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Study on physio-biochemical attributes and metallothionein gene expression affected by chromium (VI) in sugarcane (*Saccharum* spp. hybrid)

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Abstract

Single bud setts of sugarcane (*Saccharum* spp. hybdrid) cultivar CoLk 94184 was grown in soil tray culture conditions at varying levels of Cr (VI) *viz*., 0, 10 and 50 ppm in the form of potassium dichromate to study the effects of Cr exposure on growth, physio-biochemical attributes and metallothionein (MT) gene expression. The results obtained showed stunted growth, leaf chlorosis, reduced bud sprouting, plant height, root and shoot weight, plant vigor due to chromium in growing medium, 50 ppm Cr exhibited marked effect. Biochemical measurements indicated reduction in chlorophyll a, b and total and carotenoids contents, accumulation of proline and induction of lipid peroxidation, in terms of high malondialdehyde content, in leaf tissues of plants exposed to excess Cr. Specific activity of peroxidase enzyme in various plant organs decreased at 50 ppm Cr, and at 10 ppm it increased in leaf and root tissues. Metallothionein (MT) mRNA expression analysis showed increased MT gene expression with increase in Cr supply in both leaf and stalk tissues however, in leaf tissues, gene expression was comparatively low. Upregulation of MT gene due to high chromium in growing medium may help sugarcane crop in tolerance to Cr toxicity.

Key words

Chromium, Metallothionein gene, Photosynthetic pigments, Sugarcane

Introduction

Environmental contamination and accumulation of heavy metals in soil and water is a widespread ecological problem affecting all the living organisms, bacteria, plants, animals and humans (Eapen and D'Souza, 2005). Chromium is a dangerous pollutant, released in the environment due to its enormous industrial use (Cervantes *et al*., 2001). In nature, Cr exists in two different stable oxidation states, Cr (III) and Cr (VI) that differ in mobility, bioavailability and toxicity (Panda and Choudhury, 2005). Cr (III) is less soluble in water and toxic than Cr (VI) and is required in very low concentrations as an inorganic nutrient for animals and also for some plants (Schiavon *et al*., 2008). Cr uptake in plants depends on its oxidation state: Cr (III) is passively taken up by plants while entry of Cr (VI) is mediated by an active process requiring metabolic energy (Kleiman and Cogliatti, 1997). Oliveira (2012) reviewed the effects of low and high concentrations of chromium on growth and metabolism of plants and narrated reduced seed germination, growth yield, inhibition of enzyme activities, nutrient imbalances and mutagenesis due to Cr toxicity. Cr at low concentration (0.05 to 1 mg l^{-1}) has been found to promote growth and increase yield but it is toxic at high level for plants, limiting their growth and triggering symptoms of toxicity such as chlorosis of young leaves, tissue necrosis and root system damage (Peralta-Videa *et al*., 2009). Another toxic effect of high concentration of Cr in plants is induction of oxidative stress, which leads to DNA and cell membranes damage and inhibition of antioxidant metabolism (Panda and Choudhury, 2005; Schiavon *et al*., 2008; Jain *et al*., 2000, 2004; Yadav *et al*., 2010). Plants have developed several homeostatic

mechanisms, both enzymatic and nonenzymatic, to prevent the damage caused by overproduction of reactive oxygen species. Accumulation of free proline is one of the example of nonenzymatic mechanisms, which is recognized as having an important protective role against heavy metal stress, being reported to act as a radical scavenger or involved in metal chelation (Andrade *et al*., 2009). In addition, lipid-soluble antioxidants like carotenoids play an important function in plant metabolism, including oxidative stress tolerance (Gill and Tuteja, 2010). Thus, quantification of membrane damage, carotenoids, and free proline levels in plants exposed to high level of metals provides indications of damaging effects caused by Cr exposure, and degree of stress imposed to plants. Because inorganic compounds cannot be degraded into simple compounds, they have to be removed from the environment and transported elsewhere, transformed into less toxic forms, or stabilized *in situ*.

Metallothioneins are cysteine rich, low-molecular-weight, and metal binding proteins, which have been found in a wide variety of organisms including animals, plants, cyanobacteria and fungi. Plant MTs are extremely diverse and can be classified into four subfamilies (MT1 to MT4) based on the arrangement of Cys-residues. Due to their ability to reversibly bind both toxic and essential metal ions, plant MTs play an important roles in detoxification, metal ion homeostasis, and metal transport adjustment. Recently, the role of MT genes in heavy metal tolerance mechanism has been demonstrated in several plant species, such as *Aradiopsis thaliana, Zea mays, Solanum*, etc. Sugarcane is one of the important plant species that contain all four types of MTs and one of most potential species for biomass production and high metal enrichment capacity. In view of the above, the present study aimed at evaluating the physiological and biochemical responses of sugarcane, *Saccharum* spp. hybrid CoLk 94184 to Cr (VI) treatment, by analyzing growth parameters, photosynthetic pigments, free proline, peroxidase activity and degree of membrane degradation by lipid peroxidation. Semi quantitative reverse transcription polymerase chain reactions (qRT-PCR) were performed to investigate the behaviour of metallothionein (MT) gene encoding transcripts in shoot and leaf tissues.

