Biospectra : Vol. 8(2), September, 2013 Spl. pp 125-132
An International Biannual Refereed Journal of Life Sciences

Plant Sciences
Abst.No.MBD- 05

ISSN : 0973-7057

Changes in Growth, Antioxidative Defence System and Osmoprotectants in *Brassica juncea* L. Plants under Cd Metal Stress

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Received 16th July, 2013; Revised 10th August, 2013

Abstract : The present work was conducted to study the effects of Cd metal on morphological parameters (root length, shoot length and number of leaves), activities of antioxidative enzymes (guaiacol peroxidase, catalase, superoxide dismutase, ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase and mono-dehydroascorbate reductase), protein content, MDA content, proline content and glycine-betaine content in 30-days old plants of Brassica juncea as these plants are hyper-accumulator of Cd metal. Findings of present study were revealed that root length, shoot length and number of leaves lowered under different concentrations of Cd treatment while activities of antioxidative enzymes and osmolytes content were found to enhance. The observations indicated that the defence system of plant was activated under metal stressed conditions.

Key Words: Cd toxicity, Oxidative stress, Antioxidative defence system, osmolytes.

INTRODUCTION

Heavy metal toxicity is one of the major environmental threats to the world. Concentration of heavy metals is increasing in the environment due to some natural sources like decaying vegetation, forest fires, wind-blown dust and sea spray and certain anthropogenic activities such as mining, wood production, use of phosphate fertilizer and metal production etc. Even their low concentration may cause serious problems to organisms (Thounaojam *et al*., 2012). Cadmium

(Cd) is considered as one of the most toxic metals for organisms because of its phytotoxicity and high water solubility (Clemens, 2006). It acts as a carcinogen for humans (Deckert, 2005). Use of phosphate fertilizers and industrial processes are the main sources by which Cd enters into the environment and which further pass into the food chain. Stunted growth, leaf chlorosis and unspecific necrosis are the main symptoms of Cd toxicity, which leads to death of plants (Baryla *et al*., 2000; Schutzendubel *et al.,* 2001). Commercial development of Cd electroplating enhanced the production of cadmium. Cd toxicity reduces stomatal density and conductance to carbon dioxide (Baryla *et al*., 2000). It causes alteration in metabolic processes of plants like photosynthesis, respiration and nutritional status (Nada *et al*., 2007). Cd stress triggers the formation of reactive oxygen species (ROS) like hydrogen peroxide, O^{2-} radicals and OH^{-} radicals and thus leads to oxidative burst, which is further responsible for the changes in the antioxidative systems.

Prevalence of oxidative stress is indicated by enhanced level of malondialdehyde (MDA) and it may be the possible mechanism by which heavy metal stress could be manifested in plant tissues. MDA content is measured in terms of level of lipid peroxidation (Pant *et al*., 2011).

To tolerate heavy metal stress, plants have evolved certain mechanisms like metal accumulation, metal exclusion and binding of heavy metal with strong ligands like metallothioneins (MTs), cysteine-rich proteins and thiol-rich peptides, phytochelatins (PCs) (Clemens, 2001). Furthermore, plants have their own antioxidant system

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which can combat the oxidative stress produced by the heavy metals (Zabalza *et al*., 2008). Antioxidative system of plants includes several ROS-scavenging enzymes like guaiacol peroxidase (GPX), catalases (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) etc.

In response to various environmental stresses like drought, salinity, extreme temperatures, UV radiation and heavy metals, two major organic osmolytes, glycine betaine (GB) and proline accumulate in a number of plant species (Ashraf and Foolad, 2007). GB is found to accumulate in many crop plants under stressed conditions, such as in spinach (*Spinacia oleracea*), sugar beet (*Beta vulgaris*), wheat (*Triticum aestivum),* barley (*Hordeum vulgare*), *and sorghum (Sorghum bicolor)* (McCue and Hanson, 1990; Rhodes and Hanson, 1993; Yang *et al*., 2003). Proline generation under heavy metal stress is also a vital response for the protection of plant cells from oxidative injury (Choudhary *et al*., 2007).

Brassica juncea is an edible crop and hyperaccumulator of Cd metal. Metal toxicity also includes reduction in growth and yield of plants. Thus present study was undertaken to analyze the growth, antioxidative defence system and level of osmoprotectants in 30-days old plants of *B. juncea* L.

