

Biochemical responses of capsicum annum under cadmium stress

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Abstract: A pot culture study was carried out to assess the antioxidant activity, lipid peroxidation and DNA damage in *Capsicum annum* (L.) var. *Vellayani athulya*, under different cadmium concentrations applied as cadmium chloride salt (10mM, 20mM, 30mM, 40mM and 50mM). Activities of superoxide dismutase (SOD), peroxidase (POX), catalase (CAT) and reduced glutathione (GSH) were observed along with lipid peroxidation and DNA damage. Results showed significant changes in the activities of above antioxidants in test plants, compared to control. A significant increase in the activity of SOD, POX and decrease in the activities of CAT and GSH were observed up to 30mM concentrations. However, at 40mM concentration, the activity of antioxidants was observed to revert to the near normal, showing a sign of recovery which may be due to the inactivation of antioxidants in the presence of excess Cd. Further, in 50mM concentration, the activity of enzymes reversed to the initial condition, i.e either increased or decreased. Nevertheless, production of malondialdehyde, which is an indicator of lipid peroxidation, also showed the same pattern of change. The results of comet assay showed that the extent of DNA damage was greater in *C. annum* as the Cd concentration increased.

Keywords: Heavy Metal, Antioxidant Enzymes, Lipid Peroxidation, DNA Damage

1. Introduction

In plants, reactive oxygen species (ROS) like super oxide, hydrogen peroxide, and hydroxyl radicals, peroxy radicals, alkoxy radicals and singlet oxygen are continuously produced as a by-product of aerobic metabolic processes. Plants possess enzymatic non-enzymatic antioxidants to scavenge the ROS and thereby protecting the plants from destructive reactions [1]. Under normal conditions, the formation and destruction of ROS are in balance; however the production of ROS is elevated under stressed conditions. The imbalance between ROS and antioxidant defence system may increase oxidative stress and lead to damage of macromolecules. Organelles with high oxidising activity or with intense rate of electron flow such as chloroplast, mitochondria, peroxysomes are the main sites of ROS production in cell. The cellular components susceptible to damage by free radicals are primarily proteins (oxidation) lipids (peroxidation), carbohydrates (oxidation) and nucleic acid (purines and pyrimidines) [1].

The presence of heavy metals in the environment is widely reported to stimulate the formation of free radicals

and accumulation of ROS inside the cell further increased antioxidant activity [2]. Cadmium is a non-redox toxic heavy metal. Though a non-biological metal, Cd is readily taken up by the roots of many plants because of its high mobility and solubility [3, 4]. Cd causes the production of ROS in plants and animal cells [5]. Excess Cd resulted in lipid peroxidation and modified the activities of antioxidants like SOD, CAT, POX and GSH. The interaction between ROS and DNA can lead to DNA damage including strand breaks and oxidation of DNA [6]. But the nature and direction of response vary with plant species, kind of tissues and intensity of stress [7]. Present study discusses the effect of different concentrations of Cd in the antioxidant defence mechanism (SOD, POX, CAT and GSH), lipid peroxidation and DNA damage in *C. annum*.

2. Materials and Methods

2.1. Experimental Plant

Capsicum annum (L.) cv. *Vellayani athulya* is a green chilli variety with light green, medium pungent fruits

having excellent fruit quality and was selected by the Agricultural University from the local collection. *Capsicum* fruits are important source of bioactive compounds and hence used as traditional medicine in addition to vegetable spice. Seeds of *C. annum* were obtained from Agriculture College, Thiruvananthapuram, India.

2.2. Experimental Set Up

Seeds of *C. annum* were washed in sterile distilled water and sown in a pot filled with potting mixture (1:1:1) of soil, cow dung and sand) and seedlings were raised. Uniform sized seedlings (40 DAG) were transplanted in pots filled with 1 kg potting mixture. Control and test plants were raised in triplicates in pots supplied with different concentrations of CdCl₂ (10mM, 20mM, 30mM, 40mM and 50mM) and designated as T1, T2, T3, T4 and T5 respectively. A control pot was also maintained with the same potting mixture without CdCl₂ and designated as T0. Preliminary studies showed that maximum cadmium uptake was in roots and in about 80 days after germination (unpublished data). Hence root and shoot samples of *C. annum* after 80 DAG and 100 DAG were taken for further observations.