Materials and Methods

Plant material and growth condition : Single bud setts of sugarcane (*Saccharum* spp. Hybrids) cultivar CoLk 94184 (an early maturing, high sugar) were planted in soil tray culture condition at Indian Institute of Sugarcane Research, Lucknow in the year 2014. Graded level of Cr (VI) was applied at the time of planting by mixing chromium in soil @ 0, 10 and 50 ppm Cr (VI) as potassium dichromate. Each treatment was replicated three times and each replicate included 5 plants per tray. These trays were kept under net house conditions (day temperature of 31.3º to 37.6 ºC, night temperature of 15.6º - 19.7ºC). About 11 and 17 days after planting, data on bud sprouting were recorded. Plants at about 20 days after planting (DAP) were sampled to determine plant height, fresh weight of shoot and root. Plant vigor was calculated as mentioned earlier (Jain *et al*., 2010).

Biochemical analysis : In fresh leaves, chlorophyll, carotenoid, proline, MDA, soluble protein contents and activity of peroxidase enzyme were determined at settling stage (18 DAP).

Determination of photosynthetic pigments : Photosynthetic pigments *viz.*, Chlorophyll a, b and total and carotenoids contents were determined in fresh leaves by the method of Arnon (1949). 100 mg fresh leaves were ground in 10 ml acetone solution (80%) with pinch of $CaCO_3$. The homogenate was centrifuged at 10,000 rpm at room temperature for 10 min. Supernatants were collected and absorbance of the supernatants was measured at 663, 645 and 470nm. Chlorophyll a, b and carotenoid contents were calculated and results were expressed in mg g^{-1} f. wt.

Determination of lipid peroxidation : Lipid peroxidation level in leaf tissues was determined by estimating malondialdehyde (MDA) content as described by Heath and Packer (1968). Fresh leaves (200 mg) were ground in 3 ml TCA (5%) solution and centrifuged at 6000 rpm for 10 min. 2 ml supernatant was mixed with 2 ml 5% TBA and incubated for 30 min at 100 ºC to develop (TBA)2-MDA complex. The mixture was cooled rapidly in an ice bath and centrifuged for 10 min. After centrifugation, absorbance was measured at 532 and 600 nm wavelength. Lipid peroxidation was expressed as µmol g^{-1} f.wt.

Quantification of proline content : Proline was determined in fresh leaves by the method of Bates *et al*. (1973). Samples (200 mg f.wt.) were extracted in 2 ml of 3% (w/v) sulphosalicylic acid. Samples were centrifuged at 6000 rpm and supernatants were collected for proline estimation. In 1 ml aliquot, 2 ml ninhydrin reagent and 2 ml acetic acid were added and heated for 30 min in boiling water bath. After cooling, color was extracted in 5 ml toluene solution by vertex mixing and the upper aqueous phase (toluene) was decanted in a dry glass tubes and absorbance was measured at 520nm using Spectrophotometer. Results were expressed in µg proline 100 mg^{-1} f. wt.

Preparation of enzyme extract : Enzyme extract for peroxidase assay was prepared by freezing fresh leaf tissues in liquid nitrogen, followed by grinding with 5 ml 0.1M phosphate buffer pH 7.5 containing 0.5mM EDTA. Homogenate was centrifuged for 10 min at 4ºC and 12,000 rpm and supernatant collected was used as enzyme extract.

Peroxidase assay : For peroxidase assay, the reaction mixture containing 5 ml 0.1 M phosphate buffer, pH 6.0, 1 ml 0.01% H_2O_2, 1 ml 0.5% *p*-phenylenediamine and enzyme extract (0.1 ml) was incubated for 5 min at 25ºC. After 5 min, reaction was stopped by adding 2 ml 5N H_2SO_4. The color developed was measured at 485 nm, and peroxidase activity was expressed as change in optical density (ΔOD) per mg protein as described earlier (Jain *et al*., 2014).

RNA extraction and qRT-PCR reactions for MT gene expression : Nucleotide sequences for candidate MT gene (gene accession no EU760482.1) and for internal control actin (gene accession no. 53759188) were obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and oligonucleotide primers were designed using Prime3 Output software (Table 1).

For gene induction studies, 30-day-old plants were sampled from control and treatment and stalk and leaf tissues were used for RNA isolation. Total RNA was isolated in stalk and leaf tissues of control and treated plants using QIAGEN RNeasy plant Mini kit as per Manufacturer instruction. DNA contamination was removed by using RNAse free DNase (QIAGEN). Purified RNA was stored at -20°C for further analysis. The quantity and quality of RNA was checked using Picodrop spectrophotometer on 1.0% agarose gel.

qRT-PCR was performed in a PCT-200 thermal cycler (BioRad). Equal amount of RNA (200ng) was used for RT-PCR reaction. Amplification was carried using QIAGEN one step RT-PCR kit as follows: 50°C for 30 min for reverse transcription reaction, 94°C for 15 min, 94°C for 1 min, 49°C for 1 min, 72°C for 1 min for 30 cycles and final extension at 72°C for 10 min. Amplification products were electrophoresed on 1.6% agarose gel. Gels were stained with ethidium bromide and visualized on gel documentation system (AlPha Innotech) after agarose gel electrophoresis. Three biological replicates were assayed for each treatment and each reaction was performed in duplicate. Gene expression, in terms of integrated density value (IDV), was determined using AlphaEase software supplied along with gel documentation system (Alpha Innotech, USA). IDV thus obtained was divided by 1,000 for ease of writing on Y-axis; thus, IDV was multiplied by 1,000 to get the original value of gene expression.

