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The impact of cadmium-zinc interactions on phytobiochemical responses in *Brassica napus* cv. Hyola

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Abstract

The activity of antioxidant enzymes in response to cadmium-zinc interactions (Cd and Zn up to levels of 80 and 800 mg.kg^{-1}, respectively) in *Brassica napus* cv. Hyola was studied using a factorial greenhouse experiment in a randomized complete block design with three replications. Results indicate that the content of hydrogen peroxide (H_2O_2) significantly increased with the increased level of Cd at a given level of Zn, particularly at high levels of Zn. However, the H_2O_2 content at controls and all levels of Cd with low supply of Zn decreased. The results clearly show that with increasing the H_2O_2 content, the activity of antioxidant enzymes including catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione S-transferases (GST), significantly increased. Moreover, the content of H_2O_2 had a positive relationship with the malondialdehyde (MDA) content. In conclusion, the activity of antioxidant enzymes not only depends on the levels of Cd but also on the level of Zn supplementation. Low levels of Zn improved biochemical activity of *Brassica napus* under both low and high levels of Cd.

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Introduction

Among abiotic stresses, heavy metal stress is known to disturb plant growth in soils from the vicinity of industrial areas in developed countries (Ona *et al*., 2006). Many plant species including crops species are able to accumulate high amounts of heavy metals in their aboveground tissues (Seregin and Kozhevnikova, 2008). Most of the heavy metals are dangerous to health or to the environment (e.g. Cd, Hg and Pb), but meanwhile some of them are quite essential in low concentrations (e.g. Cu and Zn) (Mangal *et al*., 2013).

Cadmium (Cd) is a very toxic heavy metal which induces oxidative stress in plants (Hasan *et al*., 2009). Cd easily moves through the soil to the plant, and it can cause various phytotoxic symptoms including inhibition of growth, leaf chlorosis and root putrescence (Valrntovi *et al*., 2010). The plants allow Cd to be absorbed and accumulated in their different parts which make some changes in morphological, physiological and biochemical characteristics (Benavides *et al*., 2005).Cd decreases root and shoot growth (Eshghi *et al*., 2010), by inhibition of cell growth or cell division, or both (Pal *et al*., 2006). Zinc (Zn) occurs naturally in soil but more is being released into the environment as a result of activities, such as mining, smelting, refining, steel production, coal combustion, galvanization and application of sewage sludge. Although zinc is an essential element for good health, excess zinc can cause deficiencies in other nutrients and toxicity (Benavides *et al*., 2005).

An increased production of reactive oxygen species (ROS) is one of the most common consequences of all environmental stresses. These species such as hydrogen peroxide and free radicals of hydroxyl, peroxide and superoxide could cause extremely hard damages to DNA, lipids and proteins (Schutzendubel and Polle, 2002). Plants have evolved different mechanisms to overcome ROS harmful effects mainly through enzymatic (superoxide dismutase, peroxidase, catalase, and glutathione reductase) and non-enzymatic responses (Apel and Hirt, 2004). ROS are partially reduced forms of molecular oxygen resulting from processes such as photorespiration, photosynthesis and respiration (Uchida *et al*., 2002). To produce water in these processes, four electrons are required for perfect reduction of oxygen. But, ROS typically result from the transference of one, two and three electrons, respectively, to O_2 to form superoxide, hydrogen peroxide and hydroxyl radicals (Mittler, 2002). Peroxidation of plasma-lemma leads to the leakage of cellular contents, rapid desiccation and cell death. Intracellular membrane damage can affect respiratory activity in mitochondria, causing pigment to break down and leading to the loss of the carbon fixing ability in chloroplasts (Scandalios, 1993).

Studies conducted to investigate the Cd-Zn interaction on Cd and Zn uptake and accumulation have revealed mostly antagonistic interaction between these two metals (Balen *et al*., 2011). Moreover, it was found that Zn supplementation in lower concentrations can decrease Cd-induced oxidative stress, while high levels of Zn can induce oxidative stress (Cherif, *et al*., 2011). Therefore, the objective of the present study was to investigate the effects of Cd-Zn interactions on some phytobiochemical activities (enzymatic and non-enzymatic anti-oxidative responses) in *Brassica napus*.

