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RESEARCH ARTICLE

EFFECT OF CADMIUM ON GROWTH AND ∂-AMINOLEVULINIC ACID DEHYDRATASE AND ACID PHOSPHATASE ACTIVITIES OF TOBACCO SEEDLINGS

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ABSTRACT

Nicotiana tabaccum var. souffi seedlings were grown under different cadmium (Cd) concentrations (0, 10, 20, 50 and 100 µM) in a hydroponic system during 7 d. Plant growth, micronutrient, chlorophyll and carotenoid concentrations, as well as δ-aminolevulinic acid dehydratase (ALA-D; E.C.4.2.1.24) and acid phosphatase (AP; E.C.3.1.3.2) activities were then analysed. Cadmium concentration in both shoots and roots increased with increasing external Cd levels. Metal concentration was on average7-fold greater in root than in shoot tissues. Root length was unaffected by Cd treatments. In contrast, dry weight of both shoot and roots decreased significantly with 100 µM of Cd. A micronutrient- and organ-dependent response to Cd toxicity was observed. Zinc and Cu concentrations in both shoot and roots did not alter upon treatment with Cd. Cadmium stress reduced Mn uptake but not its translocation within the plant. A synergistic effect of Cd on Fe concentration in root at 10 µM and 100 µM Cd levels was observed. The activity of AP, and especially that of ALA-D, was reduced with increasing Cd levels. At those these Cd levels, chlorophyll concentration was also reduced. There was a positive correlation between concentrations of carotenoids and chlorophylls. Our results indicate that *N. tabaccum* seems to have some degree of Cd tolerance.

INTRODUCTION

It is known that unfavourable effects of heavy metals on plants are manifested, among others, by inhibiting the normal uptake and utilization of mineral nutrients (Jiang *et al*., 2004; Dong *et al*., 2006), for instance, can interfere with mineral nutrition by hampering the uptake and translocation of essential elements (Jiang *et al*., 2004, Feng *et al*., 2010). In addition to affecting the overall cell metabolism via alterations in (i) the behaviour of key enzymes of important pathways (Verma & Dubey, 2001), (ii) membrane composition and function (Pdozza *et al*., 2005) and (iii) by lowering the control of the cell redox state, which ultimately causes oxidative stress (Gratoa *et al*. 2005). Acid phosphatases, AP (ortophosphoric-monoester phosphohydrolases; E.C.3.1.3.2) are widely distributed in plants and significantly differ from their susceptibility to inhibition by various compounds (Penheiter *et al*., 1997). Acid phosphatases nonspecifically catalyze the hydrolysis of a variety of phosphate esters in an acidic environment (Tham *et al*., 2010). Several factors have shown to influence AP activity (Tabaldi *et al*., 2007), but heavy metals effects on AP are poorly understood (Tabaldi *et al*., 2007). The enzyme δ-aminolevulinic acid dehydratase (ALA-D; E.C.4.2.1.24), which catalyzes the asymmetric condensation of two molecules of δ-aminolevulinic acid to porphobilinogen, is sensitive to metals due to its sulphydrylic nature (Pereira *et al*., 2006). The synthesis of porphobilinogen promotes the formation of porphyrins, hemes and chlorophylls, which are essential for adequate aerobic metabolism and for photosynthesis (Jaffe *et al*., 2000). Furthermore, altered ALA-D activity concomitant with reduced chlorophyll contents has been reported in many terrestrial plants exposed to various metals (Penheiter *et al*., 1997). The mechanism of action of heavy metals lies in their ability to form strong bonds with bases and phosphates of nucleic acids and with -SH groups of proteins, modifying both their structure and function. They compete with other divalent cations such as Ca, Zn and Mg replacing them in their physiological roles (Pauza *et al*., 2005, Tabaldi *et al*., 2007). In a recent study, Dixit *et al*., (2011) showed that an undetermined species of the gene TvGST exhibited high tolerance to soil contamination, growing quite abundantly in a soil mix with 90 and 1,450 mg kg^{-1} of Cd and Zn, respectively. Moreover, this species showed that, Cd content higher than 100 mg kg^{-1}, being considered a Cd hiperacumulator, and possibly contributing to phytoremediation of sites contaminated with heavy metals. Taking into account these characteristics and the high commercial value of *N. tabaccum* to the pharmaceutical industry, it is important to determine whether this species accumulates and is tolerant to Cd. If there is a tolerance or an accumulation it is of interest, what mechanisms are involved ?. The present work was therefore

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designed to analyze the growth, micronutrient, chlorophyll and carotenoid concentrations, as well as ALA-D and acid phosphatase activities in *N. tabaccum* plants during an extended 7-d period of exposure to different Cd concentrations.

