inoculated control (Table 1). Significant reduction in fresh biomass of seedlings was observed in case of Cr(VI) stress and about 6.72 and 52.7% decreases in this parameter was recorded at 300 μg ml^{-1} of Cr(III) and Cr(VI), respectively, over chromium free control

Table 1. Effect of inoculation of chromium resistant bacteria on germination and seedling length of *Helianthus annuus* var SF-187 at 0 and 300 μg ml^{-1} of $CrCl_3$ and K_2CrO_4. (Means of four replicates).

Strains	% Germination			Seedling length (cm)		
	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4
Control	100±0.0	92±8.9	80±7.2	18.1±2.7	17.4±2.8	6.06±1.5
CrT-1	100±0.0	100±0.0	92±1.6	23.0±3.4	22.4±3.3	9.30±1.98
CrT-2	100±0.0	100±0.0	91±2.5	22.0±3.3	21.4±3.1	7.84±1.72
CrT-3	100±0.0	100±0.0	89±2.8	22.3±3.34	21.8±3.12	7.52±1.78
LSD at 0.05						
For strain		3.50			1.44	
For treatment		1.45			0.58	

Table 2 Effect of inoculation of chromium resistant bacteria on fresh weight and dry weight g^{-1} fresh weight of *Helianthus annuus* var SF-187 seedlings at 0 and 300 μg ml^{-1} of $CrCl_3$ and K_2CrO_4. (Means of four replicates).

Strains	Fresh weight (g)			Dry weight g^{-1} fresh weight		
	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4
Control	4.46±0.24	4.16±0.20	2.11±0.15	0.120±0.03	0.185±0.08	0.242±0.12
CrT-1	5.96±0.31	5.76±0.29	3.16±0.20	0.118±0.01	0.151±0.05	0.163±0.06
CrT-2	5.76±0.29	5.61±0.27	2.94±0.18	0.123±0.03	0.167±0.06	0.151±0.05
CrT-3	5.68±0.30	5.68±0.28	2.81±0.17	0.114±0.02	0.159±0.05	0.131±0.03
LSD at 0.05						
For strain		0.32			0.00	
For treatment		0.12			0.00	

(Table 2). With bacterial inoculations some increment in fresh weight was recorded. Dry weight increased significantly under chromium stress as compared to control seedlings (Table 2). About 102% increment in dry weight was observed when the seedlings were grown under 300 μg ml^{-1} K_2CrO_4 as compared to control. Under hexavalent chromium stress bacterial inoculation especially CrT-3 resulted a decrease (46%) in dry weight in sunflower seedlings when compared to its respective non-inoculated control (Table 2).

Activity of auxin in *Helianthus annuus* seedlings increased at 300 μg ml^{-1} of $CrCl_3$ and K_2CrO_4 where almost 104 and 246% increases were recorded (Fig. 1). All bacterial strains promoted auxin content in sunflower seedlings when compared to their respective non-inoculated control (Fig. 1). Significant enhancement in the synthesis of this enzyme was observed in those seedlings which were inoculated with strains CrT-2 and CrT-3 where about 156 and 211% increments in auxin content, over respective non-inoculated control treatment, were observed (Fig. 1). Activity of acid phosphatase increased tremendously under chromium stress and especially in case of hexavalent chromium. About 119% and 199% increment in the activity of this enzyme was observed at 300 μg ml^{-1} of $CrCl_3$ and K_2CrO_4, respectively, when compared with control (Fig. 2). The uptake of chromium by the sunflower seedling was more in case of hexavalent chromium stress as compared to trivalent chromium (Fig. 3). Bacterial inoculations resulted low chromium uptake by the seedlings especially in case of hexavalent chromium stress. CrT-1 caused 33% inhibition in chromium uptake in seedlings as compared to its respective non-inoculated control (Fig. 3).

