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Ameliorative effects of 24-epibrassinolide and thiamine on excess cadmium-induced oxidative stress in Canola (*Brassica napus* L.) plants

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ABSTRACT

The present research investigates the interactive effects of 24-epibrassinolide (0, 0.02 and 0.5 µM) plus thiamine (0 and 200 µM) treatments on some of physio-biochemical parameters in *Brassica napus* plants under Cadmium Chloride toxic levels; 250 and 500 µM. Application of 24-epibrassinolide 0.02 µM plus thiamine 200 µM significantly increased growth and biochemical parameters such as shoot length, shoot dry weight and total chlorophyll content compared to the Cd stress conditions. While, reduced oxidative markers such as malondialdehyde, hydrogen peroxide contents and leaves Cd accumulation. Apply of 24-epibrassinolide plus thiamine increased non-enzymatic antioxidant contents and activity of antioxidant enzymes such as catalase, ascorbate peroxidase and guaiacol peroxidase. Also, enhancing the DPPH radical scavenging potential along with increasing PAL activity indicates the major influence of 24-epibrassinolide 0.02 µM and thiamine 200 µM in the reduction of Cd-induced oxidative stress in canola plants. Also In the present of 250 and 500 µM Cd, EBL 0.02 µM plus thiamine 200 µM significantly decreased H_2O_2 accumulation by 49% and 38% compared to 250 and 500 µM Cd treatment without applying the plant growth regulators respectively. Generally, Application of EBL 0.02 µM plus thiamine 200 µM improved the oxidative resistance of Canola plant under cadmium stress 250 µM.

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Brassica napus; cadmium; 24-epibrassinolide; thiamine; antioxidant enzymes; non-enzymatic antioxidants; DPPH

Introduction

Plants growth is affected by various kinds of biotic and abiotic stresses such as drought, extreme temperatures, salinity, and metal toxicity in their natural environment, as the most important factors responsible for restriction of the crop plants (Farid et al. 2013). Among heavy metals, Cadmium (Cd) is one of the most toxic heavy metals for human and plants, entering into the environment mainly through phosphate fertilizers, industrial processes, and farming practices. It has been ranked number seven among the top 20 toxins (Li et al. 2016). The uptake of Cd triggers a set of complex changes in plant growth attributes as well as modulations at biochemical and physiological levels. In general, Cd interferes with the uptake, transport, and consumption of essential nutrients, photosynthesis, respiration, protein metabolism (Martins et al. 2011), disturbed water uptake and water relations (Nagajyoti et al. 2010), declines growth, induction of lipid peroxidation and inhibition of certain enzymes activities (Farooq et al. 2016). As a result, invisible damage occurs in plants in the form of chlorosis, necrosis, browning of the root tip, and finally death (Hasan et al. 2011). Most of these changes are a consequence of the oxidative stress induced by the increase of cellular reactive oxygen species (ROS) caused by the presence of Cd in the intracellular compartment. Cd is a non-redox reactive metal and is not able to induce production of ROS. However, it induces oxidative stress in plants by blocking essential functional groups in biomolecules and by indirect mechanisms such as interaction with the antioxidative defense or disruption of the electron transport (Mishra et al. 2014).

To deal with the stress and induced damages by ROS, Plants employ their antioxidant defense machinery comprising antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), polyphenol oxidase (PPO), guaiacol peroxidase (GPX), etc and non-enzymatic antioxidants such as ascorbic acid, phenolic compounds, etc. (Gupta et al. 2013).

Brassinosteroids (BRs) are a class of plant poly hydroxyl steroids that are ubiquitously distributed in the plant kingdom. These compounds, when applied to plants, improve their quality and yield (Vardhini & Anjum 2015). These hormones at very low concentrations control numerous processes associated with plant growth and development (Yusuf et al. 2011). They have been further explored for stress- protective properties in plants against a number of stresses (Oklestkova et al. 2015). BRs have the ability to regulate the uptake of minerals into the plant cells and can be used to decrease the accumulation of heavy metals, lower the toxic effects generated by excess of heavy metals, and the synthesis of several ligands such as the phytochelatins mixed with metal ion (Sharma & Bhardwaj 2007). Another function of BR is the ability of them to improve the antioxidant system by elevating the activities and levels of enzymatic and non- enzymatic antioxidants has made them a favorite tool to increase resistance potential of important agricultural crops against various abiotic stresses such as heavy metal excess (Choudhary et al. 2011). From various analogues of BRs, 24-epibrassinolide (EBL) is known to have a significant impact on plant metabolism, growth and productivity, and experience high stability under field conditions (Fariduddin et al. 2013).

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Vitamin B_1 could be considered as bio-regulators compound which in low concentrations exerted a profound influence upon plant growth (Abdel-Aziz et al. 2009). It `participates in processes underlying adaptations to many stresses (Boubakri et al. 2013). Thiamine is the active form of vitamin B1 that serves as a coenzyme in a number of the major metabolic pathways (Rapala-Kozik et al. 2012). Some plant species can synthesize thiamin in roots, while others do not have the ability to produce it in their roots, so such plants transport thiamin from shoot to roots (Kaya et al. 2015), it also reduce oxidative stress (Zhou et al. 2013). The antioxidant role of thiamine can be indirect, by providing NADH and NADPH to the antioxidant network, or direct, by acting as an antioxidant (Asensi-Fabado & Munne-Bosch 2010).

Brassica napus is considered as the largest sources of edible oil in the world. It is not only a key economic crop but also consists antioxidants and phenolics in leaves (Jun et al. 2014). Therefore, the aim of this paper was to confirm our hypothesis that EBL and thiamine as plant growth regulators may have a positive role in controlling and alleviation of Cd-induced oxidative stress by ameliorating of antioxidant compounds accumulation, shoot Cd accumulation and enzyme activities in canola plants. In other words, the main objective of this study is to achieve a more effective combination of EBL and thiamine to increase the plant's resistance to oxidative stress resulting from cadmium toxicity.

