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Hexavalent chromium induced stress and metabolic responses in hybrid willows

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Abstract Metabolic responses to hexavalent chromium (Cr^{6+}) stress and the uptake and translocation of Cr^{6+} were investigated using pre-rooted hybrid willows (*Salix matsudana Koidz* × *Salix alba* L.) exposed to hydroponic solution spiked with K_2CrO_4 at 24.0 ± 1°C for 192 h. Various physiological parameters of the plants were monitored to determine toxicity from Cr^{6+} exposure. At Cr^{6+} treatments of ≤2.1 mg Cr/l, the transpiration rate of plants was > 50% higher than that of the non-treated control plants. As Cr concentrations were increased further, a slight increase in the transpiration rate was also observed compared with the controls. Negligible difference in the chlorophyll contents in leaves between the treated and the non-treated control plants was measured, except for willows exposed to 1.05 mg Cr/l. The response of soluble proteins in leaves of willows to Cr treatments was remarkable. Cr-induced toxicity appeared in all treatments resulting in reduced activities of catalase (CAT) and peroxidase (POD) compared to the controls. Superoxide dismutases (SOD) activity in the leaf cells showed a positive increase after Cr exposure. Of all selected parameters, soluble proteins in leaves were the most sensitive to Cr^{6+} doses, showing a significant linear correlation negatively ($R^2 = 0.931$). Uptake of Cr^{6+} by willows grown in flasks was found to increase linearly with the added Cr^{6+} (a zero order kinetics), as indicated by the high R^2 (0.9322). Recovery of Cr in different parts of plant materials varied significantly with roots being the dominant site of Cr accumulation. Although the translocation to shoots was detected, the amount of Cr translocated to shoots was considerably small. The capacity of willows to assimilate Cr^{6+} was also evaluated using detached leaves and roots in sealed glass vessels in vivo. Uptake of Cr by roots was mediated possibly through an active transport mechanism, whereas the cuticle of leaves was the major obstacle to uptake Cr from the hydroponic solution. In addition, both cysteine and ascorbic acid showed a remarkable potential to reduce Cr^{6+} at a neutral pH. Results indicated that the added Cr did not cause deleterious effects on plant physiological functions over a 192-h period of exposure. Significant removal of Cr from the hydroponic solution was observed in the presence of hybrid willows. The data also suggest that phytoremediation of Cr^{6+} is possible and ecologically safe due to the minor translocation of Cr to aerial tissues.

Keywords Accumulation · Enzyme · Hexavalent chromium (Cr^{6+}) · Oxidative stress · Phytoremediation · Translocation · Willows

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Introduction

The major sources of hexavalent chromium (Cr^{6+}) pollution are anthropogenic inputs. Cr is widely used in steel, alloys, cast iron, chrome plating, dyes and pigments, textile, leather tanning and wood preserving

(Kimbrough et al. 1999; Khan 2001; Dixit et al. 2002). Due to its unregulated disposal of effluent from industrial activities, Cr has increased the ecological risk of soil, sediment, surface water, and groundwater and become an environmental, health, economic and planning issue worldwide. Cumulative Cr production has been estimated to be 105.4 million tons globally in 2000 and has been significantly increased since the 1950s (Han et al. 2002). Currently, the regulatory limit of Cr in the environment in the US is 50 μg/kg. However, the actual Cr concentrations in soil can reach levels between 5 μg/kg and 4 g/kg (Salt et al. 1998). The maximum concentration of Cr allowed in drinking water in the US is 0.10 mg/l due to the toxic effects of Cr^{6+} and the potential for oxidation of Cr^{3+} to Cr^{6+} (Banks et al. 2006).

Cr^{6+} is considered the most hazardous to animals and plants due to its high solubility, mobility, toxicity as well as carcinogenic and mutagenic properties (Dixit et al. 2002). It was found that occupational exposure to Cr^{6+} compounds leads to a variety of clinical problems. Inhalation and retention of Cr^{6+}-containing materials can cause perforation of the nasal septum, asthma, bronchitis, pneumonitis, inflammation of the larynx and liver and increased incidence of bronchogenic carcinoma. Skin contact of Cr^{6+} compounds can induce skin allergies, dermatitis, dermal necrosis and dermal lesion (Gad 1989; Lee et al. 1989). The biotoxicity of Cr^{6+} is largely a function of its ability to cross biological membranes, its powerful oxidizing capabilities and its interference with electron transport in respiration and photosynthesis (Losi et al. 1994). The toxicity of Cr^{6+} also originates from the formation of free radicals during the reduction of Cr^{6+} to Cr^{3+} occurring inside the cell as a consequence of metal-mediated inhibition of metabolic reactions (Halliwell and Gutteridge 1984). Active production of free radicals has been reported in many plants exposed to Cr stress, leading to damage of DNA, protein, and pigments and initiated lipid peroxidation (Zayed et al. 1998). Adequate defense against oxygen toxicity requires efficient scavenging of both O^{2-} and H_2O_2 (Tsang et al. 1991). Superoxide radicals (O^{2-}) are toxic by-products of oxidative metabolism (Fridovich 1978). Their toxicity has been attributed to interactions with hydrogen peroxide to form highly reactive hydroxyl radicals (OH^-), which are thought to be largely responsible for mediating oxygen toxicity in vivo (Yu et al. 2006). The enzymatic antioxidant components, e.g., SOD, CAT, POD and glutathione, can neutralize free radicals and may reduce or even help prevent some of the potential damage they cause (Prasad 1998; Shanker et al. 2005). Furthermore, phytotoxicity of Cr^{6+} has been studied in many plants. Reduction and inhibition of plant growth, chlorosis in young leaves, transpiration, antioxidant enzymes, nutrient balance, wilting of tops and root injury have been observed frequently as an end point of toxicity determination (Hunter and Vergnano 1953; Hauschild 1993; Chatterjee and Chatterjee 2000; Dixit et al. 2002; Sharma et al. 2003; Scoccianti et al. 2006).

