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Glutathione metabolism in *Urtica dioica* in response to cadmium based oxidative stress

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Abstract

To investigate the antioxidative response of glutathione metabolism in *Urtica dioica* L. to a cadmium induced oxidative stress, activities of glutathione reductase (GR), glutathione-S-transferase (GST), and glutathione peroxidase (GSH-Px), content of reduced (GSH) and oxidized (GSSG) glutathione, lipid peroxidation (LPO), and also accumulation of Fe, Zn, Mn, Cu besides Cd were determined in the roots, stems, and leaves of plants exposed to 0 (control), 0.045, and 0.09 mM $CdCl_2$ for 58 h. Whereas the Cd content continuously increased in all organs, the Fe, Zn, Mn, and Cu content decreased in dependence on the applied Cd concentration and incubation time. The Cd treatment resulted in increased GR and GST activities in all organs, however, GSH-Px activity was dependent on Cd concentration and plant organ. The GSH/GSSG ratio maintained above the control level in the stems at both Cd concentrations. The LPO was generally close to the control values in the roots and stems but it increased in the leaves especially at 0.09 mM Cd.

Additional key words: glutathione peroxidase, glutathione reductase, glutathione-S-transferase, lipid peroxidation, nettle.

Introduction

Cadmium (Cd) occurring as trace element in soils is non-essential and toxic heavy metal for humans, animals, and plants. Non-polluted soils contain Cd at concentrations ranging from 0.04 to 0.32 mM, however, because of some human activities, its concentration may increase up to 5 mM and so it is considered as one of the widespread environmental pollutants with a long biological half-life (Wagner 1993, Ernst and Neilssen 2000). Cadmium accumulation causes reductions in water and nutrient uptake (Sanita di Toppi *et al.* 1999), induction of oxidative stress in consequence of production of reactive oxygen species (ROS; Foyer *et al.* 1997), inhibition of growth, root damage, chlorosis, browning root tips, and eventually plant death (Kahle 1993, Sneller *et al.* 1999). Unlike other heavy metals, such as manganese (Mn) and copper (Cu), Cd is redox-inert metal and does not produce the ROS directly *via* the Fenton/Haber-Weiss reaction but indirectly by impairment of some physiological processes (Sanita di Toppi and Gabbrielli 1999) or by inhibition of antioxidant enzymes (Chaoui *et al.* 1997, Stroinski 1999).

Plants respond to Cd induced stress in various ways. One possible mechanism is that Cd triggers the synthesis of phytochelatins which bind Cd in cytosol and sequester it in vacuoles (Mehra and Tripathi 1999). Reduced glutathione (GSH), which is the major non-protein thiol, is precursor for the synthesis of phytochelatins in plants. Reduced glutathione has many functions in plant metabolism including signal transduction, gene activation, and also redox regulation of the cell cycle (Gomez *et al.* 2004, Foyer and Noctor 2005). It can be certainly stated that GSH and GSH associated enzymes, such as glutathione reductase (GR; EC 1.6.4.2), GSH-S-transferases (GSTs; EC 2.5.1.18), and glutathione peroxidase (GSH-Px; EC 1.11.1.9), together with other antioxidant enzymes represent a dynamic and widely sensitive system controlling the overall cellular redox state. The cysteine (Cys) thiol group of GSH is oxidized to yield oxidized glutathione (GSSG) and reversed reaction is catalyzed by GR using NADPH. A GSH pool maintained by GR is necessary for active protein function, and millimolar concentrations of GSH act as key redox buffer forming a barrier between protein Cys groups and the ROS (Yannarelli *et al.* 2007). Another enzymes involved in the glutathione system are GSTs, best known for their ability to catalyze the conjugation of GSH to potentially dangerous xenobiotics for their detoxification. Reduced glutathione safeguards the -SH group in biomolecules during non-enzymatic or enzymatic processes involving GSTs (Marrs 1996) and

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Abbreviations: GR - glutathione reductase; GSH - reduced glutathione; GSH-Px - glutathione peroxidase; GSSG - oxidized glutathione; GST - glutathione-S-transferase; LPO - lipid peroxidation; MDA - malondialdehyde; ROS - reactive oxygen species.
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selenium-dependent glutathione peroxidase (Se-GSH-Px; EC 1.11.1.9) (Halliwell and Gutteridge 1999). Both GSH-Px are able to detoxify products of lipid peroxidation, but only Se-GSH-Px detoxifies hydrogen peroxide (H_2O_2).

Some previous studies have demonstrated that modulations of GSH content and GSH metabolism enzymes along with other antioxidant enzyme activities in various plants occur in response to Cd stress (Stroinski *et al.* 1999, Iannelli *et al.* 2002, Sun *et al.* 2007, Marquez-Garcia *et al.* 2011). Nevertheless, there is no knowledge about the behaviors of GSH and GSH related enzymes in *Urtica dioica* under Cd stress. *U. dioica*, often called common nettle or stinging nettle, is used in traditional medicine and sometimes in cheese making (Vogl *et al.* 2013).

In this study, we focused on effects of applied Cd on the content of Cd, Fe, Zn, Mn, Cu, GSH, and GSSG, activities of GR, GST, and GSH-Px, and lipid peroxidation (LPO) in *U. dioica* roots, stems, and leaves in dependence on Cd concentration and incubation time.

