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Induction of phytochelatin and responses of antioxidants under cadmium stress in safflower (*Carthamus tinctorius*) seedlings

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Abstract: We investigated the role of antioxidant compounds (e.g., α-tocopherol, phytochelatins, glutathione, and other non-protein thiols) in the cadmium (Cd) tolerance of safflower (*Carthamus tinctorius* L. cv. Arak2811) seedlings exposed to different concentrations of Cd (0-100 µM) for a week. A concentration- and tissue- dependent response to Cd was observed. Increasing Cd supply markedly reduced the dry weight of roots. Plants accumulated a substantial amount of Cd, especially in the roots. Levels of α-tocopherol showed a significant increase with an increase in the concentration of Cd in leaves. Upon Cd exposure, α-tocopherol levels followed a similar pattern in the root tissue with no significant change as compared to the control. Cadmium exposure caused a significant increase in non-protein thiols and phytochelatin levels in roots, whereas non-protein thiols and phytochelatin levels were not affected in leaves. The glutathione content in leaves significantly increased with increasing Cd concentrations, whereas in roots glutathione contents increased up to a concentration of 50 µM Cd and then decreased. The results indicate that the non-protein thiol and phytochelatin biosynthesis induction in roots and enhanced level of α-tocopherol and glutathione in leaves may be involved in Cd tolerance and hyperaccumulation in safflower.

Key words: Cadmium, safflower, phytochelatin, α-tocopherol, glutathione, non-protein thiols

Introduction

Heavy metals are major environmental pollutants. Among heavy metals, Cd is one of the most dangerous elements to plants. Cadmium accumulation in the soil may come from different sources, including air pollution and soil applications of commercial fertilisers, sewage sludge, manure, and lime (López-Milán et al., 2009). The accumulation of Cd in plant tissues may cause a variety of toxicity symptoms ranging from chlorosis, wilting, and growth reduction to cell death (Nocito et al., 2007). It is therefore important to develop methods of cleaning up Cd in contaminated soils (Liu et al., 2011). Phytoremediation, in which hyperaccumulators are used to take up large quantities of pollutant metals, has become a promising soil remediation technique. Phytoremediation is a natural process that uses various types of green plants to remove, transfer, or stabilise contaminants in soil, sediment, and ground water (Yu & Gu, 2007).

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During the last decade, a number of studies have been conducted to investigate the mechanisms responsible for enhanced metal uptake and tolerance using natural hyperaccumulators as model plant species (Mishra et al., 2006; Jin et al., 2008; Zeng et al., 2009; Liu et al., 2011). Plants have evolved a number of mechanisms to cope with heavy metal stress (Mishra et al., 2006; Bishehkolaei et al., 2011). These include the synthesis of low molecular weight antioxidants that consist of lipid-soluble membrane-associated antioxidants (e.g., α-tocopherol and β-carotene) and the S-rich metal chelators glutathione and phytochelatins (Nocito et al., 2007).

Tocopherols are lipid soluble antioxidants found in all parts and are potential scavengers of reactive oxygen species (ROS) and lipid radicals (Jaleel et al., 2009). Out of 4 isomers of tocopherols (α-, β-, γ-, δ-) found in plants, α-tocopherol has the highest antioxidative activity due to the presence of 3 methyl groups in its molecular structure.

Glutathione (GSH) plays a central role in protecting plants from environmental stresses, including oxidative stress, xenobiotics, and some heavy metals (Wu et al., 2004). Indeed, GSH acts as an antioxidant, quenching the ROS generated in response to stress before the ROS can cause damage to cells. Glutathione also serves an additional function in plant responses to heavy metal stress as a precursor of phytochelatins (Mishra et al., 2006).

Phytochelatins (PCs), with the basic structure of $(\gamma\text{- Glu- Cys})_n$-Gly, where n = 2-11, have been implicated as playing an important role in plant metal tolerance. They are glutathione-derived peptides synthesised by the transpeptidase phytochelatin synthase. Phytochelatins seems to be an intercellular mechanism for Cd detoxification by shuttling PC-Cd complexes into plant cell vacuoles (Mendoza-Cozalt et al., 2008).

