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STUDY THE EFFECTS OF $CdCl_2$ ON LIPID PEROXIDATION AND ANTIOXIDANT COMPOUNDS CONTENT IN *BRASSICA NAPUS*[*]

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Abstract – Heavy metal accumulation in plants causes induction of oxidative stress. Plants have several defense systems involved in detoxifying free radicals. In the present research we treated the 15 d. seedlings of *B. napus* grown in pots with different concentrations of cadmium (0, 10, 50 and 100 µM) as $CdCl_2$. After 3 weeks we measured malondealdehyde (MDA) content in leaves of treated plants as an indication of lipid peroxidation. We also measured ascorbate (ASC) and anthocyanin content as antioxidative compounds. Our results showed that a high concentration of Cd^{2+} causes an increase in MDA content in leaves compared with control, which is possibly due to the induction of oxidative stress and lipid peroxidation. Low levels of this ion cause an increase in ASC content, while high concentrations of this ion cause a decrease in ascorbate content. It is possible that the entrance of a substrate for ASC reduction in the pathway for phytochelatin synthesis will be the cause. Anthocyanin content increased significantly in plants treated with a high concentration of Cd^{2+}. Increase in anthocyanin content may be an important mechanism for reduction of cadmium toxicity, either by removing free radicals or compartmentation of Cd^{2+} in vacuole.

Keywords – *Brassica napus*, cadmium, oxidative stress, ascorbate, dehydroascorbate, lipid peroxidation, malondealdehyde, anthocyanin

1. INTRODUCTION

Many trace metals, when present at high concentrations, cause toxicity in plants. This applies both to those trace metals that are considered essential for plant growth and those that have no known function in plants. Cadmium has not been shown to be an essential element for physiological processes, but high amounts of this element are absorbed either by root tissues or leaves of plants [1]. The absorption coefficient is higher for cadmium when compared with other heavy metals like zinc and copper [2, 3]. Soil can be polluted by cadmium from a variety of sources. These are mainly phosphate fertilizer and sewage sludge. Excess cadmium induces toxicity in plants, the critical level varying with species. Absorption of cadmium depends on the cadmium concentration in soil as well as the amount of cadmium ions available for absorption by plants [4]. Cation exchange capacity, pH values, fertilizer application, redox state of cadmium and cation competition is the main factor affecting the availability of cadmium [5, 6]. Toxicity symptoms including chlorosis of the mature leaf and the necrotic appearance of leaf tissues, red or dark red appearance of the leaf margin, and root tips with a dark or red-brown color have been reported with high concentrations of cadmium [7, 8].

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Toxicity of cadmium for animals and plants is the result of high affinity of this ion for binding to sulfhydryl groups of enzymes and changing the protein conformation [9].

It has been proposed that Cd^{2+} and other heavy metals can induce free oxygen radical generation and cause oxidative stress. However the exact mechanism of Cd^{2+} toxicity is not clearly known in the molecular level [10]. One possibly is that cadmium ions either inhibit the activity of some antioxidative enzymes or enhances their activity. The effects of cadmium on activities of some antioxidant enzymes like peroxidase, catalase, ascorbate peroxidase and glutathione reductase have been studied [4]. Lipid peroxidation is the major index of the increase in active free radicals, and malondealdehyde (MDA) is the main by-product of the lipid peroxidation process.

Plants, like many aerobic organisms, can cope and react to the oxidative stress caused by heavy metals [7]. One of these responses to heavy metal toxicity is an increase in the production of enzymatic and non-enzymatic antioxidants. Lipophylic antioxidants include carotenoids and tocopherols, while water soluble antioxidants include ascorbate and glutathione as well as many antioxidative enzymes like superoxide dismutase, peroxidase and catalase. These antioxidants have a major role in reducing the oxidative stress and quenching free radicals [11].

