International Journal of Agriculture and Crop Sciences.
Available online at www.ijagcs.com
IJACS/2013/6-17/1186-1193
ISSN 2227-670X ©2013 IJACS Journal

IJACS

Cadmium affects glutathione-related metabolism in Vicia faba roots

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ABSTRACT : Cadmium (Cd) is one of the most toxic heavy metals in plants. To investigate whether Cd induces common plant defense pathways, different physiological and molecular reactions, including modifications in glutathione (GSH) level, activity and gene expression of glutathione reductase (GR) enzyme were characterized in roots of hydroponically cultivated-Vicia seedlings during 12, 24 and 48 h. Vicia roots which accumulated the metal, showed an increase in GR1-like gene transcripts, encoding for GR enzyme, as earlier as 12 h after the onset of the Cd treatment while the root growth was not as yet affected by the Cd treatment. When Vicia roots exposure was longer (24 h and 48 h), metal induced significant decreases in fresh (FW), dry weight (DW) and water content. These growth changes were accompanied by progressively increases in GR activity, GSH and oxidized glutathione (GSSG) level.
Keywords : cadmium, glutathione, oxidative metabolism, Vicia faba
Abbreviations: Cd, cadmium; DW, dry weight; FW, fresh weight; GR, glutathione reductase; GSH, glutathione; GSSG , oxidized glutathione; H_2O_2, hydrogen peroxide.

INTRODUCTION

Cadmium (Cd) is a toxic heavy metal and is also known as one of the major environmental pollutant. It has an important impact on agriculture, as the excessive consumption of Cd from contaminated food crops can lead to toxicity in humans. Wagner (1993) estimated that non-polluted soil solutions contain Cd concentrations ranging from 0.04 to 0.32 mM. Soil solutions which have a Cd concentration varying from 0.32 mM to about 1 mM can be regarded as polluted to a moderate level (Sanitá di Toppi and Gabrielli 1999). The degree to which higher plants are able to take up Cd depends on its concentration in the soil and its availability, modulated by the presence of organic matter, pH, Redox potential, temperature and concentration of other elements (Benavides et al. 2005). Cd can be absorbed by plants in two possible ways: passive (the metal uptake involves ion diffusion from the soil solution inside the endoderm) and active (the concentration gradient requests metabolic energy). After being absorbed by roots, metal can be spread through xylem vessels throughout the entire plant. On leaves, metal ion can be either incorporated inside the proteins or translocated by phloem, causing series of phototoxicities (Alloway 1995). In general, Cd is retained in roots and only a small amount is transported to the above ground part, essentially accumulating on leaves (Cutler and Rains 1974).

Cd is suspected to exert its toxic effect on cells through its oxidative damage involving the initial formation of reactive oxygen species (ROS) (Souguir et al. 2011). ROS (such as oxygen superoxide O_2^-) and hydrogen peroxide (H_2O_2) have a potential to generate oxidative stress within cells by reacting with macromolecules and causing damage such as mutations in DNA, destruction of protein function and structure, and peroxidation of lipids (Valko et al. 2006). Cd promotes oxidative damage by increasing the cellular concentration of ROS and by reducing the cellular antioxidant capacity (Corticeiro et al. 2006). As a predominant non-protein thiol compound, glutathione (GSH, c-glutamyl-cysteinyl-glycine) is a major intracellular antioxidant in living organisms and the first line of defense against oxidative stress. GSH is a central component in the multifaceted cellular detoxification system that constitutes an important mechanism for cellular protection against agents, such as Cd, which produce ROS. Glutathione takes part in the control of H_2O_2 levels (Shao et al. 2005; Foyer and Noctor 2005) and the change in the ratio of its reduced (GSH) to oxidized (GSSG) form during the degradation of H_2O_2 is important in certain redox signaling pathways (Millar et

