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Full Length Research Paper

Chromium stress in *Brassica juncea* L. cv. 'Pusa Jai Kissan' under hydroponic culture

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Chromium (Cr) entering plant tissue inhibits most physiological processes at all levels of metabolism including inhibition of growth, photosynthesis and nitrate assimilation. Since Cr exists in many forms, its toxicity to plants depends on its valence state, with Cr (VI) found to be highly toxic and mobile than Cr (III). Different concentrations of Cr (0, 25, 50 and 100 µM) in the form of $K_2Cr_2O_7$ was added to 30 days old *Brassica juncea* plant and harvested on the 3rd and 5th days after treatment for estimation of plant growth, chlorophyll, total soluble protein, free amino acids and nitrate reductase activity. Cr was found to cause deleterious effects on whole plant growth. The potential of plants with the capacity to accumulate or to stabilize Cr compounds for bioremediation of Cr contamination has gained interest in recent years. The biochemical aspects like photosynthetic pigments (Chl a and Chl b), total protein and amino acids content decreased with Cr concentration. A significant increase in nitrate reductase activity was observed corresponding to Cr concentration.

Key words: *Brassica juncea*, chromium, heavy metal, phytoremediation.

INTRODUCTION

The toxicity of plants due to heavy metals, particularly on the agricultural economic crops, presents a challenge to plant scientists concerned with yield and quality in crop production (Bishehkolaei et al., 2011). These metals retard farming efficiency and destroy the health of the plants and animals (Mudgal et al., 2010). Chromium (Cr) is found in all living organisms and has long been known as an essential element for man and animal (Panda and Choudhury, 2005; Balk et al., 2007), but there is not sufficient evidence of its essentiality for the normal growth of plants (Zayed and Terry, 2003). Nowadays, contamination of the environment by Cr has become a major concern and its toxicity to plants depends on its valence state with Cr (VI) being highly toxic and mobile than Cr (III) (Gupta et al., 2009).

Cr enters the food chain through consumption of plant material. A high concentration of Cr has been found to be harmful to vegetation (Faisal and Hasnain, 2005; Gbaruko and Friday, 2007). As the Cr concentration in plants increases, it adversely affects several biological parameters and ultimately there is loss of vegetation, making lands sometimes becomes barren (Dube et al., 2003). Cr as one of several heavy metals that cause serious environmental contamination in soil, sediments and ground water, is a matter of serious problem. Cr is toxic for agronomic plants at about 0.5 to 5.0 mgL^{-1} in nutrient solution, but at lower concentration it stimulates plant growth (Kumarrai and Kumar, 2010). Cr (III) and Cr (VI), the major stable chemical forms in environment are of more concern (Arduini et al., 2006). Symptoms of Cr phytotoxicity include inhibition of seed germination or of early seedling development, reduction of root growth, leaf chlorosis and depressed biomass (Sharma et al., 1995).

There are many studies on Cr toxicity in crop plants.

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Abbreviations: Cr, Chromium; **DAT,** days after treatment; **NR,** nitrate reductase.

Reports have shown that it can affect growth, water balance, pigment content and initials lipid peroxidation, causing oxidative damage to plants (Bonet et al., 1991). Cr inhibits Hill reaction, affecting both dark and light reaction (Krupa and Baszynski, 1995; Zied, 2001). Chromium significantly affects the metabolism of plants such as *Hordeum vulgare* (Ali et al., 2004), *Citrullus* (Dube et al., 2003), Cauliflower (Chatterjee and Chatterjee, 2000), vegetable crops (Zayed et al., 1998), wheat (*Triticum aestivum* cv. HD2204) (Sharma et al., 1995), maize (*Zea mays*) (Sharma and Pant, 1994) and *Brassica juncea* (Jan et al., 2010).

Phytoremediation is a term that covers many different plant-based approaches for cleaning up contaminated environments, whereas phyto-detoxification involves the ability of plants to change the chemical species of contaminant to a best toxic form for example, plants take up toxic Cr (VI) and convert it to non-toxic Cr(III) (Zhang et al., 2009). Phyto-remediation of Cr pollution can be achieved by extraction of metal from polluted soils into harvestable plant tissues (phyto-extraction), by accumulation of elements in root tissues of aquatic plants growing in contaminated water (rhizofiltration) or by in site detoxification of metal through plant based chelation, reduction and oxidation mechanisms termed phyto-detoxification (Tangahu et al., 2011). With these aspects in view, the present investigation was made to study the effect of different concentrations of chromium on the growth, pigment concentration, protein content and enzyme activities of *B. juncea* under hydroponic culture.

