Effects of extended exposure to cadmium and subsequent recovery period on growth, antioxidant status and polyamine pattern in in vitro cultured carnation

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Abstract The effect of different doses of Cd (0.05, 0.1 and 0.2 mM) and subsequent period in a Cd-free medium on growth, the antioxidant status and the polyamine (PA) pattern was studied using in vitro cultured nodal segments of carnation. The Cd within the tissues increased in parallel with its concentration in the culture medium, inhibited growth, altered the concentration of some minerals and decreased the levels of pigments and the total antioxidants. However, the concentration of ascorbate (Asc) + dehydroascorbate (DHA) and the Asc redox status remained unaffected, and malondialdehyde (MDA) increased only with 0.2 mM Cd. Cd also affected PA metabolism, decreasing the total PA concentration and disturbing the relative predominance of each PA fraction. Cd exposure increased the total putrescine (Put)/(spermidine (Spd) + spermine (Spm)) ratio, and an opposite pattern was recorded during the phase in Cd-free medium. Regarding individual amines, Cd induced significant changes mainly in the free Put levels. Our results suggest that Cd produces oxidative stress and that PA (especially free Put and the total Put/(Spd+Spm) ratio), are good indicators of the stress caused by Cd.

Keywords Cadmium · Carnation · *Dianthus caryophyllus* · Oxidative stress · Polyamines · Antioxidants

Abbreviations

Asc Ascorbate
Cad Cadaverine

Chl Chlorophyll
DHA Dehydroascorbate
DW Dry weight
MDA Malondialdehyde
MS Murashige and Skoog's medium
PA Polyamine(s)
Put Putrescine
ROS Reactive oxygen species
Spd Spermidine
Spm Spermine

Introduction

Cd is considered an environmental pollutant particularly widespread in areas with a high anthropogenic pressure (Sanità di Toppi and Gabbrielli 1999). In plants, Cd affects many physiological and metabolic processes. For instance, it causes leaf rolling and chlorosis and reduces the growth in roots and stems, it interferes with the uptake, transport and use of several mineral elements; it interacts with the water balance and damages the photosynthetic apparatus; it strongly affects the activity of several enzymes and produces oxidative stress by altering the cellular redox balance (reviewed in Das et al. 1997 and Sanità di Toppi and Gabbrielli 1999). In fact, oxidative damage is a common effect of many environmental stresses (Smirnoff 1998). Under normal growth conditions, the production of reactive oxygen species (ROS) in cells is low, but stress situations disrupt the cellular homeostasis and enhance their production (Mittler 2002). Regarding Cd, it has been reported that it produces alterations in both the enzymatic and non-enzymatic antioxidant systems (Chaoui et al. 1997; Schützendübel et al. 2002).

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Polyamines (PA) are low molecular mass polycations found in all living organisms. Their polycationic nature is believed to mediate their biological activity by interacting with proteins, phosphate groups of nucleic acids or anionic components of phospholipids and with cell wall components such as pectic polysaccharides (Kakkar and Sawhney 2002). In addition, they are also involved in many physiological and developmental processes and stress responses from both biotic and abiotic origin (Edreva 1998; Bouchereau et al. 1999; Kakkar et al. 2000; Kusano et al. 2008; Alcázar et al. 2010). Putrescine (Put) has been suggested as a physiological marker of stress situations in plants (Groppa et al. 2007a). Moreover, with the finding that spermidine (Spd) and spermine (Spm) show antioxidant properties, the hypothesis that PA may play a crucial role in stress tolerance and cell adaptation was greatly reinforced. However, the precise role of PA in stress tolerance is not clearly understood.

The comprehension of the effect of metals on PA metabolism is still scarce if we compare it with other environmental stresses. For example, the effects of Cd on PA titer and metabolism have been previously studied in sunflower (Groppa et al. 2001; 2003), wheat (Groppa et al. 2003; 2007b) and tobacco BY-2 cells (Kuthanová et al. 2004). However, in all these cases, the effects were evaluated at a short term. To the best of our knowledge, the effects of long-term exposure to Cd are less known in spite of the fact that in nature, the exposure time of plants to sub-lethal concentrations of this kind of pollutants is normally long. In addition, there is also a lack of information regarding the physiological changes that occur during the recovery process after extended exposure to Cd. Therefore, our two objectives were to describe how plants adapted to the stressful presence of Cd in the culture medium and how they eventually recover their normal growth rate and physiological status after transferring them to Cd-free medium by analyzing part of the non-enzymatic antioxidant system and the possible role played by PA in these processes.

Materials and methods

Plant material and Cd treatments

Carnation (*Dianthus caryophyllus* L.) plants regularly micropropagated at 4-week intervals by axillary bud culture were used in this study. Forty-eight nodal segments per treatment (around 1 cm in length) were transferred to an agar-solidified (0.7 % (w/v) Plant agar (Duchefa)) Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3 % (w/v) sucrose and different Cd concentrations (0, 0.05, 0.1 and 0.2 mM as CdCl₂ 2H₂O) for 30 days in test tubes, one node per tube. Shoots were

subcultured into the same medium after 15 days, and on day 30 transferred to a Cd-free medium for additional 15 (+15 in figures and tables) or 30 (+30 in figures and tables) days. The pH of all media was adjusted to 5.8 before autoclaving at 121 °C for 20 min. The Cd solution was filter-sterilized and the precise volume added to the autoclaved medium. Cultures were maintained at 24±1 °C under warm white fluorescent tubes (49 μmol m⁻² s⁻¹, 16-h light photoperiod).