Statistical analysis : The experiment was conducted in a completely randomized design (CRD) with three replications. Data were analyzed by one-way analysis of variance, according to Cochran and Cox (1957). The mean values were compared using post-hoc least significant difference (LSD) test and term significant was used to indicate differences at $P < 0.05$.

Results and Discussion

Chromium application in growing medium gradually reduced bud sprouting in a dose dependent manner, highest decrease was obtained at 50 ppm Cr (33%) (Fig.1). Reduction in bud sprouting might be due to poor setts root growth and bud injury under toxic level of Cr in growing medium. Similar to bud sprouting in sugarcane, reduced rate of bud germination has been reported in wheat, sorghum, maize and other crops exposed to higher concentration of Cr (VI) (100-500 mg l^{-1}) (Lopez- Luna *et al*., 2009; Dey *et al*., 2009; Labra *et al*., 2006). Decrease in germination is a common response upon exposure to heavy metals like Cd, Pb and Hg (Smiri *et al*., 2009; Munzuroglu and Geckel, 2002). Reduction in bud sprouting/germination due to excess Cr in growing medium can be related to decrease invertase/amylase activities under Cr stress which reduces sugar availability to developing organs (Jain *et al*., 2000; Dua and Sawhney, 1991). Increasing Cr supply decreased plant vigor, highest decrease was obtained at 50 ppm Cr supply (Fig.3). Reduction in plant vigor might be due to poor plant height (49.4 % reduction over control) (Fig.1) and root growth indicating phytotoxicity of Cr (VI) for sugarcane at 50 ppm.

Results obtained indicated decrease in fresh weight of both root and shoot due to Cr presence in growing medium; root growth was affected more (78.8%) than shoot (23.1%) at 50 ppm Cr supply (Fig.1). Plants exhibited stunted growth, reduced leaf area, root length, number, leaf chlorosis at early stage. Sett roots affected maximum at 50 ppm Cr, which in turn affected shoot growth negatively. Similar to sugarcane, other plants like maize, mung bean, sorghum, paddy, oat also showed decreased root growth when exposed to Cr (VI) (Peralta *et al*., 2001; Samantry, 2002; Lopez-Luna *et al*., 2009; Sundaramoorthy *et al*., 2010). Reduction in root growth might be due to inhibition of root cell division, elongation and lower number, which consequently resulted in reduced root capacity to absorb water and nutrients under Cr stress (Oliveira, 2012).

Reduction in shoot growth, leaf area, plant height due to Cr exposure has also been reported in mung bean, wheat

Table 1 : Gene sequence used for qRt-PCR analysis

Sequence name	Forward (5'-3')	Reverse (3'-5')
MT gene	AGATGTACCCAGACATGAGC	AGGGTTACACTTGCAGTCAG
Actin gene	GGACATCCAGCCTCTTGT	GCAAGATCCAAACGAAGAATG