Materials and methods

Growth of plants and experimental design

This experiment was conducted at the plant physiology laboratory of Shahid Bahonar University of Kerman. Canola seeds (*B. napus* L.) from Iran Falat co. were sterilized for 10 min with 0.01% (w/v) sodium hypochlorite solution, washed with distilled water and planted in height 12 cm × diameter 12 cm pots filled with perlite. During the first week of seed sowing, seedling were irrigated with distilled water and then, half strength Hoagland's nutrient solution was used to irrigated plants. The plants were grown in a growth chamber, during a 16 h light and 8 h dark cycle. Temperatures were 23 ± 2°C during the day and 16 ± 2°C at the night. The intensity of the light above the surface of the plants was 14000 Lux for 30 days. After this period, the effect of Cd exposure and other compound (EBL and thiamine) on growth and oxidative parameters were investigated by replacing the nutrient with a fresh solution containing the respective added compounds for 7 days. The indicated total treatments concentrations were supplied as $CdCl_2$ (0, 250 and 500 µM) (extra pure, Mreck Co, Germany), thiamine (0 and 200 µM) and EBL (0, 0.02 and 0.5 µM) (extra pure, Sigma-Aldrich Co) dissolved in the deionized water that adjusted to pH 5.8 with 0.1 M KOH or 0.1 M HCl as required. All 18 treatments combination were prepared by factorial test that indicated in Table 1. After treatment in this experiment, the shoot and third leaves of plants were harvested, and were frozen in liquid nitrogen and stored at −80°C for performing biochemical analyses.

Growth parameters: Shoot length and Shoot dry weights of treated plants were measured as growth Parameters.

Total chlorophyll assay: Total chlorophyll content was estimated according to the method of Lichtenthaler (1987). The absorbance of acetone extract of the leaves was measured at 2 wave lengths of (646.8 and 663.2 nm) using spectrophotometer. The concentration of the pigment fraction was calculated as mg/ml using the following equations:

Chlorophyll a (mg/ml) = (12.24 A663.2–2.79 A646.8)

Chlorophyll b (mg/ml) = (21.21 A646.8–5.1 A663.2)

Chlorophyll total (mg/ml) = (Chl.a + Chl.b)

Determination of lipid peroxidation and H_2O_2 content: The content of hydrogen peroxide (H_2O_2) was determined according to Alexieva et al. (2001). Leaf tissue (500 mg) was homogenized in an ice bath with 5 cm^3 of cold 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged and 0.5 cm^3 of the supernatant was added to 0.5 cm^3 of 100 mM potassium phosphate buffer (pH 7.0) and 1 cm^3 of 1 M KI. The absorbance was read at 390 nm.

Lipid peroxidation: The level of lipid peroxidation in plant tissues was measured by determination of malondialdehyde (MDA) production (Heath & Packer 1968) and others aldehydes (Meir et al. 1992) breakdown products of lipid peroxidation. MDA content was determined with a thiobarbituric acid (TBA) reaction. A leaf sample (0.5 g) was homogenized in trichloroacetic acid, TCA (10 ml; 0.1%). The homogenate was centrifuged (15000 g; 5 min) and supernatant was collected (1.0 ml) of the supernatant, 4 ml of 0.5% thiobarbituric acid (TBA) in TCA (20%) was added. The mixture was heated at 95°C for half an hour and then quickly cooled in an ice bath. After centrifugation, the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The MDA content was calculated by its extinction coefficient of 155 mM^{-1} cm^{-1} and of others aldehydes formed the extinction coefficient was 0.457 × 105 M^{-1} cm^{-1}. Expressed as µM MDA per gram fresh weight.

Total phenolic and flavonoid compounds and PAL activity assay: Total phenolic content was determined using the Folin-Ciocalteau method Gao (2000). Gallic acid was used for constructing the standard curve. Results were expressed as mg gallic acid (GA) per gram of the fresh weight.

Flavonoid content: The flavonoid content was estimated as per the colorimetric method of Zhishen et al. (1999). Catechin was used as standard for the calibration curve. The absorbance was measured at 510 nm and flavonoid content was expressed as mg catechin equivalents g^{-1} of extract.

Phenylalanine ammonia lyase enzyme (PAL) was extracted from a 0.3 g sample with 6.5 ml of 50 mM Tris–HCl buffer (pH 8.8) containing 15 mM *B*-mercaptoethanol in an ice-cooled mortar. The homogenate was centrifuged at 10,000 g for 15 min, and the supernatant was collected for enzyme assay. PAL activity was determined by monitoring the production of t-cinnamic acid (CA) at 290 nm (Hahlbrock & Ragg 1975). The reaction mixture contained

Table 1. The composition of the treatments used in this study which has different concentrations thiamine, EBL and CdCl.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Thiamine (µM)	0	0	0	0	0	0	0	0	0	200	200	200	200	200	200	200	200	200
EBL (µM)	0	0	0	0.02	0.02	0.02	0.5	0.5	0.5	0	0	0	0.02	0.02	0.02	0.5	0.5	0.5
CdCl (µM)	0	250	500	0	250	500	0	250	500	0	250	500	0	250	500	0	250	500

50 mM Tris-HCl buffer (pH 8.8), 20 mM L-phenylalanine and enzyme extract. Incubation was at 30°C, and the reaction was stopped by the addition of 0.5 ml 10% trichloroacetic acid. Absorbance at A290 nm was measured after 30 min. One unit of PAL activity is equal to 1 mol of CA produced per min.

Extraction and estimation of DPPH radical scavenging activity

The leaves (100 mg tissue) were crushed with 2 ml of ethanol. Homogenate was centrifuged for 20 min and the DPPH (diphenyl-picrylhydrazyl) radical scavenging activity of the supernatant was determined according to the method given by Zhu et al. (2006). A volume of 2 mL of each sample was added to 2 mL of 0.1 mM DPPH in 95% ethanol. The mixture was shaken and left for 60 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm. Triplicate tests were conducted for each sample. A lower absorbance represented a higher DPPH scavenging activity. The inhibitory percentage of DPPH was calculated according to the following equation:

$$\%\text{Inhibition} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of Control}}\right](100)$$

Determination of ascorbate (ASC) and dehydroascorbate (DHA)

The leaves plants (0.2 g) were homogenized with 5% (w/v) metaphosphoric acid at 4°C. The homogenate was centrifuged at 20,000 g for 15 min at 4°C and the supernatant was collected for analysis of ascorbate (ASC). ASC and DHA were determined spectrophotometrically at 525 nm according to the method of Hodges et al. (1996). Briefly, total ASC was determined after reduction of DHA to ASC with DTT, and the concentration of DHA was estimated from the difference between total ascorbate pool (ASC plus DHA) and ASC. A standard curve was developed based on ASA in the range of 0–50 lg/ml.

