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ORIGINAL PAPER

Antioxidant response of *Nicotiana tabacum* cv. Bright Yellow 2 cells to cadmium and nickel stress

Priscila L. Gratão · Georgia B. Pompeu · Flávia R. Capaldi · Victor A. Vitorello · Peter J. Lea · Ricardo A. Azevedo

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Abstract Plant cell cultures are a suitable model system for investigation of the physiological mechanisms of tolerance to environmental stress. We have determined the effects of Cd (0.1 and 0.2 mM $CdCl_2$) and Ni (0.075 and 0.75 mM $NiCl_2$) on *Nicotiana tabacum* L. cv. Bright Yellow (TBY-2) cell suspension cultures over a 72-h period. Inhibition of growth, loss of cell viability and lipid peroxidation occurred, in general, only when the TBY-2 cells were grown at 0.2 mM $CdCl_2$ and at 0.75 mM $NiCl_2$. At 0.1 mM $CdCl_2$, a significant increase in growth was determined at the end of the experiment. Increases in the activities of all of the four enzymatic antioxidant defence systems tested, were induced by the two concentrations of Cd and Ni, but at different times during the period of metal exposure. Overall, the cellular antioxidant responses to Cd and Ni were similar and were apparently sufficient to avoid oxidative stress at the lower concentrations of Cd and Ni. The activities of glutathione reductase and glutathione S-transferase increased early but transiently, whereas the activities of catalase and guaiacol peroxidase increased in the latter half of the experimental period. Therefore it is likely that the metabolism of reduced glutathione was enhanced during the initial onset of the stress, while catalase and guaiacol-type peroxidase appeared to play a more important role in the antioxidant response once the stress became severe.

Keywords Antioxidant enzymes · Cadmium · Lipid peroxidation · Nickel · Oxidative stress

Abbreviations

Cd	Cadmium
Ni	Nickel
ROS	Reactive oxygen species
SOD	Superoxide dismutase
APX	Ascorbate peroxidase
CAT	Catalase
GPOX	Guaiacol-type peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GR	Glutathione reductase
EDTA	Ethylenediaminetetracetic acid

P. L. Gratão · F. R. Capaldi · R. A. Azevedo (✉)
Departamento de Genética, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo (USP), Piracicaba 13418-900, SP, Brazil
e-mail: raazeved@esalq.usp.br

G. B. Pompeu
Laboratório de Ecotoxicologia, Centro de Energia Nuclear na Agricultura (CENA), Piracicaba 13416-700, SP, Brazil

V. A. Vitorello
Laboratório de Biologia Celular e Molecular, Centro de Energia Nuclear na Agricultura (CENA), Piracicaba 13416-700, SP, Brazil

P. J. Lea
Department of Biological Sciences, University of Lancaster, Lancaster LA1 4YQ, UK

TBARS	Thiobarbituric acid reactive substances
MDA	Malondialdehyde
GST	Glutathione S-transferases
DHAR	Dehydroascorbate reductase
MDHAR	Monodehydroascorbate reductase

Introduction

Cadmium (Cd), a non-essential element, is among the most hazardous environmental pollutants for humans, animals and plants even at low concentrations (Benavides et al. 2005; Fojta et al. 2006; Mobin and Khan 2007; Wahid and Ghani 2008). Cd can seriously affect plant metabolism in several ways and induce oxidative stress (Vitória et al. 2001; Noriega et al. 2007), although the intensity depends on the species, metal concentration and duration of exposure (Benavides et al. 2005). On the other hand, nickel (Ni) is not toxic at low concentrations and is required for normal plant growth due to the presence of two Ni atoms in the active site of the enzyme urease (EC 3.5.1.5.) (Bai et al. 2006). The solubility of Ni can influence its toxicity to plants (Rooney et al. 2007), although at higher concentrations Ni is a toxic pollutant for humans, animals and plants (Gomes-Junior et al. 2006a; Gajewska and Sklodowska 2007a).

When plants are grown in the presence of toxic metals, oxidative stress can be imposed on cells as a result of an imbalance between the production of reactive oxygen species (ROS) and antioxidant defences, leading to oxidative damage of proteins and DNA and lipid peroxidation, which in turn causes severe damage of cell membranes (Gratão et al. 2005; Pitzschke et al. 2006). Recently, information about the role of ROS has suggested that they are also regulatory signals for plant growth and development (Foyer and Noctor 2005; Hancock et al. 2006; Jones et al. 2007). Cd and Ni can stimulate oxidative stress, but in contrast to other toxic metals, they do not seem to stimulate the production of ROS directly (Boominathan and Doran 2002), probably because they do not undergo changes in oxidation.