MATERIALS AND METHODS

***Plant material*:** seeds of tobacco (*Nicotiana tabaccum* var souffi) were sterilized in 10 % (v/v) hydrogen peroxide for 20 minutes, and washed abundantly in distilled water afterwards. After imbibition, the seeds were germinated on moistened filter paper at 25^{O}C in the dark. After 7 days, uniform seedlings were transferred to 6 litres plastic beakers (8 plants per beaker) filled with continuously aerated, basal nutrient solutions of an initial pH 5.8-6, containing 3 mM KNO_3, 0.5 mM Ca $(NO_3)_2$, 2.4 mM KH_2PO_4, 0.5 mM $MgSO_4$, 100 µM Fe-K_2-EDTA, 30 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, and 1 µM $(NH_4)_6Mo_7O_{24}$. Plants were grown in a growth chamber (26^{O}C/70 % relative humidity during the day, 20^{O}C/90 % relative humidity during the night). The photoperiod was 16 h daily with a light irradiance of 150 µmol m^{-2}. s^{-1} at the canopy level. At the age of 10 days after transplant, cadmium was added to the medium as $CdCl_2$ at 0 to 100 µM. After one week of Cd treatment, plants were separated into shoots and roots. Samples were stored in liquid nitrogen for subsequent analysis or dried at 70^{O}C for at least three days in order to determine both dry material and ionic contents.

Growth analysis: At harvest, plants were divided into shoots and roots. Roots were rinsed twice with fresh aliquots of distilled water. Subsequently, growth and biochemical parameters were determined. Length of roots was determined according to Tennant (1975), and the length of sprouts was measured with a ruler. To obtain dry weight, the plants were left at 65°C untreaching a constant weight.

Cadmium and micronutrient concentrations: Approximately 0.2 g of roots and shoots were digested with 4 mL HNO_3 using the following stages of heating: a) 50°C for 1 h; b) 80°C for 1 h; and c) 120°C for 1 h in a digester block (Velp, Italy). The samples were then diluted to 50 mL with deionized water. Concentrations of Cd, Zn, Mn, Fe, and Cu were successively measured by atomic absorption spectroscopy (Iyengar *et al*., 1997).

Acid phosphatase (AP, E.C. 3.1.3.2) activity: Fresh root and shoot extracts were centrifuged at 40000 *g* for 30 min at 4ºC and the supernatant used for enzyme assay. Acid phosphatase activity was determined according to Tabaldi *et al*. (1949) in a reaction medium consisting of 3.5 mM sodium azide, 2.5 mM calcium chloride, 100 mM citrate buffer, pH 5.5, in a final volume of 200 µL. A 20 µL aliquot of the enzyme preparation (10-20 µg protein) was added to the reaction mixture and pre-incubated for 10 min at 35°C. The reaction was started by the addition of substrate and stopped by the addition of 200 µL of 10% trichloroacetic acid (TCA) to a final concentration of 5%. Inorganic phosphate (Pi) was measured at 630 nm using malachite green as the colorimetric reagent and KH_2PO_4 as the standard for the calibration curve. Controls were run to correct for nonenzymatic hydrolysis by adding enzyme preparation after TCA addition. Enzyme specific activities are reported as nmol Pi released min^{-1} mg^{-1} protein. All assays were performed in triplicate using PPi as substrate at a final concentration of 3.0 mM.