Safflower (*Carthamus tinctorius*) is a crop plant of the family Asteraceae, with a wide geographical distribution. It possesses interesting characteristics in terms of heavy metal accumulation. It has been reported that safflower is capable of accumulating high levels of Cd in roots and leaves without showing symptoms of toxicity (Shi et al., 2010; Namdjoyan et al., 2011). In our previous study, it was shown that *C. tinctorius* cv. Arak2811 had a strong tolerance to Cd in the nutrient medium and a strong accumulation capability for Cd in roots (Namdjoyan et al., 2011). The objectives of this study were to investigate the accumulation of α-tocopherol, PCs, GSH, and other non-protein thiols in seedlings of a cadmium-tolerant cultivar of safflower (*C. tinctorius* cv. Arak2811) in order to understand the stress exerted by the Cd and the detoxification strategy adopted by the plants.

Materials and methods

Plant material and growth conditions

The safflower cultivar *Carthamus tinctorius* cv. Arak2811 was used. The seeds were germinated in petri dishes under sterile conditions at a temperature of 25 ± 1 °C for 48 h, transferred to pots (300 mm in diameter) containing a mixture of sand and perlite (1:1, v/v), and irrigated with a nutrient solution (1 mg L^{-1} KNO_3; 250 mg L^{-1} $Ca(H_2PO_4)_2$; 250 mg L^{-1} $MgSO_4.7H_2O$; 2.3 mg L^{-1} H_3BO_3; 1.8 mg L^{-1} $MnCl_2.4H_2O$; 0.22 mg L^{-1} $ZnSO_4.7H_2O$; 0.08 mg L^{-1} $CuSO_4.5H_2O$; 0.02 mg L^{-1} H_2MoO_4; and 6.92 mg L^{-1} FeEDTA). The seedlings were grown for 10 days in a growth chamber (200 μE/($m^{-2}s^{-1}$) featuring a 12 h photoperiod, 60 ± 5% relative humidity, and a temperature of 25 ± 1 °C. Administration of the cadmium treatment ($CdCl_2.2.5H_2O$) was performed after 10 days. Different Cd concentrations (0, 50, 75, and 100 μM) were applied for 7 days. After harvesting, the plants were washed with double distilled water, separated into leaves and roots, and dried at 80 °C in order to determine their dry weights and Cd concentrations.

Cadmium concentrations in plants

Cd concentrations were determined by atomic absorption spectrophotometer (AAnalyst 300; Perkin Elmer Corporation, Germany) after wet digestion of the dried root and leaf tissue (100 mg) in a 10 mL mixture of analytical grade acids HNO_3 / $HClO_4$ at a ratio of 3:1 (v/v) as well as after dry ashing (by gradually increasing temperature from 160 °C to 500 °C over a period of 1.5 h, followed by 2 h at 500 °C). After cooling, the solution was brought to a final volume of 30 mL with deionised water. The element contents of the samples were quantified by comparison with standard solution at appropriate dilution (Merck, Darmstadt, Germany): 0.1 μg mL^{-1} Cd.

Estimation of α-tocopherol content

The α-tocopherol content of the plants was assayed as described by Backer et al. (1980). Briefly, 500 mg of fresh tissue was homogenised 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 before the supernatant was used for the estimation of α-tocopherol. To 1 mL of extract was added 0.2 mL of 2% 2, 2-dipyridyl in ethanol, and the resulting mixture was mixed thoroughly, and kept in the dark for 5 min. The resulting red colour was diluted with 4 mL of distilled water and mixed well. The resulting colour in the aqueous layer was measured at 520 nm. The α-tocopherol content was calculated using a standard graph made with known amounts of α-tocopherol.

Estimation of glutathione and other non-protein thiol contents

Reduced glutathione (GSH) content was determined by the recycling method outlined by Anderson (1985). Fresh root and leaf samples (0.5 g) were harvested after a week of growth in control and Cd media and homogenised in 0.3 mL of 5% sulfosalicylic acid under cold conditions. The homogenate was centrifuged at 10,000 rpm for 10 min. A 0.5 mL aliquot was taken in a microfuge tube, to which 0.5 mL reaction buffer [0.1 M phosphate buffer (pH 7.0), 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 50 µL of 3 mM 5,5' -dithio-bis (2- nitrobenzoic acid) (DTNB) were added. After 5 min, the absorbance was read at 412 nm using UV-vis spectrophotometer (Model UV-1601 PC, Shimadzu, Japan) in order to determine the GSH.

