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Effects of triacontanol on ascorbate-glutathione cycle in *Brassica napus* L. exposed to cadmium-induced oxidative stress

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ABSTRACT

The ability of exogenous triacontanol (TRIA), a plant growth regulator, to reduce Cd toxicity was studied in canola (*Brassica napus* L.) plants. The following biological parameters were examined in canola seedlings to investigate TRIA-induced tolerance to Cd toxicity: seedling growth, chlorophyll damage and antioxidant response. In particular, TRIA application reduced Cd-induced oxidative damage, as shown by reduction of ROS content, lipoxygenase (LOX) activity and lipid peroxidation level. TRIA pretreatment increased non-enzymatic antioxidant contents (ascorbate, AsA, glutathione and GSH), phytochelatin content (PCs) and activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), monodehydroascorbate reductase (MDHAR), dehydro ascorbate reductase (DHAR), and glutathione reductase (GR), so reducing the oxidative stress. These results clearly indicate the protective ability of TRIA to modulate the redox status through the antioxidant pathway AGC and GSH, so reducing Cd-induced oxidative stress.

1. Introduction

Cadmium toxicity leads to health impairment in living organisms, so it represents an ecologically hazardous toxic metal. As Cd enters the trophic chain through plants, it is noteworthy to know how the plants respond to Cd. Plants growing in a Cd-added growth media show biochemical and physiological disorders: chlorosis, necrosis, leaf rolling, growth inhibition, damage of membrane functions, alteration of ion homeostasis, decreased water and nutrient transportation, photosynthesis inhibition, altered metabolism, altered activities of several key enzymes, and even cell death (Ehsan et al., 2014). A common consequence of heavy metal (HM) toxicity is the excessive accumulation of reactive oxygen species (ROS) and methylglyoxal (MG), both of which can cause peroxidation of lipids, oxidation of protein, inactivation of enzymes, DNA damage and/or interact with other vital constituents of plant cells (Hossain et al., 2012). An antioxidant system is well equipped with different antioxidant components to scavenge overproduced ROS and then protects plants from oxidative injury (Hasanuzzaman et al., 2013). Furthermore, chelation, binding, exclusion, active excretion, and compartmentalization of Cd are some adaptive mechanisms by which plants avoid toxic effects of Cd (Basile et al., 2009, 2012a, 2012b, 2013, 2015; Carginale et al., 2004; Nahar et al., 2016; Zagorchev et al., 2013).

Plant hormones increase stress tolerance in plants by regulating various physiological and biochemical processes (Shahbaz et al., 2011). Their crucial roles encourage the seeking for new plant growth regulators and elucidation of their roles in regulating different plant processes (Perveen et al., 2014). One of relatively new PGRs, the Triacontanol (TRIA), is reported to stimulate plant growth even at a very low concentration, when exogenously applied to various plant species (Verma et al., 2011). TRIA has been reported to enhance photosynthesis (Eriksen et al., 1981) and water and mineral nutrients uptake (Chen et al., 2003), to regulate activities of various enzymes (Naeem et al., 2012), and to increase various organic compounds in leaf tissues (Kumaravelu et al., 2000; Chen et al., 2003). So TRIA has received much attention in recent years as a plant growth regulator. It may play an important role in resistance of some plants to abiotic stresses, such as salinity (Shahbaz et al., 2013; Perveen et al., 2014), chilling (Borowski and Blamowski, 2009) and arsenic toxicity (Asadi karam et al., 2016).

This study focuses on the response to oxidative stress in canola seedlings under Cd stress and investigates the ability of exogenous TRIA as a regulator of the glutathione-ascorbate cycle and the antioxidant metabolism, and as enhancer of tolerance to oxidative stress.