At the end of each experimental period, the length was recorded and samples were collected. A minimum of three samples, each containing four to six complete in vitro plantlets were collected, and subsequently lyophilized before the different analysis were performed. The concentration of pigments, MDA and antioxidants were analyzed after 30 days of Cd treatment and subsequent 15 days in a Cd-free medium, while PA and minerals were also quantified after 30 days of culture without Cd.

Cd and mineral analysis

Samples consisting of 0.05 g of lyophilized powdered material were incubated with a mixture of HNO₃:HClO₄ (2:1, v/v) into Pyrex[®] test tubes according to Chapman and Pratt (1961). The resulting solutions were transferred to volumetric flasks and diluted appropriately with ultrapure water. For element analysis, an Inductively Coupled Plasma—Optic Emission Spectrometer (ICP-OES) (Perkin Elmer, Optima 3000) was used. The efficiency of the acid digestion procedure was not determined, so the element concentrations reported are estimates.

MDA determination

0.01 g of lyophilized powdered tissue was homogenized in 1.75 ml of 0.1 % (w/v) trichloroacetic acid and then centrifuged at 15000 g for 15 min. The MDA concentration was determined according to Heath and Parker (1968) with some modifications. One ml of supernatant was combined with 2 ml of 0.5 % (w/v) 2-thiobarbituric acid (TBA) in 20 % (w/v) trichloroacetic acid. The mixture was incubated at 95 °C for 30 min and the reaction stopped by placing the reaction tubes directly into ice. Then, the samples were centrifuged at 10,000 g for 10 min at 4 °C and the absorbance of the supernatant was measured at 532 nm and corrected for unspecific turbidity by subtracting the absorbance of the same at 600 nm. The amount of MDA-TBA complex was calculated from the extinction coefficient 155 mM⁻¹ cm⁻¹.

Antioxidant analysis

Antioxidants were extracted according to Prieto et al. (1999) with some modifications. To extract hydrophilic

antioxidants, 0.01 g of powdered lyophilized material was homogenized in 1.5 ml of 50 mM Na-phosphate buffer (pH 7.5) in polypropylene tubes. The tubes were shaken for 1 h at 4 °C in the dark and centrifuged at 10,000 g for 15 min. The supernatant was transferred to new tubes and kept at 4 °C until its immediate use. To extract lipophilic antioxidants, the pellet was resuspended in 1 ml of pure ethanol and subjected to the treatment described. The process was repeated again with another volume of ethanol and the supernatant was combined with the first extract and kept at 4 °C until used.

The hydrophilic antioxidant capacity was measured according to Arnao et al. (2001). In a total volume of 1 ml, a reaction mixture containing 2 mM of 2, 2'-azino-bis-(3-ethylbenzthiazoline-6 sulfonic acid (ABTS), 15 μ M H₂O₂ and 0.25 μ M peroxidase type VI in 50 mM Na-phosphate buffer (pH 7.5) was prepared. The assay was performed at 25 °C, and the reaction was monitored at 730 nm until stable absorbance was obtained. Next, 10 μ l of the supernatant previously obtained was added and the decrease of absorbance was determined after 5 min. A standard curve with ascorbate (Asc) was used.

The total lipophilic antioxidant capacity was determined according to Prieto et al. (1999). Briefly, 0.1 ml of the appropriate supernatant was combined with 1 ml of reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The mixture was incubated at 95 °C for 90 min, and after the samples had cooled at room temperature, the absorbance was measured at 695 nm. A standard curve with butyl hydroxitoluene (BHT) was plotted.

For Asc and dehydroascorbate (DHA) measurements, 0.01 g of lyophilized tissue was homogenized in 1.5 ml of 6 % (w/v) trichloroacetic acid and then centrifuged at 15000 g for 5 min at 4 °C. The supernatant was kept in ice and quantified quickly according to Kampfenkel et al. (1995) using a standard curve with Asc.

Pigments analysis

The total chlorophyll (Chl), and carotenoids were extracted in 80 % acetone as described in Harborne (1998). Their concentration was analyzed at 470 nm, 646 nm and 663 nm with a spectrophotometer (Shimadzu UV-1603) according to Wellburn (1994). The entire process was performed in low light conditions and keeping the samples in ice.