Other non-protein thiols (NP-SH) were determined as described by Del Longo et al. (1993). As described above, 100 µL of the aliquot was taken in a microfuge tube, to which 0.5 mL reaction buffer [0.1 M phosphate buffer (pH 7.0), 0.5 mM EDTA] and 0.5 mL of DTNB (1 mM) were added. The reaction mixture was incubated for 10 min and absorbance was read at 412 nm using a UV-vis spectrophotometer (Model UV-1601 PC, Shimadzu, Japan). Values were corrected for the absorbance by preparing a blank without extract. A standard curve was prepared from varying concentrations of cysteine to calculate the other non-protein thiol contents in samples.

Extraction and assay of PC

Homogenate preparation and assay for PC was carried out by the method of Grill et al. (1987). To that end, 0.4 g of tissue was frozen in liquid nitrogen, pulverised, and transferred to a microfuge tube. To this, 0.4 mL of a freshly prepared alkaline solution of $NaBH_4$ was added. After thorough mixing, the solution was centrifuged at 11,000 × *g* for 5 min at 4 °C. The supernatant was collected and acidified with a 3.6 N HCl solution (ratio 5: 1). The tubes were incubated on ice for 15 min followed by centrifugation at 11,000 × *g* for 5 min at 4 °C and the supernatant was collected. The supernatant was filtered through a 0.45 µm filter and used for the analysis of PC. Phytochelatins were separated by HPLC on a Beckman Ultrasphere C_{18} µm 4.6 × 250 mm column using a gradient of acetonitrile in 0.1% trifluoracetic acid at a flow rate of 1 mL/min. The gradient program was 0% acetonitrile in 2 min, 0% to 10% acetonitrile in 2 min, and 10% to 20% acetonitrile in 20 min. The column eluent was derivatised with 75 µM DTNB in 50 mM potassium phosphate (pH 7.6) at a flow rate of 2 mL/min and monitored at 412 nm. Samples of 100 µL were injected. The PCs present in the crude samples were identified by comparison of the retention time with that of the standard PC. Quantitative determination was established by relating the peak area of detected PC to that of the standard glutathione on a percentage basis. The concentration of PC was expressed as nanomoles of GSH equivalent g^{-1} FW.

Statistical analysis

All data presented are the mean values of 2 independent sets of experiments. Each value was presented as mean ± SD from a minimum of 3 replicates. Statistical assays were carried out by one-way ANOVA using Duncan's multiple range test (DMRT) to evaluate whether the means were significantly different, taking $P \leq 0.05$ as significant.

Results

Effect of Cd on biomass and Cd concentration

With increasing Cd supplies from 0-100 μM, the leaf and root dry weights decreased at all Cd concentrations. The lowest leaf dry weight (215 mg plant^{-1}), which was 46% lower than that of the control, was noted at 100 μM Cd (Table). The root dry weight of seedlings was affected more severely as compared to the leaf dry weight at all Cd concentrations. The lowest root dry weight, 85% lower than that observed in the control, was 21 mg plant^{-1} at 100 μM Cd (Table).

Cadmium accumulation in roots and leaves showed a linear increase in response to increasing external Cd supply level. The highest Cd accumulation was detected in the roots. At 100 μM Cd, this value was 4961.83 μg g^{-1} DW, which indicated that it was a potential accumulator of Cd. Leaves treated with Cd concentrations of 100 μM reached 581.18 μg g^{-1} DW (Table). In addition, the concentrations of Cd in leaves and roots differed significantly.

Effect of Cd on α-tocopherol levels

Leaves and roots of the safflower seedlings exhibited an increase in α-tocopherol levels in a concentration-dependent manner (Figure 1). In leaves, α-tocopherol levels increased significantly at all of the tested concentrations (Figure 1). The maximum amount of α-tocopherol (208.75 μg g^{-1} FW), which was 81% higher than that of the control, was noted at 100 μM Cd. In roots, α-tocopherol levels also increased with an increase in the concentration of Cd (Figure 1). The maximum α-tocopherol level in roots (20.02 μg g^{-1} FW), about 41% higher than the control, was observed at 100 μM Cd. However, α-tocopherol levels in roots changed slightly and did not correlate with Cd concentrations (Figure 1). In general, α-tocopherol levels were higher in leaves than in roots at all of the Cd concentrations.

