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The effect of cadmium on growth and antioxidant responses in the safflower (*Carthamus tinctorius* L.) callus

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Abstract: Safflower (*Carthamus tinctorius* L.) callus cultures grown in the presence of different concentrations (0-100 µM) of cadmium were assayed for growth, Cd accumulations, and antioxidative responses. Cadmium inhibited the growth of calli by 25% and 46% at 75 and 100 µM Cd concentrations, respectively. Cadmium concentration in the calli increased with an increased Cd supply in the growth medium. Calli accumulated 334 mg Cd kg–1 of their dry weight at 100 µM Cd. Cadmium induced oxidative stress, which was indicated by modulating antioxidant (glutathione and α-tocopherol) levels and antioxidative enzymes. The effect of Cd on glutathione (GSH) was dose-dependent. The GSH content increased up to a concentration of 75 µM Cd and then decreased. Levels of α-tocopherol showed a significant increase with the increase in concentrations of Cd in the media. Antioxidant enzyme activity increased significantly up to a concentration of 75 µM Cd. Concentrations of Cd greater than 75 µM resulted in a decline in antioxidant enzyme activity. Increased antioxidant levels and antioxidant enzyme activity in Cd-treated calli indicate that Cd detoxification and accumulation in the safflower callus might be associated with the efficiency of these mechanisms.

Key words: Cadmium, callus, *Carthamus tinctorius*, glutathione, α-tocopherol

Introduction

Cadmium is an important widespread trace pollutant with high toxicity to plants, animals, and humans. Cadmium causes various phytotoxic symptoms including chlorosis, growth inhibition, water imbalance, phosphorus and nitrogen deficiency, reduced manganese transport, and accelerated senescence in plants (Mishra et al. 2006). It has been suggested that growth inhibition by Cd is due to a direct effect of Cd on the nucleus or interaction with hormones, and, in the aerial parts of the plants, it is due to the inhibition of photosynthesis (Laspina et al. 2005). Heavy metals cause molecular damage to plants, either directly or indirectly, through reactive oxygen species (ROS) formation. Although the mechanism of the metal's damaging action is not clearly understood, there is increasing evidence suggesting that, at least in part, metal toxicity is due to oxidative damage (Ganesh et al. 2008).

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High cadmium levels can cause oxidative stress by favoring the production of reactive oxygen species. Since higher plants are immobile, they cannot escape from environmental stresses. Higher plants survive in a constantly fluctuating environment, which has driven the evolution of a highly flexible metabolism and the development necessary for their sessile lifestyle (Pitzschke and Hirt 2006). The ability of higher plants to scavenge the toxic effects of active oxygen seems to be a very important determinant of their tolerance to these stresses. Antioxidants are the first line of defense against free radical damage. This antioxidant defense system mainly includes antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR) and nonenzymatic antioxidant compounds such as glutathione (GSH), ascorbic acid (ASA), and α-tocopherol. SOD is the key enzyme responsible for catalyzing the dismutation of highly reactive $O_2^{\bullet-}$ to O_2 and H_2O_2. The resulting H_2O_2 is further decomposed to water and oxygen either by the APX of the ascorbate-glutathione cycle or by the GPX and CAT localized in the cytoplasm and other cellular compartments. Tocopherol, a lipid soluble antioxidant found in all parts, is a potential scavenger of ROS and lipid radicals (Gajewska and Sklodowska 2007). Out of 4 isomers of tocopherols (α-, β-, γ-, δ-) found in plants (Desel et al. 2007), α-tocopherol has the highest antioxidative activity due to the presence of 3 methyl groups in its molecular structure.

Heavy metal stresses have been studied for their effects on callus growth and antioxidant systems (Nilesh et al. 2005; Israr et al. 2006). In order to obtain insights into the antioxidative response to Cd stress in cell cultures, safflower (*Carthamus tinctorius* L.) calli were exposed to this metal and the effect on growth and antioxidant responses were analyzed.

Safflower (*Carthamus tinctorius* L.) is a crop plant of the family Asteraceae with a wide geographical distribution; it possesses interesting characteristics useful in Cd-accumulation (Madaan and Mudgal 2009; Sayyad et al. 2009; Sayyad et al. 2010). Although many reports regarding the medicinal and agronomic aspects of this plant are available, to our knowledge, there is no study dealing with the Cd tolerance of safflower calli or the role of antioxidant systems in the expression of Cd toxicity in safflower calli. This study therefore aimed to examine the response of the safflower callus to oxidative stress posed by Cd with reference to the gross effect on biomass, antioxidant enzymes (SOD, APX, and GR), and other antioxidants (GSH and α-tocopherol) in order to see the stress exerted by the metal as well as the level of tolerance and the detoxification strategy adopted by the callus.

