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EFFECTS OF CADMIUM STRESS ON ROOT TIP CELLS AND SOME PHYSIOLOGICAL INDEXES IN *ALLIUM CEPA* VAR. *AGROGARUM* L.

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Allium cepa var. *agrogarum* L. seedlings grown in nutrient solution were subjected to increasing concentrations of Cd^{2+} (0, 1, 10, 100 μM). Variation in tolerance to cadmium toxicity was studied based on chromosome aberrations, nucleoli structure and reconstruction of root tip cells, Cd accumulation and mineral metabolism, lipid peroxidation, and changes in the antioxidative defense system (SOD, CAT, POD) in leaves and roots of the seedlings. Cd induced chromosome aberrations including C-mitoses, chromosome bridges, chromosome fragments and chromosome stickiness. Cd induced the production of some particles of argyrophilic proteins scattered in the nuclei and even extruded from the nucleoli into the cytoplasm after a high Cd concentration or prolonged Cd stress, and nucleolar reconstruction was inhibited. In Cd^{2+}-treated *Allium cepa* var. *agrogarum* plants the metal was largely restricted to the roots; very little of it was transported to aerial parts. Adding Cd^{2+} to the nutrient solution affected mineral metabolism. For example, at 100 μM Cd it reduced the levels of Mn, Cu and Zn in roots, bulbs and leaves. Malondialdehyde content in roots and leaves increased with treatment time and increased concentration of Cd. Antioxidant enzymes appear to play a key role in resistance to Cd under stress conditions.

Key words: Cadmium, chromosome aberration, mineral metabolism, antioxidative enzymes, *Allium cepa* var. *agrogarum* L.

INTRODUCTION

Cadmium (Cd), a non-essential element, is among the most hazardous environmental pollutants for humans, animals and plants even at low concentrations (Benavides et al., 2005; Fojta et al., 2006; Mobin and Khan, 2007; Wahid and Ghani, 2008). Cd is not an essential nutrient for plants, and it can accumulate at higher levels in aerial organs (Wang et al., 2007; Zou et al., 2008), inducing phytotoxicity manifested in leaf roll, chlorosis, growth reduction, and eventually death (Benavides et al., 2005; Zou et al., 2008). It has been demonstrated that Cd affects a wide range of physiological and metabolic activities in plants: for example, Chl *a* and *b* content (Mobin and Khan, 2007), the activities of photosynthetic carbon reduction cycle enzymes (Burzyński and Zurek, 2007), mineral distribution (Wang et al., 2007; Zou et al., 2008), photosynthetic processes (Zou et al., 2009; Chen et al., 2010) and oxidative stress (Zhang et al., 2009; Markovska et al., 2009). The intensity of the effects depends on the species, metal concentration and duration of exposure (Benavides et al., 2005).

Free Cd ions in the cytosol can be toxic to plant cells. In plant cells, cadmium tends to be stored in the apoplast and in vacuoles, which may contribute to Cd tolerance in hyperaccumulator plants and common crops (Boominathan and Doran, 2003; Ma et al., 2005; Liu et al., 2007). Cadmium may cause growth inhibition related to reduction of mitotic activity, induction of chromosome aberrations, toxicity to nucleoli in apical meristems (Liu et al., 2003/2004; Zhang et al., 2009; Qin et al., 2010), damage of macromolecules, mainly proteins and lipids (Skórzyńska-Polit and Krupa, 2006) and DNA (Li et al., 2005), and disturbance of the organization of the microtubular cytoskeleton in interphase and mitotic cells (Jiang et al., 2009; Liu et al., 2009).

Cadmium is a non-redox metal, but it leads to the formation of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{-\bullet}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH) (Verma et al., 2008; Muñoz et al., 2008). One

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of the most deleterious effects induced by Cd is lipid peroxidation, which can cause biomembrane deterioration. In fact, cell membranes are major targets of environmental stresses. Cd is known to modify membrane composition and integrity by altering the total lipid composition. Malondialdehyde (MDA), one of the decomposition products of membrane polyunsaturated fatty acids, is regarded as a reliable indicator of oxidative stress (Xu et al., 2008; Zhang et al., 2009; Zou et al., 2009). Plants have evolved protective enzymatic and non-enzymatic mechanisms to scavenge ROS and alleviate their effects. The protective enzymes include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.16), peroxidase (POD, EC 1.11.1.11), and glutathione reductase (GR, EC 1.6.4.2), while several molecules such as glutathione, ascorbate and carotenoids provide non-enzymatic protection (Tiryakioglu et al., 2006). Overproduction of ROS and the occurrence of oxidative stress in plants may be an indirect consequence of Cd toxicity. Some of these effects are mediated through some general actions on membrane structure and behavior resulting from alteration of the lipid composition. Alterations in the composition of the plasma membrane may change its permeability and consequently net metal ion uptake, and may disrupt the electron transport chain (Qadir et al., 2004) or disturb the metabolism of essential elements (Zou et al., 2009). Although extensive studies have shown that Cd can change the activities of antioxidant enzymes, the results have varied between plant species or cultivars, and have been reported mostly from exposure of plants to Cd for short periods of time (Tiryakioglu et al., 2006; Ekmekçi et al., 2008).

In this study we examined the response of *Allium cepa* var. *agrogarum* plants against oxidative stress evoked by various Cd concentrations. We studied the toxic effects on cell division, nucleoli, uptake and translocation of cadmium and minerals (Fe, Mn, Cu and Zn), the activities of antioxidant enzyme such as SOD, CAT and POD, as well as lipid peroxidation in the roots and leaves in order to determine the level of tolerance and identify the detoxification strategy adopted by the plants under that stress.