The use of plants to remove inorganic chemicals from contaminated sites has received considerable attention over the last decade. Phytoextraction is a process of assimilating soil pollutants and subsequent translocation to harvestable plant parts (Kumar et al. 1995). A number of works have been focused on Cr bioaccumulation with emphasis on the selection of hyperaccumulator species of plants (Lytle et al. 1998; Boonyapookana et al. 2002; Aldrich et al. 2003; Zayed and Terry 2003). Most plants selected belong to either grasses or cultivated species, which directly serve as food sources for numerous higher animals. Therefore, screening appropriate plants with poor translocation towards aerial tissues is of critical concern for field implementation. It is evident that there are significant differences in the degree of tolerance, uptake and accumulation of Cr among different plant species (Shahandeh and Hossner 2000). Plants with a fast growth and a high biomass are preferred over those with somewhat higher accumulation capacity, but a small biomass. Currently, there is very little data available on accumulation of Cr in woody plants. In this investigation, the effects of Cr^{6+} on transpiration rates, chlorophyll contents, soluble protein and antioxidative systems in hybrid willows, a woody plant from the *Salicaceae* family were evaluated, with the objectives to compare the toxic effects and to offer additional information whether subsequent tests will be needed at a more intensive level. Uptake and bioaccumulation of Cr^{6+} in willow cuttings and detached plant materials and the reduction of Cr^{6+} by exogenous organic acids were also determined to provide quantitative information for risk assessment whether Cr phytoremediation is ecologically safe in field application.

Materials and methods

Trees specimens and exposure regimes

Hybrid willows (*Salix matsudana Koidz* × *Salix alba* L.) were taken from those grown naturally on the Dongting Lake, Hunan, P.R. China. Tree cuttings of 40 cm long were removed from a mature tree and all cutting used in

this study were obtained from a single tree. They were placed in buckets of tap water at room temperature of 15–18°C under natural sunlight until new roots and leaves appeared. After a two-month period of growth, one young rooted cutting was transferred to a 250 ml Erlenmeyer flask filled with approximately 200 ml modified ISO 8692 nutrient solution (Table 1), which was prepared from reagent grade chemicals and deionized water. The flasks were all sealed with cork stoppers and silicon sealant (Dow Chemical Co, Midland, Michigan) to prevent escape of water, and wrapped with aluminum foil to inhibit potential growth of algae. For each treatment concentration, nine replicates were prepared. All flasks were housed in a climate control chamber maintained at a constant temperature of 24.0 ± 1°C under natural sunlight (light: dark cycle 14:10 h). The plants were conditioned for 48 h first to adapt to the new environmental conditions. Then, the weight of the plant–flask system was measured and recorded individually. The flasks including the tree cuttings were weighed again after 24 h. By doing this way, the transpiration rate of each flask was determined. Trees with a similar transpiration rate were selected and grouped for the tests. The nutrient solution in each flask was replaced by spiked solution, except for the controls. Cr used was in the form of potassium chromate (K_2CrO_4) of analytical grade with ≥95% purity. Several concentrations (1.05, 2.10, 4.20, 6.40 and 12.6 mg Cr/l) were prepared by adding the required aliquots of 1.0 g Cr/l stock solution of K_2CrO_4 to the modified ISO 8692 standard nutrient solution.

The effect of Cr^{6+} was quantified by measuring the transpiration rate of the pre-rooted trees in flasks. The weight loss of the plant-flask system was expressed as the transpiration rate.

Chlorophyll measurement

The chlorophyll content in leaves was determined spectrophotometrically at the end of the experiments (192 h). Plant leaves were cut into small pieces, precisely weighed (0.5 g fresh weight) and placed in 25 ml flasks. Then, 80% acetone was added to the mark of 25 ml. Three separate flasks were conducted for each treatment group. All flasks were placed in the dark for 24 h. During this period, flasks were shaken twice. The absorption of light at 645 and 663 nm was measured in a cell with an optical path of 10 mm against 80% acetone as a blank. The amount of chlorophyll a and chlorophyll b in plant leaves was calculated by the following formulae of Maclachalam and Zalik (1963):

$$C_a = \frac{(12.3D_{663} - 0.86D_{645}) \cdot V}{d \cdot 1000 \cdot W}$$

$$C_b = \frac{(19.3D_{645} - 3.60D_{663}) \cdot V}{d \cdot 1000 \cdot W}$$

Where C_a is the concentration of chlorophyll a (mg/g FW), C_b is the concentration of chlorophyll b (mg/g FW), D is the optical density (OD) at the specific wave length indicated, V is the final volume (ml), W is the fresh weight of leaf materials (g), and d is the length of the light path in cm.

