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Plant Physiology and Biochemistry 42 (2004) 149–156
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Original article

Cytological changes and alterations in polyamine contents induced by cadmium in tobacco BY-2 cells

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Received 20 August 2003; accepted 7 November 2003

Abstract

Changes in cell viability, proliferation, cell and nuclear morphology including nuclear and DNA fragmentation induced by 0.05 and 1 mM $CdSO_4$ (Cd^{2+}) in tobacco BY-2 cell line (*Nicotiana tabacum* L.) were studied in the course of 7 days. Simultaneously changes in endogenous contents of both free and conjugated forms of polyamines (PAs) were investigated for 3 days. The application of 0.05 mM Cd^{2+} evoked decline of cell viability to approximately 60% during the first 24 h of treatment. Later on degradation of cytoplasmic strands, formation of the stress granules and vesicles, modifications in size and shape of the nuclei, including their fragmentation, were observed in the surviving cells. Their proliferation was blocked and cells elongated. Beginning the first day of treatment TUNEL-positive nuclei were detected in cells cultivated in medium containing 0.05 mM Cd^{2+}. Treatment with highly toxic 1 mM Cd^{2+} induced fast decrease of cell viability (no viable cells remained after 6-h treatment) and cell death occurred before DNA cleavage might be initiated. The exposure of tobacco BY-2 cells to 0.05 mM Cd^{2+} resulted in a marked accumulation of total PAs (represented by the sum of free PAs and their perchloric acid (PCA)-soluble and PCA-insoluble conjugates) during 3-day treatment. The increase in total PA contents was primarily caused by the increase in putrescine (Put) concentration. The accumulation of free spermidine (Spd) and spermine (Spm) at 12 and 24 h in 0.05 mM Cd^{2+} treated BY-2 cells and high contents of Spd and especially Spm determined in dead cells after 1 mM Cd^{2+} application was observed. The participation of PA conjugation with hydroxycinnamic acids and PA oxidative deamination in maintaining of free PA levels in BY-2 cells under Cd^{2+}-induced oxidative stress is discussed.

Keywords: BY-2 cells; Cadmium; DAO; PAL; Polyamines; Tobacco; TUNEL

1. Introduction

Cadmium is one of the most toxic metals to both animals and plants. Its effects are not only physiological, but also genotoxic, inducing the broad spectrum of mutations (point mutation, chromosomal aberrations) which could even lead to immediate or stepwise cell death [8,35]. The exposure of plants to Cd^{2+}, even at low concentration, induces an increased production of reactive oxygen species, which cause unspecific oxidation of proteins and membrane lipids and results in heavy metal (HM) stress, affecting many metabolic pathways and the key physiological processes (for review see [12,28]).

The pleiotropic effects of HM stress on the intact plant makes difficult both (a) the investigation of their primary interaction with plant cells and (b) the study of the mechanisms which plants use to eliminate/decrease actual HM toxicity. As the simplified experimental model plant cell cultures of various origin have been used. Among others Domažlická and Opatrný [9] characterised the effect of Cd^{2+} on the growth parameters, viability and morphology of tobacco cell line VBI-0. The authors proved the possibility to select in vitro the sublines expressing increased tolerance to relatively high Cd^{2+} doses. The exposure of tobacco BY-2

Abbreviations: DAO, diamine oxidase; FDA, fluorescein diacetate; HM, heavy metal; PAL, phenylalanine ammonia-lyase; PAs, polyamines; PCA, perchloric acid; PCD, programmed cell death; Put, putrescine; Spd, spermidine; Spm, spermine; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-uridinetriphosphate-nick labelling.

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doi:10.1016/j.plaphy.2003.11.003

cells to Cd^{2+} induced some symptoms characteristic for programmed cell death (PCD), i.e. fragmentation of nuclei, DNA oligonucleosomal fragmentation and stepwise death of cells [11]. The results of Piqueras et al. [26] and Olmos et al. [23] indicated that the oxidative burst and rapid generation of H_2O_2 represent the early steps of tobacco BY-2 cell response to Cd^{2+} application. However, studies of both metabolic and cytological changes induced by Cd^{2+} treatment are, to our knowledge, still rare.

Plant cells can be protected against the oxidative damage by a broad spectrum of radical-scavenger systems including antioxidant enzymes (ascorbate peroxidase, glutathion reductase and superoxide dismutase; [34]) and a number of biologically active substances as glutathion, carotenoids, ascorbate or phytochelatins that may prevent the free radical-induced cellular damage [10]. During the last few years polyamines (PAs) have been reported as efficient antioxidants in many experimental systems and various kind of environmental stresses [18,19]. However, the PA role in the protection systems still remains unclear. The cationic nature of free PAs (especially putrescine (Put), spermidine (Spd) and spermine (Spm)) at physiological pH allows them to interact with negatively charged molecules such as nucleic acids, phospholipids and proteins and to protect them from metal-induced oxidative damage [27]. PAs have been also suggested to function as metal chelators [18] and as direct or indirect free radical scavengers [13].