Materials and methods

Plant growth conditions and Cd^{2+} treatment: The seeds of nettle (*Urtica dioica* L.) were obtained from Dokuz Eylul University, the Fauna and Flora Research and Application Center, Izmir, Turkey. The seeds were disinfected with 10 % (v/v) H_2O_2 for 20 min and washed thoroughly with distilled water and germinated between wet paper towels at 25 °C in the dark for 3 d. The seedlings were transferred to Hoagland's nutrient solution which included [g dm^{-3}]: KNO_3 (202.0); $Ca(NO_3)_2 \cdot 4\,H_2O$ (472.0); Fe-EDTA (15.0); $MgSO_4 \cdot 7\,H_2O$ (493.0); NH_4NO_3 (80.0); H_3BO_3 (2.86); $MnCl_2 \cdot 4\,H_2O$ (1.81); $ZnSO_4 \cdot 7\,H_2O$ (0.22); $CuSO_4 \cdot 5\,H_2O$ (0.051); $H_3MoO_4 \cdot H_2O$ (0.09). They were cultivated in a growth chamber at a 16- h photoperiod, an irradiance of 150 μmol m^{-2} s^{-1}, day/night temperatures of 25/20 °C, and a relative humidity of 65 ± 5 %. The pH of the nutrient solution was adjusted to 6.1 which is appropriate for plant growth (Hipps *et al.* 2013). These solutions were permanently aerated and renewed three to four times a week to minimize a pH shift and nutrient depletion. The seedlings having eight leaves were selected for experiments based on a uniform appearance, average height, and total number and size of leaves. These seedlings were then transferred to the nutrient solution at pH 6.1, and $CdCl_2$ to a final concentration of 0 (control), 0.045, and 0.090 mM was added. The roots, leaves, and stems were sampled at 12, 26, 34, 50, and 58 h after the start of the treatments.

Preparation of crude extracts: To prepare the extracts of *U. dioica* organs, 1 g of leaf (without the main midribs), stem, and root material was homogenized in 8 cm^3 of a 20 mM phosphate buffer (pH 7.4) containing 50 mM β-mercapto-ethanol. The homogenate was filtered through a single-layer cheesecloth and then centrifuged at 15 000 *g* for 15 min. All operations were carried out at 0 to 4 °C. β-mercaptoethanol was not included in the homogenization buffer for determination of the LPO.

Enzyme activity assays: Activity of GR was assayed as reported by Massey and Williams (1965) with slight modifications. Absorbance was determined at 340 nm (UV-Vis spectrophotometer, *Shimadzu*, Hayward, USA) by using NADPH as substrate, and the activity was calculated (coefficient of absorbance of 6.22 mM^{-1} cm^{-1}). One unit (U) of activity was defined as the amount of the enzyme that oxidized 1 nmol of NADPH to $NADP^+$ per minute at 25 °C.

Activity of GST was measured as reported by Habig *et al.* (1974) using chlorodinitrobenzene as substrate. A reaction rate at 340 nm was determined by using a coefficient of absorbance of 9.6 mM^{-1} cm^{-1}. One U was defined as the amount of the enzyme that conjugated 10.0 nmol of chlorodinitrobenzene with reduced glutathione per minute at 25 °C.

Activity of GSH-Px was measured as reported by Paglia and Valentine (1967) with some modifications. A reaction rate at 340 nm was determined by using the NADPH coefficient of absorbance of 6.22 mM^{-1} cm^{-1}. One U was defined as the amount of the enzyme that oxidized 1.0 μmol of GSH to GSSG per minute at pH 7.0 and 25 °C.

Specific activities were expressed as U per mg of protein, and protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard.

GSH and GSSG content and LPO: Content of GSH and GSSG were measured as reported by Teare *et al.* (1993). This method is based on the reduction of GSSG to GSH in the presence of GR and NADPH and on the formation of a colored product by the reaction of GSH with 5,5'-dithiobis-(2-nitrobenzoic acid). The formation of the product was followed by measuring absorbance at 412 nm.

Lipid peroxidation was estimated according to malondialdehyde (MDA) production using a spectrophotometric assay for thiobarbituric acid (TBA) according to Buege and Aust (1976). Absorbance was measured at 532 nm and a coefficient of absorbance of 153 mM^{-1} cm^{-1} was used.

Metal content was measured by inductively coupled plasma spectroscopy (*ICP Model-8410*, *Labtam*, Malcolm, Australia). A fresh tissue (1 g) was digested in 2 cm^3 of concentrated HNO_3 followed by 2 cm^3 of concentrated $HClO_4$. All glassware and apparatus were washed with ultra-pure water and 0.1 M HNO_3 before use.

Statistical analysis: Tukey's test, one of the multiple comparisons, was used for statistical significance analyses. The values are means of three separate experiments.