Materials and methods

Establishment of safflower cell suspension culture

The seeds of *Carthamus tinctorius* L. (cv. Arak2811) were surface sterilized with a 0.1% (w/v) $HgCl_2$ solution for 8 min. After rinsing 4-5 times with sterile distilled water, seeds were aseptically germinated on semisolid Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) without growth regulators. The cultures were incubated at 25 ± 2 °C under a 16-h photoperiod of cool white fluorescent lights (55 μmol m^{-2} s^{-1}). In subsequent experiments, 8-day-old seedlings were used. Leaf blade segments (3-5 mm) derived from 8-day-old seedlings were used as explants (Gao et al. 2000). These segments were placed on semisolid basal MS medium supplemented with various concentrations of α-naphthaleneacetic acid (NAA) (0.0, 0.5, 1.0, 2.0, and 3.0 mg L^{-1}) and benzylaminopurine (BA) (0.0, 0.25, 0.5, and 1.0 mg L^{-1}) alone or in combination for the induction of calli. Preliminary experiments showed that the rate of callus growth was better on the MS medium containing 0.25 mg L^{-1} (1.109 μM) BA and 1 mg L^{-1} (5.37 μM) NAA (data not shown), so this treatment was used in subsequent experiments. After 4 weeks of callus proliferation, 10 g of callus mass was homogenized aseptically in 50 mL of MS medium and used as a stock culture to initiate suspension cultures in 250-mL flasks. Equal volumes (5 mL) of the stock cultures were transferred to 100 mL of MS growth medium containing 0.25 mg L^{-1} BA and 1 mg L^{-1} NAA. Flasks containing cultures were placed on a shaker (125 rpm) and incubated for 4 weeks at 25 ± 2 °C under a 16-h photoperiod with cool white fluorescent lights (55 μmol m^{-2} s^{-1}).

Cadmium treatment and growth assay

Exponentially growing cells were filtered through 3 layers of sterile cheese cloth and resuspended in fresh growth medium at a concentration of 200 mg cell mL^{-1}. This suspension was continuously stirred while pipetting 1-mL aliquots onto the surface of 9-cm disks of Whatman No. 2 qualitative filter paper that had been placed on 25 mL of agar (0.8%) medium in 100 mm × 25 mm plastic petri plates. The agar growth medium contained 1.109 µM BA and 5.37 µM NAA and different concentrations of $CdCl_2$ (0, 25, 50, 75 and 100 µM $CdCl_2.H_2O$). The fresh weight of the culture was measured using filter paper growth assay (Horsch et al. 1980). Culture growth was recorded during weeks of 1-3 and expressed as a percentage increase in the fresh weight (FW).

Estimation of Cd

Calli were harvested after 3 weeks of growth under control and Cd media, thoroughly washed with deionized water, and oven-dried at 70 °C for 2 days. Dried callus samples (100 mg) were digested in a $HNO_3/HClO_4$ (3:1 v/v) mixture at 160 °C. Digested material was diluted with deionized water and the Cd concentration was determined using an atomic absorption spectrophotometer (PerkinElmer, Germany).

Nonenzymatic antioxidant assays

Reduced (GSH) and oxidized (GSSG) glutathione contents were determined by the recycling method described by Anderson (1985). Fresh callus (0.5 g) was homogenized in 0.3 mL of 5% sulfosalicylic acid under cold conditions. The homogenate was centrifuged at 10,000 rpm for 10 min and a 0.5-mL aliquot was taken in a microfuge tube, to which 0.5 mL of reaction buffer [0.1 M phosphate buffer (pH 7.0), 0.5 mM ethylenediaminetetraacetic acid (EDTA)] and 50 µL of 3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were added. After 5 min, absorbance was read for the determination of GSH at 412 nm using a UV-Vis spectrophotometer (Model UV-1601 PC, Shimadzu Corporation, Japan). To the same tube, 100 µL of nicotinamide adenine dinucleotide phosphate (NADPH) (0.4 mM) and 2 µL of GR were added for the determination of total glutathione; the reaction was allowed to run for 20 min and the amount of GSSG was calculated by subtracting the GSH from the total glutathione concentration. A standard curve was prepared from varying concentrations of reduced glutathione. The α-tocopherol content was assayed as described by Backer et al. (1980). Briefly, 0.5 g of fresh callus was homogenized with 10 mL of a mixture of petroleum ether and ethanol (2:1.6 v/v) and the extract was centrifuged at 10,000 rpm for 20 min; the supernatant was then used for the estimation of α-tocopherol. To 1 mL of extract, 0.2 mL of 2% 2,2-dipyridyl in ethanol was added, mixed thoroughly, and kept in the dark for 5 min. The resulting red product was diluted with 4 mL of distilled water and mixed well. The resulting color in the aqueous layer was measured at 520 nm. The α-tocopherol content was calculated using a standard graph made with a known amount of α-tocopherol.