PA determination

Samples were analysed for their concentration in free, conjugated and bound PA according to Sharma and Rajam (1995). Samples of 0.01 g of lyophilized material were

homogenized in 1 ml of 5 % (v/v) perchloric acid (PCA) with 1,6-diaminehexane as internal standard and left to stand for 1 h in an ice bath at 4 °C. The homogenate was centrifuged at 15,000 g for 20 min and the supernatant and pellet were used separately. The supernatant was used in the determination of free and conjugated (PCA-soluble) PA fractions, whereas the pellet was used to extract bound (PCA-insoluble) PA fraction. In order to extract conjugated PA, 0.150 ml of the supernatant was mixed with 0.150 ml of 12 N HCl and heated at 110 °C for 14–16 h in tightly capped glass test tubes. After acid hydrolysis, the sample was evaporated from the tubes by further heating at 80 °C and the residue was resuspended in 0.150 ml of 5 % PCA. This solution was used as a source of total (free and conjugated) soluble PA. To extract bound PA, the pellet was rinsed four times with 5 % PCA to remove any trace of soluble PA and then dissolved by vigorous vortexing in 1 ml of 1 N NaOH. The mixture was centrifuged at 15,000 g for 20 min and the supernatant, including the solubilised bound PA, was hydrolysed as described above. All different PA fractions were derived with N-9-fluorenylmethylchloroformiate (FMOC) (Yokota et al. 1994) and measured by HPLC with fluorescence detection (excitation wavelength at 260 nm and emission wavelength at 313 nm). Samples were eluted from a reverse phase C-18 column (particle size 5 μ m; 4.6 x 200 mm) with a programmed acetonitrile-acetic acid (100 mM, pH 4.4) solvent gradient changing from 50 to 95 % over 60 min at a flow rate of 1 ml min⁻¹. PA in the extracts were identified by comparison of their retention times with those of authentic PA (Sigma Chemical Co., USA).

Statistical analysis

Data were subjected to analysis of variance and significant differences were determined using Duncan's multiple range test at 5 % level of probability, using SPSS version 11.5 (SPSS Inc., Illinois, USA).

Results

Effect of Cd on plant growth and mineral concentrations

In a preliminary trial, Cd was applied at a wider concentration range (0.05–10 mM) in order to evaluate its effects on in vitro-grown carnation shoots. With Cd concentrations above 0.5 mM, the plant material was seriously damaged and died. The rest of the Cd concentrations tested (0.05, 0.1 and 0.2 mM) were not lethal and caused evident effects on the growth and morphology of the carnation shoots (Fig. 1; Table 1). These concentrations were therefore used in the subsequent experiments.

Fig. 1 Influence of Cd on in vitro growth of carnation plants. **a** Plants after 30 days of exposure to different concentrations of Cd; **b** plants after 15 days in Cd-free medium after 30 days of exposure to Cd; **c** plants after 30 days in Cd-free medium after 30 days of exposure to Cd; **d** close up of a plant cultured for 30 days in medium containing 0.1 mM Cd; **e** close up of an in vitro plant cultured for 15 days in Cd-free medium after 30 days of exposure to 0.1 mM Cd

Cd caused a dose-dependent reduction in shoot growth (Table 1) and in the development of the root system (Fig. 1). Leaves showed chlorosis, rolling edges and size reduction (Fig. 1) with all the Cd concentrations tested. All these effects were correlated to the endogenous Cd level within the tissues, that increased proportionally to the Cd concentration applied (Table 1).

After the treatments, shoots were transferred to a Cd-free medium and the morphological response of the explants

evaluated again after 15 and 30 days (Table 1). A reduction of the Cd concentration within the tissues was observed, but it was still significantly higher compared to untreated controls in a concentration-dependent way. Normal growth and morphology was noticed after 15 days without Cd in the newer parts formed in the explants previously treated with 0.05 and 0.1 mM Cd (Fig. 1). A normal size and the absence of chlorosis was also recorded in the new leaves formed, although these symptoms progressed in the leaves

that were already affected. Only some of the plants treated with the highest Cd concentration began to display a normal morphology after 30 days without Cd (Fig. 1) while most died. At that point, the endogenous concentration of Cd within the plant tissues was near to $300 \mu\text{g g}^{-1}$ DW while after 15 days without Cd, these plants still contained a concentration of Cd ($480.8 \mu\text{g g}^{-1}$ DW) similar to the growth-inhibiting concentrations found in plants after 30 days of treatment with 0.1 mM Cd ($452.7 \mu\text{g g}^{-1}$ DW) (Table 1).

The presence of 0.2 mM Cd for 30 days also influenced the concentration of minerals such as K (Table 1). Fifteen days after the removal of Cd from the culture medium, differences were still observed in the Cd-treated plants. Indeed, Fe increased in plants pre-treated with the metal, while Mn and Zn decreased in plants treated with 0.2 mM Cd . This latter element was also reduced in the 0.1 mM Cd -treated plants. Finally, after 30 days in a Cd-free medium, significant changes were recorded in the plants previously treated with 0.1 mM Cd , where Fe and Mn increased if compared to the controls. Fe also increased in the plants pre-treated with 0.2 mM Cd . No changes were observed in the concentration of Mg, Cu and Ca throughout the experiment irrespective of the treatment applied (data not shown).

Stress markers

Total Chl and carotenoids were reduced concomitantly to a Cd increase within the tissues after 30 days of Cd exposure

(Table 2). After subculturing to a Cd-free medium for 15 days, low levels of total Chl and carotenoids were still observed in the Cd-pretreated plants in relation to the untreated plants.

On the other hand, after 30 days of treatment, the MDA level was significantly enhanced (+21 %) in plants exposed to 0.2 mM Cd while a decrease was observed in relation to the controls when 0.1 mM Cd was applied (Table 2). No differences in the MDA concentration were observed after the period in Cd-free medium for 15 days.