MATERIALS AND METHODS

PLANTS, GROWTH CONDITIONS, TREATMENTS

After removing the dry scales of healthy and equal-sized onion bulbs (*Allium cepa* var. *agrogarum*) which had not started green leaves or any root growth, we germinated and grew the bulbs in plastic containers at 27°C for 3 days by dipping the base in tap water. Then we selected seedlings of uniform appearance, transferred them to a hydroponic system and grew them in a climate chamber under a 14 h photoperiod at 25/20°C and 55/75% relative humidity. Groups of seedlings were placed on a polystyrol plate in a pot containing 2 L modified half-strength Hoagland's nutrient solution (Stephan and Prochazka, 1989) comprised of 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 1 mM KH_2PO_4, 50 μM H_3BO_3, 1 mM $MgSO_4$, 4.5 μM $MnCl_2$, 3.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_24$ and 10 μM Fe-EDTA, pH 5.5. The seedlings grew in this solution for 1 week, and were stressed with different concentrations of Cd^{2+} (1 μM, 10 μM, 100 μM) for 12 days. Cadmium was given as $Cd(NO_3)_2$. The Cd^{2+} solutions were prepared in deionized water and were added to full-strength Hoagland's nutrient solution. Full-strength Hoagland's solution without Cd^{2+} was used for the control plants. The seedlings treated with different concentrations of Cd^{2+} grew in the same climate chamber. In each treatment group, 24 treated seedlings were examined and recorded for antioxidant enzyme activities and MDA content every 3 days (3 d, 6 d, 9 d and 12 d). Seedlings were harvested after 12 days of stress to determinate metal content. All treatments were done in five replicates. The nutrient solutions were continuously aerated and changed regularly every 5 days until the seedlings were harvested.

CYTOLOGICAL ASSAYS

The bulbs were germinated in half-strength Hoagland's nutrition solution at 25°C, producing roots about 0.6 cm long. Then the roots were treated in Petri dishes with different concentrations of Cd^{2+} solutions (1 μM, 10 μM and 100 μM) for 24 h, 48 h and 72 h. Half-strength Hoagland's solution without Cd^{2+} was used for the control plants. The test liquids were changed regularly every 24 h. For cytological studies, 20 roots in each treatment group were cut and fixed in 95% 3:2 ethanol:acetic acid for 2–3 h at room temperate and hydrolyzed in 1 M 5:3:2 hydrochloric acid:95% ethanol:acetic acid for 6–6.5 min at 60°C. For observations of chromosome morphology, 10 root tips were squashed in Carbol Fuchsin solution. For observations of changes in the nucleolus, 10 root tips were squashed in 45% acetic acid, dried, and stained with silver nitrate after 2 days.

PLANT GROWTH AND ESTIMATION OF Cd AND SEVERAL MINERALS

Macroscopic observations were made at the end of 3, 6, 9 and 12 days. In each treatment, 10 plants were examined and the root length and leaf length were measured every 3 days. The seedlings were separately removed from their solution and washed

thoroughly with running tap water for 30 min and then with deionized water to remove traces of nutrients and Cd^{2+} ions on the root surface. The samples were dried for 3 days at 45°C, 1 day at 80°C, and dried again for 12 h at constant 105°C in an oven.

All dried plant samples were prepared using wet-digestion methods (Liu et al., 2008). Concentrations of Cd, Mn, Fe, Cu and Zn were analyzed using inductively coupled plasma atomic emission spectrometry (ICP-AES, LEEMAN LABS Inc., NH, U.S.A.) as described by Duan (2003).

ESTIMATION OF LIPID PEROXIDATION

Levels of lipid peroxidation are expressed as the content of malondialdehyde. MDA was determined as described by Li (2000). Fresh leaf or root material (0.2 g) from each treatment was homogenized in 5 mL 10% TCA with a mortar and pestle every 3 days (3 d, 6 d, 9 d, 12 d). The homogenates were centrifuged at 10,000 × g for 20 min. To each 2 mL aliquot of the supernatant, 2 mL 0.6% 2-thiobarbituric acid (TBA) in 10% TCA was added. The mixtures were heated in a boiling bath for 15 min and then quickly cooled in an ice bath. After centrifugation at 4000 × g for 10 min, the absorbance of the supernatant was recorded at 600, 532 and 450 nm. Calculation of MDA was based on this formula: $C\ (\mu mol \cdot L^{-1}) = 6.45(A_{532} - A_{600}) - 0.56 \cdot A_{450}$. Lipid peroxidation was expressed as MDA content (μmol per g fresh weight).

ANTIOXIDANT ENZYME ASSAYS

Enzyme extraction and preparation were carried out at 4°C. Fresh leaf material (0.2 g) was harvested, washed with deionized water and homogenized with a mortar and pestle with 5 mL chilled sodium phosphate buffer (50 mM, pH 7.8). The homogenates were centrifuged at 10,500 × g for 20 min and the supernatant was stored at 4°C prior to enzyme analyses.

Superoxide dismutase activity was estimated according to a modification of Beauchamp and Fridovich's (1971) method. The reaction mixture (3 mL) contained 1.5 mL 0.05 M sodium phosphate buffer (pH 7.8), 0.3 mL 130 mM methionine, 0.3 mL 750 μM nitroblue tetrazolium chloride (NBT), 0.3 mL 0.1 mM EDTA-Na_2, 0.3 mL 20 μM riboflavin, 0.01 mL enzyme extract, 0.01 mL 4% (w/v) insoluble polyvinylpyrrolidone (PVPP) and 0.28 mL deionized water. The reaction was started by placing the tubes below two 15 W fluorescent lamps for 10 min, and stopped by keeping the tubes in the dark for 10 min. Absorbance was recorded at 560 nm. One unit of SOD enzyme activity was defined as the quantity of SOD enzyme required to produce 50% inhibition of NBT reduction under the experimental conditions, and specific enzyme activity is expressed as units per mg fresh weight of leaf.

Catalase activity was assayed according to the method of Beers and Sizer (1952). CAT activity was determined by UV-Vis spectrophotometry (UV-2550, Shimadzu, Japan) at 25°C in 2.8 mL reaction mixture containing 1.5 mL 200 mM sodium phosphate buffer (pH 7.8), 1.0 mL deionized water and 0.3 mL 0.1 M H_2O_2 prepared immediately before use. The reaction was initiated by adding 0.2 mL enzyme extract. CAT activity was measured by monitoring the decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption. Activity is expressed as units per minute per g leaf fresh weight; one CAT activity unit was defined as a 0.1 change in absorbance at 240 nm.

Peroxidase activity was determined following a modification of Kato and Shimizu's (1987) method. The reaction mixture in a total volume of 25 mL 100 mM sodium phosphate buffer (pH 6.0) containing 9.5 μL H_2O_2 (30%) and 14 μL guaiacol was prepared immediately before use. The reaction was initiated by adding 1 mL enzyme extract to 3 mL reaction mixture, and the increase in absorbance was monitored at 470 nm at 0.5 min intervals up to 2 min with a UV-Vis spectrophotometer (UV-2550, Shimadzu, Japan). POD activity was defined as units per g fresh weight (one POD activity unit was defined as a 0.1 change in absorbance at 470 nm).

