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## Cadmium and copper toxicity for tomato seedlings

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**Abstract** – We studied cadmium (Cd) and copper (Cu) toxicity in tomato, their accumulation in plant organs and their ability to induce phytochelatin synthesis. The seedlings were cultivated in nutrient solution supplemented with increased concentrations of CdCl<sub>2</sub> or CuSO<sub>4</sub> from 0 to 50 µM. After 7 days of treatment, plants were harvested and the dry weight, the amount of thiobarbituric acid-reactive substances (TBARS), Cd and Cu contents and SH groups rich peptides were determined. We found that Cd and Cu decrease tomato growth, notably at high Cu levels. Cd and Cu concentrations in plant organs increased with applied amounts. Cd and Cu concentrations were the highest in roots. The amount of thiobarbituric acid-reactive substances, that estimate the lipid peroxidation, was increased with heavy metal exposure and was, mainly, higher in roots with Cu treatment.

**Cadmium / copper / *lycopersicon esculentum* / phytochelatins / thiobarbituric acid-reactive substances**

### 1. INTRODUCTION

Heavy metals constitute a heterogeneous group of elements, including essential, such as copper (Cu) and non-essential ones, such as cadmium (Cd). In general, the metal ions will be taken up by plants and can be accumulated in the roots and shoots. When these metals are in excess their concentrations may become toxic. Several studies have revealed that Cd induces growth inhibition and chlorosis in plants (Jiang et al., 2004). Prolonged exposure to copper caused gross perturbations of root morphology, reduced root and shoot growth and leaf chlorosis (Wisniewski and Dickinson, 2003). That heavy metals disturb almost all physiological processes in plants is well established; however, there is relatively little information on the response of plants to the same rate of cadmium or copper exposure. The few reports available suggest that plants were more sensitive to copper than to cadmium (Xia et al., 2004).

Heavy metals toxicity has been related to their ability to induce oxidative stress. Heavy metals cause oxidative stress either by inducing oxygen free-radical production or by decreasing enzymatic and non-enzymatic antioxidants due to their high affinity towards sulfur-containing peptides and proteins (Mishra et al., 2006). Reactive oxygen species may lead to unspecific oxidation of proteins and membrane lipids, resulting in an increased concentration of malondialdehyde (Cho and Seo, 2005). Malondialdehyde, a product of lipid peroxidation, has been seen to be greatly accumulated after copper (Groppa et al., 2001) or Cd exposure (Smeets et al., 2005).

The concentration of heavy metals within cells must be carefully controlled. So plants and other organisms possess a range of potential mechanisms for metal ion homeostasis and tolerance, such as complexation in the cytoplasm by amino acids, organic acids and tripeptide GSH or by specific metal-binding ligands, the phytochelatins (Hall, 2002). Phytochelatins (PCs) or poly( $\gamma$ -glutamyl-cysteinyl)glycines are small metal-binding peptides with the structure ( $\gamma$ -Glu-Cys)<sub>n</sub>-Gly, in which n varies from 2 to 11 (Rauser, 1995). They are synthesized from reduced glutathione (GSH) by the transpeptidation of  $\gamma$ -glutamyl-cysteinyl dipeptides through the action of the constitutive enzyme phytochelatin synthase (Rauser, 1995).

There has been considerable debate concerning the function of phytochelatins. They are essential for normal tolerance to several non-essential metals, particularly Cd. In fact, *Arabidopsis thaliana* cad-1 and cad-2 mutants, which are deficient in phytochelatin biosynthesis, are significantly sensitive to Cd (Van Vliet et al., 1995). On the other hand, phytochelatins have been assumed to function in the cellular homeostasis or trafficking of essential heavy metal nutrients, particularly Cu and Zn (Cobbett, 2000).

