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Review

Interaction of triacontanol and arsenic on the ascorbate-glutathione cycle and their effects on the ultrastructure in *Coriandrum sativum* L.

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ABSTRACT

Exogenous application of triacontanol (TRIA) has the ability to mitigate the adverse effects of abiotic stresses by modulating a number of physio-biochemical processes in different plants. However, information about how its effects may be mediated under heavy metal stress is scanty. In this study, we evaluated how TRIA exerted its role against the toxicity of sodium arsenate in coriander (*Coriandrum sativum* L.). The activities of enzymes, including ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione-S-transferase (GST), were measured. In addition, the contents of ascorbate (ASC), dehydroascorbate (DHA), reduced glutathione (GSH) and some elements including both As and the nutrients Ca, Mg, Zn, K, P were determined. Results suggested that As decreased GSH, ASA and DHA contents, a clear indication of oxidative stress, but their amounts were raised by TRIA treatment. Also, As stress decreased plant Ca, Zn, K, Mg and P contents, while TRIA improved their uptake and inhibited As accumulation. As 200 μM treatment inhibited the activities of APX, MDHAR, DHAR, and GR, enzymes of the ascorbate-glutathione cycle (AGC). TRIA supplementation restored and even enhanced the activity of all the AGC enzymes. 10 μM TRIA treatment increased GST gene expression and activity to a greater extent than under only As treatment. TRIA-alone treatments did not change the mentioned parameters.

Transmission electron microscopy (TEM) observations showed that TRIA was able to protect cells, and most of all chloroplasts, from As-induced damage.

These results clearly indicate the protective role of TRIA in modulating the redox status of the plant system through the antioxidant AGC and GSH enzymes, which could counteract arsenic-induced oxidative stress.

1. Introduction

Heavy metals are important environmental pollutants and many of them are toxic even at very low concentrations. Arsenic (As), one of the most toxic metalloids, is widely distributed in the environment and is non-essential for plants (Farooq et al., 2016). The presence of As in irrigation water or soil could hamper normal growth of plants inducing biomass reduction (Finnegan and Chen, 2012). Arsenic is known to induce production of reactive oxygen species (ROS), which are counteracted by plant antioxidant enzymes and compounds (Farooq et al., 2016). ROS, generated in the cell wall as well as inside the cell, affect membrane permeability, enzyme activity, metabolic pool, plant biomass, inducing leaf chlorosis and necrosis (Upadhyaya et al., 2014). To scavenge ROS, plants involve strong antioxidant defense system, comprising antioxidant molecules, such as glutathione, vitamin C, polyphenols, flavonoids, and antioxidant enzymes, such as superoxide dismutase, catalase, guaiacol peroxidases, glutathione reductase, ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase (Asadi karam et al., 2017). The non-enzymatic antioxidants include ascorbate (ASC) and GSH, two main constituents of the ASC-GSH cycle, also involved in detoxification of H_2O_2 in chloroplasts and cytosol (Sinha and Saxena, 2006). Scavenging H_2O_2 by ascorbate peroxidase (APX) is the first step of the ASC-GSH cycle, which maintains the ASC pool in its reduced form (Foyer and Halliwell, 1976). Plant dehydroascorbate reductase (DHAR) is an important reducing enzyme, involved in the ascorbate-glutathione recycling reaction. DHA must be converted to AsA by DHAR in presence of glutathione (GSH) as a reducing agent. Thus, DHAR is a key factor in maintaining a reduced

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AsA level in the adaptation to environmental conditions. In plants, the monodehydroascorbate reductase (MDHAR) is an enzymatic component of the glutathione-ascorbate cycle, which is one of the major antioxidant systems of plant cells for protection against ROS-induced damages (Aravind and Prasad, 2005; Pandey et al., 2009). Glutathione reductase (GR) is a key enzyme for maintaining the GSH pool (Rennenberg, 1982). Glutathione S-transferases (GST), induced by both toxic metals and oxidative stress, are ubiquitous enzymes, which perform functional roles using the tripeptide glutathione (GSH) as a co-substrate or coenzyme (Ghelfi et al., 2011). The GSH-dependent catalytic functions include the conjugation and resulting detoxification of cytotoxic products. It has been observed that As induces the GST activity in mesquite and maize plant (Mokgalaka-Matlala et al., 2009; Mylona et al., 1998).

Plant hormones increase stress tolerance in plants by regulating various physiological and biochemical processes (Shahbaz et al., 2011). Research on their crucial roles led to the discovery of new plant growth regulators (PGRs) and the elucidation of their roles in modulating plant processes (Perveen et al., 2012). One of relatively new PGRs, Triacontanol (TRIA), is reported to stimulate plant growth at a very low concentration when exogenously applied to various plant species like groundnut (Verma et al., 2009), rice, maize, and wheat (Perveen et al., 2011). TRIA has been reported to enhance photosynthesis (Eriksen et al., 1981) and water and mineral nutrient uptake (Chen et al., 2003), to regulate activities of various enzymes (Naeem et al., 2011), and to increase the amounts of organic compounds in leaf tissues (Kumaravelu et al., 2000; Chen et al., 2003). TRIA has received much more 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 in coriander (Asadi karam and Keramat, 2017) and *Triticum aestivum* (Perveen et al., 2014), and cadmium toxicity in *Erythrina variegata* (Muthuchelian et al., 2001). Our previous report has shown that TRIA mitigates As-induced oxidative stress in coriander plants (Asadi karam et al., 2016).

The aim of this paper is to confirm the hypothesis that TRIA is able to mitigate As-induced oxidative stress by stimulating the ASC-GSH cycle and the activity of GST enzyme. Furthermore, we aimed at evaluating the protective ability of TRIA against As-induced alterations of the cell ultrastructure.

