

Ultrastructural localisation of chromium in *Ocimum basilicum*

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Abstract: *Ocimum basilicum* L. seedlings were exposed to different concentrations of Cr (0-8 mg L⁻¹ of Cr³⁺) to evaluate the cellular localisation of chromium, plant accumulating capacity, and potential cellular defence mechanisms against Cr stress. Dried plant tissues (roots and shoots) were exposed to X-ray microanalysis for element determination (including Cr, Cu, Zn, Co, Ni, and Mo). Results showed that the highest amount of Cr had accumulated in the roots of the plants treated with 8 mg L⁻¹ of Cr³⁺, while less Cr was transported to the shoots. TEM micrographs of *Ocimum basilicum* root cortex cells exposed to 4, 6, and 8 mg L⁻¹ of Cr³⁺ revealed dense granular metal deposits in the periplasmic zone along the cell walls; such deposits were not observed in leaf mesophyll cells. Root cortical cells of the control seedlings grown in the absence of Cr³⁺ exposures were void of such granular metal deposits. While Cr was the predominant element, lower amounts of Cu, Zn, Co, Ni, and Mo were also detected in the chromium bearing deposits analysed by X-ray microanalysis. Formation of Cr bearing deposits in the root cells of *Ocimum basilicum* may have the effect of maintaining relatively low cytoplasmic concentration of the element and possibly reduce the toxic effects of chromium on cellular metabolism as a detoxification mechanism.

Key words: *Ocimum basilicum*, Cr, X-ray microanalysis, TEM

Introduction

Chromium is found in all living organisms and has long been known as an essential element for man and animal (Bahijiri & Mufti, 2002; Panda & Choudhury, 2005; Balk et al., 2007), but there is not sufficient evidence of its essentiality for the normal growth of plants (Mei et al., 2002; Zayed & Terry, 2003). Chromium is important for metallurgical industry. Cr salts are used in many industrial process and products such as leather tanning, electroplating, steel production, metal finishing, catalyst application, pigment manufacturing, and metal corrosion

inhibitors. Therefore, they are present in the effluents of those industries and in municipal sewage (Zayed & Terry, 2003; Nath et al., 2005; Babel & Opiso, 2007; Venkateswaran et al., 2007). Chromium is one of the most common heavy metal contaminants in the environment, ground water, soil, and sediments (Srivastava & Thakur, 2006; Shrestha et al., 2007; Kar et al., 2008; Ogundiran & Afolabi, 2008). Chromium salts, which are more or less soluble in water and soils, can create potentially toxic environments for plants (Srivastava & Thakur, 2006). Anthropogenic chromium sources contribute greatly to the current

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Cr pollution in the environment. The global industrial-age cumulative Cr production in 2000 has been estimated at 105.4 million tons and has been significantly increasing since the 1950s (Han et al., 2002). In nature, chromium is largely found in either + 3 or + 6 oxidation states, as it is unstable and short-lived in other oxidation states (Arduini et al., 2006). Phytotoxicity of chromium has been considered to be inhibitory for plant growth. Its presence in excessive amounts within the plant can cause stunted growth of shoots and roots (Citterio et al., 2003; Faisal & Hasnain, 2005; Gbaruko & Friday, 2007). Chromium toxicity in plants may lead to chlorosis of leaves, tissue necrosis, membrane damage, and changes in soluble protein content as well as the activity of various antioxidant enzymes and diminished photosynthesis (Parmar et al., 2002; Du et al., 2003; Scoccianti et al., 2006; Munir & Aftab, 2009). The toxicity of chromium to plants depends on its valence state. Both hexavalent Cr (VI) as well as trivalent Cr (III) forms of Cr are phytotoxic and Cr (VI) is more toxic than Cr (II) (Mei et al., 2002; Han et al., 2004; Shanker et al., 2005; Karuppanapandian & Manoharan, 2008; Gupta et al., 2009; Zhang et al., 2009). The toxicity of chromium and Cr content in plants is species specific. Low concentrations of chromium ($0.1\text{--}0.75\text{ mg L}^{-1}$ of Cr^{3+}) have been reported to have a stimulatory effect and increase the normal growth of plants (Qian et al., 1999; Chen et al., 2001; Han et al., 2004), but higher concentrations have toxic effects (Chatterjee & Chatterjee, 2000; Scoccianti et al., 2006; Sinha et al., 2006; Juarez et al., 2008; Subrahmanyam, 2008). The interest in chromium metallome for practical considerations would be driven by the following goals: (I) to reduce its uptake in crop plants so it does not affect growth and yield, (II) to increase uptake in hyperaccumulator plants and keep toxicity to a minimum for the completion of its lifecycle (Hema et al., 2008; Shanker et al., 2009). In order to identify an ideal plant system that can be appropriately exploited to clean up the sites contaminated with chromium, it is vital to understand the basic mechanism(s) that promote uptake and detoxification of the element (Zayed & Terry, 2003; Arduini et al., 2006). The interaction of chromium and other elements is important for plant growth and development, but principally it depends on the availability of the metal. However, availability in the soil depends on several