Ascorbate is the major antioxidant compound found in plants [4, 12]. This compound, through the ascorbate-glutathione cycle, is converted to monodehydroascorbate (MDHA) and dehydroascorbate (DHA) and generated by MDHA reductase and DHA reductase and glutathione. Therefore, an increase in the activity of ascorbate peroxidase and glutathione reductase in plants which are under stress cause the balance of ascorbate in plants [12]. Another way to reduce cadmium toxicity is the compartmentation of cadmium. Hale and collegues reported that anthocyanins have a major role in the sequestration of Cd^{2+} to the vacuoles [13]. It has also been reported that anthocyanin has a role in quenching free radicals. The reaction between anthocyanin and metals depends on the redox state of metal. This reaction is reversible. Marss and Walbot [3] reported that Cd^{2+} causes an increase in the antioxidative content of *Zea mays*. They also reported that Cd^{2+} significantly increased the expression of the *Bronze2* gene. This gene is probably responsible for encoding the Glutathione-S-Transferase (GST) enzyme. The GST enzyme performs the last genetically defined step in anthocyanin biosynthesis, allowing for recognition and entry of anthocyanins into the vacuoles [14].

In this research the effects of different concentrations of cadmium on MDA as an index of oxidative stress induced by Cd^{2+}, ASC and anthocyanin content as detoxifying compounds in *Brassica napus* plant were studied.

2. MATERIAL AND METHODS

a) Plant material and growth condition

Seeds of *B. napus* were surface sterilized in 0.1% sodium hypochlorite solution and germinated on vermiculite. Seeds were irrigated with distilled water. After one week seedlings were irrigated with the Long-Ashton nutrient solution [15]. Solutions were changed twice a week. Plants were grown in a growth chamber in which they received 800 µmol photon. $m^{-2}. s^{-1}$. photosynthetically active radiation, during a photoperiod of 16/8 day/night and temperature was 23ºC. The visible light was supplied by a halogen (Osram, HAL-R, 150 W) and sodium halide (Philips, SON-H, 220 W) lamp. When the seedlings were 14 days old they were exposed to a different cadmium concentration. Cd^{2+} was added as $CdCl_2$ in five concentrations; 0, 10, 50,100 and 500 µm in irrigated water. The treatments were repeated 3 times.

The plants were treated with cadmium for 21 days before they were harvested. The treatments were replicated 3 times in a random design.

b) MDA measurement

0.25g of leaf tissue of plants were homogenized in 5 ml of 0.1% (w/v) trichloroaceticacid (TCA) and then centrifuged at 14000 g for 10 minutes. 1 ml of supernatant was then vortexed with 4 ml of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituric acid (TBA), and the solution was heated for 20 minutes at 95°C. The samples were cooled on ice for 5 minutes and recentrifuged at 14000g for 5 minutes at 10000 g. The non-specific absorbance of supernatant at 600 nm was subtracted from the maximum absorbance at 532 nm for MDA measurement [16]. For MDA calculation, an extinction coefficient (E) of 1.55 mM^{-1} cm^{-1} was used at 532 nm.

c) Ascorbic acid, dehydroascorbic acid and total ascorbate

ASC and DHA were determined as described by Mc de Pinto et.al, [17]. 0.2g of leaf tissue were homogenized in 10 ml ice-cold metaphosphoric acid 5% and centrifuged for 5 minutes at 10000g.

300µl of supernatant was used for ASC assay and these solutions were added to the extract, respectively: 750µl potassium phosphate buffer and 300 µl distilled water. 300 µl of supernatant was used for DHA assay and to these solutions were added: 750 µl potassium phosphate buffer (pH 7.2) and 150 µl of 10 mM dithiothreitol. The mixture was incubated at room temperature for 10 minutes and then 150 µl of 0.5% N-ethylmaleimide was added. Both samples were vortexed and incubated at room temperature for 10 minutes. To each sample 600 µl of 10% (w/v) TCA, 600µl of 44% (v/ v) H_3PO_4, 600µl of 4% (w/v) bipyridyl in 70% (v/v) ethanol and 10 µl of 3% $FeCl_3$ were then added. After vortex-mixing, samples were incubated at 40ºC water bath for 20 minutes, and then samples brought up and vortexed again and incubated at 40ºC water bath for 20 minutes. Absorbance of samples at 532 nm was recorded. A standard curve of ASC and DHA was used for calculation of ASC and DHA concentration. Total ascorbate was obtained by the summation of the ASC and DHA content.

d) Anthocyanin analysis by HPLC

Crude anthocyanin extract was made from fresh plant tissues in a 99% methanol and 1% HCl solution. These extracts were used to compare total anthocyanin profiles by HPLC.