MATERIALS AND METHODS

Plant culture and Cr treatment

Mustard seeds were germinated in paper towels and germinating seedlings of similar size were placed in half strength Hoagland's solution (Hoagland and Arnon, 1950) containing (mM): 2.4 Ca $(NO_3)_2$, 1.0 KH_2PO_4, 3.0 KNO_3, 1.0 $MgSO_4$ and 0.5 NaCl and (µM) 23.1 H_3BO_3, 4.6 $MnCl_2$, 0.38 $ZnSO_4$, 0.16 $CuSO_4$, 0.052 H_2MoO_4 and 44.8 $FeSO_4$ (as ferric sodium-EDTA complex) on perforated polystyrene floats. The nutrient solution was bubbled with sterile air and changed on alternate days. Growth chamber conditions were: photosynthetic photon flux density of 430 µM m^{-2} s^{-2}, 14 h of light, 10 h of dark and a relative humidity of 60%. Subsequent to day 7, plants were subjected to three chromium treatments. Chromium was supplied as $K_2Cr_2O_7$ as T_0=0 µM; T_1=25 µM; T_2=50 µM, T_3=100 µM.

After 3 and 5 days of treatment, plants were harvested for estimation of antioxidant enzymes and other biochemical studies. The whole results in this work are mean of three replicates per treatment followed by standard deviation (Mean ± SD) and percentage variation (PV). The experiments were conducted in completely randomized design (CRD).

Fresh and dry weight of root and shoot

Fresh weight of roots and fully expanded leaves was taken immediately after harvesting. Dry weight of roots, stem and leaves was determined after placing samples in hot air oven at 60°C till they dried to constant weight.

Estimation of chlorophyll

Chlorophyll a and b were extracted by non-maceration of tissue using dimethylsulphoxide (DMSO) by the method of Hiscox and Israelstam (1979).

Chlorophyll extraction

Freshly harvested leaves (0.05 g) were finely chopped and collected in test tubes containing 5.0 ml DMSO. The test tubes were covered with black paper and incubated at 65°C for 40 min. The reaction mixture was transferred to a graduated tube and the final volume was made up to 10.0 ml by adding DMSO. The chlorophyll content was then measured immediately or the reaction mixture without leaves was stored at 4°C in dark until analyzed.

Chlorophyll estimation

Briefly, 3.0 ml sample of chlorophyll extract was transferred to cuvette and the absorbance was taken at 645 and 663 nm on UV-visible spectrophotometer (Model DU 640B, Beckman, USA). The chlorophyll content was expressed as mg g^{-1} fw. Chlorophyll content was calculated following the equation given by Arnon (1949).

$$\text{Chlorophyll a (mg g}^{-1}\text{ fw)} = (12.7 \times OD\ 663) - (2.69 \times OD\ 645) \times \frac{V \times 10}{1000 \times W \times d}$$

$$\text{Chlorophyll b (mg g}^{-1}\text{ fw)} = (22.9 \times OD_{645}) - (4.68 \times OD_{663}) \times \frac{V \times 10}{1000 \times W \times d}$$

Where, V is the volume of the extract and W is the weight of the tissue taken.

Estimation of soluble protein content

Soluble protein content was estimated in leaves by the method given by Bradford (1976). To 0.5 ml of the crude extract, 1 ml of 10% trichloroacetic acid (TCA) was added and allowed to age for 30 min in ice. After centrifugation at 5,000 rpm for 5 min, the pellet obtained was washed with 1 ml of 5 % TCA. The precipitate was then dissolved in 0.1 N NaOH. To 0.1 ml of aliquot, 5.0 ml Bradford's reagent was added and mixed vigorously. The blue colour was developed within 2 min. The absorbance was taken at 595 nm with UV-Visible spectrophotometer (Model Spekol 1200, Analytical Jena, Germany). Calibration curve was drawn using different concentrations of bovine serum albumin, treated similarly as that of aliquots to calculate protein content. The protein content was expressed as mg g^{-1} fw.

Soluble amino acids

Lee and Takahashi's method (1966) was used for the estimation of soluble amino acids.

Extraction of total free amino acids

Briefly, 100 mg of dried material was homogenized in 5 ml of 80% ethanol, refluxed for 15 min on a steam bath and centrifuged at 20,000 rpm for 20 min. The residue was further refluxed twice with

Table 1. Effect of various concentration of chromium on root length and shoot length (cm per plant), root fresh weight and shoot fresh weight (g per plant), Chlorophyll a and b (mg g^{-1} fw) content of *B. juncea* seedling.