Table 1 Composition of the modified ISO 8692 nutrient solution used in this study

Macronutrients (μmol/l)		Micronutrients (nmol/l)	
$NaNO_3$	2823.9	HBO_3	2992.1
$MgCl_2 \cdot 6H_2O$	59.0	$MnCl_2 \cdot 4H_2O$	2097.0
$CaCl_2 \cdot 2H_2O$	122.4	$ZnCl_2$	22.0
$MgSO_4 \cdot 7H_2O$	60.9	$CoCl_2 \cdot 6H_2O$	6.3
KH_2PO_4	246.0	$CuCl_2 \cdot 2H_2O$	0.1
$NaHCO_3$	1785.5	$NaMoO_4{\cdot}2H_2O$	28.9

Enzyme activity measurement

The activities of three antioxidant enzymes SOD, CAT and POD were measured in fresh leaves at the end of the experiment. Fresh leaves were taken from the shoot and 0.3 g of leaves (fresh weight) was precisely weighted and placed in a triturator. 1.4 ml of phosphate buffer solution (pH 7.8, containing NaH_2PO_4, Na_2HPO_4, PVPP, EDTA and mercapto-ethanol) was added before trituration. Trituration was performed in an ice-bath and then centrifuged at 8 000 rpm for 15 min, the supernatant was collected and stored at 4°C, and used for enzyme assays. Each enzyme was assayed independently. SOD, POD and CAT activities in leaf cells of plants were determined spectrophotometrically as described by Yu et al. (2006).

Assay of SOD activity: The reaction mixture (3 ml) was composed of 13 mM methionine, 0.075 mM NBT, 0.1 mM EDTA, 0.002 mM riboflavin, and 0.1 ml of enzyme extract in 50 mM phosphate buffer (pH 7.8). The mixture in the test tube was placed on a rotating tube holder at 25°C for 10 min. The absorbance was measured spectrophotometrically at 550 nm in a cell with an optical path length of 10 mm against the reaction mixture without enzyme extract as blank. The unit of SOD activity (U/g FW) was defined as the amount of enzyme, which caused 50% inhibition of the initial rate of reaction in the absence of the enzyme.

Assay of CAT activity: The enzyme extract (0.1 ml) was added to 2 ml assay mixture (50 mM Tris–HCl buffer pH 6.8, containing 5 mM H_2O_2). The reaction was terminated by adding 0.1 ml of 20% titanic tetrachloride after incubation for 1 min at 25°C. The absorbance of the reaction solutions was measured at 405 nm spectrophotometrically against the reaction mixture without enzyme extract. One unit of CAT activity (U/g FW) was defined as the amount of CAT, which decomposed 1 μmol hydrogen peroxide in one minute at 25°C.

Assay of POD activity: The reaction mixture (3 ml) was composed of 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 65 mM H_2O_2 and 0.1 ml enzyme extract. Changes in absorbance were recorded spectrophotometrically at 470 nm against the reaction mixture without enzyme extract at 25°C for 3 min. One activity unit of POD (U/g FW) was defined as the amount of enzyme that caused an increase of 0.001 unit of absorbance per minute.

Soluble protein measurement

The soluble protein content was determined spectrophotometrically on fresh leaves from the top shoot as described by Jin and Ding (1981). Three separate measurements were conducted for each treatment. At the end of the experiments (192 h), 0.5 g of tissue materials (fresh weight) was precisely weighted and placed in a triturator. 2.5 ml of 65 mM phosphate buffer solution (pH 7.8) containing 0.4 % mercaptoethanol (v/v) was added before trituration. Triturating was performed in an ice-bath and then centrifuged at 12,000 rpm for 15 min. The supernatant was stored at 4°C before analyzing the soluble protein in leaves. 0.1 ml aliquot of the samples was pippetted into a vessel and 5 ml Coomassie Brilliant Blue G-250 solution (Sigma-Aldrich Inc., St. Louis, Missouri) were added. After mixing, the vessel was left standing for 2 min. The absorption of light at 595 nm was measured spectrophotometrically against water as reference. Albumin Bovine V solution from bovine serum (Sigma-Aldrich Inc., St. Louis, Missouri) was used as a standard. The Coomassie Brilliant Blue G-250 solution was prepared as follow: 100 mg Coomassie Brilliant Blue G-250 were dissolved in 50 ml 90% ethanol, followed by 100 ml of 85% phosphoric acid (v/v), and then diluted to 1000 ml with deionized water as described by Cheung and Gu (2005). This solution was finally filtered through 0.45 μm membrane filter (Gelman Science, Ann Arbor, Michigan) and stored at room temperature with a maximum holding time of one month.