Materials and methods

Plant material and treatments

An experiment was conducted during 2014 in the greenhouse of *Agriculture and Natural Resources Research Center of East Azarbayjan*, Tabriz, Iran, in order to determine the effects of cadmium (Cd) and zinc (Zn) interactions on some phytobiochemical responses in *Brassica napus* (cv. Hyola). Range of temperature and day light intensity in the greenhouse were 15-25°C and 15-25 mol. $m^{-2}.day^{-1}$, respectively. The soil used in this study with low available contents of Cd (lower than 0.06 $mg.kg^{-1}$) and Zn (lower than 1 $mg.kg^{-1}$) was obtained from Khalat-Pooshan station, Tabriz, Iran (latitude 38.05°N, longitude 46.17°E,

altitude 1360 m above sea level).

In this research, according to the results of some previous researches (14, 21, 27, 43, 57), eight levels of Cd (0, 0.5, 2.5, 5, 10, 20, 40 and 80 mg.kg^{-1} from $3CdSO_4.8H_2O$), eight levels of Zn (0, 5, 25, 50, 100, 200, 400 and 800 mg.kg^{-1} from $ZnSO_4.7H_2O$) and combination of them as Cdx-Zny, were used. To contaminate the soil to attain the desired levels of Cd and Zn, 64 plastic bags containing 12 kg of the soil were sprayed with 1200 ml of different treatment solutions. After 30 days incubation under wetting-drying conditions, the soils were fertilized based on soil testing results as 180 mg N.kg^{-1} ($(NH_2)_2CO$), 50 mg P.kg^{-1} ($Ca(H_2PO_4)_2.H_2O$), 100 mg K.kg^{-1} (K2SO4), 10 mg Fe.kg^{-1} ($FeSO_4.7H_2O$), 10 mg Mn.kg^{-1} ($MnSO_4.H_2O$), 5 mg Cu.kg^{-1} ($CuSO_4.5H_2O$) and 2 mg B.kg^{-1} (H_3BO_3). Then, in each plastic pot containing 4.0 kg (±3 g) of the treated soil, 8 germinated seeds of rapeseed (*Brassica napus* cv. Hyola) were sown at a depth of 2 cm and tap water (0.8 dS m^{-1}) was added to achieve 100% field capacity.

Enzyme assays

In vegetative growth stage (40 days after emergence), young leaves of rapeseed plants were collected and enzyme activities were assayed. Leaf samples were placed in an ice bucket. Leaves were then washed with distilled water and surface moisture was wiped out. Leaf samples (0.5 g) were homogenized in ice-cold 0.1 M phosphate buffer (pH 6.8) containing 0.5 mM EDTA in a pre-chilled pestle and mortar. The homogenates were centrifuged at 21000×g for 15 min at 4°C in a Beckman refrigerated centrifuge. The supernatants were used for enzyme assay (Srivastava 2010).

Hydrogen peroxide (H_2O_2) levels were determined according to Sergive *et al.* (1997). Leaf tissues (0.5 g) were homogenized in ice bath with 5 ml 0.1% (w/v) TCA. The homogenates were centrifuged at 12000×g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of supernatant was measured at 390 nm and the content of H_2O_2 was obtained from a standard curve.

Catalase (CAT) activity was measured according to Aebi (1984). The reaction mixture contained 1.5 ml of 100 mM potassium phosphate buffer (pH=7), 0.5 ml of 75 mM H_2O_2, 0.05 ml of enzyme extract and distilled water to make up the volume to 3 ml. The reaction was started by adding H_2O_2 and the decrease in absorbance was recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed.

Ascorbate peroxidase (APX) activity was determined according to Yoshimura *et al.* (2000) by monitoring the rate of ascorbate oxidation at 290 nm (ε=2.8 mM^{-1} cm $^{-1}$). The reaction mixture contained 25 mM phosphate buffer (pH=7), 0.1 mM EDTA, 1 mM H_2O_2, 0.25 mM AsA and the enzyme sample. Glutathione peroxidase (GPX) activity was measured according to Panda *et al.*, (2003). Reaction mixture contained 100 mM potassium phosphate buffer (pH 7), 0.1 mM EDTA, 5mM guaiacol, 15 mM H_2O_2 and enzyme sample. The enzyme produced a colorful product by using H_2O_2 and guaiacol as substrates. The absorbance of the product was monitored at 470 nm (ε= 26.6 mM^{-1}cm^{-1}), and peroxidase activity was expressed as units/mg protein.min.