2.3. Preparation of Enzyme Extract

0.5 g plant samples were homogenised in a chilled mortar and pestle with 100mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA, 3mM DL-dithiothreitol and 5%(w/v) insoluble polyvinyl pyrrolidone. The extract was centrifuged at 10000rpm for 30min at 4° C [8]. After separation of supernatant, the residue was again extracted with known volume of extraction buffer, centrifuged and the combined supernatant served as the enzyme source for SOD, POX, CAT and GSH. Proteins in the sample were determined by Lowry's method using Bovine Serum Albumin (BSA) as standard [9].

2.4. Enzyme Assay

2.4.1. Superoxide Dismutase

(E.C.1.15.1) was estimated following the method of [10]. The activity of SOD was assayed by measuring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) and was expressed as per cent inhibition mg-1 protein. The assay mixture contained 50mM potassium phosphate buffer (pH 7.8), 45μM methionine, 5.3mM riboflavin, 84μM NBT and 20μM potassium ferricyanide and 50μL of enzyme extract. The tubes were incubated at 25°C for 10 minutes and read the absorbance at 600 nm.

2.4.2. Peroxidase

(E.C.1.11.1.7) activity was measured by the method of [11]. A100 mL of reaction mixture contained 10mL of 1 per cent guaiacol (v/v), 10mL 0.3 per cent H₂O₂ and 80mL of 50mM phosphate buffer (pH 6.6). 75μL enzyme extract was added to the reaction mixture. The increase in

absorbance due to oxidation of guaiacol (extinction coefficient 26.6mM-1cm-1) was monitored at 470nm. Enzyme activity was expressed as units min-1 mg-1 protein.

2.4.3. Catalase

(E.C.1.11.1.6) activity was determined by the method of [12]. 3mL of reaction mixture contained 50mM sodium phosphate buffer (pH 7), 20mM H₂O₂ and 50μL of enzyme extract. Decrease in the absorbance was taken at 240nm.

(Molar extinction coefficient of H₂O₂ was 0.04 mM-1cm-1). The enzyme activity was expressed as units min-1 mg-1protein.

2.4.4. Reduced Glutathione

Activity was measured by the method of [13]. 1mL of enzyme extract was taken in a test tube. 0.5mL of 0.2M phosphate buffer (pH 8), 1.3mL of distilled water and 0.2mL of 0.6mM DTNB were added and mixed well. The absorbance was read at 420nm and activity was expressed as unit's g-1 fresh weight.

2.5. Estimation of Lipid Peroxidation

Lipid peroxidation was measured by the estimation of Malondialdehyde (MDA) content by the thiobarbituric acid method (TBARS) as described by [14]. 1mL of plant sample was treated with 2mL of TCA-TBA-HCl (0.37% TBA, 15%TCA and 0.25N HCl) and placed in a boiling water bath for 15min. The mixture was cooled, centrifuged and absorbance of supernatant was measured at 535nm against blank. MDA concentration was expressed as mMgm-1 tissue.

Statistical analysis was done using SPSS 17.0. Difference between control and test plants were analysed by one way ANOVA taking significant level at p<0.001. Pairwise comparison was done using Bonferroni test.

2.6. Assesment of DNA Damage

DNA damage in *C. annum* was assessed using alkaline version of comet assay by the method of [15]. The images of Comets were analysed through a computer (Olympus CKX 41) assisted image analysis (Tri Tek Comet ScoreTM Freeware v 1.5) by measuring the comet area, tail length and per cent of DNA in tail. The observations were made at a magnification of 200X.

3. Results and Discussion

3.1. Antioxidant Enzyme Activities (SOD, POX and CAT)

The heavy metal induced stress results in the production of free radicals which are scavenged by the antioxidative defence mechanism. Nevertheless, plants contain a number of enzymes that convert the ROS into less reactive products. The effect of heavy metal cadmium on antioxidant enzyme in *C. annum* is presented and discussed.

3.1.1. Effect of Superoxide Dismutase (SOD)

SOD is the first enzyme in the detoxifying process which converts the superoxide radicals to H_2O_2 immediately after its formation [16]. Treatment with heavy metal cadmium showed statistically significant differences in the SOD activity of *C. annum* among different treated plants compared to control ($p < 0.001$). In both roots and shoots (80 and 100 DAG), SOD activity increased up to 30mM Cd concentration (Fig.1 & 2).

