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Morphophysiological responses and programmed cell death induced by cadmium in *Genipa americana* L. (Rubiaceae)

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Abstract Cadmium (Cd) originating from atmospheric deposits, from industrial residues and from the application of phosphate fertilizers may accumulate in high concentrations in soil, water and food, thus becoming highly toxic to plants, animals and human beings. Once accumulated in an organism, Cd discharges and sets off a sequence of biochemical reactions and morphophysiological changes which may cause cell death in several tissues and organs. In order to test the hypothesis that Cd interferes in the metabolism of *G. americana*, a greenhouse experiment was conducted to measure eventual morphophysiological responses and cell death induced by Cd in this species. The plants were exposed to Cd concentrations ranging from 0 to 16 mg l^{-1}, in a nutritive solution. In TUNEL reaction, it was shown that Cd caused morphological changes in the cell nucleus of root tip and leaf tissues, which are typical for apoptosis. Cadmium induced anatomical changes in roots and leaves, such as the lignification of cell walls in root tissues and leaf main vein. In addition, the leaf mesophyll showed increase of the intercellular spaces. On the other hand, Cd caused reductions in the net photosynthetic rate, stomatal conductance and leaf transpiration, while the maximum potential quantum efficiency of PS2 (*Fv/Fm*) was unchanged. Cadmium accumulated in the root system in high concentrations, with low translocation for the shoot, and promoted an increase of Ca and Zn levels in the roots and a decrease of K level in the leaves. High concentrations of Cd promoted morphophysiological changes and caused cell death in roots and leaves tissues of *G. americana*.

Keywords Anatomy · Apoptosis · Heavy metal · Mineral nutrients · Photosynthesis

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Introduction

Anthropogenic activities have led to an increase of heavy metal concentrations in the biosphere, which, when compared with other pollutants, are not biodegradable and thus persist in the environment (Prasad and Freitas 2003). Among them, cadmium (Cd) is pointed out since it is a highly toxic motile element,

which has unknown physiological functions both in plants and in animals (Ma et al. 2003).

Excessive Cd in plant tissues may stimulate the formation of free radicals and of reactive oxygen species (ROS), causing severe oxidative stress. Such disorder of the redox control of cells brings about several reactions, causing inhibition of growth, stimulation of secondary metabolism, lignification and cell death (Schützendübel and Polle 2002). The Cd-induced cell death may present apoptotic aspects, such as condensation of chromatin, nuclear DNA cleavage into oligonucleosomes and formation of apoptotic bodies. Usually, when plants are exposed to high Cd concentrations, a necrotic process is started (Berboodi and Samadi 2004).

The main anatomical changes observed in plants submitted to Cd-stress include cell wall thickening due to the deposit of phenolic compounds, collapse of phloem cells and tissue necrosis (Schützendübel et al. 2001; Vollenweider et al. 2006). In addition, Cd causes reduction of leaf transpiration and photosynthesis (Mendelssohn et al. 2001; Mobin and Khan 2007), interferes with the absorption of essential mineral nutrients, such as Cu, Fe, Zn and Mn due to site competition or processes shared by these cations (Almeida et al. 2007), promotes leaf chlorosis, inhibits root and shoot growth (Sanitá di Toppi and Gabbrielli 1999; Soares et al. 2005), among other symptoms.

In this context the selection of woody species with hyperaccumulative capacity for heavy metals, to be used as phytoremediators, is practical, ecologically and economically viable, due to the low cost of implantation, promoting soil stabilization that limits the migration of metallic contaminants (Almeida et al. 2007). Woody phytoremediator species should present mainly: (i) high growth rate; (ii) high biomass production; (iii) deep root system, to explore a large soil volume; (iv) high capacity to grow in soils low in nutrients; and (v) high capacity to immobilize metals in the trunk (Pilon-Smits 2005; Almeida et al. 2007).

Genipa americana L. is a fruit and woody species of the Rubiaceae family, with wide dispersion in neotropical forests and intense regeneration in areas which have high anthropogenic activity. The fruits are highly pursued and its wood is of high quality. Moreover, this species is considered to be tolerant to flooding, thus indicated for the recomposition of ciliary forests (Mielke et al. 2003), presenting potential as phytoremediator, as a rhizofilterer and phytostabilizerer of chromium (Cr) (Barbosa et al. 2007).

We tested the hypothesis that Cd interferes in the metabolism of *G. americana* by performing greenhouse experiments in which plants grown in nutrient solution were exposed to a range of Cd concentrations and tracking of: (i) cell changes, that are typical for programmed cell death; (ii) anatomical changes; (iii) leaf gas exchange; (iv) chlorophyll fluorescence emission; and (v) chemical composition of plant tissues.

Materials and methods

Growth conditions and plant material

The seeds, obtained from sub-spontaneous trees in the Banco do Pedro district, Ilhéus, Bahia, Brazil, were planted in black plastic tubes with capacity of 288 cm^3, containing as substrate *Pinus* shell and turf + triturated coconut fiber (1:1), enriched with mineral macro- and micronutrients. Tubes were placed in perforated plastic trays, used as support, which could hold 54 units and kept in a plant nursery, with 50% of shadow and intermittent irrigation through micro-aspersion with 20 l h^{-1} flow, automatically operating for 30 s at 10 min intervals. At the age of 4 months plants were transplanted to 3 dm^3 tubes, with the same substrate, and kept in the same environment.

When plants were 2.8 years old they were transferred to a greenhouse (14°45′15″S, 39°13′59″W, a.m.s.l.) and transplanted to 30 l polyethylene trays (8 plants/tray) with a nutritive solution of ¼ of ionic force, prepared according to Hoagland and Arnon (1950), where they stayed for 70 days for acclimatization. During this period, the constantly aerated solution was changed weekly and its level was kept by daily replacement of volume with de-mineralized water. After the acclimatization period, the nutritive solution was exchanged and the treatments with increasing concentrations of Cd (0.5, 1, 2, 4, 8 and 16 mg l^{-1}) were applied, together with the control (without Cd), using $CdCl_2 \times 5/2\ H_2O$. The pH of the solution was monitored and adjusted to 5.9, using either NaOH or HCl.