The accumulation of phytochelatins under copper stress has been demonstrated in a large number of algae and plants. However, more or less precise quantitative information with regard to the dose-effect relationships of this phenomenon is scarce. The threshold exposure levels for phytochelatin accumulation appeared, in general, to be lower for Cd than Cu (Rijstenbil and Wijnholds, 1996). In addition, phytochelatin accumulation has

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been shown to be lower with copper than with cadmium in *Phaeodactylum tricornutum* exposed to the same concentrations of the two metals (Kawakami et al., 2006). The synthesis and implication of phytochelatin in cadmium tolerance have been well established. However, the implication of the same mechanism in copper tolerance has not been clearly investigated. Here, we compared in tomato plants the effect of two heavy metals (Cd and Cu) and the ability of these two metals to induce metal-binding peptides that can be implicated in their intracellular chelation.

## 2. 2. MATERIALS AND METHODS

### 2.1. Plant material and growth conditions

The seeds of tomato (*Lycopersicon esculentum* Mill. cv. Ibiza F1) were sterilized in 10% hydrogen peroxide for 20 min. The seeds were then thoroughly washed with distilled water and germinated on moistened filter paper at 25 °C in the dark. The uniform seedlings were then transferred to continuously aerated nutrient solutions containing  $\text{KH}_2\text{PO}_4$ , 0.5 mM;  $\text{Ca}(\text{NO}_3)_2$ , 1.25 mM;  $\text{KNO}_3$ , 1 mM;  $\text{MgSO}_4$ , 0.5 mM; Fe-K-EDTA, 50  $\mu\text{M}$ ;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 5  $\mu\text{M}$ ;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1  $\mu\text{M}$ ;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1  $\mu\text{M}$ ;  $\text{H}_3\text{BO}_3$ , 30  $\mu\text{M}$ , and  $(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ , 1  $\mu\text{M}$ . After an initial growth period of ten days, cadmium and copper were separately added to the medium, respectively, as  $\text{CdCl}_2$  and  $\text{CuSO}_4$  at 1, 5, 10, 25 and 50  $\mu\text{M}$ .

Plants were grown and treated in a growth chamber (26 °C/70% relative humidity during the day, 20 °C/90% during the night). A 16-h (daily) photoperiod was used with a light irradiance of 150  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at the canopy level. After 7 days of heavy metal treatment, plants were assorted into shoots and roots. Roots were rapidly washed three times in 1 L of distilled water and samples were desiccated at 60 °C. The fresh and dry weights of each sample were determined before chemical analysis. For the determination of TBARS contents and identification of metal-polypeptide complexes, plants were cultivated in the absence of metals for the control, or in the presence of 10  $\mu\text{M}$   $\text{CdCl}_2$  or  $\text{CuSO}_4$ .

### 2.2. Determination of metal contents

Cadmium and copper contents in various plant tissues were analyzed by digestion of dried samples with an acid mixture ( $\text{HNO}_3/\text{HClO}_4$ , 4/1 v/v). Metal concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer, AAnalyst 300).

### 2.3. Lipid peroxide determination

Lipid peroxidation in leaves and roots was determined by measuring the concentration of thiobarbituric acid-reactive substances (TBARS), as described by Buege and Aust (1972). The concentration of thiobarbituric acid-reactive substances was calculated using an extinction coefficient of 155  $\text{mM}^{-1}\cdot\text{cm}^{-1}$ .

### 2.4. Extraction, protein determination and Sephadex G50 chromatography

Frozen root material was ground to a fine powder in the presence of liquid nitrogen. The powder was then homogenized with extraction buffer (20 mM Tris-HCl pH 8, 20  $\mu\text{M}$  KCN, 0.5 mM ascorbate and 1 mM  $\beta$ -mercaptoethanol) using 1  $\text{mL}\cdot\text{g}^{-1}$  plant material. After clearing by filtration through four layers of cheesecloth and subsequent centrifugation at 25 000 g for 30 min at 4 °C, the extract was subjected to a gel filtration on Sephadex G50 (fine). The control extract was charged with an amount of metal (60  $\mu\text{g}$  of  $\text{CdCl}_2$  or 35  $\mu\text{g}$  of  $\text{CuSO}_4$ ) to detect unspecific binding. Elution was performed using Tris-HCl 20 mM pH 8 and fractions of 5 mL were collected. SH groups and metal contents in collected samples were determined according to Jemal et al. (1998). Protein content in root preparations was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