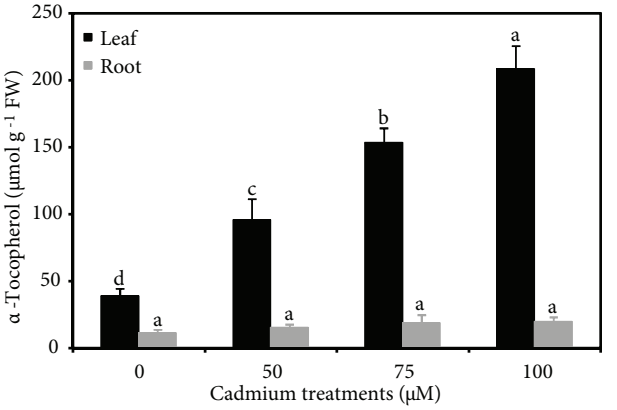

Figure 1. The effect of Cd treatment on levels of α-tocopherol in the roots and leaves of *C. tinctorius*. Each value represents the mean ± SD of triplicate measurements. The mean values of a particular tissue type (either root or leaf) followed by the same letter are not significantly different (P ≤ 0.05; DMRT).

Table. The effect of Cd treatment on the dry weight and Cd accumulation of roots and leaves of *C. tinctorius* grown in the presence of different Cd concentrations.

Cd conc. (μM)	Dry weight (mg plant^{-1})	Cadmium concentration (μg g^{-1} DW)
Leaf		
0	**403 ± 17 ^{a}**	**nd**
50	**386 ± 12 ^{a}**	**91.22 ± 12 ^{c}**
75	**371 ± 2 ^{a}**	**196.84 ± 2 ^{b}**
100	**215 ± 8 ^{b}**	**581.18 ± 8 ^{a}**
Root		
0	**121 ± 2 ^{a}**	**nd**
50	**96 ± 5 ^{b}**	**1277.25 ± 12 ^{c}**
75	**52 ± 1 ^{c}**	**2043.94 ± 2 ^{b}**
100	**17 ± 3 ^{d}**	**4961.83 ± 8 ^{a}**

Each value represents the mean ± SD of triplicate measurements. The mean values of a particular tissue type (either root or leaf) followed by the same letter are not significantly different (P ≤ 0.05; DMRT).

Effect of Cd on glutathione and other non-protein thiol levels

Cadmium treatments altered the levels of GSH in leaves and roots (Figure 2). In leaves, GSH levels increased significantly at all Cd concentrations. A 75% increase in GSH level in leaves was observed at 100 μM Cd, with respect to the control (Figure 2). In roots, the maximum GSH level, which was about 38% higher than the control, was observed at 50 μM Cd. At higher concentrations (75 and 100 μM Cd), GSH levels decreased slightly.

With increasing Cd concentrations, the level of NP-SH in leaves showed a slight increase at all concentrations. The highest NP-SH level in leaves, which was 42% higher than that of the control, was found at 100 μM Cd. However, NP-SH levels in leaves changed only slightly and did not correlate with Cd concentrations (Figure 2).

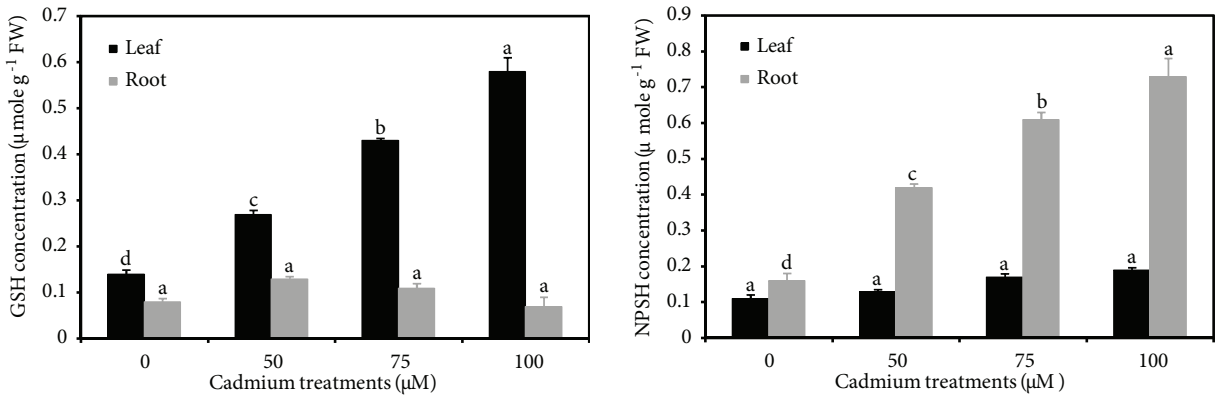

Figure 2. The effect of Cd treatment on levels of GSH and NP-SH in the roots and leaves of *C. tinctorius*. Each value represents the mean ± SD of triplicate measurements. The mean values of a particular tissue type (either root or leaf) followed by the same letter are not significantly different (P ≤ 0.05; DMRT).