Effect of Cd on the concentration of antioxidants

The exposure to 0.2 mM Cd for 30 days significantly reduced the concentration of total antioxidants in carnation explants (Fig. 2a). This overall trend was the consequence of a dramatic decrease affecting the most abundant lipophilic antioxidant fraction, while the hydrophilic antioxidants increased by 62 % in relation to the untreated plants (Fig. 2a). A similar pattern was observed after 15 days in Cd-free medium, but only the plants pre-treated with 0.05 mM Cd showed a significant increase in total and lipophilic antioxidants (Fig. 2b).

Since Asc is probably the most abundant compound within the hydrophilic antioxidant fraction, we quantified it separately. Interestingly, the 30 day-period of growth in the presence of different Cd concentrations did not result in significant changes in the concentration of Asc+DHA or in the DHA/(Asc+DHA) ratio (Fig. 3a).

Table 1 Effect of 30 days of Cd treatment and subsequent 15 (+15) and 30 days (+30) in a Cd-free medium after the treatments on the morphological response and Cd and mineral concentrations within the tissues of carnation plants

Cd (mM)		Length (cm.)	Concentration ($\mu\text{g g}^{-1}$ DW)				
			Cd	Mn	K	Fe	Zn
30 days	0	$6.0 \pm 1.2 \text{ a}$	0.0 d	154.6 a	32847.2 bc	382.0 a	135.6 ab
	0.05	$3.0 \pm 0.5 \text{ b}$	226.5 c	113.2 a	29559.2 c	562.7 a	144.8 ab
	0.1	$2.3 \pm 0.3 \text{ c}$	452.7 b	122.4 a	41577.0 ab	492.0 a	112.8 b
	0.2	$1.8 \pm 0.4 \text{ c}$	1037.6 a	167.4 a	45435.9 a	514.6 a	149.6 a
+15 days	0	$9.8 \pm 2.3 \text{ a}$	0.0 d	242.2 a	39888.7 a	446.6 b	176.0 a
	0.05	$6.1 \pm 0.6 \text{ b}$	183.1 c	266.1 a	41515.9 a	635.3 a	156.6 ab
	0.1	$3.8 \pm 0.6 \text{ c}$	301.4 b	227.9 ab	40565.2 a	640.8 a	134.6 c
	0.2	$2.2 \pm 0.5 \text{ d}$	480.8 a	192.0 b	40901.4 a	744.9 a	137.2 bc
+30 days	0	$14.7 \pm 1.4 \text{ a}$	0.0 c	221.9 b	33831.0 b	356.8 c	332.8 a
	0.05	$11.1 \pm 1.5 \text{ b}$	74.3 b	221.5 b	39205.3 b	549.3 bc	198.6 a
	0.1	$6.9 \pm 1.6 \text{ c}$	261.7 a	291.0 a	48144.0 a	1083.0 ab	244.1 a
	0.2	$3.7 \pm 1.2 \text{ d}$	316.9 a	222.7 b	32464.5 b	1565.1 a	156.6 a

Values followed by the same letter were not significantly different ($\alpha=0.05$) as determined by the Duncan's multiple-range test. The letters next to the values refer to each subset of data within same experimental stage and can be compared only vertically, and not across columns. Data are mean $\pm$ SD ($n=3$ for element analysis and $n=48$ for length determination)

Table 2 Total Chl, carotenoids and MDA concentrations in carnation plants after 30 days of treatment with different concentrations of Cd and after subsequent culture for +15 days in a Cd-free medium

	Cd (mM)	Total Chl (mg g ⁻¹ DW)	Carotenoids (mg g ⁻¹ DW)	MDA (μmol g ⁻¹ DW)
30 days	0	5.49±0.17 a	0.20±0.02 a	0.23±0.02 b
	0.05	4.83±0.40 b	0.18±0.05 a	0.26±0.02 ab
	0.1	3.22±0.03 c	0.08±0.03 b	0.18±0.04 c
	0.2	1.48±0.05 d	0±0 b	0.28±0.02 a
+15 days	0	5.84±1.13 a	0.37±0.09 a	0.25±0.03 a
	0.05	4.75±0.10 ab	0.26±0.03 b	0.27±0.06 a
	0.1	3.97±0.35 b	0.20±0.04 b	0.28±0.02 a
	0.2	2.15±0.42 c	0.04±0.02 c	0.22±0.02 a

Values followed by the same letter were not significantly different ($\alpha=0.05$) as determined by the Duncan's multiple-range test. The letters next to the values refer to each subset of data within same experimental stage and can be compared only vertically, and not across columns. Data are mean±SD ($n=3$)

However, after 15 days without Cd, the Asc reduced and the DHA increased in the plants that had been treated with the two lowest Cd concentrations, thus leading to a change in the redox status, as shown by the increased DHA/(Asc+DHA) ratio in these plants in relation to both of the controls and the 0.2 mM Cd-treated plants (Fig. 3b).