δ-aminolevulinic acid dehydratase (ALA-D, E.C. 4.2.1.24) activity: Shoot tissue was homogenized in 10 mM Tris-HCl buffer, pH 9.0 (1:1, w/v). The homogenate was centrifuged at 12000 *g* at 4°C for 10 min to yield a supernatant (S1) that was used for the enzyme assay. The S1 was pre-treated with 0.1% Triton X-100 and 0.5 mM DTT. The ALA-D activity was assayed as described by Pauza *et al* (2005) by measuring the rate of porphobilinogen (PBG) formation. The incubation medium for the assays contained 100 mM Tris-HCl buffer, pH 9.0. For the enzyme assay, the final concentration of ALA was 3.6 mM. Incubation was started by adding 100 µL of the tissue preparation to a final volume of 400 µL. The product of the reaction was determined with the Ehrlich reagent at 555 nm using a molar absorption coefficient of 6.1×10^4 L $mol^{-1}cm^{-1}$ for the Ehrlich-porphobilinogen salt. Activity of ALA-D was expressed as nmol PBG mg^{-1} protein h^{-1}.

Protein extraction: In all the enzyme preparations, protein was determined by the method of Bradford (1976) using BSA as standard and was expressed in mg mL^{-1}.

Chlorophyll and carotenoid concentrations: Chlorophyll and carotenoids were extracted and estimated following Arnon (1949). Briefly, chopped fresh shoot sample (0.1g) was incubated at 65°C in dimethylsulfoxide (DMSO) until tissues were completely bleached. Absorbance of the solution was then measured at 470, 645, and 663 nm in order to determine the concentrations of carotenoids, chlorophyll a, and chlorophyll b, respectively. Chlorophyll and carotenoid concentrations were expressed as mg g^{-1} fresh weight.

Statistical analysis: The analyses of variance were computed for statistical significance based on the appropriate *F*-tests. The results are the means ± SD of at least three independent replicates. Significance was determined at $P < 0.05$. The mean differences were compared utilizing Duncan's multiple range test.

RESULTS AND DISCUSSION

Cadmium concentration under Cd exposure

Cd concentration in both shoots (Fig. 1a) and roots (Fig. 1b) increased with increasing Cd levels. It is noteworthy that external Cd concentrations ranging from 20 to 50 µM brought about the same enhancement of Cd concentration in both shoots and roots (36 and 12.5-fold greater than controls, respectively). Cadmium concentration in roots was on average 12-fold greater than in the shoot. The maximum concentration of Cd in shoot and roots was 305 mg kg^{-1} DW and 2222.2 mg kg^{-1} DW, at the 100 µM Cd level, respectively. Our data (Fig. 1) demonstrate that higher metal exposures led to remarkable Cd accumulation in both root and shoot tissues, hence leading to a high degree of toxicity, which is in agreement with results reported by other authors (Lima *et al*., 2006, Mishara *et al*., 2006). Meanwhile, no significant difference in both shoot and root Cd concentrations was found

between the 10 to 50 µM Cd treatments. We also checked Cd concentration in the nutrient solution at 0 and 7 d after applying the treatments, and it was found that external Cd was not significantly depleted during the experiment (data not shown). These data suggest that, up to a certain level of metal concentration, roots of *N. tabaccum* have some mechanism to avoid excess of Cd uptake. Cadmium confinement in the root tissues may be due to an efficient binding and sequestration to the vacuoles by glutathione and phytochelatins, or by imobilization of Cd by cell wall and extracellular carbohydrates (Mishara *et al*., *2006*, Almeida *et al*., 2007).