The toxicity of ROS explains the evolution of a complex array of non-enzymatic and enzymatic detoxification mechanisms in plants capable of quenching ROS without themselves undergoing conversion to a destructive radical, thus preventing the formation of cascades of uncontrolled oxidation (Hassan 2006; Pitzschke et al. 2006). ROS-scavenging mechanisms of plants include enzymes such as superoxide dismutase (SOD, EC 1.15.1.1) which dismutates $O_2^{\bullet-}$ to H_2O_2. Subsequently, H_2O_2 may be detoxified to H_2O by ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, 1.11.1.6) and glutathione peroxidase (GPX, EC 1.11.1.9) (Gratão et al. 2005). In addition, for the detoxification of H_2O_2, phytophenolics can act as antioxidants by donating electrons to guaiacol-type peroxidases (GPOX, EC 1.11.1.7) (Sakihama et al. 2002). The ascorbate–glutathione cycle is closed by regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG) by glutathione reductase (GR, EC 1.6.4.2) using NAD(P)H as a reducing agent (Moller et al. 2007).

Plant cell lines can be considered good systems for the study of antioxidative responses due to their capacity to grow under high oxidative stress conditions (Kim et al. 2004). The TBY-2 cell line is relatively well understood and is often used as a model system for higher plants due to the exceptionally high homogeneity of the cells and high growth rate (Olmos et al. 2003; Saito et al. 2005) In the case of heavy metals, a more uniform exposure can be obtained than when working with other plant tissues. Furthermore, the sensitivity to metals depends on the physiological and developmental stages of the cells. For instance, TBY-2 cells exhibit distinct alterations in sensitivity to aluminium (Al) during growth of the culture (Vitorello and Haug 1996).

The aim of this work was to study the effect of Cd and Ni on TBY-2 cell metabolism, through physiological parameters related to cell growth, lipid peroxidation and enzymatic antioxidant system. The information available in this work aims to improve our understanding about some of the basic physiological mechanisms related to phytotoxicity caused by Cd and Ni through the measurements of antioxidants as stress markers.

Material and methods

Cell culture

Tobacco TBY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cells were cultured as described by Nagata

et al. (1992). The cell culture was maintained in a medium consisting of MS basal salts supplemented with sucrose (30 g/l), KH_2PO_4 (370 mg/l), myo-inositol (100 mg/l), thiamine (1 mg/l), 2,4-D (0.22 mg/l) and MES (0.5 g/l). The pH was adjusted to 5.7 before autoclaving. Subcultures were carried out every week by transferring 2 ml inoculum of stationary-phase culture (equivalent to about 1.5 ml of packed cells) into 50 ml of fresh medium contained in 250 ml Erlenmeyer flasks. Cultures were grown in the dark on a rotary shaker at 160 rpm at 27°C and growth was evaluated by packed cell volume following centrifugation at 500 rpm for 5 min.

Evaluation of cell viability

TBY-2 cells samples were incubated for 5 min in a 1:1 (v/v) mixture of cell suspension and 0.4% (w/v) trypan blue. Approximately 500 cells per replicate were examined from nine fields of view, from three different slide preparations through optical microscopy. Cell viability was evaluated by plasma membrane permeability to trypan blue.

Exposure to Cd and Ni

TBY-2 cells were maintained in liquid medium for 7 days and 3 ml of cells were subcultured into fresh medium (50 ml). Two-day-old cell cultures were submitted to preliminary assays on the effect of several $CdCl_2$ and $NiCl_2$ concentrations (0–1 mM) and $CdCl_2$ (0.1 and 0.2 mM) and $NiCl_2$ (0.075 and 0.75 mM) concentrations were chosen for the main experiments. Two experiments were carried out for each metal element separately. Cells harvested at distinct periods during the growth cycle (6, 12, 24, 36, 48 and 72 h), were suction-dried and weighed for packed cell volume and stored at −80°C for further analyses.

Lipid peroxidation

Lipid peroxidation in TBY-2 cells was determined by estimating the content of thiobarbituric acid reactive substances (TBARS) as described by Gomes-Junior et al. (2006a). The concentration of equivalent malondialdehyde (MDA) was calculated using an extinction coefficient of 155 mM/cm.