soil conditions, such as pH or redox potentials (Han et al., 2004). The increase of heavy metals in soil can lead to certain irreversible cytogenetic effects in plants and higher organisms (Kumarrai & Kumar, 2010). Excess Cr interferes with the uptake of some elements, such as Fe, Mo, Cu, Zn, Mn, P, and N by plants (Chatterjee & Chatterjee, 2000; Shanker et al., 2005).

Ultrastructural localisation of chromium in plants exposed to high concentrations of chromium and the formation of metal deposits of various shapes and sizes are characteristic features of plant cell ultrastructure treated with high concentrations of chromium as well as other heavy metal ions (Chatterjee & Chatterjee, 2000; Han et al., 2004). The purpose of the present study was to investigate cellular localisation of chromium to understand the tolerance and detoxification/compartimentalisation mechanisms of *Ocimum basilicum* L. exposed to this heavy metal in order to improve the quality or productivity of the vegetable under investigation in natural environments.

Materials and methods

Hydroculture experiment and Cr exposure

The seeds of *Ocimum basilicum* L. (cv. Qarakhale) were collected from the Agricultural Research Institute, Sari, Iran, washed thoroughly with distilled water, placed in petri dishes with a double layer of filter paper soaked in distilled water, and germinated in a laboratory germinator at $20\text{ }^{\circ}\text{C}$. After 7 days, seedlings of similar size were carefully transferred to polyethylene containers filled with 3 L of modified Hoagland's culture solution (Mei et al., 2002). Chemical grade $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ was used for Cr^{3+} treatments. Different concentrations of Cr^{3+} , including 0, 2, 4, 6, and 8 mg L^{-1} , was introduced to nutrient solutions and transferred to phytotron with controlled temperature ($24 \pm 1\text{ }^{\circ}\text{C}$), 16 h day^{-1} white light ($700\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) and 70% relative humidity. Treatments were performed with 3 replicates and 9 plants per replicate. The culture solutions were renewed every 5 days to maintain a precise element concentration. After 6 weeks of Cr exposure, the roots and shoots of the seedlings were harvested and immersed in 20 mmol L^{-1} of ice-cold EDTA solution

for 15 min to displace extracellular Cr^{3+} . The seedlings were rinsed with deionised water and blotted to remove excess water before further experiments took place.

X-ray microanalysis and elements determination

Harvested seedlings were oven dried at 60 °C for 24 h and weighed. Dried roots and shoots were powdered and prepared for determination by energy dispersive X-ray fluorescence technique (Cortizas et al., 2007), an analytical method for the determination of a wide range of elements with detection limits at the sub-ppm level. Standards were defined for the method for Cr, Cu, Zn, Co, Ni, and Mo as follows:

Cr: 0, 2, 5, 9, 134, 410, 1000, 1200 ppm; Mo: 0, 1, 2, 4, 6, 8, 18, 34 ppm; Zn: 0, 2, 4, 8, 16, 22, 44, 88 ppm; Cu: 0, 2, 4, 6, 12, 24, 48 ppm; Ni: 0, 1, 2, 4, 8, 16, 32 ppm; and Co: 0, 1, 2, 4, 8, 16, 32 ppm. The elements were measured using an energy dispersive multielement XRF analyser (Philips-X unique II, Dy 673).

TEM procedure

Transmission electron microscopy of root and leaf tissues was performed using segments of tap root and leaf tissues (approximately 3×2 mm) of *Ocimum basilicum* L. seedlings treated with 0, 2, 4, 6, and 8 mg L^{-1} of Cr^{3+} . The tissues were fixed in an ice-cold 3% glutaraldehyde plus 0.05 M potassium phosphate buffer (pH 7.1) for 2 h, and post fixed in a 2% osmium tetroxide solution for 3 h. The samples were dehydrated in ethanol series and embedded in Spurr's epoxy resin. Unstained specimens were sectioned by an ultramicrotome and examined with LEO, 914-AB transmission electron microscope (Han et al., 2004).