al. 2003). It has been suggested that the GSH/GSSG ratio, indicative of the cellular redox balance, may be involved in ROS perception (Millar et al. 2003; Foyer and Noctor 2005). In many reactions involving GSH, the Cys thiol group is oxidized to yield GSSG, and the reverse reaction is catalysed by glutathione reductase (GR), using NADPH. The highly reduced glutathione pool maintained by GR, which is encoded by two genes (GR1 and GR2) (Mhamdi et al. 2010), is necessary for active protein function and avoids unspecific formation of mixed disulphide bonds that cause protein inactivation or aggregation. Stable protein disulphide bonds are relatively rare except in quiescent tissues such as seeds, where GSSG is allowed to accumulate. In metabolically active tissues, millimolar concentrations of GSH act as a key redox buffer, forming a barrier between protein Cys groups and ROS. Moreover, GSH is a substrate for several reductive enzymes, including enzymes that reduce peroxides.

Regarding glutathione, several studies have shown its role in defense mechanisms and adaptation of cells to oxidative stress (Dixit et al. 2001; Mishra et al. 2006; Liu et al. 2007). This metabolite is involved in the synthesis of phytochelatins which play an essential role in the plant homeostasis of metal ions, such as Cd, in the cytoplasm. ATP-dependent tonoplast carriers convey the complex to vacuoles where they will be stored. Phytochelatins are synthesized in the roots (Zhu et al. 1999) and induction by Cd has been extensively studied (Srivastava et al. 2004; Mishra et al. 2006). Glutathione creates complexes with heavy metals and an induction of glutathione as well as cysteine synthesis has been documented in plants as a response to heavy metals stress (Arya et al. 2008).

Main aim of the present study was to assess changes in GSH levels and some other biochemical parameters in Vicia faba, plant high consumed in Tunisia, that might have an effect on signaling and production of GSH following exposure to different doses of Cd for 12, 24 and 48 h.

MATERIALS AND METHODS

PLANT MATERIAL AND TREATMENTS

Seeds of V. faba var. Aguadulce were surface sterilized with 10% sodium hypochloride, rinsed several times with water and placed on moistened filter paper at 25°C for 4-5 days. The germinated seeds were then transferred in hydroponic conditions as described by Souguir et al. (2008). In order to determine growth disruption threshold under Cd stress in Vicia, plant responses were observed in a range of Cd concentrations and incubation time. Moreover, by using a transient period we were able to determine the early responses of the plant toward Cd pollutant. The hydroponic nutrient solution was supplemented with 50, 100 or 200 µM $CdCl_2$ in distilled water, at 12-day-old seedlings for 12, 24 or 48 h. For each treatment, three replicates were performed.

Enzyme Assay

1g of fresh root samples were homogenised in 5ml of 50 mM potassium phosphate buffer (K_2HPO_4/KH_2PO_4; pH 7.0), 5 mM sodium ascorbate and 0.2 mM EDTA using a chilled mortar and pestle. The homogenate was centrifuged at 12,000 g for 10 min at 4 °C and after dialysis supernatant was used for enzyme assays.

In all the enzymatic preparations protein was determined by the method of Bradford (1976) using bovine serum albumin (BSA, Sigma) as standard. The GR assay was performed according to Albrecht et Wiedenroth (1994). The reaction mixture contained 100 mM Tris-HCl (pH 9), 0.2 mM NADPH and 0.5 mM GSSG. The reaction was started by addition a suitable protein volume and the NADPH oxidation (e = 6.2 mM^{-1} cm^{-1}) was recorded as the decrease in absorbance at 340 nm for 1 min.

Rna Isolation And Semi Quantitative Reactions

RNA extraction was performed using Tri-reagent (Euromedex) and the Euroscript Reverse Transcriptase (Eurogentec) was used for cDNA synthesis, both according to manufacturer's instructions. RNA integrity was verified on 1% agarose gel by detecting the three bands corresponding to ribosomal RNA 28S, 18S and 5S.