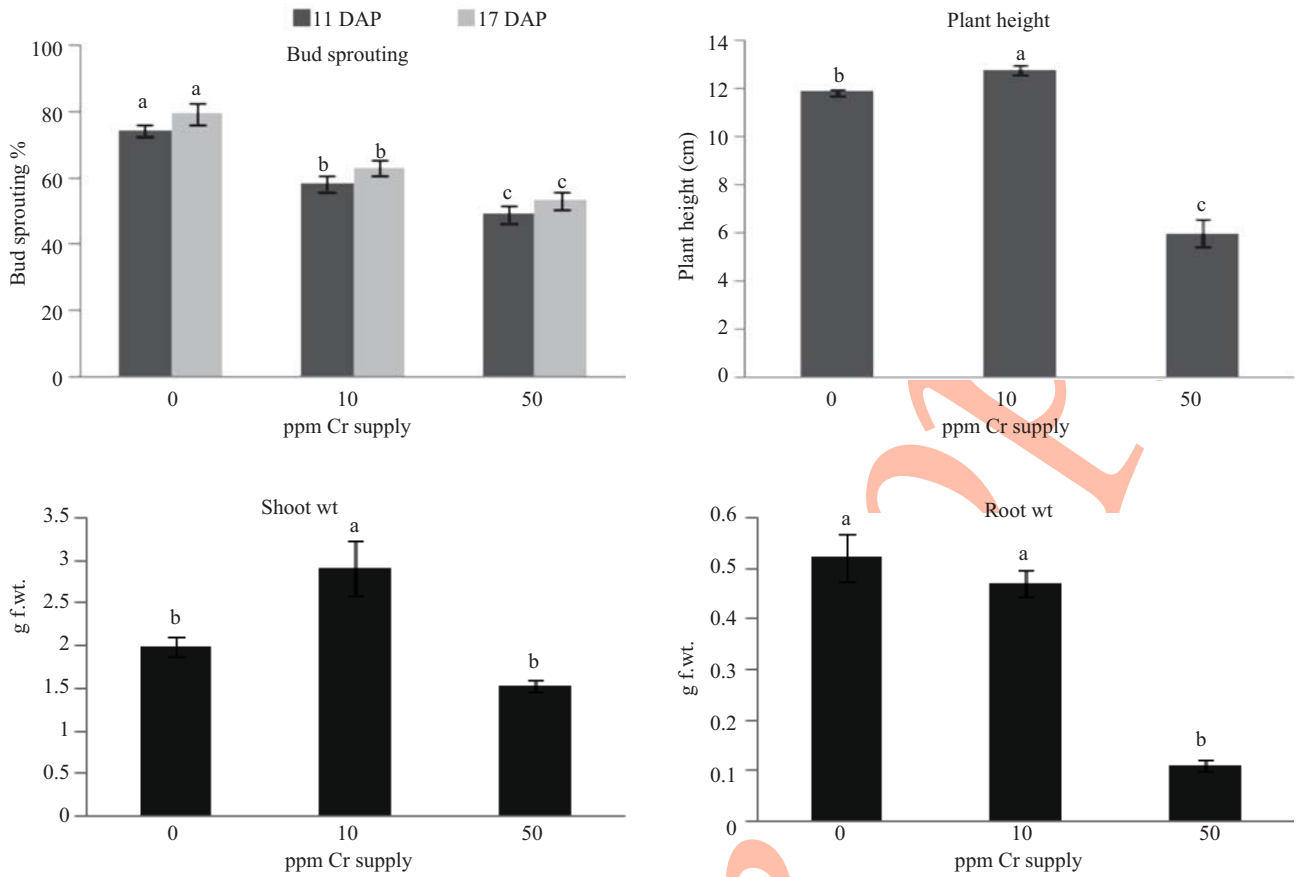


Fig 1 : Values of bud sprouting (%), plant height (cm), shoot and root weight (Wt-gfwt) in sugarcane (*Saccharum* spp. hybrid) cultivar CoLk 94184. The vertical bars indicate ± SD. Mean values with different letters indicate significant ($p \leq 0.05$) differences between treatments

and oat by several workers (Lopez-Luna *et al*., 2009; Dey *et al*., 2009; Rout *et al*., 2000). Growth inhibition of sugarcane root and shoot growth at higher level of Cr might be due to higher solubility and easily translocation of hexavalent chromium to growing shoot resulting in oxidative stress (reactive oxygen species (ROS) formation) which causes cell membrane damage (Schiavon *et al*., 2008). Reduction in leaf area might be due to reduced cell number and size. Chlorosis in upper part has been considered primarily due to toxic effects of chromium in roots and central parts of stem (Sharma *et al*., 1995).

Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents were comparatively lower in Cr treated plants as compared to control; highest reduction was observed at 50 ppm Cr supply (Fig. 2). Reduction in chlorophyll content was also reported earlier by other workers in several monocots and dicots (Tiwari *et al*., 2009; Zeid , 2001; Scoccianti *et al*., 2006; Amin *et al*., 2013), which might be due reduced chlorophyll synthesis, chlorophyllase activity, degradation of δ-aminolaevulinic acid dehydrates and inhibition of assimilation and transport of Mg and Fe to growing leaves (Vernay *et al*., 2007; Vajpayee *et al*., 1999; Pandey *et al*., 2009).

Lipid peroxidation determined in terms of MDA content was significantly increased in leaf tissues due to Cr toxicity growing medium; maximum content was observed at 50 ppm Cr level (8.02% over control) (Fig.3). In contrast to these results, MDA content decreased in root tissues of *Solanum nigrum* under low level of Cr supply (Teixeira *et al*., 2013).

Proline content showed maximum increase at 50 ppm Cr concentration (263% increase over control) (Fig.3). It is well known that proline accumulates in plants during adaptation to various types of environmental stresses such as drought, high temperature, nutrient deficiency and exposure to heavy metals (Oncel *et al*., 2000). Proline can eliminate singlet oxygen and OH radicals. It plays an important role to stabilize protein structures, DNA, as well as membranes and sub-cellular structures against denaturation (Kavi Kishor *et al*., 1995). These results suggest that stress-inducible proline accumulation in sugarcane plants under chromium acts as a