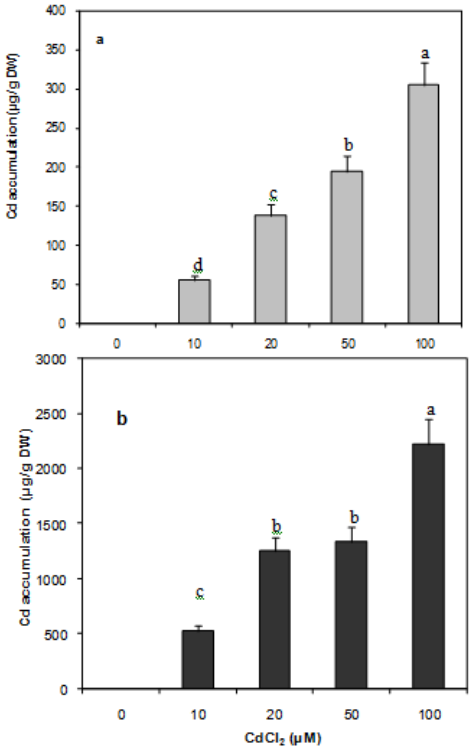


Figure 1. Cadmium content in shoot (A) and root (B) tissues from control and cadmium treated plants. Shoot and root samples from 17-days-old tobacco Cadmium content was expressed as µg/g DW. Values represent the means of analysis from five independent plants. DW, dry weight

Characterisation of Cd tolerance

There was no general pattern of plant growth responses to Cd stress. Number of leaves per plant was slightly, but not significantly, reduced by Cd concentrations up to 20 µM, whereas at the 100 µM Cd level it was reduced by 22% compared to control plants (Fig. 2a). In addition, length of shoots (Fig. 2b) and length of the root system (Fig. 2c) per plant were unaffected by Cd. On the other hand, the root length/shoot length ratio increased significantly at the 10 µM Cd level and this ratio was slightly, but not significantly, increased upon addition of Cd exceeding 10 µM, when compared to control plants (Fig. 2d). Our data demonstrate that the root length/shoot length ratio increased after treatment with all studied Cd concentrations (Fig. 2d), which is not consistent with the results of Guo and Marschner (1995), who reported that usually the inhibition of root elongation of different plant species is the most sensitive parameter of Cd toxicity. In addition, our results also indicate that root elongation was much less affected than the decrease in biomass, which is not consistent with the earlier results of Lima *et al.* (2006) for *Pisum sativum* and Meuwly & Rauser (1992) for *Zea mays*. According to Meuwly & Rauser (1992), since most of the root elongation is located in the first 10 mm of the root apex, the contribution of the biomass to this portion is probably too small to allow the detection of any toxic symptoms during the first days, but length inhibitions are enough to be detected.

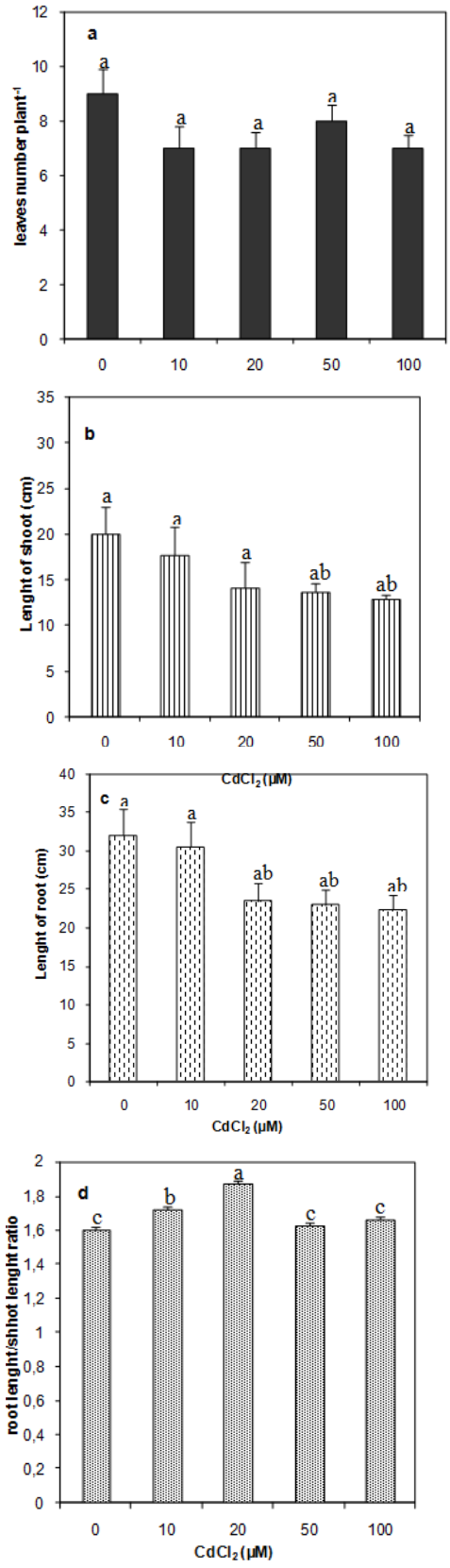
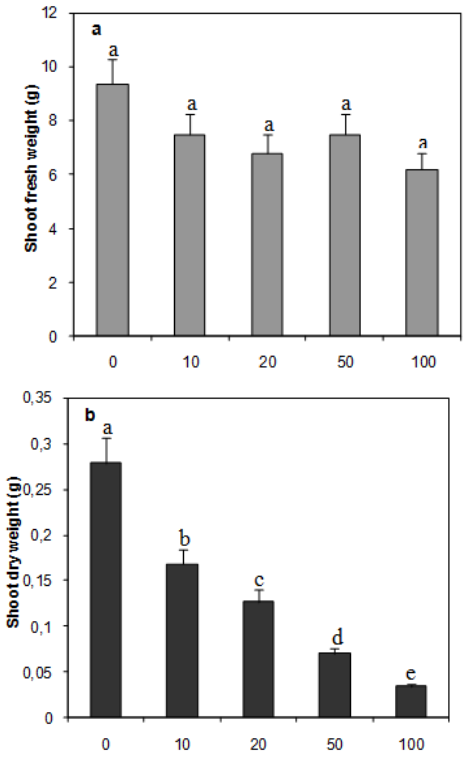


