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Different response to Cd stress in domesticated and wild safflower (*Carthamus* spp.)

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ABSTRACT

Cadmium (Cd) can stress plants by affecting various physiological functions. Cd stress-response mechanisms were investigated in two genotypes of domesticated safflower (*Carthamus tinctorius*) and a population of wild safflower (*Carthamus oxycantous*) to explore potential differences in tolerance mechanisms of these species. A hydroponic experiment was conducted with 6-day-old safflower plants. Genotypes AC-Sterling (tolerant) and Saffire (semi-tolerant) from *C. tinctorius*, and Arak (sensitive) a population from *C. oxycantous*were subjected to three concentrations of Cd (i.e., 0, 1, and 20 μM $CdCl_2$). Genotypic differences were detected in Cdtolerance index, Cd concentration in shoots and roots, Cd translocation to shoots, Cd bound to cell walls, superoxide dismutase (SOD) activity, lipid peroxidation, and phytochelatins accumulation in safflower plants upon exposure to $CdCl_2$. Results indicate that genotypic differences were more obvious in the presence of low (i.e., 1 μM) rather than high (i.e., 20 μM) $CdCl_2$ concentrations. Comparing genotypes, root and shoot Cd accumulation was highest in the semi-tolerant genotype. Cadmium translocation to shoots was increased with increasing tolerance. The percentage of Cd bound to root cell walls was higher in the tolerant genotype, but only with low $CdCl_2$ addition. Furthermore, in the tolerant genotype, SOD activity was lowest in both roots and shoots with low $CdCl_2$ addition but highest with high $CdCl_2$ addition, while the opposite was found for phytochelatins. Lipid peroxidation was decreased with Cd tolerance at both $CdCl_2$ concentrations. We conclude that safflower relies mainly on binding Cd to the cell walls and the formation of phytochelatins in root and shoot tissues, in order to handle the Cd stress, evidenced by lessening Cd-induced lipid peroxidation.

1. Introduction

Though not yet demonstrated to be an essential heavy metal, cadmium (Cd) can be easily taken up by plant roots (Vitória et al., 2001). Cd is known as a toxic heavy metal due to its high solubility in water. Heavy metals such as Cd may cause the formation of reactive oxygen species (ROS), damage plant tissue membranes, and inhibit photosynthesis, carbon dioxide assimilation, and growth (Ali et al., 2013; Moradi and Ehsanzadeh, 2015). Moreover, Cd also disturbs plant physiology by enlarging vacuoles, altering diffusion properties of the cell walls, and leading to undeveloped mitochondria in the root tip cells (Ali et al., 2014).

Various mechanisms, including metal restriction or exclusion and detoxification, are suggested to be involved in internal metal tolerance in plants. Prevention of metal absorption, restriction of metal translocation to the shoots, and restriction of metal movement across the plasma lemma are examples of exclusion mechanisms. Detoxification is a type of physiological response whereby plants absorb and accumulate high concentrations of metals (Macfie and Welbourn, 2000; Shi and Cai, 2009). Thus, species having high Cd accumulation may have high Cd resistance (Shi et al., 2016).

Cadmium-induced oxidative stress is associated with the activation of genes related to enzymatic and non-enzymatic antioxidative defence systems, related modifications in transcript concentrations and an array of metabolites (Gill et al., 2015). Cadmium that has passed through the cytoplasm can be removed by metal-binding thiol-rich peptides such as phytochelatins (PCs) (Gadapati and Macfie, 2006). Cadmium tolerance and accumulation in safflower plants may involve induction of PCs biosynthesis in roots (Namdjoyan et al., 2012). However, the exact role of PCs in heavy metal tolerance is unsettled and PCs are not necessarily

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found in all types and organs of plants (Landberg and Greger, 2004).

Plant cell walls have negatively charged sites with the capacity to bind positively charged metal ions. According to Carrier et al. (2003), cell walls may bind Cd, preventing it from being transported further into the cytoplasm. Cation uptake in plant roots, also involves the cation exchange capacity (CEC) of the cell walls. Cadmium exposure increases CEC due, likely, to the synthesis of new binding sites, representing a mechanism to cope with Cd in the plant surroundings (Nyquist and Greger, 2009).

Heavy metals are largely transported apoplastically in plant tissue. To reach the xylem vessels of roots, metals must travel across the endodermis and its suberized cell walls (Casparian bands and suberin lamellae), which is not a straightforward path (Marschner, 1995). Lux et al. (2004) demonstrated that the suberin lamellae is laid more distant from the root tip in *Salix* clones with high Cd compared to those with low Cd translocation capability.

Safflower (*Carthamus* spp.) is gaining importance as an oil seed, medicinal, and industrial plant (Lid, 1996) cultivated in dry regions and marginal areas. Safflower is known to with stand, at least in part, salinity and drought (Moradi and Ehsanzadeh, 2015) and, therefore, it is a crop of choice for cropping systems in dry regions and marginal areas typically found in countries like Iran. Cadmium is released into the soil and water and atmosphere from a variety of sources, including metallurgic industries, cement factories, urban waste materials, and phosphate fertilizers (Ali et al., 2014). With the ever-increasing trend of application of agricultural chemicals (pesticides and fertilizers), crops grown on almost all types of soils and in different regions are prone to heavy metals (e.g. Cd) pollution. Furthermore, it has been reported that safflower may be used as a hyper-accumulator crop for Cd-polluted soils (Shi et al., 2010). Despite a previous report (Pourghasemian et al., 2013) indicating that tolerance, uptake, and translocation of Cd vary among cultivars and populations within the species *C. Tinctorius* and *C. oxycantous*, very little is known about many other physiological responses of safflower to heavy metal stress.

The aim was to find an answer to the question of potential differences in Cd stress-response mechanisms in two *C. tinctorius* genotypes and one *C. oxycantous* population and unravel possible tolerance mechanisms in this oilseed plant. Safflower genotypes that are sensitive, semi-tolerant, and tolerant to Cd were investigated in terms of physiological mechanisms such as superoxide dismutase (SOD) activity, lipid peroxidation, PCs accumulation, cell wall binding of Cd, and Cd translocation to shoots.

