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Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances

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Abstract

Maize and pea plants were treated with 0.0 (control), 0.01 or 0.05 mM Cd in the growing medium for 11 d. Although the total Cd concentration was similar in shoot and root tissues of both species, pea plants showed more severe toxic symptoms. The fresh weight and percentage of water content of root and shoot decreased concomitantly to Cd supply. High Cd levels were found in the cell-wall fraction (Fraction I) and in Fraction IV (soluble) of maize plants, whereas Cd-treated pea accumulated more Cd in the soluble fraction. The protein concentration of Fraction IV of pea shoot and root significantly increased upon treatment with 0.05 mM Cd, whereas maize showed no effect. Furthermore, a previously not visible protein (~12 kDa), appeared in Fraction IV of pea root grown with the highest Cd supply. Cadmium treatment, in general, notably enhanced the concentrations of 2-thiobarbituric acid reactive material (lipid peroxidation products) in pea fractions, presumably due to Cd-induced oxidative stress.

Key words: Cadmium sensitivity, tissue fractions, stress, *Pisum sativum*, *Zea mays*.

Introduction

Most strategies of plant tolerance to Cd exposure are based on the reduction, by various mechanisms, of the cytosolic concentration of free Cd. In this way, the plant cell avoids Cd accumulation in the cytosol by compartmentalizing Cd in subcellular compartments, although this distribution is not clearly established yet. In maize plants, Khan *et al.* (1984) reported that Cd may be mainly associated with cell walls, whereas other authors (e.g. Velazquez *et al.*, 1992) observed the accumulation of Cd in vacuoles of bean roots.

Another mechanism of tolerance involves Cd complexation by organic molecules. Thus, phytochelatins were found to bind most of the Cd present in cells of Cd-treated bean plants; moreover, Cd could also be bound to high molecular weight proteins (MW > 70 kDa) (Weigel and Jager, 1980). The biosynthetic pathway of those polypeptides (~10 kDa) probably involves glutathione (GSH) or its metabolites (Scheller *et al.*, 1987; Gupta and Goldsbrough, 1991). On the other hand, GSH plays an important role in the control of oxidative stress in plant cells. Depletion of GSH in the presence of heavy metals results in an increase in the oxidative stress (De Vos *et al.*, 1992), seen as an appearance of lipid peroxidation products—a common symptom of this stress (Buege and Aust, 1978). Thus, plants exposed to toxic levels of Ni and Cu showed an increase in the concentration of thiobarbituric acid reactive material (TBA-rm) (Pandolfini *et al.*, 1992; De Vos *et al.*, 1993).

In the present work, the distribution of Cd is reported in tissue fractions of root and shoot of pea and maize plants, which have been previously shown to exhibit different sensitivity to Cd, in order to elucidate the different detoxification mechanisms in both species. In addition, TBA-rm level, altered due to Cd-induced oxidative stress, was assessed as an index of Cd toxicity.

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Abbreviations: FW, fresh weight; MW, molecular weight; GSH, reduced glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; DTT, 1,4 dithiothreitol; PVPP, polyvinylpyrrolidone; TCA, tricloroacetic acid; TBA-rm, 2-thiobarbituric acid reactive material.

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Materials and methods

Plant material

Maize (*Zea mays* cv. Dekalb Paolo) and pea (*Pisum sativum* cv. Argona) seeds were germinated on moistened paper for 4 d at 28 °C. Seedlings were cultivated hydroponically in a controlled environment chamber at 24 °C day/15 °C night, with 16 h of light (10 Sylvania cool White VHO lamps of 120 W m^{-2} each) and 70–80% relative humidity. Maize nutrient solution (pH 5.5) consisted of: macronutrients (mM): 2.0 KH_2PO_4, 1.5 $MgSO_4$, 0.2 NaCl, 1.0 $Ca(NO_3)_2$, 1.5 KNO_3; and micronutrients (μM): 44.8 Fe (Fe-EDDHA), 18.1 Mn ($MnSO_4$), 3.9 Cu ($CuSO_4$), 7.6 Zn ($ZnSO_4$), 46.2 B (H_3BO_3), and 2.1 Mo ($Mo_7O_{24}(NH_4)_6$). The nutrient solution for pea (pH 5.5) was: macronutrients (mM): 2.0 $Ca(NO_3)_2$, 1.5 KNO_3, 0.5 $Mg(NO_3)_2$, 1.0 KH_2PO_4, 0.5 $MgSO_4$, 0.1 NaCl; and micronutrients (μM): 44.8 Fe (Fe-EDDHA), 18.1 Mn ($MnSO_4$), 3.2 Cu ($CuSO_4$), 6.1 Zn ($ZnSO_4$), 0.1 Mo ($Mo_7O_{24}(NH_4)_6$), and 18.5 B (H_3BO_3).