Glutathione S-transferase (GST) activity was determined according to Carmagnol *et al.* (1981). The reaction of 1-chloro 3,4-dinitrobenzene (CDNB) with the thiol group of glutathione is catalyzed by Glutathione-S-transferase. The CDNB- glutathione conjugate absorbs light at 340nm and the activity of the enzyme is therefore estimated by measuring the changes in absorbance at this wavelength. The activity of enzyme expressed as mg protein.min^{-1}.

The malondialdehyde (MDA) content of the leaves was measured by the method of Stewart and Bewley (1980) with some modifications. 0.5 g of fresh leaves was cut into small pieces and homogenized by addition of 5 ml phosphate buffer (50 mM) in an ice

bath. Then, the homogenate was transferred into a tube and centrifuged at 14,000×g for 30 min at 4°C. 1 ml of supernatant and 1 ml of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) solution were added into a new tube. This mixture was incubated at 98°C for 30 min, then it was cooled and centrifuged at 10,000×g for 10 min. The supernatant was subjected to analysis with the spectrophotometer. The MDA content was calculated from the subtracted absorbance (A532-A600) using the extinction coefficient of 155 mm^{-1} cm^{-1}.

Statistical analysis

The experiment was conducted in factorial arrangement based on randomized complete block design with three replications. All the data were analyzed on the basis of the experimental design by ANOVA using SAS 9.1 software. Mean comparisons were performed using LSD multiple range test at $p \leq 0.05$.

Results

The content of hydrogen peroxide (H_2O_2) in all treatments significantly increased with increasing the Cd level. The highest H_2O_2 content was observed in Zn-controls at all Cd levels. The H_2O_2 content first decreased and then increased with increasing the level of Zn. However, the content of H_2O_2 at 200 mg $Zn.kg^{-1}$ was lower than 100 mg $Zn.kg^{-1}$ .Also, no significant difference was found between 100 mg $Zn.kg^{-1}$ treatments and Zn-controls at all Cd levels. The lowest H_2O_2 contents were obtained from plants under 25 mg $Zn.kg^{-1}$ treatment at all Cd levels. There was no significant difference in H_2O_2 content between 50 and 100 mg $Zn.kg^{-1}$ treatments at all Cd levels (Fig. 1).