Extraction and analysis of antioxidant enzymes

The following steps were carried out at 4°C unless stated otherwise. The TBY-2 cells were homogenized (2:1 buffer volume: fresh weight) in a mortar with a pestle with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol and 5% (w/v) insoluble polyvinylpolypyrrolidone (Azevedo et al. 1998). The homogenate was centrifuged at 10,000 rpm for 30 min and the supernatant was kept stored in separate aliquots at −80°C, prior to CAT, GR, GPOX and glutathione S-transferase (GST) analyses. CAT, GR and GPOX activities were determined as described by Gomes-Junior et al. (2007) and GST as described by Habig and Jakoby (1981).

Determination of protein concentration

Protein concentration for all samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

The experimental design was randomized with three replicates for each flask/treatment/time interval and the results were expressed as mean and standard error of mean (±SEM) of three independent replicates of cell growth, cell viability, MDA, CAT, GR, GPOX and GST activities.

Results

Cell culture growth and cell viability

Preliminary experiments with several different concentrations of Cd and Ni were carried out to determine the concentrations of Cd and Ni which cause inhibition of growth (data not shown). Based on these early tests, further experiments were carried out for periods of up to 72 h using two concentrations of Cd (0.1 and 0.2 mM) and Ni (0.075 and 0.75 mM). The two highest concentrations of Cd and Ni used resulted in cell culture growth inhibition, particularly at 0.75 mM Ni (Fig. 1d), and also in a significant reduction of cell viability (Fig. 1b and d). On the