Statistical analysis

Statistical analysis was performed using the SPSS program. Variations of the measured parameters in roots and shoots of the Cr-exposed plants at different Cr^{3+} concentrations were evaluated through the Spearman R coefficient as a non-parametric measure of the correlation between Cr, Cu, Zn, Co, Ni, and Mo accumulations and the treatments. P-values less than 0.05 were considered as statistically significant. Data shown in Tables 1 and 2 represent means \pm standard deviation (SD).

Results and discussion

TEM micrographs of root cortical cells (Figure 1) and leaf mesophyll cells (Figure 2) of *Ocimum basilicum* L. treated with 4, 6, and 8 mg L^{-1} of Cr^{3+} , respectively, showed distorted cells with poorly defined plasmalemma and cell organelles compared to the control (Figure 1). Dense granular particles (metal deposits) were observed in the enlarged periplasmic zone along the cell walls of root cortical cells of *Ocimum basilicum* L. Such dense deposits were not seen in the root cortical cells (Figure 1) or leaf mesophyll cells (Figure 2) of the seedling treated with 0 and 2 mg L^{-1} of Cr^{3+} , respectively. Granular chromium bearing deposits occurred normally in small groups along the innermost layer of the cell wall and was seldom seen in the cytoplasm of root cortical cells (Figure 1). X-ray microanalysis results proved positive identification of Cr, Cu, Zn, Co, Ni, and Mo in those deposits. In all measurements, chromium was the predominant element, while lower amounts of other mentioned elements were also present in the metal deposits (Tables 1 and 2). The concentrations and exposure responses of Cr accumulation in the roots and shoots of basil plants are presented in Tables 1 and 2. The variations of the measured element content in the roots and shoots of Cr exposed plants at different concentrations were evaluated through the Spearman R coefficient, as a non-parametric measure of the correlation between Cr accumulation and Cu, Zn, Co, Ni, and Mo concentrations. The results showed that excess chromium ($\geq 4 \text{ mg L}^{-1}$ of Cr^{3+}) caused a decrease in the accumulation of Cu, Zn, Co, Ni, and Mo in roots and shoots of basil plants. Data analysis results showed that Cr ($R = 0.986$), Cu ($R = -0.917$), Zn ($R = -0.873$), Co ($R = -0.786$), and Mo ($R = -0.884$) accumulation in roots and Cr ($R = 0.786$), Cu ($R = -0.918$), Zn ($R = -0.906$), Co ($R = -0.918$), Ni ($R = -0.799$), and Mo ($R = -0.876$) accumulation in shoots were significantly correlated ($P < 0.05$) with the respective Cr- treatments. One of the reasons for the decreased uptake and accumulation of Cu, Zn, Co, Ni, and Mo in Cr stressed plants could have been the inhibition of the activity of the plasma membrane H^{+} -ATPase as noted by Shanker (2005). Chromium can affect mineral uptake of plants in a complex way. The competitive interaction of chromium with the uptake and accumulation of other mineral elements has received attention by several researchers (Moral