### 2.5. Statistics

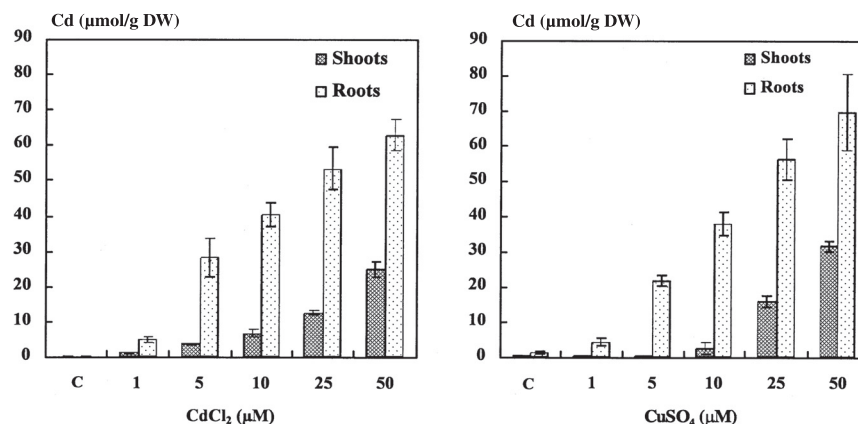
The experiment was repeated at least twice. The data presented here correspond to a representative experiment. The mean values  $\pm$  standard error are reported in the figures. The significance of differences between control and treatments was determined at the 0.05 level of probability.

## 3. RESULTS AND DISCUSSION

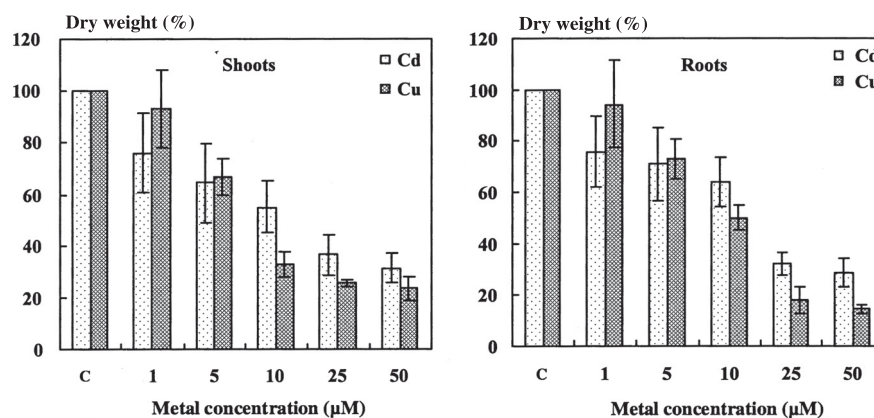
Tomato plants exposed to  $\text{CdCl}_2$  or  $\text{CuSO}_4$  in the nutrient solution accumulated substantial amounts of either Cd or Cu in the roots and shoots (Fig. 1). At the different concentrations tested, accumulation of both metals increased with increasing external supply. The distribution pattern between the two plant organs is, approximately, the same for the two metals. At 5  $\mu\text{M}$ , about 90% of total plant content of either Cd and Cu was found in the roots. At 50  $\mu\text{M}$ , this proportion decreased to 70% for Cu and to 75% for Cd.

The higher accumulation of Cd and Cu in roots than in shoots may be due to the direct exposure of roots to the metals. On the other hand, higher plant roots are considered as barriers of heavy metal translocation to shoots. This is an important factor to protect several physiological and metabolic processes active in aerial organs (Krupa et al., 1993). The presence of either Cd or Cu in the culture medium affected the growth of the tomato plants. Chlorosis and necrosis of leaves and reduced length and browning of roots are the main visual toxicity symptoms. Such effects were observed, especially, at the metal concentrations up to 10  $\mu\text{M}$  (data not shown).