Results and discussion

In the roots, stems, and leaves of *U. dioica* grown hydroponically in the medium supplemented with 0.045 and 0.09 mM $CdCl_2$ Cd content increased in dependence on the Cd concentration and incubation time (Table 1). Among *U. dioica* organs, the order of magnitude of the Cd accumulation was: root > stem > leaf for the 0.09 mM $CdCl_2$ treated samples for 58 h and reached 1012 μg g^{-1}(f.m). *Raphanus sativus* and *Pisum sativum* roots accumulate lower amounts of Cd compared to *U. dioica* (Dixit *et al.* 2001, Vitoria *et al.* 2001). Whereas Cd content is 27.5 μg g^{-1}(f.m.) in the roots of *Raphanus sativus* exposed to 1 mM Cd for 48 h, it is 640 μg g^{-1}(f.m.) in the roots of *Pisum sativum* after 7-d exposure to 0.04 mM Cd. All these results support an idea that ability to uptake metals differs in plant species and depends on its binding to extracellular matrix, root efflux, and intracellular detoxification (Marchiol *et al.* 1996). In the stems of *U. dioica*, the highest Cd content was 24.3 μg g^{-1}(f.m.) in the presence of 0.09 mM $CdCl_2$ for 50 or 58 h. It was reported that Cd hyper-accumulators can have more than 100 μg(Cd) g^{-1}(f.m.) in their stems without showing any signs of phytotoxicity (Baker and Brooks 1989). Acoording to these results and many other data, it is certain that *U. dioica* is not a Cd hyper-accumulator (Gjorgieva *et al.* 2013). It is well known that Cd is readily taken up by plant roots and translocated to shoots, on most occasions to stems (Leita *et al.* 1991, Chardonnens *et al.* 1998, Hart *et al.* 1998). According to the results, Cd content in the roots of *U. dioica* was much higher in comparison with the stems and leaves, and this suggests that the capacity of *U. dioica* to transport Cd from the roots to the stems and leaves was poor like in

Table 1. The content of different metals [μg g^{-1}(f.m.)] in the roots, stems, and leaves of *U. dioica* in dependence on an external Cd concentration (0, 0.045, and 0.090 mM) and incubation time (12 - 58 h).

Metal	[h]	Roots			Stems			Leaves		
		0	0.045	0.090	0	0.045	0.090	0	0.045	0.090 [mM]
Cd	12	1.11±0.14	180.21±8.68	206.13±14.26	0.24±0.05	13.51±3.84	16.12±2.33	0.011±0.01	6.32±0.58	8.01±1.38
	26	1.13±0.12	205.45±15.51	303.44±10.80	0.23±0.01	16.00±0.88	19.32±3.55	0.010±0.01	7.00±0.77	9.03±0.83
	34	1.14±0.11	280.47±5.79	381.87±12.51	0.22±0.04	16.50±2.03	22.54±2.81	0.012±0.004	7.51±0.44	10.04±1.32
	50	1.17±0.19	410.78±14.29	620.22±18.29	0.20±0.02	17.82±3.08	24.34±2.13	0.012±0.004	8.53±0.95	10.97±2.25
	58	1.19±0.15	580.96±8.39	1012.31±24.39	0.29±0.03	17.83±2.16	24.35±3.56	0.013±0.003	9.01±0.67	11.31±0.61
Fe	12	85.63±3.29	53.25±4.67	49.12±9.77	75.21±4.52	75.45±4.55	65.16±3.27	98.32±2.35	82.45±2.78	64.47±1.86
	26	85.63±4.87	50.11±4.59	39.33±2.89	75.35±3.79	67.85±2.29	58.89±3.65	98.34±2.23	80.74±1.58	58.98±2.33
	34	85.67±3.03	45.45±3.29	31.47±1.89	75.47±5.53	63.63±2.95	54.49±3.84	98.31±5.05	77.45±2.12	53.12±2.05
	50	85.68±3.94	39.36±2.49	26.49±3.73	75.78±3.43	59.41±3.76	50.51±2.73	98.33±7.42	74.12±5.36	51.54±3.67
	58	85.68±4.43	34.47±3.54	22.54±2.98	75.63±3.23	56.69±4.10	48.00±3.01	98.33±3.24	71.69±3.60	50.47±1.81
Zn	12	73.10±2.62	51.94±2.55	40.94±1.25	62.81±2.58	46.44±2.31	41.30±2.17	57.10±2.92	49.74±3.58	45.12±2.71
	26	73.13±2.52	49.54±4.08	37.81±3.42	62.83±2.83	43.81±3.68	37.00±3.68	57.12±1.70	44.93±4.19	38.82±2.14
	34	73.14±7.08	47.63±1.77	37.25±3.85	62.84±2.98	41.00±1.96	31.60±2.69	57.12±2.35	42.48±1.88	35.11±4.14
	50	73.14±5.85	46.23±5.29	35.26±3.16	62.85±5.31	39.90±2.84	30.01±3.92	57.11±2.53	38.22±5.18	30.94±1.19
	58	73.15±3.32	44.31±4.20	33.82±3.84	62.89±3.94	38.75±3.64	28.90±3.86	57.13±2.54	36.31±3.13	30.15±2.89
Mn	12	45.20±1.17	39.82±5.95	37.52±6.18	55.21±3.29	41.69±1.87	31.30±3.02	48.64±3.88	38.73±2.41	32.48±1.96
	26	45.21±4.80	37.13±2.59	33.33±1.85	55.45±2.11	35.11±2.41	26.21±1.46	48.59±2.47	35.39±2.93	28.96±1.34
	34	45.24±2.61	36.22±2.40	30.81±2.54	55.78±3.42	32.31±1.95	21.90±2.85	48.58±3.06	32.11±3.55	26.77±1.85
	50	45.24±3.31	34.81±1.67	29.90±1.40	55.45±1.77	30.03±3.04	20.80±1.96	48.62±1.99	31.47±3.72	22.64±2.61
	58	45.26±4.28	30.44±3.47	28.70±1.89	55.69±3.74	28.30±1.18	19.40±2.66	48.61±2.94	31.22±5.34	22.21±1.34
Cu	12	14.30±1.77	13.11±2.51	11.10±1.83	11.21±1.94	7.50±1.06	7.23±0.37	12.30±2.60	9.83±1.16	9.75±0.46
	26	14.32±1.73	11.21±2.00	9.31±1.79	11.19±1.07	7.20±0.35	6.60±0.26	12.31±1.98	9.67±1.37	8.89±0.67
	34	14.35±2.68	10.01±1.46	8.01±1.41	11.18±1.28	7.00±0.50	6.00±0.54	12.30±1.96	9.01±0.86	8.87±0.79
	50	14.35±0.71	8.91±1.99	7.03±0.18	11.16±1.86	6.70±0.53	5.90±0.19	12.33±1.19	8.91±0.94	8.84±1.19
	58	14.38±2.56	8.61±0.91	7.04±1.36	11.24±1.87	6.20±0.41	5.90±0.32	12.34±3.14	8.77±0.66	8.41±0 53