Antioxidant enzyme activity assays

Fresh callus samples from different treatments (500 mg) were harvested and homogenized with a mortar and pestle in 100 mM cold phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpolypyrrolidone (PVP) at 4 °C. After homogenization in cold phosphate buffer, homogenates were centrifuged at 15,000 rpm at 4 °C for 15 min to remove plant debris. The supernatant was used for the following antioxidant enzyme activity assay. All enzymatic activities were measured at 25 °C with a UV-Vis spectrophotometer (Model UV- 1601 PC, Shimadzu Corporation).

SOD (EC 1.15.1.1) activity was measured according to the method of Beauchamp and Fridovich (1971). A single unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition of the nitroblue tetrazolium (NBT) reduction rate at 560 nm. APX activity (EC 1.11.1.11) was measured according to the method of Nakano and Asada (1987) by monitoring the rate of ascorbate oxidation at 290 nm ($E = 2.8\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0, containing 0.1 mM EDTA), 0.1 mM H_2O_2, 0.5 mM sodium ascorbate, and the enzyme aliquot. One unit of enzyme is the amount necessary to decompose 1 µmol of substrate per minute at 25 °C. GR activity (EC 1.6.4.2) was assayed following the method of Rao et al. (1996) by monitoring the decrease in absorbance at 430 nm caused by NADPH

oxidation (E = 6.22 mM^{-1} cm^{-1}). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5, containing 1 mM EDTA), 0.5 mM oxidized glutathione (GSSG), 0.2 mM NADPH, and the enzyme aliquot. One unit of enzyme is the amount necessary to decompose 1 μmol of NADPH per minute at 25 °C.

Determination of soluble protein contents

Protein content determination in the current study followed the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Statistical analysis

Values in the figures and tables are mean values ± standard error (SE) of 2 independent experiments performed in 3 replicates (n = 6). Duncan's multiple range test was also performed in order to determine the significant difference between treatments.

Results

Effect of Cd on growth and the accumulation of Cd in safflower calli

The safflower calli grew and proliferated over the course of time on increasing concentrations of $CdCl_2.H_2O$ (Figure 1). While the growth of tested calli was comparable to that of the control at lower concentrations of Cd (25 and 50 μM Cd), higher concentrations (75 and 100 μM Cd) reduced callus growth. Compared to the control, reductions of 25% and 46% were observed in callus growth at concentrations of 75 and 100 μM Cd, respectively.

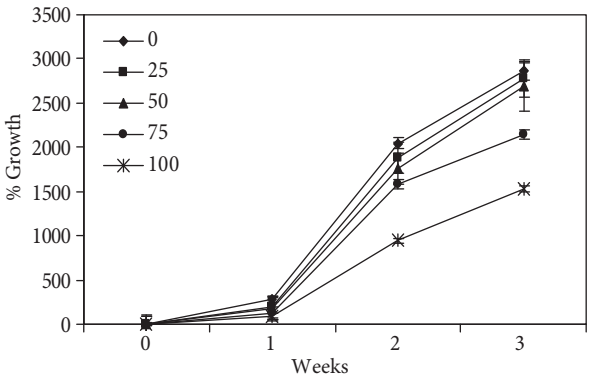

Figure 1. The growth of *Carthamus tinctorius* L. calli in different concentrations of Cd. Vertical bars represent SE (n = 6).

Cadmium concentrations in safflower calli revealed a linear increase in response to increasing external Cd supply levels (Table 1). Cadmium concentrations of calli treated with 75 and 100 μM Cd reached 173 and 334 mg Cd kg^{-1} dry weight (DW), respectively (Table 1).