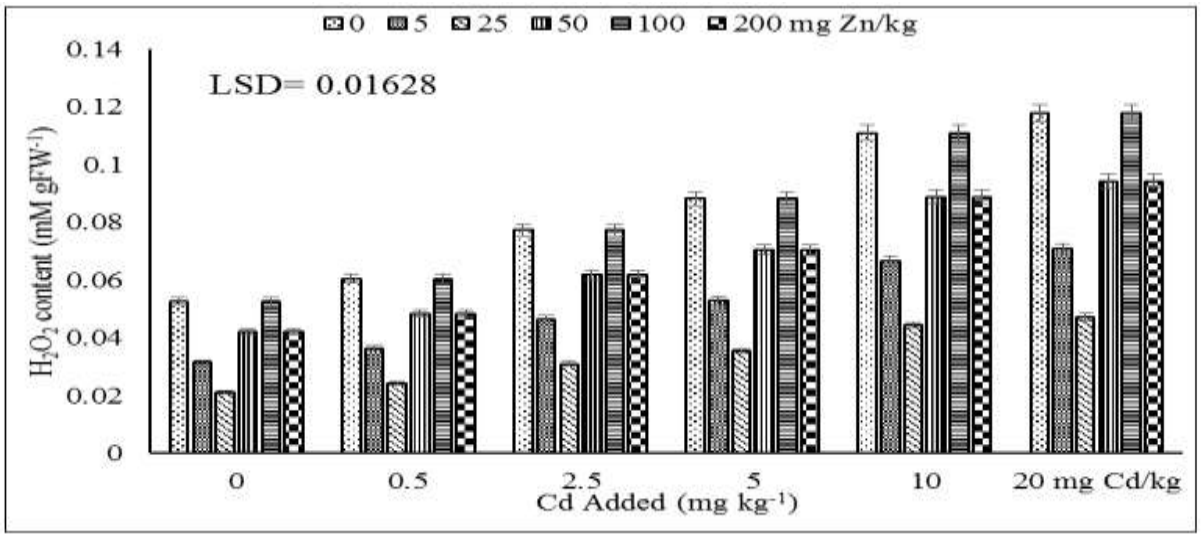

Fig. 1. Means of hydrogen peroxide (H_2O_2) content in rapeseed leaves in response to Zn-Cd interaction.

Catalase (CAT) activity at elevated levels of Cd (2.5, 5, 10 and 20 mg $Cd.kg^{-1}$) was higher than 0.5 mg $Cd.kg^{-1}$ and Cd-control. At Cd-control and 0.5 mg $Cd.kg^{-1}$, the activity of CAT significantly increased with an increase in Zn supply.

In contrast, there was no difference in the activity of CAT between the levels of Zn supplementation at 2.5, 5, 10 and 20 mg $Cd.kg^{-1}$ (Fig. 2). An exception was observed at 2.5 mg $Cd.kg^{-1}$, in which the CAT activity decreased at 100 and 200 mg Zn kg^{-1}.

Ascorbate peroxidase (APX) and glutathione peroxidase (GPX) activities showed similar trends to that of CAT (Fig. 3 and 4).

Similarly, at Cd-control and 0.5 mg $Cd.kg^{-1}$ (in contrast to other levels of Cd) the activities of APX and GPX increased with an increase in Zn supply.

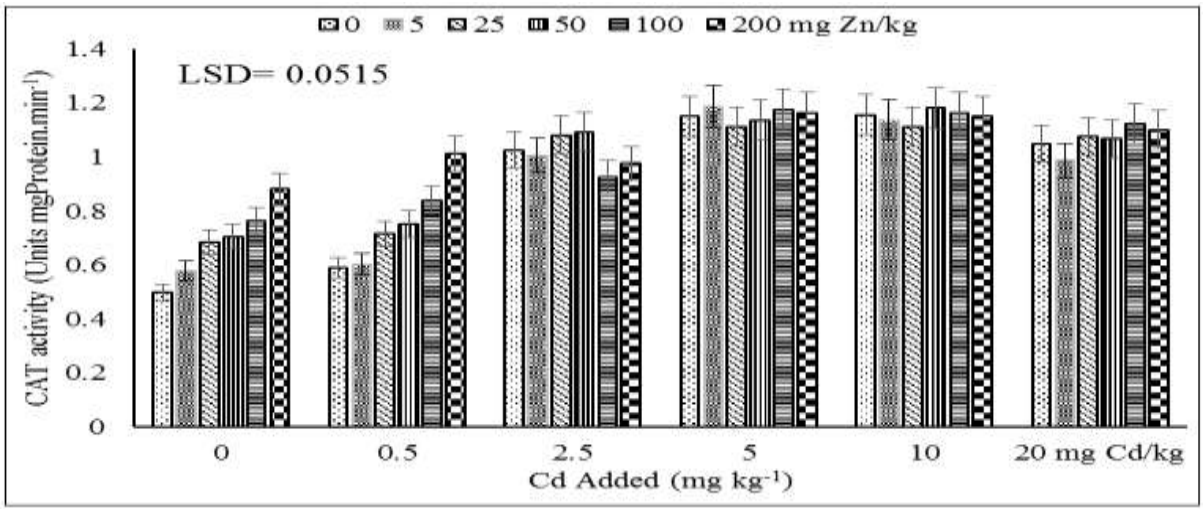

Fig. 2. Means of catalase (CAT) activity in rapeseed leaves in response to Zn-Cd interaction.