Antioxidant enzymes activity: For the assay of antioxidative enzymes activity, the extracts of frozen leaf tissue prepared in a 50 mM potassium phosphate buffer (pH 7) containing 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM sodium ethylene diaminetetraacetic acid (Na2EDTA), and 1% (w/v) polyvinylpyrrolidone (PVP) were centrifuged at 15,000 g at 4°C for 15 min and the supernatants were used for the estimation of enzyme activities.

Catalase (CAT) activity was assayed with spectrophotometry by monitoring the decrease in absorbance of H_2O_2 at 240 nm using the method of Dhindsa et al. (1981). The assay solution contained 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H_2O_2. The reaction was started by the addition of 100 µL of enzyme extract to the reaction mixture and the change in absorbance was followed 1 min after starting of the reaction. Unit of activity was taken as the amount of enzyme that decomposes 1 mM of H_2O_2 in 1 min.

Ascorbate peroxidase (APX) activity was determined by following the decrease in the absorbance at 290 nm for 3 min in a 1 ml mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA, and 100 mM of enzyme extract. One unit of APX activity was defined as the amount of enzyme that decomposed 1 mM of ascorbate for 1 min (Nakano & Asada 1981).

Peroxidase (POD) activity was determined using the guaiacol test (Plewa et al. 1991). The tetraguaiacol formed in the reaction has a maximum absorption at 470 nm and thus the reaction can be readily followed photometrically. The enzyme was assayed in a solution that contained 50 mM phosphate buffer (pH 7.0), 0.3% H_2O_2, and 1% guaiacol. The reaction was started by the addition of 20 µL of the enzyme extract at 25°C and was followed 3 min after starting the reaction. The enzyme unit was calculated for the formation of 1 mM tetraguaiacol for 1 min.

Element analysis by ICP-OES: After treatments, shoot samples were oven dried at 70°C for 72 h; then 0.5 g of dry shoot were dissolved in 10 ml 65% (w/v) nitric acid (supra pure, Merck). After digestion, the volume of each sample was adjusted to 50 ml using double deionized water. Total concentration of Cd was determined by inductively coupled plasma atomic emission spectroscopy (ICP, OES, Varian CO). The stability of the device was evaluated after every ten sample determination by examining the internal standard. Reagent blanks were also prepared to detect potential contamination during the digestion and analytical procedure. The samples were analyzed in triplicates. For quality control, we used standard solutions with Cd concentrations in the range of our experiment (standard solution, MERCK) (Sagner et al. 1998).

Statistical analysis

The experimental design was completely randomized with 18 treatments, one cultivar and three replications per treatment. Data were analyzed by using the analysis of variance (Two-way ANOVA) followed by the Duncan test at a 0.05 probability level. All statistical analyses were done using the software SPSS package, v. 18.0 for Windows.

Results

Generally, based on ANOVA Table 3; the main effects of thiamine, EBL and Cd on the antioxidative system showed significant differences among treatments ($P \leqq 0.01$ and 0.05) by measuring the changes in growth parameters and antioxidant contents. In this regard, there were significant differences in the contents of polyphenol, flavonoid, ASA, percentage inhibition of DPPH and PAL activity under our treatments. Additionally, the antioxidative components accumulation exhibited a significant difference in the interaction effect (thiamine × EBL × Cd), except (thiamine × Cd) for DHA compared to stress conditions.

Growth parameters and total chlorophyll

The effects of treatments on the canola plants were evaluated by measuring the changes in morphological parameters and photosynthetic pigments content. The evaluation of interactive effects of thiamine, EBL and Cd on the morphological parameters and photosynthetic pigments contents showed significant differences among treatments compared to the separate treatments or control ($P \leqq 0.01$ and 0.05) (Table 2).

Table 2. ANOVA of main effects of Thiamine, EBL and Cd treatment and their interaction on growth and total chlorophyll content in canola plant.

Treatment μM		Shoot length (cm/plant)	Shoot dry weight (g/plant)	Total chlorophyll content (mg/g FW)
Thiamine	1	7.78**	0.13**	0.82**
EBL	2	19.09**	0.16**	4.56**
Cd	2	56.89**	0.47**	14.34**
EBL + Cd	4	0.50**	0.00*	0.37**
Thiamine + EBL	2	1.39**	0.02**	0.04**
Thiamine + Cd	2	0.23	0.01**	0.08**
Thiamine + EBL + Cd	4	0.46**	0.00**	0.01
Error	36	0.11	0.00	0.01

**: $P \leqq 0.01$; *: $P \leqq 0.05$.

Additionally, the significant difference between the plants growth parameters of the project was observed by the treatments interaction effect especially for shoot dry weight.

However, shoot length under Cd 250 μM without thiamine and EBL application, consistently decreased significantly by 33% when compared with the control condition. Thus, the application of 250 μM Cd containing EBL 0.02 μM plus thiamine 200 μM increased shoot length under Cd stress as control plants (Table 4). Also as it is shown in Table 3 when external thiamine and EBL are low at each Cd levels, an increase from 0 to 0.02 μM EBL and thiamine 200 μM significantly increased shoot dry weight and total Chlorophyll content in canola plants compared to the stress conditions (Table 4).

The 250 μM Cd stress caused a significant decrease in shoot dry weight by 37% as compared to control (Table 4). The data showed that the application of EBL and thiamine enhanced the shoot dry weight in the tested plants, and this increment was greater in plants treated with Cd (250 μM) along with EBL (0.02 μM) and thiamine (200 μM) by 53% compared to stressed plants.

In comparison to control plants, the stress treatments; Cd 250 and 500 μM induced significant reductions in total chlorophyll contents in the canola leaves by 27% and 83%, respectively (Table 4). Application of thiamine, 200 μM and EBL, 0.02 μM in *B. napus* plants under Cd stress resulted in significant rises of total chlorophyll contents by 29% and 113%. In all the treatments, the minimum content for total chlorophylls was obtained in EBL 0.5 μM along with Cd 500 μM treatment as 0.13 mg/g FW (Table 4).