Primers used for the amplification of target cDNA were designated according to their availability in the data bank (http://www.ncbi.nlm.nih.gov/). Both forward and reverse primers must frame a relatively short sequence (approximately 150 bp) suitable for qPCR, designed with a GC percentage of around 60% and a Tm of between 58 and 60°C. Primers for the internal control Actin were designated on sequences of V. faba genes:
VfActin Fw: 5'- CAGCAGAGCGGGAAATTGTGAGGG,
VfActin Rev : 5'- AGGGCATCTGAATCTTTCAGCACCG,

Primers for GR1 were designated based on the sequence of the related species Pisum sativum and using sequence alignment with ClustalW software (http://align.genome.jp/) in order to locate highly conserved regions:

PsGR Fw: 5'- CGGATGTTGTGCTATTTGCCACTGG,
PsGR Rev: 5'- TCACCCACAGCCCATATGCTAGGG,

The identity of each reverse-transcription PCR product was confirmed by direct sequencing. PCR products were resolved on a 1.2% (w/v) agarose gel for size verification, purified with a DNA gel band purification kit (GFX^{TM} PCR, Amersham Pharmacia Biotech) according to manufacturer's protocol and then sequenced using Genome Express services. Sequence homology searches were carried out using the BLAST search facility available through NCBI (http://www.ncbi.nlm.nih.gov) Amplifications were performed with the following thermal profile: 30 s at 95°C, 30 s at a temperature chosen according to the Tm of the primers, 30 s at 72°C using Taq DNA polymerase (Sigma) for a number of cycles to generate detectable signal for every sequence.

Semi-quantitative PCR reactions visualized on agarose gels were carried out as follows: 30 s at 94°C for denaturation, 30 s at 59°C (with primers) and 30 s at 72°C for 22 cycles, and 10 min at 72°C for final extension. The optimal numbers of PCR cycles were established to generate unsaturated (linear phase) but detectable signals. These PCR were performed in parallel on the same cDNA than the ones performed for real-time quantitative PCR.

The quantitative assessment of mRNA levels was performed using the iCycler iQv3 (BIO-RAD). Real time quantitative PCR based on the fluorescence emitted by the amplification products in the presence of SYBR Green, allows quantification of the accumulation of the target transcript relative to the Actin transcript taken as reference. The reactions were prepared using the qPCR kit Mastermix for SYBR green (Eurogentec) according to the manufacturer's protocol. The cDNA concentration used produced a CT (threshold cycle) between 15 and 30 cycles. The abundance of targeted gene transcripts was normalized to Actin mRNA and set relative to control plants (no heavy metal exposure) according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Glutathione Content

To determine the contents of GSH and GSSG, frozen roots were ground and homogenized in ice-cold 5% metaphosphoric acid (1:4 w/v), then centrifuged at 2,000 g for 35 min at 4 °C. Total (GSH + GSSG) and GSSG contents were estimated by the method of Anderson (1985). The assay was based on sequential oxidation of GSH by DTNB to produce TNB and reduction of GSSG by NADPH in the presence of GR. To determine GSSG content, 2-vinylpyridine was added to the extract. GSH content was obtained from the difference between the total glutathione and GSSG.

Statistical Analysis

The results presented are the values (± SD) obtained from at least three replicates. Significant differences between treated and control plants are determined using ANOVA test ($P < 0.05$).