Chemical analysis

The concentration of Cr in the aqueous solution was analyzed by flame atomic absorption spectrophotometry at the end of experiments. Preparation and extraction of root, stem and leaf samples for total Cr were conducted according to the method described by Banks et al. (2006). Plant materials from the treated and the non-treated plants were harvested after 192 h of the treatment. The plants were washed with tap and distilled water followed by thorough rinsing, and then oven dried at 90°C for 48 h. Dried plant samples were ground in an electrical blender, except for the roots due to the small quantity of the total harvested materials. The biomass was sieved to pass 2 mm before use. Then, the ground tissue were placed in clean glass bottles and dried for an additional 24 h at 65°C to remove any moisture absorbed during the processing step. The bottles were sealed and placed in a desiccator. Root, stem and leaf samples were extracted for total Cr using a nitric/perchloric acid digestion method. Exactly 0.25 g of oven dried and ground plant materials was placed in 50 ml digestion tubes, mixed with 8 ml of 1:1 $HNO_3/HClO_4$, and allowed to stand overnight. The samples were then placed in a digestion block and heated for 2 h at 200°C until the digested liquid was clear. The digested liquid was diluted to 25 ml with deionized water and filtered (Whatman #1 filter paper Fisher Scientific, Pittsburgh, Pennsylvania) into 120 ml Erlenmeyer flasks. The filtrates were analyzed by the flame atomic absorption spectrophotometry. The detection limits, determined as three times the standard deviation of ten replicates of blank, was 0.001 mg Cr/l for water samples and 0.005 mg Cr/kg DW for plant materials, respectively. The sample preparation methods used were also checked against the spiking samples with certified solution standards; mean recovery was 96.49 %. The precision of Cr determination, based on variations of replicate analyses ($n = 2$) for the same sample, was <15%.

Experiments with detached leaves and roots

To further clarify the uptake mechanism of Cr^{6+}, additional experiments were performed. Sealed glass vessels containing Cr^{6+} solution and plant materials were used. Plant leaves and roots were cut into small pieces, precisely weighted (1.0 g fresh weight) and placed in 100 ml flasks. Then 100 ml of spiked aqueous solution (deionized oxygen-saturated water) were added. The flasks were closed with glass stoppers and all placed at an incubator with a constant temperature of 24°C for 24 h. The initial concentration of Cr spiked

solution was 2.17 mg Cr/l. Three separate measurements were conducted for roots and leaves, respectively. The concentrations of Cr in solutions and plant materials were measured after 24 h of exposure. Preparation and extraction of plant materials for Cr analysis was performed as described above.

Reduction of Cr^{6+} by organic acids and amino acids

Exactly 25 ml of potassium chromate solution (15 mg Cr/l, which is equal to 0.29 mM) were placed in 50 ml test tubes, mixed with 2.25 ml of 10 mM test chemical. The concentration of Cr in solution was measured after 24 h. Six different organic compounds were used, namely citric acid, oxalic acid, malic acid, glycine, cysteine and ascorbic acid (Sigma-Aldrich Inc., St. Louis, Missouri). The pH of the solution was approximate 6.8. This experiment was performed in a temperature-controlled room at 22°C in replicate.

Results

Effects of Cr^{6+} on the transpiration rate of hybrid willows

Transpiration rate of hybrid willows grown in hydroponic solution spiked with various concentrations of Cr^{6+} was measured (Table 2). The transpiration rate of plants is coupled to the photosynthesis, and an inhibition of transpiration is a reliable and quick measure of toxic effects (Trapp et al. 2000). At low Cr treatments (≤2.1 mg Cr/l), Cr application showed a positive effect on the water permeability of root cell membranes and the transpiration rate of the treated willows was higher than that of the non-treated control plants by 50%. This is largely due to the positive effect of low concentrations of Cr on CO_2 fixation during the plant photosynthesis (Shanker et al. 2005). Negligible increase of the transpiration rate was detected for hybrid willows exposed to Cr from 4.2 to 12.6 mg Cr/l. Visible toxic symptom, e.g., chlorosis of leaves was not observed in all treatment groups in the whole duration of the test.

Effects of Cr^{6+} on the content of chlorophylls in plant leaves

Both chlorophyll a and b contents in leaves were not significantly affected by the increase in Cr^{6+} concentraions (Table 2), except for the willows exposed to 1.05 mg Cr/l. A remarkable increase of 100% in chlorophyll was observed after 192 h of exposure to 1.05 mg Cr/l compared with the controls. This was in agreement with other findings. Zeid (2001) found that low concentrations of Cr (10^{-6} and 10^{-4} M) enhanced the content of chlorophyll. Increased chlorophyll concentrations caused by exposure to low concentrations of Cr were also reported in bean plants (Bonet et al. 1991). The positive effect of low and moderate concentrations of Cr on chlorophyll synthesis may be attributed to the increased transport of Mg^{2+} which is an essential component of the chlorophyll molecule (Barcelo et al. 1985). No significant difference in chlorophylls was observed between the other treated and the non-treated control plants.

Effects of Cr^{6+} on the content of soluble proteins in plant leaves

Soluble protein concentrations in leaf cells of hybrid willows were significantly reduced by the presence of

Table 2 Effects of various chromium (Cr^{6+}) treatments on transpiration rate, soluble protein, chlorophyll contents, and activities of superoxide dismutases (SOD), peroxidase (CAT) and catalase (POD). The exposure period was 192 h (see Materials and Method for details). Values are the mean of three replicates for both the treated and non-treated control plants, except the transpiration rate (nine replicates for the treated plants and six for the non-treated plants). Numerical values in brackets represent standard deviation FW = fresh weight