2. Materials and methods

2.1. Growth condition

Two safflower genotypes from *C. tinctorius* and a population from *C. oxycantous,* differing in Cd tolerance and translocation capabilities upon exposure to Cd (Pourghasemian et al., 2013) were studied. Saffire, which is semi-tolerant to and has a high uptake and modest translocation of Cd to shoots (ST-HuSt) and AC-Sterling, which is tolerant with a low uptake but high translocation of Cd to shoots (T-LuHt) were genotypes belonging to domesticated *C. tinctorios*. The third (i.e. Arak) was a population from the wild species of safflower (*C. oxycantous*); it is sensitive to and has a modest uptake and low translocation of Cd to shoots (S-SuLt). Hereafter, for convenience, these plant materials are being collectively referred to as safflower genotypes. Seeds were surface sterilized for 10 min in 1% (W/V) calcium hypochlorite and thereafter sown on paper moistened with distilled water. After six days of germination, seedlings with 2–3 leaves were formed. The 2–3 leaved seedlings were transferred to Styrofoam plates floating on 300 mL of Hoagland nutrient solution in plastic containers. Each container had six plants and the experiment was run in five replicates; all containers were placed in a climate chamber with 50% relative humidity kept at 23 °C during the 16 h light period and 20 °C during the dark period. The light was given at a photon flux density of 600 ± 20 μ mol m^{-2} s^{-1} from metal halogen lamps (Powerstar HQI-R; Osram, Munich, Germany).

Six-day-old plants were treated for 14 days with 0 (control), 1, and 20 μM $CdCl_2$. These concentrations were selected based on a previous study (Pourghasemian et al., 2013), where eight safflower genotypes had been exposed to nine concentrations of $CdCl_2$ (0–500 μM $CdCl_2$). They had demonstrated that the growth of the studied genotypes was greatly reduced in concentrations of $CdCl_2$ exceeding 20 μM and the largest difference between genotypes was observed at 1 μM $CdCl_2$.

When the volume of the nutrient solution in the containers had decreased by 10%, water was added to maintain the initial volume. The pH was 6.3 and did not change during the experiment, shown by measuring the pH every second day. The nutrient solution was renewed every six days, i.e. the nutrient solution was renewed twice over the course of experiment.

2.2. Experimental design and harvest of plants

A factorial randomized complete block design experiment with five replicates was used to study the effect of Cd (in three concentrations) on three safflower genotypes. Each replicate encompassed two pots; one pot for traits measured on dry plant material and another pot for traits measured on fresh plant material. When nothing else is specified, five replicates were used for each treatment.

At the end of $CdCl_2$ treatment, plants were harvested and the roots were washed in distilled water. Then, half of the plants were separated into roots and shoots, which were dried at 105 °C for 24 h to determine the dry weight for tolerance index, Cd concentration and cation exchange capacity. Other half of the plants were separated into roots and shoots, kept in liquid nitrogen, and then stored in freezer for one week to determine superoxide dismutase activity, lipid peroxidation, Cd bound to the cell walls, and phytochelatin formation. After harvesting of plant localization suberin lamellae in root was measured immediately.

2.3. Analysis of Cd content, tolerance index and translocation Cd to shoots

The tolerance index was calculated using the total dry weight of the plants according to Eq. (1) (Wilkins, 1978). The dried plant materials were wetdigested in HNO_3:$HClO_4$ (7:3, v/v) according to Frank (1976). The Cd content in roots and shoots was analysed using a Spectra A 55B atomic absorption spectrophotometer (Agilent, Santa Clara, CA, USA) using a flame atomizer and a GTA 100 graphite furnace (Agilent). Using this instrument enabled us to detect 0.1 ng/g (i.e. 0.1 μg kg^{-1}) of the target metal in the examined samples. Matrix-standard addition was applied to eliminate matrix effects from the samples. The translocation of Cd from roots to shoots was calculated using the Eq. (2) (Mattina et al., 2003).

$$\text{Tolerance Index} = \frac{\textit{Dry weight of Cd} - \textit{treated plant}}{\textit{Dry weight of untreated plant}} \times 100 \quad (1)$$

$$\text{Translocation of Cd to shoots}(\%) = \frac{\textit{Cd concentration in shoot} \quad (\mu g g^{-1} DW)}{\textit{Cd concentration in root} \quad (\mu g g^{-1} DW)} \times 100 \quad (2)$$

2.4. Quantifying superoxide dismutase (SOD)

Milled fresh plant material was mixed in 50 m mol L^{-1} KH_2PO_4/K_2HPO_4 buffer (pH 7.0) and 10 g L^{-1} PVP-10 (polyvinyl pyrrolidone) using a Polytron PT 2000 ultramixer (Kinematica, Lucern, Switzerland) and then centrifuged at 10,000 g for 10 min. Superoxide dismutase (SOD, EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of epinephrine at 480 nm, according to Beauchamp and Fridovich (1971). The amount of SOD required for 50%

inhibition of epinephrine was defined as one unit of SOD activity.

2.5. Measurement of lipid peroxidation

The oxidative damage (observed as the degeneration of lipids), depicted in malondialdehyde (MDA) concentration, by the Cd was measured and expressed as TBA-rm, according to Heath and Packer (1968). Fresh plant material was mixed with 0.1% trichloric acid (TCA) using a Polytron PT 2000 ultramixer (Kinematica). Samples were centrifuged for 5 min at 10,000*g*. The supernatant was added to 0.5% thiobarbituric acid (TBA) in 20% TCA and then heated at 100 °C for 30 min. The reaction was stopped by placing the tubes on ice. Samples were then centrifuged for 5 min at 10,000*g*. The absorbance was measured at 532 nm and adjusted for non-specific absorbance at 600 nm (the extinction coefficient was 155 mmol L^{-1} cm^{-1}) in the supernatant.