MATERIAL AND METHODS

To study the effects of Cd metal on growth, antioxidative defence system and level of osmoprotectants in *Brassica juncea* plants, a field experiment was conducted in Botanical Garden of Guru Nanak Dev University, Amritsar. 20 X 20 feet area was taken for the experimentation and soil: manure in a ratio of 3:1 was added into it. The certified and disease free seeds of *Brassica juncea* L. var. RLC-1 were procured from Punjab Agricultural University, Ludhiana, Punjab and surface sterilized with 0.01% mercuric chloride solution, followed by the repeated washing of sterile double distilled water (DDW). Seeds were sowed in different blocks of field. Randomized block design was made in which different treatments of Cd metal was given (0, 0.2, 0.4 and 0.6 mM Cd). Plants were then harvested after 30-days of germination to study following parameters:

Growth Parameters:

Root length, shoot length and number of leaves were observed after 30 days. Root and shoot lengths were measured by using a metre scale and number of leaves of all treatments were counted.

Antioxidative Enzymes:

1 g of plants was homogenized in 3 mL of 100 mM potassium phosphate buffer (pH 7.0). The homogenates were centrifuged at 4°C for 20 min at 13,000 rpm. Supernatant was taken for estimation of protein content (Lowry *et al.*, 1951) and antioxidative enzyme activities.

Guaiacol peroxidase (POD) was estimated according to the method given by Putter (1974). Activity of this enzyme was determined by using the guaiacol as substrate. The reaction mixture consists of 3 ml phosphate buffer, 50 ml guaiacol solution, 100 ml enzyme sample and 30 ml H_2O_2 solution. The rate of formation of guaiacol dehydrogenation products (GDHP) was measured at 436 nm by spectrophotometre. Activity of enzyme was calculated by using the extinction coefficient of 25 mM^{-1} cm^{-1}.

Catalase (CAT) activity was measured by the method of Aebi (1974). Reaction mixture containing 100 mM K-phosphate buffer (pH 7.0), 150 mM H_2O_2 and 300 ìL of enzyme extract. The decomposition rate of H_2O_2 was followed by reduction in absorbance at 240 nm. Activity of enzyme activity was estimated by using the extinction coefficient of 6.93 x 10^{-3} mM^{-1} cm^{-1}.

Superoxide dismutase (SOD) activity was determined according to Kono (1978). The reaction mixture contains 1.3 ml sodium carbonate buffer, 500 ml NBT and 100 ml Triton X-100. 100 ml hydroxylamine hydrochloride was used to initiate the reaction. Then 70 ml of the enzyme extract was added after 2 minutes. The increase in absorbance was recorded at 540 nm with the percent inhibition at the reduction rate of NBT.

Ascorbate peroxidise (APOX) activity was measured by method of Nakano and Asada (1981), followed by the reduction in absorbance at 290 nm. Reaction mixture containing 100 mM K-phosphate buffer (pH 7.0), 5 mM ascorbate, 0.5 mM H_2O_2 and 600 ìL of enzyme extract. Enzyme activity was determined using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Glutathione reductase (GR) activity was estimated by the method given by Carlberg and Mannervik (1975) followed by the oxidation of NADPH at 340 nm. The

assay mixture containing 50 mM K-phosphate buffer (pH 7.6), 3 mM EDTA, 0.1 mM NADPH, 1 mM oxidized glutathione (GSSG) and 150 ìL enzyme extract. Enzyme activity was determined using the extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Dehydroascorbate reductase (DHAR) activity was measured by the method of Dalton *et al.* (1986). The reaction mixture consists of 1.5 ml phosphate buffer, 300 ìL glutathione reduced, 300 ìL dehydroascorbate and 400 ìL enzyme extract. Increase in absorbance was recorded at 265 nm and enzyme activity was determined by extinction coefficient 14 mM^{-1} cm^{-1}.

Mono-dehydroascorbate reductase (MDHAR) activity was estimated by Hossain *et al.* (1984) method. Reaction mixture was followed by 1.8 ml of phosphate buffer, 300 ìL EDTA, 200 ìL NADH, 250 ìL ascorbate oxidase and 300 ìL enzymes extract. Enzyme activity was determined by extinction coefficient 6.22 mM^{-1} cm^{-1}. Decrease in absorbance was measured at 340 nm.

Malondialdehyde (MDA) content:

MDA content was determined by Heath and Packer (1968) method. The harvested plants of *Brassica juncea* were homogenized in 1% trichloroacetic acid and then centrifuged at 13,000 rpm for 20 minutes. Supernatant was collected and heated with thiobarbituric acid for 30 min at 95^0C. The absorbance was taken spectrophotometrically at 532 and 600 nm.

Proline content:

Proline was analyzed by Bates *et al.* (1973). The plant samples were homogenized in 10 ml of 3% sulfosalicylic acid. Centrifuged was done at 10,000 rpm for 10 min. Then 2 ml of ninhydrin with 2 ml glacial acetic acid were added into 2ml of supernatant and incubation was given at boiling temperature for 1h. Mixture was extracted with toluene, and proline was analyzed spectrophotometrically at 520nm.