many other plant species (Sbartai *et al.* 2012, Saidi *et al.* 2013). In addition, a low accumulation of Cd in leaves may be a strategy to protect photosynthetic function (Dixit *et al.* 2001).

It is known that Cd changes directly or indirectly the properties of membranes including their permeability and limits uptake of certain indispensable metals by interfering with regulation of transporter gene expression (Siedlecka and Krupa 1999, Andresen and Küpper 2013). According to our results, the accumulation of Fe, Zn, Mn, and Cu decreased with the increasing Cd concentration in a time-dependent manner in all parts of *U. dioica*. The highest decrease of Fe, Zn, and Cu was in the roots, and of Mn in the stems in the presence of 0.09 mM $CdCl_2$ for 58 h. Wu *et al*. (2010) similarly found that Zn and Cu content in *Osmanthus fragrans* shows a decreasing tendency with an increase in Cd supply.

Glutathione reductase catalyzes reduction of GSSG to the GSH, which is critical in resisting oxidative stress. Its activity is modulated under different stresses including heavy metals (Gallego *et al.* 1996, Chaoui *et al.* 1997, Schickler and Caspi 1999). We observed that the Cd treatment resulted in increases in GR activity in all organs (Fig. 1). In the *U. dioica* roots, GR activities of the 0.045 and 0.09 mM Cd treated samples reached their maxima 34 and 26 h after the treatment and these values were 67.3 and 67.8 % higher than in the control, respectively (Fig. 1). Glutathione reductase activities of the 0.09 mM Cd treated samples were significantly higher in comparison with the 0.045 mM Cd treated ones up to 34 h of the incubation period. Similarly, GR activity in the roots of *Phragmites australis* increases up to 108.8 % after treatment with 0.05 mM Cd for 21 d (Iannelli *et al.* 2002). The higher induction in GR activity of *Phragmites australis* roots than of *U. dioica* may be a result of a longer exposure to Cd. Water hyacinth, *Raphanus sativus*, and *Pisum sativum* are some other plants whose GR activities in their roots are induced with varying concentrations of Cd in a manner consistent with our results (Dixit *et al.* 2001, Vitoria *et al.* 2001, Vestena *et al.* 2011). On the other hand, *Crotalaria juncea* roots show little increase in GR acitivity in the presence of 2 mM $CdCl_2$ (about 22-fold higher than the highest concentration used in this study) for 48 h compared to a control (Pereira *et al.* 2002). In contrast, Schützendübel *et al.* (2001) and Li *et al*. (2013) observed that GR activity decreased with increasing concentrations of Cd in the roots of Scots pine or kenaf.

Glutathione reductase activities in the stems of *U. dioica* treated with both 0.045 and 0.09 mM Cd were significantly higher than in the control during all treatment periods (Fig. 1) and the maximum GR activity was reached at 0.09 mM Cd after 50 h. This value was 40.7 and 145.3 % higher compared to the 0.045 mM Cd treated and control samples for the same time, respectively. Similarly, Iannelli *et al*. (2002) demonstrated that GR activity increases by 283.8 % in *Phragmites australis* stems exposed to 0.05 mM $CdSO_4$ for 21 d.

Glutathione reductase activity of *U. dioica* leaves generally showed a considerable increase in the presence of Cd compared to the control. The maximum GR activity was observed after 50 h for both 0.045 and 0.09 mM Cd, and the increase was 69.3 and 88.2 % in comparison with the control, respectively. A significant increase in GR activity under a low Cd accumulation in leaves may be indicative of a rapid response. Pietrini *et al.* (2003) showed that GR activity slightly increases in *Phragmites australis* leaves treated with 0.05 mM Cd for 21 d, whereas considerably (73.9 %) increases at 0.1 mM Cd. It was, on the other hand, found that GR activity