Anatomical aspects: About 18% and 102% reduction in the diameter of cortical region was observed at μg ml^{-1} of $CrCl_3$ and K_2CrO_4, respectively, when compared with chromium free control (Table 3a). In inoculated seedlings, some increment in cortical cells was observed. Adverse effects of chromium salts were also seen in vascular region. At 0 μg ml^{-1} of chromium, diameter of vascular bundle was 330 μm which was slightly altered with $CrCl_3$ and K_2CrO_4 stresses where it was 300 μm and 290 μm respectively (Table 3a). Bacterial inoculation resulted an improvement in the diameter of vascular bundle in all cases. Maximum increase in vascular bundle was observed in seedlings inoculated with strain CrT-1 where 15.5% increase over non-inoculated respective treatment, was observed. Internal morphology of shoot showed variables effects with the application of chromium salts on the various tissue parameters as compared to those of chromium free control (Table 3b). Under Cr(III) stress, strains CrT-1 (26.6%), CrT-2 (32.1%) and CrT-3 (39.3%) caused expansion in the diameter of cortical

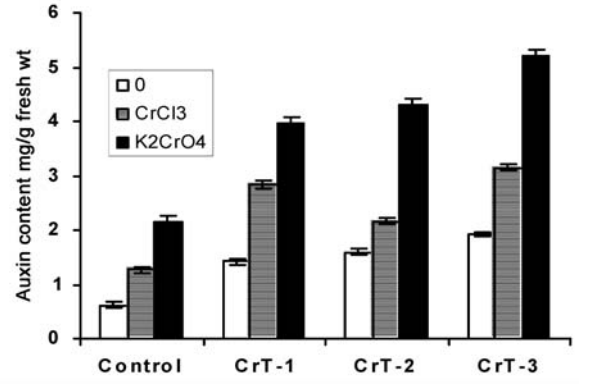

Figure 1. Effect of bacterial inoculation on auxin content of *Helianthus annuus* var SF-187 seedlings at 0 and 300 µg ml^{-1} of $CrCl_3$ and K_2CrO_4. (Means of four replicates).

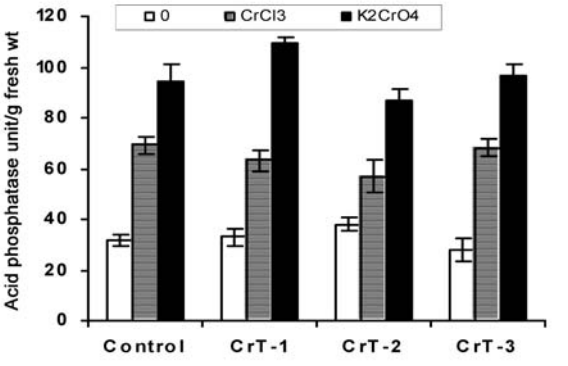

Figure 2. Effect of bacterial inoculation on acid phosphates of *Helianthus annuus* var SF-187 seedlings at 0 and 300 mg ml^{-1} of $CrCl_3$ and K_2CrO_4. (Means of four replicates).

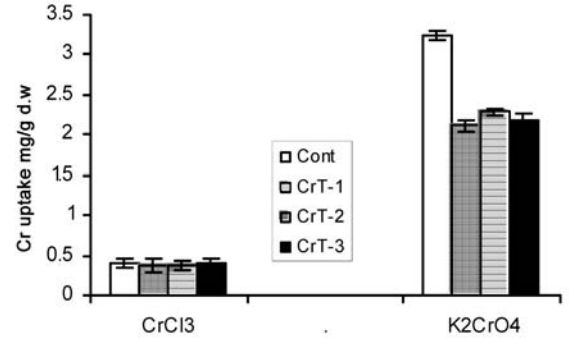

Figure 3. Effect of bacterial inoculation on chromium uptake in the *Helianthus annuus* var SF-187 seedlings at 300 µg ml^{-1} of $CrCl_3$ and K_2CrO_4. (Means of four replicates).