2.6. Measuring cation exchange capacity

The cation exchange capacity (CEC) of the root material was analysed using the method of Crooke (1964), with some modifications. Hydrochloric acid, 0.01 M, was added to 10 mg (dry weight) of safflower roots while stirring the mixture continuously for 5 min. The plant material was then rinsed with 250 mL of water in a Büchner funnel using a 25-µm nylon cloth. Thereafter, the plant material was placed in 1 M KCl at pH 7.00 for 30 min. The plant material was removed from the medium and titrated with 0.001 M KOH until pH 7.00 was reached. By calculating the amount of potassium needed to increase the pH of the medium to 7.00, the hydrogen equivalent (m Eq./100 g DW) was determined.

2.7. Measurement of Cd bound to the cell walls

Cell wall-bound Cd was measured according to Lozano-Rodríguez et al. (1997). Fresh root tissue was homogenized, first with a mortar and pestle in liquid nitrogen and then with a Polytron PT 2000 ultramixer (Kinematica) in an extraction buffer comprising 500 mM sucrose, 50 mMHepes, 5.0 mM ascorbic acid,1.0 mM DTT (dithiothreitol), and 1.0% (w/v) PVP (polyvinylpyrrolidone), adjusted to pH 7.5 with NaOH. The homogenate was sieved through a nylon cloth (10 µm) and washed with extraction buffer; this residue, together with the pellet retained after centrifugation of the filtrate at 10,000*g* (Sigma 3E-1 centrifuge; Sigma Laborzentrifugen, Osterode am Harz, Germany) for 5 min, consisted of the cell wall-bound metals. The material was dried and wet digested in HNO_3:$HClO_4$ (7:3, v/v) according to Frank (1976).

The Cd content in cell walls was related to that in the whole plant tissue and was calculated as follows:

$$\text{Cd in cell walls}(\%) = \frac{\textit{Cd concentration in planttissue}}{\textit{Cd concentration in plantroot tissue}} \times 100 \quad (3)$$

2.8. Localization of suberin lamellae in roots

A minimum of six root samples (average length, 100 mm) from each treatment were used to prepare free-hand sections (Ruzin, 1999). For detection of suberin lamellae development, the roots were cut into 5-mm sections from the tip extending to 100 mm from the tip (Lux et al., 2005). Free-hand sections of roots were stained with 0.2% Fluoral Yellow 088 in poly (ethylene glycol) and glycerol (Brundrett et al., 1991). Free-hand sections were also stained for Casparian bands using 0.1% toluidine blue and 0.2% berberine (Brundrett et al., 1988). Sections were observed using an Axioskop 2 Plus microscope (Zeiss, *Oberkochen*, Germany) and a BX 60 fluorescence microscope (Olympus, Tokyo, Japan) with illumination and filters (excitation filter, BP330–385; barrier filter, BA-420; dichroic mirror, DM-400) allowing > 400 nm wavelengths to pass to the viewer

2.9. Analysis of phytochelatin formation

Phytochelatins (PCs) were analysed in the leaves and roots of the treated plants according to Sneller et al. (2000) using monobromobimane (mBrB) to detect the thiol groups. Plant materials (i.e., roots and shoots separately) were sonicated with an ultrasonic homogenizer (UW2070;Bandelin Electronics, Berlin, Germany) for 10 s in 12.6 m M diethylene-triamine-pentaacetic acid (DTPA) with 0.1% trifluoroacetic acid (TFA). After centrifugation (Biofuge A 1230; Heraeus, Hanau, Germany) at 16,000*g* for 5 min at 4 °C, the supernatant was filtered through a 0.45 µm Millex-HA filter (Millipore, Burlington, MA, USA). Thereafter, the supernatant was mBrB-derivatized for 30 min at 45 °C with a mixture of 200 mM HEPES, 6.3 mM DTPA, and 50 µM mBrB (pH 8.2). The reaction was stopped with 1 M methane sulphonic acid. All work was done on ice.

Analysis was performed on an LC20AD HPLC equipped with an SIL-20A/AC auto sampler and an RF-10Axf fluorescence detector (Shimadzu, Kyoto, Japan). A 100-mm Chromolith Performance RP-18e column (Merck, Darmstadt, Germany) was used to analyse PCs after being equilibrated with 0.1% TFA in water. Samples (50 µL) were injected and the column was developed with a linear gradient from 0% to 60% (v/v) methanol with 0.1% TFA for 70 min. The emission and the fluorescence excitation wavelengths were 470 and 380 nm, respectively. Cadmium-treated *Thlaspi caerulescens* was analysed to identify PCs (Lindberg et al., 2007); for the quantification of PCs, glutathione was analysed as a standard.

2.10. Statistical analysis

In all examined traits, data were subjected to analysis of variance (ANOVA) using the SAS statistical program (SAS Institute, Gary, NC, USA) and the mean comparisons were performed using the least significant difference (LSD) test, where the *F*-value was significant.

3. Results

3.1. Cd tolerance index

The tolerance index was significantly affected by the genotype, $CdCl_2$ concentration, and interaction effect of genotype × $CdCl_2$ (Table 1). Tolerance indices for AC-Sterling (tolerant), Saffire (semi-tolerant), and Arak (sensitive) were 81%, 62%, and 44%, respectively, when exposed to 1 µM $CdCl_2$. AC-Sterling (tolerant) and Arak (sensitive) had the highest and lowest tolerance index values, respectively, averaged over the two concentrations of $CdCl_2$. The tolerance index value of Saffire (semi-tolerant) did not differ significantly from that of Arak (sensitive) or AC-Sterling (tolerant) at the 20 µM concentration of $CdCl_2$, while 1 µM $CdCl_2$ treatment caused significant differences in tolerance index between all three genotypes. With an increase in the $CdCl_2$ concentrations from 1 to 20 µM, the tolerance index values of AC-Sterling (tolerant), Saffire (semi-tolerant), and Arak (sensitive) were decreased by 71%, 66%, and 58%, respectively.