The level of NP-SH was always higher in roots as compared to leaves (Figure 2). Levels of NP-SH showed a remarkable increase in roots under Cd treatments (Figure 2). The highest NP-SH level, which was 78% higher than that of the control, was noted at 100 µM Cd.

Effect of Cd on phytochelatin levels

No phytochelatin synthesis was observed in either tissue in the absence of Cd. However, Cd treatment led to the synthesis of PCs in both root and leaf tissues (Figure 3). The amount of PCs synthesised in response to Cd was dependent on the concentration of Cd in roots. In leaves, PCs level also increased, but not significantly. Interestingly, induction of PC_2 and PC_3 was higher in roots than in leaves. In roots, with Cd exposure increasing from 0 to 100 µM Cd, levels of PC_2 and PC_3 were significantly increased at all Cd concentrations (Figure 3). At 100 µM Cd the highest levels of PC_2 and PC_3 were noted, 90% and 74% higher, respectively, than those recorded in leaves at the same Cd concentration. In leaves, levels of PC_2 and PC_3 also increased with increases in the Cd concentrations (Figure 3). PC_2 and PC_3 levels in leaves changed slightly and did not correlate with the Cd concentrations (Figure 3). However, in the case of safflower, PC_2 seems to be the major form of PC.

Discussion

In the present study, biomass was greatly reduced with external exposure to Cd, and roots were more affected than leaves, especially at higher Cd concentrations (Table). Similarly, Mishra et al. (2006) reported that

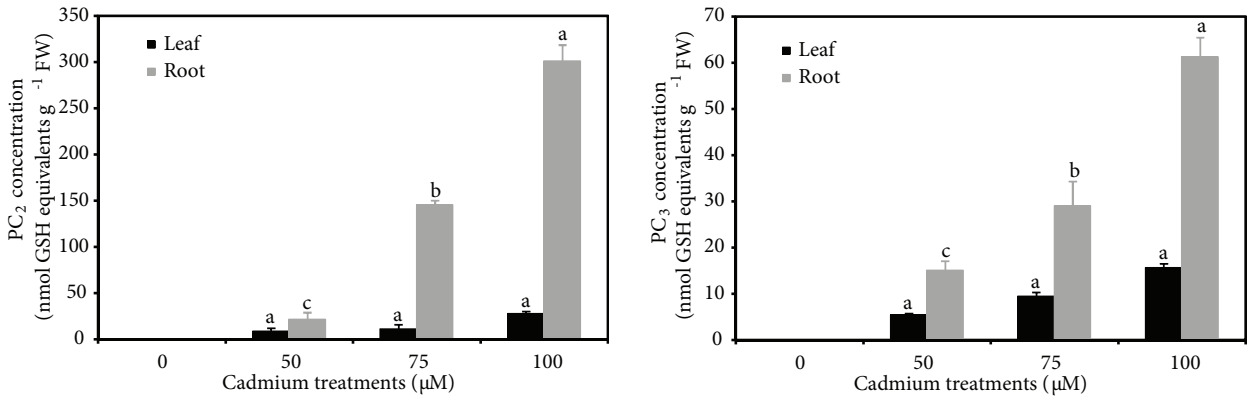

Figure 3. The effect of Cd treatment on levels of PCs in the roots and leaves of *C. tinctorius*. Each value represents the mean ± SD of triplicate measurements. The mean values of a particular tissue type (either root or leaf) followed by the same letter are not significantly different (P ≤ 0.05; DMRT).

Cd affected all of the growth parameters and that root growth was the most affected. The toxic effect of Cd was evident from the reduced biomass, especially at higher Cd concentrations. Roots are the first site of exposure and toxicity to the metal; thus, root biomass was severely affected.

Cadmium accumulation in roots and leaves increased with increasing levels of Cd in the medium (Table). The Cd concentration was lower in leaves than in roots, indicating that a higher proportion of the Cd taken up by plants remained in the roots. This was in agreement with a number of recent reports on plants such as *Lonicera japonica* Thunb. (Liu et al., 2011). Such a high metal confinement in the root tissues may be due to its efficient binding and sequestration to the vacuoles by PCs.

Cadmium treatments significantly affected the contents of α-tocopherol in the leaves of safflower. In roots, α-tocopherol levels also increased, but not significantly (Figure 1). The synthesis of low-molecular-weight antioxidants, such as α-tocopherol, has been previously reported in stressed plants (Jaleel, 2009; Yusuf et al., 2010). Oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in plants (Yusuf et al., 2010). Based on the above trends, this study suggests that α-tocopherol plays an important role in Cd detoxification.