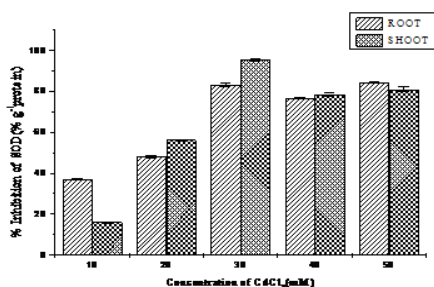


Figure 1. Effect of different concentrations of $CdCl_2$ on SOD (80DAG)

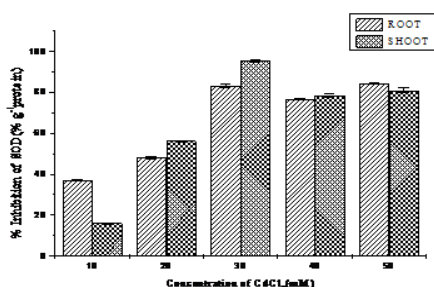


Figure 2. Effect of different concentrations of $CdCl_2$ on SOD (100DAG)

However, SOD activity decreased in treatment 4 (40mM) followed again by an increase in 50mM concentration. The increase in SOD activity may be due to increased production of ROS and a decrease in activity may be due to the inactivation of the enzyme by H_2O_2 [17]. The results were similar to the observations of [18, 19] that above a certain heavy metal concentration, antioxidant enzymes are inhibited.

3.1.2. Effect of Catalase (CAT)

Catalases are the important scavengers of H_2O_2 which are generated during photorespiration and β -oxidation of fatty acids [20]. Results of the present study showed a significant difference in catalase activity in the root and shoot samples of (80DAG and 100DAG) *C. annum* ($p < 0.001$). A gradual increase in catalase activity was noticed up to 30mM concentration, which declined thereafter in both roots and shoots of *C. annum* (80DAG and 100DAG) (Figure 3 & 4).

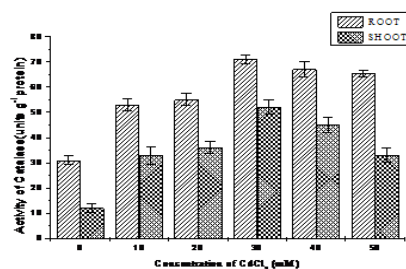


Figure 3. Effect of different concentrations of $CdCl_2$ on CAT (80DAG)

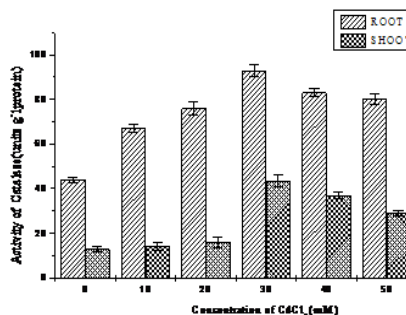


Figure 4. Effect of different concentrations of $CdCl_2$ on CAT (100DAG)

The decrease in catalase activities may be due to the harmful effect of the overproduction of H_2O_2 and other ROS radicals. Similar results were observed by [8, 17, 7, 21].

3.1.3. Effect of Peroxidase (POX)

Peroxidases catalyse the reduction of H_2O_2 which is a common end product of oxidative metabolism, to water rendering it harmless. The results of the peroxidase activity in *C. annum* showed significant difference between the shoots of control and test plants (80DAG) (Figure 5).