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2. Material and methods

2.1. Plant growth and treatments

Seeds of canola (*Brassica napus* L.) were sterilized using 0.1% sodium hypochlorite solution, washed with distilled water and planted in pots filled with perlite. Pots were transferred in growth chamber with day/night temperature of 25/20 °C and a 16 h light/8 h dark photoperiod, with 70% relative humidity. During the first week from the sowing, seedlings were irrigated with distilled water; then half strength Hoagland's nutrient solution (pH 5.7 ± 0.1) was used to irrigate plants every other day. The 21-day-old seedlings, at the three leaf stage, were exposed to TRIA (Sigma Aldrich) and Cd treatments. Two concentrations of TRIA in ethanol solutions, at10 and 20 µM, were applied as foliar spray at the three leaf stage for 7 days. After pretreatment, plants were irrigated with Hoagland's solution containing 1.5 mM $CdCl_2$ for 7 days. At the end of experiment, leaves of both the treated and the untreated samples (control) were collected, immediately frozen in liquid nitrogen and then stored at −80 °C for the analyses.

2.2. Plant growth evaluation

At the end of the experiment, the weights of 10 randomly selected fresh seedlings from each treatment were measured and expressed as fresh weight (FW) grams.

2.3. Physiological and biochemical parameter evaluation

The level of lipid peroxidation in plant tissues was evaluated by measuring the malondialdehyde (MDA) content by using thiobarbituric acid (Heath and Packer, 1968).

The H_2O_2 content in the plants was measured by reaction with potassium iodide (KI), according to Velikova et al. (2000). The amount of H_2O_2 was extrapolated from the standard curve.

A fluorescence technique using 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) has been used for quantitative measurement of ROS production. DCFH-DA is de-esterified intracellularly and turns to non-fluorescent 2′, 7′-dichlorofluorescin (DCFH). DCFH is then oxidized by ROS to the highly fluorescent 2′, 7′-dichlorofluorescein (DCF) (LeBel et al., 1990). Briefly, after collection leaf samples were immediately frozen in liquid nitrogen and thoroughly ground with a pestle in a prechilled mortar. The resulting powder (150 mg) was then suspended in TrisHCl 40 mM at pH 7.4, sonicated and centrifuged at 12,000g for 30 min. The supernatant (500 µL) was collected and protein content determined. An aliquot (10 µL) of each sample was incubated with 5 µM DCFH-DA for 30 min at 37 °C, followed by recording of the final fluorescence value, which was detected at 488 nm excitation and 525 nm emission. DCF formation was quantified from a standard curve (0.05–1.0 µM).

The ASC and dehydroascorbate (DHA) contents were measured as described by De Pinto et al. (1999). Briefly, total ASC was determined after reduction of DHA to ASC with dithiotreitol (DTT), and the content of DHA was estimated by the difference between the total ASC pool (ASC plus DHA) and ASC.

The GSH content was determined by the spectrophotometric method of Ellman (1959), where GSH was oxidized in 2.6 ml of a sodium phosphate buffer (pH 7.0) containing 0.2 ml of a sample extract and 0.2 ml of 6 mM 5,5′-dithiobis-(2 nitrobenzoic) acid (DTNB). The absorbance was monitored at 412 nm. The GSH content was calculated from a standard curve constructed using GSH over the range 0–100 µM.

Measurement of non-protein thiol content was measured according to Sedlak and Lindsay (1968). Samples were homogenized in 0.02 M EDTA in an ice bath. Aliquots of 5 ml of the homogenates were mixed with 4 ml of distilled water and 1 ml of 50% TCA. After 15 min resting, the mixtures were centrifuged for 15 min. Two ml of the supernatant was mixed with 4 ml of 0.4 M Tris buffer (pH 8.9) and 0.1 ml of DTNB; absorbance was read within 5 min at 412 nm wavelength against a reagent blank. Total phytochelatin concentrations were calculated by subtracting the amount of GSH from the total amount of NPT, according to De Vos et al. (1992).