H_2O_2 content, MDA and other aldehydes

Metal stress caused a significant increase in the content of MDA, other aldehydes, and H_2O_2 in both Cd concentrations than the control plants (Figures 1–3). However, a different pattern of response was detected when studying these parameters in EBL and thiamine-treated plants in the presence of Cd stress. Under Cd stress condition, exogenous addition of EBL 0.02 μM and thiamine 200 μM could considerably decrease the content of MDA, other aldehydes and H_2O_2 under Cd-stressed, compared with the plants grown under stress alone.

In the present of 250 and 500 μM Cd, EBL 0.02 μM plus thiamine 200 μM significantly decreased H_2O_2 accumulation by 49% and 38% compared to 250 and 500 μM Cd treatment without applying the plant growth regulators respectively (Figure 1).

Table 4. The effects of exogenous thiamine and EBL on growth and total chlorophyll content in canola plants under Cd stress.

Treatment Thiamine + EBL + Cd (μM)	Shoot length (cm/plant)	Shoot dry weight (g/plant)	Total chlorophyll content (mg/g FW)
0 + 0 + 0	7.17 ± 0.29bc	0.60 ± 0.02cd	2.21 ± 0.14c
0 + 0 + 250	4.83 ± 0.29ef	0.38 ± 0.02hi	1.61 ± 0.08g
0 + 0 + 500	2.83 ± 0.29i	0.23 ± 0.02k	0.37 ± 0.04l
0 + 0.02 + 0	7.00 ± 0.50bc	0.62 ± 0.01bc	2.53 ± 0.01b
0 + 0.02 + 250	5.67 ± 0.29d	0.40 ± 0.01h	1.79 ± 0.01f
0 + 0.02 + 500	4.00 ± 0.50h	0.21 ± 0.01k	0.58 ± 0.01k
0 + 0.5 + 0	5.33 ± 0.29de	0.45 ± 0.01fg	1.22 ± 0.01h
0 + 0.5 + 250	4.17 ± 0.29gh	0.30 ± 0.02j	0.88 ± 0.01j
0 + 0.5 + 500	2.67 ± 0.29i	0.15 ± 0.01l	0.13 ± 0.01m
200 + 0 + 0	7.50 ± 0.50b	0.66 ± 0.02b	2.52 ± 0.08b
200 + 0 + 250	5.67 ± 0.29d	0.54 ± 0.00e	1.94 ± 0.01e
200 + 0 + 500	3.83 ± 0.29h	0.35 ± 0.02i	0.44 ± 0.11l
200 + 0.02 + 0	9.17 ± 0.29a	0.70 ± 0.03a	3.06 ± 0.02a
200 + 0.02 + 250	6.83 ± 0.29c	0.58 ± 0.02d	2.08 ± 0.20 d
200 + 0.02 + 500	4.67 ± 0.29fg	0.41 ± 0.01gh	0.79 ± 0.09 j
200 + 0.5 + 0	5.83 ± 0.29d	0.47 ± 0.05f	1.50 ± 0.01 g
200 + 0.5 + 250	4.33 ± 0.29fgh	0.31 ± 0.03j	1.04 ± 0.02j
200 + 0.5 + 500	2.67 ± 0.29i	0.20 ± 0.01k	0.17 ± 0.02m

Means ± SD of 3 replicates for biochemical parameters and different letters indicate significant differences ($P < 0.05$) according to Duncan test.

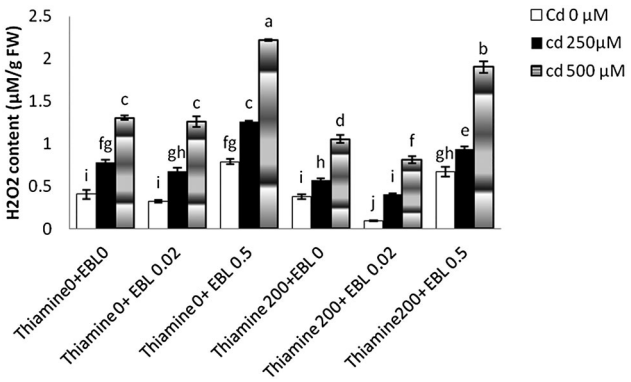

Figure 1. Effect of EBL and thiamine on H_2O_2content in canola leaves under Cd stress. Data are means ± SE of three replicates. Different letters indicate the significance of difference at $P \leq 0.05$ levels by Duncan test.

Table 3. ANOVA of main effects of thiamine, EBL and Cd treatment and their interaction on contents of phenols, flavonoids, ASC, DHA, DPPH and PAL activity in canola plants.

Treatment μM		Phenol (mg/g FW)	Flavonoids (mg/g FW)	PAL activity (U/mg protein)	ASC (mg/g FW)	DHA (mg/g FW)	DPPH activity (%)
Thiamine	1	26.41**	42.36**	3377.67**	1.28**	10.77**	2269.69**
EBL	2	12.07**	19.48**	1567.23**	2.59**	1.53**	664.14**
Cd	2	33.66**	49.73**	3534.06**	13.77**	27.65**	5401.23**
EBL + Cd	4	2.36**	2.25**	263.93**	0.10**	0.90**	66.73**
Thiamine + EBL	2	5.22**	4.02**	602.79**	1.15**	1.65**	63.09**
Thiamine + Cd	2	4.10**	3.11**	320.67**	0.09*	0.17	93.16**
Thiamine + EBL + Cd	4	0.85**	3.37**	109.35**	0.10**	0.67**	48.68**
Error	36	0.07	0.17	2.97	0.02	0.07	3.70

**: $P \leqq 0.01$; *: $P \leqq 0.05$.

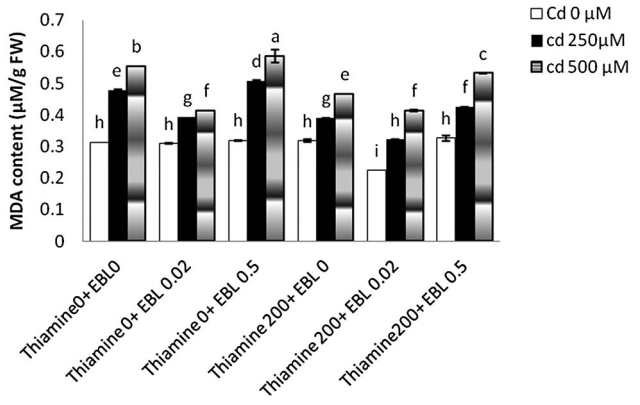

Figure 2. Effect of EBL and thiamine on MDA content in canola leaves under Cd stress. Data are means ± SE of three replicates. Different letters indicate the significance of difference at $P \leq 0.05$ levels by Duncan test.