Effect of Cd on PA metabolism

Free PA

The presence of Cd for 30 days also altered the levels of free PA, Put being the most affected amine (Table 3). Put concentration increased by 63 % in the 0.05 mM Cd-treated plants in relation to the untreated ones and reduced by 37 % and 69 % in the plants exposed to 0.1 and 0.2 mM Cd, respectively. Spd, which was found to be the major amine in all cases stayed almost unchanged irrespective of the Cd treatment. Spm also showed a strong reduction of almost 40 % in relation to the untreated plants, but only in the treatment with the highest Cd concentration tested.

After 15 days in Cd-free medium, the PA distribution was similar to that caused by the treatments. Put concentration remained higher (55 % over the controls) in the plants treated with 0.05 mM Cd, while with 0.1 mM and 0.2 mM Cd it decreased 45 % and 63 % on average, respectively (Table 3). No significant differences were detected in Spd and Spm concentrations in relation to the controls (Table 3).

After 30 days without Cd, Put levels of the Cd-treated plants continued with the same trend in relation to the controls. However, at this moment, only the plants previously exposed to 0.05 mM or 0.2 mM Cd showed significant differences in relation to the control plants. Furthermore, Put also became the major free PA in all the treatments except in the highest Cd concentration tested.

Conjugated PA

The analysis of the conjugated forms of PA revealed the presence of conjugated cadaverine (Cad) as its most relevant feature (Table 3). After 30 days growing in the

Fig. 2 Effect of 30 days of Cd exposure **a** and subsequent 15 days in Cd-free medium after the treatments **b** on the concentration of antioxidants in carnation plants. Values followed by the same letter were not significantly different ($\alpha=0.05$) as determined by the Duncan's multiple-range test. Data are mean±SD ($n=3$)

Fig. 3 Effect of 30 days of Cd exposure **a** and subsequent 15 days in Cd-free medium after the treatments **b** on Asc, DHA, Asc+DHA concentration and the DHA/(Asc+DHA) ratio in carnation plants. Values followed by the same letter were not significantly different ($\alpha=0.05$) as determined by the Duncan's multiple-range test. Data are mean $\pm$ SD ($n=3$)

presence of Cd the results revealed increases in conjugated PA only in plants treated with 0.05 mM Cd, in which a significant rise in conjugated Spd (2.8-fold) and Spm (8-fold) was recorded.

After 15 days in Cd-free medium, the plants pre-treated with the two highest Cd concentrations significantly reduced the concentration of conjugated Put in comparison to the untreated carnations. Cad concentrations were also significantly lower in all the Cd-treated plants. The concentration of conjugated Spd was however significantly higher only in the 0.2 mM Cd-treated plants (Table 3).

After 30 days in Cd-free medium, all the previous differences in the PA concentration disappeared and no significant differences were recorded in the Cd-treated plants in relation to the controls (Table 3).

Bound PA

The analysis of bound polyamines showed a high concentration of Cad as was described in conjugated PA (Table 3). After 30 days of growth in the presence of Cd, the most relevant findings were the reduction in bound Spd in the plants treated with the two highest Cd concentrations and in Spm with 0.05 and 0.1 mM Cd (Table 3). No significant

changes were detected after 15 days in Cd-free medium except for the significant increase of Spm in the plants treated with 0.05 mM Cd (Table 3). Relevant changes in bound PA titers were observed after 30 days in Cd-free medium. In this case, Put and Cad concentrations were reduced by around 50 % in all the Cd-treated plants (Table 3). No significant changes affecting Spd or Spm were detected in this period, with the exception of the plants treated with 0.1 mM Cd that showed a reduced concentration of Spm in relation to the control plants.

Total PA concentration

By gathering together the data presented in Table 3, we were able to depict the evolution of the total PA concentration (free + conjugated + bound forms) (Fig. 4). The amount of total PA was reduced by Cd in a dose-dependent manner, and this drop was maintained once the carnation plants were transferred to a Cd-free medium. The partition of PA in free, conjugated, and bound forms was particularly altered in those plants treated for 30 days with the lowest Cd concentration, showing a higher concentration of free and conjugated PA, and therefore less bound PA in relation to both of the controls and the rest of the Cd concentrations assayed. This trend in the free fraction was observed in each time period studied. On the other hand, it is important to note that 15 days after the Cd removal, control plantlets showed a higher percentage of conjugated forms when compared with the Cd-treated plants. It is also worth mentioning that the major fraction found in all cases corresponded to bound PA irrespective of the treatment applied.

In order to display the effects of Cd on PA metabolism better, we calculated the total concentration of each detected PA separately, the sum of ubiquitous ones (Put+Spd+Spm) and some ratios according to the Cd treatments (Table 4). Cd decreased the total concentration of the ubiquitous PA and regarding individual PA, a slight accumulation of Put and Cd concentration-dependent Spd and Spm reductions were observed. However, a reverse trend was observed after 15 days of culture in a Cd-free medium. The total concentration of Put decreased, except in the plants treated with 0.05 mM Cd, whereas Spd and Spm concentrations were higher than in the controls with the exception of the plants treated with 0.2 mM Cd for Spm. After 30 days without Cd, the Put concentration clearly diminished depending on the Cd concentration applied. Taken together, all these results implied that the total Put/(Spd+Spm) ratio was below 1 in the controls and the 0.05 mM Cd-treated plants, and above 1 in the other Cd treatments. Regarding the evolution of this ratio, the tendency observed both in control plants and those treated with 0.05 mM Cd was to increase with time. On the contrary, the two highest Cd