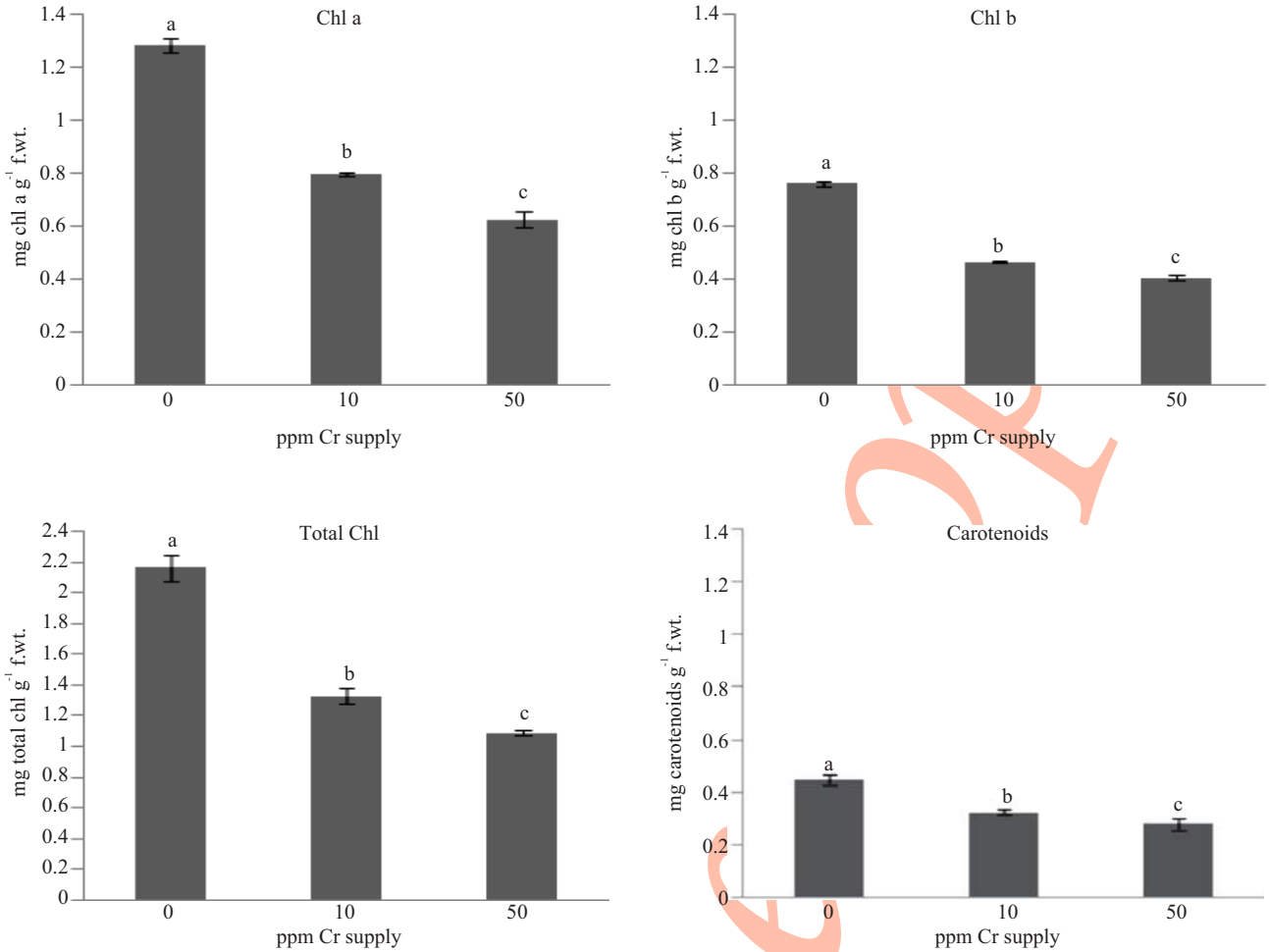


Fig 2 : Chlorophyll a, b, total and carotenoid (mg g^{-1} f.wt.) in sugarcane (Saccharum spp. hybrid) cultivar CoLk 94184. The vertical bars indicate ± SD. Mean values with different letters indicate significant ($p \leq 0.05$) differences between treatments

component of antioxidative defense system rather than as an osmotic adjustment mediator (Teixeira *et al*., 2013).

Peroxidase activity was determined in leaf, stalk and root tissues of chromium treated plants. Result obtained indicated increased activity of peroxidase enzyme in leaf and root tissues at 10 ppm Cr, and decreased markedly at 50 ppm Cr in all the plant parts (Fig.3). Increased activity of antioxidant enzyme at low Cr level protected sugarcane plants from membrane damage caused by free redicals due to Cr toxicity (Lin and Kou, 2007), and at higher level reduction might be due to leaf chlorosis.

Accumulation of MT-related transcripts occurred in both shoot and leaf tissues of treated plants and these transcript level was comparatively low in control plants (Fig. 4). This increase was about 63% in stalk and 535% in leaf over control at 50 ppm Cr supply. Sugarcane like other plants may have also used other antioxidant defence mechanisms, including chelating agents such as metallothioneins, phytochelatins or organic acids (Cobbett and Goldsbrough, 2002) and enzymatic defenses (Andrade *et al*., 2010; Fidalgo *et al*., 2011; Teixeira *et al*., 2013). Increase in free proline content in leaves of Cr treated plants may be result of metal uptake, indicating involvement of proline in homeostasis of heavy metals in plants, as suggested earlier in sugarcane and other plants (Mehta and Gaur, 1999; Pandey and Sharma, 2003; Jain *et al*., 2000). MT gene transcripts accumulated in both leaf and stalk tissues at 10 and 50 ppm Cr level which is in accordance with the results reported by Teixeira *et al*. (2013).

Findings obtained in the present study suggest poor bud sprouting, reduced root and shoot growth, leaf chlorosis, and lower content of photosynthetic pigments due to chromium in growing medium; 50 ppm Cr level showed significant reduction. Increased MDA content under chromium excess indicates oxidative stress and this might be