Characteristic	Chromium concentrations (mg Cr/l)					
	0.00	1.05	2.10	4.20	6.40	12.60
Transpiration rate (g/d)	4.13 (1.210)	6.50* (2.184)	6.27* (1.542)	4.37 (1.151)	4.25 (0.973)	4.05 (0.889)
Chlorophyll a (mg/g FW)	0.23 (0.009)	0.51* (0.149)	0.28 (0.046)	0.22 (0.045)	0.23 (0.032)	0.24 (0.048)
Chlorophyll b (mg/g FW)	0.25 (0.005)	0.57* (0.174)	0.29 (0.046)	0.23 (0.046)	0.25 (0.026)	0.27 (0.059)
Soluble protein (mg/g FW)	5.50 (0.291)	5.23 (0.542)	5.20 (0.672)	5.00 (0.265)	3.36* (0.567)	2.10* (0.170)
Superoxide dismutases (U/g FW)	330.92 (3.743)	310.62* (10.816)	319.44 (18.303)	334.74 (9.984)	336.21 (2.078)	336.50 (14.144)
Peroxidase (U/g FW)	34.65 (13.364)	40.83 (2.034)	39.43 (6.445)	36.01 (3.269)	33.83 (4.872)	35.23 (6.726)
Catalase (U/g FW)	88.30 (8.178)	116.56* (6.660)	103.61* (0.833)	101.55 (24.559)	70.25 (24.159)	65.94 (25.224)

* Significantly different to the controls on 95% significance level (one-tailed *t*-test)

Cr^{6+} (Table 2). A slight reduction of soluble proteins (≤10%) was observed for the willows exposed to Cr^{6+} concentrations ≤4.2 mg Cr/l in comparison with the non-treated control plants. When exposed to high concentrations ≥6.4 mg Cr/l, the effect was remarkable. The observed decline in soluble proteins was correlated with the decrease in nitrogen uptake in the presence of the Cr^{6+}, resulting in an inhibition of assimilation of nitrogen into amino acids and proteins. Cr exposure caused a decrease of soluble proteins was also reported by Rai et al. (1992) and Vajpayee et al. (2000).

Effects of Cr^{6+}on the activities of antioxidant enzymes in plant leaves

Activities of SOD, CAT and POD in the leaf cells of hybrid willows were also measured at the end of Cr exposure (Table 2). At low Cr concentration of 1.05 mg Cr/l, activity of SOD was slightly decreased compared to the controls, whereas with increasing Cr addition, SOD activity gradually increased to the level of the controls. Our observations contradicted with early findings by Gwozdz et al. (1997) and Dixit et al. (2002) in which, at a low Cr concentration of 20 μM, higher SOD activity was found, whereas 200 μM Cr^{6+} produced a significant inhibition. The difference is probably due to the different species of plants selected in different investigations.

Activity of CAT in leaves varied with the doses of Cr (Table 2). At low concentrations of Cr^{6+} (≤4.2 mg Cr/l), CAT activities were higher than the controls, implying that Cr^{6+} may increase the presence of CAT in leaf cells. However, significant decline of CAT activity was correlated with an increase in Cr present in the growth medium. That Cr exposure decreased the activity of CAT coincided with other findings. Chatterjee and Chatterjee (2000) observed that excessive Cr (0.5 mM) restricted the activity of CAT in leaves of cauliflower. A decline in the CAT activity was also reported when Cr concentrations were increased from 20 to 80 ppm (Jain et al. 2000). CAT is an iron–porphyry biomolecule, the decreased activity of CAT indicated that Cr is either interacting with iron in metabolic pool or affecting the availability of active form of iron (Sharma et al. 2003).

POD activity of willows was not significantly affected by the increase in Cr^{6+} concentrations (Table 2). The mean of the five treatments was 37.07 U/g FW (SD 2.9459), which was slight higher than the controls (34.65 U/g FW). Similar results were observed by Sen et al. (1994) in which POD activity was the least affected by Cr^{6+} at lower concentrations. Sharma and Sharma (1996) reported results to the contrary. In wheat cultivar, the application of 0.05–0.5 mM Cr^{6+} decreased the activity of POD significantly.

Cr^{6+} uptake from hydroponic solution by hybrid willows

Figure 1 shows the measured Cr concentrations in the hydroponic solutions at different treatment concentrations after 192 h of exposure. The amount of applied Cr in the growth medium was significantly reduced by the presence of hybrid willows. Nearly all Cr added into the hydroponic solution was removed by plants exposed to 1.05 mg Cr/l. At the highest concentration of 12.6 mg Cr/l, 42.35% of the total applied Cr in the solution were removed by willows over a 192-h period of exposure. When expressed by mg Cr per kg plant dry weight, the Cr uptake rate by plants was highly correlated with Cr concentraions in the hydroponic solution as shown in Fig. 2 (the linear trend line and the R-square value are given), which agreed with others (Lytle et al. 1998; Boonyapookana et al. 2002; Aldrich et al. 2003).