Figure 2. The effect of different cadmium concentrations in leaves number (A), length of shoots (B), length of roots (C) and root/shoot length ratio (D) of tobacco plants. Values are the means ± SE of triplicates from five independent experiments

Therefore, since root biomass of *N. tabaccum* was significantly reduced upon addition of Cd levels exceeding 10 µM, this result suggests that *N. tabaccum* has some degree of Cd tolerance. Shoot fresh weight was reduced significantly only upon adding 100 µM of Cd (Fig. 3a). In contrast, dry weight of both shoot (Fig. 3b) and roots (Fig. 3c) decreased significantly upon addition of 10 and 50 µM Cd. Conversely, root dry weight decreased significantly upon addition of Cd at levels exceeding 50 µM (Fig. 3d). Meanwhile, there was an increase in both root and shoot biomass (Fig. 3) at lower Cd levels 10 µM. It should be emphasized that experiments of the present study were carried out three times, and results were almost the same for most parameters analyzed. The positive effects of low levels of Cd on plant growth have been poorly discussed in the literature, and the mechanisms are not well understood. This phenomenon is normally related to a so-called hermetic effect that probably represents an "overcompensation" response to a disruption in the homeostasis of the organism (Aina *et al.*, 2007). Khan *et al.* (2008) observed similar phenomena in sand culture where 10 µM Cd enhanced the activities of leaf superoxide dismutase, ascorbate peroxidase, glutathione reductase and carbonic anhydrase, net photosynthetic rate and plant dry mass of *Triticum aestivum* at low Zn level. These authors suggested that the synergies among the activities of antioxidative enzymes helped to maintain carbonic anhydrase and thus photosynthesis and plant biomass at low Cd levels under low Zn concentration. Taking into account the fact that the total dry biomass of *N. tabaccum* was only significantly reduced at the 100 µM Cd level and such a concentration is within that observed in highly polluted soils, these results further indicate that *N. tabaccum* seems to have some degree of Cd tolerance, as was found for an undetermined species belonging to the genus TvGST, reported by Dixit *et al.* (2011). However, additional experiments should be performed in order to allow a better understanding of the mechanism of the effect of Cd toxicity on growth, photosynthesis and antioxidative mechanisms of *N. tabaccum.*