Histochemical and anatomical analysis

At the end of the experiment, 120 h after treatment application (ATA), root tip and the median portion of the second mature and completely expanded leaf from the apex was collected in groups of three plants per treatment. The plant material was fixed with 3% glutaraldehyde in PBS buffer (pH 7.4) for 4 h and then transferred to 70% ethanol and kept at 4°C. Samples of treated plants were dehydrated in butanolic series, included in paraffin, cut into sections with a microtome (thickness of 7 μm) and submitted to a staining process with safranin and 1% astra blue (Kraus and Arduin 1997). For histochemical tests freehand razor blade cuts were used; lugol to identify starch (Johansen 1940); floroglucin 2% diluted in 95% ethanol to identify lignin; sudan III to identify lipid substances, to suberize and cutinize the cell walls; and ferric chloride to identify phenolic compounds (Sass 1951). Four slides were observed from each of the three different plants per treatment. The results of the analyses were documented using a photomicroscope (model Olympus 50).

TUNEL analysis (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling)

The utilization of plant material for analysis through TUNEL method followed the same procedures described for anatomical and histochemical analysis. Samples of roots and leaves which had been submitted to concentrations of 4, 8 and 16 mg Cd l^{-1} and the treatment control were selected, dehydrated in butanolic series and included in paraffin. Transverse sections were made on a rotary microtome (7 μm thick) and mounted on slides with glycerine albumin (Kraus and Arduin 1997). Paraffine was removed by submersion in xylol (twice for 15 min) and rehydration in decreasing concentrations of ethanol (2 × 5 min in 100, 95, 75% and 3 min in distillated water).

In order to permeabilize the cuttings and to detect the DNA fragmentation, we followed the procedures described by Ceita et al. (2007) and Deng et al. (2001). The TUNEL procedure was applied, using the "In situ Cell Death Detection Kit, AP" (Boehringer Mannheim) according to the manufacturer's instructions. Slides were examined using a fluorescence microscope Leica DM RXA2, equipped with a digital camera Leica MPS 60. The images were captured with the filter I3 (450–490 nm of excitation). The number of TUNEL-positive cells in root tip and leaves were counted in five areas of 1 mm^2 in groups of three plants per treatment and statistically analyzed.

Photosynthetic parameters

The net photosynthetic rate by unit of leaf area (*A*), stomatal conductance to water vapor (*gs*) and leaf transpiration (*E*) were measured 5 days after application of treatment (DAAT), between 08:00 a.m. and 12:00 a.m., on one mature and completely expanded leaf from the apex using five plants per treatment, with a portable photosynthesis system LI-6400 (Li-Cor, Nebraska, USA), connected to a fluorescence/gas exchange camera model 6400-40. During the leaf gas exchange measurements, the artificial light source was adjusted to provide values of the photosynthetic photon flux density of 800 μmol m^{-2} s^{-1}. The mean values of atmospheric CO_2 concentration, air temperature and air vapor pressure deficit during the leaf gas exchange measurements were 390.9 ± 1.1 μmol mol^{-1}, 28.6 ± 0.1°C and 2.04 ± 0.01 kPa, respectively (mean ± SE, $n = 35$, corresponding to 7 treatments × 5 repetitions).

Chlorophyll fluorescence emissions were measured together with the gas exchanges of the same leaf. Before they were measured, a clip was placed in the median region of the leaf, so that such region would be in the dark for at least 20 min to assure that all reaction centers would acquire oxidized conditions. The fluorescence signals were registered in the data acquisition system of LI-6400, which automatically calculated the minimum (*Fo*), maximum (*Fm*), variable (*Fv*) fluorescence and the maximum potential quantum efficiency (*Fv*/*Fm*).

Chemical composition

The dry matter of different parts of the Cd-treated plants (roots, stems and leaves) were analyzed for their, contents of Cd, P, K, Mg, Ca and Zn by using HR-ICP-MS (Inductively Coupled Plasma sector type Mass Spectrometer), using the methodology described by Severo et al. (2004). The plant samples (50 mg) were digested with the Teflon closed digestion bombs in the microwave (Ethos Plus) using the

super analytical reagent grade of the following sequence: 4 ml HNO_3 + 1 ml HCl. All measurements were performed with a PQ Excell from VG Elemental 2 (Finnigan MAT) of the Lab. Service Central d'Analyses (Lyon, France). Signal drift due to matrix effects was monitored by adding internal standards (In, 10 μg l^{-1}) to sample and standard solutions, according to the established protocols of the manufacturer. All estimations were conducted in triplicate and the concentrations were expressed as microgram per gram of dry weight, with the overall recovery ranging between 87 and 95%.

Statistics analysis

The experiment was conducted in a totally random way, comparing seven levels of Cd, each one containing five repetitions, but a different number of experimental units, according to the variables assessed. The results were analyzed based on linear and non-linear mathematical models, with variation of Cd concentration in a nutrient solution as independent variable and leaf gas exchange, chlorophyll fluorescence emission and chemical composition the dependent variables. Regression analyses and analyses of variance to define the best fitting model were done utilizing the General Linear Model (GLM) procedure of the 'Statistical Analysis System' (SAS Institute, 1994), using methodology outlined in Stell and Torrie (1980). The coefficients of the equations were tested using Student's *t*-test ($P < 0.05$). The data TUNEL histochemistry were analyzed by Tukey's test ($P < 0.05$).

Results

TUNEL analysis

The TUNEL reaction, on transversal sections of *G. americana* roots and leaves which were submitted to different Cd concentrations, showed the presence of TUNEL-positive nucleus which presented a shiny yellowish-green fluorescence (Figs. 1, 2). The number of TUNEL-positive cells increased in root tip and leaf proportional to the increase of Cd in nutrient solution (Table 1).

Epidermis, exodermis and vascular cylinder cells of the roots were TUNEL-positive in the absence of Cd treatment as they undergo developmental PCD (Fig. 1a, e). An increase of TUNEL-positive cells was observed in the *G. americana* roots with the increase of Cd concentration (Fig. 1a–d). In addition, some cells presented nucleus condensed and with an elliptical format for the concentrations of 4, 8 and 16 mg Cd l^{-1} (Fig. 1e–h).

In the adaxial surface of the leaf main vein, the cells located in the collenchyma and in the epidermis presented TUNEL-positive nuclei, mainly for concentration 8 mg Cd l^{-1} (Fig. 2a–d). In the abaxial surface there was a predominance of TUNEL-positive nucleus in the epidermis and in the collenchyma with different morphological patterns (Fig. 2e–g). The analysis of the morphological pattern of the abaxial cells showed, for the concentrations of 4 and 8 mg Cd l^{-1}, nuclei with spheres of condensed chromatin, moved to the peripheral area of the cell (Fig. 2h, j) when compared to the control (Fig. 2a). In some cases, the loss of nuclear architecture was observed, presenting nuclei with elliptical format (Fig. 2i). Still, at 8 mg Cd l^{-1}, micronuclei and completely fragmented nuclei could be observed (Fig. 2i).