Growing tomato plants for 7 days on nutrient solution supplemented with different concentrations of  $\text{CdCl}_2$  or  $\text{CuSO}_4$  resulted in the inhibition of shoot and root growth (Fig. 2). Decrease in the biomass production occurred in spite of the low metal concentrations, and it varied as a function of the nature and the concentration of the metal added to the nutrient solution. At 1  $\mu\text{M}$  of Cd treatment, growth of leaves and roots was reduced by 24% compared with the untreated plants. The same concentration of Cu did not induce a significant decline in the biomass production of leaves and roots.



**Figure 1.** Cadmium and copper contents in shoots or roots of tomato seedlings not treated (C) or treated for 7 days with increasing concentrations of CdCl<sub>2</sub> or CuSO<sub>4</sub>. Data are means ± SE (n = 7).

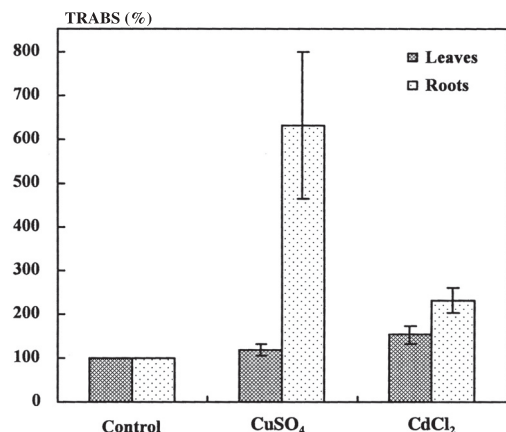


**Figure 2.** Dry weights (DW) of shoots or roots from tomato seedlings not treated (C) or treated for 7 days with increasing concentrations of CdCl<sub>2</sub> or CuSO<sub>4</sub>. Data (given in % of the control) are means ± SE (n = 7). The DW (mg) of the untreated plants (C) is: 0.232 ± 0.037 for Shoots and 0.035 ± 0.005 for roots.

At low concentrations, Cd seems to have a more depressive effect than Cu. Similarly, decrease in biomass production of Arabidopsis plants grown in hydroponic culture was more accentuated by the presence of low cadmium doses than with copper (Maksymiec and Krupa, 2002). This could be due to the fact that Cu is known to be an essential oligonutrient for plant growth. It is involved in numerous physiological functions as a component of several plant enzymes (Marschner, 1995). On the other hand, Cd is known to be a non-essential element for growth, and the plant may actively exclude or sequester such a metal to minimize its toxicity. However, at high concentrations copper appeared to be more toxic than cadmium. Treatment of tomato plants with 25 µM of CuSO<sub>4</sub> resulted in a decline in growth of leaves and roots of, respectively, 74% and 82% versus 64% and 68% with the use of an equivalent dose of CdCl<sub>2</sub>. The higher toxicity of Cu compared with Cd at elevated concentrations was also observed in *Gracilaria lemaneiformis* (Xia et al., 2004) and in *Halophila ovalis* (Ralph and Burchett, 1998).

The reduction in growth could be a consequence of the heavy metals' interference with a number of metabolic processes