2.4. Enzyme extraction and activity evaluation

For protein extraction and analysis, the extracts of frozen samples prepared in a 50 mM potassium phosphate buffer (pH 7) containing 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM sodium ethylene diaminetetraacetic acid (Na_2EDTA), and 1% (m/v) polyvinylpyrrolidone (PVP) were centrifuged at 15,000*g* at 4 °C for 15 min and the supernatants were used for the estimation of protein content and enzyme activities. The total protein content was measured according to the method of Bradford (1976) using bovine serum albumin as standard. All spectrophotometric analyses were conducted in a final volume of 3 ml by using a *Cary 50* UV/visible spectrophotometer.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT) (Giannopolitis and Ries, 1977). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction at 560 nm. Catalase activity (CAT, EC 1.11.1.6) was assayed by monitoring the decrease in the absorbance of H_2O_2 within 30 s at 240 nm. Unit of activity was taken as the amount of enzyme that decomposes 1 µmol of H_2O_2 in 1 min (Dhindsa et al., 1981). The decrease in hydrogen peroxide was inferred from the decline in absorbance at 240 nm.

Ascorbate peroxidase (EC 1.11.1.11) was assayed by monitoring the decrease in absorbance at 290 nm due to ASC oxidation (Nakano and Asada, 1981). One unit of APX activity was defined as the amount of enzyme that decomposed 1 mmol of ascorbate per minute. Guaiacol peroxidase (GPX; EC 1.11.1.7) activity was determined by a method derived from Plewa et al. (1991). One unit of GPX activity was defined as the amount of enzyme that produced 1 mmol of tetraguaiacol per minute. For the measurement of the LOX (EC 1.13.11.12) activity, we used the Minguez-Mosquera et al. (1993) method. The enzyme unit was defined as 1 µmol of the product formed per min. Glutathione reductase (GR; EC1.6.4.2) activity was determined following the decrease in absorbance at 340 nm associated with the oxidation of NADPH (Foyer and Halliwell, 1976). One unit of GR was defined as the amount of enzyme that oxidized 1 µmol of NADPH per minute.

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was assayed at 340 nm with microplate assay kit (Mybiosource) according to the manufacturer's instructions. One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 µmol NADH per minute. A molar coefficient of 6.2 mM^{-1} cm^{-1} was used for the calculation of enzyme activity. Dehydroascorbate reductase (DHAR, EC 2.5.1.18) activity was measured at 265 nm with microplate assay kit (Mybiosource) according to the manufacturer's instructions. One unit of DHAR activity was defined as the amount of enzyme that produces 1 µmol of AsA per minute.

2.5. Element analysis by ICP-OES

After treatments, shoot samples were oven dried at 70 °C for 72 h; then 0.5 g of dry leaves 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).

Table 1
Effects of cadmium and triacontanol on the growth and the biochemical parameters in canola leaves. Note: values are expressed as mean ± SE (n = 3). The different letters indicate significant differences among treatments at $p \leq 0.05$ according to Duncan's multiple range tests.

Treatments		Cd contents (mg g^{-1} dw)	Shoot fresh weight (g $seedling^{-1}$)	Total Chl (a + b) (mg g^{-1} FW)	MDA content (μmol g^{-1} FW)	H_2O_2 content (μmol g^{-1} FW)	ROS content (Fluorescence intensity)	LOX activity (U/mg protein)
Cd (mM)	TRIA (μM)							
0	0	0.0002 ± 0.00001d	4.39 ± 0.22a	3.73 ± 0.03a	0.61 ± 0.08d	4.6 ± 0.22c	2231 ± 345.12c	15.11 ± 2.08c
1.5	0	0.119 ± 0.027a	3.18 ± 0.14d	2.14 ± 0.06e	0.97 ± 0.15a	6.85 ± 0.30a	6436 ± 594.07 a	25.64 ± 3.77a
0	10	0.001 ± 0.0004d	4.29 ± 0.92a	3.65 ± 0.05a	0.63 ± 0.07d	5.03 ± 0.24c	1884 ± 215.20d	16.35 ± 1.91c
1.5	10	0.085 ± 0.012 bc	3.45 ± 0.38c	2.64 ± 0.11c	0.86 ± 0.04b	5.35 ± 0.31b	3796 ± 443.89b	23.70 ± 2.68b
0	20	0.0004 ± 0.00002d	3.57 ± 0.13b	2.81 ± 0.03b	0.75 ± 0.09c	4.29 ± 0.26d	2728 ± 362.71c	18.49 ± 1.46c
1.5	20	0.090 ± 0.003ab	3.53 ± 0.32c	2.38 ± 0.02d	0.92 ± 0.14a	5.15 ± 0.19c	3433 ± 221.32b	24.85 ± 3.47ab