Effect of Cd on glutathione and α-tocopherol levels in safflower callus

Cadmium addition was found to significantly increase GSH levels when compared to the control. The maximum GSH content in the calli was observed at 75 μM Cd, which was 71% higher than in the control (Table 2).

An increase was recorded for GSSG but this was observed to be significant only for the higher rate of 100 μM Cd (Table 2).

Under Cd treatment, the GSH-to-GSSG ratio increased in the calli, but no significant difference was observed between Cd-treated tissues (Table 2).

Levels of α-tocopherol also revealed a remarkable increase in calli grown under Cd stress (Table 2). The application of Cd resulted in a significant enhancement of α-tocopherol levels in the safflower calli (Table 2). Cadmium caused increases in α-tocopherol levels by 46% and 68% at 75 and 100 μM Cd, respectively, in comparison to the control.

Effect of Cd on antioxidative enzyme activities

The effect of varying concentrations of Cd on antioxidative enzyme activities during the growth of safflower calli revealed an increase in activity in

Table 1. The accumulation of Cd in the calli of *Carthamus tinctorius* L.

Cd conc. (μM)	Callus Cd content (mg kg^{-1} DW)
0	2.34 ± 0.01 a
25	39.03 ± 0.07 b
50	86.47 ± 2.4 c
75	173.12 ± 4.3 d
100	334.57 ± 6.9 e

Each value represents means ± SE (n = 6). Different letters indicate significant differences between treatments at $P \leq 0.05$ using Duncan's multiple range test.

Table 2. Levels of reduced and oxidized glutathione, reduced and oxidized glutathione ratios, and α-tocopherol in the calli of safflower.

Cd conc. (μM)	GSH (nmol g^{-1} FW)	GSSG (nmol g^{-1} FW)	GSH-to-GSSG ratio	α-tocopherol (μg g^{-1} FW)
0	58.8 ± 2.4 a	15.7 ± 1.3 a	3.7 ± 0.13 a	43 ± 1.3 a
25	109.1 ± 4.7 b	16.5 ± 3.4 a	6.6 ± 0.28 b	52 ± 3.6 b
50	172.6 ± 6.9 c	24.1 ± 2.8 ab	7.1 ± 0.19 b	64 ± 9.2 c
75	203.4 ± 5.2 d	27.2 ± 4.6 ab	7.4 ± 0.24 b	81 ± 7.1 d
100	199.5 ± 10.3 d	29.4 ± 5.8 b	6.8 ± 0.29 b	137 ± 12.8 e

Each value represents means ± SE (n = 6). Different letters within columns indicate significant differences between treatments at $P \leq 0.05$ using Duncan's multiple range test.

a concentration-dependent manner up to 75 μM Cd (Figure 2). These activities tended to decrease at a concentration of 100 μM Cd. In every case, however, the activities were appreciably higher than those found in the absence of Cd (control). The highest SOD activity, 79% higher than that of the control, was noted at 75 μM Cd. This trend declined slightly at 100 μM Cd (Figure 2).

As shown in Figure 2, increasing Cd concentrations (from 0 to 100 μM Cd) caused an increase in APX activity by 85% at 75 μM Cd when compared with the control. However, a slight decrease in APX activity was observed in the calli at 100 μM Cd, which was higher than the control by 82% (Figure 2).

GR activity showed a similar trend as observed for SOD and APX activity in response to increasing concentrations of Cd in media (Figure 2). The maximum GR activity was observed at 75 μM Cd, with results that were about 41% higher than those of the control (Figure 2).