Table 3 Free, conjugated and bound PA concentrations ($\mu\text{mol g}^{-1}$ DW) in carnation in vitro plants after the treatment with different concentrations of Cd for 30 days and after a subsequent period of +15 and +30 days in a Cd-free medium

Cd (mM)	Free PA				Conjugated PA				Bound PA			
	Put	Spd	Spm		Put	Cad	Spd	Spm	Put	Cad	Spd	Spm
30 days	0	0.16±0.03b	0.74±0.05a	0.37±0.04a	0.13±0.05a	0.46±0.20b	0.16±0.02b	0.03±0.03b	1.58±0.18a	2.92±0.95a	1.72±0.85a	0.81±0.19a
	0.05	0.26±0.03a	0.80±0.02a	0.37±0.05a	0.13±0.03a	0.73±0.21a	0.46±0.11a	0.23±0.06a	1.76±0.70a	1.74±0.75a	0.91±0.47ab	0.13±0.25b
	0.1	0.10±0.00c	0.69±0.05a	0.38±0.04a	0.09±0.07a	0.37±0.09b	0.13±0.07b	0.01±0.03b	1.94±0.91a	2.93±1.10a	0.43±0.13b	0.16±0.02b
	0.2	0.05±0.02d	0.70±0.09a	0.22±0.03b	0.07±0.03a	0.35±0.06b	0.09±0.03b	0.05±0.04b	1.86±1.00a	2.93±1.29a	0.39±0.02b	0.27±0.61ab
+15 days	0	0.38±0.03b	0.68±0.10a	0.40±0.07a	0.31±0.16a	2.20±1.16a	0.08±0.02b	0.02±0.04a	1.48±0.46a	1.72±0.58a	0.30±0.06a	0.13±0.05b
	0.05	0.59±0.01a	0.82±0.02a	0.41±0.01a	0.37±0.05a	0.49±0.06b	0.20±0.03ab	0.12±0.09a	1.40±0.06a	1.63±0.31a	0.52±0.16a	0.37±0.08a
	0.1	0.21±0.03c	0.78±0.06a	0.43±0.06a	0.14±0.08b	0.41±0.14b	0.22±0.05ab	0.07±0.02a	1.13±0.29a	1.16±0.37a	0.45±0.01a	0.14±0.06b
	0.2	0.14±0.05d	0.72±0.11a	0.38±0.05a	0.05±0.06b	0.80±0.61b	0.54±0.34a	0.04±0.04a	1.29±0.15a	1.33±0.64a	0.49±0.22a	0.11±0.04b
+30 days	0	1.05±0.33b	0.66±0.13a	0.30±0.04b	0.25±0.15a	0.23±0.04a	0.02±0.02a	0.03±0.03a	2.04±0.74a	2.45±0.72a	0.47±0.28ab	0.31±0.04a
	0.05	1.55±0.29a	0.63±0.08a	0.33±0.04b	0.35±0.26a	0.23±0.03a	0.05±0.01a	0.00±0.00a	1.02±0.07b	1.42±0.18b	0.72±0.19a	0.25±0.07a
	0.1	0.71±0.09b	0.70±0.07a	0.39±0.02a	0.15±0.04a	0.35±0.06a	0.04±0.05a	0.03±0.05a	1.08±0.26b	1.22±0.56b	0.30±0.18b	0.11±0.04b
	0.2	0.22±0.03c	0.59±0.05a	0.34±0.02b	0.11±0.02a	0.31±0.17a	0.08±0.04a	0.04±0.07a	1.31±0.30b	1.76±0.06ab	0.78±0.15a	0.32±0.19a

Values followed by the same letter were not significantly different ($\alpha=0.05$) as determined by the Duncan's multiple-range test. The letters next to the values refer to each subset of data within same experimental stage and can be compared only vertically, and not across columns. Data are mean±SD ($n=3$)

concentrations provoked an inverse trend, being the total Put/(Spd+Spm) ratio initially much higher than in the controls and decreasing after 15 days without the metal. In the last 15-day period, this ratio seemed to initiate a rising tendency in those plants treated with 0.1 mM Cd but not in the plants treated with 0.2 mM Cd (Table 4).

Discussion

In vitro carnation plantlets showed dose-dependent growth inhibition and chlorosis, coinciding with many other previous reports on the effects of Cd in plants (Das et al. 1997; Sanità di Toppi and Gabbriellini 1999; Demirevska-Kepova et al. 2006). The subsequent culture in a Cd-free medium resulted in the recovery of normal growth, morphology and colouring after 15 days by the plants treated with 0.05 and 0.1 mM Cd and after 30 days by the plants treated with 0.2 mM Cd. This fact demonstrates that: 1) these plants recovered from an extended Cd stress because endogenous Cd concentration did not produce irreversible damage and; 2) in our system, plants containing up to 300 $\mu\text{g Cd g}^{-1}$ DW were able to overcome the effects of the metal.