RESULTS AND DISCUSSION

Recognized as an extremely significant pollutant due to its high toxicity and large solubility in water (Pinto et al. 2004), Cd is rather readily translocated throughout the plant following its uptake by the roots, the first entry point for Cd uptake. According to a previously study of Souguir et al. (2011), Vicia roots accumulated highly Cd. The metal accumulation in roots was demonstrated after 12 h of treatment by 50, 100 and 200 µM of $CdCl_2$ (Souguir et al. 2011). Once Cd enters into root cells, its movement through the root symplasm to the xylem can be restricted by its sequestration in the vacuoles (Nocito et al. 2011). Apoplastic movement of Cd to the xylem can also be restricted by development of the endodermal suberin lamellae in roots exposed to Cd (Lux et al. 2011). Our results confirmed the high sensitivity of V. faba to Cd. Indeed, accumulation of Cd affected drastically root growth, which was measured by fresh weight (FW), dry weight (DW) and water content. As seen in Table 1, there are no significant changes in fresh and dry weight after 12 h of exposure to different Cd concentrations. The depressive effect of Cd on growth was observed at 24 h and 48 h of treatment. At lower Cd exposures, no significant changes of FW, DW and water content were noticed at 12 h of treatment while the reduction of growth parameters were marked after long exposure (24 or 48 h) for 50, 100 and 200 µM of Cd. Cd content in 12 h treated roots might not be high enough to significantly inhibit root growth. With longer treatment (24 or 48 h), gradually accumulated Cd might affect root growth. It is well known that Cd inhibited growth (Tamás et al. 2008; Lux et al. 2011). The growth reduction might be due to mitotic index decreases and abnormal chromosome formations (Liu et al. 2003; Souguir et al. 2008; Souguir et al. 2011).

Table 1. Fresh weight (FW), Dry weight (DW) and H_2O content in roots of hydroponically cultivated V. faba plants treated for 12, 24 and 48 h with different concentrations of $CdCl_2$ (Data represent mean ± SD of three replicates (* P < 0.05; ** P < 0.01 compared to control).

		Treatment (h)		
		12	24	48
FW (g)				
	Control	0.40±0.04	1.01±0.05	1.18±0.09
	50 µM	0.42±0.14	0.69±0.09**	0.51±0.01**
	100 µM	0.34±0.04	0.43±0.09**	0.58±0.08**
	200 µM	0.36±0.07	0.43±0.05**	0.57±0.07**
DW (g)				
	Control	0.035±0.004	0.070±0.004	0.077±0.006
	50 µM	0.032±0.0087	0.054±0.006*	0.044±0.007**
	100 µM	0.027±0.004	0.035±0.006**	0.048±0.005**
	200 µM	0.028±0.008	0.035±0.004**	0.045±0.004**
H_2O (%)				
	Control	12.49±0.04	13.4±0.05	14.11±0.13
	50 µM	11.77±0.68	11.79±0.08**	10.67±0.09**
	100 µM	11.84±0.54	11.29±0.08**	11.13±0.10**
	200 µM	11.98±0.17	11.43±0.04**	11.68±0.06**

The toxicity of Cd is associated with production of reactive oxygen species (ROS). Relationships between Cd toxicity and oxidative stress caused by ROS have been studied in many systems and heavy metal contamination has often been implicated as the root cause of oxidative injury to the plants. The key step in oxidative stress is the production of ROS which initiate a variety of auto-oxidative chain reactions on membrane unsaturated fatty acids, producing lipid hydroperoxides and thereby cascade of reactions ultimately leading to destruction of organelles and macromolecules (Shaw et al. 2004). However, it is only when production of ROS exceeds the capacity of scavenging systems that oxidative damage occurs. Previously, an increase in H_2O_2 production was noticed throughout the growth period (12 - 48 h) of Cd-treated Vicia and a significantly positive correlation was found between Cd uptake and H_2O_2 accumulation at every time point of treatments (Souguir et al. 2011). Similarly, Populus cathayana was shown to display a high cadmium concentration in tissues, with a ROS production in roots and leaves (He et al. 2013).