In plant cells, PAs do not only occur as free molecular bases but can also be covalently linked to phenolic acids, mostly hydroxycinnamic acids (soluble conjugated PAs), as well as to high molecular-mass substances like hemicelluloses and lignins and in small amounts also to proteins (insoluble conjugated PAs). In recent years, attention has been focused on possible roles of conjugated forms of PAs in plants exposed to unfavourable environmental conditions [6].

Since PAs have a lot of important physiological functions their endogenous levels have to be tightly regulated. Apart from PA biosynthesis, the intracellular concentrations of free PAs could be maintained by conjugation with hydroxycinnamic acids and by PA degradation through oxidative deamination [1].

The present work is focused on the role of PAs in Cd^{2+}-induced stress response in tobacco cell line BY-2. Simultaneously with the analysis of their endogenous levels, the changes in cell viability, cell and nuclear morphology and growth parameters were investigated. Possible relationships between the dynamics of these metabolic and structural changes is discussed.

2. Results

2.1. Cytological changes

The used $CdSO_4$ concentrations in the culture medium induced either very fast or stepwise death of the cell population. Cd^{2+} cytotoxic effect was manifested in three different but causally connected cytological parameters: the decrease of cell viability, the inhibition of cell division and, after longer treatment the inhibition of the accumulation of total biomass. The extent of these changes mostly correlated with the dose of the treatment.

2.1.1. Cell viability

The viability of cells cultured in the control medium was sustainable high over the whole culture period (about 98%, Fig. 1A). Treatment with Cd^{2+} in 1 mM concentration caused total and rapid cell death already after 6 h while application of 0.05 mM Cd^{2+} induced a marked decline of cell viability during the first 24 h of the cultivation (Fig. 1A). Following that, the viability did not change significantly until day 3 and then gradually decreased till day 5 when almost all cells were dead. The initial cell density of the control cultures increased during 3-day subculture interval ca. five times, up to 2.5×10^6 cells ml^{-1}. Treatment with Cd^{2+} at 0.05 mM appeared to be inhibiting cell division and inducing stepwise either "mitotic" or total cell death in the more sensitive cell subpopulation. As shown in Fig. 1A, inserted picture, the initial fresh

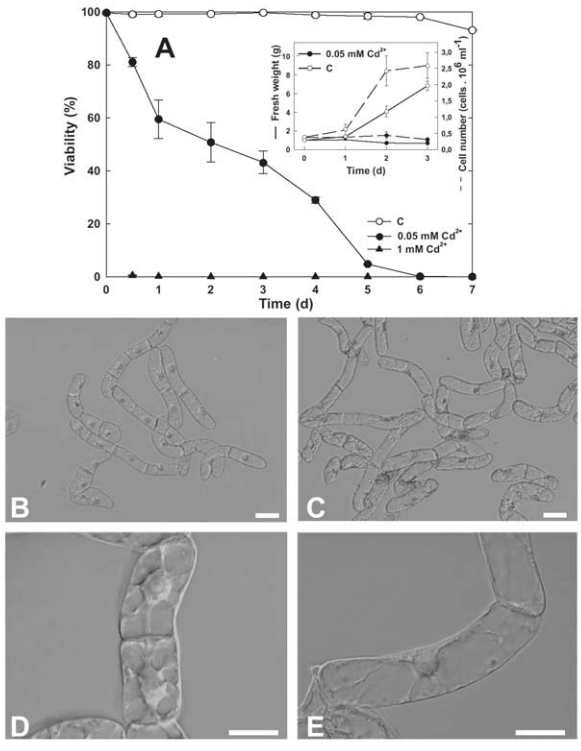

Fig. 1. Cell viability and proliferation changes in tobacco BY-2 cell line induced by treatment with 0 (control), 0.05 and 1 mM Cd^{2+} during 7-day cultivation interval. (A) Viability of the cell populations. Each value represents the mean of three independent experiments ± S.E. ((A) inserted) Changes in fresh weight and cell number in tobacco BY-2 cells during 3-day culture. (B, D) The phenotype of control cells after 6-h cultivation. (C, E) Elongated cells cultured in 0.05 mM Cd^{2+} after 6-h treatment. Nomarski DIC, Bars, 25 μm.