2. Material and methods

2.1. Plant growth and treatments

Seeds of coriander (*Coriandrum sativum* L.) were sterilized using 0.1% sodium hypochlorite solution, washed by distilled water and planted in pots filled with perlite. Fresh Hoagland's nutrient solution (pH 5.7 ± 0.1) was prepared for irrigation (Hoagland and Arnon, 1950). The plants were kept in a greenhouse with 16 h light/8 h dark photoperiod, 25 °C day −20 °C night, and a relative atmospheric humidity of 70%. Triacontanol (TRIA, Sigma–Aldrich) was dissolved in ethanol. At the three-leaved stage, seedlings were sprayed with TRIA at concentrations of 0, 5, 10, and 20 μmol L^{-1} once a day for 72 h. Our preliminary experiment showed that these three concentrations created the most measurable effects on As-stressed coriander seedlings. After TRIA treatments, seedlings were irrigated by Hoagland's solution containing sodium arsenate salt (Na_2HASO_4) at 0, 100 and 200 μM. After a 6 day-treatment with As, shoots were uprooted, fresh weight (FW) was recorded and the plant material was immediately frozen in liquid nitrogen and stored at −80 °C for the next analyses. All treatments were replicated three times.

2.2. Determination of biochemical parameters

The ascorbate (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); DHA content 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.

2.3. Element analysis by ICP-OES

Samples of shoot were oven dried at 70 °C for 72 h. After determination of the dry biomass, 0.5 g samples were dissolved in 10 ml of 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 As, Ca, P, K, Mg and Zn were determined by inductively coupled plasma atomic emission spectroscopy (ICP, OES, Varian CO). The stability of the device was evaluated after determination of every ten samples 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 also used standard solutions with As, Ca, P, K, Mg and Zn known concentrations within the range of plant analyzed solutions (As standard solution, MERCK) (Sagner et al., 1998).

2.4. Enzyme extraction and activity determination

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. 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 the spectrophotometric analyses were conducted in a final volume of 3 ml by using a *Cary 50* UV/visible spectrophotometer.

Ascorbate peroxidase (APX; EC 1.11.1.11) was assayed by monitoring the decrease in the absorbance at 290 nm due to ASC oxidation (Nakano and Asada, 1981). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 0.15 mM H_2O_2, 0.5 mM ASC, and 0.15 ml of the enzyme extract. One unit of APX activity was defined as the amount of enzyme that decomposed 1 mmole of ascorbate per minute.

The glutathione reductase (GR; EC1.6.4.2) activity was determined following the decrease in the absorbance at 340 nm associated with the oxidation of NADPH (Foyer and Halliwell, 1976). The assay contained 50 mM Tris–HCl (pH 7.8), 150 μM NADPH, 500 μM oxidized glutathione (GSSG) and 0.05 ml of the enzyme extract. One unit of GR was defined as the amount of enzyme that oxidized 1 μmole 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 μmole of 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 μmole of AsA per minute.

Glutathione S-transferase (GST, EC 2.5.1.18) activity was measured using a commercial kit (CS0410, Sigma). The conjugation of GSH to 1-

chloro-2,4-dinitrobenzene (CDNB) catalyzed by GST was monitored at 340 nm for 4 min. The reaction mixture contained 4 µl of extract and 196 µl of reaction solution (200 mM GSH and 100 mM CDNB in Dulbecco's buffer at pH 7). The activity was calculated with $\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$ (Habig and Jakoby, 1981). A GST unit is defined as the amount of enzyme that catalyses the formation of 1 µmole of the GS-DNB conjugate per minute at 25 °C and pH 7.

2.5. Transmission electron microscopy

For conventional Transmission Electron Microscopy (TEM), sections from leaflets of *Brassica napus* samples were fixed with 3% glutaraldehyde in phosphate buffer (65 mM, pH 7.2–7.4) at room temperature for 2 h, post-fixed with 1% osmium tetroxide in the aforementioned phosphate buffer for 1.5 h at room temperature, dehydrated with ethanol up to propylene oxide and finally embedded in epoxy Spurr resin. After sectioning, the 40 nm thick slices were mounted on copper grids, stained with UAR stain (Electron Microscopy Sciences) and Reynold's lead citrate and observed under an EM 208S FEI TEM, using an accelerating voltage of 80 kV.

2.6. Gene expression

One hundred mg of leaf tissue was ground thoroughly in liquid nitrogen using a pre-chilled mortar and pestle. Total RNA was extracted with Trizol reagent, according to the manufacturer's instructions (Invitrogen). The concentration of the RNA was read using a NanoDrop ND-1000 spectrophotometer, working at 260 nm. The quality of the RNA was checked by both 1% agarose gel and NanoDrop at the 260/280 ratio. The expression of genes was analyzed using reverse transcription polymerase chain reaction (RT-PCR).

First strand cDNA synthesis was performed from preheated and snap cold treated 5 µl of total RNA, using an oligo (dT) primer in a 20 µl reaction containing: 10x reverse transcription reaction buffer, HyperScript ™ reverse transcriptase, ZymAll™ RNase Inhibitor and dNTPs. The reaction was carried out at 55 °C for 60 min, followed by a 5 min step at 85 °C and then by cooling to 4 °C. We performed the PCR reactions using glyceraldehyde phosphate dehydrogenase gene (GAPDH) as internal reference. The following specific primers were used and checked for dimer formation:

F-TGAPDH: CTCGCGCTATGAATGTCGCC

R-TGAPDH: TTCGCTCAGTCTGAGCAGAC

F-GST: AGCTCGTCGCCTTCAAGTTC

R-GST: ACATCCTTAAGCTCGGCAAG

Three different and independent cDNA sets were used. To determine the expression of GST, 2 µl of the cDNA was used in the real-time PCR assay, using 1 µl of each forward and reverse primer in 20 µl as a final volume. PCR reaction was performed in duplicate for 35 cycles under the following conditions: denaturation at 95 °C, for 3 min; annealing at 60 °C, for 1 min; extension at 72 °C, for 10 min. Each experiment was repeated at least three times in order to ensure reproducibility. A no reverse transcriptase control (No-RT) was performed for all samples to monitor DNA contamination. PCR products were detected on 1% agarose gels by ethidium bromide staining. Interpretation of differential expression was performed in an optical density of electrophoresis PCR. In particular, the RT-PCR was screened by using the software ImageJ: the intensity of each band was escorted as an area interposed under Gaussian curve.