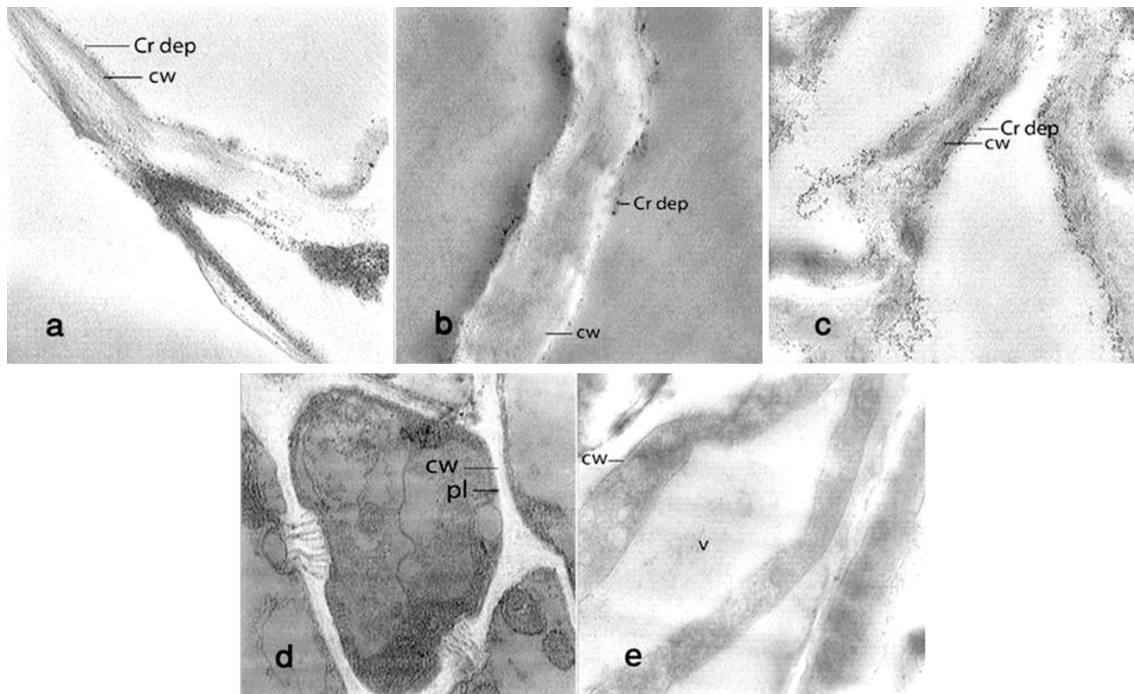


Figure 1. TEM micrographs of *Ocimum basilicum* root cortical cells treated with a: 4 mg L⁻¹ Cr³⁺, b: 6 mg L⁻¹ Cr³⁺, c: 8 mg L⁻¹ Cr³⁺ showing chromium bearing granular deposits along the innermost layer of cell wall, d: untreated showing some cytoplasmic organelles with no metal deposits, e: 2 mg L⁻¹ Cr³⁺ showing no chromium bearing deposits in cytoplasm and along the cell wall. cw: cell wall; v: vacuole; pl: plasmalemma; Cr dep: chromium bearing deposits.