STATISTICAL ANALYSIS

Each treatment was run in triplicate for statistical validity. Data from this investigation were analyzed with standard statistical software (Sigma Plot 9.0) using means ±SE. For equality of averages the t-test was applied. Results were considered statistically significant at $p<0.05$.

RESULTS

EFFECTS OF Cd^{2+} ON ROOT TIP CELLS

The mitotic index (number of dividing cells per 1000 observed cells) was used for the investigation. The mitotic index reflects the frequency of cell division and is an important parameter when determining the rate of root growth. As seen from Table 1, the mitotic index decreased progressively with increased Cd^{2+} concentration and duration of treatment. At 1 μM Cd^{2+} the mitotic index was slightly higher than the control in the first 24 hours, but lower than the control with increasing duration of treatment. At 10 μM Cd^{2+} the mitotic index was lower than the control during the whole experiment. At 100 μM Cd^{2+} the mitotic index was extremely low.

TABLE 1. Effects of Cd^{2+} on cell division in root tip cells of *Allium cepa* var. *agrogarum*

Time (h)	Cd^{2+} (μM)	Mitotic index (‰)	No. of dividing cells observed	Normal dividing cells (%)		Anomalous dividing cells (%)				
				Metaphases	Anaphases	C-mitosis	Chromosome bridges	Chromosome fragments	Chromosome stickiness	Anomalous mitoses (%)
24	Control	215	500	48.3	44.4	6.3	0.6	0.4	0.0	7.3
	1	232	500	53.8	35.3	6.9	1.5	1.4	1.1	10.9
	10	198	500	49.7	32.5	12.1	0.8	0.8	4.1	17.8
	100	132	500	41.5	32.6	16.3	0.4	0.6	8.6	25.9
48	Control	236	500	47.3	42.5	7.4	0.9	0.9	1.0	10.2
	1	207	500	45.4	41.3	6.3	2.3	1.5	3.2	13.3
	10	116	500	44.5	36.9	10.5	0.6	0.8	6.7	18.6
	100	59	238	36.8	28.2	13.8	0.5	1.2	19.5	35.0
72	Control	232	500	41.5	47.5	7.2	1.0	0.9	1.9	11.0
	1	192	500	42.1	43.8	5.6	1.7	0.6	6.2	14.1
	10	101	500	40.4	34.0	11.8	0.8	1.4	11.6	25.6
	100	180	65	13.0	7.2	3.3	1.0	2.3	73.2	79.8

Normal cell mitoses showed mitosis prophase (Fig. 1a,b), metaphase (Fig. 1c), anaphase (Fig. 1d,e), telophase (Fig. 1f) and intermitosis (Fig. 1g). C-mitoses, chromosome bridges, chromosome fragments and chromosome stickiness were observed in the root tip cells of all treated groups after treatment with Cd^{2+} (Tab. 1, Fig. 1). The frequency of C-mitoses increased with increasing Cd^{2+} concentration and treatment time. Highly condensed chromosomes were randomly scattered in the cell (Fig. 1h,i). Chromosome fragments were also found (Fig. 1j,k). Chromosome bridges involving one or more chromosomes (Fig. 1l-n) were found after Cd^{2+} treatment; some chromosome bridges developed chromosome stickiness (Fig. 1n,o). Chromosome stickiness, usually irreversible, reflected highly toxic effects and probably led to cell death. The frequency of cells with chromosome stickiness increased with increasing Cd^{2+} concentration (Tab. 1). In addition to the aberrations mentioned above, a micronucleus beside the normal nucleus was also found in the cytoplasm (Fig. 1p).

Normally the nucleus of *A. cepa* var. *agrogarum* contained one nucleolus (Fig. 2a). The toxic effects of Cd^{2+} on nucleoli varied depending on the concentration and treatment time. Some tiny particles containing argyrophilic proteins were observed in the nucleus of root tip cells exposed to 1 μM Cd^{2+} for 24 h (Fig. 2b,c). More particles accumulated in the nucleus with increasing Cd^{2+} concentration and treatment time, for example at 1 μM Cd^{2+} for 48 h (Fig. 2d,e) and 10 μM Cd^{2+} for 24 h (Fig. 2f,g). With increasing duration of treatment the observed effects involved mainly the nucleoli. In the group treated with 10 μM Cd^{2+} for 48 h we noted that some particles containing argyrophilic proteins were extruded from the nucleus into the cytoplasm (Fig. 2h,i). Nucleolar material gradually accumulated in the cytoplasm over time. Under 10 μM Cd^{2+} for 72 h, nucleolar material finally occupied all of the cytoplasm (Fig. 2j,k), at which time Cd stress induced cell death (Fig. 2l).

After treatment with Cd^{2+}, abnormal phenomena of the nucleolar cycle during mitosis were observed in some cells. First, nucleoli did not disaggregate normally and retained their characteristic structures during metaphase, referred to as persistent nucleoli (Fig. 2m). Second, some particles of silver-stained material were still localized on chromosomes (Fig. 2n,o). Third, there were no NORs but many silver-stained particles were localized on sticky chromosomes (Fig. 2n). Fourth, nucleolar reconstruction was inhibited, and there were still many small silver-stained particles in the nuclei (Fig. 2p).