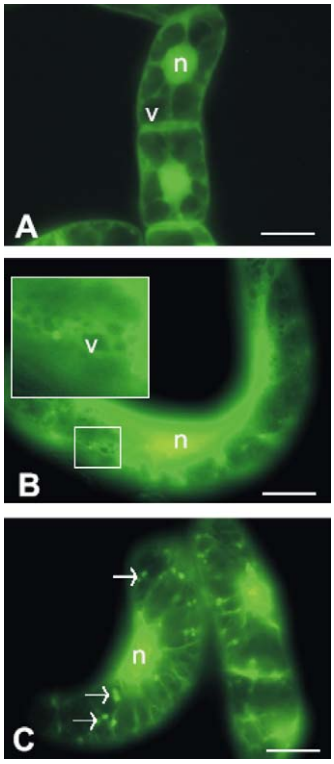

Fig. 2. Internal architecture of tobacco BY-2 cells. (A) Exponential 3-day old control cells. FDA is located in the peripheral protoplasmic layer, in central nuclei with perinuclear zones and in numerous transvacuolar strands. (B, C) Cells after 3-day exposure to 0.05 mM Cd^{2+}; (B) numerous small vesicles/vacuoles in perinuclear cytoplasmic zone, big nucleus with perinuclear zone is located in the cell periphery; (C) large fluorescein-labelled bodies/granules (arrows) near cytoplasmic strands. Nuclei with perinuclear zones located in the cell periphery. *n*, nucleus with perinuclear zone; *v*, vacuoles. FDA, Bars, 50 μm.

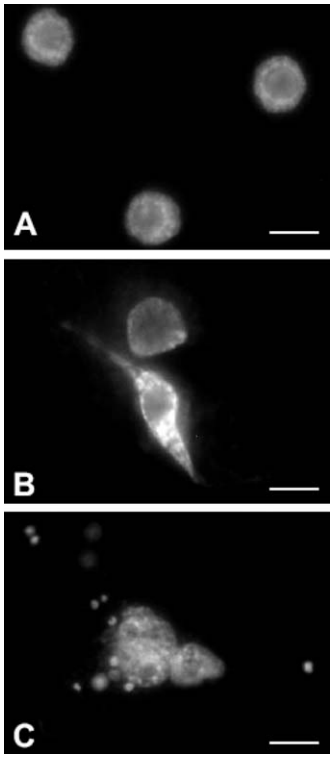

Fig. 3. Changes in nuclear morphology of BY-2 cells treated with 0 (control) and 0.05 mM Cd^{2+}. (A) Typical round shape of nuclei of control cells after 3-day cultivation. (B) Extremely elongated nuclei of cells after 1-day exposure to 0.05 mM Cd^{2+}. (C) Fragmentation of interphasic nuclei into several micronuclei after 3-day exposure to 0.05 mM Cd^{2+}. Hoechst 33258, Bars 25 μm.

mass of the control BY-2 biomass was increased ca. four times, while that of Cd^{2+}-treated variant exhibited almost no increase.

Cells treated with Cd^{2+} highly phytotoxic concentration (1 mM) represented the "negative" control in the studies of deterioration of the cell structure and changes in polyamine metabolism evoked by toxic impact of 0.05 mM Cd^{2+}.

2.1.2. Cytological and morphological alterations

Remarkable phenotypical deviations induced by Cd^{2+} sublethal concentration were observed. The mitotic death of some cells resulted in their abnormal longitudinal growth (Fig. 1B,D and C,E control and 0.05 mM Cd^{2+}-treated cells, respectively) and was frequently accompanied with changes in their internal architecture. The system of numerous cytoplasmic strands which was typical for the exponentially growing culture (Fig. 2A) was modified. In the part of cell population (up to 70–80% in 3 days) the incidence of either numerous small vesicles/vacuoles in the perinuclear cytoplasmic zone (Fig. 2B) or large granules closed to cytoplasmic strands was observed (Fig. 2C).

2.1.3. Nuclear and DNA fragmentation

The large rounded nuclei containing uniformly stained chromatin, typical for control cells (Fig. 3A), had the tendency to elongate under 0.05 mM Cd^{2+} treatment and after 3 days almost 30% of nuclei were considerably elongated (Fig. 3B). The granular structure of chromatin occurred in all 0.05 mM Cd^{2+}-treated cells (Fig. 3B). The fragmentation of nuclei and the formation of micronuclei which was observed in about 15% of treated cells on day 3 (Fig. 3C) increased to ca. 30% on day 5. Both rapid and very drastic malformations of the nuclei were induced by very short (1 h) treatment with 1 mM Cd^{2+}. Abnormal filamentous nuclei were observed in 70% of cells after 1 h treatment (data not shown).

The internucleosomal fragmentation of DNA which produces 3′OH ends was indicated by means of in situ TUNEL assay (Fig. 4A–J). Comparing with the control cells, which showed maximally 1% of cells with TUNEL-positive nuclei during the whole period of cultivation (Fig. 4A–C,J), cell populations treated with 0.05 mM Cd^{2+} exhibited up to 10% of the TUNEL-positive nuclei after 24 h (Fig. 4D–F,J). The percentage of these cells in cell population increased gradually to about 45% after 7-day treatment. The cells treated with Cd^{2+} in 1 mM concentration probably died too rapidly to pass through the process of PCD. They showed the original level (1–2%) of TUNEL-positive nuclei during the whole studied period (Fig. 4G–J).