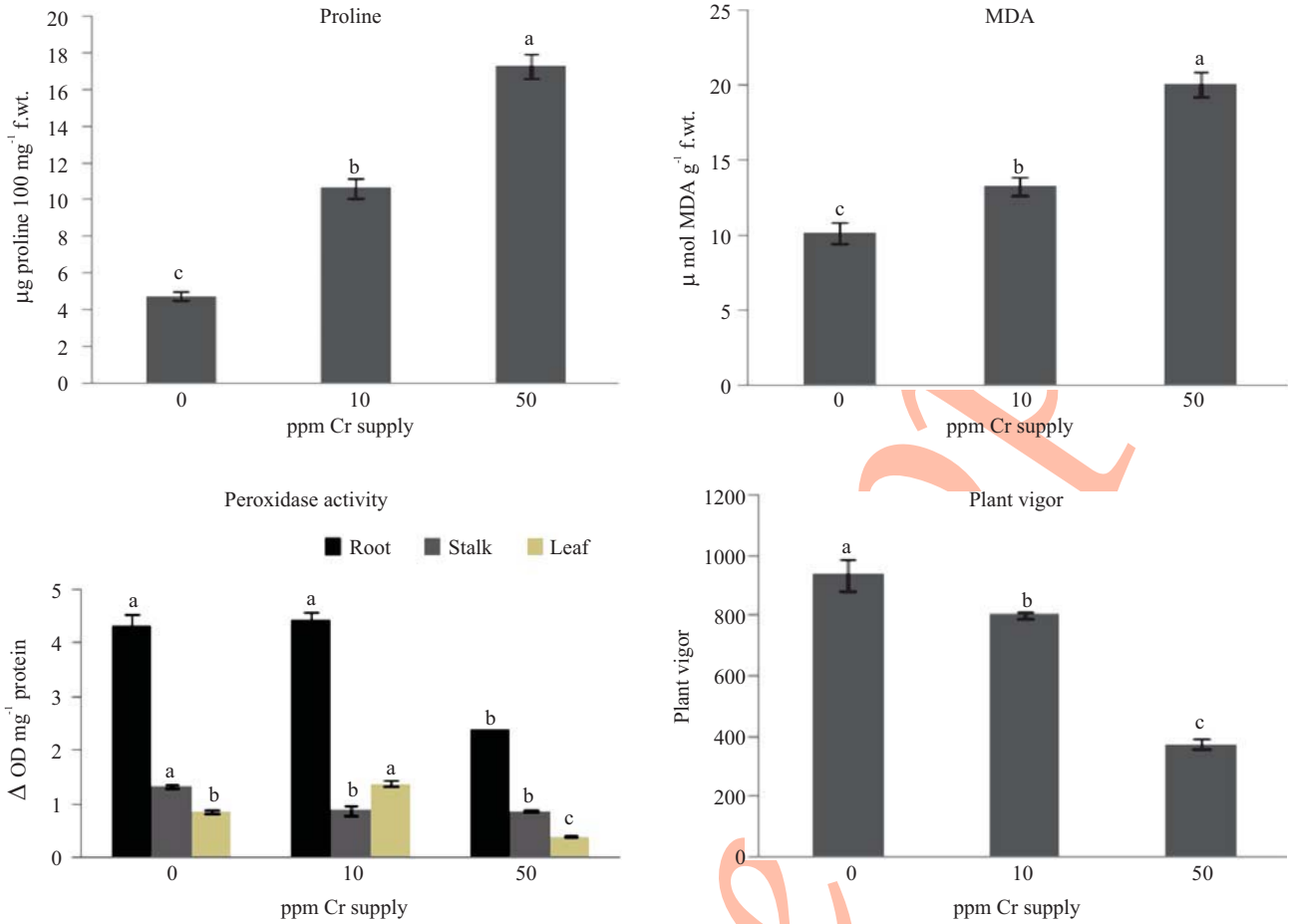
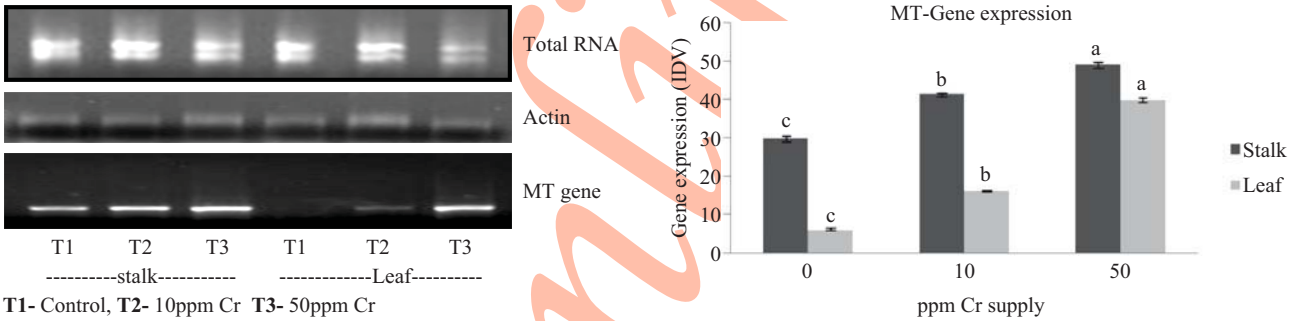


Fig. 3 : Values of Proline (µg 100 mg^{-1} f.wt.), MDA (µmol g^{-1} f.wt.), peroxidase activity (ΔOD mg $protein^{-1}$) and plant vigor in sugarcane (Saccharum spp. hybrid) cultivar CoLk 94184. The vertical bars indicate ± SD. Mean values with different letters indicate significant ($p \leq 0.05$) differences between treatments



T1- Control, **T2-** 10ppm Cr **T3-** 50ppm Cr

Fig. 4 : Expression pattern of MT gene in response to chromium in stalk and leaf tissues of sugarcane (Saccharum spp. hybrid) cultivar CoLk 94184. The vertical bars indicate ± SD. Mean values with different letters indicate significant ($p \leq 0.05$) differences between treatments

one of the potential mechanisms by which toxicity due to heavy metals is manifested in plant tissues. Accumulation of proline content and upregulation of MT gene may help sugarcane plants in tolerance to chromium toxicity.