Histochemical and anatomical analysis

Cell wall of epidermis and exodermis cells of root tip became more lignified in the presence of Cd when compared to the control plants (Fig. 3). Besides, for concentrations 4, 8 and 16 mg Cd l^{-1}, the pith was totally sclerenchymatous (Fig. 3). In addition, a high lignification of vascular tissues of the main vein was also observed at the leaf level (Fig. 4a–d) and also the appearance of fibers in the parenchymatous rays and in the phloem (Fig. 4e–h), with higher intensity in concentrations 4 (Fig. 4f) and 16 mg Cd l^{-1} (Fig. 4h). It was observed, in the leaf mesophyll, that there was an increase of intercellular spaces with increasing Cd concentrations (Fig. 5a–d).

Leaf gas exchanges and chlorophyll fluorescence emission

The leaf gas exchanges in *G. americana* were affected with the increased Cd concentrations in nutritive solution. There were decreases in *A*, *gs* and *E* up to 8 mg Cd l^{-1}. The reduction in *A* was proportional to

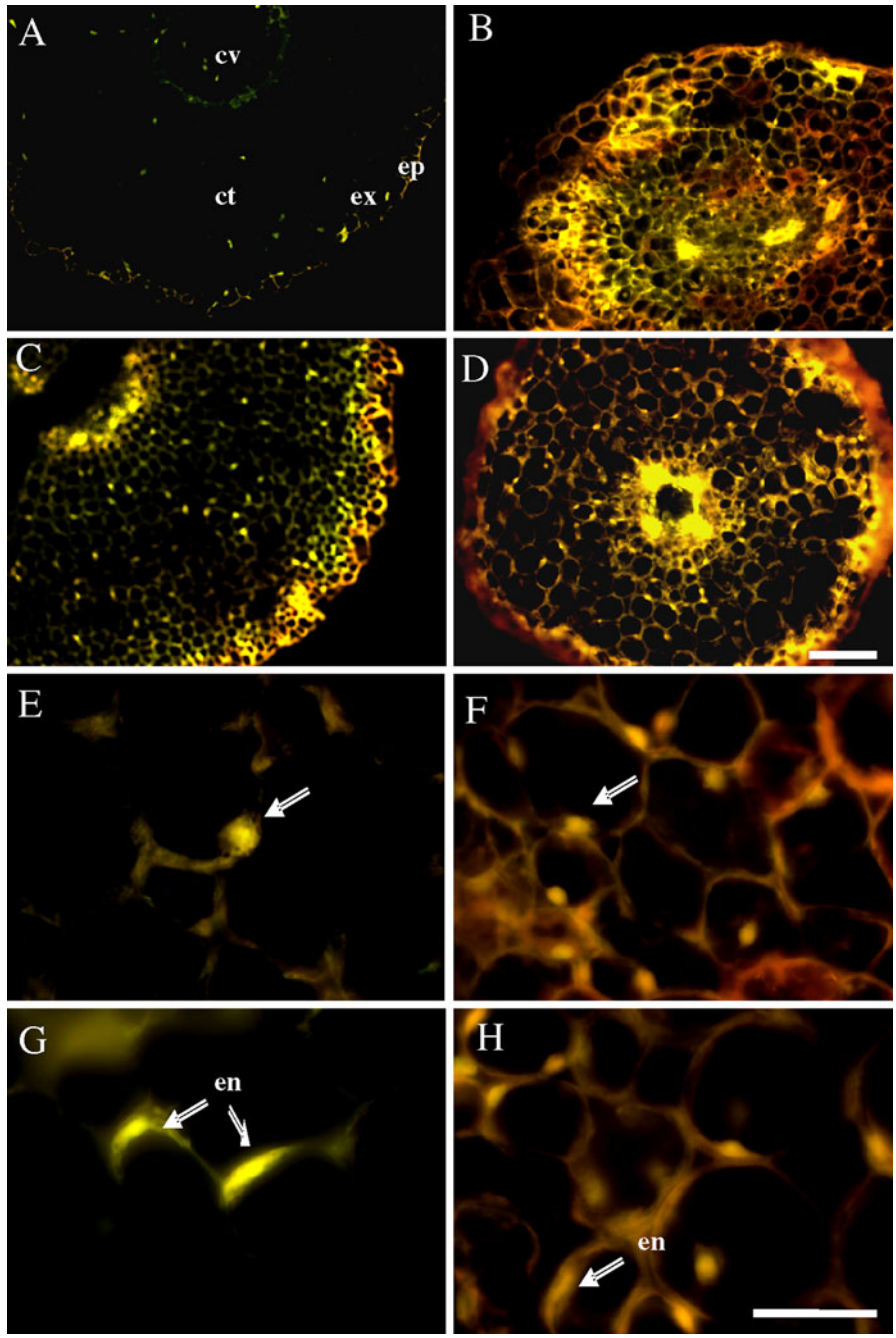

Fig. 1 Transversal section of the root of *G. americana* plants grown in nutrient solution for 120 h in different Cd concentrations. TUNEL assay: control (**a**), 4 (**b**), 8 (**c**) and 16 mg Cd l^{-1} (**d**). Detail of nucleus of the cortex. TUNEL assay: control (**e**), 4 (**f**), 8 (**g**) and 16 mg Cd l^{-1} (**h**). *Arrows* indicate TUNEL-positive nuclei. *ep* epidermis, *ex* exodermis, *ct* cortex, *en* elliptical nuclei, *cv* cylinder vascular. Bar: 30 μm (**a**–**d**), 10 μm (**e**–**h**)

the reduction in *gs*. The highest values of *A*, *gs* and *E* were 9.4 μmol CO_2 m^{-2} s^{-1}, 0.06 mol H_2O m^{-2} s^{-1} and 1.22 mmol m^{-2} s^{-1}, respectively, for the control, while the lowest values were 2.8 μmol CO_2 m^{-2} s^{-1}, 0.02 mol H_2O m^{-2} s^{-1} and 0.38 mmol m^{-2} s^{-1}, respectively, for 8 mg Cd l^{-1} (Fig. 6).

Our results showed a directly proportional relationship between *A* and *gs* (Fig. 7). It was verified, in *G. americana*, that there was no variation in the chlorophyll fluorescence emission with the increase of Cd concentration in the nutritive solution (Fig. 8), since the mean values of *Fv*/*Fm* proportion varied between 0.79 and 0.81.

