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Modulation of Cd-induced Oxidative Stress in *Brassica juncea* Plants involves Elemental Analysis, Osmolytes, Photosynthesis and Antioxidative Defence System

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ABSTRACT

Heavy metals are emerging environmental contaminants. Accumulation of heavy metals beyond critical levels produces oxidative stress in plants. This stress is usually conquered by antioxidant defence system and compatible solutes. Thus, the present study has been focused to analyze the effect of Cd metal on level of elements (carbon, hydrogen, nitrogen and sulfur) & osmolytes (proline and glycine-betaine), antioxidant assays (ferric ion reducing antioxidant power and molybdate ion reduction assay), antioxidative enzyme activities (GPOX, GST, DHAR, MDHAR and PPO), photosynthetic pigments (anthocyanin and xanthophyll) and gaseous exchange parameters (transpiration rate, vapour pressure deficit and intrinsic mesophyll rate). Seeds of Brassica juncea var. RLC-1were given Cd metal (0, 0.2, 0.4 and 0.6mM) treatment. Toxic effects of Cd were observed in terms of inhibition of transpiration rate, vapour pressure deficit and intrinsic mesophyll rate, whereas improved level of compatible solutes and antioxidant potential of Brassica juncea plants helped in overcoming the adverse effects of metal.

Keywords: Cd toxicity, *Brassica juncea*, compatible solutes, photosynthetic system, antioxidative defence system
Abbreviations: *GPOX- Glutathione peroxidase, GST- Glutathione-S transferase, DHAR- Dehydroascorbate reductase, MDHAR- Monodehydroascorbate reductase, PPO- Polyphenol oxidase, FRAP- Ferric ion reducing antioxidant power, IRGA- infra red gas analyzer*

INTRODUCTION

In recent decades, heavy metal stress has become one of the major abiotic stresses that cause environmental pollution. These metals unlike other organic pollutants are neither degraded nor even converted into harmless compounds via biological processes. They persist for a long duration in the environment and moreover, they enter into the food chain (1). Heavy metals make a considerable contribution to environmental pollution as a consequence of human activities like mining, electroplating, intensive agriculture, smelting, fuel production, power trans-mission, military operations and sludge dumping. Conversely, elevated concentrations of both essential and non-essential heavy metals in the soil can cause toxicity symptoms like growth inhibition in most plants (2). Toxicity results from the metal binding to sulphydryl groups in proteins, triggering the reduction of their activity or disruption of structure and displaces the essential elements, resulting in deficiency effects. A most frequent feature of heavy metal stress is their capacity to produce toxic oxygen derivatives (3).

Cd is one of the non-essential heavy metal pollutants occurring naturally in the environment. This metal is also released anthropogenic sources like Cd-containing phosphate industrial emissions, fertilizers and sewage sludges. Mining and smelting industries also release generous quantities of Cd into the environment. Exposure to high levels of Cd reduces the rates of photosynthesis, leads to chlorosis, growth inhibition and decrease in water and nutrient uptake and finally death of plants (4).

Toxicity of heavy metals can bring forth a range of adaptive responses in plants. Among the most common responses in plants to abiotic stresses is the production of different types of organic solutes including osmoprotectants and antioxidant such as proline during stress (5). *Brassica juncea* plant is oftenly meeting the stress of heavy metals specifically Cd (6), so the present work was planned to study the influence of Cd on various defence responses.

EXPERIMENTAL SECTION

To study the effects of Cd metal on *Brassica juncea* plants, a field experiment was conducted in the 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 sown in different blocks. Different treatments of Cd metal were given (0, 0.2, 0.4 and 0.6 mM Cd). Plants were then harvested after 60-days of germination to study following parameters:

1.1 Elemental analysis

The percentage of carbon, hydrogen, nitrogen and sulphur in 60- days old plants were determined with the help of CHNS analyzer (Elementar Vario ELIII). Samples were dried completely in oven at 80^0C temperature. They were crushed to make fine powder. 10 mg of powdered samples was used to analyze the carbon, hydrogen, nitrogen and sulphur content by vario micro cube instrument run at CHNS mode. CHNS content was displayed in percentage (%).