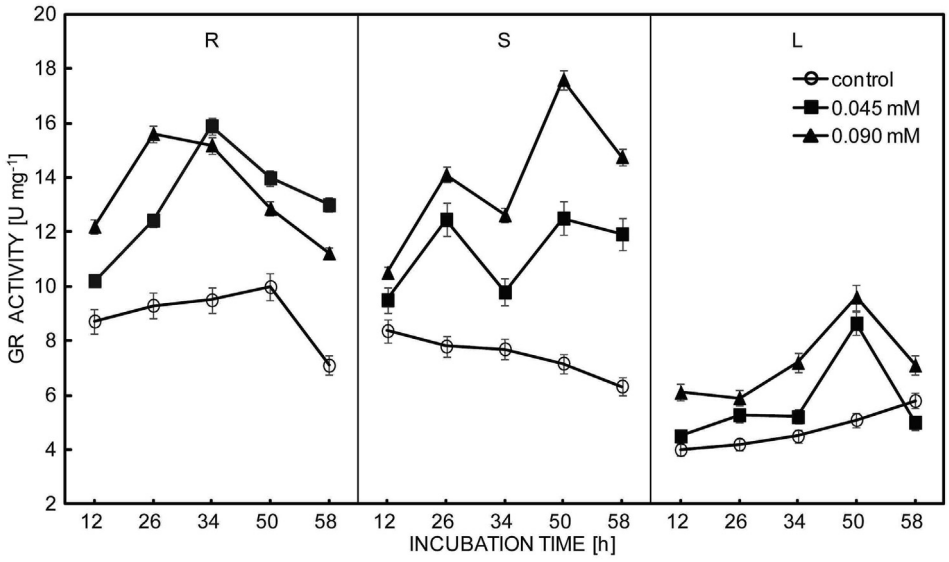

Fig. 1. The activity of GR in roots (R), stems (S), and leaves (L) of *U. dioica* plants grown for 58 h in three different concentrations of $CdCl_2$. Means ± SEs, $n = 3$.

mostly remains lower than control in pea leaves exposed to 0.04 mM Cd in a hydroponic culture for 7 d (Dixit *et al.* 2001).

The results show that the maximum GR activity of *U. dioica* in the presence of 0.09 mM Cd was after 26 h in the roots and after 50 h in the stems and leaves. All the above mentioned data clearly support an idea that GR activity is not only related to different organs and plant species but also to Cd concentration and/or a given period of exposure.

Glutathione-S-transferases are known as enzymes that catalyze the conjugation of Cd with GSH and facilitate the transfer of the Cd-GSH complex to plant vacuoles (Mohanpuria *et al.* 2007). In the *U. dioica* control roots and leaves, GST activity increased by about 30 % during the experimental period. Unlike in the roots and leaves, there was no significant change in stem GST activity except a slight increase of 13 % after 58 h (Fig. 2).

Glutathione-S-transferase activity of the *U. dioica* roots was significantly higher at both Cd concentrations than in the control roots. Glutathione-S-transferase activity reached its maximum after 26 h in the root samples treated with 0.045 mM Cd. This activity was 93.6 and 49.3 % higher than the GST activities of the control and 0.09 mM Cd treated samples for the same time, respectively ($P < 0.01$).

Glutathione-S-transferase activities of both the Cd treated *U. dioica* stem samples showed similar trends during the treatment period (Fig. 2). It was observed that GST activities of both the 0.045 and 0.09 mM Cd treated samples reached their maxima 34 h after the treatment by increasing 30.4 and 46.3 % compared to the control, respectively ($P < 0.01$).

Similarly as in the stems, GST activities in the leaves of *U. dioica* showed their maximum after 34 h for both Cd concentrations but without a significant difference (Fig. 2). The maxima of the 0.045 and 0.09 mM Cd treated samples were 96.5 and 113.7 % higher than the control, respectively.

Glutathione-S-transferases, a group of multiple

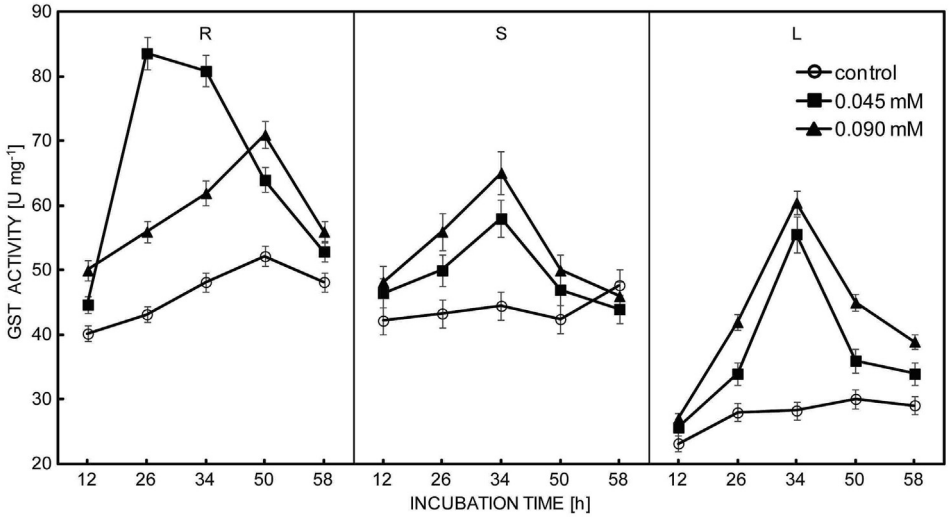

Fig. 2. The activity of GST in roots (R), stems (S), and leaves (L) of *U. dioica* plants grown for 58 h in three different concentrations of $CdCl_2$. Means ± SEs, $n = 3$.