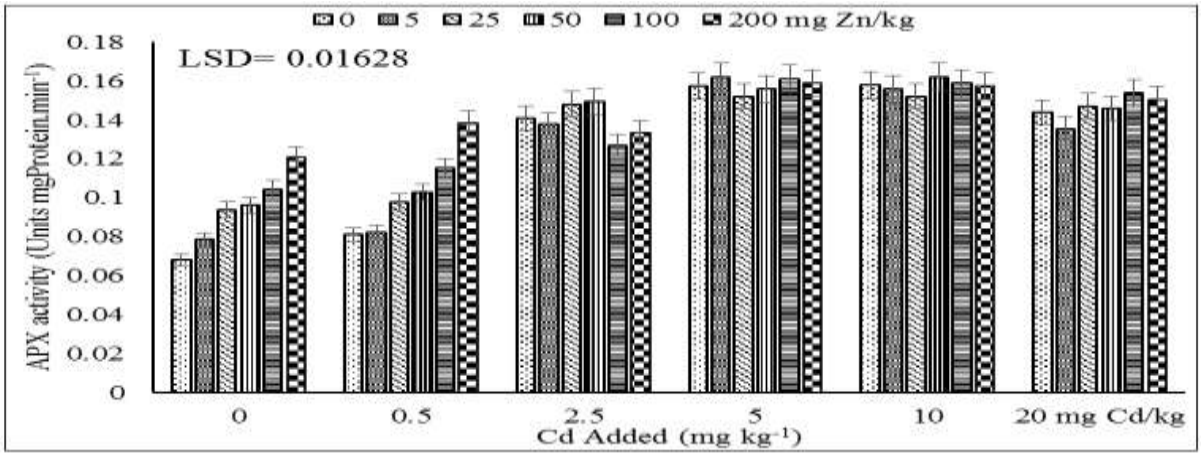

Fig. 3. Means of ascorbate peroxidase (APX) activity in rapeseed leaves in response to Zn-Cd interaction.

The activity of glutathione S-transferases (GST) significantly increased with increase in Cd level. At Cd-control and low levels of Cd (0.5 and 2.5 mg $Cd.kg^{-1}$) the lowest activity of GST was observed at 25 mg $Zn.kg^{-1}$. In contrast, with the middle and high levels of Cd (5, 10 and 20 mg $Cd.kg^{-1}$), the lowest activity of GST was obtained at 5 mg $Zn.kg^{-1}$. Maximum activity of GST was recorded at highest level of Cd (20 mg $Cd.kg^{-1}$) and highest level of Zn (200 mg $Zn.kg^{-1}$) (Fig. 5). The content of malondialdehyde (MDA) showed similar trend to that of H_2O_2. Similarly, the lowest contents of MDA were obtained at 25 mg $Zn.kg^{-1}$ in all Cd levels. (Fig. 6).

Discussion

Many degenerative reactions associated with the abiotic stresses like heavy metal stress in plants result from the production of ROS, causing oxidative stress. Plants can protect themselves by synthesizing antioxidant enzymes under such a conditions (Tkalec *et al.*, 2014; Lotfi *et al.*, 2015_a; Lotfi *et al.*, 2015_b).Based on the results, there was a positive relationship between the H_2O_2 content and the MDA content, but meanwhile a negative relationship was observed between the antioxidant enzyme activities and the lipid peroxidation (expressed as the MDA content) (Fig.1-6). Furthermore, the contents of H_2O_2 and MDA, and also the activities of CAT, APX, GPX and GST enzymes were increased with increase in Cd level (Fig. 2-5). However, combined treatments of Zn and Cd showed that Zn in low supply significantly decreased H_2O_2 and MDA contents and increased antioxidant enzyme activities (Fig. 1-6). The majority of the studies reported an antagonistic Cd×Zn

interaction on oxidative stress (Aravind and Prasad, 2003; Balen *et al.*, 2011), but others observed a synergistic interaction as well (Nan *et al.*, 2002). In tobacco plants, the supplementation of Zn had no significant effect on Cd uptake, whereas Cd addition had a significant adverse effect on Zn content (Tkalek *et al.*, 2014). Reduced concentration of Zn in Cd treated plants was also reported by Balen *et al.* (2011) and Cakmak (2000).