2.6. Statistical data analysis

Data were analyzed by using two-way analysis of variance (ANOVA). Differences between means were considered significant at a confidence level of $P \leq 0.05$. All statistical analyses were done using the software SPSS package, version 18.0. The Duncan test analysis was done to determine the significant difference between treatments.

3. Results

3.1. Cd accumulation, plant growth and chlorophyll content

Canola plants exposed to $CdCl_2$ accumulated substantial amounts of Cd in leaves (Table 1). There was a positive correlation between Cd contents in the culturing solutions and plant leaves. After a 7 day-exposure to 1.5 mM $CdCl_2$, the young leaves contained 0.119 mg g^{-1}dw. Applications of 10 and 20 μM TRIA decreased Cd contents by 28% and 24%, respectively, compared to Cd treatment only. Application of 1.5 mM $CdCl_2$ decreased shoot fresh weight of seedlings by 27%, compared to control. The exogenous TRIA mitigated the negative effects of Cd and increased the growth (Table 1). The reduction of total chl (a + b) in Cd concentration indicates destructive nature of Cd. Exogenous TRIA pretreatment increased chl content under Cd stress. Total chl increased after TRIA addition by 23% and 11% at 1.5 mM $CdCl_2$ (Table 1).

3.2. Oxidative stress evaluation

Oxidative stress of Cd-treated canola seedlings was shown by increased ROS content, H_2O_2 generation and lipid peroxidation. Cd stress increased lipid peroxidation or MDA level, H_2O_2 content and ROS amount by 59%, 48% and 188%, respectively, compared to control seedlings. The activity of LOX increased under Cd stress, which partly contributed to the oxidative stress. TRIA pretreatment decreased ROS and H_2O_2 contents, LOX activity, and subsequently the lipid peroxidation or MDA level, in comparison to Cd-treated samples with no TRIA pretreatment (Table 1).

3.3. Ascorbate and glutathione pool, phytochelatin content

Under Cd stress, in comparison to control samples, ascorbate content decreased by 38% and DHA increased by 25%, which resulted into a decrease of the AsA/DHA ratio (Fig. 1A–D). Cadmium also induced increase of the endogenous GSH level, in comparison to control. TRIA pretreatment on the Cd-treated samples increased AsA content, GSH level and ASA/DHA ratio, in comparison to TRIA-untreated, Cd-exposed samples (Fig. 1A–D). In the leaves,treatment with 1.5 mM Cd caused an increase in PC content (Fig. 1E). PC content increased from 15 μmol g^{-1}(protein) in the leaves of control plants to 22 μmol g^{-1}(protein) in 1.5 mM Cd-treated plants. The PC synthesis was associated with GSH increase. During the experiment, the GSH content increased and high PC content was detected in leaves of TRIA-untreated plants (Fig. 1C-E). Addition of 10 and 20 μM TRIA led to an immediate and significant increase in the GSH content. At the same time, there was a marked increase in PC contents.

3.4. Antioxidant enzyme evaluation

Superoxide dismutase activity increased under Cd stress and in TRIA-added Cd treatments. Under Cd stress, CAT activity decreased by 37%, compared to control; TRIA pretreatment restored CAT activity (Table 2).

The activity of GPX increased by 129%, under Cd stress, compared to control. When seedlings were supplied with exogenous TRIA 10 and 20 μM and Cd, GPX activity increased by 112% and 113%, respectively, compared to control (Table 2).

The activity of APX decreased under Cd stress, compared to control. Exogenous TRIA pretreatment increased its activity under Cd stress (Table 2).