1.2 Estimation of Osmolytes

2.2.1 Proline content

Proline was estimated by the method of Bates et al. (7). The plant samples were homogenized in 3% sulfosalicylic acid and then centrifuged at 10,000 rpm for 10 min. 2 ml of ninhydrin was added with 2 ml glacial acetic acid into 2ml of supernatant and incubation was given at boiling temperature for 1h. Extraction of mixture was done with toluene, and proline was analyzed spectrophotometrically at 520nm. A graph of absorbance vs concentration was plotted for the standard solutions of L-proline and the amount of proline in the sample was calculated from the graph.

2.2.2 Glycine-Betaine (GB) content

GB content was estimated by following the method of Grieve and Grattan (8). 1g of dried plant material was homogenized in 10 ml of distilled water and filtered. After filtration, 1 ml of the extract was mixed with 1 ml of 2M HCl. 0.2 ml of potassium tri-iodide solution was added to the 0.5 ml of this mixture. The contents were shaken and cooled in an ice bath for 90 min with shaking. Then 2.0 ml of ice cooled distilled water and 20 ml of 1-2 dichloromethane were added to it. Two layers thus formed in the mixture were mixed by passing a continuous stream of air for 1-2 min while tubes were still in ice bath (4^0C). Optical density of the organic layer was measured at 365 nm and upper aqueous layer was discarded. The concentrations of the betaine were calculated against the standard curve.

1.3 Antioxidative Defence system

1.3.1 Antioxidative Enzymes

Activities of antioxidative enzymes were determined by the standard methods of Dalton et al. (9) for Dehydroascorbate reductase (DHAR), Hossain et al. (10) method for Mono-dehydroascorbate reductase (MDHAR), Kumar and Khan (11) method for Polyphenol oxidase (PPO), Habig et al. (12) method for Glutathione-S-transferase (GST) and Flohe and Gunzlar (13) method for Glutathione peroxidase (GPOX) activity.

1.3.2 Antioxidant assays

2.3.2.1 Ferric ion reducing antioxidant power assay (FRAP)

Reducing power assay was performed by the method given by Oyaizu (14). To 1 ml of plant extract, 2.5 ml of phosphate buffer and 2.5 ml of 1% potassium ferricyanide were added and incubated at 50ºC for 20 minutes. To the supernatant, 2.5 ml of 10% TCA, 2.5 ml distilled water and 0.5 ml $FeCl_3$ was added. The absorbance was taken at 700 nm.

Calculations

The reducing potential (%) was calculated by following formula

$$\text{Reducing potential (\%)} = \frac{Ac-As}{Ac} \times 100$$

2.4.2.2 Molybdate ion reduction assay
Molybdate ion reduction assay was done by Prieto et al. (15). In 0.3 ml of plant extract, 3 ml of reagent solution was added and incubation was given for for 1.5 hours at 95^0C. Absorbance was taken at 695 nm.

Calculations
The reducing potential (%) was calculated by following formula:
Reducing potential (%) = $\frac{Ac-As}{Ac}$ x100

1.4 Photosynthetic system
1.4.1 Photosynthetic Pigments
2.5.1.1 Total Anthocyanin Content
Total anthocyanin content was determined by method given by Macinelli (16). 1gm of fresh plant tissue was homogenized in chilled pestle and mortar with 3ml of extraction mixture consisting of acidified methanol (methanol: water: HCl, 79:20:1). The crushed material was then centrifuged for 20 minutes at 13,000 rpm in Eltek cooling centrifuge for 20 minutes at 13,000 rpm at a temperature of 4^oC. The supernatant from the plant extract was collected for the analysis of anthocyanin content. The absorbance of the supernatant was taken at 530 and 657nm.

Calculations:
Total anthocyanin content = Absorbance $_{530}$ – 0.25 Absorbance $_{657}$

2.5.1.2 Xanthophyll Content
Xanthophylls content was estimated by following the method given by Lawrence (17).

Preparation of sample:
Dried plant sample was homogenized well into fine powder using pestle and mortar. Then 0.05g of dried plant material was weighed and sample was transferred to 100ml flask. 30ml of extract was pipetted into the flask and shook well for 10-15 minutes.