Cadmium toxicity is usually checked by various endogenous antioxidants whereby the thiol pool of plants plays an important role (Potters et al. 2002). It has been shown that reduced glutathione (GSH), an important component of the thiol pool plays several and important roles in cell protection under most of the environmental stresses, such as direct neutralization of free radicals and reactive oxygen compounds, regulation of nitric oxide cycle, DNA synthesis and repair, protein synthesis, amino acid transport and enzyme activation (Foyer and Noctor, 2005; Sharma and Dietz, 2009). Glutathione is also involved in the formation of phytochelatins and together with its oxidized form (GSSG) make up the redox couple (GSH/GSSG), which plays an essential role in the maintenance of the cellular homeostasis and signaling system in plants (Ha et al. 1999; Clemens, 2006; Srivalli and Khanna-Chopra, 2008). This leads to the suggestion that the GHS/GSSG ratio, indicative of the cellular redox balance, may be involved in ROS perception (Shao et al. 2008). Under Cd stress, GSH is considered a primary defense mechanism against Cd, since its cysteine thiol group rapidly reduces the metal by forming a stable GS-Cd complex (Wang et al. 2008). Our results of glutathione (GSH - GSSG) estimation were shown in table 2. There are no significant changes in GSH levels at 12 h of treatment with all metal concentrations. Extending the period of Cd stress induced significantly ($P < 0.05$) increase in GSH content. At 48 h, the GSH content increased 104, 204 and 255% in root cells treated with 50, 100 and 200 µM Cd, respectively. The drastic increase in GSH level at 24 h of metal exposure was probably occurred after increases in Cd/H_2O_2 level (at 12 h of exposure) (Souguir et al. 2011). Oxidized glutathione (GSSG) increased lately at the end of experiment under higher Cd concentrations (100 and 200 µM). Despite the increase in GSSG level, the GSH/GSSG ratio increased due to the higher level of GSH. It was reported that increased availability of H_2O_2 was among specific situations where the glutathione pool becomes preferentially oxidized (Noctor et al. 2012). This could be a key property of pathway regulation, allowing altered glutathione status to transmit signaling information.

Table 2. Glutathione redox state in roots of hydroponically cultivated V. faba plants treated for 12, 24 and 48 h with different concentrations of $CdCl_2$. Data represent mean ± SD of three replicates (* P < 0.05 compared to control).

		Treatment (h)		
		12	24	48
GSH	Control			
(µmol g^{-1} FW)		0.90±0.04	0.91±0.15	0.89±0.21
	50 µM	0.81±0.16	1.65±0.17*	1.83±0.23*
	100 µM	0.83±0.09	1.47±0.18*	3.08±0.72*
	200 µM	1.09±0.16	1.50±0.21*	3.19±0.35*
GSSG	Control	0.49±0.07	0.40±0.03	0.49±0.08
(µmol g^{-1} FW)	50 µM	0.43±0.09	0.35±0.02	0.40±0.12
	100 µM	0.58±0.01	0.34±0.03	0.94±0.18*
	200 µM	0.44±0.08	0.35±0.09	0.89±0.14*
GSH/GSSG	Control	1.83±0.42	2.29±0.31	1.95±0.31
	50 µM	1.88±0.19	4.98±0.26*	4.56±0.42*
	100 µM	1.27±0.22	4.31±0.66*	3.28±0.29*
	200 µM	2.46±0.48	4.23±0.01*	3.57±0.73*

The reduction of GSSG to GSH was catalyzed by GR. Intracellular specific activity of GR was also assayed and results were shown in Fig.1. No significant GR enzyme increase was observed in Cd treated plants during early period of growth (12 h). Increase of GR activity was observed in correlation with times and cadmium treatments. Increase in GR activity was increased, by about 208 - 233 %, in 48 h grown seedling roots treated with 50, 100 and 200 µM Cd.