Activity of MDHAR and DHAR decreased under Cd stress, compared to control seedlings. MDHAR and DHAR activity increased by 39.11% and 273%, respectively, when the seedlings were pretreated with 10 μM TRIA before Cd treatments.

Activity of GR increased under severe Cd stress, compared to control. Under Cd stress, exogenous TRIA pretreatment increased GR activity, compared to Cd-only treatment (Table 2).

4. Discussion

Cadmium toxicity is responsible for reduction of nutrient uptake, inhibition of cell division and elongation, damaging photosynthetic pigments and net photosynthesis, which ultimately results in strong inhibition of plant growth (Dias et al., 2013; Ehsan et al., 2014). In our study on canola seedlings, shoot fresh weight decreased in Cd-treated samples; TRIA pretreatment reduced Cd-induced growth inhibition. The decrease in plant growth was proportional to the uptake of Cd. Our data showed that Cd supply, significantly increasing Cd accumulation in canola plants, is related to canola growth reduction. That is in accordance with other studies on *Lepidium sativum, Brassica juncea*, and *Lycopersicon esculentum* (Gill et al., 2011; Gratão et al., 2012). Reduction in cell growth could be due to Cd-mediated cell damage, inhibition of mitosis, decreased cell wall synthesis and lignin deposition in the cell wall (Kumari et al., 2015). On the other hand, TRIA, decreasing Cd accumulation in canola plants, reversed Cd-induced growth inhibition. The ability of TRIA to modulate plant physiology and biochemistry so as to improve growth under metal stress was reported in an our previous work (Asadi karam et al., 2016). The growth promoting ability of TRIA in crop plants has been especially focused on its synergistic interaction with phytohormones and induction of 9-b-L (+) adenosine, which has a structure similar to cytokinin (Naeem et al., 2012). The induction of 9-b-L (+) adenosine by TRIA is thought to be one of the reasons for the increase in dry matter and other growth parameters, as