isozymes, are responsible for catalyzing the conjugation of electrophilic xenobiotics with the tripeptide GSH to form polar, non-toxic peptide conjugates (Cummins *et al.* 1997). In addition, GST has also peroxidase activity and can reduce hydroxyl-peroxide toxicity (Marrs 1996). Compared with the stems and leaves, the *U. dioica* roots had a generally higher GST activity. Zhang *et al.* (2013) found that GST activity in *Oryza sativa* stems is similar to that in roots but decreases after incubation in 0.05 mM Cd for 7 d. Glutathione-S-transferase activities in the roots, stems, and leaves of *Phragmites australis* treated with 0.05 mM Cd for 21 d increase by 219.04, 51.47, and 85.71 %, respectively, compared to their controls (Iannelli *et al.* 2002). In *Pisum sativum*, the highest GST activity is in roots treated with 40 μM Cd for 3 d (Dixit *et al.* 2001). According to these results, *U. dioica* is one of the plants whose ability of Cd detoxification is much higher in roots by comparison with the other two organs. On the other hand, a prolonged Cd exposure results in a reduced GST activity in all organs of *U. dioica*, probably because the accumulation of Cd-SH conjugates in cytosol (Grzam *et al.* 2006).

In plants, GSH-Px is a powerful scavenger of H_2O_2 and lipid hydroperoxides with the help of GSH and protects cells against low levels of oxidative stress (Mates 2000). In the *U. dioica* roots, GSH-Px activity for both investigated Cd concentrations showed significant increases after 50 h compared to the control (Fig. 3). However, it was close to the control in the rest of the incubation period.

Although GSH-Px activity of the stem samples treated with 0.045 mM Cd was mostly higher than in the control, the reverse situation was observed for the 0.09 mM Cd treated ones. It was determined that GSH-Px activity in

the presence of 0.045 mM Cd increased by 76.61 % after 50 h compared to the control. Conversely, GSH-Px activities of the 0.09 mM Cd treated stem samples which were significantly lower than the control up to 34 h approached to the control in the later hours of the treatment period (Fig. 3).

In the leaves of *U. dioica*, GSH-Px activity was considerably lower compared to the other two organs. Leaf GSH-Px activities for both investigated Cd concentrations were lower than in the control in all treatment periods. Activities of GSH-Px determined after 34 h at 0.045 and 0.09 mM Cd were 18.54 and 37.51 % lower than in the control, respectively (Fig. 3). Aravind and Prasad (2005) also reported that GSH-Px activity in *Ceratophyllum demersum* considerably decreases after 0.01 mM Cd application for 7 d.

Plants respond to an increased Cd exposure by activating mechanisms involved in the reduction of free cellular Cd and also by activating defence against oxidative stress. Reduced glutathione is an important component of cellular defence where it gets oxidized to GSSG that should be converted back to GSH by GR to perform normal physiological functions (Cuypers *et al.* 2010, Gill and Tuteja 2011). Reduced glutathione is also involved in the formation of phytochelatins and together with GSSG makes up a redox couple (GSH/GSSG) which plays an essential role in the maintenance of cellular homeostasis (Clemens 2006, Srivalli and Khanna-Chopra 2008). Reduced glutathione has a relatively high affinity for binding Cd^{2+} making it a potential cytosolic chelator of this metal (Perrin and Watt 1971, Vogeli-Lange and Wagner 1993). Therefore, Cd-induced depletion of the GSH pool has been shown in many studies (Rauser 1995, Zenk 1996, Lopez *et al.* 2006). On the contrary, the amount of both GSH and GSSG in the roots of *U. dioica* significantly increased at 0.09 mM Cd by 1.73- and 2.04-fold after 50 h, respectively, compared with the control (Fig. 4). Although GSH content was significantly higher at 0.045 mM Cd than in the control, GSSG content was similar to the control up to 50 h of the incubation period. Mohamed *et al*. (2012) also found that GSH is about 5.5- and 6.4-fold higher in the roots of Indian mustard treated with 0.05 and 0.20 mM Cd for 7 d compared with the controls. In contradiction to our results, the same researchers stated that no significant difference between the applied Cd concentrations exists. Also in the *Phragmites australis* roots exposed to 0.050 mM $CdSO_4$ for 21 d, GSH and GSSG content increased 5.69- and 2.74-fold, respectively (Iannelli *et al.* 2002). A GSH/GSSG ratio in the roots of *U. dioica* significantly decreased at 0.090 mM Cd from 34 h in comparison with the control ($P < 0.01$). On the other hand, a GSH/GSSG ratio was significantly higher after 34 h in the 0.045 mM Cd treated root samples than in the control (Fig. 4).