Cadmium (cadmium sulphate) was supplied at three concentrations: 0.0 (control), 0.01 and 0.05 mM Cd. Shoots and roots were collected from 100 plants of each treatment after 15 d of growth; ten plants were used to determine total Cd concentration and the rest were taken for tissue fractionation.

Tissue fractionation

Shoot and root tissues were homogenized in extraction buffer (50 mM HEPES, 500 mM sucrose, 1.0 mM DTT, 5.0 mM ascorbic acid and 1.0% (w/v) Polyclar AT PVPP, and adjusted to pH 7.5 with NaOH) with a chilled mortar and pestle. The homogenate was sieved through a nylon cloth and the residue constituted the cell wall-containing fraction or Fraction I. The filtrate was centrifuged at $10\,000 \times g$ for 30 min and the pellet retained was the organelle-rich fraction or Fraction II. The supernatant was then centrifuged at $100\,000 \times g$ for 30 min and the pellet designated as the membrane-containing fraction or Fraction III and the supernatant as the soluble fraction or Fraction IV. The resultant pellets were resuspended in extraction buffer. All steps were performed at 4 °C and the fractions were stored at −20 °C for further analysis.

Protein determination

Bio-Rad Coomassie-blue assay reagent was used according to the method of Bradford (1976), using thyroglobulin as standard to determine the protein concentration of extracts.

Malondialdehyde assay

TBA-rm was assayed according to Buege and Aust (1978) using 1.0 cm^3 of biological sample (0.1–2.0 mg of protein) with 2.0 cm^3 of TCA–TBA–HCl reagent (15% (w/v) trichloroacetic acid [TCA], 0.37% (w/v) 2-thiobarbituric acid [TBA] and 0.25 M HCl) and mixing thoroughly. The solution was heated for 30 min in a sand bath at 90 °C. Butylated hydroxytoluene (0.01%, w/v) was added to the reagent to avoid the metal-catalysed autoxidation of lipids during heating. After cooling, the flocculent precipitate was removed by centrifugation at $2000 \times g$ for 15 min. The absorbance of the samples was measured at 535 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm (Heath and Packer, 1968). The level of lipid peroxidation products in roots and shoots was expressed as TBA-rm (nmol malondialdehyde g^{-1} FW). The malondialdehyde concentration was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Wills, 1969).

Protein dialysis

An aliquot of Fraction IV (1.0 cm^3) was dialysed against 2.0 dm^3 hypo-osmotic solution (5 mM TRIS–HCl and 10 mM mercaptoethanol, pH 7.5) for 48 h, using a dialysis membrane which excluded molecules below 6–8 kDa of molecular weight (Spectra/Por® membrane with diameter 6.4 mm, volume length 0.32 cm^3 cm^{-1}). Sodium azide (0.01 g dm^{-3}) was added as antibacterial agent. The final volumes of the dialysates were measured and samples were stored at −20 °C until further analysis.

Cadmium analysis

Cadmium was determined directly in the resuspended Fractions II and III and in Fractions IV and IV-dialysed, by atomic absorption spectrophotometry (AAS) (Perkin-Elmer 2100), using an air-acetylene flame and Cd hollow-cathode lamp.

Fraction I was dried at 70 °C to constant weight. After milling with a mortar and pestle, samples were digested in an autoclave for 30 min at 125 °C, 24.5×10^4 N m^{-2}, with an acid oxidative mixture H_2O : HNO_3 : H_2O_2 (5 : 4 : 2, by vol.) (Lozano-Rodriguez *et al.*, 1995). Whole shoots and roots of maize and pea plants were dried at 70 °C. After homogenization, samples were digested under pressure, following a similar method to that described above, but the acid oxidative mixture employed was H_2O : HNO_3 : H_2O_2 (5 : 3 : 2, by vol.). Cadmium concentration was measured in the digests by AAS.