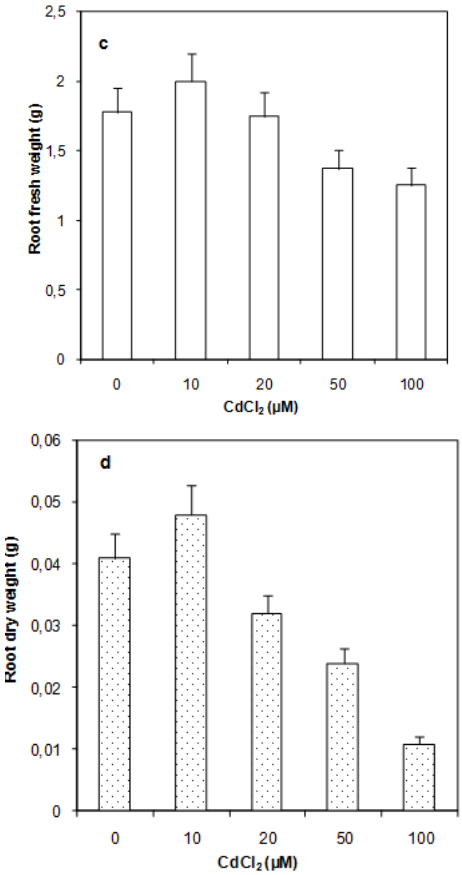
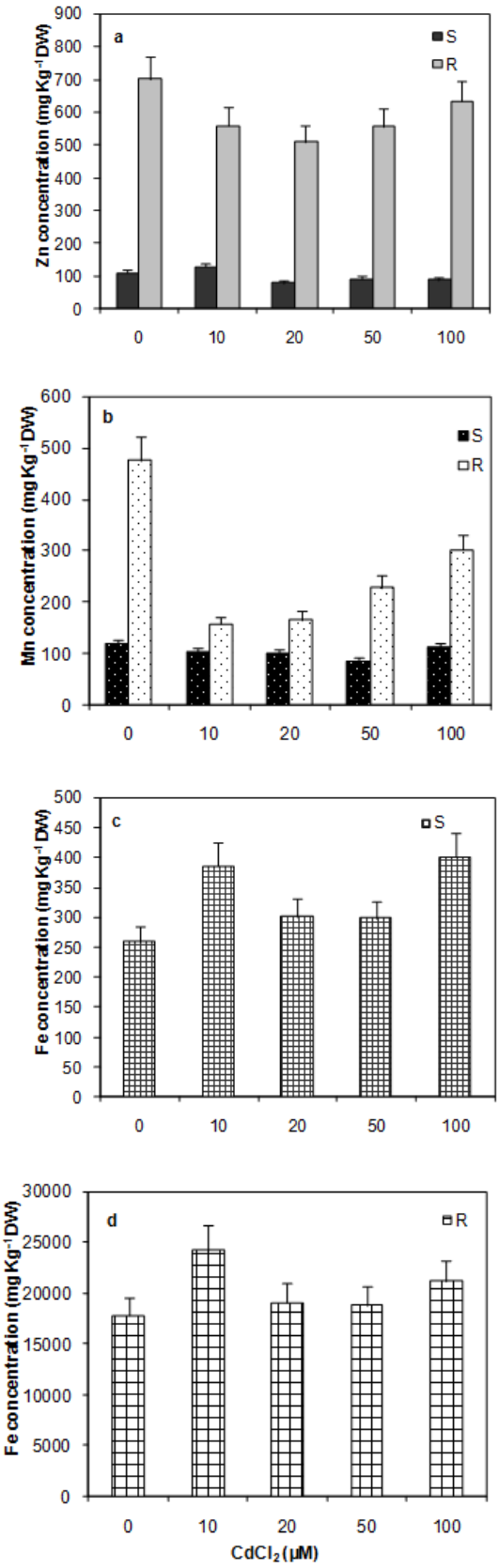


Figure 3. The effects of different cadmium concentrations on shoots fresh weight (A), shoot dry weight (B), root fresh weight (C) and root dry weight (D). Values are the means ± SE of triplicates from five independent experiments