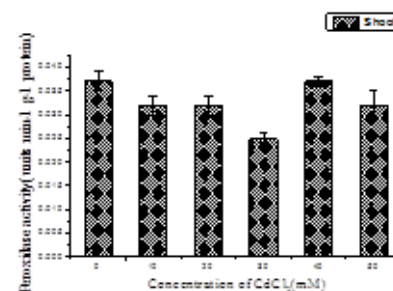


Figure 5. Effect of different concentrations of $CdCl_2$ on POX (80DAG)

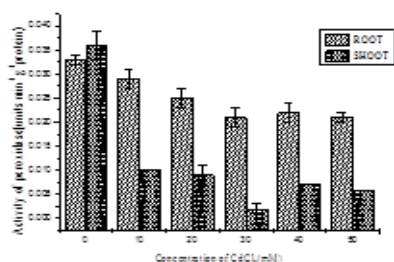


Figure 6. Effect of different concentrations of CdCl_2 on POX (100DAG)

But roots of plants taken after 80DAG showed no significant difference in the enzyme activity between the control and test plants (Figure 6). Roots and shoots of control as well as test plants showed significant difference in the enzyme activity. Peroxidase activity showed a decrease up to 30mM Cd concentration both in roots and shoots of *C. annum* (80DAG and 100DAG). In 40mM concentration, peroxidase activity increased which further decreased in 50mM concentration.

3.2. Non-Enzymatic Antioxidant Activities

3.2.1. Effect of Reduced Glutathione (GSH)

GSH is an important antioxidant counteracting the effects of free radicals produced by oxidation. It exists in two forms- reduced and oxidised forms. In the reduced state it is readily available to neutralize free radicals by bonding with them. From the data, it was observed that in roots (80DAG), GSH activity showed significant difference $p < 0.01$ between the control and test plants (Figure 7 & 8).

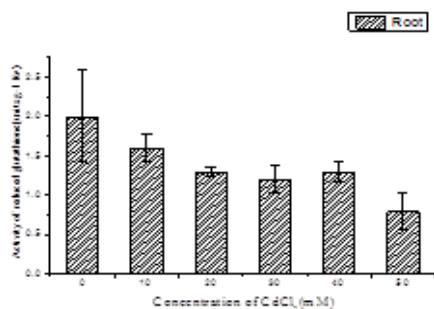


Figure 7. Effect of different concentrations of CdCl_2 on Reduced Glutathione activity (80DAG)

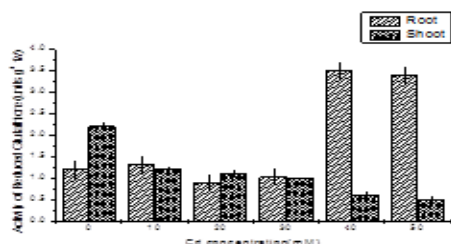


Figure 8. Effect of different concentrations of CdCl_2 on Reduced

Glutathione activity (100DAG)

But in the case of shoot (80DAG), no significant difference was noticed among the control and test plants. A significant difference in GSH activity was noticed in the root and shoot samples (100DAG) of plants ($p < 0.001$). In roots (80DAG), GSH activity decreased up to 30mM Cd concentration which was observed to increase at 40mM ($p < 0.01$).

3.3. Estimation of Lipid Peroxidation

Lipid peroxidation caused as a result of increase in H_2O_2 is the major cause of cell membrane damage leading to lysis of cell. Malondialdehyde (MDA), which is a toxic product of lipid peroxidation reaction, is an index of lipid peroxidation. The result of this study showed significant difference in MDA content among the control and test plants ($p < 0.001$) and a gradual increase in the production of MDA was noticed up to T3 plants (30mM) and T4 plants showed decrease in MDA content. In 50mM concentration, MDA content was again found to increase (Figure 9). The results were similar to the report of [17, 22].

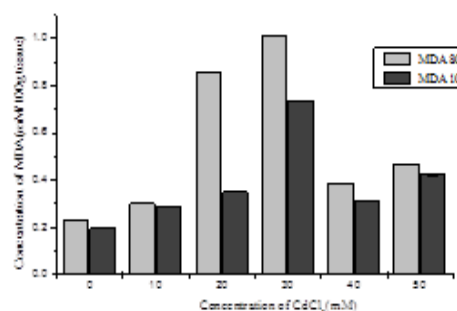


Figure 9. Effect of different concentrations of CdCl_2 on MDA

3.4. Effect of Cd on DNA Damage

Comet assay was the technique used to measure various types of DNA damage and repair in *C. annum* due to the effect of various concentrations of cadmium. Mean \pm standard deviation of the results of comet area, tail length and per cent DNA were calculated. Increased comet area, tail length and percentage of DNA in tail indicate the extent of DNA damage. There was difference in the extent of DNA damage in *C. annum* root cells between the control and test plants. However the extent of DNA damage was greater in test plants with increase in concentration of cadmium. However per cent DNA in tail showed a decrease in T5 plants (50mM) which may be attributed to DNA repair. Cadmium chloride induced DNA damage as evaluated by comet assay was previously studied in broad bean (*Vicia faba*) by [23, 24, 25].