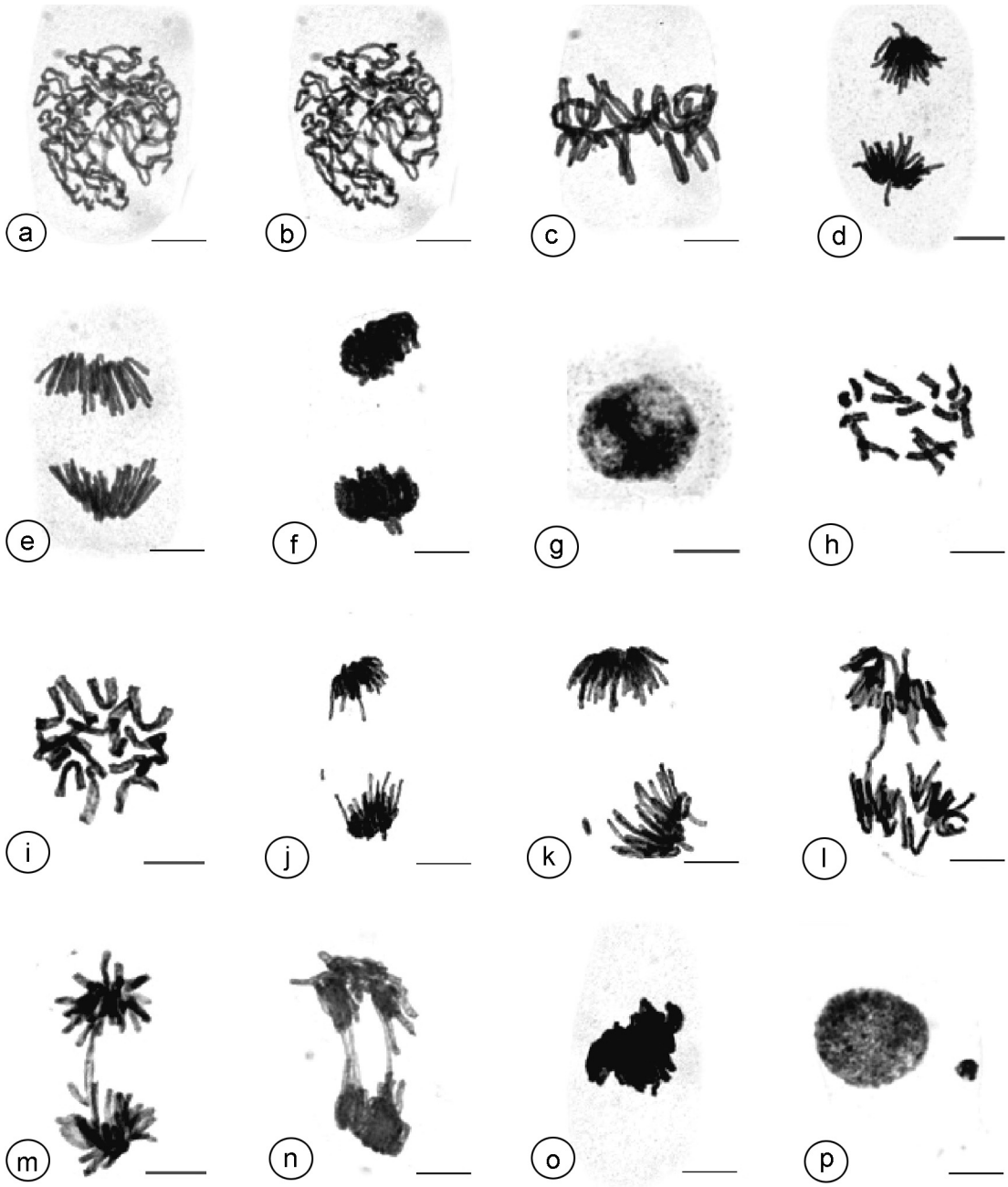

Fig. 1. Effects of Cd^{2+} on root tip cell division in *Allium cepa* var. *agrogarum*. (**a–g**) Normal mitotic stages. (**a,b**) Prophase, (**c**) Metaphase, (**d,e**) Anaphase, (**f**) Telophase, (**g**) Interphase, (**h,i**) C-mitosis after treatment with 1 μM Cd for 24 h (h) and with 10 μM Cd for 24 h (i), (**j,k**) Chromosome fragments after treatment with 1 μM Cd for 48 h (j) and with 10 μM Cd for 24 h (k), (**l–n**) Chromosome bridges after treatment with 10 μM Cd for 24 h (l) and with 10 μM Cd for 48 h (m), (**n–o**) Chromosome stickiness after treatment with 10 μM Cd for 72 h, (**p**) Micronucleus after treatment with 10 μM Cd for 72 h. Bar = 10 μm.

EFFECTS OF Cd^{2+} ON SEEDLING LEAF AND ROOT LENGTH

Throughout the 12 days, 1 and 10 μM Cd^{2+} exposure had little effect on root length. In the 100 μM Cd^{2+} treatment, root length decreased 13.1%, 34.5% and 42.4% in seedlings after exposure for 6, 9 and 12 days respectively (Fig. 3a).

The first visible symptom of Cd^{2+} exposure to *A. cepa* var. *agrogarum* appeared in the form of leaf