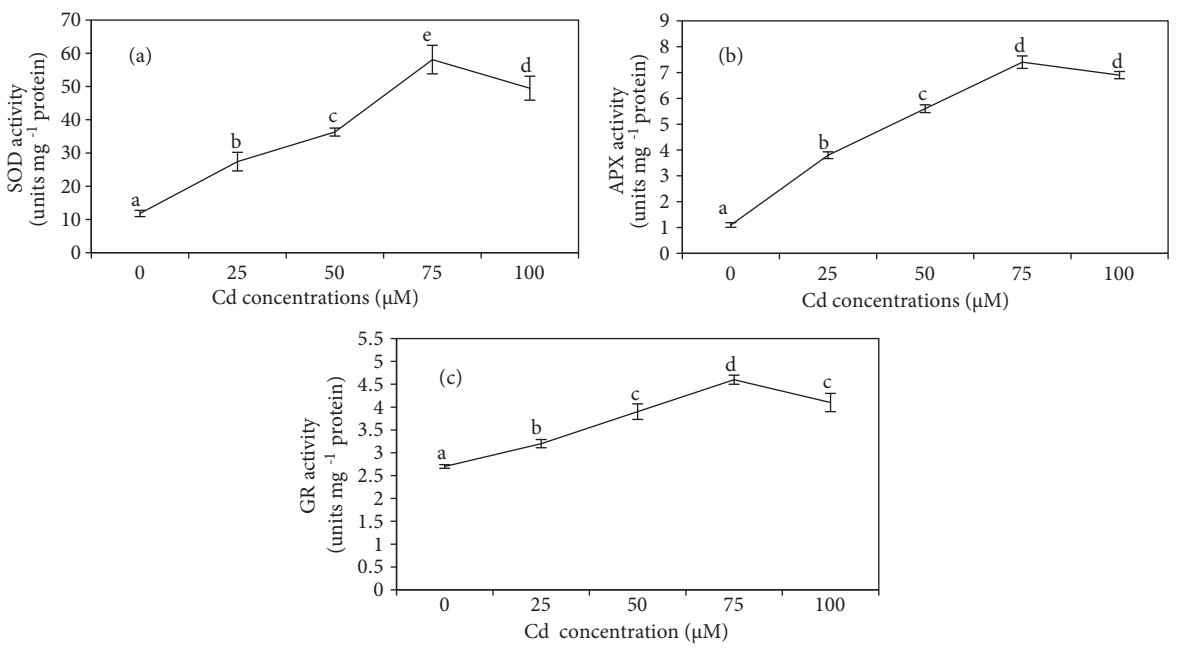

Figure 2. The effect of Cd treatments on SOD (a), APX (b), and GR (c) activities of safflower calli. Vertical bars represent SE (n = 6) and different letters indicate significant differences between treatments at $P \leq 0.05$ using Duncan's multiple range test.

Effect of Cd on soluble protein contents

Increasing the Cd concentration in the growth medium caused significant protein accumulation ($P \leq 0.05$) in safflower calli. A maximum protein concentration 63% higher than the control was noted at 100 μM Cd (Figure 3).

Discussion

Cadmium exposure led to the inhibition of callus growth in a concentration-dependent manner. The toxic effect of the Cd was evident from the reduced biomass seen at higher Cd concentrations. The reduction of callus growth at high levels of Cd may be correlated with high Cd accumulations by the calli (Table 1). This result was in agreement with previous studies that have observed that, in *Sesbania drummondii* (Nilesh et al. 2005; Israr et al. 2006) and *Cuscuta reflexa* (Srivastava et al. 2004), callus growth was significantly inhibited at higher concentrations of Cd. In contrast, a Cd-induced decrease in growth has been observed in soybean cells at lower concentrations (Sobkowiak et al. 2004).

The accumulation of Cd by the calli was found to be concentration-dependent (Table 1). Particularly at the highest concentration of Cd (100 μM Cd), cadmium accumulation was enormous, and this affected callus growth dramatically. In fact, 334.57 mg Cd kg^{-1} DW can be considered hyperaccumulation (Brooks 1998), which has never been reported for this species.

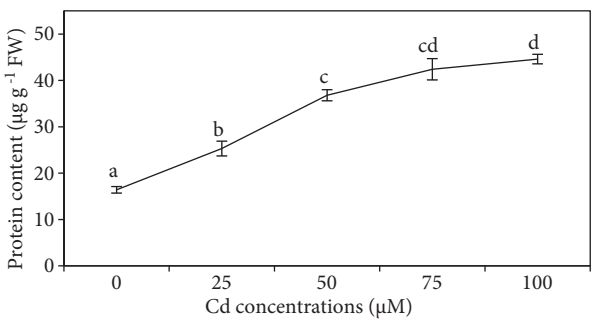

Figure 3. The protein content (μg g^{-1} FW) of *Carthamus tinctorius* L. calli exposed to different concentrations of Cd. Vertical bars represent SE (n = 6) and different letters indicate significant differences between treatments at $P \leq 0.05$ using Duncan's multiple range test.

In this study, the effect of Cd on GSH concentrations was found to be dose-dependent (Table 2). The high content of GSH in Cd-treated calli, as compared to the control, confirms the active participation of GSH in the detoxification of oxygen species and free radicals (Mendoza-Cozatl et al. 2006). An increased concentration of GSH has been observed with increasing Cd concentrations in *Sedum alfredii* (Sun et al. 2007), while the decay of GSH concentrations has been reported in *Helianthus annuus* L. (Gallego et al. 2002).