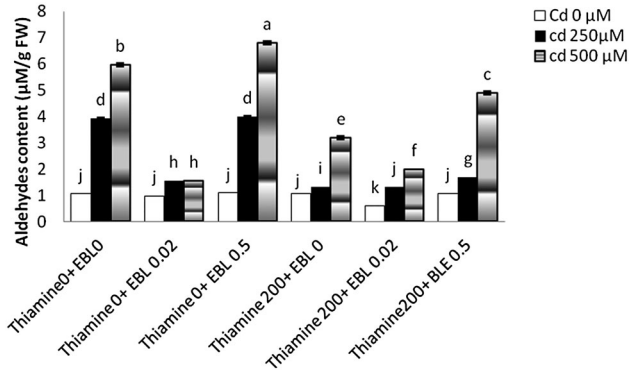

Figure 3. Effect of EBL and thiamine on Aldehydes in canola leaves under Cd stress. Data are means ± SE of three replicates. Different letters indicate the significance of difference at $P \leq 0.05$ levels by Duncan test.

The application of recent levels of EBL and thiamine with cadmium 250 µM in the plants adversely decline MDA concentration of leave by 33% compared to Cd 250 µM stress (Figure 1).

EBL and thiamine treatments clearly decreased other aldehydes production under the Cd levels in canola leaves, but under cadmium 250 µM, application of EBL 0.02 µM with thiamine 200 µM has better effect than separate effect of EBL or thiamine (Figure 3).

However, the interaction between EBL 0.02 µM and thiamine 200 µM had a significant effect on decreasing these oxidative stress parameters in stressed conditions than control plants at 0.05 levels statistically (Figures 1–7).

Activitiy of PAL enzyme and total phenolic and flavonoid contents

The results show that the activity of PAL, total phenolic and flavonoids content increased in canola treated with Cd 250 µM compared to the control samples by 166%, 34% and 137%, respectively. Thus, increasing percentage for these parameters under Cd 500 µM stress was 233%, 75% and 236% in comparison to control respectively.

Based on ANOVA analysis and data tables, the PAL activity was strongly stimulated in leaves exposed to the Cd levels, depending on its dosage. As shown in Table 4, the activity of this antioxidative enzyme was enhanced more by EBL and thiamine under both Cd levels (Tables 3 and 5).

The highest increase in PAL activity and total phenolic content was observed in treated plants at 500 µM Cd supplemented with 200 µM thiamine and 0.5 µM EBL by 231% and 168%, respectively, compared with seedlings treated with Cd 500 µM solution. On the other hand, the highest total flavonoid content was obtained from 200 µM thiamine and 0.02 µM EBL applications, under 500 µM Cd by 119% (Table 5). These findings indicate that probably EBL plus thiamine play substantial roles as a mediator in defensive reactions to deal with Cd stress in canola plants.

DPPH assay

DPPH is a stable free radical which is used as a substrate to evaluate antioxidant activity. A dramatic enhance in free radical scavenging activity was recorded under 250 and 500 µM cadmium levels by 101% and 149% respectively when compared with the plants control. The plants treated with EBL 0.02 µM and thiamine 200 µM combination supplemented with Cd 500 µM solution showed most enhancement (49%) in DPPH radical scavenging activity when compared with Cd-stress alone (Table 5).

Under Cd toxicity 500 µM, the amount of DPPH was markedly increased by separate application of EBL

Table 5. The effects of exogenous thiamine and EBL on contents, phenols, flavonoids, ASC, DHA, DPPH and PAL activity in canola plants under Cd stress.

Treatment Thiamine+ EBL + Cd (µM)	Phenol(mg/g FW)	Flavonoids (mg/g FW)	PAL activity (U/mg protein)	ASC(mg/g FW)	Dehydro ascorbate (DHA) (mg/g FW)	DPPH activity (%)
0 + 0 + 0	1.61 ± 0.14h	1.42 ± 0.22i	6.77 ± 0.7j	7.57 ± 0.23b	4.72 ± 0.02f	22.82 ± 0.86k
0 + 0 + 250	2.16 ± 0.15g	3.37 ± 0.22g	17.99 ± 0.9g	7.08 ± 0.09c	5.32 ± 0.14e	45.87 ± 2.03g
0 + 0 + 500	2.81 ± 0.42ef	4.77 ± 0.31def	22.51 ± 0.77f	5.91 ± 0.18e	7.26 ± 0.40c	56.88 ± 2.60e
0 + 0.02 + 0	1.63 ± 0.13h	2.52 ± 0.45h	7.06 ± 0.52j	7.58 ± 0.14b	4.77 ± 0.08f	29.48 ± 2.09j
0 + 0.02 + 250	2.39 ± 0.23fg	4.55 ± 0.10def	19.21 ± 0.80g	6.66 ± 0.16d	6.06 ± 0.47d	51.62 ± 0.88f
0 + 0.02 + 500	3.73 ± 0.27d	5.20 ± 0.34d	25.92 ± 2.39e	5.96 ± 0.23e	7.29 ± 0.16c	65.28 ± 1.81d
0 + 0.5 + 0	1.70 ± 0.07h	3.36 ± 0.23g	14.67 ± 1.6h	7.10 ± 0.12c	5.31 ± 0.15ef	39.09 ± 2.40h
0 + 0.5 + 250	2.80 ± 0.22ef	4.77 ± 0.19def	22.71 ± 2.87f	6.66 ± 0.11d	5.91 ± 0.29d	51.21 ± 1.87f
0 + 0.5 + 500	3.73 ± 0.30d	5.19 ± 0.25d	38.91 ± 1.73d	5.07 ± 0.03f	7.20 ± 0.19c	58.31 ± 1.62e
200 + 0 + 0	1.69 ± 0.13h	3.02 ± 0.28gh	11.04 ± 0.31i	7.59 ± 0.17b	4.84f ± 0.19g	33.71 ± 3.02i
200 + 0 + 250	2.31 ± 0.18g	4.20 ± 0.14ef	19.40 ± 2.06g	6.65 ± 0.15d	7.18 ± 0.24c	52.37 ± 1.27f
200 + 0 + 500	3.10 ± 0.19e	4.77 ± 0.31de	25.15 ± 0.98ef	5.85 ± 0.24e	8.21 ± 0.36b	65.55 ± 2.05d
200 + 0.02 + 0	2.29 ± 0.43g	3.71 ± 0.40fg	22.51 ± 1.41f	8.52 ± 0.12a	6.08 ± 0.36d	38.32 ± 2.40h
200 + 0.02 + 250	4.17 ± 0.27d	6.19 ± 0.41c	42.68 ± 1.02c	7.57 ± 0.06b	7.19 ± 0.18c	70.11 ± 1.39c
200 + 0.02 + 500	6.82 ± 0.44b	10.46 ± 0.55a	61.57 ± 2.28b	6.65 ± 0.08d	9.20 ± 0.35a	84.93 ± 1.06a
200 + 0.5 + 0	2.43 ± 0.25g	4.54 ± 1.06def	18.47 ± 1.59g	7.20 ± 0.06c	5.89 ± 0.30d	43.36 ± 2.10g
200 + 0.5 + 250	4.81 ± 0.40c	6.08 ± 0.25c	42.70 ± 2.29c	6.64 ± 0.12d	6.04 ± 0.20d	68.14 ± 1.63cd
200 + 0.5 + 500	7.53 ± 0.20a	8.12 ± 0.58b	74.59 ± 3.16a	5.69 ± 0.25e	7.24 ± 0.29c	80.77 ± 1. 97b