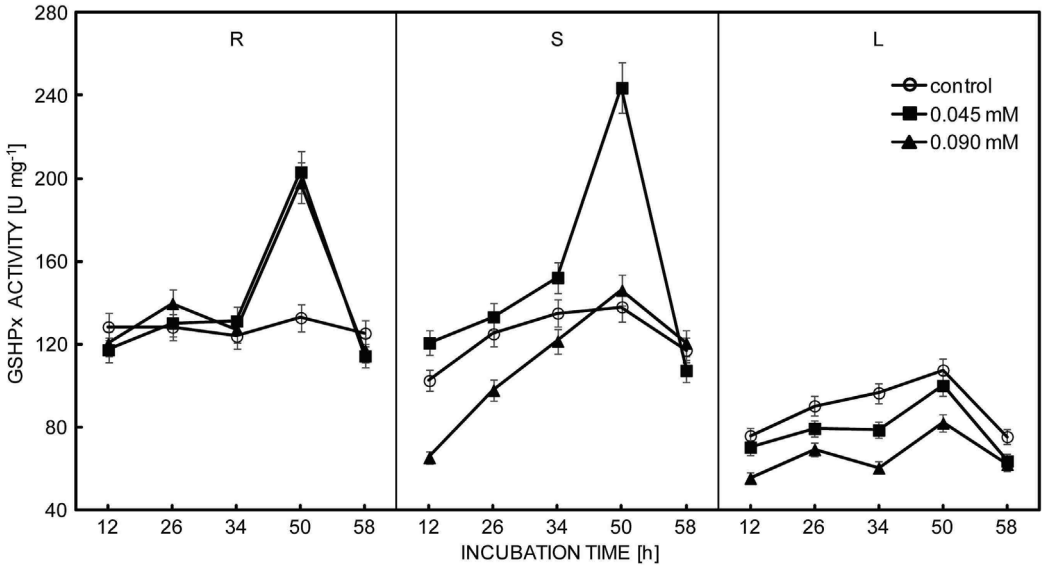

Fig. 3. The activity of GSH-P_x in roots (R), stems (S), and leaves (L) of *U. dioica* plants grown for 58 h in three different concentrations of $CdCl_2$. Means ± SEs, $n = 3$.

Reduced glutathione content in the control *U. dioica* stems and leaves was significantly lower than in the roots. Reduced glutathione content did not show any significant difference up to 34 h for both Cd concentrations and was slightly higher than in the control only after 34 and 50 h in the 0.045 mM Cd treated stem samples (Fig. 4).

It was determined that root GSSG content at 0.045 mM Cd was similar to that in the control, but at 0.09 mM Cd it was higher (Fig. 4). The sharp increase observed in GSH-Px activity of the *U. dioica* stems in the presence of 0.045 mM Cd after 50 h could be related to GSSG content which approached to the control for the same time. In the stems of *U. dioica*, GSH/GSSG ratios

were slightly higher than the control for both Cd concentrations from 26 h of the incubation period (Fig. 4).

The highest increase in GSH content was observed in the leaves of *U. dioica*. After 50 h, GSH content at 0.045 and 0.09 mM Cd increased by 1.87- and 2.02-fold compared to the control, respectively. In the leaves of *U. dioica*, GSSG content similarly reached their maxima after 50 h and was 2.67- and 3.12-fold higher than the control, respectively (Fig. 4). Ratios of GSH/GSSG of the *U. dioica* leaves were at both Cd concentrations generally lower than in the control (Fig. 4). In the study conducted by Shen *et al.* (2012), a decrease in GSH/GSSG ratio was observed in rice leaves with increasing external Cd concentrations.

In this study, MDA content as indicator of LPO in the *U. dioica* roots, stems, and leaves treated with both Cd concentrations was investigated. Whereas MDA content in the 0.045 mM Cd treated roots was lower than in the control, it was higher than in the control at 0.09 mM Cd for all incubation periods (Fig. 5). This result is compatible with the GSH/GSSG ratios which were significantly higher and lower than the control for the 0.045 and 0.09 mM Cd treated root samples, respectively. In the roots of *U. dioica*, the maximum decrease and increase in MDA content were after 34 and 58 h of the Cd treatment for the 0.045 and 0.09 mM Cd exposed samples, respectively. In a previous study, an apparent increase (178 %) in MDA content of *Vicia faba* roots treated with 0.1 mM Cd for 48 h was found (Souguir *et al.* 2011).