Parameter	Treatment (µM)			
	T_0	T_1	T_2	T_3
Root Length (cm per plant)	16.0 ± 1.00 (100)	11.0 ± 1.00 (69)	10.66 ± 0.577 (67)	9.50 ± 0.500 (60)
Shoot Length (cm per plant)	10.33±0.577 (100)	10.0 ±1.00 (97)	8.33 ± 0.510 (81)	6.66 ± 0.570 (65)
Root Fresh Weight (g per plant)	0.050 ± 0.01 (100)	0.043 ± 0.005 (86)	0.037 ± 0.055 (74)	0.025 ± 0.006 (50)
Shoot Fresh Weight (g per plant)	0.173 ± 0.03 (100)	0.150 ± 0.052 (87)	0.110 ± 0.003 (64)	0.083 ± 0.020 (48)
Chlorophyll a after 3 Days Treatment	0.050 ± 0.01 (100)	0.043 ± 0.005 (86)	0.037 ± 0.055 (74)	0.025 ± 0.006 (50)
Chlorophyll a after 5 days treatment	0.173 ± 0.032(100)	0.150 ± 0.052(87)	0.110 ± 0.003 (64)	0.083 ± 0.020 (48)
Chlorophyll b after 3 Days Treatment	4.58 ± 0.169 (100)	4.20 ± 0.100 (91.7)	4.50 ± 0.100 (98)	3.60 ± 0.152 (79)
Chlorophyll b after 5 days treatment	4.00 ± 0.260 (100)	3.60 ± 0.170 (90)	3.30 ± 0.301 (82)	3.20 ± 0.115 (80)

Data represents average of three samples analyzed ± S.D, Values in brackets represent percent variation compared to control. Plants were subjected to four concentrations of Cr: T^0 = 0 µM; T_1= 25 µM; T_2= 50 µM and T_3 =100 µM.

80% ethanol. The supernatants were pooled together for free amino acids estimation.

Estimation of total free amino acids

Total free amino acids were estimated using a reagent of pH 6.0. This reagent was prepared by mixing the following constituents (A, B and C) in the ratio of 5: 12: 2; (A) 1% ninhydrin in 0.5 M citrate buffer (pH 5.5); (B) pure glycerol; (C) 0.5 M citrate buffer (pH 5.5). Furthermore, 0.2 ml of extract was added to 3.8 ml of ninhydrin reagent. The contents were heated in boiling water bath for 12 min and cooled to room temperature. The purplish blue colour was read at 570 nm. The total free amino acids was calculated from the standard curve prepared by using glycine (5 to 50 mg) and expressed as mg amino acids per mg dry weight (DW) of tissue.

Nitrate reductase activity

This was determined by the method of Klepper et al. (1971). Vials containing 0.3 g fresh leaves with 3 ml phosphate buffer (Ph. 7.2) and 3ml of KNO_3 (0.4M) each were kept in vacuum desiccator and vacuum infiltration was done for 30 s. The vials were kept in water bath incubator for 1 h at 33°C under dark conditions and then in hot water for 5 min to stop the reaction. 1 ml of sulphanilamide (1% in 1 N-HCl) and 1 ml of N-(1-napthyl)ethylenediamine dihydrochloride (NEDD) (0.02%) were added into a 0.2 ml aliquot. The vials were kept in dark for 30 min for colour development and the final volume was then made up to 6 ml by adding diethylpyrocarbonate-treated distilled water (DDW). Absorbance was read at 540 nm on SPEKOL 1200 spectrophotometer. The corresponding concentrations of nitrite were determined against the standard curve prepared using sodium nitrite ($NaNO_2$) solution.

RESULTS AND DISCUSSION

Effect on growth

The presence of Cr in the external environment led to changes in the growth and development pattern of the plant (Table 1). The whole plant growth was decreased. The reduction of root length was 31, 33 and 40% by treatment of T_1, T_2 and T_3, respectively, as compared to T_0 (control) after 3 days of treatment (DAT). However, significant reduction in shoot length was at T_3 treatment. The significant reduction of shoot length was 97, 81 and 65% by treatment of T_1, T_2 and T_3, respectively, as compared to T_0 (100%) after 3 days of treatment (DAT). Our results comply with that of Jan et al. (2010) who reported that Cr toxicity in *B. juncea* leads to stunted growth, leaf chlorosis and alteration in the activity of many enzymes of various pathways.