3.2. Root and shoot Cd concentrations

The root and shoot Cd concentrations were significantly affected by the $CdCl_2$ concentration, genotype, and interaction effect of genotype × $CdCl_2$ (Table 1). Increasing the $CdCl_2$ concentration in the nutrient solution enhanced the internal Cd concentrations in shoots and roots. The three genotypes displayed significant differences in root and shoot Cd concentrations. At the 1 µM $CdCl_2$ concentrations, saffire (semi-tolerant) had 11% and 68% higher shoot Cd concentrations than Arak (sensitive) and AC-Sterling (tolerant), respectively, whereas it outnumbered the latter genotypes by 18% and 55%, respectively, in terms of root Cd concentration. Saffire (semi-tolerant) plants exposed to the 20 µM $CdCl_2$ concentration also had significantly higher concentrations

Table 1
Effect of $CdCl_2$ on tolerance index, Cd concentration in roots and shoots, Cd bound to root cell walls, and Cd translocation from roots to shoots in three safflower genotypes, i.e., Saffire (ST-HuSt), AC-Sterling, and Arak (S-SuLt), grown for 14 days in the presence of three concentrations of $CdCl_2$, i.e., 0, 1, and 20 μM. Different letters within a column represent significant differences. ANOVA showed F-value of Genotype, $CdCl_2$ and Genotype × $CdCl_2$.

$CdCl_2$ concentration (μM)	Genotype	Tolerance index, %	Root Cd concentration, μg/g^{-1} DW	Shoot Cd concentration, μg/g^{-1} DW	Cd bound to root cell wall, %	Cd translocation,%
1	Arak (S-SuLt)	$44^{c} \pm 4.2$	$77^{e} \pm 2.2$	$30^{e} \pm 2.81$	$13^{c} \pm 1.42$	$38^{c} \pm 2.31$
	Saffire (ST-HuSt)	$62^{b} \pm 3.3$	$87^{d} \pm 3.8$	$36^{d} \pm 1.11$	$18^{bc} \pm 1.59$	$41^{bc} \pm 4.8$
	AC-Sterling (T-LuHt)	$81^{a} \pm 4.8$	$28^{f} \pm 2.81$	$16^{f} \pm 0.88$	$28^{a} \pm 4.3$	$57^{a} \pm 6.01$
20	Arak (S-SuLt)	$18^{e} \pm 1.9$	$1727^{b} \pm 13.1$	$440^{b} \pm 18.12$	$16^{bc} \pm 2.6$	$25^{d} \pm 3.8$
	Saffire (ST-HuSt)	$21^{ed} \pm 3.4$	$1835^{a} \pm 47.6$	$553^{a} \pm 26.6$	$22^{ab} \pm 2.9$	$30^{cd} \pm 2.9$
	AC-Sterling (T-LuHt)	$24^{d} \pm 1.6$	$694^{c} \pm 32.14$	$387^{c} \pm 8.91$	$19^{b} \pm 3.8$	$48^{b} \pm 3.1$
ANOVA	Genotype	17.1**	349**	18.6**	26.8**	4.9*
	$CdCl_2$	194**	4396**	1186**	287**	2.05 ns
	Genotype × $CdCl_2$	9.71**	258**	11.7**	24.1**	0.86 ns

Each value is a mean of three replicates ± SE.; ns: not significant.
* $p < 0.05$.
** $p < 0.01$.

of Cd in the roots and shoots than did the other genotypes. The lowest root and shoot Cd concentrations were found in the AC-Sterling (tolerant) genotype at both concentrations of $CdCl_2$ treatment.

3.3. Cell wall-bound Cd

Cell wall-bound Cd in safflower roots was significantly affected by the genotype and the interaction effect of genotype × $CdCl_2$ (Table 1). At the 1 μM concentration of $CdCl_2$, the greatest and smallest amounts of cell wall-bound Cd were found in AC-Sterling (tolerant) and Arak (sensitive), respectively; however, at the 20 μM concentration of $CdCl_2$, the cell wall-bound Cd in safflower roots did not differ among the genotypes. The 20 μM concentration of $CdCl_2$ led to significantly less cell wall-bound Cd in the roots of AC-Sterling (tolerant) than the 1 μM.

3.4. Cd translocation

Only the genotype significantly affected the Cd translocation (Table 1). The greatest Cd translocation from roots to shoots was observed in AC-Sterling (tolerant). Saffire (semi-tolerant) tended to have higher Cd translocation than did Arak (sensitive), but this difference was not statistically significant (Table 1). However, Cd translocation to the shoots was increased with increasing tolerance of Cd in both $CdCl_2$ treatments.

3.5. Lipid peroxidation

The shoot MDA concentration was significantly affected by the genotype, $CdCl_2$ concentration, and interaction effect of genotype × $CdCl_2$ (Fig. 1). For Arak (sensitive), the shoot MDA concentration was increased with increasing $CdCl_2$ concentration from 0 to 1 μM. For the remaining genotypes, however, no significant changes in shoot MDA concentration versus that of controls could be observed at the 1 μM concentration. The shoot MDA concentration for Arak (sensitive), AC-Sterling (tolerant), and Saffire (semi-tolerant) was increased by 93%, 49%, and 32%, respectively, in the 20 μM $CdCl_2$ treatment versus the control treatment.

The concentration of MDA concentration in roots was significantly affected by the genotype, $CdCl_2$ concentration, and interaction effect of genotype × $CdCl_2$ (Fig. 1). At 1 μM $CdCl_2$, Arak (sensitive) and Saffire (semi-tolerant) displayed the greatest and smallest increases, relative to the controls, in root MDA concentration, respectively. However, in AC-Sterling (tolerant), no significant changes in root MDA concentration were detected at the 1 μM $CdCl_2$. When grown at 20 μM $CdCl_2$, root MDA concentration in AC-Sterling (tolerant), Saffire (semi-tolerant), and Arak (sensitive) was increased by 65%, 74%, and 160%, respectively, compared to the controls.

3.6. SOD activity

SOD activity in both roots and shoots was affected by the genotype, $CdCl_2$ concentration, and interaction effects of $CdCl_2$ × genotype (Fig. 2). In contrast to the controls, 1 μM $CdCl_2$ significantly increased shoot and root SOD activity in Arak (sensitive) and Saffire (semi-tolerant); however, 20 μM $CdCl_2$ did not lead to a significant change in the SOD activity of these genotypes. In AC-Sterling (tolerant), no significant change was observed in root and shoot SOD activity at 1 μM $CdCl_2$, whereas at the 20 μM concentration of $CdCl_2$, the shoot and root SOD activity indicated significant increases, relative to the controls.