The disturbance in mineral concentration is another consequence of the presence of Cd within the tissues (Larsson et al. 1998; 2002; Sandalio et al., 2001). In our study, Cd led to significant changes only in the concentration of K at the highest Cd concentration tested. The fact that we sampled the whole plant could be a possible reason for these minor changes when compared with previous reports. However, after subculturing the explants to a Cd-free medium, mineral uptake was still affected in some Cd-treated plants in relation to the controls. The Cd dose-

Fig. 4 Distribution of total PA concentration (sum of all PA included in each fraction) in carnation plants after 30 days of treatment with different concentrations of Cd and subsequent +15 and +30 days without Cd

Table 4 Total PA concentration and total Put/(Spd+Spm) ratio in carnation plants after 30 days of treatment with different concentrations of Cd and subsequent culture in a Cd-free medium for +15 and +30 days. Total PA concentration was calculated for each individual PA as the sum of its free + conjugated + bound forms

Cd (mM)		Total PA concentration ($\mu\text{mol g}^{-1}$ DW)					Total Put/(Spd+Spm)
		Put	Cad	Spd	Spm	Put+Spd+Spm	
30 days	0	1.87	3.39	2.63	1.20	5.70	0.49
	0.05	2.15	2.47	2.17	0.75	5.07	0.74
	0.1	2.13	3.29	1.25	0.55	3.93	1.18
	0.2	1.98	3.28	1.18	0.54	3.70	1.15
+15 days	0	2.17	3.92	1.04	0.55	3.76	1.36
	0.05	2.36	2.12	1.54	0.90	4.80	0.97
	0.1	1.48	1.56	1.44	0.71	3.63	0.69
	0.2	1.48	2.13	1.75	0.53	3.76	0.65
+30 days	0	3.34	2.68	1.16	0.64	5.14	1.86
	0.05	2.91	1.64	1.40	0.58	4.89	1.47
	0.1	1.93	1.57	1.04	0.53	3.50	1.23
	0.2	1.65	2.06	1.45	0.70	3.80	0.77

dependent inhibition in the formation and development of roots (see Fig. 1) was probably the main reason for this alteration in the mineral uptake in our system. This is also important regarding the Cd uptake during the treatments since, when present, the roots are the organs that primarily accumulate the metal (Vitória et al. 2001; Zhang et al. 2005) and only the explants treated with 0.05 mM had some roots after 30 days when compared with the rest of the Cd-treated plants.

The induction of oxidative stress (Cho and Seo 2005; Smeets et al. 2008), the disturbance of photosynthesis (Chugh and Sawhney 1999; Sanità di Toppi and Gabbriellini 1999) and changes in the antioxidant systems (Vitória et al. 2001; Ianelli et al. 2002; Zhang et al. 2005) are common events produced by Cd stress in plants. Our results support the view that these symptoms are still evident even after 30 days of exposure to Cd. However, in our study, the MDA concentration increased only with 0.2 mM Cd, suggesting that after 30 days of Cd treatment, the cell membranes were not damaged by lower concentrations of Cd.

Among the non-enzymatic antioxidants, Asc is highly abundant in plants, and can act eliminating ROS directly or through indirect mechanisms (Conklin 2001; Potters et al. 2002). Asc-dependent defenses are involved in the stress response evoked in plants by Cd (Di Cagno et al. 2001; Hatata and Adel 2008). However, a clear correlation between the toxicity symptoms and/or the concentration of Cd within the tissues with the Asc or the DHA concentration was not found in carnation. The concentration of Asc+DHA in plants after 30 days growing in the presence of Cd remained constant irrespective of the treatment applied. These results suggest that 1) *in vitro* carnation plants coped with long exposure to Cd without increasing Asc biosynthesis and 2) the enhanced hydro-

philic antioxidants recorded after 0.2 mM Cd treatment were different from Asc. We hypothesize that this rise could be associated to an increase in the concentration of glutathione (GSH) as was previously reported (Gupta et al. 2002; Ianelli et al. 2002; Pietrini et al. 2003). This molecule is the monomer of phytochelatin and has antioxidant properties, so it is considered of great importance against Cd toxicity (Noctor and Foyer 1998; Ianelli et al. 2002). In addition, high levels of GSH could contribute to keep Asc in the reduced form through the Halliwell-Asada cycle (Noctor and Foyer 1998). In this respect, the redox status of Asc (revealed by the DHA/(Asc+DHA) ratio) in all the Cd-treated plants was maintained at the levels found in the untreated controls.

After 15 days in Cd-free medium, the concentration of MDA and total antioxidants in the Cd-treated plants showed no differences in relation to the controls, except in plants previously treated with 0.05 mM Cd in the latter case. However, during this period, alterations in the Chl, Asc and DHA concentrations together with changes in the DHA/(Asc+DHA) ratio in the Cd-pre-treated plants reflect that some influences in the oxidative status still existed in spite of a Cd absence. Again, no correlation between the observed data and the toxicity symptoms and the Cd concentration within the tissues was evident. It is known that in addition to its antioxidant role, Asc is also involved in other physiological and metabolic roles in plant growth and development (Conklin 2001; Potters et al. 2002). Hence, the observed changes in carnation may be part of a physiological adjustment occurring after a stressful situation rather than being linked only to the detoxification of ROS. The elucidation of a more detailed role of Asc and its redox status in the recovery process should still be a matter of further research.