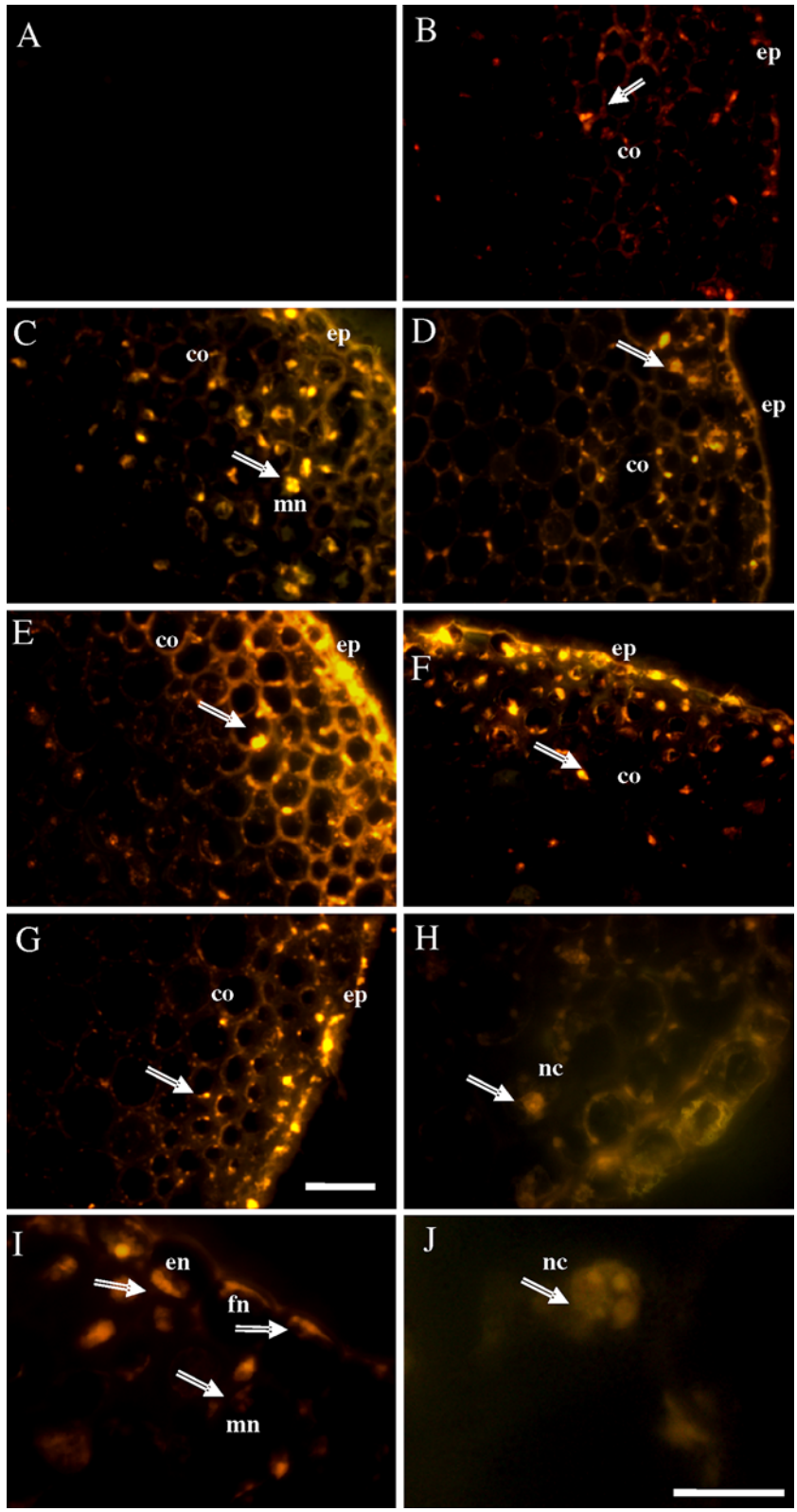

Fig. 2 Transversal section of the leaf main vein of *G. americana* plants grown in nutrient solution for 120 h in different Cd concentrations. In detail, epidermis and collenchyma. Upper epidermis and collenchyma TUNEL assay: control (**a**), 4 (**b**), 8 (**c**) and 16 mg Cd l^{-1} (**d**). Lower epidermis and collenchyma TUNEL assay: 4 (**e**), 8 (**f**) and 16 mg Cd l^{-1} (**g**). *Arrows* indicate TUNEL-positive nucleus. Detail of nucleus of the cortex and abaxial epidermis. TUNEL assay: 4 (**h** and **j**) and 8 mg Cd l^{-1} (**i**). *Arrows* indicate TUNEL-positive nuclei in different apoptotic phases for concentrations 4 and 8 mg l^{-1}. *ep* epidermis, *co* collenchyma, *en* elliptical nuclei, *fn* fragmented nuclei, *mn* micronuclei, *nc* nuclei with condensed chromatin. Bar: 20 μm (**a**–**g**), 10 μm (**h**–**j**)

Chemical composition

The chemical analysis showed that the accumulation of Cd in *G. americana* plants was proportional to the increase of Cd in the nutritive solution. Around 99% of the Cd absorbed was retained in the root system. The accumulation of Cd in the roots after 5 days of exposure to 16 mg Cd l^{-1} was 1318.3 mg Cd kg^{-1} DW (Fig. 9). There was an incipient translocation of this metal for the shoots, and in the stem the highest levels were obtained for the exposure concentration of 16 mg Cd l^{-1}, corresponding to 4.3 mg Cd kg^{-1} DW. In the leaves, Cd was only detected after treatment with 8 and 16 mg Cd l^{-1}, corresponding to 0.7 mg Cd kg^{-1} DW (Fig. 9).

Table 1 TUNEL-positive cells in transversal section of roots and main leaf vein of *G. americana* plants grown in nutrient solution for 120 h in different Cd concentrations, n = 3, ±SE

Cd (mg l^{-1})	TUNEL-positive cells (number per mm^2)	
	Root	Leaf
0	1 ± 0.33 A	18 ± 2.08 A
4	30 ± 0.88 B	32 ± 2.52 B
8	37 ± 1.45 C	47 ± 2.02 C
16	31 ± 1.33 B	45 ± 2.9 C

Means followed by the same letter inside of organ are not significantly different at $P \leq 0.05$ according to Tukey's test

Increasing of Cd levels in the nutritive solution interfered with the uptake of K, Ca and Zn. There was no significant variation in the levels of P, Mg and Fe in different organs of *G. americana*. A decrease of K levels was observed in the leaf that was proportional to the increase of Cd concentrations in nutritive solution. The highest and lowest values of K levels observed in the leaves were 19.37 and 10.92 g K kg^{-1} DW (Fig. 10), which corresponded to a reduction of 8 and 48%, respectively, in relation to the control. This decrease was also observed in the stem, but in smaller proportion. On the other hand, the Ca levels in the root were higher at higher Cd exposure, while a decrease of this nutrient was observed in the stem. The maximum and minimum Ca levels observed in the root were 3.67 and 2.28 g Ca kg^{-1} DW, which corresponded to an increase of 74 and 8.6%, respectively, in relation to the control treatment (Fig. 10). The Zn levels presented