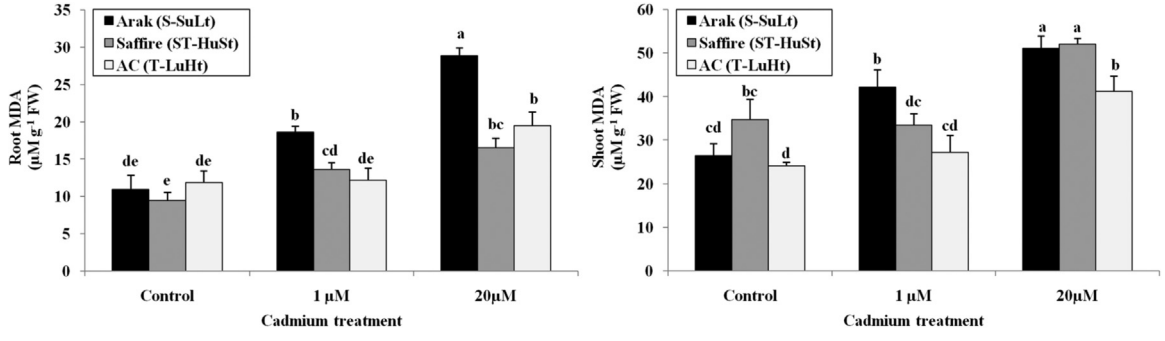

Fig. 1. MDA concentration in shoots and roots (nmol g^{-1}FW) of three genotypes, i.e., Saffire (ST-HuMt), AC-Sterling (T-LuHt), and Arak (S-SuLt), of safflower grown for 14 days in three concentrations of $CdCl_2$ (i.e., 0, 1, and 20 μM). Each value is a mean of three replicates ± SE. The letters above the bars indicate significant differences according to the LSD ($p < 0.05$); *$p < 0.05$, **$p < 0.01$. F-value in shoot MDA concentration for genotype, $CdCl_2$ and genotype × $CdCl_2$ are 17.2**, 24.1** and 3.8* respectively. F-value in root MDA concentration for genotype, $CdCl_2$ and genotype × $CdCl_2$ are 22.9**, 62.9 ** and 7.5* respectively.

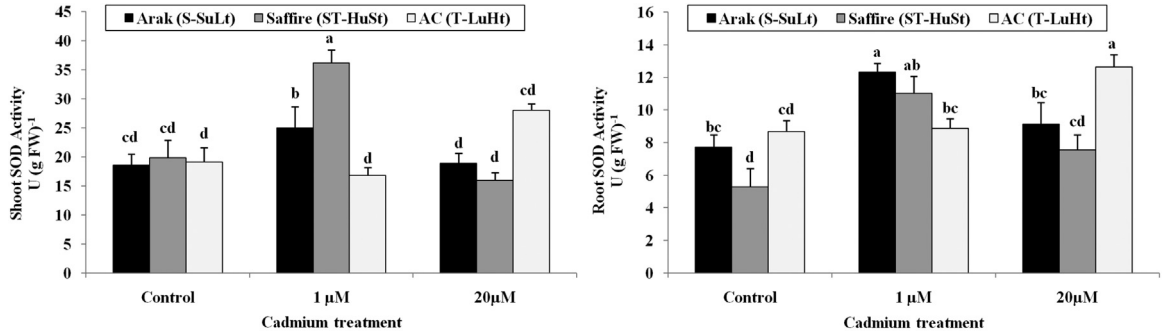

Fig. 2. Activity of SOD enzyme [U(gFW)$^{-1}$] in shoots and roots of three genotypes, i.e., Saffire (ST-HuSt), AC-Sterling (T-LuHt), and Arak (S-SuLt), of safflower grown for 14 days in three concentrations of $CdCl_2$ (i.e., 0, 1, and 20 µM). Each value is a mean of three replicates ± SE. The letters above the bars indicate significant differences according to the LSD ($p < 0.05$); ns: not significant, $*p < 0.05$, $**p < 0.01$. F-value in shoot SOD activity for genotype, $CdCl_2$ and genotype × $CdCl_2$ are 2.07^{ns}, 16.8** and 10.7** respectively. F-value in root SOD activity for genotype, $CdCl_2$ and genotype × $CdCl_2$ are 5.02*, 13.7** and 5.4** respectively.

3.7. Cation exchange capacity

The cation exchange capacity of roots (root CEC) (m Eq./100 g DW) was significantly affected by $CdCl_2$ concentration; however it was not significantly affected by the genotype or interaction effect of genotype × Cd (Fig. 3). Compared with the controls, the root CEC at 20 µM $CdCl_2$ was increased significantly by 14%, while at the 1 µM $CdCl_2$, no significant increase was detected (Fig. 3).

3.8. Suberin lamellae

The distance between the suberin lamellae and root tips did not differ between the safflower genotypes (data not shown) at the two concentrations of $CdCl_2$ application.

3.9. Phytochelatins

The concentrations of PCs in both roots and shoots were affected by the genotype, $CdCl_2$ concentration, and interaction effect of genotype × $CdCl_2$ (Fig. 4). Under $CdCl_2$ stress, a significant increase in PCs concentrations (calculated from the SH concentration) was detected in the shoots and roots (Fig. 4). PC3 and PC4 were not detected in either the roots or shoots of safflower plants at 0 µM Cd. However, PC2 was detected in the control plants in both roots and shoots. At the 1 and 20 µM $CdCl_2$ concentrations, the predominant form of these peptides was PC2, in both the roots and shoots of all three genotypes.

The concentration patterns of the different PCs in response to the $CdCl_2$ concentration in the medium differed between the three genotypes. The accumulation of PCs in the roots and shoots of the AC-Sterling (tolerant) genotype was decreased with increasing $CdCl_2$ concentration in the medium (Fig. 4). Increased concentrations of PCs in the roots and shoots of the Saffire (semi-tolerant) genotype were detected with increasing $CdCl_2$ concentration. The PCs concentration in roots and shoots of Arak (sensitive) was significantly increased and decreased by 87% and 27%, respectively, when grown in 20 µM versus the 1 µM $CdCl_2$ medium.