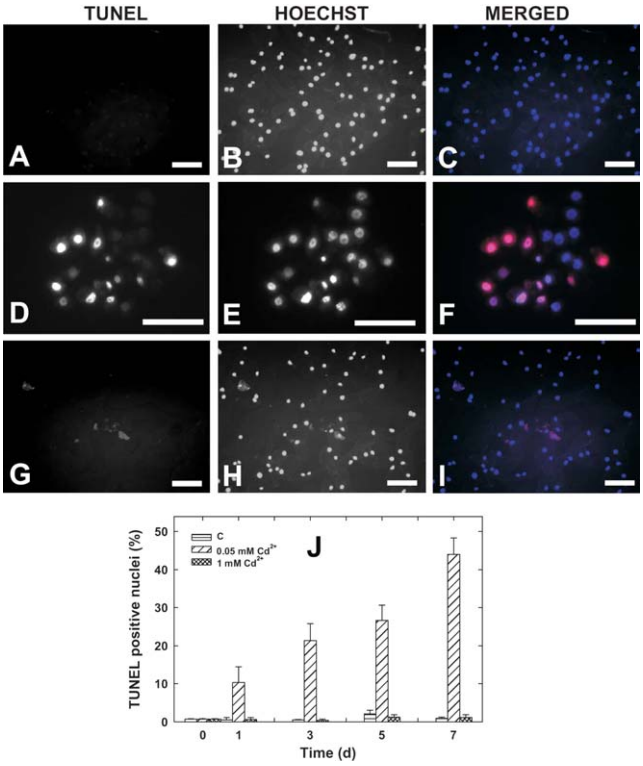

Fig. 4. TUNEL reaction of tobacco BY-2 cell line treated with 0 (control), 0.05 and 1 mM Cd^{2+}. (A–C) Untreated control cells after 24 h of cultivation. (D–F) Cells treated with 0.05 mM Cd^{2+} for 24 h. (G–I) Cells treated with 1 mM Cd^{2+} for 24 h. (A, D, G) TUNEL-positive nuclei; (B, E, H) Hoechst 33258; (C, F, I) merged pictures. Bars, 50 μm. J, the incidence of TUNEL-positive nuclei in BY-2 cells treated with 0 (control), 0.05 and 1 mM Cd^{2+} during 7-day culture. Each value represents the mean of three independent experiments ± S.E.

2.2. Biochemical changes

2.2.1. Free and conjugated polyamine contents

The contents of Put, Spd and Spm in control and Cd^{2+}-treated BY-2 cells were measured over the period of 3 days. The comparison of the total contents of PAs (represented by the sum of free PAs and their PCA-soluble and PCA-insoluble conjugates) in the cell population cultured for 12, 24 and 72 h is presented in Fig. 5. In the control Cd^{2+}-untreated variant the total content of PAs doubled during the 3-day culture and this increase correlated with the rate of cell division and fresh mass increases of control culture (Fig. 1A,

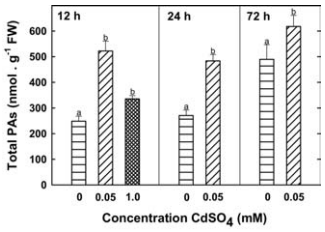

Fig. 5. Total contents of polyamines (represented by the sum of their values of free Put, Spd and Spm and their PCA-soluble and PCA-insoluble conjugates) in tobacco BY-2 cell line treated with 0 (control), 0.05 and 1 mM Cd^{2+} during 3-day culture. Bars represent S.E. of four replicates. Different letters above the bars indicate significant differences from the control ($P < 0.05$).

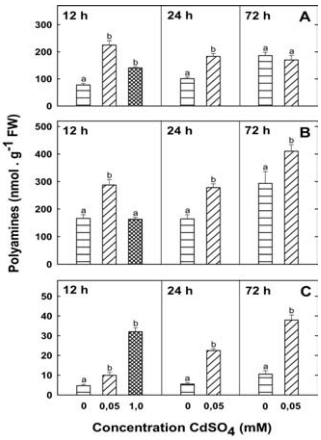

Fig. 6. Contents of polyamines (sum of the values of Put, Spd and Spm) in tobacco BY-2 cell line treated with 0 (control), 0.05 and 1 mM Cd^{2+} during 3-day culture. (A) Free forms. (B) PCA-soluble conjugates. (C) PCA-insoluble conjugates. Bars represent S.E. of four replicates. Different letters above the bars indicate significant differences from the control ($P < 0.05$).

inserted). The application of Cd^{2+} at 0.05 mM resulted in significant increase in the content of PAs 12 h after the transfer of cells to this semilethal Cd^{2+} medium and coincided with the first wave of the decrease in the cell viability. Further, not so pronounced increase in the total PAs content was observed till the end of 3-day culture period.