2.7. Statistical data analysis

Data were analyzed using one-way analysis of variance (ANOVA). Differences between means were considered as 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 different treatments.

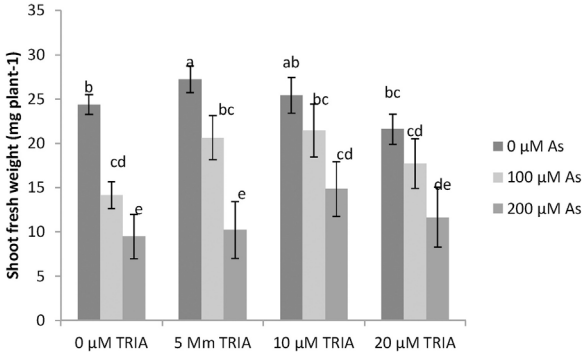

Fig. 1. Effects of As and TRIA on shoot fresh weight of *Coriandrum sativum*. Values are means ± SE ($n = 3$). Bars with different letters are statistically different ($P < 0.05$) according to Duncan's multiple range tests.

3. Results

3.1. Plant growth

Shoot fresh weights from As-treated plants decreased significantly with increasing As concentration. As at 200 µM had the maximum effect on shoot fresh weight, decreasing it by 157.7% in comparison to the As-untreated control seedlings. TRIA pretreatment alleviated reductions in shoot fresh weight under As-treatment (Fig. 1).

3.2. Ions accumulation

The results from ICP, OES analysis showed that As ions accumulated significantly in the shoots treated with As only and with no TRIA, in comparison to As-untreated plants (Table 1), the maximum effect being at 200 µM As, increasing bioaccumulation by 339.9%. Application of TRIA decreased shoot As content at both 100 and 200 µM. As-treatment at 200 µM without TRIA induced the greatest decrease in the Ca and P contents in the shoots. Generally, in this condition application of TRIA increased Ca and P contents. The lowest arsenic concentration 100 µM gave not significant difference with the untreated plants as for Ca and P contents.

Table 1 show the effects of As-treatments on the concentration of some nutrients, such as Mg, K and Zn, in the shoots. In general, these data demonstrated that foliar application of TRIA considerably improved nutrient uptake and accumulation in As-stressed plants, while As treatments, at 100 and 200 µM without TRIA, decreased accumulation of these elements in comparison to the As-untreated samples.

3.3. Ascorbate, dehydroascorbate and glutathione content

The ASC, DHA and GSH contents of *C. sativum* leaves exposed to As, with or without TRIA pretreatments, are showed in Fig. 2. As caused a significant decrease in ASC and GSH contents in absence of TRIA pretreatment. Differently, these effects were not observed in plants pretreated with TRIA, where the ASC content even increased. The maximum ASC content was observed under 100 µM As + 10 µM TRIA (Fig. 2A).

In the only As-treated samples, the DHA content increased after 100 µM As and decreased after 200 µM As. Decrease at 200 µM As was preserved by the pretreatments with 10 and 20 µM TRIA. The highest DHA values were observed in 10 µM TRIA + As-treated samples (Fig. 2B).

The results indicate that the application of TRIA improved the ASC pool under As stress.

Table 1
Effects of As and TRIA application on As, Ca, P, K, Mg, Zn (mg/gr DW) contents in coriander leaves. Values with different letters are statistically different.

Treatments		As	Ca	P	K	Mg	Zn
As	TRIA						
0	0	0.0001 ± 0.0 fg	26.84 ± 1.54 d	4.79 ± 0.10 ab	42.57 ± 3.05 a	15.02 ± 1.11 a	0.13 ± 0.004 a
0	5	0.0005 ± 0.0 fg	28.53 ± 2.72 c	5.67 ± 0.51 a	42.42 ± 2.53 a	11.48 ± 1.08 ab	0.098 ± 0.001 b
0	10	0.001 ± 0.0 fg	28.5 ± 2.36 c	4.43 ± 0.26 b	40.46 ± 2.75 ab	11.42 ± 2.25 ab	0.096 ± 0.003 b
0	20	0.001 ± 0.0 fg	28.24 ± 1.90 c	4.61 ± 0.16 ab	40.47 ± 3.44 ab	12.58 ± 2.29 ab	0.88 ± 0.005 bc
100	0	0.17 ± 0.02 c	27.02 ± 2.82 cd	3.96 ± 0.29 b	15.43 ± 1.05 e	5.21 ± 0.74 d	0.024 ± 0.001 e
200	0	0.34 ± 0.012 a	6.91 ± 0.64 g	0.44 ± 0.04 e	6.63 ± 0.84 g	0.42 ± 0.08 g	0.001 ± 0.0001 i
100	5	0.077 ± 0.004 e	40.31 ± 3.02 b	4.58 ± 0.25 ab	22.73 ± 2.06 d	7.58 ± 0.34 c	0.042 ± 0.002 e
200	5	0.26 ± 0.02 b	15.77 ± 1.84 f	1.46 ± 0.13 d	13.99 ± 0.74 ef	1.15 ± 0.73 f	0.007 ± 0.0001 hi
100	10	0.076 ± 0.019 e	44.68 ± 3.69 a	3.96 ± 0.64 b	25.18 ± 1.08 c	9.48 ± 1.06 bc	0.061 ± 0.001 d
200	10	0.21 ± 0.010 c	19.08 ± 1.22 e	1.82 ± 0.12 d	21.26 ± 1.17 d	4.05 ± 0.69 de	0.022 ± 0.0003 ef
100	20	0.10 ± 0.006 d	43.03 ± 2.59 a	4.45 ± 0.46 b	25.83 ± 1.45 cd	5.77 ± 0.70 d	0.011 ± 0.0001 g
200	20	0.24 ± 0.015 b	23.71 ± 1.31 d	2.54 ± 0.22 c	27.92 ± 2.06 c	5.47 ± 0.95 d	0.001 ± 0.0001 i