Anthocyanin purification from *B. napus* was performed essentially as described by Lange *et al.* [18]. Anthocyanins were separated by a reverse-phase HPLC by a C18 column (zorbax 300sb, 250×4.6 cm) connected to an Align HPLC system. Elution was monitored by simultaneously measuring absorbance at 530 nm. 5% formic acid was used as solvent A and HPLC grade methanol as solvent B. The flow rate was 0.98 ml min^{-1}. The following elution profile was used; 10% solvent B from 0 to 3 min., 10-25% linear gradient from 3 to 18 min., 25-50% B from 18 to 33 min., then up to 75% from 1 min, and back to 10% for 4 min.

The anthocyanin content in treatments was compared based on peak areas.

e) Determination of cadmium

At the end of the 21 day cadmium stress period the plants were divided into different portions (leaves, petiole and roots), dried at 80ºC for 48h and the dry weight measured. The cadmium determinations were made on nitric-perchloric acid (3:1 v:v) digests of three replicate tissue samples. Cadmium concentration was determined by atomic absorption spectroscopy (Varian, GTA110) [19].

f) Statistical analysis

Data reported in the Figures. were all analyzed using the statistical program SPSS 9.0 (SPSS, Chicago). Significance of difference was tested at $P = 0.05$ using ANOVA and Duncan LSD. The data are means ± SE from three determinations.

3. RESULTS

MDA content increased in those plants which were treated with 10, 50, 100 and 500 µM cadmium, significantly (Fig. 1).

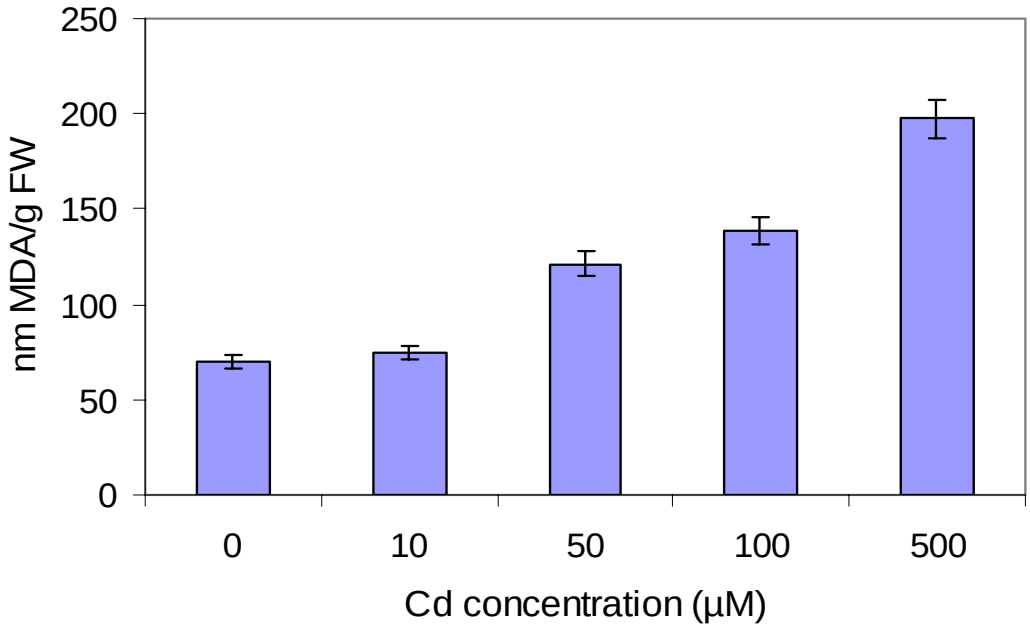

Fig. 1. The effect of different cadmium concentrations on TBA-MDA content in leaf, LSD= 37.11. P= 5%. Each value is mean ± std of three replicates

No significant decrease between total ascorbates was observed either in plants which were treated with different concentrations of cadmium, or plants that were not treated (Fig. 2).

Dehydroascorbate (DHA) content increased in plants which were treated with 50, 100 and 500 µM cadmium when compared with the control group. This increase was accompanied by a decrease in ascorbic acid. Increase in DHA and decrease in ASC content were significant when plants were irrigated with 500 µM cadmium (Fig. 2).