Owing to the growth of culture the levels of free Put, Spd and Spm and their PCA-soluble and PCA-insoluble conjugates continually increased in control cells after the subculture (Fig. 6A–C, Tables 1–3). Significant increase in the total amounts of free and conjugated forms of PAs on 12 and 24 h related to the control values was observed in 0.05 mM Cd^{2+}-treated cells (Fig. 6A–C). There was no difference between the total contents of free PAs in control and 0.05 mM Cd^{2+}-treated cells on day 3, while the level of soluble conjugates further increased on day 3 in treated culture (Fig. 6A,B). The content of PCA-insoluble conjugates in dead cells (1 mM Cd^{2+}) was, probably due to the cell decompartmentation, many times higher than the content in control and 0.05 mM Cd^{2+}-treated cells. The decline in cell viability on day 3 in 0.05 mM Cd^{2+}-treated cells coincided with the increase in the level of PCA-insoluble conjugates (Fig. 6C).

Table 1
Contents of free putrescine (Put), spermidine (Spd) and spermine (Spm) in tobacco BY-2 cell line treated with 0 (control), 0.05 and 1 mM Cd^{2+} during 3-day culture

Treatment		Polyamines (nmol g^{-1} FW)		
(h)	Cd^{2+} (mM)	Put	Spd	Spm
12	0.00	67.9 ± 7.2	8.0 ± 0.1	1.2 ± 0.0
	0.05	197.5 ± 20.8 *	32.1 ± 3.9 *	4.0 ± 0.5 *
	1.00	81.1 ± 8.0	43.6 ± 3.6 *	15.5 ± 1.6 *
24	0.00	77.4 ± 6.6	19.3 ± 2.4	4.1 ± 0.1
	0.05	142.3 ± 17.9 *	34.5 ± 4.0 *	5.9 ± 0.9 *
72	0.00	136.6 ± 11.4	40.4 ± 4.0	9.1 ± 1.0
	0.05	148.3 ± 18.2	27.3 ± 4.6 *	4.2 ± 0.6 *

Mean values of three experiments with 2–4 parallel analyses ± S.E. Asterisk (*) indicates the results obtained from Cd^{2+}-treated BY-2 cells that significantly differ from the corresponding control values at $P < 0.05$.

Table 2
Contents of PCA-soluble conjugates of putrescine (Put), spermidine (Spd) and spermine (Spm) in tobacco BY-2 cell line treated with 0 (control), 0.05 and 1 mM Cd^{2+} during 3-day culture

Treatment		Polyamines (nmol g^{-1} FW)		
(h)	Cd^{2+} (mM)	Put	Spd	Spm
12	0.00	121.8 ± 9.8	20.9 ± 2.4	23.9 ± 1.9
	0.05	252.2 ± 28.5 *	20.5 ± 2.4	14.9 ± 1.9 *
	1.00	138.8 ± 14.1	24.6 ± 2.3	2.1 ± 0.2 *
24	0.00	123.3 ± 9.9	20.0 ± 2.9	20.9 ± 2.2
	0.05	234.0 ± 27.9 *	32.0 ± 4.2	12.3 ± 1.8 *
72	0.00	208.0 ± 18.6	41.4 ± 4.1	43.7 ± 4.3
	0.05	348.4 ± 36.2 *	26.0 ± 2.4 *	35.8 ± 4.9

Mean values of three experiments with 2–4 parallel analyses ± S.E. Asterisk (*) indicates the results obtained from Cd^{2+}-treated BY-2 cells that significantly differ from the corresponding control values at $P < 0.05$.

Table 3
Contents of PCA-insoluble conjugates of putrescine (Put) and spermidine (Spd) in tobacco BY-2 cell line treated with 0 (control), 0.05 and 1 mM Cd^{2+} during 3-day culture

Treatment		Polyamines (nmol g^{-1} FW)		
(h)	Cd^{2+} (mM)	Put	Spd	Spm
12	0.00	4.5 ± 0.0	0.2 ± 0.0	–
	0.05	9.5 ± 1.2 *	0.5 ± 0.1 *	–
	1.00	27.4 ± 3.0 *	4.6 ± 0.1 *	–
24	0.00	5.2 ± 0.1	0.3 ± 0.0	–
	0.05	22.0 ± 2.4 *	0.5 ± 0.1 *	–
72	0.00	9.9 ± 1.0	0.7 ± 0.1	–
	0.05	37.0 ± 3.6 *	0.9 ± 0.1	–

Concentration Spm was below the detection limit by using the described method. Mean values of three experiments with 2–4 parallel analyses ± S.E. Asterisk (*) indicates the results obtained from Cd^{2+}-treated BY-2 cells that significantly differ from the corresponding control values at $P < 0.05$.