3.4. Response of antioxidant enzymes

The activities of APX and GR after As- and TRIA-treatments are shown in Fig. 3. In the As-exposed plants with no TRIA pretreatment, there was a significant decrease in the activities of APX and GR in comparison to the As-unexposed ones (control) (Fig. 3). However, after As-treatments TRIA-pretreatments significantly increased the activities of these enzymes. Without As treatment, TRIA had negligible effects on these activities, but a great induction of TRIA could be observed under As supply (Fig. 3).

The activities of MDHAR and DHAR were found to decrease with increasing As concentrations. Differently, the TRIA-pretreatments before As-exposure enhanced the MDHAR and DHAR activities. The maximum enhancement in their activities was observed after 5 and 10 μM TRIA-pretreatments under As-exposure (Fig. 4).

3.5. GST activity and gene expression

With increasing As concentration from 0 to 200 μM, GST activity increased rapidly, reaching the maximum at 200 μM As. Exogenous TRIA addition along with As decreased GST activity, in comparison to the As-only treatment (Fig. 5). Transcript level of GST gene was altered in response to As 200 μM and TRIA 10 μM and their combination (Fig. 6). The value from the As-untreated control samples was comparable to that from the only TRIA-treated samples. Differently, the only As-treatment produced a band with an area two fold the As-untreated control. This gene expression increased further in the sample treated with both As and TRIA, where the intensity reached 3 fold the untreated control sample. This finding was according to the GST activity.

3.6. Ultrastructural observations

TEM observations of untreated control samples of *Coriandrum sativum* L. showed cells with a large and electron clear, central vacuole surrounded by a thin layer of cytoplasm lying beneath a thin cell wall (Fig. 7a). The cytoplasm contained numerous lenticular chloroplasts (Fig. 7a and b). The chloroplasts had a well-developed thylakoid system

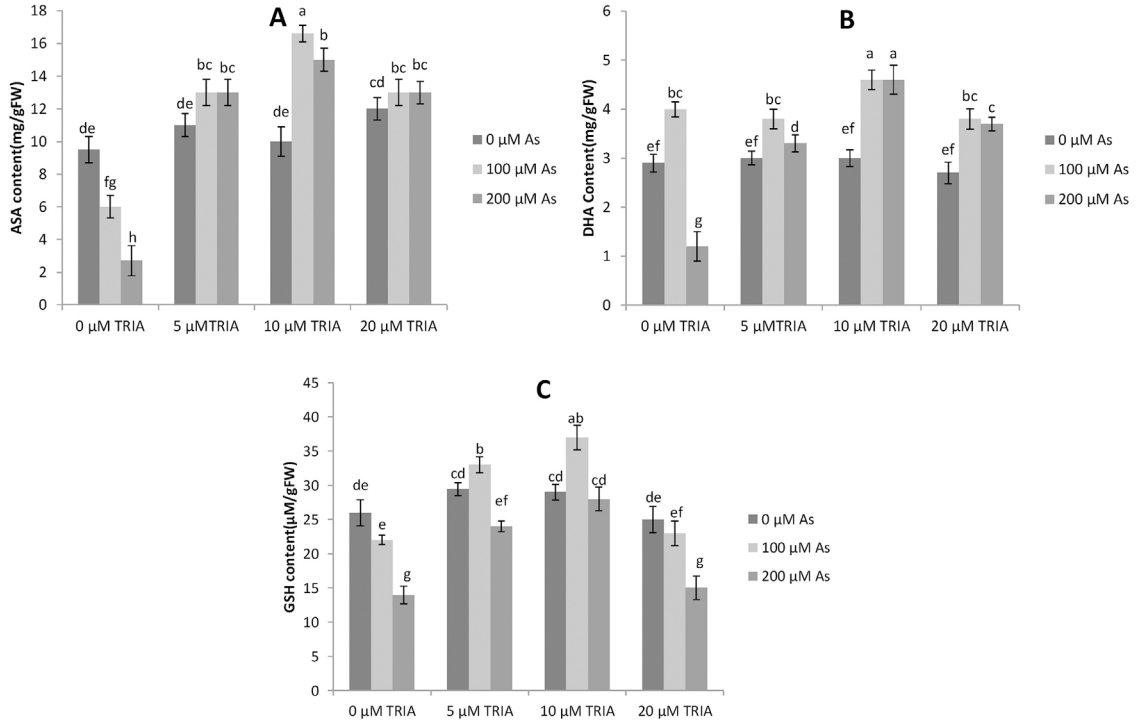

Fig. 2. Effects of As and TRIA on (A) ASA, (B) DHA, and (C) GSH contents in *Coriandrum sativum*. Values are means ± SE (n = 3). Bars with different letters are statistically different ($P < 0.05$) according to Duncan's multiple range tests.