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SDS-PAGE of proteins

SDS-polyacrylamide gel electrophoresis of root-Fraction IV of pea and maize plants was carried out according to the method of Laemmli (1970).

Statistic analysis

Data are the mean of at least three independent experiments. Statistical significance was calculated by the Duncan's test of analysis of variance (at $P \leqslant 0.05$ and $P \leqslant 0.10$), using the SAS statistical software package (SAS, 1986).

Results

The total Cd concentration of shoot and root in maize and pea plants increased concurrently with the treatments applied and no significant differences were found between the two species, Cd accumulation being approximately 10 times higher in root than in shoot (Table 1). Although 0.05 mM Cd treatment was 5 times higher than 0.01 mM Cd treatment, the concentration of total Cd in maize and pea plants increased just 2-fold, which probably indicates an efficient Cd exclusion both at the root surface and from the shoot.

The presence of Cd in the nutrient solution caused an evident reduction in the size of roots and shoots of maize and pea plants. Fresh weight (FW) reduction was calculated for both plants (Fig. 1); in pea, the shoot FW decreased by 70% in plants treated with 0.05 mM Cd, whereas the equivalent reduction for roots was 80%. Lower response to Cd exposure was observed in maize, where both shoot and root FW decreased only 20–30% in Cd-treated plants. Therefore, pea plants were more sensitive to Cd than maize plants.

Table 1. *Total Cd concentration ($\mu g\ g^{-1}$ FW) and percentage of water content (WC) of shoot and root of maize and pea plants, treated with 0.0 (control), 0.01 and 0.05 mM Cd*

Samples were taken after 15 d of growth. The values represent the mean (maize $n=3$ and pea $n=4$) ±SD

	Maize		Pea	
	Cd	%WC	Cd	%WC
Control				
Shoot	<0.05 a[a]	91.37±0.07 a	<0 05 a	89.95±0.44 a
Root	<0.05 a	93.44±1.09 a	<0.05 a	93.62±0.64 a
0.01 mM Cd				
Shoot	9.72±0.49 b	91.09±0.17 a	10.07±3 36 b	88.02±0.12 b
Root	76.05±20.38 b	92.53±1.12 a	63.83±6.64 b	91.73±0.52 b
0.05 mM Cd				
Shoot	21.13±8.87 c	89.70±0.59 b	24.11±5.81 c	83.83±0.37 c
Root	113.55±11.85 c	91.11±1.08 a	117.79±39 02 c	89.77±0.68 c

[a]Different letters show significant treatment differences ($P \leq 0.05$).

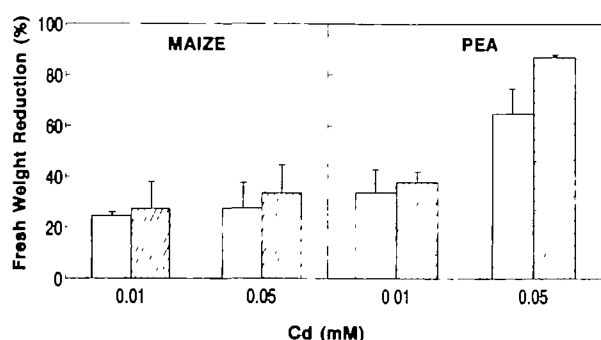

Fig. 1. Effect of Cd supply (mM) on fresh weight reduction (%) in maize and pea plants (shoot, □; root, ▨) treated with 0.0, 0.01 and 0.05 mM Cd, with regard to control plants.

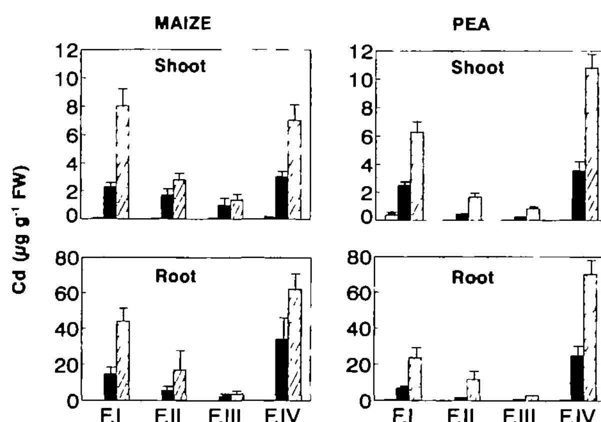

Fig. 2. Cd concentration ($\mu g\ g^{-1}$ FW) in shoot and root fractions of maize and pea plants, grown under three Cd treatments: 0.0 (control, □), 0.01 (■) and 0.05 mM Cd (▨).