Hot saponification:
2.0 ml of 40 % methanolic KOH was added in the flask containing extract. The flask was refluxed in water bath at 56^oC, followed by cooling the samples. Samples were then kept in dark for 1 hour then 30ml of hexane was added in the flask. Flask was then shaken for 1 minute and the volume was made up with 10 % sodium sulphate solution and further the flask was shaken for 1 minute. The samples were again kept in dark for 1 hour. Upper phase was collected in 50 ml volumetric flask and hexane was added, it was mixed well and measured at 474 nm.

Calculations:
Total xanthophylls (g/kg sample) = Absorbance$_{474}$ × D/ w × 236

Where,
w = weight of samples in grams
D = final dilution (50 × 100/ 3)
236 = translation specific absorbivity for 1g/litre xanthophylls yield (Kg/ha)

1.4.2 Gaseous Exchange Parameters
Gaseous exchange of plants like transpiration rate, vapour pressure deficit, and intrinsic mesophyll rate were measured with the help of (IRGA) infra red gas analyzer (Li-COR 6400). The measurement was performed within the time period (9.00–11.00) h maintaining the air temperature, air relative humidity, CO_2 concentration and photosynthetic photon flux density (PPFD) at 25 °C, 80–90%, 400 μmol mol^{-1} and 1000 μmol $m^{-2}s^{-1}$ respectively.

Statistical analysis
Each experiment was conducted in three replicates. Data was expressed in Mean±SE. To check the statistical significant difference between the treatments, one way-ANOVA was carried out by using Assistat version 7.7 beta.

RESULTS AND DISCUSSION

1.5 CHNS analysis
Control plants of 60 days old plants showed minimum value of carbon (C) (36.06%) (Table 1). It was noted that with Cd stress, C was enhanced and its highest value was found in 0.4mM Cd treated plants (54.94%). Similarly, rise in hydrogen (H) level was observed in 0.2 and 0.6mM Cd stressed plants (6.33 and 7.83% respectively). Sharp

decline was observed in nitrogen (N) and sulphur (S) contents with Cd treatments. Highest values of N were reported in control plants (1.66 %), which further reduced from 1.00 (0.2mM Cd) to 0.40% (0.6mM Cd). A rise in S level was noticed in 0.2mM Cd treatment (0.65%) as compared to control plants (0.50%), then decrease was recorded from 0.31 (0.4mM Cd) to 0.11% (0.6mM Cd). In present investigation, level of elements was found to alter during Cd stressed conditions in 60 days old *Brassica juncea* plants. Metal toxicity also alters various physiological processes at cellular and molecular level by displacing or substituting for essential elements, makes changes in the enzyme activities and blocks the functional groups of metabolically important molecules (18). High element content in the shoots of *B. juncea* might be allied to the tolerance of metal in accumulators/hyperaccumulators. The enhanced level of elements by higher metal doses in plants lead to the fact that *B. juncea* might have a specific physiological requirement for those elements when exposed to potential metal stress.

Table 1. Effect of Cd on Carbon, Hydrogen, Nitogen and Sulphur Content of 60 days old *B. juncea* Plants

Treatments	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulphur (%)
0.0 mM	36.06±5.0 ^{ab}	5.45±0.5 ^{b}	1.66±0.2 ^{a}	0.50±0.002 ^{a}
0.2mM	43.43±4.6 ^{b}	6.33±0.9 ^{a}	1.00±0.05 ^{a}	0.65±0.1 ^{a}
0.4mM	54.94±3.5 ^{a}	5.16±0.5 ^{b}	1.00±0.1 ^{a}	0.31±0.0 ^{ab}
0.6mM	37.67±5.0 ^{ab}	7.83±1.0 ^{a}	0.40±0.05 ^{b}	0.11±0.008 ^{b}

Data presented in mean ±SE. Different letters (a, b, c & d) within various concentrations of Cd (0, 0.2, 0.4 and 0.6mM) are significantly different (Fisher LSD post hoc test, p≤0.05) and signify the effect of Cd metal on Elemental analysis.