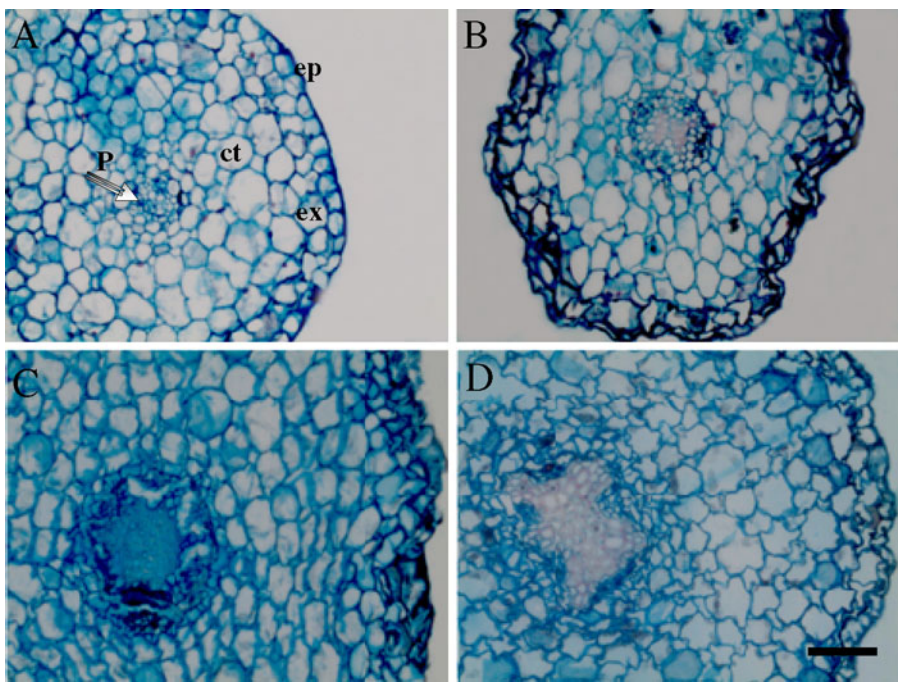

Fig. 3 Transversal section of *G. americana* root tip treated with Cd for 120 h. Control (**a**), 4 (**b**), 8 (**c**) and 16 mg Cd l^{-1} (**d**). In the roots treated with 4, 8 and 16 mg l^{-1} Cd pith with sclerified cells (*arrows*), epidermis and exodermis with lignified cells when compared to the control were observed. *ep* epidermis, *ex* exodermis, *ct* cortex, *p* pith. Bar: 30 µm