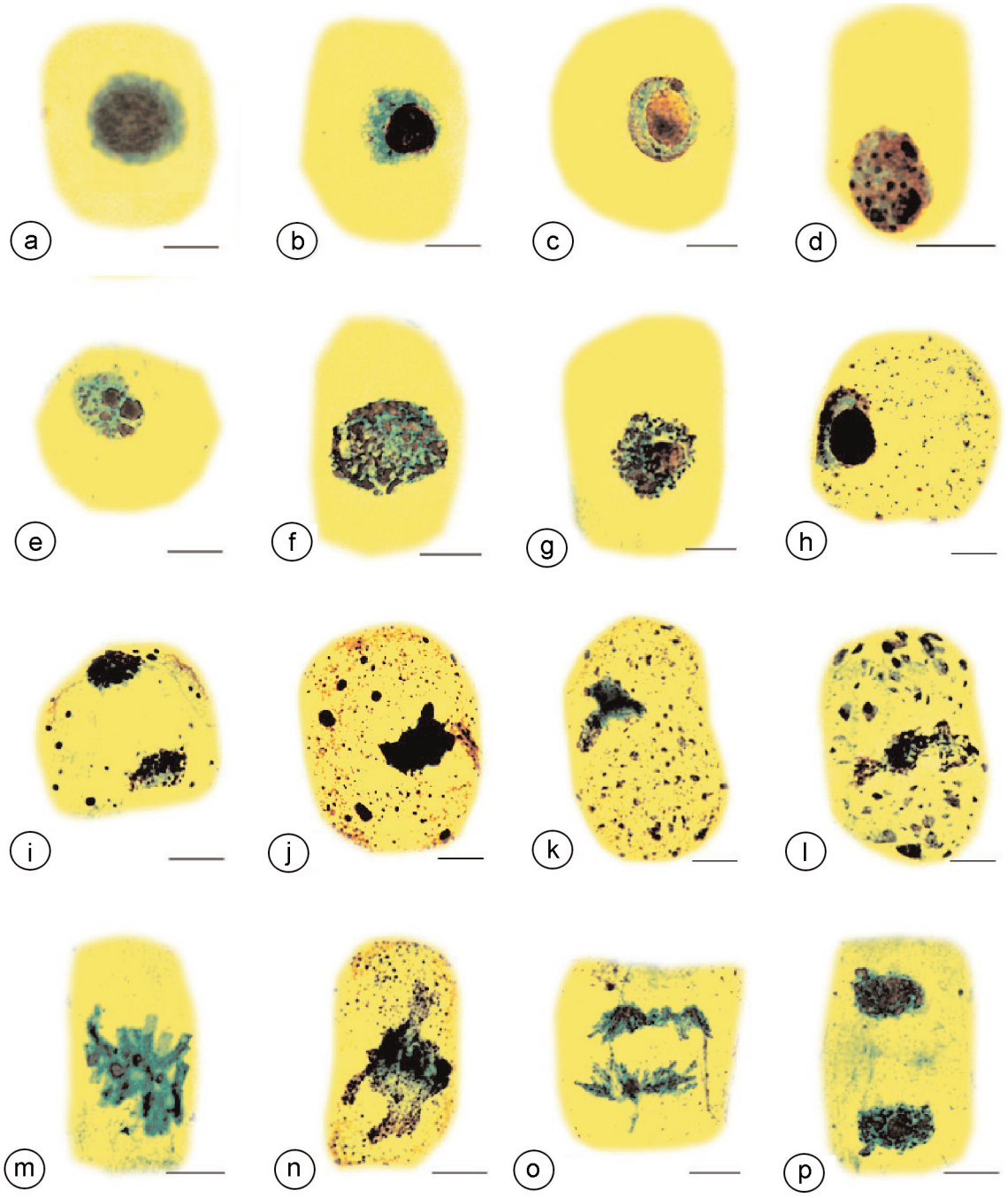

Fig. 2. Effects of Cd^{2+} on nucleoli in root tip cells of *Allium cepa* var. *agrogarum* (**a**) Control cell, (**b,c**) A few silver-stained particles scattered in the nucleus after treatment with 1 μM Cd for 24 h, (**d–g**) Numerous silver-stained particles scattered in the nucleus after treatment with 1 μM Cd for 48 h (d–e) and with 10 μM Cd, 24 h (f–g), (**h–i**) Small amount of nucleolus material extruded from the nucleus into the cytoplasm after treatment with 10 μM Cd for 48 h, (**j–k**) Nucleolus material increased in the cytoplasm with increasing Cd concentration and duration of treatment (10 μM Cd for 72 h), (**l**) Cell death after treatment with 10 μM Cd for 72 h, (**m**) Nucleoli still existing after treatment with Cd during metaphase (10 μM Cd for 48 h), (**n,o**) Particles of silver-stained material located on chromosomes after treatment with 10 μM Cd for 48 h, (**p**) Nucleolar reconstruction inhibited after treatment with 10 μM Cd for 72 h. Bar = 10 μm.

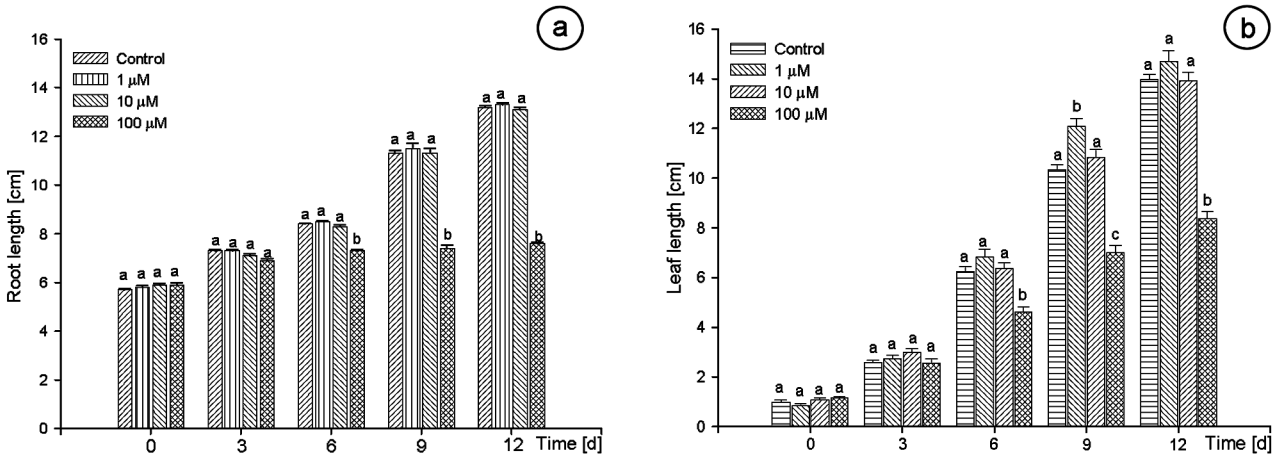

Fig. 3. Effects of different concentrations of Cd^{2+} on root and leaf length in *Allium cepa* var. *agrogarum*. (**a**) Root length, (**b**) Leaf length. Vertical bars denote SE, n = 10. Values with different letters differ significantly at $p<0.05$ (t-test).

length reduction (Fig. 3b). Reduction of leaf length in 100 μM Cd^{2+} treatments was significant after treated for 6 days, stimulation was observed in seedlings of 1 μM Cd^{2+} treatments cultivated for 9 days, and other treatments had no serious changes versus the control.

UPTAKE AND TRANSLOCATION OF CADMIUM

Cadmium accumulation in leaves and roots of *A. cepa* var. *agrogarum* treated with different Cd^{2+} concentrations is shown in Table 2. The amount of Cd detected in control roots, bulbs and leaves was quite low. Cd content in roots, bulbs and leaves increased with Cd^{2+} supply and treatment duration. Plants exposed to increasing Cd^{2+} concentrations showed increased Cd uptake by roots, reaching a maximum 2107.902±10.541 μg g^{-1} DW at 100 μM Cd^{2+} after 12 days of cultivation.

Accumulation of Cd in leaves and roots was proportional to the increase in the concentration of Cd^{2+} in the solutions. In Cd^{2+}-treated plants the metal was largely restricted to the roots, with very little of it transported to aerial parts. Nevertheless, the Cd concentration in leaves of Cd-treated plants rose to 264.149±1.106 μg Cd g^{-1} DW in the 100 μM Cd^{2+} treatment. The Cd content in leaves in the 10 μM Cd^{2+} treatment was only 10.642±0.095 μg Cd g^{-1} DW, and the shoot/root ratio was 2.5%.

The tissue levels of Cd in treated seedlings increased with increasing Cd^{2+} concentration in the nutrient solutions, and reached levels in the following order: roots > bulbs > leaves at 1 μM Cd^{2+}; roots > leaves > bulbs at 10 μM and 100 μM Cd^{2+}. By day 12 the root-to-whole plant Cd percentages were 88.1%, 96.4% and 85.9% under exposure to 1, 10 and 100 μM Cd^{2+}, respectively (Tab. 2).