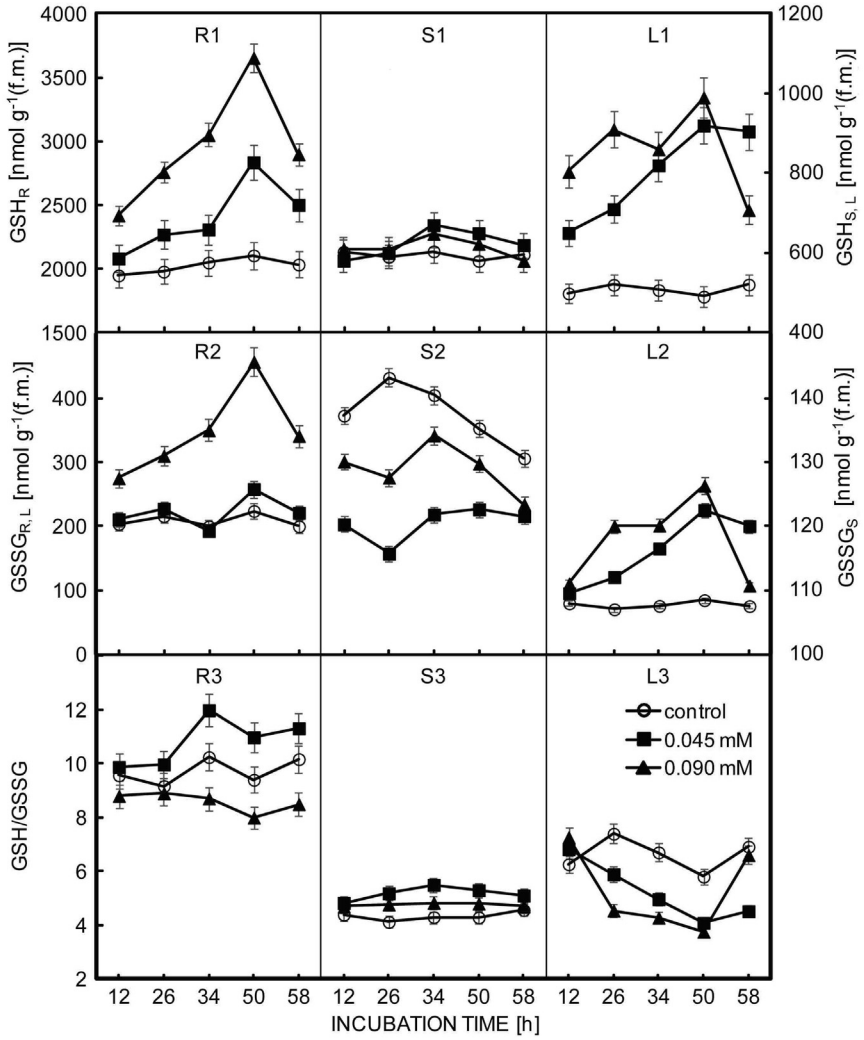

Fig. 4. The variations of GSH and GSSG content and GSH/GSSG ratio in roots (R1-R3), stems (S1-S3), and leaves (L1-L3) of *U. dioica* plants grown for a 58 h in three different concentrations of $CdCl_2$. Means ± SEs, $n = 3$.

Content of MDA in the *U. dioica* stems exposed to 0.045 and 0.09 mM Cd was lower than in the control throughout all incubation periods but not significantly from 34 h in the 0.09 mM Cd treated ones (Fig. 5). The highest decreases in MDA content was by 26.3 and 13.7 % after 26 and 12 h for the 0.045 and 0.09 mM Cd treated samples, respectively, compared to their controls. In contrast to the stems, MDA content was higher at both

Cd concentrations than in the control, and the highest increase was found after 58 h (40.3 % for 0.045 mM and 54.4 % for 0.09 mM Cd treated samples). There were significant differences in LPO between 0.045 and 0.09 mM Cd for all incubation periods. From these results and also considering the GSH/GSSG ratios, it can be deduced that although the capacity of *U. dioica* transporting Cd from the roots to stems and leaves was poor, the leaves suffered from oxidative stress under the Cd treatments.

In conclusion, the data presented in this study demonstrate that in consequence of external Cd, this metal mostly accumulated in the roots of *U. dioica* and reduced the accumulation of Fe, Zn, Mn, and Cu. Although the Cd treatment resulted in the increase in GR and GST activities in all the organs, GSH-Px activity in the leaves was lower than in the control for both applied Cd concentrations. The decrease in GSH/GSSG ratio together with the significant increase in LPO clearly show the Cd induced oxidative stress in the leaves of *U. dioica*.

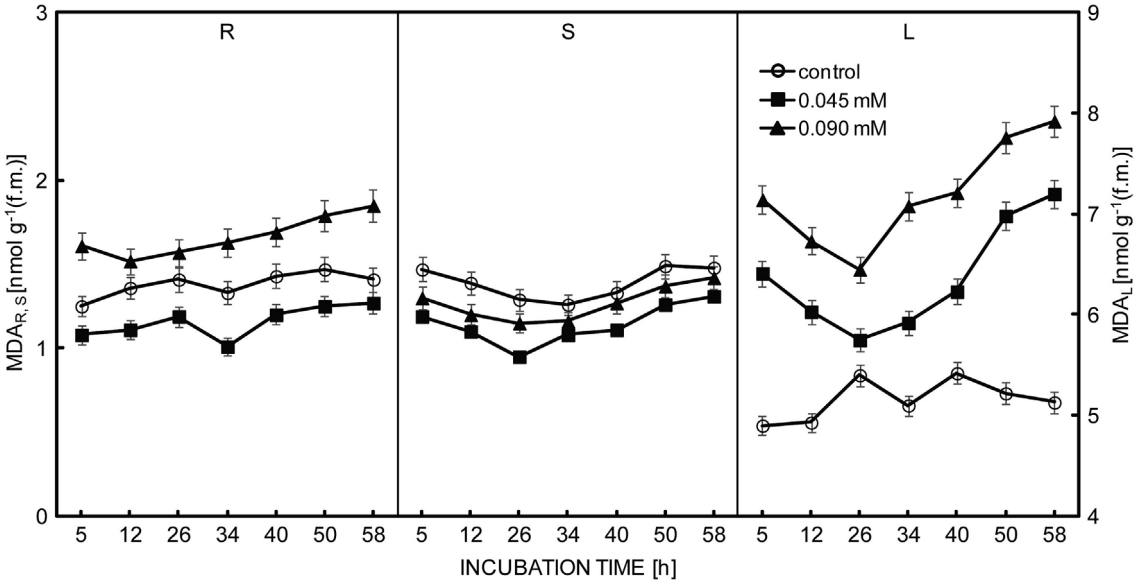

Fig. 5. The variations of LPO (MDA content) in roots (R), stems (S), and leaves (L) of *U. dioica* plants grown for a 58 hour period in three different concentrations of $CdCl_2$. Means ± SEs, $n = 3$.

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