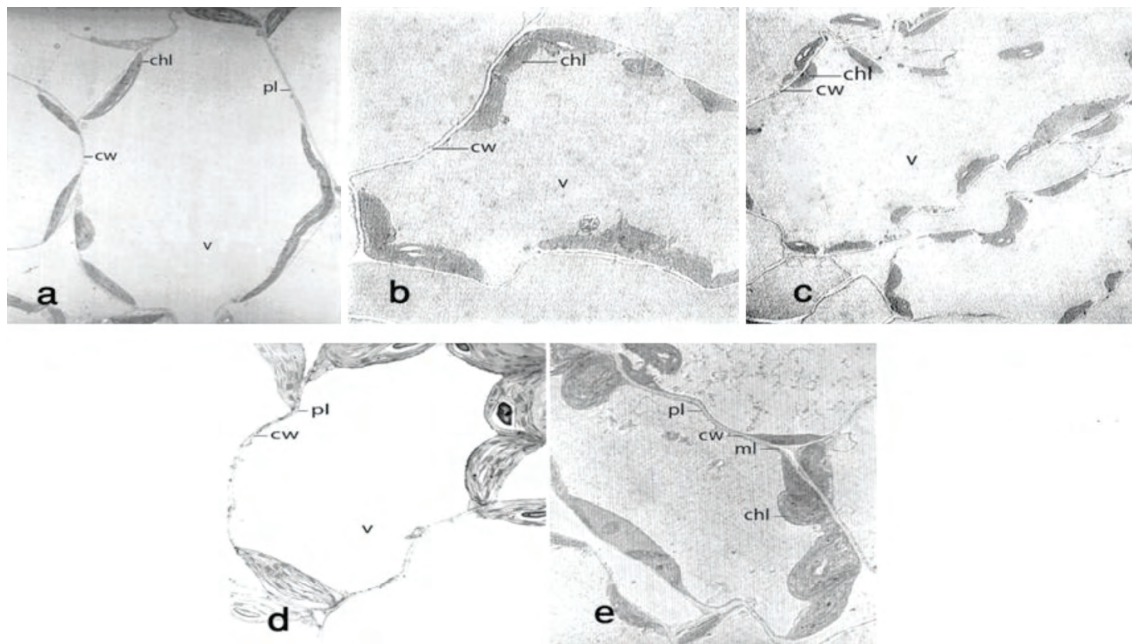


Figure 2. TEM micrographs of *Ocimum basilicum* leaf mesophyll cell treated with a: 4 mg L⁻¹ Cr³⁺, b: 6 mg L⁻¹ Cr³⁺, c: 8 mg L⁻¹ Cr³⁺, d: untreated showing some cytoplasmic organelles and no Cr bearing deposits in cytoplasm or along the cell wall, e: 2 mg L⁻¹ Cr³⁺ showing no Cr bearing deposits in cytoplasm or along the cell wall. cw: cell wall; ch: chloroplast; pl: plasmalemma; v: vacuole; ml: middle lamella.

Table 1. X-ray microanalysis of elements in *Ocimum basilicum* roots under exposure to different concentrations of Cr³⁺.

Cr ³⁺ concentrations (mg L ⁻¹)	microanalysis of element (mg g dw ⁻¹)					
	Cr	Cu	Zn	Co	Ni	Mo
0	2.1 ± 0.1	35.3 ± 2.1	78.8 ± 4.04	13.6 ± 1.7	5.1 ± 1.1	18.3 ± 1.7
2	294.3 ± 28.4	38.8 ± 2.1	81.2 ± 5.8	18.5 ± 1.6	7.6 ± 0.8	23.6 ± 1.8
4	475.9 ± 39.1	24.8 ± 1.8	72.5 ± 4.2	11.3 ± 0.7	7.1 ± 1.3	15.3 ± 0.5
6	793.8 ± 69.6	17.1 ± 1.2	51.7 ± 3.1	7.2 ± 0.5	4.8 ± 0.6	11.4 ± 0.6
8	1130.9 ± 51.5	11.7 ± 1.5	50.9 ± 3.5	8.1 ± 0.9	4.5 ± 0.7	9.5 ± 0.7
R	0.786	-0.917	-0.873	-0.786	-0.502	-0.884
P	0.001	<0.001	<0.001	0.001	0.057	<0.001

Values represent the mean of 9 repetitions from triplicate cultivations ± SD;

P-values less than 0.05 are significant; R = Spearman correlation.

Table 2. X-ray microanalysis of elements in *Ocimum basilicum* shoots under exposure to different concentrations of Cr³⁺.

Cr ³⁺ concentrations (mg L ⁻¹)	microanalysis of element (mg g dw ⁻¹)					
	Cr	Cu	Zn	Co	Ni	Mo
0	nd	8.3 ± 1.7	21.8 ± 1.2	2.8 ± 0.1	1.25 ± 0.1	3.3 ± 0.09
2	11.9 ± 1.5	9.2 ± 0.5	23.5 ± 1.8	2.8 ± 0.3	1.79 ± 0.1	3.2 ± 0.06
4	22.7 ± 1.1	5.4 ± 0.4	19.2 ± 1.7	1.4 ± 0.2	1.15 ± 0.3	2.9 ± 0.07
6	29.6 ± 1.8	3.7 ± 0.3	10.7 ± 0.9	nd	nd	1.4 ± 0.02
8	57.2 ± 2.7	2.5 ± 0.3	8.6 ± 1.1	nd	nd	nd
R	0.986	-0.884	-0.906	-0.918	-0.794	-0.876
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Values represent the mean of 9 repetitions from triplicate cultivations ± SD; nd, not detected;

P-values less than 0.05 are significant; R = Spearman correlation.

et al., 1996; Chatterjee & Chatterjee, 2000; Khan et al., 2001). Cr⁺³ and Cr⁺⁶ are taken up by the plants by different mechanisms. The uptake of Cr(VI) is suggested to be active, involving carriers of essential anions such as sulphate, while Cr(III) is absorbed

through a passive mechanism, being retained by cation exchange sites on the cell wall (Han et al., 2004; Karuppanapandian & Manoharan, 2008; Gheju et al., 2009). It has been suggested that both species can interfere with the uptake of several other mineral

elements like Fe and S in barley (Skeffington et al., 1976); Fe, Mo, P, and N in sugar beet (Adriano, 1986); P, K, Zn, and Cu in bean (Barceló et al., 1985); and Mn, Cu, Zn, Fe, and Al in rye grass (Ottabbong, 1989). Competitive interaction between Cr and Cu, Mn, Fe, and S in the roots, stems and leaves of tomato was confirmed by Shanker et al. (2005). They noted that B and Cr had a synergistic interaction in the roots, but an antagonistic effect in the stem and leaves of tomato plants under Cr stress. Therefore, it is possible that Cr effectively competes with several elements to gain rapid entry into the plant system.