1.6 Osmoprotectants

Treatment of Cd metal enhanced the proline content in 60 days old plants of *B. juncea* (Fig 1). It was observed maximum in 0.6mM Cd treatment (17.52µ mol g^{-1} FW). Minimum content was found in control plants (13.94µ mol g^{-1} FW). Very less difference in proline content was seen in 0.2mM (15.69µ mol g^{-1} FW) and 0.4mM Cd (16.23µ mol g^{-1} FW) treatment. Glycine-betaine content was increased with increasing Cd metal concentration. Highest value was noticed in 0.4mM Cd treated plants (19.18µ mol g^{-1} FW) as compared to its control (10.21µ mol g^{-1} FW). An increase in GB content with respect to control plants was observed in 0.2mM (13.15µ mol g^{-1} FW) and 0.6mM Cd (17.07µ mol g^{-1} FW) metal treatment (Fig 2). During Cd toxicity compatible solutes such as proline and glycine-betaine were observed to get accumulated in *Brassica juncea* plants. Under stress conditions, Δ1pyrroline-5-carboxylate synthase enzyme get activated, which are the main cause of rise in proline content. Glycine-betaine is also accumulated more during the stress conditions as it is formed by choline and GB substrates. Toxic intermediate betaine aldehyde stimulates the two-step oxidation of choline and these reactions are catalysed by choline monooxygenase (CMO) and NAD+- dependent betaine aldehyde dehydrogenase (BADH), activated under stress conditions (19). These compatible solutes provide protection against stress; they act as antioxidant by scavenging ROS and stabilizing the membranes. Activities of antioxidative enzymes and antioxidant potential of *Brassica juncea* plants were noticed to enhance during Cd stress conditions. There are several reports which support the results of present study.

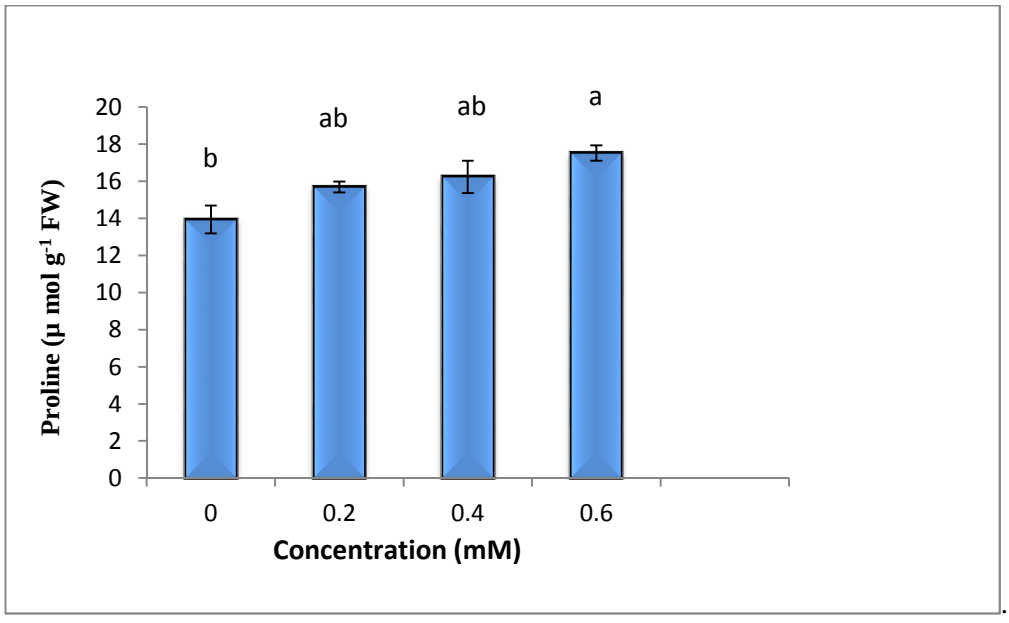

Fig 1. Effect of Cd on Proline content (µ mol g^{-1} FW) in 60-days old Plants of *Brassica juncea*