All three PAs, i.e. Put, Spd and Spm participated on the enhancement of PAs observed in untreated cells on day 3 (exponential phase of growth, Fig. 1A, inserted) (Tables 1–3), whereas in 0.05 mM Cd^{2+}-treated cells mainly Put, both in free and conjugated forms, was responsible for the rise of the total PA concentration. The elevated level of free Spd in cells after 12 h exposure to Cd^{2+} (0.05 mM) was significantly higher than the level of its PCA-soluble conjugates. Both free Spd and Spm contents in control cells were much lower than that of corresponding soluble conjugates (Tables 1–3).

2.2.2. Diamine oxidase and phenylalanine ammonia-lyase activities

The subculture of BY-2 cells into the control medium caused the rise of diamine oxidase (DAO) activity with a maximum between 12 and 24 h and its decline on day 3. The increase in DAO activity in cells subcultured into the medium containing 0.05 mM Cd^{2+} was much more pronounced at 12 and 24 h of culture as compared to the control cells. Nevertheless, there was no significant difference between the DAO activity of treated and untreated cells on day 3 (Fig. 7).

The increase in phenylalanine ammonia-lyase (PAL) activity with a maximum 12 h after inoculation was found in control BY-2 cells. Following that, the activity decreased to the basal level on day 3. The PAL activity showed an earlier

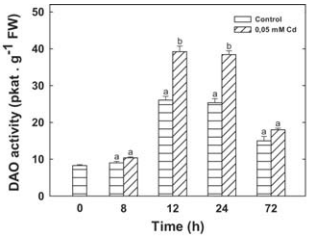

Fig. 7. Activity of diamine oxidase (DAO) in tobacco BY-2 cell line treated with 0 (control) and 0.05 mM Cd^{2+} during 3-day culture. Bars represent S.E. of four replicates. Different letters above the bars indicate significant differences from the control ($P < 0.05$).

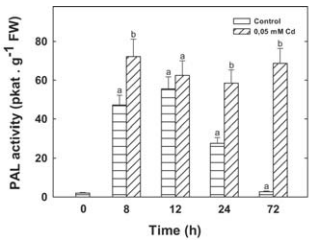

Fig. 8. Activity of phenylalanine ammonia-lyase (PAL) in tobacco BY-2 cell line treated with 0 (control) and 0.05 mM Cd^{2+} during 3-day culture. Bars represent S.E. of four replicates. Different letters above the bars indicate significant differences from the control ($P < 0.05$).

rise in 0.05 mM Cd^{2+}-treated cells in comparison with the control cells. The treatment significantly stimulated PAL activity during the whole culture period which was 25 times higher on day 3 than in the control cells (Fig. 8).

3. Discussion

Tobacco BY-2 cell line cultured in liquid medium responded to the application of $CdSO_4$ by the growth inhibition either accompanied or followed by the cell death. The effect was dose-dependent both in time and Cd^{2+} concentration applied. Stepwise decrease of cell viability coincided with characteristic changes in the cell morphology. The results obtained in this study are consistent with some previous observations. Similar dose-dependent response was described in tobacco cell line VBI-0 [9] and in tobacco cell line BY-2 [11,23]. However, it is not clear, whether the surviving cells differed a priori from the more susceptible part of the cell population or if some initiated adaptive mechanisms were more capable under these conditions.

As mentioned in the results, the treatment with 1 mM Cd^{2+} killed most of the cells in hours and rapidly destroyed their internal architecture. The stepwise degradation or even loss of the network of cytoplasmic strands in cells exposed to Cd^{2+} 0.05 mM was accompanied by alterations in vacuolar system and malformations of the nuclear shape and size.

In a part of treated cell population atypical, vacuole-like vesicles were formed in the homogenous, perinuclear cytoplasma (Fig. 2B). The nature of FDA-positively stained bodies/granules (Fig. 2C) have not yet been proved, however, both in size and shape they resembled “ER bodies”, detected

under stress conditions in the leaf epidermal cells of *Arabidopsis thaliana* [21].

Numerous observed malformations of the interphasic nuclei, i.e. the elongation and the fragmentation of nuclei (Fig. 3B,C) can be interpreted as the early symptoms of PCD of treated cells. PCD-related DNA nucleosomal fragments cannot be detected earlier than some days after the PCD process started ([11], Opatrný et al., unpublished results). The TUNEL technique enabled us to detect single strand DNA breaks 24 h after the Cd^{2+} application.