associated with normal development such as nitrogenous and lipid metabolisms and photosynthesis. Therefore, difference in heavy metals' toxicity can be due to a difference in their effect on such metabolisms. In fact, Mosulén et al. (2003) reported that the presence of high concentrations of cadmium or copper in the *Chlamydomonas reinhardtii* growth medium induced a NO<sub>3</sub><sup>-</sup> uptake inhibition, more pronounced with the supply of Cu<sup>2+</sup>. SO<sub>4</sub><sup>2-</sup> absorption is stimulated by Cd treatment, but it seems to be not affected by Cu treatment. Moreover, sulfate is required for full expression of nitrate reductase (NR), Nitrite reductase (NiR) and glutamate synthase (GOGAT) activities, enzymes involved in nitrate assimilation (Mosulén et al., 2003). Furthermore, activity of nitrate reductase appeared to be more sensitive to Cu<sup>2+</sup> ions than Cd<sup>2+</sup> ones (Singh et al., 1994). Studies carried out by Ouariti et al. (1997), showed that excess copper led to an efficient reduction of lipid content as well as great changes in fatty acid composition, compared with excess cadmium. Those changes, particularly in the leaves, would mainly concern the photosynthetic apparatus, therefore reducing the redox power and the metabolic energy for the assimilation of sulfate and nitrate (Mosulén et al., 2003).



**Figure 3.** Thiobarbituric acid-reactive substances (TBARS) in leaves and roots from tomato plants treated with 10  $\mu\text{M}$  of  $\text{CdCl}_2$  or  $\text{CuSO}_4$ . Data (given in % of the control) are means  $\pm$  SE ( $n = 5$ ). TBARS ( $\text{nmol.g FW}^{-1}$ ) content in the control is:  $17.031 \pm 4.309$  in leaves and  $5.259 \pm 0.586$  in roots.

It is generally accepted that high heavy metal concentrations lead to photosynthetic depression. Photosynthetic depression may be due to the disruption of electron transport in photosystem II and to the inhibition of chlorophyll synthesis (Ralph and Burchett, 1998). The photosynthetic  $\text{O}_2$  evolution and the yield PSII photochemistry appeared to be more repressed by Cu exposure compared with Cd treatment (Xia et al., 2004). Moreover, copper diminished, more than cadmium, the content of photosynthetic pigments (Ralph and Burchett, 1998).

On the other hand, photosynthetic apparatus alteration may be the consequence of the disturbance of the chloroplast structure via a degradation of polar lipids (Maksymec et al., 1995). In fact, heavy metals are known to involve a breakdown of cell membrane lipid, evidenced by accumulation of lipid peroxidation products. Lipid peroxidation has often been estimated by measuring thiobarbituric acid-reactive substances (TBARS) that assess the amount of malonyldialdehyde (MDA), a major product of polyunsaturated fatty acid oxidation (Buege and Aust, 1972).

Our assays in TBARS determination were performed in tomato plants exposed to 10  $\mu\text{M}$  of  $\text{CdCl}_2$  or  $\text{CuSO}_4$  for 7 days and the results obtained are shown in Figure 3. Compared with the control, the TBARS contents in the leaves are 153% and 118% with cadmium and copper exposure, respectively. Cd- or Cu-induced TBARS in the roots are 231% and 630%, respectively.