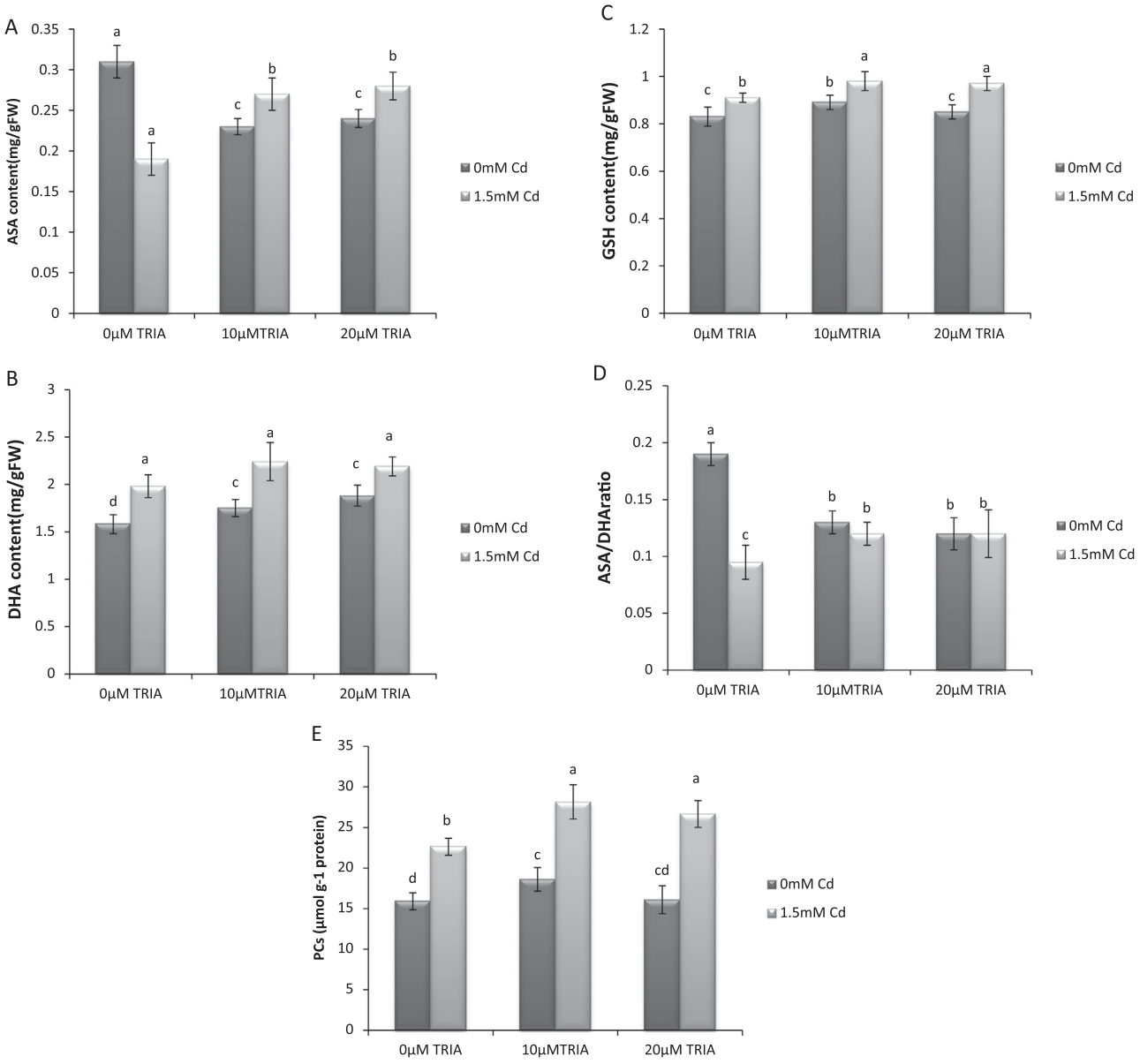

Fig. 1. Effects of cadmium and triacontanol on (A) Ascorbate, (B) Dehydroascorbate, (C) Reduced glutathione content, (D) ASA/DHA ratio and (E) Phytochelatin content in *Brassica napus* leaves. Values are means ± SE (n = 3). In the individual column, bars with different letters are statistically different ($P < 0.05$) according to Duncan's multiple range tests.

it turns on a rapid cascade of metabolic events throughout the plant within 1 min (Naeem et al., 2012).

Chlorophyll content in canola leaf declined with increasing Cd as also reported in Dias et al. (2013). Cadmium-induced reduction in chlorophyll content and chlorosis might be due to the reduction of Fe in leaves and to the negative effects of Cd on chlorophyll metabolism (Chaffei et al., 2004). Cadmium-induced degradation of chlorophyll, due to the high activity of chlorophyll-degrading enzyme and/or the

Table 2
Effects of cadmium and triacontanol application on Catalase, Superoxide dismutase, Ascorbate peroxidase, Guaiacol peroxidase, Glutathione reductase, Monodehydroascorbate reductase and Dehydroascorbate reductase activities in *Brassica napus* leaves.

Treatments		CAT activity (U/mg protein)	SOD activity (U/mg protein)	APX activity (U/mg protein)	GPX activity (U/mg protein)	GR activity (U/mg protein)	MDHAR activity (U/mg protein)	DHAR activity (U/mg protein)
Cd (mM)	TRIA (µM)							
0	0	31.31 ± 1.62cd	19.96 ± 0.14e	1.53 ± 0.13a	6.38 ± 0.89d	0.8 ± 0.08c	6.05 ± 0.21d	24.93 ± 3.10d
1.5	0	19.48 ± 0.84f	33.03 ± 0.13b	0.94 ± 0.11c	14.67 ± 1.65a	1.05 ± 0.15b	11.48 ± 0.68c	15.18 ± 2.26ef
0	10	35.24 ± 2.02c	21.95 ± 0.24d	1.07 ± 0.15b	9.33 ± 0.47d	1.12 ± 0.10b	13.06 ± 1.2b	19.61 ± 1.02e
1.5	10	42.68 ± 2.81b	35.62 ± 0.1ab	1.54 ± 0.11a	13.56 ± 1.36b	1.8 ± 0.35a	15.97 ± 1.70ab	56.74 ± 4.37a
0	20	29.98 ± 1.43e	24.79 ± 0.12c	0.91 ± 0.09c	10.45 ± 0.78c	0.93 ± 0.20c	17.39 ± 2.60a	42.07 ± 2.65b
1.5	20	49.60 ± 2.07a	38.58 ± 0.25a	1.45 ± 0.22ab	13.62 ± 2.6b	1.91 ± 0.18ab	12.65 ± 1.35c	36.16 ± 3.47c