PAs play multiple essential functions both in plant and animal cells. On one hand, they facilitate cell division and growth and on the other hand they support overexpression of some apoptotic genes, facilitating cell death. The basis of these diverse cellular responses is currently not known. The effect of different PAs may differ, in particular Spm was referred as compound preventing ionomycine induced apoptosis in T-cells [7]. In our experiments, the increase in the contents of free and conjugated forms of PAs in control BY-2 cells during 3-day culture correlated with the onset of increase in the cell number and fresh mass (Tables 1–3, Fig. 1A, inserted). These results are consistent with the PA role in stimulation of cell division as demonstrated in numerous studies both in animal (reviewed in [33]) and plant [2,24] experimental systems. On the contrary, abnormally high levels of PAs, especially of Put that accumulated in Cd^{2+}-treated cells (Fig. 5, Tables 1–3), might be involved in the process of cell death. Changes in PA homeostasis have been reported in cell death of nerve cells and in various in vitro models of apoptosis in animal system [29]. The marked increase in the level of PAs 12 h after the transfer to semilethal Cd^{2+} medium coincided with the first wave of the decrease in the cell viability (Figs. 1A and 5). As both the original cell number and the biomass mass were unchanged, these results may document also massive relative increase of PAs per cell. High contents of free PAs were detected in Cd^{2+}-treated BY-2 cells even sooner than any other convincing cytological symptoms of the PCD, i.e. the fragmentation of nuclei and the incidence of TUNEL positive nuclei occurred.

The plasma membrane may be regarded as the first cell structure that is in contact with the heavy metal toxicity. In this connection, it is noteworthy that Spd and Spm may exert different physiological effects on cell membranes compared with Put. A key role in preventing the injury of thylakoid membranes of osmotically stressed oat leaves seemed to play Spd and Spm [5]. The accumulation of free Spd and Spm in BY-2 cells exposed for 12 and 24 h to 0.05 mM Cd^{2+} and high contents of Spd and especially Spm in dead cells after 1 mM Cd^{2+} application (Table 1) might correspond with PA function in stabilisation of membranes. However, cells exposed to lethal concentration of Cd^{2+} were either not capable or not fast enough to eliminate these highly phytotoxic conditions.

Another reason for the increase in the amount of free PAs in cells under the Cd^{2+} stress is their ability to act as free radical scavengers. This opinion was supported by experiments with paraquat, well-known oxidative stress inducer [3]. Application of 0.05 mM Cd^{2+} to tobacco BY-2 cells significantly increased the levels of free Put, Spd and Spm after 12 h treatment (by 295%, 400% and 310%, respectively, related to the controls, Fig. 6A, Table 1). These findings are in a good agreement with the results reported for 0.1 mM Cd^{2+} treated seedlings of *Vigna radiata* [14].

The large intracellular accumulation of free PAs would have harmful effects on the maintenance of cellular pH, ion homeostasis and on a number of physiological functions where PAs are implied. PA levels are therefore precisely controlled. The rise in the amount of free PAs in BY-2 cells treated with Cd^{2+} 0.05 mM for 12 and 24 h (related to the controls, Fig. 6A, Table 1) positively correlated with the stimulation of DAO activity (Fig. 7). The enzyme has been reported to be substrate inducible and elevated levels of PAs stimulated DAO activity in germinating pea seedlings [32]. However, the enzyme activity in Cd^{2+}-treated BY-2 cells declined on day 3 although the content of free Put did not change significantly (Fig. 7, Table 1). Similarly, Choudhary and Singh [14] have shown that PA accumulation in Cd^{2+}-treated mungbean seedlings (0.1–1.5 mM $CdCl_2$) did not correlate with the activity of DAO. These results are in line with finding that the enzyme activity is inhibited by its product H_2O_2 (reviewed in [17]).

In addition to the oxidative deamination the endogenous PA levels could be regulated in plants by conjugation with hydroxycinnamic acids. Significant increase in PCA-soluble conjugates in 0.05 mM Cd^{2+}-treated cells correlated with the stimulation of the activity of PAL, phenylpropanoid biosynthesis key enzyme (Figs. 6B and 8). The pronounced accumulation of soluble conjugates on day 3, when the activity of DAO declined, point to the important role of PA conjugation in the control of free PA levels in cells under oxidative stress. Different metabolic roles of PA conjugates in cells are proposed. Besides their importance in the regulation of free PAs, as the results presented in this paper also indicate, the conjugation reactions may regulate PA interactions with Ca^{2+}, which might have an implication for the proposed role of PAs on membrane stabilisation [20]. As shown in Fig. 6C, Cd^{2+}-treated cells contain also high amounts of PCA-insoluble conjugates, especially that of Put (Table 3). Decompartmentation which accompanies the cell death might lead to the marked increase in the amount of PAs bound to high molecular-mass substances like proteins and nucleic acids. Another possible explanation for the rise of insoluble conjugates in cells after Cd^{2+} application in 0.05 mM concentration might be the Put and Spd interactions with cell wall components and increase in cell wall rigidity under Cd^{2+} stress conditions [4].