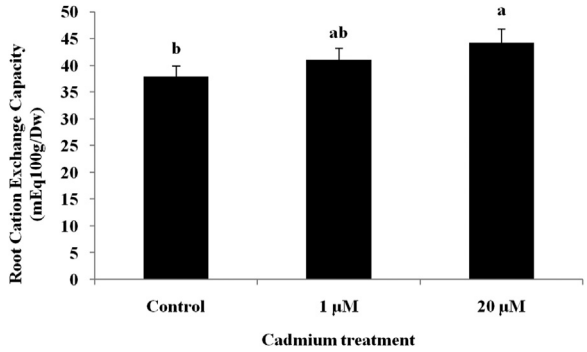

Fig. 3. Root cation exchange capacity (meq. 100gDW^{-1}) of safflower genotypes when grown for 14 days in three concentrations of $CdCl_2$ (0, 1, and 20 µM). Each value is a mean of three genotypes, i.e., Saffire (ST-HuMt), AC-Sterling (T-LuHt), and Arak (S-SuLt), of safflower and three replicates ± SE. The letters above the bars indicate significant differences according to the LSD ($p < 0.05$); ns: not significant, $*p < 0.05$, $**p < 0.01$. F-value in root cation exchange capacity for genotype, $CdCl_2$ and genotype × $CdCl_2$ are 28.69^{ns}, 63.19* and 20.72^{ns} respectively.

4. Discussion

This study found genotypic differences in Cd sensitivity in safflowers, with AC-Sterling (tolerant) and Arak (sensitive) displaying the greatest and smallest tolerance of Cd, respectively (Table 1). Plant species and even genotypes of the same species often vary greatly in Cd tolerance index, for example, in safflower (Pourghasemian et al., 2013; Shi et al., 2010), barley (Tiryakioglu et al., 2006; Wu et al., 2003), and willow (Greger and Landberg, 1999; Landberg and Greger, 1994, 2002).

There was a considerable genotypic difference in the tolerance index at low (1 µM) versus high (20 µM) $CdCl_2$ concentrations in the medium (Table 1). Confirming results have been found in barley (Wu et al., 2003); Tiryakioglu et al. (2006) demonstrated that the decrease in shoot dry matter production due to Cd toxicity was similar between two barley genotypes at a low Cd concentration, but that the same two genotypes behaved differently at a higher Cd concentration. Of course, certain inconsistencies in Cd effect in different studies are anticipated, due to the differences in plant organ examined, duration and concentration of Cd applied, and genotypes or plant species studied (Tiryakioglu et al., 2006). In our study, the considerable genotypic difference in tolerance index for low versus high concentrations of $CdCl_2$ could be attributed to the fact that the low concentration of $CdCl_2$ (i.e., 1 µM) was not detrimental to these safflower genotypes, and tolerance mechanisms adopted by the studied tolerant genotypes were effective, at least in part, in ameliorating Cd stress. Since the high $CdCl_2$ concentration was not tolerable to any of the studied genotypes, the genotypic difference in tolerance index at the high concentration of $CdCl_2$ was very little.

The Cd-tolerant genotype, i.e. AC-Sterling, displayed the greatest decrease in tolerance index with increasing $CdCl_2$ concentration from 1 to 20 µM, compared with the other genotypes (Table 1). It seems that in the AC-Sterling genotype, tolerance mechanisms at the low $CdCl_2$ concentration were more efficient than at the high concentration, while the other two genotypes were already affected at 1 µM $CdCl_2$.

The Cd concentration in the roots and shoots of the studied genotypes was increased with increasing $CdCl_2$ supply (Table 1). The highest root and shoot Cd concentrations were found in the Saffire (semi-

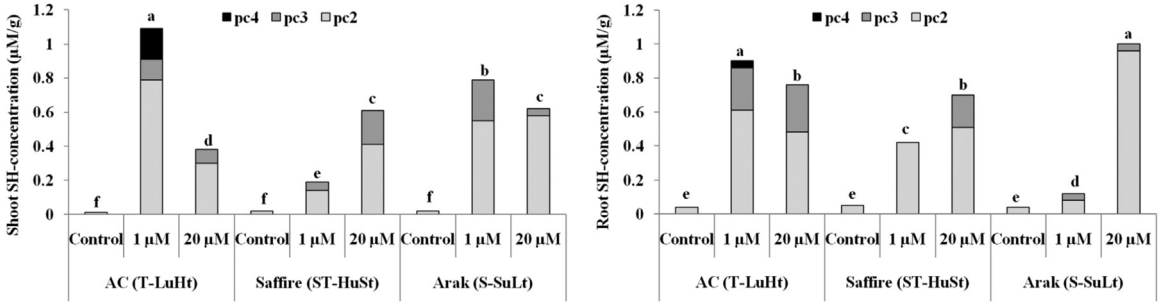

Fig. 4. Phytochelatin accumulation calculated from the SH concentration (µM g^{-1}) in shoots and roots of three genotypes, i.e., (Saffire (ST-HuSt), AC-Sterling (T-LuHt), and Arak (S-SuLt), of safflower grown for 14 days in three concentrations of $CdCl_2$ (i.e., 0, 1, and 20 µM). Each value is a mean of five replicates. The letters above the bars indicate significant differences according to the LSD ($p < 0.05$); $^{*}p < 0.05$, $^{**}p < 0.01$. F-value in SH concentration for genotype, $CdCl_2$ and genotype × $CdCl_2$ are 0.135**, 1.120** and 0.451** respectively. F-value in root SH concentration for genotype, $CdCl_2$ and genotype × $CdCl_2$ are 0.099**, 1.360** and 0.479* respectively.

tolerant) and the lowest in the AC-Sterling (tolerant) genotype at both concentrations of Cd. The latter discrepancy in tissue Cd concentrations of the Cd-exposed safflower plants leads us to propose that AC-Sterling is capable of restricting Cd absorption. The Saffire (semi-tolerant) genotype appeared to be a somewhat tolerant genotype, as it displayed the highest concentrations of Cd in its roots and shoots. In line with our findings, Wójcik et al. (2005) demonstrated that Cd tolerance is independent of metal accumulation in the hyper accumulator *Thlaspi caerulescens*.