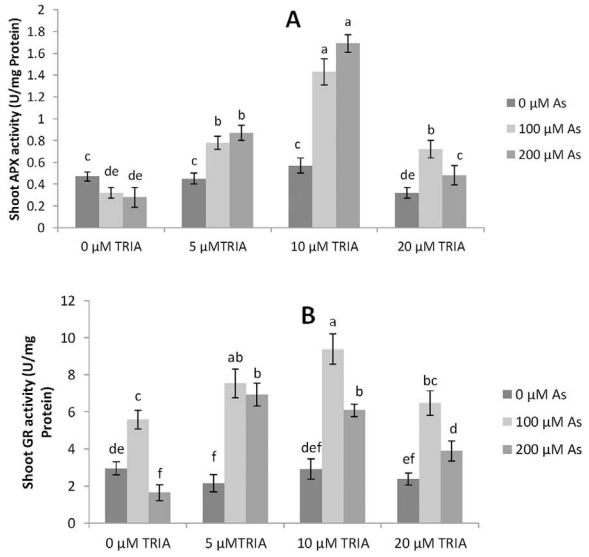

Fig. 3. Effects of As and TRIA on (A) ascorbate reductase and (B) glutathione reductase in *Coriandrum sativum*. Values are means ± SE (n = 3). Bars with different letters are statistically different (P < 0.05) according to Duncan's multiple range tests.

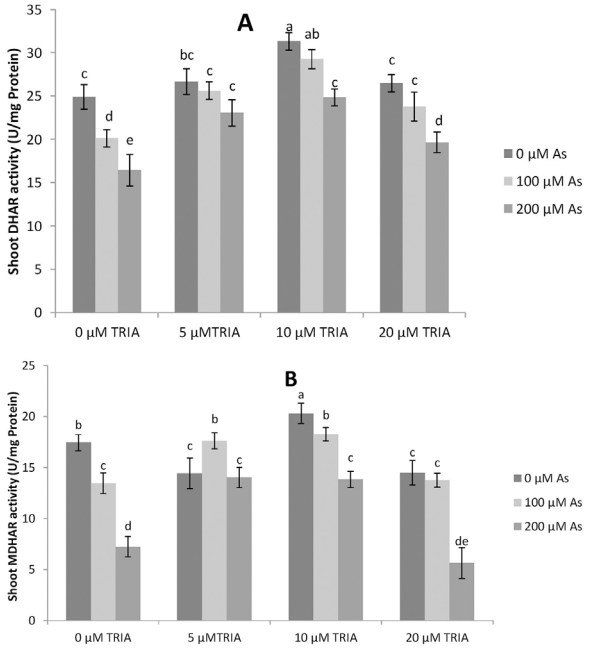

Fig. 4. Effects of As and TRIA on (A) dehydroascorbate reductase activity and (B) monodehydroascorbate reductase activity in *Coriandrum sativum* leaves. Values are means ± SE (n = 3). Bars with different letters are statistically different (P < 0.05) according to Duncan's multiple range tests.

with grana and intergrana membranes submerged in an abundant stroma (Fig. 7b and c). Mitochondria had numerous, electron clear cristae contained in the matrix (Fig. 7d).

TRIA only-treated samples gave electron dense micrographs. Like in control samples, the whole cells had a large, electron clear, central vacuole surrounded by a thin cytoplasm arranged beneath the cell wall. The cytoplasm contained numerous large chloroplasts with well-developed thylakoid systems, nuclei and large lipid droplets (Fig. 7e). The large chloroplasts had the same arrangement as the control (Fig. 7f and g). The mitochondria contained numerous cristae in the matrix (Fig. 7h), just like in the untreated control samples.

The As-treated samples, compared to control untreated samples, appeared changed. Cells were plasmolyzed; the cytoplasm was poorly

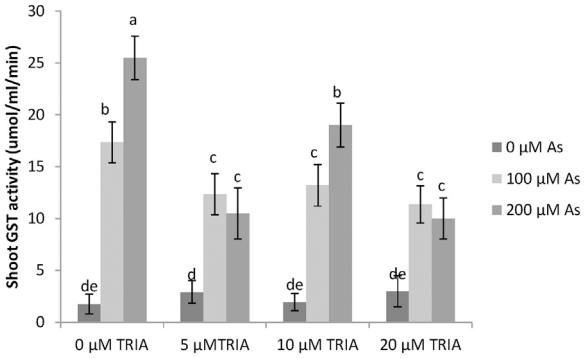

Fig. 5. Effects of As and TRIA on GST activity in *Coriandrum sativum*. Values are means ± SE (n = 3). Bars with different letters are statistically different (P < 0.05) according to Duncan's multiple range tests.

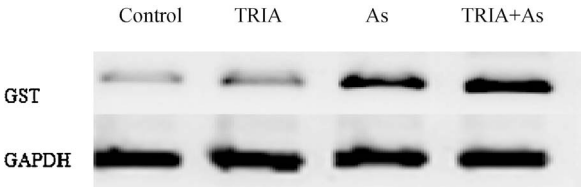

Fig. 6. Analysis of mRNA expression of the GST gene by semi-quantitative RT-PCR. Effects of 10 µM TRIA, As 200 µM and their combination on the gene expression in coriander. Glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as internal control to normalize different samples.

electron dense and contained swollen chloroplasts (Fig. 7i). The swollen chloroplasts contained a poor thylakoid system with grana and intergrana membranes (Fig. 7j and k). Mitochondria still featured cristae, but electron clear areas were visible inside (Fig. 7l and m). Cytoplasm showed multivesicular bodies (Fig. 7m).

The samples treated with both TRIA and As had an appearance comparable to the TRIA only-treated samples (Fig. 7n). The chloroplasts appeared just like those from TRIA only-treated samples (Fig. 7o and p). Mitochondria, even though preserving cristae, showed electron clear areas inside, just like those from As only-treated samples (Fig. 7q).