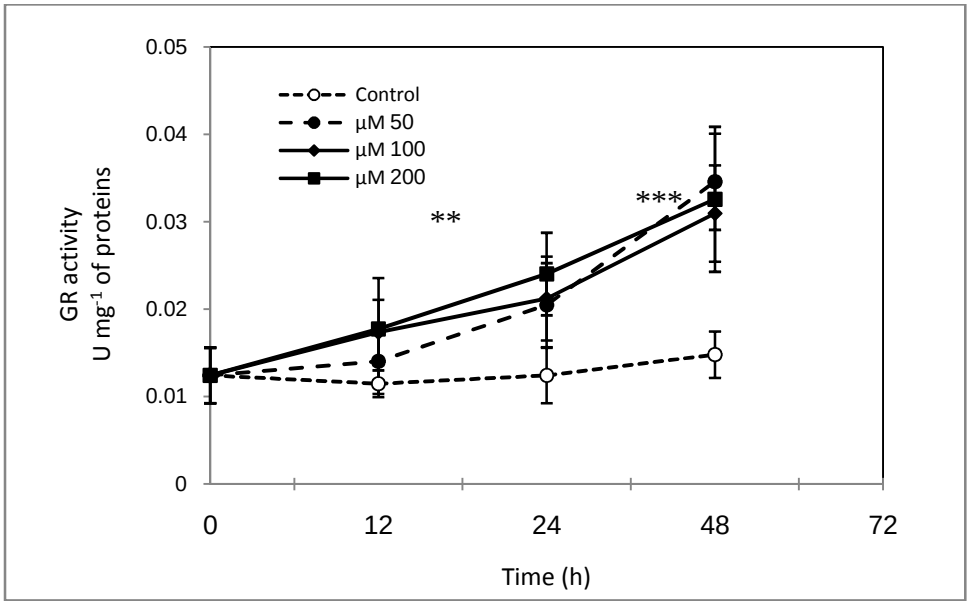

Figure 1. GR activity in roots of hydroponically cultivated Vicia plants exposed for 12, 24 and 48 h to 50, 100 and 200 µM of exogenous Cd. (** P < 0.01; *** P < 0.001 compared to control).

GR1-like gene transcripts accumulated after Cd treatments (Fig. 2). Transcript accumulation displayed a maximum at 12 h of treatment, especially for the highest concentration (200 µM), then decreased. In contrast, GR activity (Fig.1) and growth parameters (Table 1) went on increasing. For the rest of the treatment period, GR1-like gene transcript accumulation increased slightly in the presence of 50 and 100 µM of Cd. It is possible that the regulatory influence of glutathione is mediated by direct control of protein thiol-disulfide status through redox potential (Meyer et al. 2007; Han et al. 2013a). This notion receives some support from the observation that plants lacking expression of GR1 also show repression of the JA pathway (Mhamdi et al. 2010, Han et al. 2012). Recently, it was shown that in parallel to its antioxidant role, GSH acts independently of NPR1 to allow increased intracellular H_2O_2 to activate SA signaling, a key defense response in plants (Han et al. 2013b).