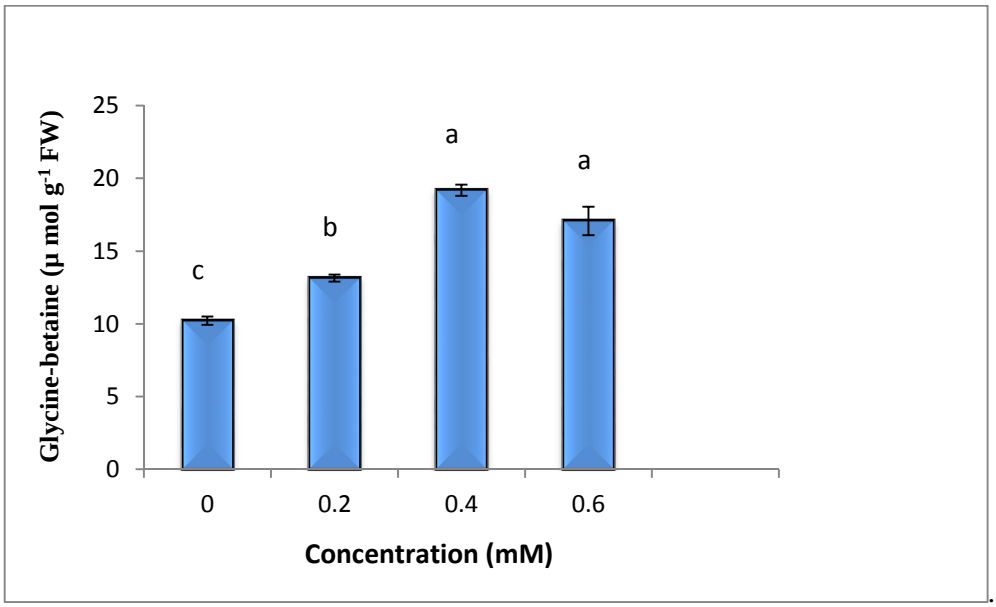

Fig 2. Effect of Cd on Glycine-betaine content (µ mol g^{-1} FW) in 60-days old Plants of *Brassica juncea*

Bars presented in mean ±SE. Different letters (a, b, c & d) within various concentrations of Cd (0, 0.2, 0.4 and 0.6mM) are significantly different (Fisher LSD post hoc test, p≤0.05) and signify the effect of Cd metal on Osmoprotectants.

1.7Antioxidant Enzymes and Assays

Activities of DHAR and PPO first decreased at 0.2mM Cd treatment (Table 2) and then got increased with increasing metal concentrations in dose dependent manner with respect to control (21.26 and 4.64UA mg^{-1} protein respectively). A continuous rise in enzyme activities were found from control to 0.6mM Cd stressed plants. Results revealed increase in activity of DHAR from control (21.26) to 0.6mM Cd treatment (23.59UA mg^{-1} protein) and activity of PPO from control (4.64) to 0.6mM Cd treatment (5.95UA mg^{-1} protein). Lowest enzyme activities were observed at 0.2mM Cd stress i.e., 20.67UA mg^{-1} protein (DHAR activity) and 4.05UA mg^{-1} protein (PPO activity). Activities of antioxidative enzymes like GST and GPOX were increased maximum with 0.2mM Cd treatment (7.3 and 7.89UA mg^{-1} protein respectively). Enzyme activities got increased with increasing metal doses, but the increase was less than 0.2mM Cd and higher than the control plants. Rise in GST activity was observed from 5.92 (control) to 6.99UA mg^{-1} protein in 0.4mM Cd and 7.24UA mg^{-1} protein in 0.6mM Cd stress. In 0.4mM and 0.6mM Cd toxicity, activity of GPOX was elevated from 6.28 (control) to 0.4mM Cd treatment (7.61UA mg^{-1} protein). Specific activity of MDHAR enzyme was first enhanced in 0.2mM Cd treatment as compared to control plants. In 0.2mM Cd treated plants, MDHAR activity got increased from 14.41 (control) to 15.86UA mg^{-1} protein. Very slight reduction in activity of MDHAR was observed i.e., 14.92UA mg^{-1} protein in comparison to 0.2mM Cd stressed plants. Maximum activity of MDHAR was recorded in plants exposed to 0.6mM Cd stress (17.2UA mg^{-1} protein). Activity of Ferric ion reducing antioxidant power assay (FRAP) was also inhibited in Cd stressed plants (Table 3). In 0.2mM Cd treated plants, first inhibition of FRAP was reduced to 49.77% with respect to control (59.29%). Maximum % inhibition was seen in 0.6mM Cd treated plants (73.28%). It was recorded that 0.4mM Cd treatment was proved to be most effective Cd concentration and enhanced the scavenging activity of *B. juncea* plant as compared to untreated plant. Whereas, 0.2mM and 0.6mM Cd treated plants also scavenged the molybdate ion more in comparison to control plants.