4. Discussion

The present study shows that one of the most visible effects of As-treatments is a decrease of shoot fresh weights. Our data suggest that the decrease in plant growth is related to the uptake of As and nutrition elements. Some Authors reported that the excess heavy metal induce disturbance in mineral nutrition (Finnegan and Chen, 2012). Arsenate is easily incorporated into plant cells through the high-affinity phosphate transport system (Finnegan and Chen, 2012). Competition between As and P physiologically results in blocking the electron transport chain and, therefore, inhibiting ATP synthesis (Pigna et al., 2009). This leads to a disruption of energy flow in cells and finally inhibits plant growth and development. As also influences the uptake of other mineral nutrients in plants. In fact, it was reported that As addition impairs the uptake of K, Ca, Mg, Mn and Zn (Pigna et al., 2009). That is according with our finding showing that As highest concentration, 200 µM, decreases the uptake of K, Ca and Zn. Similar changes have been observed in other plants in presence of the same metal (Pigna et al., 2009). Our data show that Mg uptake decreased with increasing As (Table 1). One major role of Mg is to act as a cofactor in enzymes activating phosphorylation processes; Mg is also the central atom of the chlorophyll molecule. The found decrease in Mg uptake could probably depend on the ability of As to uncouple the oxidative phosphorylation with a consequent decrease in the chlorophyll content. In addition, a reduction in its uptake may also be a result of the toxic effect of As on plant mineral nutrition (Marschner, 1995).