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References

Amin, H., B.A. Arain, F. Amin and M.A. Surhio: Phytotoxicity of chromium on germination, growth and biochemical attributes of *Hibiscus esculentus* L. *American J.Plant Sci.,* **4**, 2431-2439 (2013).

Andrade, S.A., P.L. Grat~ao, M.A. Schiavinato, A.P. Silveira, R.A. Azevedo and P. Mazzafera: Zn uptake, physiological response and stress attenuation in mycorrhizal jack bean growing in soil with increasing Zn concentrations. *Chemosphere,* **75**, 1363 1370 (2009).

Andrade, S.A., P.L. Grat~ao, R.A. Azevedo, P.D. Silveira, M.A. Schiavinato and P. Mazzafera: Biochemical and physiological changes in jack bean under mycorrhizal symbiosis growing in soil with increasing Cu concentrations. *Environ. Exp. Bot.,* **68**,198–207 (2010).

Arnon, D.I.: Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. *Plant Physiol.,* **24**,1–15 (1949).

Bates, L.S., R.P. Waldren and I.D. Teare: Rapid determination of free proline for water-stress studies. *Plant Soil,* **39**, 205–207 (1973).

Cervantes, C.J., J.C. Garcia, S. Devars, F. Gutierrez-Corona, H. Loza-Tavera, J.C. Torres-Guzman and R. Moreno-Sanchez: Interactions of chromium with microorganisms and plants. *FEMS. Microbiol. Rev.* **25**: 335–347 (2001).

Cobbett, C. and P.B. Goldsbrough: Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol*., **53**, 159–182(2002).

Cochran, W.G. and G.M. Cox: Experimental designs. Wiley, New York (1957).

Dey, S.K., P.P Jena and S. Kundu: Antioxidative efficiency of *Triticum aestivum* L. exposed to chromium stress. *J. Environ. Biol.,* **30**, 539–544 (2009).

Dua, A. and S.K. Sawhney: Effect of chromium on activities of hydrolytic enzymes in germinating pea seeds. *Environ. Exp. Bot.,* **31**, 133-139 (1991).

Eapen, S. and S.F. D'Souza: Prospects of genetic engineering of plants for phytoremediation of toxic metals. *Biotechnol. Adv.,* **25**, 97–114 (2005).

Fidalgo, F., R. Freitas, R. Ferreira, A.M. Pessoa and J. Teixeira: *Solanum nigrum* L. antioxidant defence system isozymes are regulated transcriptionally and post translationally in Cd-induced stress. *Environ. Exp. Bot.,* **72**, 312–319 (2011).

Gill, S.S. and N. Tuteja: Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.,* **48**, 909–930 (2010).

Heath, R.L. and L. Packer: Photoperoxidation in isolated chloroplasts. *Arch. Biochem. Biophys.,* **125**, 189–198 (1968).

Jain, R., A. Chandra, V.K. Venugopalana and S. Solomon: Physiological changes and expression of SOD and PCS genes in response to water deficit in sugarcane. *Sugar Tech.* **17**, 276-282 (2014).

Jain, R., S. Srivastava and V.K. Madan: Influence of chromium on growth and cell division of sugarcane. *Indian J. Plant Physiol.,* **3**, 228-231(2000).

Jain, R., A.K. Shrivastava and S. Srivastava: Heavy metals in industrial wastes and their effect on sugarcane. *Intern. Sugar J.,* **22**, 23-27 (2004).

Jain, R., S. Srivastava, S. Solomon, A.K. Shrivastava and A. Chandra: Impact of excess zinc on growth parameters, cell division, nutrient accumulation, photosynthetic pigments and oxidative stress of sugarcane plants (*Saccharum* spp.). *Acta Physiol. Plantarum,* **32**, 979-986 (2010).

Kavi Kishor, P.B., Z. Hong, G.H. Miao, C.A. Hu and D.P.S. Verma: Overexpression of $^{\Delta 1}$ - pyrroline - 5 - carboxylate synthase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol*., **108**, 1387-1394 (1995).

Kleiman, I.D. and D.H. Cogliatti: Uptake of chromate in sulfate deprived wheat plants. *Environ. Pollut.,* **97**, 131–135 (1997).

L´opez-Luna, J., M.C. Gonz´alez-Ch´avez, MC, F.J. Esparza-Garc´ıa and R. Rodr´ıguez-V´azquez: Toxicity assessment of soil amended with tannery sludge, trivalent chromium and hexavalent chromium, using wheat, oat and sorghum plants. *J. Hazard. Mate.,* **163**, 829–834 (2009).

Labra, M., E. Gianazza and R. Waitt et al.: *Zea mays* L. protein changes in response to potassium dichromate treatments. *Chemosphere,* **62**, 1234–1244 (2006).

Lin, Y.C. and C.H. Kao: Proline accumulation induced by excess nickel in detached rice leaves. *Biol. Plant.,* **51**, 351– 354 (2007).