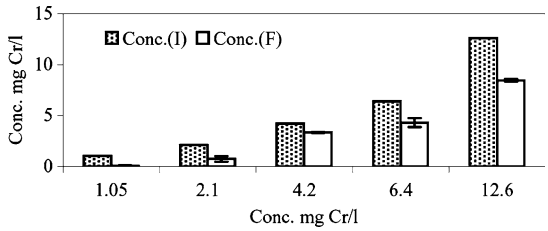

Fig. 1 Measured total chromium concentrations (mg Cr/l) in aqueous solution at different treatments. The exposure period was 192 h. The values are the mean of three replicates. Vertical lines represent standard deviation (I, initial concentration; F, final concentration)

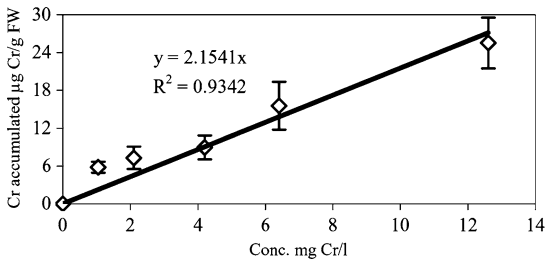

Fig. 2 Measured total chromium (μg Cr/g FW) accumulated in plant materials at different treatments. The exposure period was 192 h. The values are the mean of three replicates. Vertical lines represent standard deviation

The mass balance for total Cr

Cr contents in the various plant compartments are presented in Fig. 3. The background Cr in non-treated control trees was 0.05 mg Cr/kg DW for roots, 0.03 mg Cr/kg DW for leaves and 0.02 mg Cr/kg for stems ($n = 2$ for all controls), respectively. Compared to the controls, Cr concentrations in all plant materials of exposed plants at the end of exposure were higher than the detection limit, indicating uptake and translocation of Cr from hydroponic solution into plants. However, substantial differences existed in the distribution of Cr in plant materials: roots being the major sink for Cr accumulation, followed by stems and the least amounts of Cr supplied was found in leaves. It is interesting to note that in our study Cr concentrations in plant materials were strongly correlated to the Cr application, with R^2 values of 0.9387, 0.7163 and 0.7374 for roots, stems and leaves, respectively. Our results were partially in agreement with other findings. Aldrich et al. (2003) found that the desert plant species mesquite (*Prosopis* spp.) had higher metal accumulation potential. When plants were exposed to Cr^{6+} at 75 ppm over a 28-day period of exposure, Cr was detected to be 7636, 889, 323 mg Cr/kg DW in roots, stems, and leaves, respectively. A different conclusion was reached by Chatterjee and Chatterjee (2000) in which the cauliflower *Brassica oleracea* L. accumulated 775, 7.2, and 10.6 mg Cr/kg DW in roots, stems, and leaves, respectively. Data presented here and other observations indicated that the uptake and translocation of Cr varied with different plant compartments and also depended upon the genus and species of the different plants.

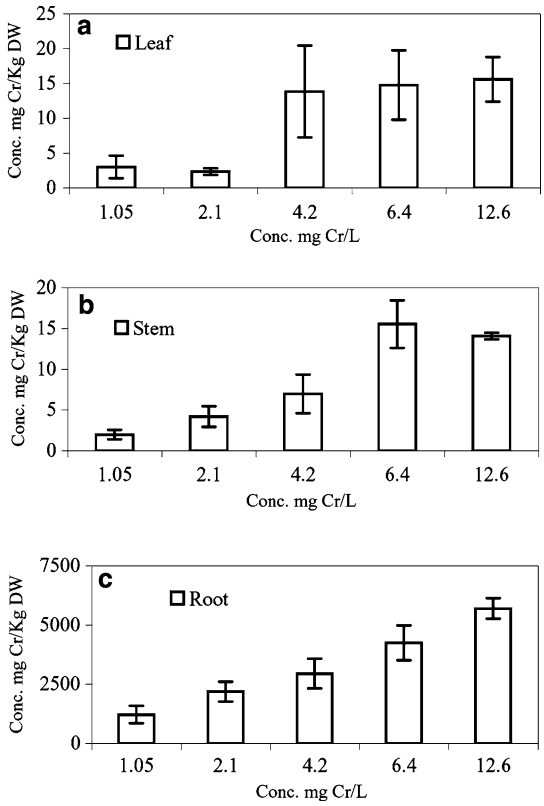

Fig. 3 Measured total chromium concentrations (mg Cr/kg DW) in roots, stems and leaves of hybrid willows (*Salix matsudana Koidz* × *Salix alba* L.) at different treatment concentrations. The exposure period was 192 h. The values are the mean of three replicates. Vertical lines represent standard deviation; DW = dry weight

The mass balance of Cr was made from tissue total Cr and the solution Cr data (Table 3). A good recovery of Cr was obtained in all treatment within the cumulative ranges of measurement errors in water and biomass. The mean recovery was 90.05% with a standard deviation of 7.76%. At low Cr concentration of 1.05 mg Cr/l, the Cr loss from the growth medium was primarily recovered in roots (87.26%) whereas lower Cr recovered in roots (67.20%) and higher recovered in stems (30.33%) was found at the higher Cr treatment of 4.2 mg Cr/l. These observations indicated that Cr translocation to stems from roots was possible. However, only approximate 1.26% of the applied Cr was detected in leaves of the treated plants, implying minimum translocation to leaves.

The bioconcentration factor (BCF) is defined as the ratio of metal concentration in the biomass in the initial concentration of metal ion in the feed solution (Raskin et al. 1994). It is interesting to note that at low Cr exposure (≤4.2 mg Cr/l), BCF values dropped from 0.76 to 0.30 with an increasing concentrations of Cr. However, the BCF values were a constant (the mean was 0.35 with a standard deviation of 0.0586) when plants were exposed to higher Cr concentrations. Therefore, hybrid willows were unable to accumulate more Cr in their biomass due to Cr biotoxicity and the limited metal accumulation potential.