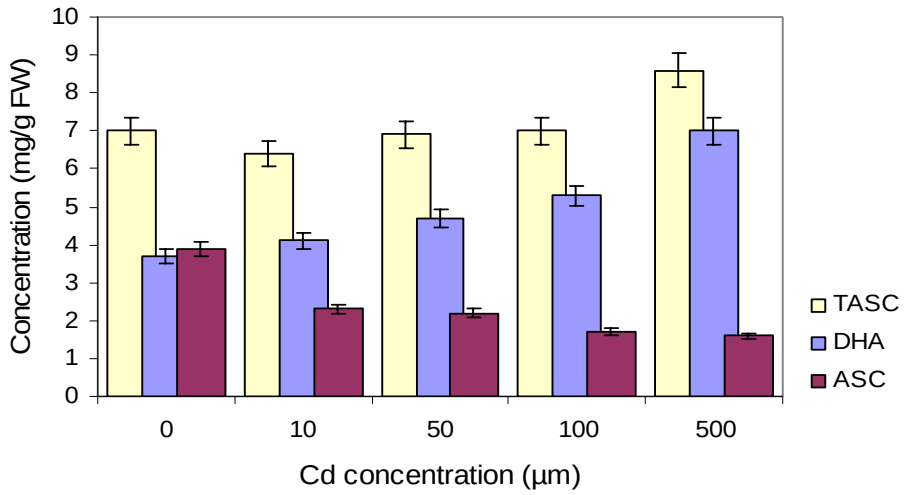

Fig. 2. The effect of different cadmium concentrations on ASC, DHA and total ascorbate content in leaf, LSD (ASC) = 0.074, LSD (DHA) = 0.037, LSD (TASC) = .072, P: 5%, Each value is mean ± std of three replicates

Anthocyanin content was observed to increase in the presence of different concentrations of Cd^{2+} based on a peak area separated by HPLC (Fig. 3).

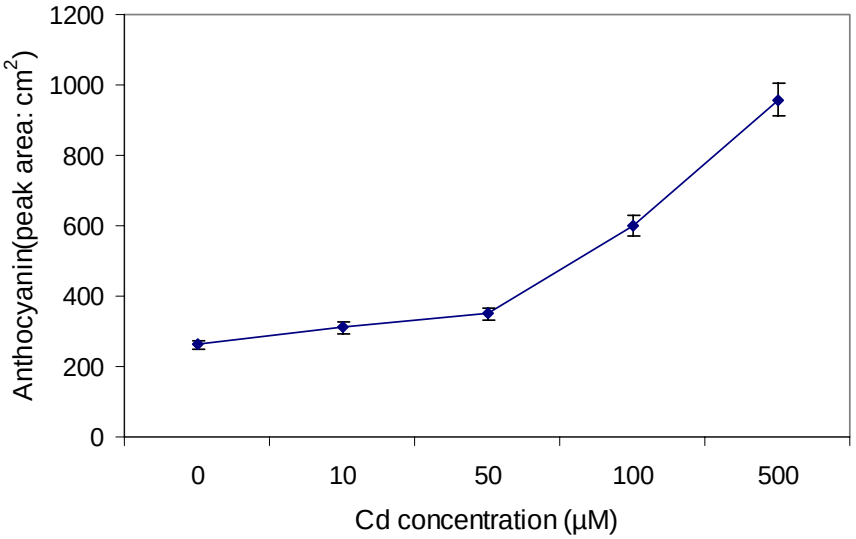

Fig. 3. The effect of different cadmium concentrations on anthocyanin content in leaf, LSD: 173.25, P: 5%. Each value is mean ± std of three replicates

We observed that the most and least Cd^{2+} was concentrated in roots and petiole, respectively (Fig. 4). There was a correlation between Cd^{2+} content concentrated in plants and the Cd^{2+} content of irrigated water.