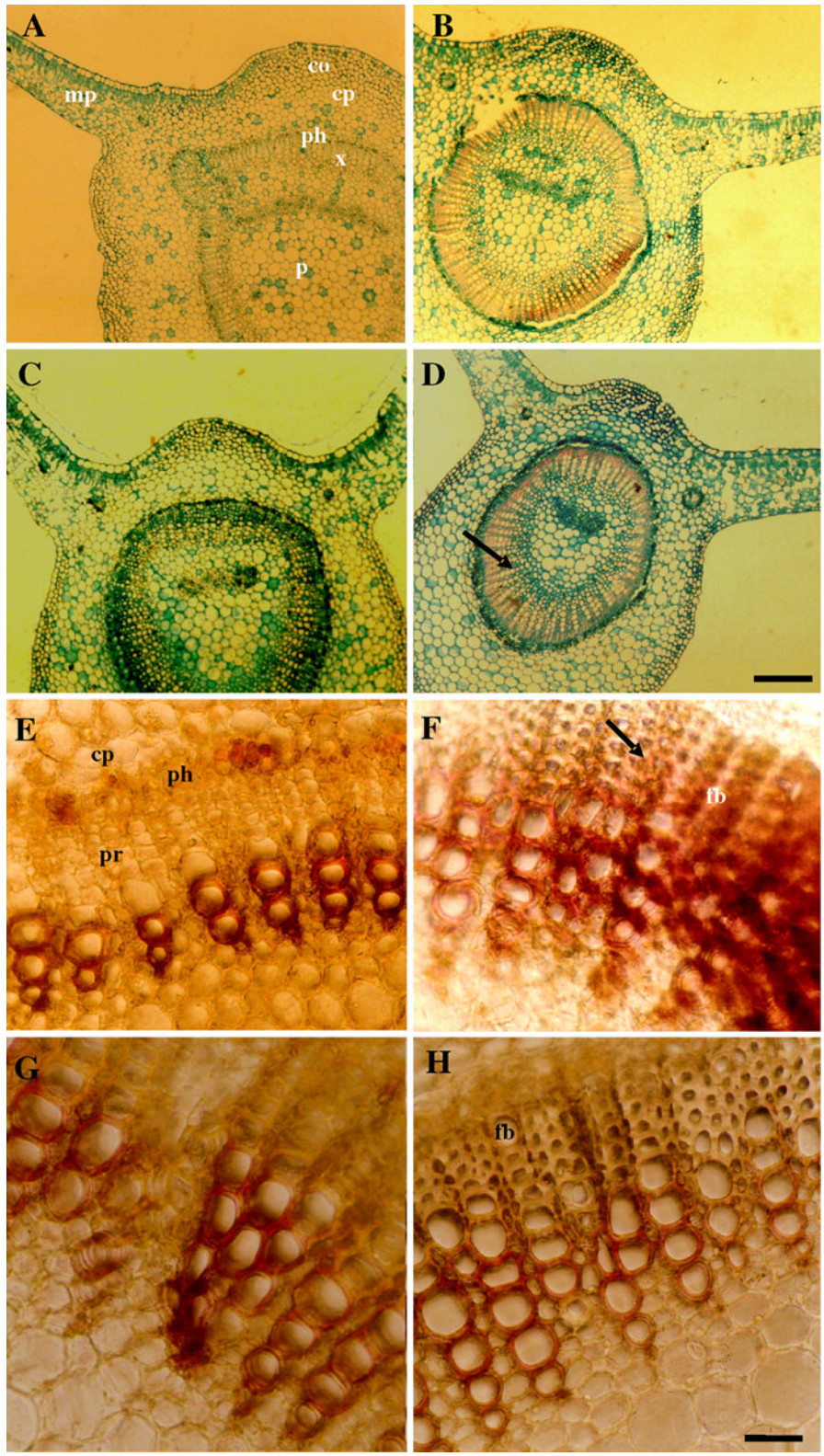

Fig. 4 Transversal section of the leaf main vein of plants exposed to different Cd concentrations. Control (**a**), 4 (**b**), 8 (**c**) and 16 mg Cd l^{-1} (**d**), respectively. Detail of the vascular tissue of the leaf vein, control (**e**), 4 (**f**), 8 (**g**) and 16 mg Cd l^{-1} (**h**), respectively. The formation of fibers is verified in the parenchyma rays. *Arrows* indicate fibers. *co* colenchyma, *cp* cortical parenchyma, *ph* phloem, *x* xylem, *p* pith, *mp* mesophyll, *fb* fibers, *pr* parenchyma rays. **a**-**d** Stained with astra blue and 1% safranine, **e**–**h** histochemical test with acidified floroglucin. Bars: (**a**–**d**) 170 μm, (**e**–**h**) 30 μm

significant variation only in the root. The increase in Zn content was proportional to the Cd increase in the solution. The maximum and minimum Zn levels observed in the root were 21.33 and 11.67 mg Zn kg^{-1} DW, which corresponded to an increase of 137 and 29%, respectively, in relation to the control plants (Fig. 10).

Discussion

Changes in the cell nucleus are one of the most important indicators of apoptosis (Berboodi and Samadi 2004). The observed changes in the cell nuclei of root and leaf tissues of *G. americana* were similar to those found in root tissue of *Allium cepa* plants submitted to different concentrations of Cd for 72 h. In the case of *A. cepa*, there were changes in the cellular morphology, including the presence of a marginal and condensed nuclei, condensed chromatin spheres, nuclei fragmentation and apoptotic bodies, that were similar to the ones found in animal cells (Berboodi and Samadi 2004). Furthermore, the nuclear changes induced by Cd, in leaf tissue cells of *G. americana*, were similar to those in *Nicotiana tabacum* BY2 cells cultivated in suspension and submitted to treatments with H_2O_2 (Houot et al. 2001). The first event observed in *N. tabacum* cells, after exposure to H_2O_2, was chromatin condensation. After that, the cells presented condensed chromatin at the periphery of the nuclei (pre-apoptotic nuclei) or the presence of micronuclei (apoptotic-like nuclei), loss of nuclear architecture (lace chromatin and stretched nuclei) or empty cells with completely lysed nuclei.

The lignification process, verified in root and leaf tissues of *G. americana*, was also a metabolic response which leads to programmed cell death. When Cd causes programmed cell death in plants, chromatin condensation and nuclear fragmentation precede the increase of lignin synthesis (Schützendübel et al. 2001; Berboodi and Samadi 2004). Consequently, lignification reduces the plasticity of cell walls, thus reducing the growth of cells and blocking plant development (Chaoui and El Ferjani 2005).

The increased intercellular spaces in the leaf mesophyll of *G. americana* after Cd exposure may be caused by cell death and leads to increased aeration of such tissue. In the leaf mesophyll of Cd-treated woody species *Salix viminalis* vitality of cells that make spongy and palisade parenchyma was reduced, and such cells had intense vacuolization and condensed nuclei (Vollenweider et al. 2006). However, in *Brassica juncea* no change was observed in this tissue under Cd stress (Sridhar et al. 2005).

Although Cd causes increase in the accumulation of phenols in cells of different tissues (Schützendübel

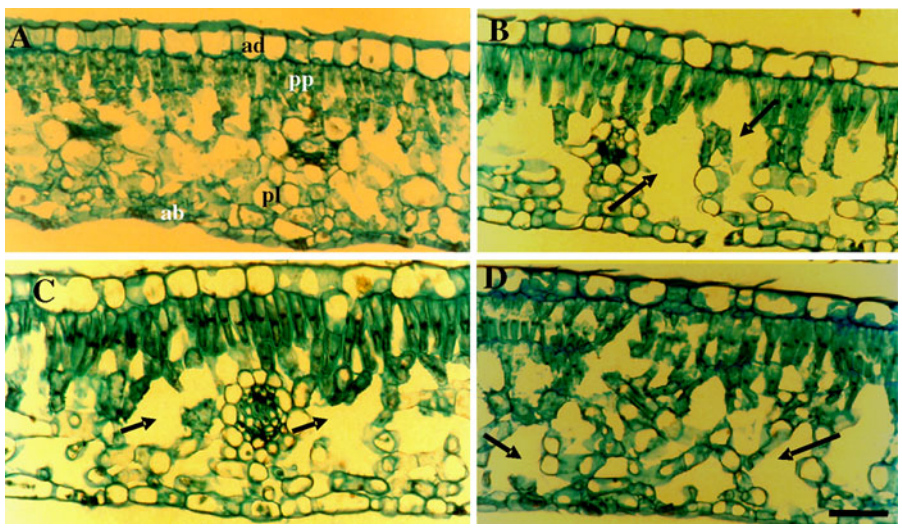

Fig. 5 Transversal section of the leaf mesophyll after plant exposure to different concentrations of Cd in nutritive solution. Extension of intercellular spaces of lacunar and palisade parenchymas was verified when compared to the control. Control (**a**), 4 (**b**), 8 (**c**) and 16 mg l^{-1} (**d**). The *arrows* indicate deformed cells and intercellular spaces. *pp* palisade parenchyma, *lp* lacunar parenchyma, *ad* adaxial epidermis, *ab* abaxial epidermis. Bar: 30 μm