The 20 µM $CdCl_2$ treatment led to a significant increase in root CEC compared with that of the controls (Fig. 3). Nyquist and Greger (2009) demonstrated that CEC was increased with increasing medium Cd concentration in a submerged plant species (i.e., *Elodea canadensis*), but not in an emergent plant species (i.e., *Carex rostrata*). They surmised that new cell wall-binding sites are synthesized in response to increasing tissue concentrations of Cd in *E. canadensis*, and therefore proposed that root CEC may be the major reason for the differences between Cd tolerances of the Cd-exposed plants. However, in our study, because root CEC was not significantly affected by genotype and genotype × $CdCl_2$ and because the correlation between CEC and the tolerance index was not significant in any of the genotypes (data not shown), the difference in the Cd sensitivity of these genotypes is apparently related to other strategies and mechanisms rather than increased CEC.

Cell wall-bound Cd in safflower root was not significantly affected by $CdCl_2$ concentration. Hall (2002) maintained that the binding property of the cell wall and its role as a mechanism of metal tolerance was a matter of controversy. They claimed that because the root cell walls are directly in contact with the metals in the soil solution, metals exert a restrictive effect on the plasmalemma surface (Hall, 2002). However, Bringezu et al. (1999) demonstrated that the heavy metal-tolerant *Silene vulgaris* accumulated high concentrations of metals in its cell walls. Cell wall-bound Cd in safflower roots did not play a significant role in genotypic differences in Cd tolerance, at least at the high $CdCl_2$ concentration in the nutrient solution (Table 1), though it appeared to play a role at the low $CdCl_2$ concentration. Since AC-Sterling (tolerant) had the highest concentration of cell wall-bound Cd in the low-$CdCl_2$ treatment, it seems that this genotype uses this mechanism at the low $CdCl_2$ concentration. The latter notion is supported by the positive correlation found between tolerance index and cell wall-bound Cd in AC-Sterling (tolerant) (data not shown).

Our data suggests that safflower plants synthesize PCs in response to $CdCl_2$ in a genotype-specific manner. AC-Sterling (tolerant) displayed the highest and lowest PCs concentrations, among the genotypes studied, at the 1 µM and 20 µM concentrations of $CdCl_2$, respectively. Although PC4 was detected only in AC-Sterling (tolerant) at the 1 µM concentration of $CdCl_2$, this genotype displayed a significant decrease in PCs concentration at the 20 µM versus 1 µM concentration of $CdCl_2$. This suggests that AC-Sterling (tolerant), at least when exposed to a low $CdCl_2$concentration, relies on PCs for Cd detoxification. On the other hand, PCs concentrations in Arak (sensitive) and Saffire (semi-tolerant) plants grown in the presence of 20 µM $CdCl_2$ (versus 1 µM) were increased significantly in both roots and shoots. Our data suggests that in these two genotypes, PCs do not play an important role in Cd tolerance. The latter finding could be taken as further evidence of the genotype-specificity of the reliance of safflower on PCs for Cd detoxification. Other studies have also found no correlation between PCs concentrations and tolerance of Cd (Schat et al., 2002; Wójcik et al., 2005).

Normally, a smaller shoot tissue Cd concentration is known to be associated with greater tolerance of this metal by different species, as it has been found by Ali et al. (2014) in two rapeseed cultivars. Data provided herein, hence, depicts AC-Sterling as a genotype that maintains a low shoot Cd concentration (at least in comparison to the remaining two genotypes), irrespective of $CdCl_2$ concentration applied. Therefore, the latter genotype is expectably more tolerant to Cd stress. AC-Sterling, the tolerant genotype, displayed the greatest Cd translocation to the shoots. Negative correlations are often found between metal tolerance and root-to-shoot metal transport. However, if the increased translocation of metal to the shoots is accompanied by some Cd detoxification mechanisms it may brings about metal tolerance in plants (Harmens et al., 1993; Wójcik et al., 2005). Mechanisms such as cell wall binding, chelating in the cytoplasm, or compartmentation in vacuoles are responsible for Cd retention and detoxification in roots (Wójcik et al., 2005). In AC-Sterling (tolerant), we observed high amounts of PCs and root cell wall-bound Cd at low concentration of $CdCl_2$. Key to the discrepancy found between non-accumulator and heavy-metal accumulator plants is differential regulation and expression of genes found in both kinds of plants (Rascio and Navari-Izoo, 2011). It is the constitutive over expression of genes encoding trans membrane transporters that play a determinant role in driving the uptake of heavy metals by roots, translocation to shoots and, subsequently, compartmentalization in vacuoles or cell walls of an accumulator kind of plant. Even though data gathered in the present study does not support a Cd accumulation proposition in any of the examined genotypes, reliance of AC-Sterling (tolerant) on Cd compartmentalization in the vacuole and hence a greater tolerance cannot be ruled out. We propose that the latter Cd-translocating genotype possesses an ability to pump this heavy metal into a safe compartment, i.e. vacuole. The yeast cadmium factor (YCF1) is a tonoplastic transporter that catalyses the transport of bis (glutathionato) cadmium (Cd-GS_2) into vacuoles (Li et al., 1997). Proper functioning of this type of transporter requires formation of GSH–metal complexes that may have been accomplished by the production of the previously discussed chelating peptide material, i.e. PCs, in the AC-Sterling (tolerant). Therefore, the most significant novelty of this work, i.e. the co-occurrence of high Cd-translocating and tolerance, is justified by the presumed concomitant

functionality of PCs formation and compartmentalization phenomena in the latter Cd-tolerant genotype.