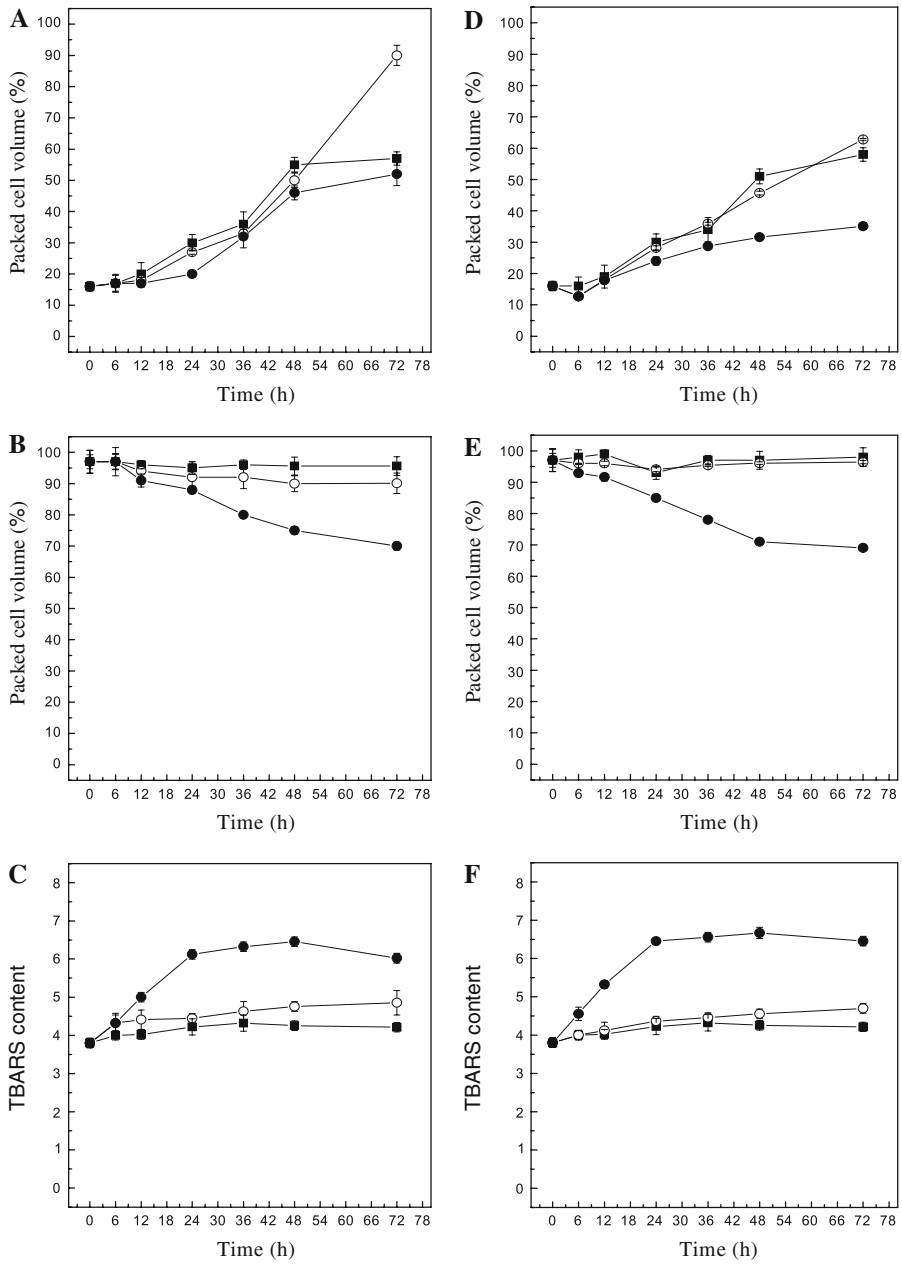

Fig. 1 Cell growth (percentage of packed cell volume): Cd (**a**) and Ni (**d**). Cell viability (%): Cd (**b**) and Ni (**e**). TBARS content (nmol g^{-1} fr. wt): Cd (**c**) and Ni (**f**) in tobacco cells grown for a 72 h period in two concentrations of $CdCl_2$ and $NiCl_2$. Control (zero $CdCl_2$ and $NiCl_2$) (■), 0.1 mM $CdCl_2$ and 0.075 mM $NiCl_2$ (○), 0.2 mM $CdCl_2$ and 0.75 mM $NiCl_2$ (●). Values are the means of 3 replicates ±SEM

other hand, 0.1 mM $CdCl_2$ stimulated cell culture growth at the end of the experimental period (Figs. 1a and 2).

Lipid peroxidation

ROS can react with unsaturated fatty acids to cause peroxidation of lipids in the membranes. Evidence that Cd and Ni can induce lipid peroxidation was determined by the concentration of TBARS in the cells. An increase in TBARS was observed in Cd and Ni treatments and was more significant at the highest concentrations used (Fig. 1c and f).

Antioxidant enzyme responses

Following a lag period of approximately 24 h, the specific CAT activity was shown to be strongly increased by both metals (Fig. 3a and b). After 36 h, Cd induced a sustained increase in CAT activity

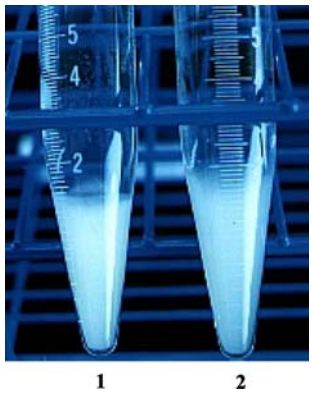

Fig. 2 Tobacco cells suspension cultures grown for 72 h in control zero $CdCl_2$ (1) and 0.1 mM $CdCl_2$ (2), following centrifugation at 500 rpm for 5 min

which was more significant at the concentration of 0.2 mM. Stimulation of CAT activity by Ni occurred earlier than that observed for Cd but, in contrast to the latter, was not sustained up to 72 h. At 0.75 mM Ni, CAT activity peaked at 36 h whereas at 0.075 mM Ni, CAT activity exhibited essentially the same behaviour, but with a slight delay in maximum activity (48 h) (Fig. 3b).

GR activity was immediately stimulated by Cd and Ni when compared to the control (Fig. 3c and d), but particularly by Cd and at the higher concentration of metal. Maximum GR activity was reached at 24 and 6 h for Cd and Ni treatments, respectively, which was followed by a drastic reduction in activity to near control levels at 48 h (Fig. 3c and d).

The trend in the response of GST activity to Cd and Ni-induced stress was similar to that observed for GR activity, with a rapid increase in activity early on followed by a rapid decrease in activity to near control levels at 36 h (Cd) and 48 h (Ni) (Fig. 4a and b). Two differences, however, were observed. In the Cd treatments, a 6 h lag period was observed before stimulation of activity and in the Ni treatments, the peak in induction was broader, similar to the response in CAT activity.

GPOX was also shown to be increased by the Cd and Ni treatments, however as observed for CAT activity in Cd-treated cells, the observed increases were continuous and a clear distinction between Cd and Ni treatments to the control GPOX activity was only observed at 36 h of treatment. Control values of GPOX increased until the end of the experiment. Again, the highest Cd and Ni concentrations used produced the highest increases in enzyme activity (Fig. 4c and d) and particularly at 72 h for Ni.