Mehta, S.K. and J.P. Gaur: Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in *Chlorella vulgaris*. *New Phytol.,* **143**, 253–259 (1999).

Munzuroglu, O. and H. Geckil: Effects of metals on seed germination, root elongation, and coleoptile and hypocotyls growth in *Triticum aestivum* and *Cucumis sativus. Arch. Environ. Contam. Toxicol.* **43**, 203–213 (2002).

Oliveira, H.: Chromium as an environmental pollutatnt: Insights on induced plant toxicity. *J. Bot.,* **2012**, (2012). (DOI 10.1155/2012/375843).

Oncel, I., Y. Keles and A.S. UStun: Enteractive effects of temperature and heavy metal stress on the growth and some biochemical compounds in wheat seedlings. *Environ. Poll.,* **107**, 315-320 (2000).

Panda, S.K. and S. Choudhury: Chromium stress in plants. *Braz. J. Plant Physiol.,* **17**, 95–102 (2005).

Pandey, V., V. Dixit and R. Shyam: Chromium effect on ROS generation and detoxification in pea (*Pisum sativum*) leaf chloroplasts. *Protoplasma,* **236**, 85–95 (2009).

Pandey, N. and C.P. Sharma: Chromium interference in iron nutrition and water relations of cabbage. *Environ. Exp. Bot.,* **49**, 195–200 (2003).

Peralta, J.R., J.L. Gardea-Torresday, K.J. Tiemann, E. Gomez, S. Arteaga, E. Rascon and G. Carillo: Uptake and effects of five heavy metals on seed germination and plant growth in alfalfa (*Medicago sativa*) L. *Bull. Environ. Contamin. Toxicol.,* **66**, 727–734 (2001).

Peralta-Videa, J.R., M.L. Lopez, M. Narayan, G. Saupe and J. Gardea-Torresdey: The biochemistry of environmental heavy metal uptake by plants: implications for the food chain. *Inter. J. Biochem. Cell Biol.,* **41**, 1665–1677 (2009).

Rout, G.R., S. Samantaray and P. Das: Effects of chromium and nickel on germination and growth in tolerant and non-tolerant populations of *Echinochloa colona* (L.) link. *Chemosphere,* **40**, 855–859 (2000).

Samantary, S.: Biochemical responses of Cr-tolerant and Cr sensitive mung bean cultivars grown on varying levels of chromium. *Chemosphere,* **47**, 1065–1072 (2002).

Schiavon, M., E. Pilon-Smits, M. Wirtz, R. Hell and M. Malagoli: Interactions between Chromium and Sulfur Metabolism in *Brassica juncea*. *J. Environ. Qual.,* **37**, 1536– 1545 (2008).

Scoccianti, V., R. Crinelli, B. Tirillini, V. Mancinelli and A. Speranza:

Uptake and toxicity of Cr(III) in celery seedlings. *Chemosphere,* **64**, 1695–1703 (2006).

Sharma, D.C., C. Chatterjee and C.P. Sharma: Chromium accumulation and its effects onwheat (*Triticum aestivum* L. Ev.HD 2204) metabolism. *Plant Sci.,* **111**, 145-151(1995).

Smiri, M., A. Chaoui, E. El Ferjani: Respiratory metabolism in the embryonic axis of germinating pea seed exposed to cadmium. *J.Plant Physiol.,* **166**, 259–269 (2009).

Sundaramoorthy, P., A. Chidambaram, K.S. Ganesh, P. Unnikannan and L. Baskaran: Chromium stress in paddy: nutrient status of paddy under chromium stress; phytoremediation of chromium by aquatic and terrestrial weeds. *Comptes Rendus Biologies* **333**, 597–607 (2010).

Teixeira, J., P. Ferraz, A. Almeida, N. Verde and F. Fidalgo: Metallothionein multigene family expression is differentially affected by Chromium (III) and (VI) in *Solanum nigrum* L. plants. *Food and Energy Security,* **2,** 130–140 (2013).

Tiwari, K.K., S. Dwivedi, N.K. Singh, U.N. Rai and R.D. Tripathi: Chromium (VI) induced phytotoxicity and oxidative stress in pea (*Pisum sativum* L.): biochemical changes and translocation of essential nutrients. *J. Environ. Biol.,* **30**, 389–394 (2009).

Vajpayee, P., S.C. Sharma, SC, R.D. Tripathi, U.N. Rai and M. Yunus: Bioaccumulation of chromium and toxicity to photosynthetic pigments, nitrate reductase activity and protein content of *Nelumbo nucifera* Gaertn. *Chemosphere,* **39**, 2159–2169 (1999).

Vernay, P., C. Gauthier-Moussard and A. Hitmi: Interaction of bioaccumulation of heavy metal chromium with water relation, mineral nutrition and photosynthesis in developed leaves of Lolium perenne L. *Chemosphere,* **68**, 1563–1575 (2007).

Yadav, D.V., R. Jain and R.K. Rai: Impact of heavy metals on sugar cane. In: Soil heavy metals: Soil Biology series, 19 (Eds: I. Sherameti and A. Varma). Verlag Berlin, Heidelberg, pp. 339–367 (2010).

Zeid, I.M.: Responses of *Phaseolus vulgaris* to chromium and cobalt treatments. *Biol. Plant.,* **44**, 111–115 (2001).