Effect of Cd on micronutrient concentrations

A nutrient- and organ-dependent response to Cd toxicity was observed (Fig. 4). Zinc remained unaltered after applying Cd treatments (Fig. 4a). Conversely, in roots, Mn concentration was reduced significantly over all Cd levels added (Fig. 4b), Fe concentration increased significantly upon addition of 10 and 100 µM Cd (Fig. 4c and 4d), whereas Zn and Cu levels did not respond to the treatments (Fig. 4e). It is well known that many toxic effects of Cd action result from interaction with micronutrients, in particular those with the same valence as Cd, such as Zn, Mn, Fe, and Cu. Our results showed that Zn and Cu concentrations in both shoot and roots remained unaltered upon Cd addition. Both synergistic and antagonistic effects of Cd on Zn and Cu were found in other studies (Jiang *et al.*, 2004, Dong *et al.*, 2006). Our data indicate that Mn uptake was affected by Cd stress but not the translocation of Mn within *N. tabaccum* plants. An inhibition in Mn uptake and transport by Cd has been reported by Dong *et al.* (2006) for *Lycopersicon esculentum.* In contrast, an increase in Mn uptake and translocation to the shoots was observed in *Lactuca* sp. exposed to Cd stress and there was a higher Mn accumulation in chloroplasts when Cd was present in the growth medium (Ramos *et al.*, 2002). Some studies showed that there is an antagonistic relationship between Cd and Fe (Sharma *et al.* 2004). *Arabidopsis* plants that overexpressed the *IRT1* gene, a major transporter responsible for high-affinity iron uptake from the soil, accumulated higher levels of Cd and Zn than the wild type, indicating that *IRT1* is responsible for the uptake of these metals (Cannoly *et al.*,

2002). Our data demonstrate that a synergistic effect of Cd on Fe concentration in root at the 10 and 100 µM Cd concentrations is likely to occur. Some of these conflicting results found in our study in relation to others might be presumably due to the differences in the culture methods, species, as well as growth conditions including Cd and micronutrient levels in medium, growth period, temperature and light.



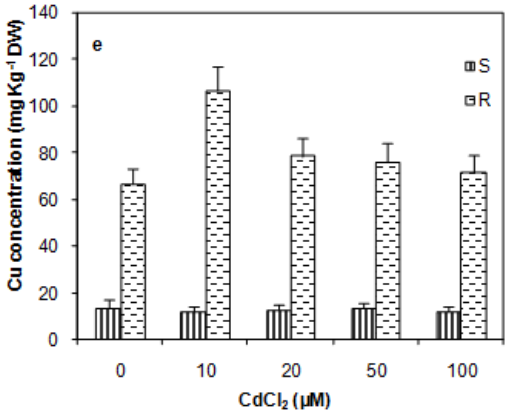


Figure 4. The effect of different cadmium concentrations on shoots and roots zinc content (A), on shoots and roots manganese content (B), on shoot (C) and root (D) Fe content and shoot and roots Cu content (E). Values are the means ± SE of triplicates from five independent experiments

Acid phosphatase activity (AP)

Acid phosphatase activity was reduced in shoot (29%) and roots (36%) with increasing Cd levels. No difference in AP activity in both shoot and roots was found for Cd treatments ranging from 10 to 100 µM (Fig. 5). Our results showed that under Cd stress, AP activity in both shoot and roots was similarly reduced regardless of the amount of Cd added. Conversely, Tabaldi *et al.* (2007) found that Cd, Mn and Na did not significantly alter the AP activity in *Cucumis sativus*. Phosphatases are generally metalloenzymes depending on Ca^{2+} or Mg^{2+}. A possible mechanism explaining Cd-toxicity at high concentrations can be the replacement of Ca^{2+} and Mg^{2+} by Cd in the active site of enzyme, or the Cd can be interfering with the PO_4^{3-} binding sites. Other metals such as Hg and Zn also inhibited AP activity of cucumber, possibly through this mechanism (Tabaldi *et al*., 2007). Therefore, inhibition of AP activity in *N. tabaccum* caused by Cd stress may impair phosphate mobilization, since this enzyme is involved in P metabolism, an essential element for plant growth and development (Duff *et al*., 1994).