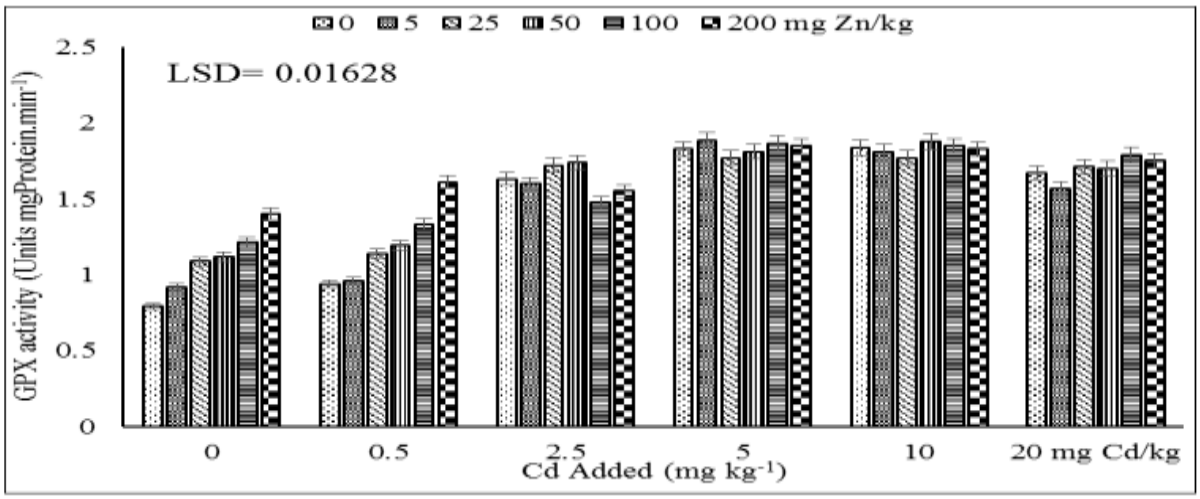

Fig. 4. Means of glutathione peroxidase (GPX) activity in rapeseed leaves in response to Zn-Cd interaction.

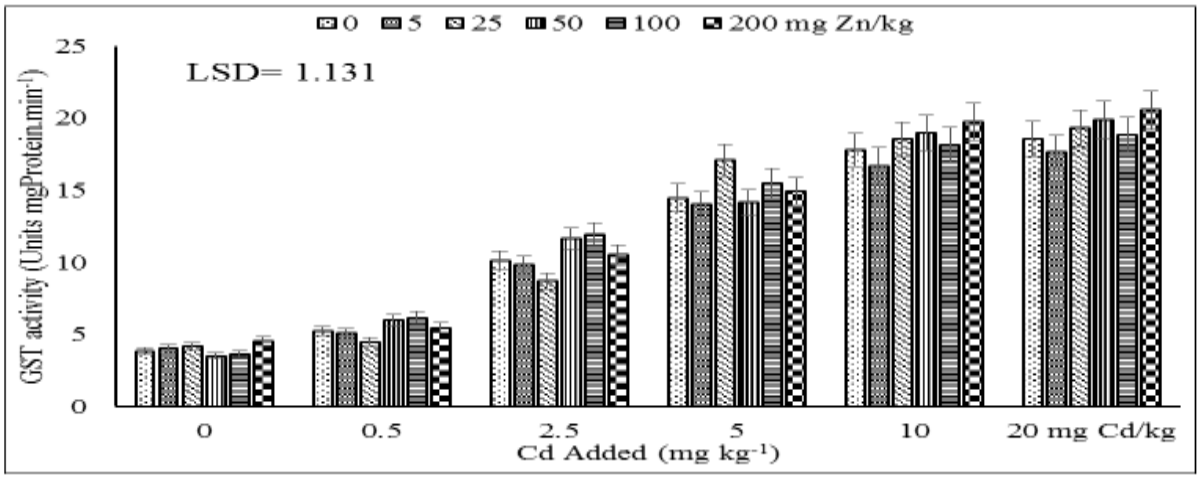

Fig. 5. Means of glutathione S-transferases (GST) activity in rapeseed leaves in response to Zn-Cd interaction.