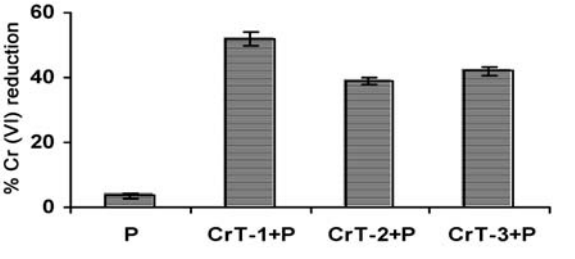

Figure 4. Reduction of Cr(VI) by the seedlings alone and in the presence of bacterial strains. Initial Cr(VI) used was 300 µg ml^{-1} and P, sunflower seedlings.

region as compared to non-inoculated control. Little reduction in the diameter of vascular bundle was observed with Cr(III) stress while the effect of Cr(VI) salt was almost negligible. Bacterial inoculation registered some expansion both in the presence and absence of chromate salts.

Cr (VI) reduction

Figure 4 showed that under control conditions where no bacterial inoculation was applied, little reduction (3.52%) of Cr(VI) was occurred with *Helianthus annuus* seedlings. In inoculated seedlings all bacterial strains caused significant reduction of toxic Cr(VI) to less toxic Cr(III) (Fig. 4). CrT-1 reduced almost (57%) of Cr (VI) in to Cr (III) while this effect was only 3.52% in non-inoculated control seedlings.

DISCUSSION

In the present study seed germination of sunflower was severely affected with the application both chromium salts. Effects of hexavalent chromium were much severe as compared to control. Many workers also reported the adverse effects of chromium salt on seed germination (Khalid *et al.*, 2004).

Under chromium stress, different growth parameters (seedling length, fresh weight, dry weight g^{-1} fresh weight) of sunflower were also severely affected. Decreased growth of *Azolla caroliniana* under hexavalent chromium stress was reported by Wilson and Al-Hamdani (1997). Nichols *et al*. (2000) also observed that under Cr (VI) stress different growth parameters decreased in *Salvinia minima*. Majority of bacterial

Table 3. Effect of inoculation of chromium resistant bacteria on the internal anatomy of root (a) and shoot (b) of *Helianthus annuus* var SF-187 seedlings at 0 and 300 µg ml^{-1} of $CrCl_3$ and K_2CrO_4. (Means of four replicates).

a.

Strains	Diameter of cortex (µm)			Diameter of vascular bundle (µm)		
	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4
Control	390±25	320±28	350±24	330±36	300±24	290±29
CrT-1	491±47	380±43	460±15	380±29	330±23	330±34
CrT-2	512±15	390±26	450±24	371±42	340±20	326±38
CrT-3	485±26	410±24	460±26	375±15	340±25	338±26
LSD at 0.05						
For strains		26.8			19.5	
For Treatment		10.5			7.50	

b.

Strains	Diameter of cortex (µm)			Diameter of vascular bundle (µm)		
	0	$CrCl_3$	K_2CrO_4	0	$CrCl_3$	K_2CrO_4
Control	280±14	260±17	230±18	360±21	340±21	360±15
CrT-1	360±15	370±20	310±15	370±18	350±24	390±17
CrT-2	370±16	360±14	300±14	380±15	360±15	380±16
CrT-3	390±20	340±15	290±16	380±12	370±18	400±18
LSD at 0.05						
For strains		28.5			14.5	
For Treatment		11.4			5.62	