As noted, plant root growth was more susceptible compared to shoot growth (Figure 1a and b). Decrease in leaf area and curling of leaves may be due to high levels of Cr accumulation in them. This interferes with the function of genes that govern the synthesis of enzymes, which in turn control the chemistry of cell; all this must somehow account for growth and development (Karuppanapandian and Manoharan, 2008; Gheju et al., 2009). Cr (VI) seems to act principally on plant roots, resulting in intense growth inhibition. Increasing concentration of Cr caused significant reduction in root length and shoot length (Gani, 2011). Adverse effects of Cr on plant height and shoot growth have been reported earlier by Rout et al. (1997). Cr transport to the aerial part of the plant can have a direct impact on cellular metabolism of shoots, contributing to the reduction in plant height. Cr exposure at micro molar range can lead to serve phototoxic symptoms in plant cell, which can result in inhibition of seed germination, degrade pigments status, nutrient balance and antioxidant enzymes (Panda and Khan, 2003; Panda, 2003). The general response of decreased root growth due to Cr toxicity could be as a result of inhibition of root cell division, root elongation or the extension of cell cycle in root (Chen et al., 2001). Decrease in root growth is a well documented effect due to heavy metals in trees and crops (Breckle, 1991; Goldbold and Kettner, 1991; Prasad et al., 2001).

a b

Figure 1. (a) Cr stress on root length of *B. juncea*. L. cv; (b) Cr stress on shoot length of *B. juncea*. L. cv.

Effect on chlorophyll

The pigment concentration significantly decreased with the increase in Cr concentration. Chl a in plant was decreased to 86, 74 and 50% with the treatments of T_1, T_2 and T_3, respectively, as compared to T_0 after 3 days of treatment (DAT). However, after 5 days of treatment (DAT), Chl a content decreased by 87, 64 and 48%. Also, the Chlorophyll b values decreased from 91.7, 98 and 79% after 3 DAT to 90, 82 and 80% after 5 DAT with the treatments of T_1, T_2 and T_3, respectively (Table 1). The chlorophyll pigments are present in thylakoid within chloroplast, and any damage brought to these structures can lead to denaturation of these pigments. It may be suggested that observed decrease in chlorophyll content at higher concentration of chromium may be due to breakdown of thylakoid and chloroplast envelope as was previously reported (Dodge and Law, 1974). There were also significant decreases in chlorophylls a and b content of the *B. oleracea* var. *acephala* plants treated with Cr (Ozdener et al., 2011). Levels of assimilatory pigments viz. chl a and b are often related to tissue damage in higher plants.

The observed decrease in leaf area can be correlated to reduced total chlorophyll content. Decrease in total chlorophyll, chlorophyll a, b and carotenoids have been well documented under Cr stress in plants (Panda and Patra, 1998, 2000; Tripati and Smith, 2000; Panda, 2003; Panda and Khan, 2003; Panda et al., 2003). Cr possesses the capacity to degrade δ-aminolevulinic acid dehydrates, an important enzyme involved in chlorophyll biosynthesis reactions (Shi and Dalal, 1990). Besides these effects, Cr can alter chloroplast and membrane ultra structure in plants (Choudhury and Panda, 2004). It can cause also ultra structural changes in chloroplast leading to inhibition of photosynthesis. Such alterations in chloroplast have been observed in case of plants like *Lemna minor*, Pistia species, *Taxithelium neplense*, and such change in chloroplast membrane structure is accompanied by changes in thylakoid arrangement. Moreover, at high concentration (1 mM), complete distortion of chloroplastic membrane was observed together with severe disarrangement of thylakoids indicating that Cr in hexavalent form can replace many Mg^+ ions from active enzymes and cause severe phytotoxic effects (Choudhury and Panda, 2004).

Effect on amino acids and protein content

The percentage increase in amino acids in T_1, T_2 and T_3 was observed after 3 and 5 days of treatment (DAT) of plants, respectively (Table 2). Protein content of leaves was significantly increased over control values at T_1, T_2 and T_3 treatments and over T_0; after 3 DAT and after 5 DAT, it resulted in significant decrease in protein content (Table 2). The amino acid content increased with increase in Cr concentration. The protein concentration was found to decrease with the increase in Cr concentration as earlier reported by Jan et al. (2010). Cr decreased

Table 2. Effect of various concentrations of chromium on Nitrate reductase (µmole $NO_2^-h^{-1}g^{-1}$ f.w.), Amino acid content (µ mole g^{-1} f.w.), Protein Content (mg g^{-1} f.w.) of *B. juncea* seedlings.