The percentage water content (WC) was also reduced in both maize and pea plants upon Cd treatment, being more evident in roots treated with 0.05 mM Cd (Table 1). Pea plants were clearly more affected by Cd exposure than maize plants, which showed only a significant reduction in shoot WC for the 0.05 mM Cd treatment.

Cadmium concentration in all fractions obtained from both species increased consistently with the concentration of Cd supplied (Fig. 2). Little Cd was observed in Fractions II (organelle-containing) and III (membrane-containing), whereas most Cd was found in Fractions I (cell wall-containing) and IV (soluble) the Cd concentration being 5–10 times higher in root- than in shoot-fractions. These results were in agreement with those of Vogeli-Lange and Wagner (1990), who observed that in isolated vacuoles of Cd-treated tobacco plants, the vacuolar sap contained most of the total Cd content of the cell but this was not associated with the tonoplast. In addition, Ros *et al.* (1992) found that in rice tissue, 1000 times less Cd was associated with plasma membrane vesicles than the total Cd-concentration. Moreover, Fractions II and III of both species appeared to be Cd-saturated, as only slight differences between Cd treatments were shown (Fig. 2). The highest subcellular Cd concentrations were observed, respectively, in root and shoot Fractions IV of pea (3 and 2 times higher than Fraction I); Fraction IV of maize root also showed slightly the same tendency, but in maize shoot the Cd concentration in Fractions I and IV were similar.

There were no significant differences in protein content between Cd treatments in all fractions studied for maize plants, whereas pea plants treated with 0.05 mM Cd showed higher protein concentration in Fractions II and IV (significant at $P \leqslant 0.10$) (Fig. 3). In the SDS–PAGE of Fraction IV proteins of pea root treated with 0.05 mM Cd a band of 12 kDa appeared, which was not present in control root (Fig. 4). No similar band was observed in Fraction IV of Cd-treated maize roots. When these root fractions from both plants were dialysed the content of protein decreased severely (Table 2), but the new band remained in the pea root extract (Fig. 4). Furthermore, most of the Cd could not be removed by dialysis (Table 3). These results suggest that in those fractions Cd was mainly associated with compounds with MW higher than 6–8 kDa, with the exception of Fraction IV

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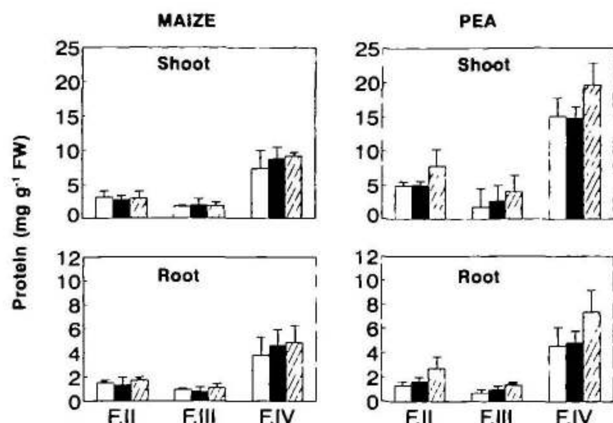

Fig. 3. Protein content (mg g^{-1} FW) in shoot and root liquid fractions of maize and pea plants, grown under three Cd treatments: 0.0 (control, □), 0.01 (■) and 0.05 mM Cd (▨).