inoculation increased the seedling length both under chromate stress and control conditions. According to reports growth stimulatory bacteria released some chemotaxis, which helps plant for better growth. Rhizospheric bacteria (*Pseudomonas* spp., *Azospirillum* spp., *Agrobacterium* spp.) improved plant growth and nutrient uptake of maize, wheat and legumes (Hoflich and Metz, 1997). The reduction in fresh weight of *Helianthus annuus* seedling under hexavalent chromium stress might be due to reduced uptake of water. Kastori *et al*. (1997) also observed that heavy metal toxicity hampered cell division decrease turgor pressure of plant cells. Under Cr(VI) stress, dry weight and dry mass accumulation increased significantly. It was reported that Cr (VI) uptake by *Salvinia minima* (mg Cr g^{-1} d.w) increased as the Cr (VI) concentration increased in the growth medium (Nichols *et al*., 2000). In the present study at 300 µg ml^{-1} of Cr (VI), majority of bacterial inoculation resulted a decrease in chromate uptake into seedlings as compared to their respective non-inoculated control. In this way bacterial strain helps plants growth by reducing the availability of Cr (VI) to plants.

Several bacterial strains are capable of producing auxin, gibberellins, ethylene or abscisic acid (Vessey, 2003). In the present study all bacterial strains caused an increment in auxin content in inoculated seedling when compared with respective non-inoculated control. High activity of auxin was also observed in chromium treated seedlings by many workers (Ben-Efraim *et al*., 1990; Hasnain and Sabri, 1997). Mutaftchier *et al*. (1993) describe the action of growth hormones and explained that auxin improves plant growth through different mechanism by combining with obigosaccharides, proteins, cell wall fragments and other biological components. Enzyme study of *Helianthus annuus* seedlings revealed that the activity of acid phosphatase was affected in chromium stress. The activity of acid phosphatase is related with metal accumulation by the cell (Macaskie, 1995). Increased acid phosphatase activity with bacterial strains was also reported by Preneta *et al*. (2002). Bacterial strains in addition to stimulate the acid phosphatase activity in inoculated plants may also secrete acid phosphatase as reported by Saleh and Belisle (2000). Hexavalent chromium caused some disintegration of the root and shoot cells but bacterial inoculations give some alleviation by minimizing the toxicity of chromate. In conclusion, bacterial strains promoted sunflower growth mainly by reducing toxic Cr(VI) into less bioavailable Cr(III).

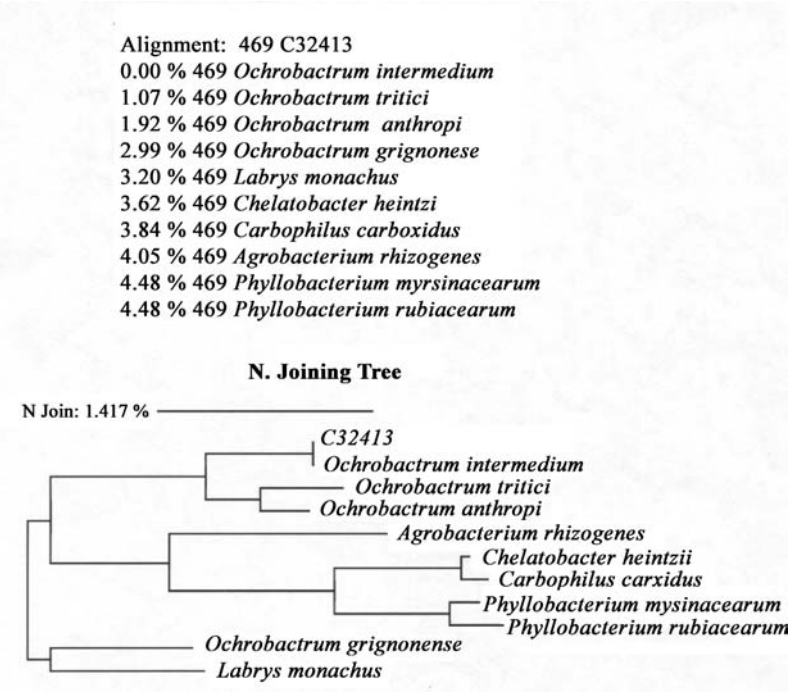

Figure 5. Phylogenetic dendrogram of strain CrT-1 now identified as *Ochrobactrum intermedium* C32413.

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