EFFECTS OF Cd^{2+} ON UPTAKE AND TRANSLOCATION OF Fe, Mn, Cu AND Zn

As seen from Table 2, the different concentrations of Cd^{2+} treatments increased tissue Fe content except for leaf Fe content in the 1 μM Cd^{2+} treatment. Leaf Fe content increased 23.2% in the 10 μM and 21.4% in the 100 μM Cd^{2+} treatment versus the control.

The Mn concentration was highest in leaves and lowest in roots (Tab. 2) in both the control and the Cd^{2+}-treated plants. Mn accumulation was affected by the different concentrations of Cd^{2+} in the nutrient solution. Versus the control, Mn content in roots and stems increased significantly at low Cd^{2+} concentration (1 μM), and decreased significantly in roots in the 10 μM treatment and in roots and leaves in the 100 μM treatment (Tab. 2).

Cd^{2+} led to marked reduction of Cu and Zn levels in roots, bulbs and leaves. The reduction increased with increasing Cd^{2+} concentration.

EFFECTS OF Cd^{2+} ON LIPID PEROXIDATION AND MEMBRANE PERMEABILITY

The effects of Cd^{2+} on malondialdehyde (MDA) concentration are presented in Figure 4. MDA concentrations of both roots and leaves increased in a manner dependent on Cd^{2+} concentration and day. Versus the control, root MDA content in the 100 μM treatment increased by 102.6% at 9 days and 110.2% at 12 days.

EFFECTS OF Cd^{2+} ON SOD, CAT AND POD ACTIVITY

Superoxide dismutase activity increased in both roots and leaves during the first 3 days of treatment.

TABLE 2. Content of Cd and several minerals in roots, bulbs and leaves of *Allium cepa* var. *agrogarum* after treatment with different concentrations of Cd^{2+}

	Treatment	μg/g DW±SE [a]		
	(μM)	Root	Bulb	Leaf
Cd	Control	0.124±0.062a	0.662±0.070a	0.095±0.004a
	1	28.367±0.069b	2.150±0.046b	1.678±0.024b
	10	427.804±1.457c	5.462±0.010c	10.642±0.095c
	100	2107.902±10.541d	81.461±0.093d	264.149±1.106d
Fe	Control	90.093±1.016a	54.831±0.132a	88.331±2.450a
	1	92.945±0.322a	61.517±0.467b	85.033±0.204a
	10	94.181±0.602a	67.920±0.283c	108.825±0.721b
	100	196.988±0.360b	59.325±0.069b	107.255±0.625b
Mn	Control	10.143±0.092a	13.747±0.215a	36.420±0.490a
	1	10.923±0.156b	17.423±0.111b	38.057±0.198b
	10	8.150±0.104c	16.191±0.238c	41.568±0.168c
	100	7.560±0.088d	14.370±0.539a	15.034±0.295d
Cu	Control	13.784±0.316a	4.376±0.065a	9.344±0.269a
	1	9.308±0.145b	3.723±0.068b	6.442±0.078b
	10	8.225±0.176c	3.253±0.158b	6.322±0.250b
	100	5.775±0.412d	1.721±0.118c	4.233±0.072c
Zn	Control	250.305±1.604a	48.211±0.342a	67.533±0.796a
	1	231.624±0.502b	49.692±0.388a	47.945±1.526b
	10	154.559±2.303c	40.318±0.398b	41.764±0.143c
	100	87.085±0.954d	30.631±0.263c	50.483±0.423b

DW – dry weight; SE – standard error. Means with different letters differ significantly at $p<0.05$ (t-test), $n = 3$.

SOD activity increased markedly in roots at 1 and 10 μM Cd^{2+} on days 9 and 12. At 100 μM Cd^{2+}, however, SOD activity declined significantly in roots at day 12 of treatment (Fig. 5a). In leaves, SOD activity clearly increased versus the control throughout the experiment at all levels of Cd^{2+} supply, except on day 9 (Fig. 5b).

In the control plants, CAT activity was higher in leaves than in roots during the duration of the experiment (Fig. 5c,d). There was a direct correlation between Cd^{2+} concentration and CAT activity in both roots and leaves: higher concentrations of Cd produced a greater increase in CAT activity (Fig. 5c,d). CAT activity increased versus the control in roots of Cd^{2+}-treated plants up to day 9. At 100 μM Cd^{2+}, CAT activity showed its maximum increase in the first 6 days but decreased significantly by day 12 (Fig. 5c). CAT activity in leaves at 100 μM and 10 μM Cd^{2+} were higher than the control from days 9 to 12 (Fig. 5d). In the 10 μM and 100 μM Cd^{2+} treatments, CAT activity markedly increased in roots in the first 6 days and in leaves during the whole treatment.

Peroxidase activity in roots was not significantly affected by treatment with 1 μM and 10 μM Cd^{2+} but increased markedly in the 100 μM Cd^{2+} treatment; that increase lasted from days 3 to 12 (Fig. 5e). In leaves, POD activity was boosted throughout the treatments at all three levels of Cd^{2+} supply (Fig. 5f).

DISCUSSION

The most common effects of Cd toxicity in plants are stunted growth, leaf chlorosis and altered activity of