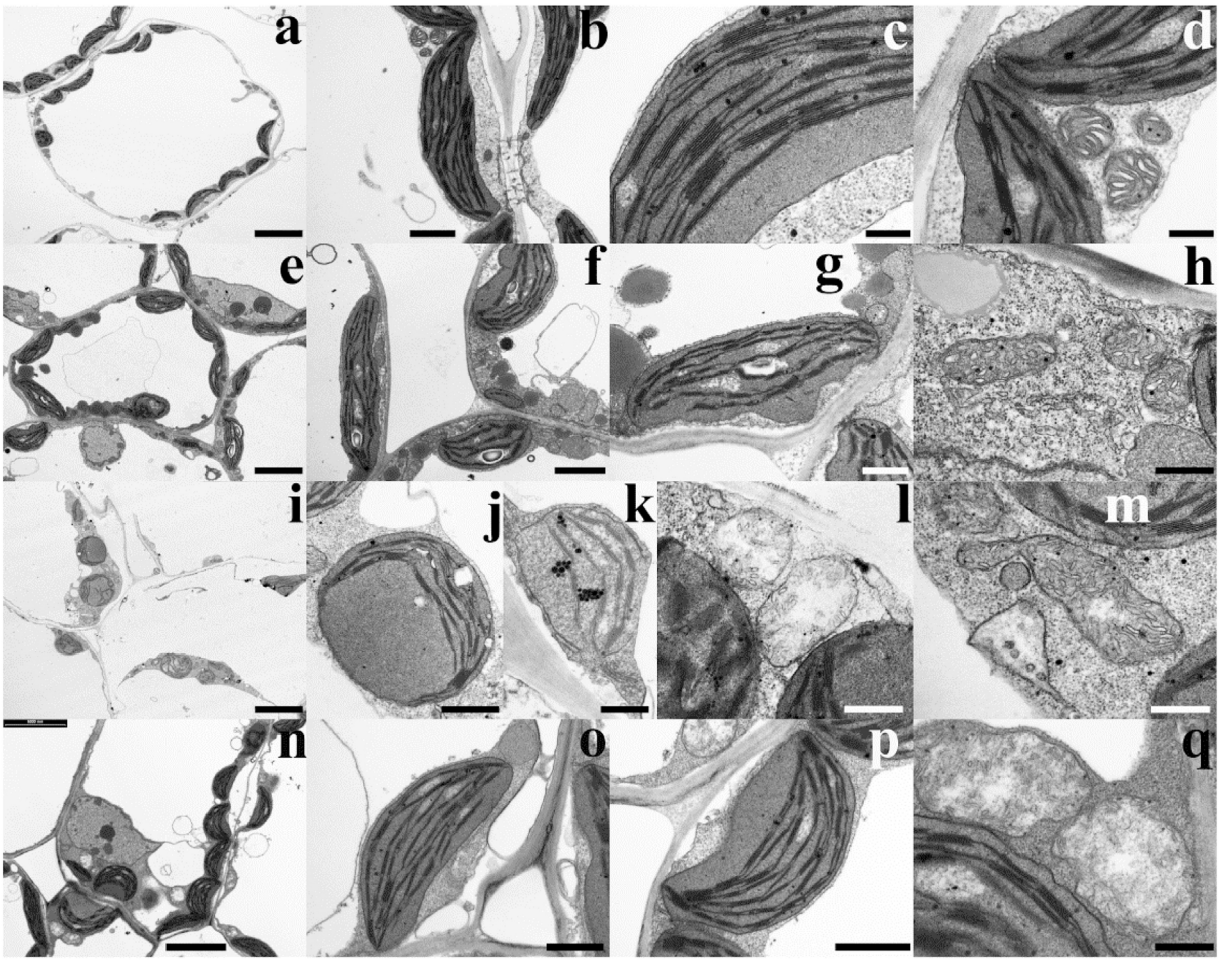

Fig. 7. The table shows TEM micrographs of *Coriandrum sativum* L. samples. (a–d) Untreated control samples. (a) A whole cell with a large central vacuole and chloroplasts. (b) Typical lenticular chloroplasts with thylakoid system and stroma. (c) Detail of a chloroplast with thylakoid system featuring grana and intergrana membranes. (d) Mitochondria with well-developed cristae next to chloroplasts. (e–h) Triacontanol-treated samples. (e) A whole cell showing a central vacuole, large chloroplasts, cytoplasmic lipid droplets and nuclei. (f–g) Chloroplasts with a well-developed thylakoid system. (h) Mitochondria with well-developed cristae. (i–m) As-treated samples. (i) The low magnificated micrograph shows plasmolyzed cells with swollen chloroplasts. (j–k) Swollen chloroplasts with thylakoid system and plastoglobules. (l) Altered mitochondria with a poor cristae system and electron clear, degeneration areas. (m) A multivesicular body next to mitochondria. The large mitochondrion, even though preserving cristae, has an inner electron clear degenerated area. (n–q) Triacontanol + As-treated samples. (n) The whole cell exhibits an appearance comparable to the triacontanol-treated samples. (o–p) Control-like chloroplasts with no evident alterations. (q) Mitochondria with few cristae and electron clear degenerated areas. Scale bars: 5μ (a, e, i, n), 2μ (f), 1μ (b, g, j, o, p), 500 nm (h, k, l, m, q), 300 nm (c, d).

TRIA is a plant growth regulator able to modulate various growth processes under normal or stress conditions (Asadi karam et al., 2016). In the present study, pretreatment with TRIA mitigates the adverse effects of As on growth plant. 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., 2011). 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 (Naeem et al., 2011). Data in Table 1 show that TRIA application proved effective at increasing Mg, Zn, P, K and Ca contents in the shoots. Enhancement in leaf nutrients due to TRIA application could be attributed to the compositional or chemical changing in plants, leading to alterations in nitrogen concentration (Knowles and Ries, 1981). Probably, increased uptake of nutrients enhanced photosynthesis and improved translocation of photosynthates and other metabolites, which may contribute to the improved growth of TRIA-treated plants. These findings are in accordance with data on TRIA ability at improving the contents of essential nutrients, i.e. N, P, K, and Ca in plant tissues (Naeem et al., 2009, 2011; Khandaker et al., 2013). Our previous data suggested that TRIA have played a key role in protecting the structure and function of cell membranes and improving the uptake of other mineral nutrients in plants under heavy metal toxicity (Asadi karam et al., 2017).

In this our previous experiment, induction of oxidative stress under As toxicity was partially alleviated by applying TRIA. That was evidenced by the decreased amounts of ROS, such as H_2O_2, and increased activities of superoxide dismutase and catalase enzymes in the leaves of TRIA-treated coriander (Asadi karam et al., 2016). Such a process was facilitated by the active oxygen scavenging system, which includes several antioxidant enzymes, and is able to enhance membrane stability. The ASC-GSH cycle is involved in scavenging H_2O_2 in plant cells (Wu et al., 2017). For the study of non-enzymatic antioxidant defense, we measured ASC, DHA and GSH in shoots under As stress. In the present study, reduced amounts of ASC and GSH were observed in As-treated plants. This result is according to Hasanuzzaman and Fujita (2013), who reported a decrease in ASC and GSH contents and the GSH/GSSG ratio in As-treated wheat (*Triticum aestivum*). This might be attributed to As toxicity (Sanchez-Viveros, 2010). Therefore, the measured decline in the content of ASC and GSH in *C. sativum* could be partially due to its consumption, while acting as antioxidant and limiting lipid peroxidation. In this study, TRIA enhanced the content of ASA, DHA and GSH by regulating the activities of enzymes of the ASA–GSH cycle, such as APX, MDHAR, DHAR, and GR. All that is in agreement with literature data reporting a stimulating effect of TRIA (Li

et al., 2006; Barrameda-Medina et al., 2014). ASC and GSH are able to detoxify ROS by a direct scavenging or by acting as substrate in the enzymatic reactions (APX and GR). So an increase or protection of their contents by TRIA pretreatment is able to enhance tolerance against As-induced oxidative stress in *C. sativum*. Tolerance of some plants to heavy metals is associated with increases in both APX and GR activities (Madhava Rao and Sresty, 2000), while we observed a decrease in the GR, APX activity under As stress (Fig. 3), which was alleviated by the TRIA applications. This result is according to Zare Dehabadi et al. (2014), who reported a decrease in GR activity in sweet basil seedling under As stress. The activities of MDHAR and DHAR (Fig. 4) in the coriander seedlings decreased with increasing As; differently higher activities of the same enzymes under stress conditions were found in other plants (Mittova et al., 2002; Arora et al., 2010). Some of the enzymes are sensitive to inhibition by heavy metals, like Cu and As, which react with thiol groups at the active sites (Garg and Singla, 2011). Thus, the reduced activity of enzymes, such as GR, may be due to their inactivation by As ions. Some researchers think that this reduction could be due to different effects of heavy metals, like As and Cd, at the transcriptional and post transcriptional levels (Romero-Puertas et al., 2007; Gupta et al., 2013). In addition, considerable decrease in GR, which acts in GSH regeneration in the ASC–GSH cycle, affects the decrease in GSH content under As stress (Zare Dehabadi et al., 2014). Our data provided that GSH content is increased in plants by TRIA application. This result is similar with those of Aziz and Shahbaz (2015), who reported an increase of GR activity in TRIA-treated sunflower. TRIA might have signaling roles in enhancing biosynthesis of APX, MDHAR, DHAR, and GR. Otherwise, TRIA might also have other roles leading to the increase of their activities (Asadi karam et al., 2017). On the other hand, increasing the activities of enzymes involved in the ASA–GSH cycle by TRIA treatment could be maintained by regulation of the amounts of defense hormones. For example, Waqas et al. (2016) have reported that TRIA treatment enhanced jasmonic acid (JA) in mungbean under heat stress. An increase in the activities of enzymes involved in the ASA–GSH cycle was observed in JA-treated wheat seedlings (Shan et al., 2015).