Discussion

Plants require an adequate supply of water (Kirda et al. 2007; Lea and Azevedo 2007) and other minerals, and a soil environment that is free or at least contains very reduced concentrations of toxic compounds. The majority of the studies involving oxidative stress induction by toxic metals have been carried out with whole plants. In this study, we have shown that the growth of TBY-2 cell cultures were inhibited when exposed to the highest Cd and Ni concentrations (Fig. 1a and d), as also demonstrated the significant inhibitory effect on viability (Fig. 1b and e) and induction of lipid peroxidation (Fig. 1c and f) indicating the establishment of stressful conditions by both metals particularly at the highest concentrations tested. Cd can inhibit growth (Fornazier et al. 2002; Liu et al. 2007) and stimulate ROS production, resulting in several metabolic perturbations (Durcekova et al. 2007). Cd can interfere with morphogenesis, by inhibiting cell division and cell enlargement (Dalla Vecchia et al. 2005). Cd has been shown to induce cell death accompanied by an increased production of H_2O_2 within several days of exposure, such as that observed in tobacco TBY-2 cells (Fojta et al. 2006) and suspension cultures of *L. esculentum* (Yakimova et al. 2006). The exposure of TBY-2 tobacco cells to millimolar concentrations of Cd resulted in cell death preceded by the accumulation of $O_2^{\bullet-}$ of mitochondrial origin and membrane lipid peroxidation (Garnier et al. 2006). Olmos et al. (2003) have also investigated the action of Cd on BY-2 cells and suggested that H_2O_2 production in Cd-treated cells is a regulated event involving calmodulin and protein phosphorylation. In the presence of 5 mM Cd, BY-2 cells responded with a rapid (4–10 min) generation of H_2O_2, but such a Cd-induced oxidative burst could be detected at concentrations as low as 200 μM. Although we have not determined ROS production, it is probable that the BY-2 cells have undergone a similar rapid oxidative burst in response to Cd, since we have used Cd concentrations within the same range used by Olmos et al. (2003) and for up to 72 h of exposure to the metal.

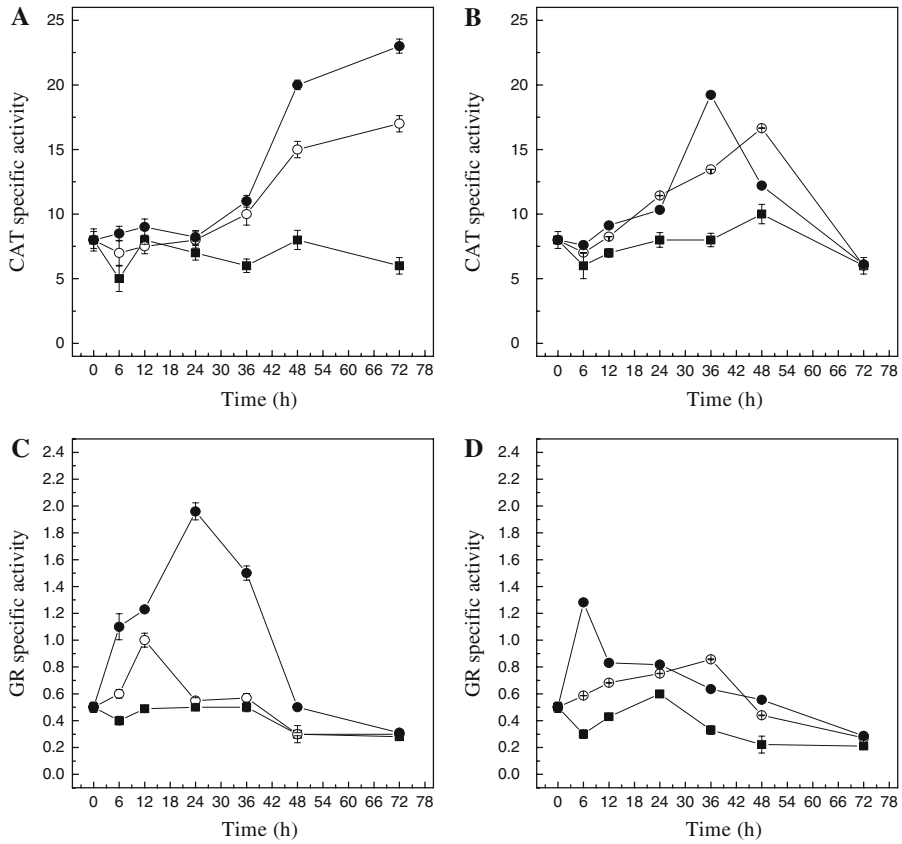

Fig. 3 Specific activity of CAT (μmol min^{-1} mg^{-1} protein): Cd (**a**) and Ni (**b**); GR (μmol min^{-1} mg^{-1} protein): Cd (**c**) and Ni (**d**) in tobacco cells grown for a 72 h period in two concentrations of $CdCl_2$ and $NiCl_2$. Control (zero $CdCl_2$ and $NiCl_2$) (■), 0.1 mM $CdCl_2$ and 0.075 mM $NiCl_2$ (○), 0.2 mM $CdCl_2$ and 0.75 mM $NiCl_2$ (●). Values are the means of 3 replicates ±SEM