Parameter	Treatment (µM)			
	T_0	T_1	T_2	T_3
NR Activity after 3 Days Treatment	1.45 ± 0.026 (100)	1.51 ± 0.030 (105)	1.35 ± 0.031 (93)	1.16 ± 0.036 (80)
NR Activity after 5 days treatment	1.49 ± 0.010 (100)	1.33 ± 0.020 (89)	1.27 ± 0.025 (85)	1.146 ±0.045 (77)
Amino acid after 3 Days Treatment (µ mole g^{-1} fw)	104.0 ± 19.07 (100)	148.0 ± 15.60 (142)	149.3 ± 7.57 (144)	174.0 ± 5.03 (167)
Amino acid after 5 days treatment (µ mole g^{-1} fw)	129.3 ± 9.01 (100)	109.3 ± 8.80 (84)	63.3 ± 3.05 (49)	48.0 ± 10.58 (37)
Protein content after 3 Days Treatment (mg g^{-1} fw)	1.34 ± 0.034 (100)	1.57 ± 0.080 (117)	1.40 ± 0.055 (104)	1.61 ± 0.046 (120)
Protein content after 5 days Treatment (mg g^{-1} fw)	1.44 ± 0.089 (100)	1.19 ± 0.066 (83)	0.99 ± 0.117 (69)	0.61 ± 0.144 (42)

Data represents average of three samples analyzed ± S.D, Values in brackets represent percent variation compared to control. Plants were subjected to four concentrations of Cr: T^0 = 0 µM; T_1= 25 µM; T_2= 50 µM and T_3 =100 µM.

protein in dose- and time-dependent manner. Degradation of proteins in plants can result in inhibition of nitrate reductase activity (Solmonoson et al., 1999; Choudhury and Panda, 2004). The correlation between nitrate and protein is well documented in plants (Rai et al., 1992). The significant decrease in the sulphate uptake rates was observed in Cr treated plants in *B. juncea*, which was accompanied by expression of root low affinity sulphate transporter: a reason for decrease of protein activity and attained levels of reducing sugars in Cr treated *Brassica* plants (Michela et al., 2008).

The increased amino acid content with Cr treatment may be due to the increased proteolysis of the cellular proteins or *de novo* synthesis of amino acid. Accumulation of amino acids may be consequence of one of the several possibilities: (1) it might be the result of malfunctioning of respiratory activity due to membrane damage (Vazquez et al., 1987), resulting in the accumulation of several TCA cycle compounds, such as 2-oxyglutarate that promote the synthesis of specific amino acids; (2) increase in the levels of sulphur amino acids, activates the enzymes involved in their synthesis (Rauser, 1999) and (3) a reduced protein synthesis that contribute to the accumulation of amino acid, particularly at high concentration (Costa et al., 1997)

Effect on nitrate reductase (NR)

Nitrate reductase (NR) activity of leaves was significantly increased over control values at T_1 treatment after 3 days of treatment (DAT). Further increase in Cr concentrations resulted in significant inhibition of NR activity. NR activity also decreased in T_2 and T_3 in 3 DAT. After 5 days of treatment (DAT), there was a sharp decrease in NR activity, but a significant decrease was found at T_3 (Table 2). Seedlings treated with Cr resulted in increased NR activity, whereas higher Cr concentrations were toxic and reduced the enzyme activity significantly in wheat (Panda and Patra, 2000). Hamid et al. (2010) also found the decrease in enzyme activities in *B. juncea* upon Cr treatment under hydroponic culture. The inhibition of NR activity by Cr also affects ammonia assimilation pathway. Lower Cr concentration containing nitrogen supplemented in medium as reported by Panda and Patra (1988) in wheat might have helped in *de novo* synthesis of NR isoenzymes. For high concentration of metals like Cr, SH-group of NR enzyme is affected, resulting in a decline of its activity. Nitrate reductase (NR) activity of leaves was significantly increased over control values and negatively correlated with root and shoot length, leaf area and biomass of the plants, indicating stress due to Cr (VI) as reported in *Albizia lebbek* (Tripathi et al., 1999). On the other hand, lower concentrations of Cr resulted in significant inhibition of NR activity in *Nelumbo nucifera* (Vajpayee et al., 1999) and *Nymphaea alba* (Vajpayee et al., 2000).

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