Bioaccumulation of chromium in the shoots and roots of *O. basilicum* was up to 57.2 ± 2.7 and 1130.9 ± 51.5 mg g dw⁻¹; respectively, ($P < 0.05$) under 8 mg L⁻¹ of Cr exposures. Results showed that while Cr accumulation in the roots was relatively high, lower amounts of Cr were transported to the shoots (Tables 1 and 2). Similar results have been reported in vegetable crops by Zayed et al. (1998) and in black gram seedlings by Karuppanapandian and Manoharan (2008). Poor translocation of chromium to the shoots could be due to sequestration of most of the Cr⁺³ in the vacuoles of the root cells to render it non-toxic, which may be a natural toxicity response of the plant. It must be noted that chromium is a toxic and nonessential element to plants; therefore, the plants may not possess any specific mechanism for the transport of Cr⁺³. On the other hand, transfer factors of Cr from roots to leaves decrease with increases in total chromium concentration in nutrient solutions. At low concentrations, Cr has higher transfer mobility from roots to leaves and when roots take up more Cr from nutrient solutions, transfer efficiency from roots to leaves decreases. This may also be one of the mechanisms plants have to tolerate and survive in highly Cr contaminated media. This has also been noted by Han et al. (2004).

TEM micrographs of root cortical cells of *Ocimum basilicum* exposed to 8 mg L⁻¹ of Cr³⁺ revealed that chromium was mainly deposited in the cytoplasm and enlarged periplasmic zone along the innermost layer of the cell wall, while ultrastructural studies of algal cells exposed to high concentrations of copper (Cu) showed that the element mainly formed nuclear inclusions, and these deposits were rarely found in the cytoplasm (Silverberg et al., 1976). Lead

(Pb) has been reported to be deposited within the nuclei of moss leaf cells (Skaar et al., 1973) and in some wetland plants (Qian et al., 1999). Formation of metal deposits of various shapes and sizes is a characteristic feature of the ultrastructure of plant cells exposed to high concentrations of heavy metal ions. However, the exact location of the electron dense deposits seems to depend on the element and the species under investigation (Han et al., 2004). X-ray microanalysis confirmed the presence of Cr, Cu, Zn, Co, Ni, and Mo in the granular metal deposits and Cr was the predominant element. Leaf mesophyll cells of *Ocimum basilicum* were void of any metal deposits as seen in Figure 2, possibly due to very little chromium being transported from the roots to the leaves. TEM micrographs of root (Figure 1) and leaf cells (Figure 2) of the plants exposed to Cr⁺³ show the overall toxicity effects of Cr⁺³ on intracellular organelles, disorganisation of the cytoplasm, formation of several metal deposits in the vacuole, cytoplasm along the cell wall, and abnormalities in the chloroplast ultrastructure; like a poorly developed lamellar system with widely spaced thylakoids and fewer grana. These Cr induced toxicity and abnormalities can cause severe toxic effects, both in the dark and light reactions of photosynthesis and photosynthetic pigments. Bioaccumulation of Cr and its toxicity to photosynthetic organelle and pigments in various crops and trees is well documented (Panda & Choudhury, 2005; Shanker et al., 2005; Sinha et al., 2006). Bera et al. (1999) studied the effect of Cr present in tannery effluent on the chloroplast pigment contents in mung bean and reported that irrespective of concentration, chlorophyll a, chlorophyll b, and total chlorophyll decreased in 6-day-old mung bean seedlings as compared to controls. Disorganisation of the chloroplast ultrastructure and the inhibition of electron transport processes due to Cr toxicity is a possible explanation for Cr induced decreases in the photosynthetic rate (Samantaray et al., 2001; Shanker et al., 2005). It can be concluded that the formation of chromium bearing deposits in root cells of *Ocimum basilicum* may have the effect of maintaining relatively low cytoplasmic concentrations of Cr, and presumably reduces the toxic effects of the element on cellular metabolism as a natural detoxification and cellular defensive mechanisms of basil plant under Cr stress.

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