Glutathione (GSH), a sulphur-containing tripeptide, is considered a very important antioxidant involved in cellular defence against toxicants. Glutathione levels are constitutively higher in plants adapted to stress conditions (Mishra et al., 2006; Jin et al., 2008). It is also the precursor for PCs that act as heavy metal-binding peptides in plants (Heiss et al., 2003). Increased concentrations of GSH have been observed with increasing Cd concentrations in *Sedum alfredi* Hance leaves (Jin et al., 2008) and a decay in GSH concentrations has been reported in *Oryza sativa* leaves under Cd stress (Hsu & Kao, 2004). In the present study, GSH levels in the leaves were significantly increased at all Cd concentrations (Figure 2). This may be associated with more active GSH synthesis due to induced transcription of the genes responsible for this process, such as glutathione synthetase and glutathione reductase (Hall, 2002). In *Arabidopsis thaliana* (L.) Heynh., the gene responsible for GSH synthesis increased when plants were exposed to Cd, resulting in higher GSH production (Cobbett, 2000). An increased GSH concentration seems to be an optimal defence strategy.

Levels of GSH in roots increased when exposed to up to 50 μM Cd and then decreased (Figure 2). A decline in the levels of GSH might be attributed to its increased utilisation for direct interaction with Cd (Mishra et al., 2006). In roots, the depleted levels of GSH at higher concentrations of Cd may also be due to consumption for the synthesis of PC. Accordingly, as discussed below, PC levels showed marked increases in roots in response to the Cd supply (Figure 3). The depletion of GSH in response to metal exposure has been reported in many earlier studies (Srivastava et al., 2004; Mishra et al., 2006).

Accumulations of Cd in safflower root were accompanied by a concomitant induction in the levels of NP-SH. In our study, a significant increase in NP-SH levels in roots was observed with Cd treatments (Figure 2). This indicates the active participation of NP-SH in the detoxification of Cd. This result was in agreement with those observed in *Sesbania drummondii* (Rydb.) Cory (Zeng et al., 2009). The elevated levels of other NP-SH in plants may be associated with enhanced S assimilation due to the overexpression of genes responsible for this process.

In the present study, Cd strongly induced the accumulation of PC_2 and PC_3 in roots but not in leaves (Figure 3). PCs have been the most widely studied in plants, particularly in relation to Cd tolerance (Heiss et al., 2003; Srivastava et al., 2004; Mishra et al., 2006; Sun et al., 2007). One very important mechanism for heavy metal detoxification and tolerance in plants is the chelation of the metal ions by a ligand and, in some cases, the subsequent compartmentalisation of the ligand-metal complex (Hall, 2002). It has been argued that PCs are involved in the chelation of Cd ions entering the roots. These chelated ions are compartmentalised into vacuoles and could be the cause of the high content of Cd found in roots (Molina et al., 2008). Increased PC accumulation in the roots, as seen in our study, may be responsible for increased Cd accumulation in the roots.

In this study, the accumulation of PC_2 and PC_3 was very low in leaves at all of the Cd concentrations tested (Figure 3). This may be due to the binding of metal with GSH or to the cell wall (Vecchia et al., 2005), thus making the amount of free metal too low to induce PC to significant levels.

Conclusion

It can be concluded that *C. tinctorius* cv. Arak2811 has a high ability to adapt to Cd toxicity and Cd hyperaccumulation. Cd accumulation was significantly enhanced with increasing concentrations of Cd in the media and the fast growth and easy harvesting of the plant further implicate its usefulness in phytoremediation research. A coordinated increase in α-tocopherol, GSH, and NP-SH was noted under Cd stress, consistent with leaf and root Cd concentrations. This indicates the role these compounds play in supporting Cd tolerance in safflower seedlings. On the basis of Cd-induced PC synthesis in safflower seedlings, it can be suggested that Cd probably causes the increased formation of ROS; consequently, various antioxidative compounds (e.g., α-tocopherol, GSH, and NP-SH) are activated synchronously to mitigate the damaging effect of ROS. Metal-binding peptides (PCs) are also synthesised to chelate and sequester these toxic ions. The fact that Cd exposure increased the concentration of PCs in the roots implies the potential role of PC in Cd accumulation and Cd complexation in the roots of safflower.

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