In a manner similar to Cd, the high Ni concentration used also caused a significant inhibition of TBY-2 cell growth (Fig. 1d). Exposure of plants and plant cells to high concentrations of Ni resulted in a variety of effects such as reduction of growth (Nakazawa et al. 2004; Gajewska et al. 2006b), inhibition of ribonuclease and protease activities (Maheshwari and Dubey 2007), decrease of dry matter of roots and shoots (Rao and Sresty 2000) and decrease of soil microbial biomass (Berton et al. 2006), among others. Although lower concentrations of Ni in the nutrient medium have previously been shown to have a favourable effect on the growth of a number of plants species (Kevresan et al. 2001), we have not clearly observed such an effect (Fig. 1d).

It is suggested that at concentrations above 1 μM, Cd inhibits cell growth and DNA synthesis in a wide variety of cell types. Interestingly, growth stimulation was observed in the lower Cd concentration (0.1 mM) used (Fig. 2), however, such an effect has also been observed in in vitro cell culture of sugarcane (Fornazier et al. 2002) and coffee (Gomes-Junior et al. 2006b), where low Cd concentrations stimulated the growth, whilst higher Cd concentrations caused a drastic reduction of growth. It appears that our results could be linked to a hormetic dose-response relationship induced by inorganic agents, when small concentrations of toxic elements appear to stimulate growth (Calabrese and Baldwin 2003). It is also possible that other key metabolites required for plant growth such as nitric oxide, brassinosteroids and polyamines, may be involved in the stimulatory effect of the lower Cd concentration used in similar manner to a hormetic model. Brassinosteroids are a group of plant steroidal hormones, which regulate processes as diverse as cell elongation, xylem differentiation, and fruit ripening (Li and Jin 2007; Symons et al. 2008). These hormones act synergistically, or at least additively, with several other hormones such as auxin and the gibberellins, but at far lower concentrations. Polyamines, which are involved in the control of numerous cellular functions, including free radical scavenging and antioxidant activity, have been found to confer protection from abiotic stresses