Moreover, Glutathione as a sulfur-containing tripeptide thiol is involved in plant protection against heavy metals as a precursor in the synthesis of phytochelatins (PCs) (Xiong et al., 2010). In our study, decreasing GSH content may be caused by increased conversion of GSH to PC in response of As toxicity. GSH can directly bind with ROS and detoxify them through a reaction catalyzed by glutathione-S-transferases (GSTs). GST catalyzes the conjugation of various electrophiles with reduced glutathione, detoxifying both exogenously and endogenously derived toxic compounds (Dixit et al., 2011). An increase in GST activity was observed in pumpkin (*Curbita maxima*) seedlings subjected to Cd, Cr, Mn, and As stress (Fujita and Hossain, 2003; Hossain et al., 2006) and in rice (*Oryza sativa* L.) seedlings in response to Cd (Hu et al., 2009). Similarly, our results showed that As toxicity enhanced GST activity and gene expression in coriander plants; TRIA + As treatment increased the gene expression more than As only and TRIA only treatments. The present work is the first study concerning the effect of TRIA on GST activity in plants, so the mechanism leading to the increase of this enzyme is not yet clear. This work is an important progress to better understand the matter. Increasing the capacity of ROS quenching could be maintained by over-expression of glutathione-S-transferases.

TEM observations of untreated control samples revealed the typical appearance of mature cells from the leaf photosynthetic parenchyma. Both chloroplasts and mitochondria had a typical appearance. In literature no electron microscopy data are available on the application of TRIA to plants. TEM observations of TRIA-treated plants showed a healthy appearance of cells, which micrographs are well-electron dense and show large chloroplasts, several nuclei and cytoplasmic lipid droplets. There are not any reports on the influence of TRIA on cell structure. The cytoplasmic lipid droplets could also be interpreted as an accumulation of intermediate metabolites, which might be effect of the already known stimulating activity of TRIA on metabolic pathways (Ivanov and Angelov, 1997). TEM observations on As-treated plants demonstrate ultrastructural damage. Plasmolysis of the whole cell, swelling of chloroplasts with increased plastoglobules and thylakoid system depletion, mitochondrion electron clear areas, multivesicular bodies are all frequent damages reported in heavy metal-treated plants, from angiospermophyta (Dalla Vecchia et al., 2005; Basile et al., 2012a, 2015) to lichens (Sorbo et al., 2011; Paoli et al., 2013, 2014), passing through bryophytes (Basile et al., 2012a,b, 2013).

Heavy metal toxicity was suggested to be possibly related to oxidative stress on tissues (Stohs and Bagchi, 1995). Higher doses of arsenate produced oxidative damage in clover plants (Mascher et al., 2002). Hartley-Whitaker and Meharg (2001) reported significant lipid peroxidation in As-exposed *Holcus lanatus* due to an increase in reactive oxygen species. All that could suggest that membranes, particularly in organelles involved in reactive oxygen species production, are common targets of heavy metal damage. Chloroplast is known to produce superoxide radical during electron transport and mitochondrion is the main site of oxidative metabolism. All that is consistent with our finding that chloroplasts and mitochondria are main targets of As-induced damage.

Plasmolysis and swelling of chloroplasts were also observed in As-treated *Pteris vittata* (Li et al., 2006). These phenomena can be explained by a metal-induced damage to the membrane selective permeability causing drifting of ions and the accompanying solvent across the membranes (Schwartzman and Cidlowsky, 1993). All that finally ends up causing swelling or shrinkage of organelles or the whole cell, due to the filling or depriving the cell compartments.

Oxidative damage can also explain the occurrence of multivesicular bodies. That ultrastructure was already observed in heavy metal-treated plants (Basile et al., 2012b, 2013, 2015; Esposito et al., 2012) and related to autophagic and endocytic phenomena (Thompson and Vierstra, 2005; Todeschini et al., 2011). Chiarelli and Roccheri (2012) reported As to enhance autophagy via ROS, which could account for the occurrence of multivesicular bodies in our As-treated samples. Micrographs of the whole cells from As- and TRIA-treated samples are comparable to those from the TRIA only-treated plants and quite different from the As-treated ones. The appearance is electron-dense and healthy; the chloroplasts are large and well-equipped with thylakoids; nuclei are frequently visible. Even though the overall arrangement of the cells and the chloroplasts are healthy, mitochondria are not. Just like in As-treated samples, they still exhibit electron clear areas, which may be regarded as degenerated areas with no cristae and weak matrix. So, our TEM results show that TRIA has an overall protective effect against the As damage. That is consistent with our biochemical results, showing an enhancement of antioxidant activity, and also agrees with an our previous work, reporting a protective effect of TRIA in *Coriandrum sativum* under As toxicity (Asadi karam et al., 2016). We reported that As acts most of its toxic effects also via an oxidative stress, which is counteracted by TRIA. The remaining damage in the mitochondria of the TRIA + As-treated samples could be due to the abundant ROS production in the main site of the oxidative metabolism, which summarized with As-induced oxidative chemicals.

5. Conclusions

In conclusion, the coriander tolerance to As could dependent upon the efficiency of the antioxidant system, which maintained the redox homeostasis and integrity of cellular components. So, the ASC-GSH cycle enzymes probably played major roles in the As-stressed coriander plants. So we can conclude that our findings support the hypothesis that the higher efficiency of the antioxidant system after TRIA-treatment could explain coriander tolerance to As.

Furthermore, TRIA influence on ROS quenching could be also maintained by over-expression and/or enhancing GST activity along

with stimulating ASC-GSH cycle enzymes, which may also contribute to As tolerance of coriander.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2017.07.012.

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