Note: Values are means ± SE (n = 3). The different letters indicate significant differences among treatments at p ≤0.05 according to Duncan's multiple range tests.

inhibition of its biosynthesis, was proposed to reduce both photosynthesis and growth in other studies (Dias et al., 2013). Oxidative damage of photosynthetic pigments is also a common deleterious effect of cadmium (Nahar et al., 2016). Similar reasons are supposed to be involved in decrease of the chlorophyll content in canola leaf exposed to Cd stress. However, chlorophyll content increased after TRIA pretreatment, compared to Cd stress only. TRIA is reported to affect various plant characteristics, increasing CO_2 assimilation rate (Haugstad et al., 1983), size and number of chloroplasts (Chen et al., 2002), and chloroplast membrane viscosity (Ivanov and Angelov, 1997). Among these, regulation of photosynthesis is a complex process, which is known to be modulated by TRIA; that can be explained by different mechanisms such as increased Hill reaction activity (Verma et al., 2011), specific activity of Rubisco, activity of photosystem (PS) I and II complexes (Moorthy and Kathiresan, 1993), up-regulation of genes (*rbc*S isogene profile) related to photosynthesis and suppression of stress-related genes in rice (Chen et al., 2002). In our previous experiment, the reduction in biomass and induction of lipid peroxidation under toxicity condition were partially alleviated by applying TRIA (Asadi karam et al., 2016). Our data suggested that TRIA might have played a key role in protecting the structure and function of cell membranes and pigments under Cd toxicity.

Impairing the antioxidant system (Nahar et al., 2016), the electron transport chain, and the metabolism of essential elements (Dong et al., 2006), Cd causes oxidative stress. Cadmium slows down or holds or blocks the photoactivation of PSII by inhibiting electron transfer, leading to the generation of ROS (Sigfridsson et al., 2004). That metal was also suggested to stimulate ROS production in the mitochondrion electron transfer chain (Heyno et al., 2008). This experiment showed that cadmium increased ROS, lipid peroxidation and LOX activity, the last being reduced by exogenous TRIA application. Our finding that exogenous TRIA reduced ROS and lipid peroxidation is in agreement with our previous work (Asadi karam et al., 2016). Being AsA the most abundant antioxidant, it directly quenches many ROSs (Gill and Tuteja, 2010a, 2010b); so, its reduction after Cd treatments is supposed to be responsible for ROS generation and oxidative stress. Decreased AsA and increased DHA levels observed in Cd-treated canola seedlings. Reduced AsA content is correlated to APX activity and reduction of MDHAR and DHAR activities which recycle AsA, but exogenous addition of TRIA with Cd decreased DHA and increased AsA level by increasing MDHAR and DHAR activities, which increased the ratio of AsA/DHA (compared to Cd stress alone). GSH has a role in ROS detoxification, conjugation of metabolites, and detoxification of xenobiotics, and signaling action, which triggers adaptive responses under stress condition (Foyer and Noctor, 2005). Plant heavy metal stress response is often associated with increased GSH level (Gill and Tuteja, 2010a, 2010b; Kanwar et al., 2015). In our experiment the contents of GSH increased under Cd stress, compared to control. During ROS scavenging GSH is oxidized to GSSG and then GR recycles GSH. In our Cd-treated seedlings, exogenous TRIA increased both the activity of GR and GSH level, in comparison to Cd-only treated seedlings. In our canola samples TRIA pretreatment increased GSH level and decreased H_2O_2 content. TRIA might have a role in GSH biosynthesis or regeneration, which increased the GSH level. In the present study exogenous TRIA also enhanced other components of AsA-GSH cycle. TRIA might have signaling roles in enhancing biosynthesis of APX, MDHAR, DHAR, and GR. Otherwise, TRIA might have other roles leading to the increase of their activities. There are no data on the effect of TRIA on the activity of these enzymes, so the mechanism leading to the increase of these enzymes is not yet clear. This work is an important progress to better understand the matter.