Means ± SD of 3 replicates for biochemical parameters and different letters indicate significant differences ($P < 0.05$) according to Duncan test.

(0.02 µM) and thiamine (200 µM) comparing to stress condition without EBL and thiamine (Table 5). In cadmium treatment 250 µM containing EBL (0.02 µM) and thiamine (200 µM), EBL plus thiamine effect substantially elevated concentration of DPPH than stress condition. Thus, under Cd 500 µM stress, interactive effect between EBL (0.5 µM) and thiamine (200 µM) was observed by 42% in comparison to stress condition alone (Table 5).

Interaction effect between thiamine, EBL and Cd on ASC and DHA contents

As it is shown in Table 4, Cd-stressed plants especially 500 µM, showed a marked decrease (22%) in the contents of ASC with a simultaneous increase (54%) in DHA over untreated control. Utilization of exogenous thiamine (200 µM) and EBL (0.02 µM) improved ASC pool significantly under stress particularly 500 µM Cd. While we determined highest concentration of ASC with treatment thiamine 200 µM and EBL 0.2 µM under non-Cd conditions than control samples by 12% significantly. However, the maximum DHA content was observed by the interactive effect of thiamine (200 µM) puls EBL (0.2 µM) supplemented with Cd 500 µM solution in comparison to stressed plants.

Our findings illustrated that both thiamine 200 µM and EBL 0.2 µM could up-regulate the antioxidant mechanisms in the *B. napus* under Cd 250 and 500 µM toxicity conditions.

Activities of antioxidative enzymes

The activities of the enzymes CAT, APX, and GPX increased significantly with using the Cd concentrations in *B. napus*. The most enhancement in the activities of the above-mentioned antioxidative enzymes were observed due to Co-application 200 µM thiamine and 0.02 µM EBL in conjunction with Cd 500 µM compared with Cd treatment alone by 64%, 45% and 43%, respectively. The dependent effects of thiamine and EBL on elevating the enzymes activity were more pronounced in comparison to the separate effects of both. The control plants presented the lowest activities of CAT, APX, and GPX enzymes (Figures 4–6).

When the plants were exposed to 500 µM Cd, the leaf CAT activity was 99% higher than the control (Figure 4). Also, the activity of recent enzyme increased in the plants under the Cd 500 µM containing 200 µM thiamine plus 0.02 EBL compared to 500 µM Cd stress conditions. However similar changes were obtained about 250 µM Cd stress treatments (Figure 4).

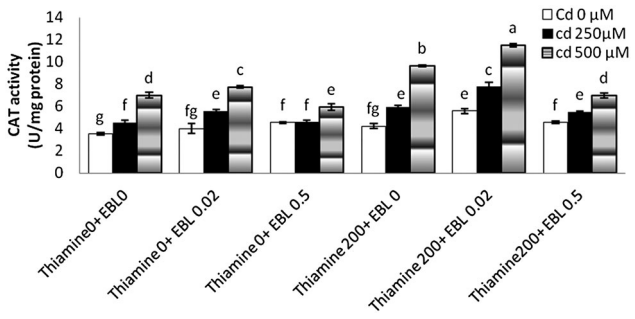

Figure 4. Effect of EBL and thiamine on CATactivity in canola leaves under Cd stress. Data are means ± SE of three replicates. Different letters indicate the significance of difference at $P \leq 0.05$ levels by Duncan test.

Remarkable elevation in the APX activities were approximately 23% and 62% under 250 and 500 µM Cd compared to the control plants, respectively. Under both Cd concentration 250 and 500 µM, the activity of APX increased by using 200 µM thiamine plus 0.02 EBL treatments compared to the recent Cd concentrations significantly (Figure 5).

Also, GPX activity increased by 32% due to 500 µM Cd stress alone but it considerably improved and increased with the thiamine and EBL applications compared to the stress conditions. While, the combination 200 µM thiamine and 0.02 µM EBL with 250 µM Cd increased the GPX activity by 25% than recent stress conditions (Figure 6).

Generally, our findings illustrated that both 200 µM thiamine and 0.02 µM EBL could up-regulate the antioxidant enzymes activities in the canola plants under control and Cd toxicity conditions.