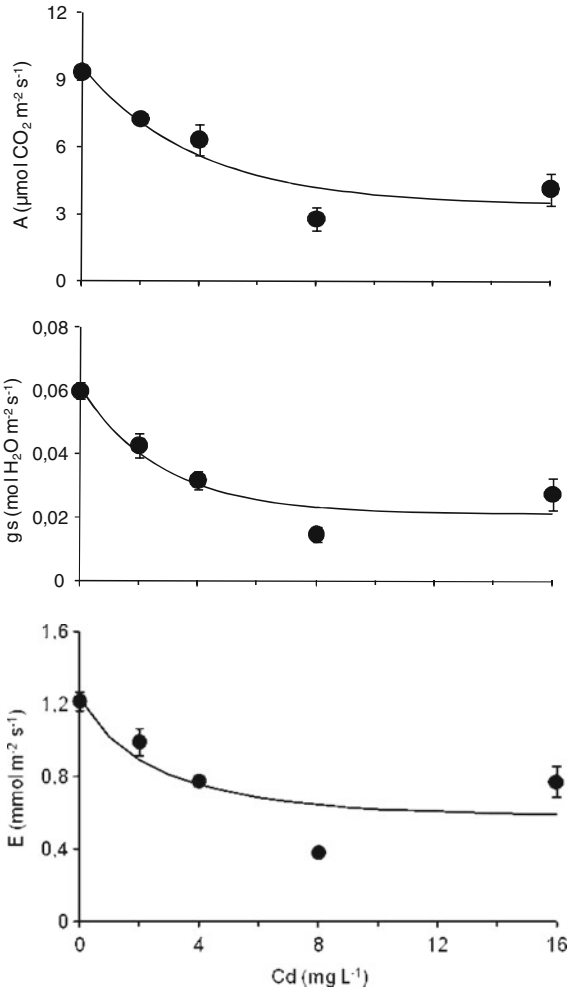

Fig. 6 Changes in net photosynthesis rate (*A*), stomatal conductance to water vapor (*gs*), transpiration (*E*) in leaves of *G. americana* plants exposed to concentrations of 2, 4, 8 and 16 mg Cd l^{-1} together with the treatment of 'zero', in nutrient solution during 72 h, n = 4, ±SE ($P \leq 0.01$ according to Student's *t*-test). The regression curve equations were y = −5.48 (−0.73 − exp (−0.20)) ($r^2 = 0.87$) for *A*; y = −0.034 (−0.78 − exp (−0.33x)) ($r^2 = 0.83$) for *gs*; and y = 0.58/(1 − 0.53 exp (0.20x)) ($r^2 = 0.77$) for *E*. The measurements were made at PPFD of 800 µmol m^{-2} s^{-1}

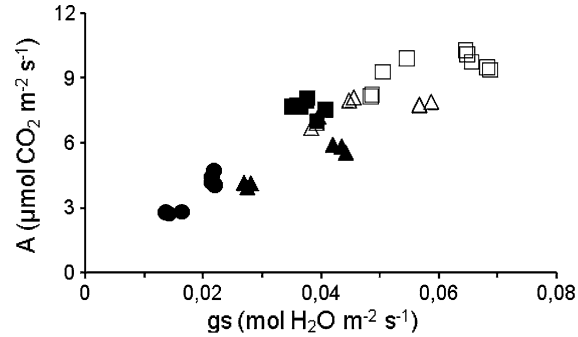

Fig. 7 Relationship between net photosynthesis (*A*) and the stomatal conductance to water vapor (*gs*) in leaves of *G. americana* plants exposed to concentrations of 2 (*open triangle*), 4 (*filled square*), 8 (*filled triangle*) and 16 (*filled circle*) mg Cd l^{-1}, together with the treatment of 'zero' (*open square*), in nutrient solution for 72 h, n = 4, ±SE. The measurements were made at PPFD of 800 µmol m^{-2} s^{-1}

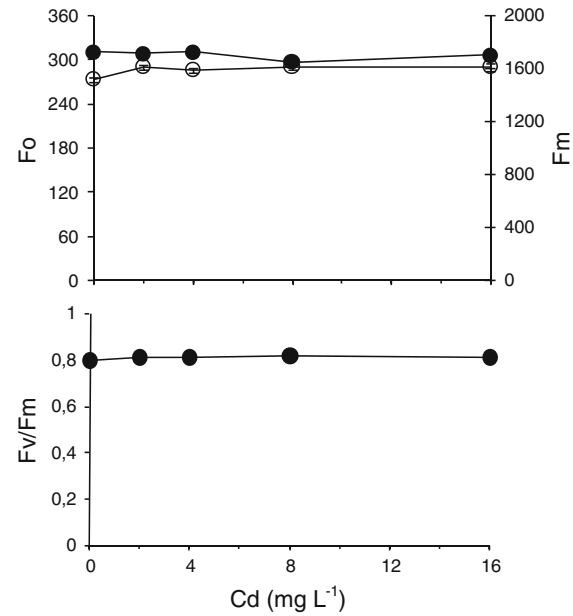

Fig. 8 Initial fluorescence (*Fo*), maximum fluorescence (*Fm*) and maximum quantum efficiency of PS-II (*Fv/Fm*) of *G. americana* plants grown in nutrient solution for 72 h with different Cd concentrations. *Fo* (*filled circle*) and *Fm* (*open circle*), n = 4, ±SE

et al. 2001; Vollenweider et al. 2006), the histochemical test with ferric chloride did not detect the presence of phenol in the leaf tissue cells of Cd-treated plants. Also, cells of the tissues of stem buds did not present modifications that could be related to Cd stress.

As a result of the defense reactions induced by Cd, the lignification and death of *G. americana* root tip tissue cells (Figs. 1, 3) may have promoted a decrease of the root system ability in absorbing and transporting water and substances which are dissolved in it through xylem. That, in turn, caused stomata closure and, consequently, affected *A* and *E* at the leaf level.

According to Mendelssohn et al. (2001), the photosynthetic activity significantly decreases with increasing Cd concentrations and thus is considered

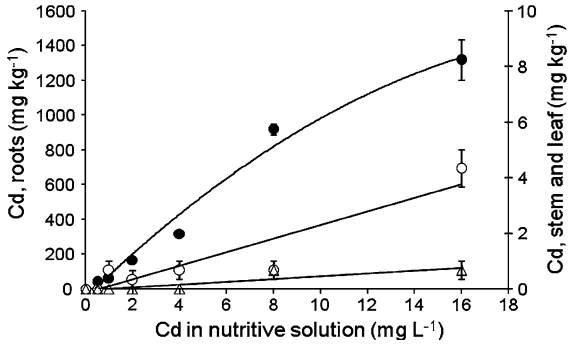

Fig. 9 Variation in the Cd contents in roots, stems and leaves of *G. americana* plants grown for 120 h in nutrient solution with different Cd concentrations. The regression curve equations were $\hat{y} = -51.32 + 65.70^{**}x - 0.69^{**}x^2$ ($r^2 = 0.98$) for roots (*filled circle*); $\hat{y} = -0.14 + 0.12^{**}x$ ($r^2 = 0.85$) for stems (*open circle*); and $\hat{y} = -0.03 + 0.02^{**}x$ ($r^2 = 0.79$) for leaves (*open triangle*). Each point represents the mean ± SE, n = 3 (** $P \leq 0.01$ according to Student's *t*-test)

to be a sensitive indicator of stress caused by this metallic element. Mobin and Khan (2007) observed in a seedling of *B. juncea*, that the reduction in *A* was followed by increase in *gs* and *E.* According to these authors, such change was caused by the activity reduction of the enzymes carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Chugh and Sawhney (1999), in studies done with *Pisum sativum*, evidenced that Cd caused a deleterious effect over photosynthetic enzymes and that *A* progressively reduced with the increase of the concentration of this metal.