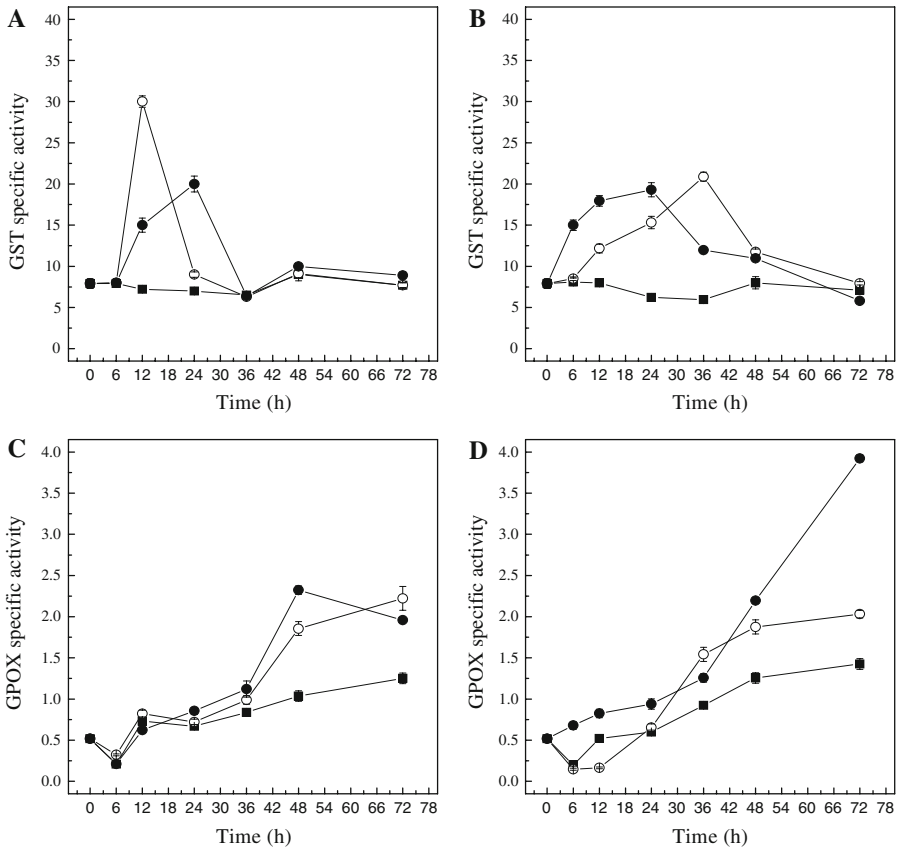

Fig. 4 Specific activity of GST (μmol min^{-1} mg^{-1} protein): Cd (**a**) and Ni (**b**) and GPOX (μmol mg^{-1} protein): Cd (**c**) and Ni (**d**) in tobacco cells grown for a 72 h period in two concentrations of $CdCl_2$ and $NiCl_2$. Control (zero $CdCl_2$ and $NiCl_2$) (■), 0.1 mM $CdCl_2$ and 0.075 mM $NiCl_2$ (○), 0.2 mM $CdCl_2$ and 0.75 mM $NiCl_2$ (●). Values are the means of 3 replicates ±SEM

but their mode of action is not fully understood (Groppa and Benavides 2008). Polyamines have also been shown to play a role in cell culture induction, growth and development (Chiancone et al. 2006; Santa-Catarina et al. 2007; Steiner et al. 2007). In sunflower, polyamines have been shown to increase dramatically in response to high Cd concentrations (1 mM) (Groppa et al. 2007), which may be a mechanism of cell defense to the oxidative stress induced by Cd. However, in rice leaves, spermidine and spermine, but not putrescine, were shown to increase in response to Cd, indicating that the former may be able to protect against Cd-induced oxidative damage and that this protection is most likely related to the avoidance of H_2O_2 generation and the reduction of Cd uptake (Hsu and Kao 2007). When in vitro cell cultures are concerned, red spruce cell culture in the presence of Cd exhibited a trend for decrease in spermidine level (Thangavel et al. 2007).

Under regular growth conditions, the production of ROS in cells is a normal occurrence, however, adverse environmental factors can disrupt cellular homeostasis and enhance the levels of ROS (Gratão et al. 2005; Moldes et al. 2008). In tobacco cells, Cd and Ni concentrations induced oxidative stress, but almost exclusively at the highest concentrations tested, which is consistent with the effects on growth and cell viability. The fast increase in TBARS (Fig. 1c and f) was likely correlated with an increase in electrolyte leakage, contributing to a process of oxidative damage leading to cell growth inhibition and reduction of cell viability as observed in this study. Malondialdehyde (MDA) is one of several low molecular weight products formed via the decomposition of primary and secondary lipid peroxidation products, being the most frequently used indicator of lipid peroxidation, a consequence of oxidative damage (Dewir et al. 2006). Cd and Ni can induce increased levels of H_2O_2, which appear to increase MDA levels (Gomes-Junior et al. 2006a, b; Mishra et al. 2006; Skorzynska-Polit and Krupa 2006; Ben Ammar et al. 2007; Hsu and Kao 2007).

Cellular enzymatic and non-enzymatic mechanisms are important for scavenging and quenching

of reactive oxygen. We have tested several antioxidant enzymes in response to Cd and Ni treatments and, different from growth, cell viability and lipid peroxidation, all of them responded positively to both concentrations of metals. This probably indicates that at the lower concentrations of metal, the antioxidant system was capable of avoiding oxidative stress. In general, the antioxidant response was similar in both Cd- and Ni-treated cells. Furthermore, the tobacco cells were grown in the dark and the response to ROS may have been more prominent in the mitochondria. Although these cells contain numerous plastids, antioxidant activity would be expected to be low, since no photochemical reactions would occur.