Table 2. Effect of Cd on Specific Activities of DHAR, MDHAR, PPO, GST and GPOX in 60 days old *B. juncea* Plants

Treatments	DHAR (UA mg^{-1} protein)	MDHAR (UA mg^{-1} protein)	PPO (UA mg^{-1} protein)	GST (UA mg^{-1} protein)	GPOX (UA mg^{-1} protein)
0.0 mM	21.26±0.29 ^{b}	14.41±0.79 ^{c}	4.64±0.52 ^{ab}	5.92±0.60 ^{c}	6.28±0.71 ^{b}
0.2mM	20.67±1.66 ^{c}	15.86±0.90 ^{b}	4.05±0.21 ^{b}	7.3±0.46 ^{a}	7.89±0.44 ^{a}
0.4mM	21.81±2.16 ^{b}	14.92±0.79 ^{c}	5.32±0.36 ^{ab}	6.99±0.5 ^{b}	7.61±1.23 ^{a}
0.6mM	23.59±1.47 ^{a}	17.2±0.23 ^{a}	5.95±0.04 ^{a}	7.24±0.18 ^{a}	7.32±0.35 ^{a}

Data presented in mean ±SE. Different letters (a, b, c & d) within various concentrations of Cd (0, 0.2, 0.4 and 0.6mM) are significantly different (Fisher LSD post hoc test, p≤0.05) and signify the effect of Cd metal on Enzyme activities.

Increase in the activities of antioxidant enzymes was observed under Ni stress in *Nasturtium officinale* (20). These enzymes help in the generation of antioxidants that scavenge the reactive oxygen species produced during metal

stress like MDHAR regenerates AA and DHAR regenerates AA utilizing GSH to form GSSG at the cost of nicotinamide adenine dinucleotide phosphate (NAD(P)H) and cause removal of the free radicals (21).

Table 3. Effect of Cd on Scavenging Activities of FRAP and Molybdate ion in 60 days old *B. juncea* Plants

Treatments	FRAP (%)	Molybdate ion (%)
0.0 mM	59.29±2.13 ^{ab}	46.2±3.25 ^{c}
0.2mM	49.77±5.0 ^{b}	57.09±2.71 ^{bc}
0.4mM	65.34±3.44 ^{ab}	76.45±2.43 ^{a}
0.6mM	73.28±5.89 ^{a}	65.16±2.25 ^{ab}

Data presented in mean ±SE. Different letters (a, b, c & d) within various concentrations of Cd (0, 0.2, 0.4 and 0.6mM) are significantly different (Fisher LSD post hoc test, p≤0.05) and signify the effect of Cd metal on Antioxidant assays.