Moreover, Glutathione, as a sulfur-containing tripeptide thiol, is involved in the plant protection against heavy metals both as a precursor in the synthesis of phytochelatins (PCs) and as ROS scavenger by the ascorbate–glutathione cycle (Xiong et al., 2010). In fact PCs bind and transport metals to vacuole and are the most effective Cd chelators (Zagorchev et al., 2013). In this study, increasing PC content in canola may be caused by increased GSH content in response to Cd toxicity. Recently, Gupta et al. (2009) demonstrated that PC synthesis is stimulated by heavy metal supply in *Brassica juncea* due to the overexpression of PC gene. The PC–metal complex is often sequestered in the vacuoles (Sharma et al., 2010). Increase of GSH content and PC content in Cd-affected canola plant (compared to control) is corroborating the findings of previous studies (Najmanova et al., 2012; Nahar et al., 2016). After TRIA application along with Cd stress, the GSH and PC contents increased; that probably indicates upregulation of Cd chelation and sequestration capacity by TRIA.

SOD provides the first line of defense against ROS (Gill et al., 2015). In Cd-treated samples the SOD reduced activity was not enough to counteract the occurring oxidative load. When exogenous TRIA was applied, the activity of SOD increased significantly, which reduced H_2O_2 generation. Exogenous TRIA application was reported to increase SOD activity in salinity-affected *Triticum aestivum* (Perveen et al., 2014). These findings support the results of the present study. The activity of CAT is involved in converting H_2O_2 to H_2O and O_2. Cadmium, replacing Fe from the active center of CAT, inhibits the enzyme functioning (Nazar et al., 2012). In Cd-treated samples, the Cd-induced reduction of the CAT activity was probably one of the reasons accounting for the increase of H_2O_2. As our results showed, exogenous TRIA significantly increased CAT activity, in comparison to Cd treatment alone. Furthermore, TRIA application increased CAT activity also in salinity-affected *Triticum aestivum* (Perveen et al., 2014). That finding supports the results of the present study. Glutathione dependent conjugation of lipid hydroperoxides and endobiotic substrates by GPX and GST contributes in defending plant from metal toxicity effects. Scavenging of peroxides and other electrophiles, GPX and GST protect cell components from oxidative damage (Gill and Tuteja, 2010a, 2010b). In our experiment, under Cd stress, the activity of GPX increased in comparison to control. GPX activity was also significantly improved by TRIA in Cd-stressed *Zea mays,* which supports the roles of TRIA at improving GPX activity (Ahmed et al., 2012).

5. Conclusion

In conclusion, the tolerance of canola to the Cd-induced oxidative stress seems to be dependent upon the efficiency of the antioxidant system, which maintains the redox homeostasis and integrity of cellular components. Then, the ASC-GSH cycle enzymes might play major roles in preserving the Cd-stressed canola plants. On the whole, our findings support the hypothesis that the higher efficiency of the antioxidants after TRIA application could be responsible for the increased tolerance to Cd. Exogenous application of TRIA improved the non-enzymatic antioxidant level, phytochelatin content and increased the activities of antioxidant enzymes and reduced oxidative damage. Exogenous TRIA alleviated growth inhibition and improved chlorophyll content. Finally, the ASC-GSH cycle enzymes under the Cd stress and TRIA applications may have a significant role in canola heavy metal tolerance.

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