Cadmium did not have any effect on the photochemical phase of photosynthesis in *G. americana*. Similar results were found by Mendelssohn et al. (2001) in studies done with *Thypha domingesis* and *Spartina alterniflora.* These authors concluded that the *Fv*/*Fm* ratio is less sensitive to the stress imposed by Cd, when compared to *A*. Consequently, this ratio is not a good physiological indicator for evaluating Cd stress. However, Chugh and Sawhney (1999) observed in in vitro experiments with isolated chloroplasts of *P. sativum* that the photosystem 2 (PS-2) was highly sensitive to Cd and that its functionality was more affected than that of photosystem 1 (PS-1). However, results with isolated chloroplasts are not very reliable because the isolation of this organelle necessitates a chemical process.

The low Cd translocation to the shoots and its retention in the root system was is good for

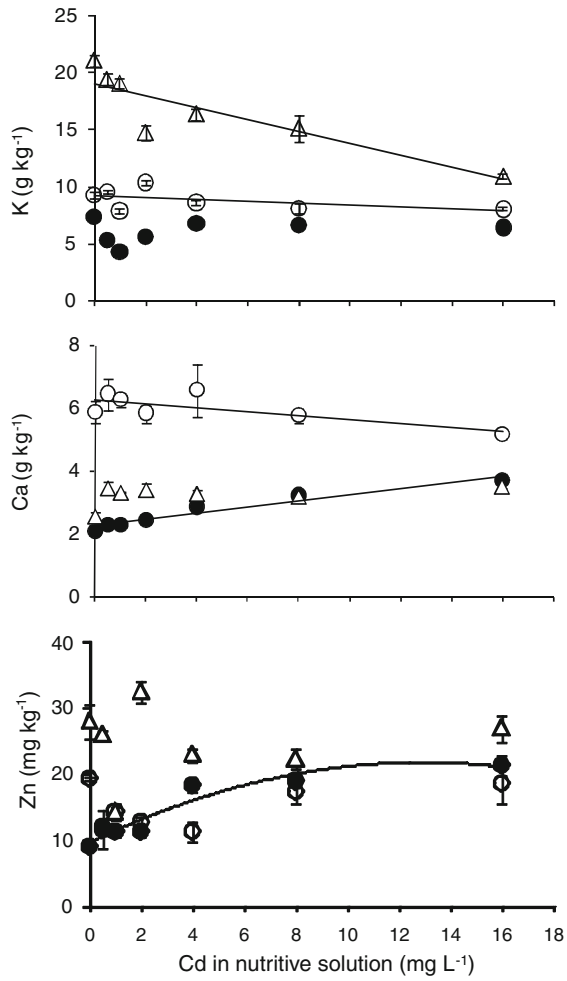

Fig. 10 Variation in K, Ca and Zn contents in roots (*filled circle*), stems (*open circle*) and leaves (*open triangle*) of *G. americana* plants grown for 120 h in nutrient solution with different Cd concentrations. The regression curve equations for K were $\hat{y} = 5.81$ for roots; $\hat{y} = 9.20 - 0.042^{*}x$ ($r^2 = 0.27$) for stems and $\hat{y} = 19.03 - 0.26^{**}x$ ($r^2 = 0.78$) for leaves; for Ca were $\hat{y} = 2.26 + 0.05^{**}x$ ($r^2 = 0.94$) for roots; $\hat{y} = 0.62 - 0.31^{*}x$ ($r^2 = 0.56$) for stems and $\hat{y} = 3.13$ for leaves; for Zn were $\hat{y} = 9.62 + 0.94^{**}x - 0.02^{**}x^2$ ($r^2 = 0.92$) for roots; $\hat{y} = 13.90$ for stems and $\hat{y} = 24.4$ for leaves. Each point represents the mean ± SE, n = 3 (** $P \leq 0.01$; * $P \leq 0.05$ according to Student's *t*-test)

G. americana, since it is a fruit species, whose fruits are consumed by human beings, animals and birds. Cd accumulation in roots may be related to several factors, such as: (i) exposure time of this species to this metal, considering that the Cd accumulation in the tissues increases as exposure time increases (Cosio et al. 2005; Arduini et al. 2004); (ii) the Cd concentrations to which the species was exposed

(Arduini et al. 2004), since Cd started being translocated to the stem after the dose 1 mg Cd l^{-1} and to the leaf after the dose 8 mg Cd l^{-1} (Fig. 8); and (iii) the species strategy to protect its photosynthetic activity, since, according to Dixit et al. (2001) the low level of Cd in the leaves may be a strategy to protect the photosynthetic functions of oxidative stress induced by Cd. Therefore, metal accumulation in the roots and the low translocation to the shoots are considered mechanisms through which the root system may contribute to the tolerance of woody species to toxic metal concentrations (Arduini et al. 1996).

In *G. americana*, the low translocation of K to leaves after exposure to Cd may have contributed to the observed decrease in photosynthesis in this species, since K is one of the major solutes involved in stomatal movements. In *Commelina communis* the responses of stomata on detached epidermis were strongly dependent on the concentration of KCl. At lower concentrations, the ability of the stomata to open is thought to be limited by the availability of K^+ ions (Travis and Mansfield 1979). In addition, in the same species, exposed to different Cr^{3+} concentrations, there was also a reduction in the K levels in roots and shoots (Barbosa et al. 2007).

The Cd effects in the absorption of mineral macro and micronutrients are much diversified in woody species. In *Tabebuia impetiginosa*, Ca and K levels in the dry mass of the root diminished in a linear way with Cd application, while the shoot levels of such macronutrients were not affected. In this species the Zn levels in the root were reduced in presence of Cd (Paiva et al. 2004). Increase in Cd concentration reduced the Ca levels in shoots of both *Eucalyptus maculata* and *E. urophylla* (Soares et al. 2005), while K and Zn levels were not affected. In *Prunus dulcis* Cd caused the decrease of Ca and K in both root and shoots (Nada et al. 2007).

We therefore may conclude that Cd caused the process of cell death in root and leaf tissues of *G. americana* similar to the apoptosis seen in animals, showing all changing phases of cell nuclei. Different from chlorophyll *a* fluorescence emission, the leaf gas exchanges may be used as one of the indicators of tolerance to Cd stress in *G. americana*.

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