GSH can react chemically with single oxygen, superoxide, and hydroxyl radicals, and, therefore, function directly as a free radical scavenger. This confirms the suggestion that GSH can trap Cd only when in the reduced state. GSH and its oxidized form, GSSG, maintain a redox balance in the cellular compartments. The conversion of GSSG to GSH by the GR enzyme was correlated with the change in GSH-to-GSSG ratios, which play an important role in the signal transduction of several transcription and metabolic processes. GSH is also the precursor for the phytochelatins that act as heavy metal-binding peptides in plants (Ozturk et al. 2003; Ahmad et al. 2009). The high GSH-to-GSSG ratio in response to Cd therefore seems to be necessary, not only for the role of glutathione as a reductant (GSH), but also to achieve optimal protein synthesis in the plant cells (Mishra et al. 2006; Ahmad et al. 2009). In this study, the increased GSH-to-GSSG ratio (at up to 75 μM Cd) may account for the ability of safflower calli to tolerate Cd toxicity. Similar to the results observed in the present study, an increase in GR activity in calli following exposure to Cd was noticed in *Cuscuta reflexa* (Srivastava et al. 2004). GR recycles the GSSG back into GSH, which is crucial for the functioning of the ascorbate-glutathione cycle as well as for the synthesis of phytochelatins (Ahmad et al. 2009). The considerable change in GR activity observed in this study proves the above hypothesis. The increased activity of GR could also be explained by transcriptional or translational modification in order to keep an adequate GR level to protect against Cd stress (Mishra et al. 2006).

Cadmium treatments induced a change in the contents of α-tocopherol in safflower calli (Table 2). The synthesis of low-molecular-weight antioxidants,

such as α-tocopherol, has been reported in a number of stressed plants (Colin et al. 2008; Jaleel 2009; Yusuf et al. 2010). Oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in plants (Zhang et al. 2006; Yusuf et al. 2010). Based on the above trends, this study suggests that α-tocopherol also plays an important role in Cd detoxification.

The activity of SOD, which is responsible for the elimination of superoxide radicals in cells, showed a remarkable increase in safflower calli grown under Cd stress (Figure 2). However, with 100 μM Cd treatment, SOD activity decreased slightly. This was in agreement with the account of SOD activity reported in the adapted calli of sunflower cultivated under Cd treatments (Gallego et al. 2002). The increase in SOD activity may be attributed to the increased production of active oxygen species (Somashekaraiah et al. 1992) or to increased expression of the genes encoding SOD (Mishra et al. 2006). A reduction in SOD activity at higher Cd concentrations may result from the inactivation of the enzyme by H_2O_2, which is produced in different cellular compartments or from a number of nonenzymatic and enzymatic processes in cells (Dixit et al. 2001; Romero-Puertas et al. 2007).

APX activity was also induced in response to Cd treatments in the safflower calli. APX activation by Cd has been shown in the adopted calli of sunflower (Gallego et al. 2002) and in *Sesbania drummondii* (Israr et al., 2006). APX functions in chloroplasts in the ascorbate-glutathione cycle and protects the cell against oxidative damage by detoxifying toxic H_2O_2. Upon exposure to the increasing Cd supply (from 0 to 100 μM Cd), safflower calli increased the level of APX activity within 3 weeks (Figure 2). This increase possibly reflects enhanced ROS production and thus the activation of H_2O_2-scavenging enzymes of the ascorbate-glutathione cycle (i.e. APX and GR).

In this study, it was determined that Cd treatments stimulated protein accumulation in safflower calli. An increase in the protein level of the safflower calli may be attributed to the induction of stress protein (Sanita di Toppi and Gabbrielli 1999). Stress proteins constitute various antioxidant enzymes and other enzymes involved in GSH and phytochelatin biosynthesis, including some heat shock proteins.

It can be concluded that the safflower callus has a high ability to adapt to Cd toxicity and Cd hyperaccumulation. Cadmium accumulation was significantly enhanced with increasing concentrations of Cd in the medium, suggesting its usefulness in understanding the mechanism of the metal resistance. Elevated antioxidant (GSH and α-tocopherol) levels and enzymatic antioxidant (SOD, APX, and GR) activity seemed to be more important and the most promising reply to the higher Cd tolerance and accumulation in safflower calli.

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