Cadmium is translocated in the roots through apoplasmic and symplasmic pathways before entering the xylem and being translocated to the shoots (Lux et al., 2011). Plants have evolved mechanisms to limit Cd translocation to the shoots. The apoplasmic Cd translocation pathway can be restricted by the development of suberin lamellae in the endodermis. In our study, however, no difference in root suberization was observed between the three genotypes (data not shown). Therefore, we rule out the function of the suberin lamellae as a significant factor in differences in Cd translocation between the three genotypes. Similar results have been found in wheat (Greger and Landberg, 2008). In a previous study (Pourghasemian et al., 2013) of eight safflower genotypes, including AC-Sterling (tolerant), Saffire (semi-tolerant), and Arak (sensitive), it was speculated that Cd translocation in safflower may be dominated by the symplasmic pathway. Symplasmic movement of Cd through the root is likely restricted by the production of PCs, which bind to and chelate the Cd, sequestering it in vacuoles (Lux et al., 2011). This mechanism could justify the lack of differences in Casparian band formation in the endodermal cell layer in the safflower genotypes studied here.

Oxidative stress due to the existence of non-redox heavy metals can be detected by measuring the lipid peroxidation. Lipid peroxidation is often measured as the change in stress-induced malondialdehyde (MDA) concentration (Bazrafshan and Ehsanzadeh, 2016). The MDA concentration in shoot and root tissue (i.e., an indicator of oxidative destruction of cellular membranes) was substantially lower in the Cd-tolerant genotype AC-Sterling than in the less-tolerant genotypes, irrespective of $CdCl_2$ concentration of the medium (Fig. 1). This is in line with the finding of Moradi and Ehsanzadeh (2015) that a Cd-tolerant safflower genotype displayed a lower MDA concentration than did the less-tolerant genotypes. To protect against oxidative stress, plants have evolved abroad range of enzymatic and non-enzymatic ROS scavenging systems. These systems play a crucial role in protecting the structure and function of membrane systems and maintaining the cellular redox state (Chen et al., 2010).

With the increase in $CdCl_2$ concentration from the control to the 1 μM $CdCl_2$treatment, the concentration of shoot and root MDA increased in the sensitive genotype, Arak. However, only the concentration of root MDA was increased in the semi-tolerant genotype, i.e. Saffire, and neither root MDA nor shoot MDA concentrations of the tolerant genotype, i.e. AC-Sterling, indicated such changes (Fig. 1). The increase in MDA concentration was associated with lowered tolerance index values and increased SOD activity in Arak and Saffire genotypes. This association may be taken as an indication of Cd-induced oxidative stress. Guo et al. (2007) suggested that increased antioxidative activity in Cd-tolerant barley genotypes is indicative of the fact that these genotypes have a greater capacity to adapt to Cd stress by developing an antioxidant defence system. However, in the present study, higher SOD activity was observed in the Cd-sensitive safflower genotype at the 1 μM $CdCl_2$ concentration (Fig. 2), indicating that the increased SOD activity might reflect a damage response to stress factors. This notion is in line with Tiryakioglu et al. (2006), who suggested that the highly Cd-sensitive barley genotype may exhibit an enhanced production of antioxidants to handle the oxidative damage of ROS.

Considering that AC-Sterling (tolerant) displayed slight increases in MDA concentration and SOD activity and a low Cd concentration in roots and shoots in the presence of the 1 μM $CdCl_2$, one may suppose that this genotype has a greater capacity to resist moderate Cd stress (1 μM) by developing avoidance mechanisms and an antioxidative defence system. Furthermore, since root and shoot MDA concentration was increased significantly when the three examined safflower genotypes were subjected to the 20 μM $CdCl_2$, we may infer that the increasing concentration of tissue Cd led to the production of reactive oxygen species, resulting in increased MDA concentration and irreversible damage to tissue development and function in the safflower genotypes. The contrasting trends observed for cell wall bound Cd and lipid peroxidation, i.e. tissue MDA content, observed in the tolerant genotype (i.e. AC-Sterling) in response to increasing concentrations of Cd are indicative of the beneficial role of cell wall bound Cd in protecting the latter safflower genotype from the Cd damage. Plant tissue MDA content is an indicator of oxidative stress-induced peroxidation of membrane lipids and considering the meaningful modifications in SOD activity of the examined genotypes it is reasonable, therefore, to hypothesize that the Cd-initiated stress was exacerbated by the oxidative stress, leading to the growth decline of safflower genotypes in the present study. Furthermore, excessive concentration of tissue Cd may have led to the suppression of the activity of the antioxidant enzymes, i.e. the decrease in enzyme activity can be due to suppression of enzyme synthesis and change in the assemblage of enzyme subunits (Ali et al., 2014); under such circumstances, the reactive oxygen species in the plants may exceed the scavenging capacity of the antioxidant enzymes, i.e. SOD in the present case. At high $CdCl_2$ concentration compared with the control treatment, the MDA concentration was increased less in the AC-Sterling (tolerant) than in the Saffire (semi-tolerant) genotype. This may be because catalase (CAT) activity is compensated for by other isoperoxidases. Similar patterns of peroxidases (POD) and CAT activity have been reported in plant tissues subjected to NaCl salinity (Mittal and Dubey, 1991) and Fe toxicity (Hendry and Brocklebank, 1985; Wu et al., 2003).

5. Conclusion

This study demonstrated that genotypes of safflower from the *C. oxycantous* and *C. Tinctorius* differ in their Cd tolerance mechanisms, at least at a low $CdCl_2$ concentration. AC-Sterling, a Cd-tolerant genotype from *C. tinctorius* with low Cd uptake, low Cd concentration, and high translocation, relied on Cd absorption restriction in concomitance to certain detoxification mechanisms, such as cell wall-bound Cd, PCs accumulation, and perhaps Cd compartmentation when grown in a low concentration of $CdCl_2$. Since SOD activity was increased in the presence of 1 μM $CdCl_2$, compared with the control treatment, in the Arak (sensitive) and Saffire (semi-tolerant) genotypes, the increased SOD activity might reflect a damage rather than curative response to Cd stress. None of the genotypes seemed capable of relying on CEC or suberized tissues (i.e. as Cd detoxification mechanisms) to decrease Cd translocation.

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