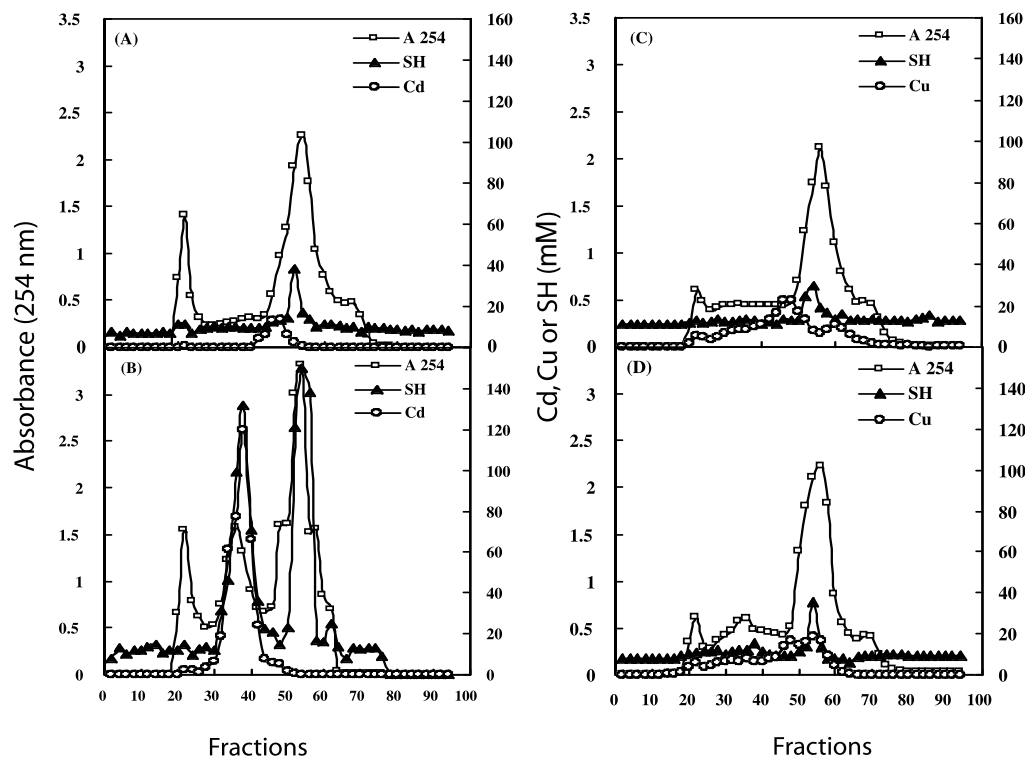
The peroxidation of unsaturated lipids in biological membranes is the most prominent symptom of oxidative stress in animals and plants (Cho and Seo, 2005). The data obtained here suggest that cadmium and copper induce an oxidative stress situation in tomato plants characterized by an accumulation of lipid peroxides. Oxidative stress is imposed by heavy metals catalyzing the formation of harmful free radicals such as hydroxyl, peroxy and alkoxy radicals, which induce lipid peroxidation (Halliwell and Gutteridge, 1984). Moreover, copper appeared to be the most effective metal at causing oxidative stress (De Vos et al., 1993). Accordingly, we found that the

most pronounced effects, in root TBARS accumulation, were obtained with copper ions.

In fact, Cu is the most powerful catalyst of free radical formation and Cu ions themselves can directly initiate oxidative breakdown of polyunsaturated lipids (De Vos et al., 1993). Conversely, Cd is not a transition metal and hence will not directly generate damaging oxygen species. However, surprisingly, cadmium induces more lipid peroxidation in leaves than copper. Similarly to our results obtained in root organs, those given by Groppa et al. (2001) in *Helianthus annuus* reported that Cu is the most important inducer of lipid peroxide accumulation. On the other hand, copper lipid peroxidation induction can be due to a significant increase in lipoxygenase (LOX) activity. In fact, it has been suggested that the formation of free radicals, which are responsible for lipid degradation during senescence, is caused mainly by the action of lipoxygenase (Thompson et al., 1998). Increase in lipoxygenase activity, in *Arabidopsis thaliana*, appeared to be more enhanced with copper ions than with cadmium ones (Skorzynska-Polit and Krupa, 2003). This can, probably, justify the excessive lipid peroxidation observed in the tomato roots under copper stress.

On the other hand, the higher toxicity induced by copper compared with cadmium can be explained by the existence of the same variability in the mechanisms of tolerance implicated by plant species to alleviate the deleterious effects imposed by heavy metals. Indeed, results obtained in *Helianthus annuus* showed that the decrease in the antioxidant enzyme activities was more pronounced with copper than with cadmium (Gallego et al., 1996). Antioxidant enzymes, such as catalase, superoxide dismutase, ascorbate peroxidase and *gaïacol* peroxidase, were implicated in scavenging of oxygen reactive species generated by heavy metals (Mishra et al., 2006). Plants can also respond to heavy metal exposure by the synthesis of phytochelatins. Phytochelatins have been considered as potential molecules at the cellular level that might be involved in the detoxification and thus tolerance of heavy metal stress (Hall, 2002). The presence of additional UV and SH peaks in the gel filtration chromatogram of root extract from Cd-stressed plants, compared with untreated ones, suggests that cadmium induces the synthesis of phytochelatins in tomato plants, estimated by SH-containing compounds (Fig. 4A, B). Moreover, the highest amount of cadmium present in the root extract (95%) was eluted in the intermediate peak that superimposed on the SH peak (Fig. 4A, B). In agreement with what has been suggested by Jemal et al. (1998), this result gives evidence that induced phytochelatins were implicated in the binding of absorbed Cd.