Uptake of Cr^{6+} by detached leaves and roots of plants

The potential of plant materials of hybrid willows to uptake Cr^{6+} was also tested. Concentrations of Cr did not change in the controls, nor in the test flasks with detached leaves. The amount of Cr in sealed vessels was significantly reduced in the presence of plant roots. Concentrations of Cr in the solution declined from 2.17 mg Cr/l initially to 1.74 mg Cr/l (SD 0.0218, $n = 3$), with 20.34 % (SD 0.6164, $n = 3$) reduction of the total Cr over a 24-h period of exposure. The recovery of Cr was 95.75% (SD 1.077 %, $n = 3$) for the

Table 3 Mass balance for hexavalent chromium in the hydroponic systems containing hybrid willows (*Salix matsudana Koidz* × *Salix alba* L.) The exposure period was 192 h. Values are mean of three replicates with standard deviation in brackets

Treatment (mg Cr/l)	Mass in solution (μg Cr)		Mass in tissues* (μg Cr)			Recovery (%)
	Initial	Final	Root	Stem	Leaf	
1.05	262.5	13.91 (8.476)	174.30* (38.134)	22.97* (9.620)	2.86* (1.855)	80.15 (15.742)
2.10	525	141.22 (47.835)	279.05* (60.886)	73.65* (23.678)	1.89* (0.530)	92.13 (3.685)
4.20	1050	734.34 (25.963)	209.76* (85.051)	95.91* (42.394)	6.36* (3.063)	97.56 (32.940)
6.40	1575	905.76 (165.792)	422.50* (246.013)	236.1* (76.563)	9.11* (3.234)	96.60 (21.067)
12.6	3159	1815.93 (122.979)	849.33* (164.00)	262.37* (29.187)	5.60* (1.105)	83.80 (12.636)

* The measured concentrations of Cr in plant materials were significantly higher than that of the background Cr in non-exposed control trees. The mass balance did not take background Cr into account

* Significantly different to the controls (data not shown) on 95% significance level (one-tailed *t*-test)

treatments with roots. These indicated that Cr was unable to across the biomembrane of the leaves, which was in good agreement with the observation obtained by Schönherr and Riederer (1989). They found that plant cuticles are the limiting barriers in foliar uptake of a wide range of chemicals. Roots of willows showed a remarkable capacity to remove and accumulate Cr, supporting that roots were the dominant sink for accumulation of the applied Cr. Cr^{6+} uptake by plants highly depends on metabolic energy (Shanker et al. 2005) and results from this test implied the Cr reduction enzyme was largely located at the root biomembrane rather than leaf biomembrane, resulting in poor translocation towards leaves.

Reduction of Cr^{6+} by organic acids and amino acid

The effects of organic acids and amino acid on the reduction of Cr^{6+} were tested in batch experiments at near neutral conditions. Cysteine among all chemicals showed a remarkable reduction of Cr^{6+} (50.38% in 24 h) while ascorbic acid was the fastest, with 99.28% reduction of Cr^{6+} in 24 h. However, no reduction of Cr was observed by oxalic acid, malic acid, glycine or citric acid in our test. Results suggested that only selective organic components are capable of acting as electron donors for the reduction of Cr^{6+} to Cr^{3+}. This was similar to the results obtained by Xu et al. (2005). Equations 1 and 2 give as illustrative examples the proposed stoichiometry of the reduction of Cr by ascorbic acid and cysteine.

$$CrO_4^{2-} + 3C_6H_8O_6 + 2H^+ \rightarrow Cr^{3+} + 3C_6H_6O_6 + 4H_2O \quad (1)$$

$$CrO_4^{2-} + RSH + H^+ \rightarrow [RS\text{-}CrO_3]^- + H_2O \quad (2)$$

Reduction of Cr^{6+} to Cr^{3+} has been demonstrated an effective means of immobilization of the toxic Cr^{6+} and can be catalyzed by inorganic, organic, or biological agents (James and Barlett 1984; Banks et al. 2006). However, additional observations (Lytle et al. 1998; Srivastava et al. 1999; Aldrich et al. 2003; Bolan et al. 2003) suggested that the reduction of Cr by organic acids was mostly confined to the acidic conditions. Bartlett and James (1988) also reported that the kinetics of Cr^{6+} reduction to Cr^{3+} was slow at near neutral pH, even though strong reducing conditions prevailed environmentally. In our study, the pH of the solution was maintained at approximate 6.8, which eventually terminated the reduction of Cr.