Glycine-betaine (GB) Content:

Glycine betaine was determined by Grieve and Grattan (1983) method. 1g of dry plant material was ground in 10 ml of distilled water and filtered. 1 ml of the filtered extract was mixed with 1 ml of 2 M HCl. Then in 0.5 ml of this mixture, 0.2 ml of potassium tri-iodide solution was added. The contents were then shaken and cooled in an ice bath. 2.0 ml of ice cooled distilled water in addition to 20 ml of 1-2 dichloromethane were added to the mixture. The optical density of lower organic layer was measured at 365 nm. Betaine concentrations were calculated against the standard curve.

The data obtained was statistically analyzed using one-way ANOVA. Data were presented as means ± SE. Each set consists of three replicates, which is represented in the form of bars. An asterisk sign indicates a significant mean difference from control at P d”0.05.

RESULTS AND DISCUSSION

The effect of different concentrations of Cd on the growth of 30 days old *Brassica juncea* plants was given in Table 1. A decrease in root and shoot lengths were observed in the Cd treated plants as compared to untreated control. With the increase in concentration of Cd, decrease in root and shoot lengths were found. Maximum inhibition in roots (2.4 Cm) and in shoots (6.6 Cm) was occurred with respect to control (14.4 Cm). At the same way, there was a slight decrease in number of leaves in metal treated plants as compared to control. In 30 days *B. juncea* plants, it was found that inhibition in shoot length was less as compared to root length , when treated with Cd metal, because of its less mobility towards the shoot. Accumulation of metal occurs in roots and thus its mobility is controlled by root cell wall. (Carrasco-Gil *et al.*, 2011). Negative effects on nutrition and water supply is produced by root damage, so growth and physiology of aerial parts of plants are affected (Hussein *et al.*, 2007).

The antioxidant enzymes play significant role in preventing oxidative stress in plants by scavenging free radicals and peroxides. During stress conditions activities of almost all the antioxidant enzymes have been greatly activated.

In the present study, it has been found that protein content decreased in Cd treated plants as compared to untreated control. As the metal stress increased, protein content also enhanced and it was found maximum (11.857 mg/g FW) in plants treated with 0.6 mM Cd (Table 2). Increase in protein content under metal stress conditions is because plants own defence system becomes activitated due to release of stress proteins namely heat shock proteins (Hsp) against the metal (Wang *et al.,* 2004).

Effect of different concentrations of Cd on antioxidative enzyme activities were shown in Table 2 and

Table 3. POD appeared as an important enzyme to combat Cd imposed oxidative stress as activity of POD has been to enhance in the stressed plants. This observation was in coherence with the report observed in *Pavlova viridis* (Mei *et al*., 2006). This antioxidant enzyme can neutralize or scavenge free radicals, mainly in the removal of H_2O_2. Increase in POD activity in the present study reflects its vital role in scavenging of H_2O_2, which is produced due to Cd stress.

CAT activity was found to enhance in plants exposed to Cd stress. As increased activity of this enzyme helped in scavenging of H_2O_2 and degrade into H_2O and water. These results are similar tothe findings of Hayat *et al*. (2007).

Activity of SOD also found to increase with Cd stress as compared to control. SOD acts as first line of defense against ROS. It dismutates superoxide radical to H_2O_2 and oxygen. Enhanced O^{2-} production stimulated the enzyme activity and thus oxidative stress tolerance. In present work steady detoxification of the generated O^{2-} have occurred. Similar results were observed in *Elsholtzia haichowensis* when exposed to Cu stress (Zhang *et al*., 2010).

Activity of APOX enzyme was also enhanced in metal stressed plants as compared to control. These results were in accordance with the observations of Drazkiewicz et al. (2003). But maximum activity was observed in plants treated with 0.4 mM Cd. As it is an important component of ASH – GSH cycle and it uses ascorbate for detoxification of H_2O_2.

Activities of GR, DHAR and MDHAR were also enhanced in the plants exposed to Cd metal stress and with increasing the concentration of metal, activities were found to stimulate. As the stress increases, plants own antioxidative defence system becomes activated due to the generation of stress proteins like hsp.

Proline content was increased in plants treated with Cd. As the concentration of metal was enhanced, proline content was also found to increase and it was observed maximum (35.904 µ mol g^{-1} FW) in the plants treated with 0.6 mM Cd (as shown in Table 4). Accumulation of proline was also observed by Alia and Pardha Saradhi (1991) under metal stressed conditions. Proline accumulation helps in the prevention of membrane distortion and scavenge the hydroxyl radical. Thus protects the plants from oxidative injury.

Content of GB was also enhanced during Cd stress as compared to control and also found to increase with increasing stress. As it is a cellular osmolyte which enhances intracellular osmolarity when a cell is exposed to stress. it has also been reported that, *in vitro,* GB helps in stabilization of structures, enzyme activities and protein complexes and protects the membranes against the detrimental effects of environmental stress (Gorham, 1995).