Our results give evidence that tomato plants, like other plant species (Mishra et al., 2006), respond to Cd stress by synthesis of  $(\gamma\text{-Glu-Cys})_n\text{Gly}$  peptides that chelate intracellular metal ions. This can diminish the availability of intracellular Cd and therefore reduce its toxicity. This seems to be in correlation with previous results, that demonstrate that Cd induces a lesser decrease in biomass production and a lower accumulation of lipid peroxidation products, especially in roots. Phytochelatin-based Cd sequestration is generally considered to be essential for normal Cd tolerance in organisms with functional phytochelatin synthase genes (Ha et al., 1999). In agreement with this point of view, a high Cd-induced phytochelatin accumulation and BSO-imposed hypersensitivity to Cd in non-metallicolous *Silene vulgaris* was observed (Schat et al., 2002). In addition,



**Figure 4.** Filtration chromatography on Sephadex G50 (fine) of root extract of untreated (A+C) and treated tomato plants with 10  $\mu\text{M}$   $\text{CdCl}_2$  (B) or  $\text{CuSO}_4$  (D). 60  $\mu\text{g}$  of  $\text{CdCl}_2$  or 35  $\mu\text{g}$  of  $\text{CuSO}_4$  was added to the control extract.

phytochelatin-deficient mutants of the yeast *Schizosaccharomyces pombe* showed a great sensitivity to Cd (Ha et al., 1999). Vatamaniuk et al. (1999) reported that overexpression of phytochelatin synthase (AtPCS1), an enzyme required for phytochelatin synthesis, in *Schizosaccharomyces cerevisiae* induced an enhanced tolerance of Cd.

The study carried out with Cu treatment (Fig. 4C,D) showed the absence of SH peaks in the gel filtration chromatogram of root extract. This gives evidence that the tomato plants did not produce phytochelatin in response to copper exposure and that phytochelatin are not implicated in the intracellular sequestration and tolerance to  $\text{Cu}^{2+}$  ions. In accord with this result, Howden and Cobbett (1992) reported that phytochelatin-deficient mutants, such as *Arabidopsis cad1*, did not show considerably increased sensitivities to Cu. Furthermore, the *Arabidopsis thaliana* cup1-1 mutant, a mutant not affected in phytochelatin biosynthesis or function, is significantly sensitive to Cu (Van Vliet et al., 1995). Moreover, variability in Cu tolerance in *Arabidopsis thaliana* ecotypes were shown to be correlated with type-2 metallothionein expression, rather than with phytochelatin accumulation rates (Murphy and Taiz, 1995). Tomato plants lacking in Cu-binding peptides can result in a great accessibility of this metal in intracellular compartments and can, therefore, lead to significant deleterious effects. This seems to be in accordance with the high Cu-induced toxicity in tomato plants under our experimental conditions compared with Cd.

#### 4. CONCLUSION

We conclude that of the two heavy metals tested, copper is the most toxic heavy metal at high concentrations. It induces more decrease in biomass production and it leads to the highest lipid peroxidation compared with cadmium. Differences in cadmium and copper toxicity in tomato seedlings might be related to the ability of the two metals to induce phytochelatin synthesis. Phytochelatin could be implicated in cadmium sequestration, which might decrease its cytosolic accessibility and thus limit its toxic effects. This may explain, at least, the lower root Cd-induced lipid peroxidation, compared with Cu.

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