Discussions

The metabolic lesions in hybrid willows due to Cr^{6+} exposure were not observed, indicating the doses of Cr used in this study did not cause visible deleterious effects on plant physiological functions over a 192-h period of exposure. The results of all measured toxic effects were also plotted and analyzed (data not shown). All linear trends were significant except chlorophyll contents, judged by judged by the critical R for given n ($\alpha = 5\%$) (Sachs 1992). Of these selected parameters, the correlation between the Cr^{6+} concentrations and the soluble proteins in leaves was the highest of all toxicity assays ($R^2 = 0.931$), indicating that the soluble proteins were the most susceptible to the changes in Cr doses than others. The susceptibility of these parameters to the change of Cr^{6+} exposure was in the order: soluble protein > CAT > transpiration rate > SOD > POD > chlorophyll *a* > chlorophyll *b*. The correlation between the Cr supplied and chlorophyll contents in leaves was weak ($R^2 < 0.32$). In our

observations chlorophyll contents were measured for leaf cells and the Cr translocation to leaves was minor throughout the Cr concentration tested. This could be an explanation for the minor effect of Cr on this parameter. Chlorosis of leaves is one of the first visible signs of metabolic stress, but decrease of chlorophylls was not found for all treatments. This also provided an additional confirmation.

As discussed above, the mechanisms that contribute to Cr uptake, transport and distribution remain to be elucidated. The movement of metals from external solution into root cells was either due to diffusion of metal iron along the concentration gradient formed or due to mass flow driven by transpiration (Greger 1999). Therefore, it is interesting and important to know by what biochemical pathway or mechanism Cr^{6+} is transported into plant tissues at the very beginning of exposure. In our observations significant removal of Cr from the hydroponic solution was observed in the presence of hybrid willows and the variations in the applied Cr concentrations affected both the transpiration rate of plants and the uptake of Cr. Cr uptake and the Cr supplied was linear ($R^2 = 0.9864$). The correlation between the initial concentrations and the transpiration rate ($R^2 = 0.6406$) were also highly significant ($p < 0.01$). Since humidity was kept constant during the entire period of experiments for all treatments, an improved correlation could be expected, regardless of the different sizes of trees. Additionally, a linear correlation between the transpiration rate and mass accumulated was established ($R^2 = 0.5792$). However, the partial correlation between the uptake of Cr and the transpiration rate, assuming the initial concentrations a constant, was very weak ($R^2 = 0.2354$). This indicated that the plant transpiration rate had a minor influence on the uptake of Cr. On the other hand, the partial correlation between the uptake of Cr and the initial Cr concentrations (assuming transpiration rates a constant) was significant ($R^2 = 0.9754$), indicating that the Cr uptake was highly dependent on concentrations of Cr supplied. This could be an explanation why Cr uptake mechanism by willows was linearly correlated to the Cr supply. This observation coincided with the finding of Scoccianti et al. (2006) in which exogenous Cr uptake by celery seedlings was in a dose-dependent manner.

It was generally accepted that the sulfate carrier could play a crucial role in the uptake and secretion of Cr^{6+}, and immediately converted to Cr^{3+} in roots, possibly by the Fe^{3+} reductase enzyme (Zayed et al. 1998). It is known that P and Cr are competitive for surface sites and Fe, S and Mn are also known to compete with Cr for transport binding (Wallace et al. 1976). Our observations indicated that large fraction of the added Cr^{6+} was taken up from the hydroponic medium in the presence of plants. It is evident that Cr effectively competed with these elements to gain rapid entry into the plant system. Only the trivalent form of chromium was detected in roots and shoots of several crop plants treated with Cr^{6+} confirmed with X-ray absorption spectroscopy (XAS) (Zayed et al. 1998, Aldrich et al. 2003). The high percentage of Cr in the roots in the form of Cr^{3+} was largely ascribed to the reduction of Cr^{6+} by the Fe^{3+} reductase enzyme occurring inside the cells. Aldrich et al. (2003) found that Cr^{3+} in plant materials exists in either the structure of a sugar or a carboxylic acid complex, which is sparingly soluble. This could be a reliable explanation for the poor translocation of Cr to the shoots from roots.

Additionally, the fractionation of Cr over the various plant compartments is of significance for field application, particularly using woody plants in phytoremediation. Stems and leaves often contain the greatest mass of contaminants due to their larger amounts of biomass (Ying 2002). In our study Cr concentrations in plant materials were found to decrease with elevation, with minor concentrations associated with leaves. Therefore, the implications of these findings are that the leaves are of greater concern as they directly serve as food sources for numerous higher animals, but accumulation of Cr was the least in them. Our observations also suggest that the higher capacity of Cr accumulation is not the decisive factor for choosing one over the other species in phytoremediation. Other aspects, such as climate, soil conditions, water regime, further use of the trees, and the potential threat to ecosystems might be more important in the choice of the appropriate species for a phytoremediation field trial.

Conclusions

Large fraction of hexavalent chromium (Cr^{6+}) was removed from the hydroponic solution in the presence of hybrid willows without showing visible lesions. The added Cr^{6+} did not induce deleterious effects on plant physiological functions over a 192 h period of exposure. Of the selected parameters, soluble protein content in leaves was the most sensitive to the changes in Cr^{6+} doses than others. The total amounts of Cr accumulated in plant biomass of hybrid willows were observed to be variable in response to Cr^{6+} application and the uptake mechanism can be described by a zero-order kinetics. The fractionation of Cr over the various

tree compartments varied with the added Cr^{6+}: roots being the major sink for metal accumulation and leaves the least. Although exogenous Cr uptake by willows is efficient, the translocation to shoots from roots is very limited. These information collectively suggest that phytoremediation of chromium (Cr^{6+}) is possible and ecologically safe due to the minor translocation of Cr to aerial tissues.

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