In short, Cd disturbs many physiological and biochemical processes in plants, being the photosynthetic apparatus one of the main targets affected. In Cd-treated pea plants, Sandalio et al. (2001) attributed the observed growth reduction in part to the decreased photosynthetic rate and reduced Chl concentration. However, because the dependence of the growth rate on photosynthesis is much less evident in *in vitro* plants, the growth depletion observed in our work should be better attributed to other causes as may be the influence of the metal on PA metabolism. In fact, we found that Cd impaired PA metabolism throughout the experiment, as it was revealed by the concentration-dependent reduction of the PA levels and the disturbance of the relative predominance of each PA fraction in the Cd-treated plants. On the other hand, changes in the free PA levels owing to Cd exposure have been reported in different species with varied results (Groppa et al. 2003). In carnation, significant changes after 30 days of Cd treatment were mainly observed in free Put titers. Interestingly, the effect was different depending on the concentration of Cd applied. The relevance of the metal concentration on PA metabolism has also been observed in long-term (16 days) Cd-treated sunflower plants (Groppa et al. 2007a) and in the nodules and roots of soybean plants (Balestrasse et al. 2005), where free PA levels were modified in a different manner depending on the concentration of Cd employed. In addition, the effects of Cd were much less pronounced on free Spd and Spm levels throughout the experiment, suggesting that free Put might be a target metabolite in the physiological response to Cd in our system. The results obtained during the subsequent phase without Cd supported this view. However, free Put changes were not correlated with the phenotypic symptoms and the Cd concentration within the tissues since they were concentration-dependent throughout the experimental stages and independent of the resultant PA levels at sampling times. Thus, this fraction of Put might be considered a biochemical marker of stress and toxicity to long-term Cd exposure in carnation rather than having a role in tolerance or protection against Cd toxicity. In sunflower plants, Groppa et al. (2007a) also suggested free Put and Spm as useful early and late markers of Cd and Cu stress, respectively.

Contrary to the free fraction, significant changes in conjugated and bound PA were recorded in Spd and/or Spm levels at the end of the Cd treatments. In spite of the fact that significant differences in conjugated Spd and Spm were only observed in plants treated with 0.05 mM, variations in the conjugated forms of these PA were similar to those observed in free Put after the Cd treatment, suggesting that part of the subsequently formed Spd and Spm was conjugated. This fact indicates that conjugation could be a mechanism to maintain the free PA levels in cells as it has been previously exposed in tobacco BY-2 cells treated with

Cd (Kuthanová et al. 2004). Nonetheless, changes in biosynthesis and PA oxidation cannot be rejected as a reason for the total reduction of PA even though the presence of 1,3-diaminopropane (a product of PA catabolism) was not detected. Indeed, Cd has been reported to affect the activity of enzymes involved in both the biosynthesis and degradation of PA (Kuthanová et al. 2004; Groppa et al. 2007a, 2007b).

The appearance of conjugated and bound Cad was also surprising. Even though the physiological relevance of Cad is not accurately known, the high concentration found and the alteration of its levels during the period without Cd in some of the Cd-treated plants suggests that this diamine may be of a great physiological importance in carnation. This is supported by previous work that correlated increases in conjugated Cad and Put with the establishment of normal growth in hyperhydric carnation plants (Piqueras et al. 2002). Nonetheless, as in the free PA fraction, significant differences found in conjugated and bound fractions in relation to the controls did not correlate directly with toxicity symptoms throughout the different stages. In our study, the PA levels observed reflects a situation where plants might have had enough time to get to a homeostasis that would have let them survive. Therefore, a more detailed research is needed to establish the specific role played by the different fractions.

On the other hand, the analysis of all PA fractions allowed interesting findings concerning the total PA level. For example, the concentration-dependent decrease of the total Put+Spd+Spm concentration correlated with toxicity symptoms. This fact was attributed to the drop of the total Spd and Spm concentration. A deduction from these last results could be an enhanced ethylene production induced by Cd. Although this hypothesis requires further experimentation, previous works showed that Cd treatments increased ethylene production (Sanità di Toppi et al. 1998; Groppa et al. 2003). The biosynthesis of both ethylene and PA share the common precursor S-adenosylmethionine (Pandey et al. 2000) and thus both compounds may be reciprocally affected. The drop of Spd and Spm was also reflected by the total Put/(Spd+Spm) ratio. At the end of the treatments, the ratio increased in contrast to the control, indicating a stress situation and a predominance of diamines over polyamines concurring with the most serious symptoms for each of the concentrations. However, the opposite pattern was recorded in the phase without Cd. This could be related to the disappearance of the stressing factor, the start of the recovery of normal morphology, and the disappearance of symptoms induced by Cd.

Finally, the results obtained suggest that in *in vitro* cultured carnation plants: 1) Cd negatively affects growth and the photosynthetic apparatus and induces oxidative damage; 2) the occurrence of oxidative stress after extended

Cd treatments is better evaluated if both MDA and antioxidants concentrations are quantified rather than with MDA alone; 3) Free Put and the total Put/(Spd+Spm) ratio, are good indicators of stress caused by Cd; 4) conjugated and bound fractions seem to be important from a physiological point of view, emphasizing that it is essential to analyse all PA fractions instead of only free PA in order to establish the role played by these molecules.

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