Cd accumulation in shoot canola plant

The results from ICP, OES analysis showed that Cd ions accumulated significantly in shoot of the canola plants under toxic levels of Cd without thiamine 200 µM and EBL 0.02 µM compared to the control (Figure 6). Maximum accumulation of Cd in shoot observed in the treatment containing Cd (500 µM) plus EBL (0.5 µM) by 12% increasing in comparison to 500 µM Cd, while interaction between thiamine 200 µM and EBL0.02 µM decreased shoot Cd content in plants under Cd stress (250 and 500 µM). One-way ANOVA exhibited that differences between the treatments were significant at $p \leq 0.01$ (Table 2).

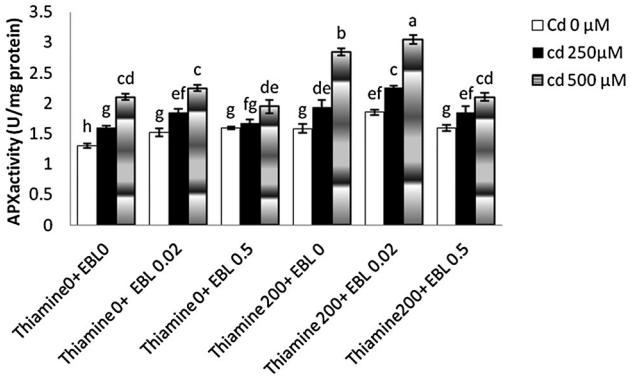

Figure 5. Effect of EBL and thiamine on APX activity in canola leaves under Cd stress. Data are means ± SE of three replicates. Different letters indicate the significance of difference at $P \leq 0.05$ levels by Duncan test.

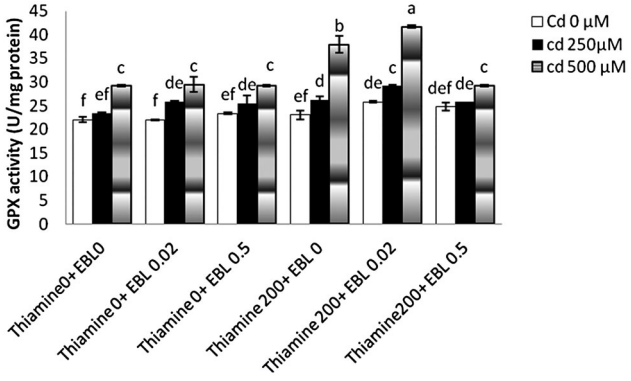

Figure 6. Effect of EBL and thiamine on GPX activity content in canola under Cd stress. Data are means ± SE of three replicates. Different letters indicate the significance of difference at $P \leq 0.05$ levels by Duncan test.

Discussion

Growth inhibition and decline of biomass production are general responses of higher plants to heavy metal toxicity. Inhibition of both cell elongation and division by heavy metals could explain, partly, the decline in biomass production (Houshm & Moraghebi 2011). Growth reduction under heavy metals toxicity conditions has been observed for numerous species tested, including *Glycyrrhiza uralensis* (Zheng et al. 2010), and safflower (Houshm & Moraghebi 2011), which is in agreement with the current data (Table 3). It can be concluded that enhancement of the growth parameters by EBL and thiamine treatments is attributed to their ability to regulate cell division and cell elongation activities and also to excite the accumulation of soluble and total carbohydrates in Cd-stressed plants. The highest growth parameters were obtained in both treatments, 0.02 µM EBL and 200 µM thiamine, in comparison to the control in canola seedling (Table 3). Studies have revealed that the ability of BRs to reduce the toxic effect of heavy metals is due to their effect on the electrical properties of membranes, their permeability, structure, and stability (Arora et al. 2010). Therefore, BRs application increases seedling growth. In a similar way, improved seedling growth has been reported by the application of BRs in various plants under heavy metal stress (Ali et al. 2008; Choudhary et al. 2011). Also, exogenous application of thiamine has been reported to increase all growth parameters of plants species (Nahed et al. 2010; Mahgoub et al. 2011; Soltani et al. 2014), in addition to its importance in maintenance growth and protection from activated status inside the plants (El-Shahawy et al. 2008). This vitamin stimulates pectin and cellulose accumulation and inhibits lignin production in plants under stress, which may be one part of the role of thiamine which is considerably helpful in defense of the plant via the cell wall (Al-Hakimi & Hamada 2011).

Chlorophyll is the most important chloroplast component for photosynthesis, and it has a positive connection with photosynthetic rate (Gubrelay et al. 2013). Cadmium toxicity is responsible for the reduction of nutrient uptake, damage photosynthetic pigments and net photosynthesis (Ehsan et al. 2014). Our data showed that Cd supply, significantly increasing Cd accumulation in canola plants, is related to canola growth reduction (Figure 6). That is in accordance with other studies on *Lepidium sativum*, *Brassica juncea*, and, *Lycopersicon esculentum* (Gill et al. 2011; Gratão et al. 2015). Various researchers have demonstrated that BRs reduce the adverse effects induced by Cd stress in plants (Janeczko et al. 2005; Anuradha & Rao 2009). Application of thiamine increases the photosynthetic pigments contents with accumulation of total carbohydrates in metal-stressed plants (Nahed et al. 2010; Mahgoub et al. 2011). The positive effect of this vitamin is due to stabilizing and protecting the photosynthetic pigments and apparatus from being oxidized (Al-Hakimi & Hamada 2011; Soltani et al. 2014). The present study showed that the BR and thiamine together are extremely useful in protecting the photosynthetic machinery and plant growth (Table 3).