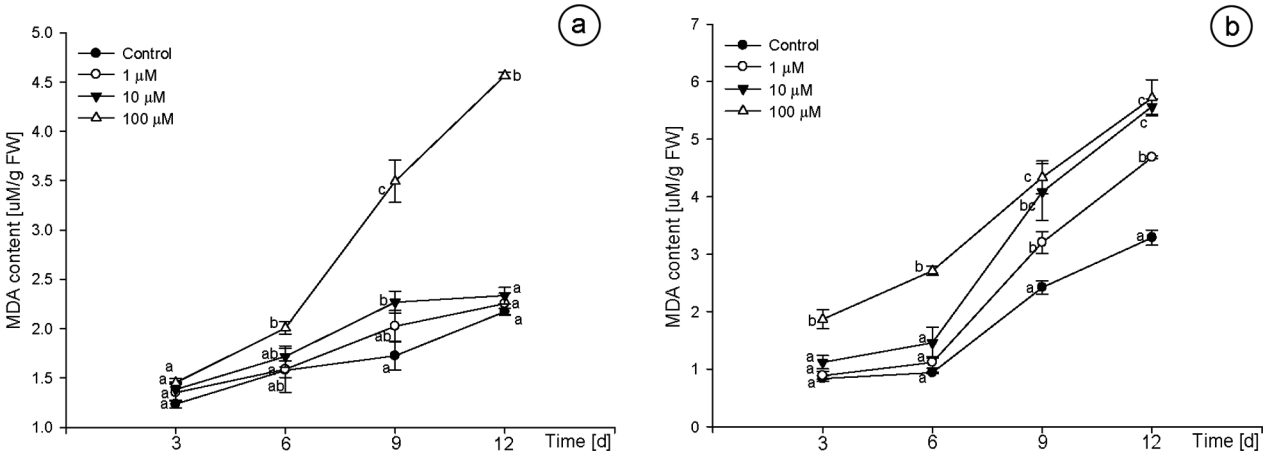

Fig. 4. MDA content of *Allium cepa* var. *agrogarum* roots and leaves after treatment with Cd^{2+} at different concentrations for 3, 6, 9 and 12 days. (**a**) MDA content in roots, (**b**) MDA content in leaves. Vertical bars denote SE, n = 5. Values with different letters differ significantly at $p<0.05$ (t-test).

many key enzymes in various metabolic pathways (Zhang et al., 2009). We observed reduction of root and leaf length of *Allium cepa* var. *agrogarum* seedlings under high concentrations of Cd treatment in this investigation (Fig. 3a,b).

Our cytological observations clearly showed that Cd had toxic effects on cell division and induced chromosome aberrations including C-mitosis, chromosome fragments, anaphase bridges and chromosome stickiness, in agreement with other reports (Liu et al., 2003/2004; Zhang et al., 2009). In cytology, chromosome stickiness is a more serious and lethal type than chromosome fragments and chromosome bridges (Liu et al., 2003/2004). We suppose that the decline in the frequency of cells with chromosome bridges and chromosome fragments with increasing Cd stress is due to poisoning of roots under high Cd concentration and duration. Cd inhibits cell division and cell expansion growth through direct or indirect effects on auxin metabolism or carriers (Prasad, 1995). Cd interferes with calmodulins located in the mitotic spindle by influencing the uptake of Ca^{2+}, causing abnormal processes of chromosome movement which lead to mitotic abnormalities (Liu et al., 2003/2004).

The nucleolus is well known as the site of transcription of ribosomal genes and further transcript processes, which contains a set of acidic nonhistone proteins that bind silver ions and are selectively visualized by silver methods. NORs are defined as nucleolar components containing a set of argyrophilic proteins, which are selectively stained by silver methods (Qin et al., 2010). Proteinic carboxyls first combine and deoxidize certain silver cations (Ag), and then more silver cations continue to be deposited at the focalization. After silver-staining, the nucleoli can be selectively stained and the NORs can be easily identified as black dots (Qin et al., 2010).

Nucleolin is one of the main proteins in the nucleolus and oxidative stress can induce cleavage of it (Qin et al., 2010). According to van der Aa et al. (2006), the nuclear pore complex (NPC) is the most important channel for nuclear material. We observed tiny particles containing argyrophilic proteins in the nucleus of the root tip cells exposed to 1 µM Cd^{2+} for 24 h (Fig. 2c). More particles gradually accumulated in the nucleus with increasing Cd^{2+} concentration and prolonged treatment time. Gradually the nucleolar material was extruded from the nucleus into the cytoplasm. This may be explained by the effects of Cd^{2+} treatment on proteins, causing the NPC to lose selectivity (Amenós et al., 2009; Qin et al., 2010). Finally the material occurred throughout the cytoplasm, eventually leading to cell death.

Persistent nucleoli do not occur during normal mitosis. An increased number of persistent nucleoli cells shows increased nucleolar activity (Vostrikova and Butorina, 2006). Sheldon et al. (1981) found that embryonal carcinoma lines exhibited persistence of nucleoli during mitotic metaphase and anaphase, indicating continuing rRNA synthesis in persistent nucleoli, increased biosynthetic activity and more protein production. We observed persistent nucleoli in the present investigation. Qin et al. (2010) suggested that this is an adaptive response to stress induced by metal poisoning.

Our results do not resolve whether or not *A. cepa* var. *agrogarum* possesses the tolerance characteristics of Cd hyperaccumulators, but the results do suggest that Cd can be effectively excluded from its shoots, and that this taxon can survive