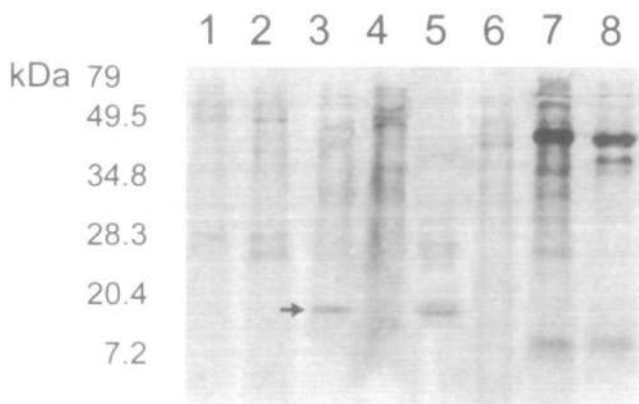

Fig. 4. SDS-PAGE of Fraction IV proteins, before and after dialysis, of maize and pea grown under two Cd treatments: 0.0 (control) and 0.05 mM Cd (+Cd). Root-maize extracts: (1) +Cd before dialysis; (2) control before dialysis; and pea extracts: (3) +Cd-root before dialysis; (4) control-root before dialysis, (5) +Cd-root after dialysis; (6) control-root after dialysis; (7) +Cd-shoot before dialysis; (8) control-shoot before dialysis. In the left side, the weight of molecular markers is shown.

of maize shoot where only 42% of Cd remained after dialysis.

Malondialdehyde and endoperoxides have mainly been identified as the products of lipid peroxidation, and are normally considered as the major TBA-rm (Buege and

Table 2. *Protein concentration (mg g^{-1} FW) in the dialysed Fraction IV of shoot and root of maize and pea plants, treated with 0.05 mM Cd, and the percentage of the remaining protein content (Rm) versus the concentration before dialysis* (n = 3)

	Maize		Pea	
	Protein[a]	Rm	Protein	Rm
Shoot	2.86 ± 0.43	31.1	7.24 ± 1.23	36.9
Root	0.43 ± 0.07	8.9	0.87 ± 0.09	11.8

[a]Mean ± SD

Table 3. *Cadmium concentration (mg g^{-1} FW) in the dialysed Fraction IV of shoot and root of maize and pea plants, treated with 0.05 mM Cd, and the percentage of the remaining Cd content (Rm) versus the concentration before dialysis* (n = 3)

	Maize		Pea	
	Cd[a]	Rm	Cd	Rm
Shoot	2.97 ± 0.80	42.2	8.96 ± 1.95	83.0
Root	56.02 ± 10.73	90.1	60.71 ± 14.81	86.4

[a]Mean ± SD

Aust, 1978). Figure 5 shows a sharp consistent increase in TBA-rm levels paralelled to increased Cd, for almost all pea fractions whereas these levels remained nearly constant in maize. All fractions of pea tissues showed good linear correlations between Cd concentration and TBA-rm level, being lower for maize (Fig. 5).

Discussion

The pea and maize cultivars studied might be considered as 'Cd-shoot excluders' (Florijn and van Beusichem, 1993), with Cd accumulated at higher concentrations in roots than in shoots (Table 1). This behaviour is one of several strategies of tolerance to Cd (Weigel and Jager, 1980; Cataldo *et al.*, 1981; Vogeli-Lange and Wagner, 1990; Metzger *et al.*, 1992; Fett *et al.*, 1994). The plant

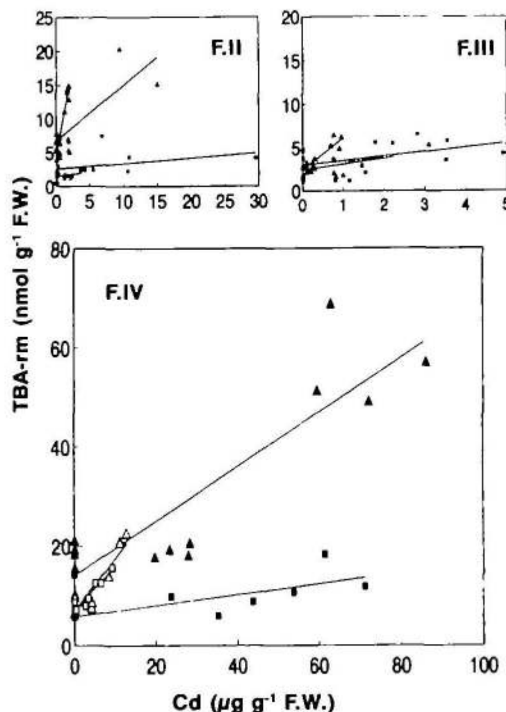

Fig. 5. Cd concentration (µg g^{-1} FW) versus lipid peroxidation product levels (TBA-rm, nmol g^{-1} FW) in fractions of maize (shoot, □; root, ■) and pea (shoot, △; root, ▲) plants.