CAT represents the major constituent of the peroxisomal matrix in photosynthetic tissues (Reumann and Weber 2006), but its presence in plant mitochondria is still an open question (Noctor et al. 2007). In this study, CAT activity in TBY-2 cells submitted to Cd and Ni stresses varied during the time course of the experiment (Fig. 3a and b). Furthermore, the response of CAT to Cd was similar to that observed for Ni treatment, but differed at the end of the experiment (increase in Cd treatment, drop in Ni treatment). CAT may be regulated by H_2O_2 levels and the activity increases observed under stress suggest that during the period analyzed significant alterations in the generation of H_2O_2 may have occurred, as discussed previously, and that other antioxidant systems were involved with the stress response, particularly other peroxidases. Once again, although we have not measured H_2O_2 or other ROS in this study, increases are very likely to have happened based on the responses of the antioxidant enzymes we measured and on the previous work by Olmos et al. (2003). Further confirmation on the production of ROS, particularly superoxide, and the response to the oxidative burst induced by Cd may be obtained by the analysis of other key enzymes such as SOD. Therefore, the increase in CAT during the final period of exposure to Cd could be related to intensification of H_2O_2 levels. During this later period, GR and GST, both related to the glutathione-ascorbate cycle, do not appear to be the main players in the defense response to the oxidative stress condition, exhibiting a drastic reduction in activity.

The ascorbate–glutathione cycle, also referred as the 'Asada-Foyer-Halliwell' pathway, plays a major role in the detoxification of reactive oxygen species (ROS) involving successive oxidation and reductions of ascorbate, glutathione and NADPH by the enzymes APX, GR, dehydroascorbate reductase (DHAR, EC 1.8.5.1), and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) (Gratão et al. 2005; Moller et al. 2007). Increases in GR activity may help maintain glutathione in the reduced form prior to incorporation into phytochelatins (PCs), and/or operation of the ascorbate–glutathione cycle in order to detoxify the ROS induced by the metals. In these experiments with TBY-2 cells, GR activity was very rapidly and significantly increased by metal stress (Fig. 3c and d). Some reports have suggested that the response of GR to Ni stress is related to the maintenance of glutathione in the reduced form, prior to the formation of a stable Ni-glutathione complex (Rao and Sresty 2000). Ni phytotoxicity in *P. sylvestris* was related to glutathione reduction and increase in the proportion of GSSG in needles (Kukkola et al. 2000), confirming the importance of glutathione and GR for Ni tolerance in plants (Gomes-Junior et al. 2006a).

The detoxification activity of GST has been shown to be related to pathogen attack, oxidative stress, xenobiotics and heavy metals (Basantani and Srivastava 2007). The results for GST may be indicating that there was a balance in glutathione utilization by antioxidants enzymes of ascorbate–glutathione cycle. It appears that both metals caused a rapid induction of both GR and GST (as compared to CAT and GPOX) indicating that GST was probably operating in the detoxification process using GSH as a substrate.

Peroxidases are a large family of important plant enzymes involved in several reactions such as ascorbate oxidation, indoleacetic acid oxidation, lignification, phenol oxidation, pathogen defence, cell wall elongation, among others (Urs et al. 2006; Passardi et al. 2007). One of these peroxidases, GPOX, utilises aromatic electron donors such as guaiacol and pyrogallol as substrates but only oxidizes ascorbate at a rate of approximately 1% that of guaiacol (Asada 1999). Increases in GPOX activity in the TBY-2 cells were detected in response to Cd and Ni, particularly at the end of the exposure period and the high Ni concentration (Fig. 4c and d), indicating a possible role in the dismutation of excess H_2O_2 produced by Cd and Ni-induced stress.

The enzymes analyzed in this work and others including peroxidases and SOD, have been examined

previously in a wide range of plant species subjected to growth in the presence of Cd and Ni. However, these investigations have concentrated mainly on the leaves and roots of plants (seedlings, young plants and adult plants) and considerable disparities in the responses have been recorded. These variations have ranged from increase, through no change, to decrease, which are probably due to variations in the plant species, tissue or organ, metal, metal concentration and length of exposure (Gajewska et al. 2006a; Garcia et al. 2006; Ghanati and Ishka 2006; Mishra et al. 2006; Rodriguez-Serrano et al. 2006; Scebba et al. 2006; Gajewska and Sklodowska 2007b; Yannarelli et al. 2007). According to results presented, the toxic action of Cd and Ni inhibited the growth and viability of TBY-2 cells and increased lipid peroxidation, leading to major increases in the four antioxidant enzymes tested. The antioxidant enzyme responses varied depending on the metal concentration and length of exposure. GR and GST activities have an early important role in the response to the oxidative stress induced by these metals in tobacco cell cultures, whereas peroxidases such as CAT and GPOX take over later in the defence process.

However, there is a large number of antioxidant enzymes and non-enzymatic antioxidants that may also be involved in the response to Cd and Ni-induced oxidative stress and should be further investigated to have a more comprehensive understanding of the mechanisms involved.

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