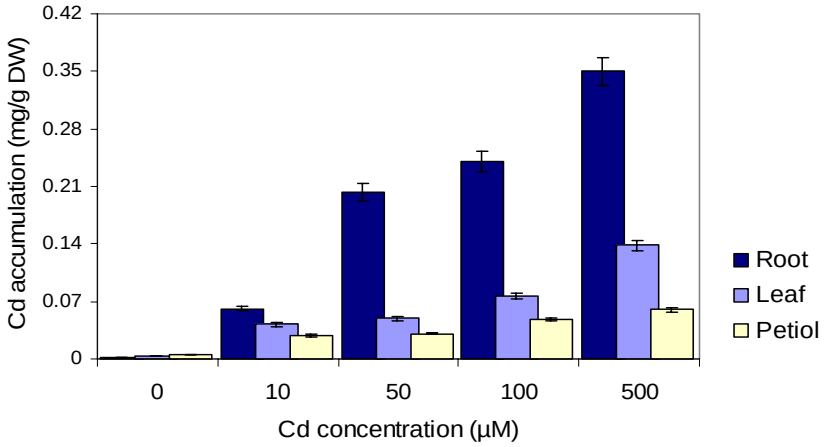

Fig. 4. Cadmium concentrations in different parts of plants treated with different concentrations of cadmium, Each value is mean ± std of three replicates

4. DISCUSSION

The toxicity of Cd^{2+} in different organs of plants has been reported to be well correlated with Cd^{2+} concentration in their environment [4]. In our experiment we measured Cd^{2+} in different organs of *B. napus*. We observed that root tissues retained much higher Cd^{2+} in comparison with other organs (Fig. 4). Many researchers reported that a decrease in transportation of Cd^{2+} from root to shoot is possibly because Cd^{2+} is present as thiol-metal complexes, and therefore can not be transported from roots [20, 7].

Malondealdehyde, which is an indication of oxidative stress, increased as Cd^{2+} concentration increased in plants (Fig. 1). One of the main mechanisms of the metal toxicity in plants is free-oxygen radical generation and oxidative stress. Oxidative stress may occur because of a disruption of detoxification mechanisms for removing free oxygen radical. There are many observations that are shown when free radicals are increased, lipid peroxidation and MDA, which is the by-product of such peroxidation, enhanced [21, 22, and 11]. Our results confirm these observations (Fig. 1). There is a report that increase in MDA content could be for the disruption of protein-lipid interaction in cell membranes. It has been reported that increase in H_2O_2 causes loss of membrane integrity and lipid peroxidation in the presence of heavy metals [23, 24].

It has been reported that the activity of the glutathione (GSH)-(ASC) cycle is responsible for an increase in tolerance of plants to oxidative stress [4, 12]. In our experiment, the ratio of ASC to DHA in plants treated with 10 and 50 µM cadmium increased, while the ratio of ASC to DHA in plants treated with 100 and 500 µM cadmium decreased (Fig. 2). The same results have been reported by Cho *et al.* in tomato plants [25]. The reason for this is because the entrance of GSH in pathway for phytochelatin synthesis and therefore the substrate for ascorbate reduction reduced. The other reason is the reduction in activity of enzymes involved in oxido-reduction of ascorbate [26, 25].

There are many documents which show that anthocyanins are able to quench the oxygen radicals [13]. Anthocyanins have been reported to increase in response to many stresses including heavy metal stress [13]. However the role of anthocyanins is not well documented in response to heavy metals, but

there is a possibility that this compound serves as a transporter of heavy metals to vacuoles. In this research we observed that when Cd^{2+} concentration increased, anthocyanin content of leaves increased (Fig. 3). It is also reported that when molybdenum (Mo) concentration increased in *Brassica rapa*, anthocyanin content increased [14]. Marrs & Walbot reported that Cd^{2+} can stimulate the synthesis of the gluthathion-S-transferase (GST) enzyme, and therefore enhance the anthocyanin synthesis [3]. This enzyme is a key enzyme which catalyzes the last step in anthocyanin biosynthesis [1].

The increase in anthocyanin, which we observed in leaves of plants treated with 100 and 500 µM Cd^{2+}, could possibly help plants to cope with stress either by compartmentation of Cd^{2+} to less sensitive parts of a cell (i.e. vacuole) or removing the free oxygen radical by quenching and detoxification of the free radicals.

5. CONCLUSION

We concluded that *B. napus*, which was used in our experiment, has not been able to increase ascorbate as an antioxidant compound; however an increase in anthocyanins can help the plant to reduce cadmium toxicity.

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