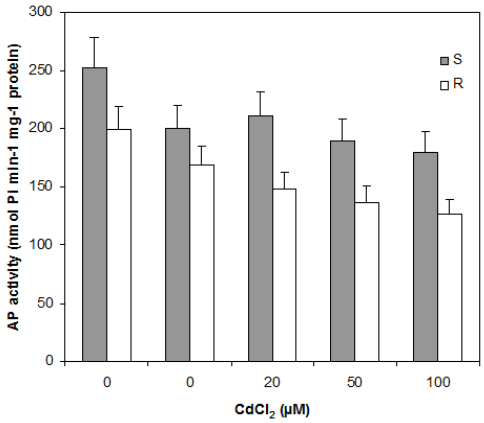


Figure 5. Effect of cadmium concentration on shoot and root acid phosphatase activity (AP) of *Nicotiana tabaccum* plants. Values are the means ± SE of triplicates from five independent experiments

ALA-D activity and concentrations of chlorophyll and carotenoids

Shoot ALA-D activity decreased with increasing Cd levels in the nutrient solution (Fig. 6a). A maximum of 87% reduction

in ALA-D activity was found at 100 µM Cd. Total chlorophylls were also reduced especially at 100 µM Cd levels (Fig. 6b). On the other hand, carotenoid concentration was significantly reduced upon addition of 50 and 100 µM Cd (Fig. 6c). Altered ALA-D activity concomitantly with reduced chlorophyll contents has been reported in many terrestrial plants exposed to various metals (Pereira *et al*., 2006). Our data also showed that total chlorophyll concentration was reduced significantly at 10 and 100 µM Cd (Fig. 5b), at which greater reduction in ALA-D activity was observed. Moreover, there was a significant positive correlation (r^2= 0.73) between concentrations of carotenoids and chlorophylls. Carotenoids play a pivotal role in photoprotection of chlorophylls against photooxidative damage by quenching reactive oxygen species (ROS) such as singlet oxygen (Behera *et al*., 2006). Similarly, other authors have found a decrease in carotenoid content in Cd-treated plants, which might be interpreted as an overproduction of ROS (Mishara *et al*., 2006). In addition, ALA-D inhibition could have led to ALA accumulation that within the cell might contribute to enhance ROS production (Noriega *et al*., 2007). Therefore, in future studies it will be necessary to analyze the effects of Cd on ROS formation and on both enzymatic and non-enzymatic antioxidant systems. These defence systems can remove, neutralise or scavenge oxy-radicals and their intermediates (Gratao *et al*., 2005).

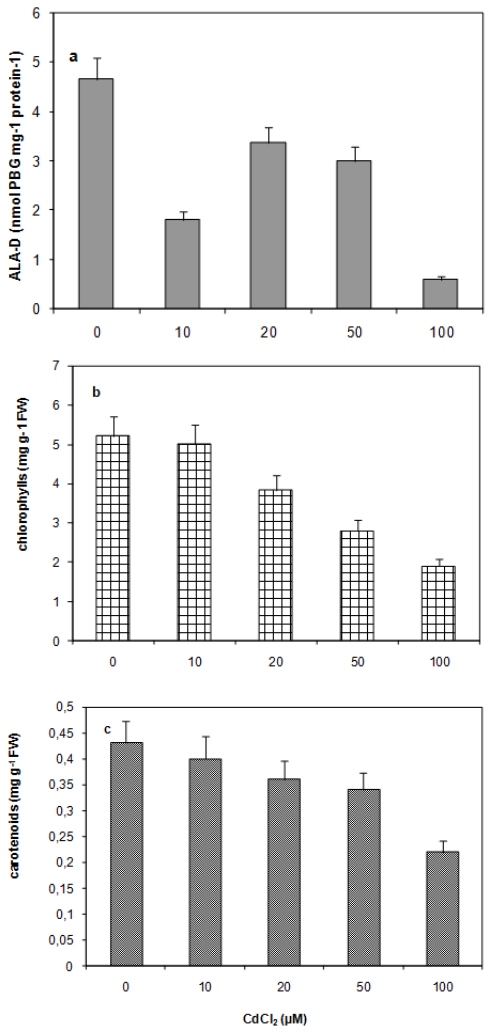


Figure 6. Effect of cadmium concentration on shoot δ-aminolevulinic acid dehydrogenase activity (A), on,chlorophyll *total* (B) and carotenoids contents (C) of *Nicotiana tabaccum lycopersicon* plants. Values are the means ± SE of triplicates from five independent experiments

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