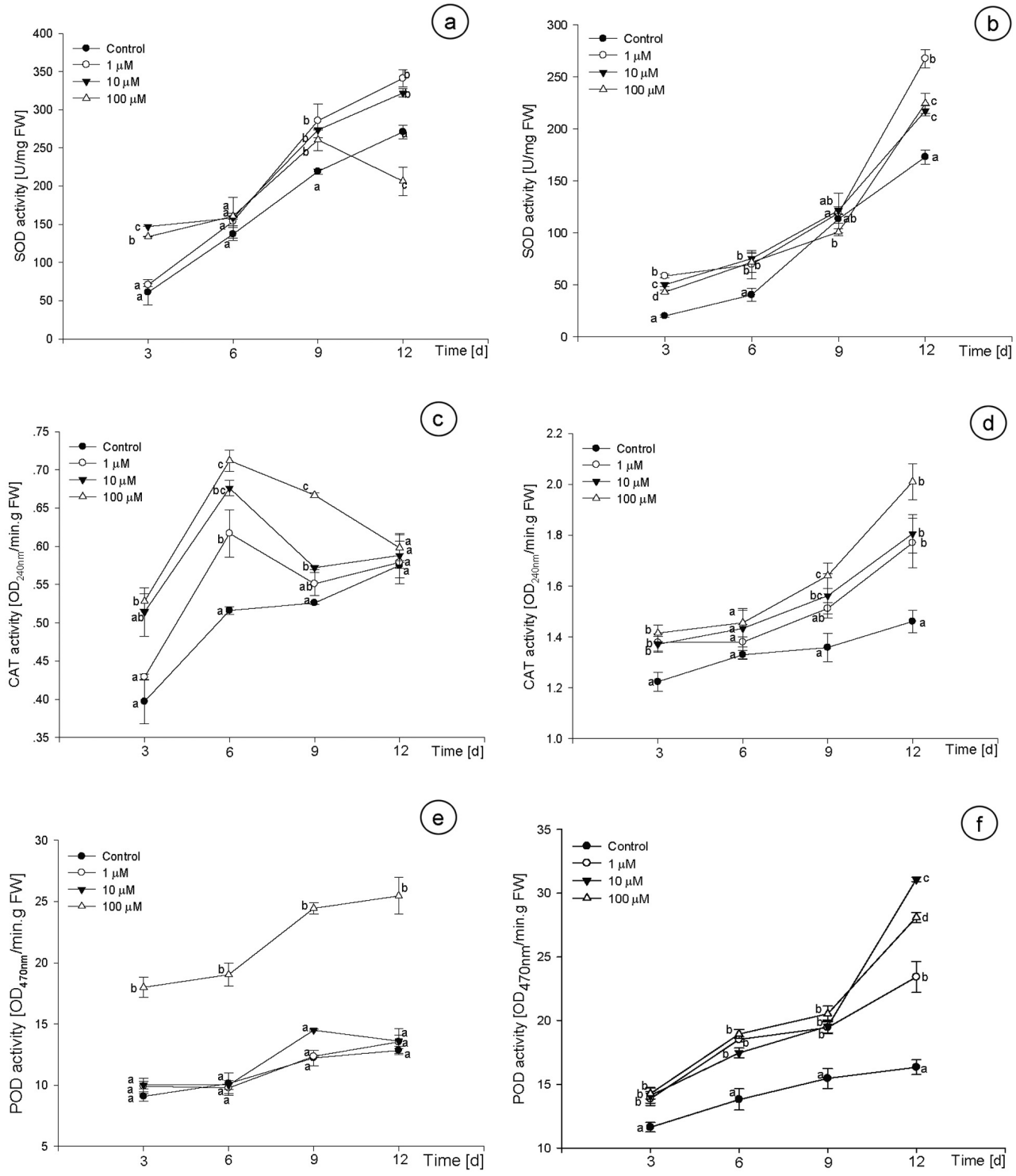

Fig. 5. Activity of three antioxidant enzymes in *Allium cepa* var. *agrogarum* roots and leaves after treatment with different concentrations of Cd^{2+} for 3, 6, 9 and 12 days. (**a**) SOD in roots, (**b**) SOD in leaves, (**c**) CAT in roots, (**d**) CAT in leaves, (**e**) POD in roots, (**f**) POD in leaves. Vertical bars denote SE, n = 5. Values with different letters differ significantly at $p<0.05$ (t-test).

as long as it is able to avoid Cd accumulation in the shoots.

Adding Cd^{2+} to the nutrient solution led to reduction in the levels of Mn, Cu and Zn in roots, bulbs and leaves, indicating that Cd^{2+} toxicity disturbed the uptake of mineral nutrients, leading to mineral nutrient deficiency. Tissue concentrations of Mn, Cu and Zn decreased, probably due to accelerated efflux of these elements from the plant. According to Broadley et al. (2001), phylogeny influences the trait of metal accumulation in angiosperms. Variation in shoot metal content occurred at the order level and above, suggesting an ancient evolution of traits. The evolution of traits influencing the shoot content of Cr, Cd and Pb also affects shoot Cu and Zn content. Our results are in accord with work by Liu et al. (2008) on *Amaranthus viridis* growing in nutrient solution with Cr(VI), in which inhibition of Cu, Zn and Fe translocation was observed. Wang et al. (2007) reported that the presence of high levels of Cd in *Zea mays* reduced the uptake of Fe, Zn and Mn.

Membrane destabilization is frequently attributed to lipid peroxidation due to enhanced production of toxic oxygen free radicals after exposure to Cd^{2+}. MDA formation has been used as a general indicator of the extent of lipid peroxidation resulting from oxidative stress. Here we showed increased MDA content in the roots and leaves of *A. cepa* var. *agrogarum* plants with increasing Cd^{2+} concentration, indicating that Cd^{2+} induced oxidative stress. This agrees with reported effects of Cd in *Brassica napus* plants: reduced formation of polyunsaturated fatty acids and a drastic alteration of membrane lipid content (Nouairi et al., 2006; Ammar et al., 2007).

Heavy metals cause oxidative damage to plants either directly or indirectly through the formation of ROS, which cause further severe oxidative damage to different cell organelles and biomolecules (Radotic et al., 2000). One of the basic mechanisms underlying Cd toxicity to plants is increased production of ROS (Romero-Puertas et al., 2004). To scavenge ROS, plants possess a well-organized antioxidative defense system comprising enzymatic antioxidants (e.g., SOD, POD, GR, GPX, CAT) and non-enzymatic antioxidants (GSH, NP-SH, PCs). The cooperative functioning of these antioxidants plays an important role in scavenging ROS and maintaining the physiological redox status of organisms (Cho and Seo, 2005). Although Cd does not generate ROS directly, it generates oxidative stress via interference with the antioxidant defense system (Shi et al., 2010) and increases MDA content in plants due to increased lipid peroxidation (Zhang et al., 2009). Here we found that SOD, POD and CAT activity in both leaf and root tissues significantly increased, except for SOD in leaves of plants exposed to 100 μM Cd^{2+} (Fig. 5). SOD catalyzes the conversion of superoxide anion to O_2 and H_2O_2, and its activity reflects the O_2 radical-scavenging ability of plants (Ekmekçi et al., 2008). POD catalyzes H_2O_2-dependent oxidation of the substrate, while CAT eliminates H_2O_2 by breaking it down directly to form water and oxygen (Ekmekçi et al., 2008).

There is evidence that the combination of SOD, CAT and POD plays an important role in plants' resistance to environmental stress (Zhang et al., 2009; Shafi et al., 2009; Chen et al., 2010). In our study the activity of SOD, CAT and POD in roots and leaves under Cd stress was enhanced, indicating their active involvement in scavenging ROS generated by Cd toxicity. These results are consistent with recent reports by Zhang et al. (2009) and Shi et al. (2010), and suggest that the plants have a high ability to tolerate Cd stress, involving an efficient antioxidant system.

The effects of Cd^{2+} on antioxidant enzymes were greater in roots than in leaves, no doubt because the roots were in direct contact with the Cd^{2+} in solution and thus subject to more oxidative stress than the leaves were. Cd^{2+} was taken up mostly through the root system and accumulated at high concentrations in roots. Only small amounts of it were transported to the leaves, as shown in our results on the Cd content of roots, bulbs and leaves.

Changes in cell division, nucleoli, antioxidant enzyme and MDA content in *A. cepa* var. *agrogarum* can serve as useful biomarkers in ecotoxicological tests with Cd. The data from these biomarkers can provide valuable information for monitoring and forecasting early effects of exposure to Cd in real conditions.

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