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species studied showed similar capacity for Cd accumulation (Table 1), in agreement with Florijn and van Beusichem (1993) for maize, and with Leita *et al.* (1993), and Landberg and Greger (1994) for pea grown under similar conditions. Cadmium retention in root might be due to cross-linking of Cd to carboxyl groups of the cell wall (Barceló and Poschenrieder, 1990) and/or to an interaction with thiol residues of soluble proteins (Leita *et al.*, 1993). That is in agreement with the results, where Cd was mostly found in the cell wall and in soluble fractions (Fig. 2).

The greatest amounts of Cd were determined in the soluble fractions of pea tissues, whereas in maize plants it mainly appeared in Fractions I and IV (Fig. 2). Maize might have a reduced level of metabolically-active Cd due to coupling to components of the cell wall (Khan *et al.*, 1984). However, pea plants retained less Cd in cell wall-containing fractions and this was associated with greater physiological damage as inferred from fresh weight reduction (Fig. 1) and lipid peroxidation (Fig. 5). This was in agreement with Cataldo *et al.* (1981) and Weigel and Jager (1980), who reported that over 50% of the total Cd concentration in leguminous plants was in the soluble fraction.

The protein with an apparent MW of 12 kDa, found in Fraction IV of pea roots treated with 0.05 mM Cd, might be a putative phytochelatin (Fig. 4). Formation of this new protein would be part of the significant increase in total protein concentration in Fraction IV of pea root (Fig. 3). Moreover, after dialysis, most of the Cd remained associated with material of higher MW than 6–8 kDa (Table 3), which supports the association of Cd with polypeptides. These results were in agreement with those reported by Rauser and Glover (1984), who suggested that up to 85% of Cd was bound to proteins of low molecular weight in roots of maize.

Production of phytochelatin proportional to the degree of Cd incorporation into the plant cell, has been widely cited (Wagner and Yeargan, 1986; Vogeli-Lange and Wagner, 1990; Gupta and Goldsbrough, 1991; Obata and Umebayashi, 1993; Fett *et al.*, 1994). Scheller *et al.* (1987) reported a decline in glutathione levels in tomato cells, which was related to the synthesis of phytochelatins in response to heavy metals. Furthermore, De Vos *et al.* (1992) noticed, in two cultivars of *Silene cucubalus* with different Cu sensitivity, that the synthesis of phytochelatins increased when symptoms of Cu toxicity appeared, but subsequently there was a reduction in the content of GSH.

Little information is available about the effect of excess concentrations of Cd on lipid peroxidation in plants. Lee *et al.* (1976) found an increment in the activity of peroxidases and hydrolytic enzymes in *Glycine max* treated with Cd. Likewise, Somashekaraiah *et al.* (1992) observed that Cd significantly reduced catalase, GSH-reductase and superoxide dismutase activities of mung bean plants, causing an increment in lipid peroxidation products. Low levels of these enzyme activities may result in the enhancement of free radical-mediated lipid peroxidation (Foyer, 1987). The accumulation of TBA-rm in pea fractions (Fig. 5) could be explained on this basis, and Cd might be considered an oxidative-stress enhancing factor, although it is not a redox-active cation (Somashekaraiah *et al.*, 1992). In addition, the formation of Cd–phytochelatin complexes to reduce Cd-free concentration in the cytosol (Fig. 4) could lead to the depletion of GSH content, causing a loss in the cellular antioxidative response (Strange and Macnair, 1991; De Vos *et al.*, 1993). Therefore, the determination of a TBA-rm response may be used as a non-specific index of Cd-phytotoxicity which is more reliable than total content of Cd (Fig. 5; Table 1) (Pandolfini *et al.*, 1992; De Vos *et al.*, 1993).

It is concluded that the different distribution of Cd among the tissues fractions studied could explain the differences in sensitivity to this toxic metal between maize and pea. Whereas maize tissues incorporate more Cd into the cell wall than pea, the latter species showed more severe damage caused by higher concentrations of putative metabolically active Cd located in the soluble fraction, in spite of phytochelatin synthesis. Moreover, this mechanism would only be exerted by pea plants in response to high concentrations of cytosolic Cd, but would disturb the redox-state of the plant cell.

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