MDA content was increased with increasing the concentration of Cd and found maximum (14.012 µ mol g^{-1} FW) in 0.6 mM Cd stressed plants (Table 4). The heavy metal caused increase in lipid-peroxidation and it is supported by the results of De Britto *et al*., (2011) in *Capsicum annum.* These findings suggest that heavy metal stress directly or indirectly triggers the production of oxygen radicals, which further leads to enhanced lipid peroxidation and oxidative burst in the plant. Thus increases the MDA content.

Growth Parameters:

Table 1. Effect of different concentrations of Cd on growth of 30 days old plants of *Brassica juncea* L.

S.No.	Treatment	Root Length (Cm)	Shoot Length (Cm)	No. of Leaves
1.	Control	14.4 ± 0.49	9.66 ± 0.32	5 ± 0.57
2.	0.2 mM Cd	4.0 ± 0.26	8.2 ± 0.17	4 ± 0.33
3.	0.4 mM Cd	3.3 ± 0.37	7.43 ± 0.18	4 ± 0.33
4.	0.6 mM Cd	2.4 ± 0.17	6.6 ± 0.32	4 ± 0.0
		F-Ratio = 258.49* HSD = 1.669	F-Ratio = 24.659* HSD = 1.254	F-Ratio = 1.266 HSD = 1.781

Antioxidative Defence System:

Table 2. Effect of different concentrations of Cd on protein and activities of antioxidative enzymes (POD, CAT and SOD) of 30 days old plants of *Brassica juncea* L.

S. No.	Treatment	Protein Content (mg/g FW)	POD (µmole UA mg protein^{-1})	CAT (µmole UA mg protein^{-1})	SOD (µmole UA mg protein^{-1})
1.	Control	9.931 ± 0.23	15.30 ± 0.429	8.651 ± 0.351	3.005 ± 0.021
2.	0.2 mM Cd	10.659 ± 0.38	22.811 ± 1.053	10.061 ± 0.514	3.189 ± 0.045
3.	0.4 mM Cd	11.266 ± 0.14	24.090 ± 0.144	10.538 ± 0.272	3.329 ± 0.17
4.	0.6 mM Cd	11.857 ± 0.089	25.963 ± 1.392	11.289 ± 0.184	3.634 ± 0.073
		F-Ratio = 11.846* HSD = 1.146	F-Ratio = 26.899* HSD = 4.31	F-Ratio = 9.944* HSD = 1.684	F-Ratio = 9.420* HSD = 0.459

Table 3. Effect of different concentrations of Cd on activities of antioxidative enzymes (APOX, GR, DHAR and MDHAR) of 30 days old plants of *Brassica juncea* L.

S. No.	Treatment	APOX (µmole UA mg protein^{-1})	GR (µmole UA mg protein^{-1})	DHAR (µmole UA mg protein^{-1})	MDHAR (µmole UA mg protein^{-1})
1.	Control	12.454 ± 0.737	54.249 ± 0.404	21.174 ± 0.225	10.068 ± 0.562
2.	0.2 mM Cd	16.433 ± 0.042	57.591 ± 2.86	21.258 ± 0.637	15.05 ± 1.154
3.	0.4 mM Cd	24.213 ± 1.105	61.171 ± 1.614	21.919 ± 0.528	17.638 ± 1.035
4.	0.6 mM Cd	23.007 ± 2.324	61.518 ± 0.133	21.549 ± 0.202	18.629 ± 1.096
		F-Ratio = 17.243* HSD = 6.398	F-Ratio = 4.262 HSD = 7.915	F-Ratio = 0.585 HSD = 2.106	F-Ratio = 14.954* HSD = 4.732

MDA Content and Osmoprotectants:

Table 4. Effect of different concentrations of Cd on MDA content and level of osmoprotectants (Proline and Glycine - betaine) of 30 days old plants of *Brassica juncea* L.

S. No.	Treatment	MDA content (µ mol g^{-1} FW)	Proline Content (µ mol g^{-1} FW)	Glycine-betaine Content (µ mol g^{-1} FW)
1.	Control	5.445 ± 0.246	14.904 ± 1.57	14.09 ± 0.979
2.	0.2 mM Cd	11.775 ± 0.271	22.19 ± 1.667	27.623 ± 1.216
3.	0.4 mM Cd	13.471 ± 0.119	35.143 ± 1.527	27.71 ± 2.06
4.	0.6 mM Cd	14.012 ± 0.180	35.904 ± 1.04	33.88 ± 1.039
		F-Ratio = 342.23* HSD = 1.017	F-Ratio = 48.305* HSD = 7.047	F-Ratio = 35.954* HSD = 6.66

AKNOWLEDGEMENTS

The authors are thankful to UGC for providing financial support.

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