CAT is an important antioxidant enzyme that converts H_2O_2 to water and molecular oxygen in the peroxysomes (Fredivich, 1989). In this organelle, H_2O_2 is produced from β-oxidation of fatty acids and photorespiration (Morita *et al.*, 1994). Application of Zn in combination with Cd increased the CAT and APX activities only at low Cd levels. Moreover, plants exposed to the individual Cd treatments revealed the highest CAT and APX activities, which was somewhat alleviated after the addition of Zn (Figs. 2 and 3). High activities of CAT and APX decreased the H_2O_2 content in cells (Fig. 1) and therefore, increased the stability of membranes (Esfandiari *et al.*, 2007). Zn supply could not alleviate the adverse effects of Cd indicating the CAT and APX enzymes available were not adequate to scavenge H_2O_2. This is the reason for finding high contents of H_2O_2 (Fig. 1) as well as elevated lipid peroxidation (Fig. 6) at high levels of Cd. High concentrations of Zn could induce oxidative stress, which has already been reported for several plant species (Balen *et al.*, 2011).

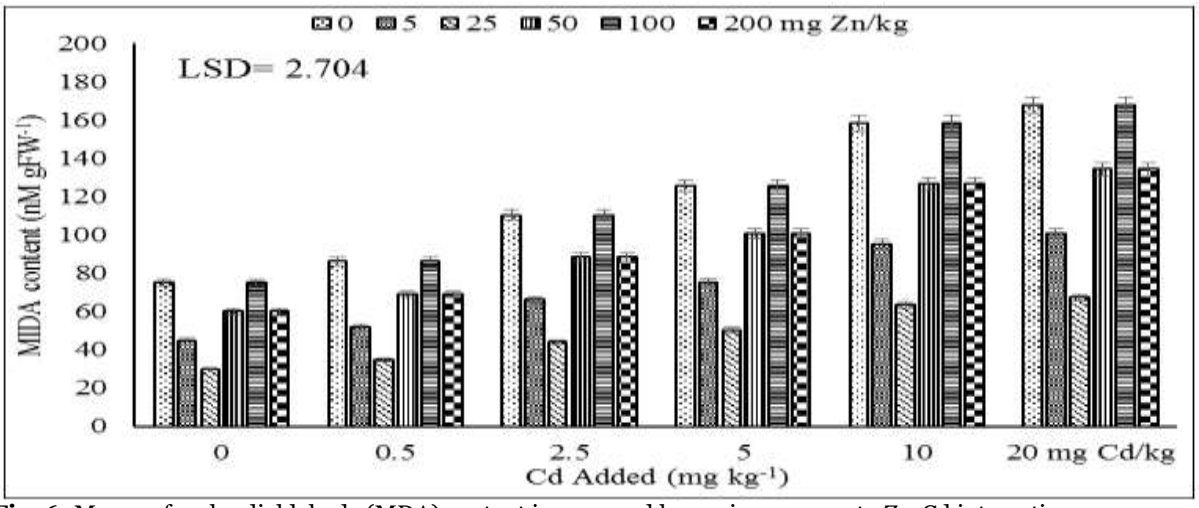

Fig. 6. Means of malondialdehyde (MDA) content in rapeseed leaves in response to Zn-Cd interaction.

Cadmium can cause oxidative stress indirectly by inhibition of metabolic reactions (Das *et al.* 1997). GST catalyzes the reaction of thiol (-SH) groups of reduced glutathione (GSH) with electrophilic reagents such as those generated by microsomal metabolism of xenobiotics, thereby neutralizing their electrophilic sites and rendering the products more water soluble (Greger *et al.*, 1995). The increase in GST activity in combined Zn-Cd treatments might be the result of high affinity of Cd for GSH. Reactions of metals with glutathione might lead to either the formation of complexes or the oxidation of glutathione. Moreover, the increase in the activity of GST would induce decreased free radicals injuring the corresponding tissues. GPX is a hydrogen peroxide degrading enzyme. Thus, the increased GPX activity in response to Cd addition in this study may be due to the maintenance of GSH homeostasis in *rapeseed* plants. This may be attributed to either free radical-dependent activation of enzyme or depletion of its co-substrate i.e., GSH and NADPH in response to Cd contamination (Bai *et al.* 2014).

Conclusion

This research put forward evidences in support of the protective role of Zn in counteracting Cd toxicity in rapeseed plants. However, the beneficial effect of Zn supplementation on Cd-induced oxidative stress was achieved at low levels of Zn and high Zn supply could induce oxidative stress.

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