Cd stress disturbs the activities of cytosolic enzymes and may cause nutritional disorders and oxidative damage, all of which drastically reduce canola yield (Ehsan et al. 2014). Production of MDA and H_2O_2 in the plants subjected to Cd stress is a major indicator of the production of toxic

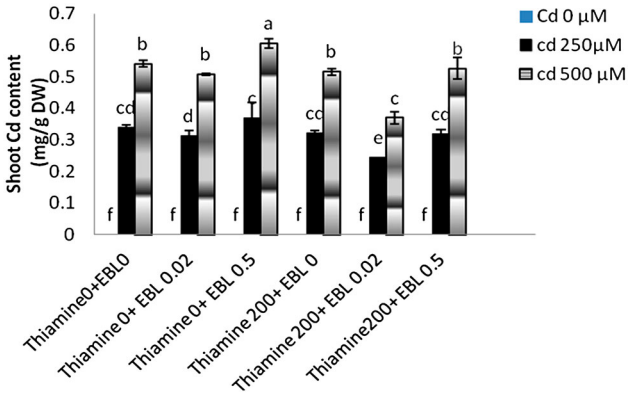

Figure 7. Effect of EBL and thiamine on Shoot Cdcontent in canola leaves under Cd stress. Data are means ± SE of three replicates. Different letters indicate the significance of difference at $P \leq 0.05$ levels by Duncan test.

oxygen species in plant (Farid et al. 2013). Increasing H_2O_2 content and lipid peroxidation with enhancing heavy metal levels in various plants is reported (Yusuf et al. 2011), which is consistent with the results of the present study (Figures 1 and 2, Figure 7). EBL can modify the membrane structure/stability under stress conditions and decreased peroxidation of membrane lipids (Surgun et al. 2016). Thiamine application was useful in alleviating the toxic effects of stress and reduced H_2O_2 and MDA contents in different plants (Kaya et al. 2015). Moreover, the exogenous application of this vitamin provides better membrane permeability. In the present study revealed that MDA and H_2O_2 content are reduced under the cooperative interaction between EBL and thiamine due to their antioxidant role in decreasing the ROS and protecting membranes against oxidative stress caused via cadmium.

Phenolic compounds act as an antioxidant and thus alleviate toxic effects of reactive oxygen radicals and able to chelate metals (Kováčik & Bačkor 2007; Mierziak et al. 2014). The PAL is a key enzyme that involved in the synthesis of phenolic compounds and can be induced by various stresses (Asghari & Zahedipour 2016; Manquián-Cerda et al. 2016). Moreover, there is a positive effect on PAL activity and total phenolics content in *Matricaria chamomilla* plant treated with Cd and Cu stress (Kováčik & Bačkor 2007). BRs regulated the secondary metabolism in tomato plant and enhanced the PAL activity and transcript levels of PAL gene under stress (Ahammed et al. 2013). It was observed that the phenolic compounds were significantly increased by application of thiamine in grapevine (Boubakri et al. 2013) and soybean plants (Abdel-Monaim 2011). The data obtained from this experiment have provided evidence that there is a high relation between increasing of PAL activity and total phenolics in the leaves of the canola seedling under Cd stress (Table 4). The results of the present study indicate that the application of exogenous BRs and thiamine obviously increased the activities of PAL and phenolic compounds under Cd stress, and these metabolites with their antioxidant properties considerably ameliorated the toxic effects of metal (Table 4).

DPPH is a stable free radical that accepts an electron or hydrogen to become a stable molecule. Thus, it is usually used as a substrate to evaluate antioxidant activity (Oueslati et al. 2010). The strong inhibition of DPPH radical may be linked to the content of phenolic compounds which are capable of donating electrons or transferring hydrogen atom to neutralize free radicals (Sharma & Ramawat 2014).

BRs could increase DPPH radical scavenging capacity under Cu stress in *Raphanus sativus* plants with increasing production of secondary metabolites related to phenylpropanoid path enzymes (Choudhary et al. 2011). Phenolic compounds were reported to be highly related with DPPH assay, suggesting that all the phenolic compounds contributed to the antioxidant potential of canola plant, which is consistent with the findings Xi et al. (2013) in grape seedling treated by EBL. The relation between increasing of DPPH and vitamin B in the many seeds were reported (Chaichana 2016). It seem, in our results, application of EBL in combination with thiamine enhanced radical scavenging capacity more considerably; there by indicating synergistic interactions of EBL and thiamine for ameliorating oxidative stress generated by Cd (Table 4).

The alteration in activities of both enzymatic (CAT, APX, GPX) and non-enzymatic (ASC) antioxidants is a general response to ROS produced by different abiotic stresses including heavy metals (Bajguz 2010). The present investigation reveals that canola plants growing under Cd stress conditions exhibited a significant increase in the activities of CAT, APX, and GPX (Figures 3–5). Also, ASC and DHA levels were respectively decreased and increased under Cd stress (Table 4). Excess metal caused a substantial reduction in ASC content in plants, probably due to the increase in oxidation of ASC or prevention of ASC synthesis under stress (Wang et al. 2009). Simultaneous application of EBL and thiamine further increases the activity of antioxidant enzymes under stress as compared to plants exposed to Cd alone (Figures 3–5). Our findings are consistent with Kanwar et al. (2013), who found the enhanced activity of antioxidant enzymes under the exogenous application of BRs in Ni stressed *B. juncea*. Similarly, Vázquez et al. (2013) reported that BRs-induced stress tolerance is associated with the enhanced expression of genes encoding antioxidant enzymes in various plants. This effect is attributed to suitable H_2O_2 accumulation in the EBL and metal-treated plants, which serves as a signal to trigger the activation of the antioxidative enzymes (Ramakrishna & Rao 2015). These data suggest that treatments with EBL and thiamine together can reduce the oxidative stress level in *B. napus* plants subjected to Cd-stress, via higher ASC and DHA levels.

Conclusion

In conclusion, exogenous EBL plus thiamine alleviated growth inhibition and improved chlorophyll content. The non –enzymatic antioxidants and free radical scavenging activity may have a significant role in canola heavy metal tolerance. Tolerance to Cd could dependent upon the efficiency of the antioxidant system, which maintained the redox homeostasis and integrity of cells. Furthermore, our findings support the hypothesis EBL + thiamine application could be responsible for the increased resistance to stress. Also In the present of 250 and 500 µM Cd, EBL 0.02 µM plus thiamine 200 µM significantly decreased H_2O_2 accumulation by 49% and 38% compared to 250 and 500 µM Cd treatment without applying the plant growth regulators respectively. generally, Application of EBL 0.02 µM plus thiamine 200 µM compared to other treatments improved the oxidative resistance of Canola plant under cadmium stress 250 µM.

Disclosure statement

This research is part of the doctoral thesis of Shima Sanjari and its research stages have been carried out in the Department of Biology of Shahid Bahonar University of Kerman, Iran.

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