1.8 Photosynthetic Pigments

Anthocyanin content enhanced from control to 0.6mM Cd treated plants (Table 4). In control plants, lowest value of anthocyanins was observed (12.97mg g^{-1} FW). In 0.6mM Cd treated plants, 17.47mg g^{-1} FW anthocyanins were found, which is 1.35 folds higher than control plants, followed by 0.2mM (14.79mg g^{-1} FW) and 0.4mM Cd (15.72mg g^{-1} FW). A very less difference in xanthophyll content was observed in control (8.98mg g^{-1} DW) and 0.4mM Cd (8.41mg g^{-1} DW) treated plants. Overall decrease in the level of xanthophyll content was noticed. 0.2mM (7.59mg g^{-1} DW) and 0.6mM Cd (6.43mg g^{-1} DW) stressed plants showed reduction in xanthophylls level in comparison to 0.4mM Cd. Further, enhanced activity of GST enzyme leads to the biosynthesis of anthocyanin pigment. Xanthophyll pigment also acts as an antioxidant and gives rise to formation of abscisic acid, which protect the plants from oxidative burst. These results are in coherence with the findings of Amiri et al. (22).

1.9 Gaseous Exchange Parameters

Transpiration rate of 60 days old plants showed the maximum decrease with 0.6mM Cd treatment (1.57m mol H_2O $m^{-2}s^{-1}$). A very less alteration was noted between control (1.91m mol H_2O $m^{-2}s^{-1}$) and 0.4mM Cd (1.90m mol H_2O $m^{-2}s^{-1}$) treated plants (Table 4). 1.83m mol H_2O $m^{-2}s^{-1}$ transpiration rate was observed when 0.2mM Cd was supplied to plants. Vapour pressure deficit was highest in control plants (0.19kPa). Cd toxicity led to decrease the value from 0.2mM (0.16kPa) to 0.6mM Cd (0.14kPa) treated plants except 0.4mM treatment. Very less alteration in intrinsic mesophyll rate was found in 60 days old plants of *B. juncea*. Decline in the values were observed from 0.024 to 0.021mgCO_2m^{-3}. Highest value was noticed in control plants (0.024mgCO_2m^{-3}). 0.4mM and 0.6mM Cd treatment showed similar values of mesophyll rate (0.021mgCO_2m^{-3}). Regarding the toxicity of Cd metal to PSII activities in plants, some researchers have investigated that Cd binds in both acceptor and the donor sides of PSII. On the donor side, Cd^{2+} exchanges the Ca^{2+} cofactor in the Ca/Mn cluster that causes reduction of photosynthetic oxygen evolution (23). Results were also supported by the observations of Stancheva et al. (24).

Table 4. Effect of Cd on Anthocyanin and Xanthophyll Content, Transpiration Rate, Vapour Pressure Deficit and Intrinsic Mesophyll Rate of 60 days old *B. juncea* Plants

Treatments	Anthocyanin (mg g^{-1} FW)	Xanthophyll (mg g^{-1} DW)	Transpiration rate (m mol H_2O $m^{-2}s^{-1}$)	Vapour Pressure Deficit (kPa)	Intrinsic Mesophyll Rate (mol $m^{-2}s^{-1}$)
0.0 mM	12.97±0.66 ^{c}	8.98±0.25 ^{a}	1.91±0.04 ^{a}	0.190±0.001 ^{a}	0.024±0.001 ^{b}
0.2mM	14.79±0.67 ^{bc}	7.59±0.79 ^{ab}	1.83±0.04 ^{a}	0.165±0.001 ^{b}	0.022±0.001 ^{ab}
0.4mM	15.72±0.62 ^{ab}	8.41±0.28 ^{ab}	1.90±0.01 ^{a}	0.189±0.001 ^{a}	0.021±0.00 ^{a}
0.6mM	17.47±0.33 ^{a}	6.43±0.29 ^{b}	1.57±0.03 ^{b}	0.142±0.004 ^{c}	0.021±0.00 ^{a}

Data presented in mean ±SE. Different letters (a, b, c & d) within various concentrations of Cd (0, 0.2, 0.4 and 0.6mM) are significantly different (Fisher LSD post hoc test, p≤0.05) and signify the effect of Cd metal on Photosynthetic system.

CONCLUSION

It was concluded from the present investigations that the defence strategies involved in heavy metal stress tolerance, particularly through improvement in antioxidative potential and level of osmolytes, may provide a novel way to enhance plant' tolerance to heavy metal stress.

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