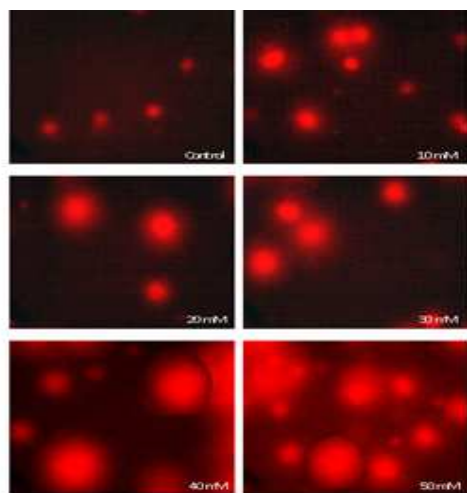


Figure 10. Dose- dependent induction of DNA damage by CdCl_2 in *Capsicum* root cells.

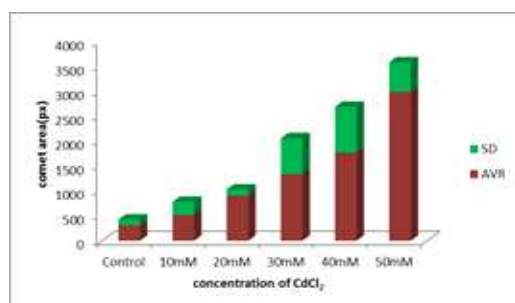


Figure 11. Area of comet under different CdCl_2 concentrations

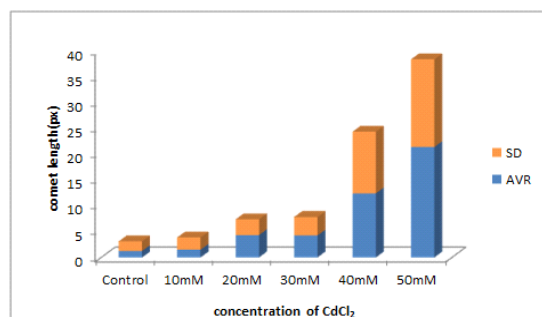


Figure 12. Length of comet under different CdCl_2 concentrations

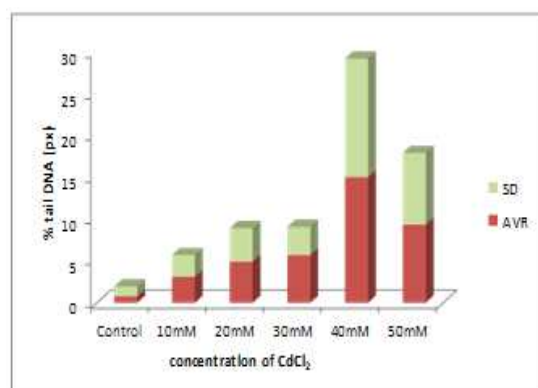


Figure 13. % tail DNA under different CdCl_2 concentrations

4. Conclusion

The present study on cadmium induced biochemical changes in *C. annum* showed significant changes in antioxidant activities (both enzymatic and non-enzymatic), lipid peroxidation and DNA damage, compared to control. Maximum changes in antioxidant activities occurred in the 30mM concentration. This reveals that, at this concentration, free radical formation in response to the oxidative stress was the maximum. Further, changes in activities (both increase or decrease) observed for all biochemical parameters showed a similar pattern in all treatments, which may be due to the inactivation of the antioxidant system by excess formation of ROS due to Cd stress. Lipid peroxidation was maximum at 30mM concentration suggesting that there is increased membrane damage at this concentration due to the increased formation of H_2O_2 . There existed difference in the extent of DNA damage between the control and test plants as the concentration of cadmium chloride increased. However, the DNA damage at high concentration (50mM) was less pronounced, which may be due to the DNA repair mechanism of the plant to cope up with the stress.

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