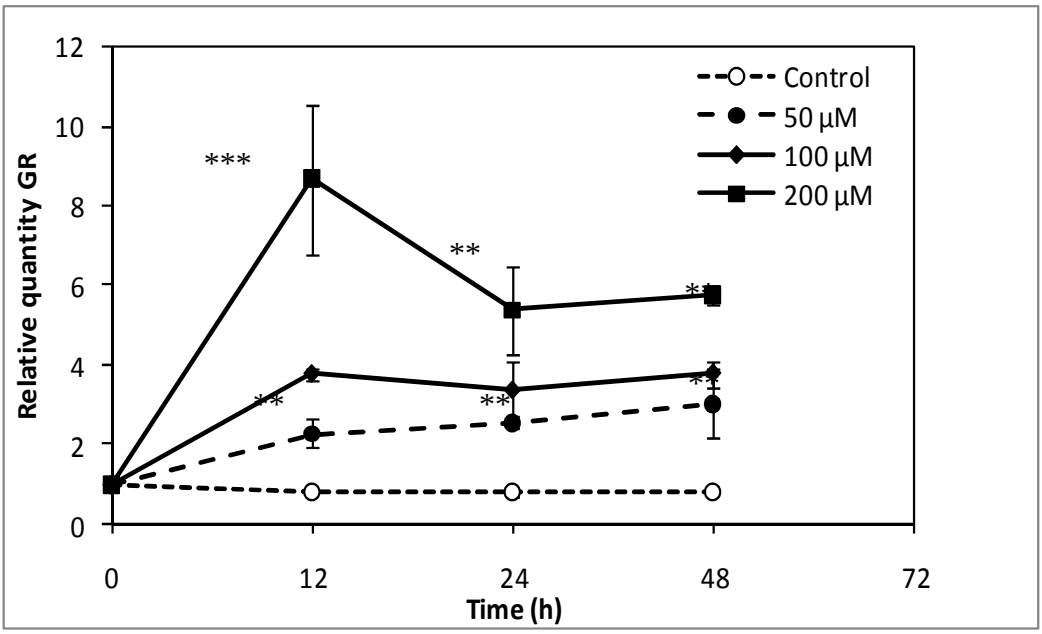

Figure 2. GR1 transcript accumulation after Cd treatments of hydroponically cultivated V. faba plants for 0, 12, 24 and 48 h. The amount of transcript encoding GR1-like gene was quantified by RT-qPCR and normalized to the amount of the housekeeping Actin transcript. Values are expressed relative to the control (no heavy metal treatment) value. Bars represent mean values ±SD from three CT values of two independent experiments. (** P < 0.01; *** P < 0.001 compared to control).

Compared with other ROS, H_2O_2 is a relatively long-lived molecule that is able to cross cell membranes (Bienert et al. 2006), and rapidly diffuses from cell to cell or can be transported long distances from its sites of origin in plants. Also, its production quickly responds to various environment stimuli (Cheng and Song 2006). Thus, H_2O_2 has all of the characteristic features of an intercellular signaling molecule and this feature is compatible with its role as a signaling molecule (Neill et al. 2002). Increasing evidence now indicates that H_2O_2 acts as a local and systemic signal that directly regulates expression of numerous genes. Some of these are involved in plant pathogen defense responses, while others are invoked during adaptation of plants to abiotic stress (Desikan et al. 2001; Wang et al. 2006; Vandenbroucke et al. 2008). Recently, Vergara et al. (2012) showed that H_2O_2 and ethylene act as signalling molecules and activate genes related to the antioxidant defense system in grapevine buds under hypoxia.

A variation in the antioxidant level, which was dependent on metal treatment, was recently noticed in Vicia faba (Nadgórska-Socha et al. 2013). In addition to enzymatic antioxidants, non-enzymatic antioxidants are important in heavy metals plants defense (Gill and Tuteja 2010). An increase in GSH in the leaves of plants cultivated in soil contaminated with Pb and Zn was found. Glutathione content in Vicia leaves positively correlated with the available Zn in soil (Wang et al. 2010; Nadgórska-Socha et al. 2013). Nocito et al. (2006) found that Cd and Zn affected the GSHt content of maize roots in different ways.

In the present study, we showed that transcripts of GR1-like gene accumulation were a sensitive response to Cd exposure. Indeed, at the beginning of our experiment (12 h of Cd exposure), Cd induced increases in H_2O_2 contents (Souguir et al. 2011) and GR1 transcripts and the most increases were observed under the highest Cd concentration (200 µM). It seems likely that H_2O_2 play an important role in regulation of GR1 gene and act as signaling molecules in the response of Vicia roots to Cd stress at this stage of experiment (12 h). As early as 24 h after the onset of heavy metal treatment, GR activity and GSH/GSSG level were going on with cadmium concentration increase. Thus, it could be suggested that H_2O_2 might act as a signal molecule to promote the transcription of gene encoding the GR enzyme. Activity of GR was maintained higher the GSH/GSSG ratio level which plays an essential role in the maintenance of the cellular homeostasis.

ACKNOWLEDGMENT

This work was supported by a grant from ADEME ‘‘Bioindicateurs de Qualite´ des Sols’’ (PNETOX 2003).

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