In summary, we have shown that tobacco BY-2 cells responded to the Cd^{2+} treatment with cytological changes that may be considered as an initial onset of stress-induced PCD. Our results suggest that PAs might be implicated in the protection response of cells to Cd^{2+}-induced stress. However, it is difficult to judge, whether the increase in PA contents reflects the activation of stress-defending mechanisms which

should prevent the cells before PCD or if it represents the early markers of PCD itself. Our results confirm the importance of PA conjugation with hydroxycinnamic acids for the maintenance of PA homeostasis in BY-2 cells under oxidative stress.

4. Methods

4.1. Plant material and cadmium treatment

The tobacco BY-2 cell line (*N. tabacum* L. cv. Bright Yellow 2, [22]) was maintained as previously described [30]. The cells were regularly subcultured once a week and cultivated at 25 °C in darkness on an orbital shaker IKA 125B (IKA Labortechnik Staufen, Germany) at 150 rpm (orbital diameter 4 mm). For cadmium stress studies, the cells in the exponential phase of growth (3 days old cell culture) were inoculated into culture medium containing 0 (control), 0.05 and 1 mM $CdSO_4$ (Cd^{2+}). Control and Cd^{2+}-treated cells were harvested for cytological studies after 12, 24 h and then every second day of culture. Biochemical determinations were performed after 0, 8 (PAL and DAO activities), 12, 24 and 72 h. The samples were stored at –70 °C before analyses.

4.2. Determination of cell population density and cell viability

Cell densities were determined by counting cells in at least 10 aliquots of each culture sample using a Fuchs-Rosenthal haemocytometer chamber. For the cell viability determination fluorescein diacetate staining (FDA—Molecular Probes Inc., Eugene, USA), as indication of esterase activity of the cells was used: 40 μl from 0.2% w/v stock solution of FDA in acetone were added into 7 ml of culture medium and this staining solution was mixed 1:1 with BY-2 cells. At least 500 cells in each sample was counted immediately after FDA addition and their fluorescein-labelled endogenous architecture was observed in three repeated experiments.

4.3. Determination of nuclear structure

To visualise nuclear morphology Hoechst 33258 substance (Molecular Probes Inc., Eugene, USA) (10 mg ml^{-1} stock solution of Hoechst 33258 in dimethylsulphoxide (DMSO), dilution 1:10 000 to final concentration 0.1 μg ml^{-1}) was used. Two microlitre of stock solution was added to 1 ml of the suspension culture. At least 500 cells were counted in each of three replicate slides per sampling time per treatment.

4.4. TUNEL assay

A terminal deoxynucleotidyl transferase (Tdt)-mediated deoxy-uridinetriphosphate (dUTP)-nick labelling (TUNEL) method was used to detect free 3′OH termini in nuclear DNA. Using TMR-red (red fluorescence) in situ cell death detection kit (Roche Diagnostic GmbH, Manheim, Germany), the procedure was exactly performed according to the method described by Jones et al. [16]. At least 500 cells were counted in each of three replicate slides per sampling time per treatment. The TUNEL reaction has been reported to give variable results due to, for example, the fixation method. False positives can occur because of DNAse activity during different washing procedures in the assay, so the absolute values should be interpreted with care. However, in this study positive and negative controls were included. The cells treated with DNAse I, which induced strand breaks, were used as the positive control. Cells treated separately with Label Solution (without terminal transferase) instead of TUNEL reaction mixture were used as the negative control.

4.5. Microscopy and image analyses

Cells were examined with an epifluorescent microscope (Olympus Provis AX 70) equipped with Nomarski differential interference contrast (DIC). The cell images were collected and processed using image analysis LUCIA 4.51 (G, G/F) system (Laboratory Imaging, Prague, Czech Republic).

4.6. Polyamine analysis

The cells were ground in liquid nitrogen and extracted overnight at 4 °C with 5% (v/v) perchloric acid (PCA) (100 mg fresh mass tissue ml^{-1} 5% PCA). The extracts were centrifuged at 21 000 × *g* for 15 min, and then PCA-soluble free PAs were determined in one-half volume of the supernatant. The remaining supernatant and pellet were acid hydrolysed in 6 M HCl for 18 h at 110 °C to obtain PCA-soluble and PCA-insoluble conjugates of PAs as described by Slocum et al. [31]. Standards (Sigma-Aldrich, St Louis, MO, USA), PCA-soluble free PAs, and acid hydrolysed PA conjugates were benzoylated. HPLC analysis of benzoyl-amines was performed on Pye Unicam PU 4002-Video Liquid Chromatograph with a C_{18} Spherisorb 5 ODS2 column (particle size 5 μm, column length 250 × 4.6 mm) according to the method of Slocum et al. [31].

4.7. Diamine oxidase assay

Diamine oxidase (DAO, EC 1.4.3.6) activity was assayed by a spectrophotometric method based on detection of the aldehyde with *cis*-1,4-diamino-2-butene as the substrate [25]. Samples were homogenised in 0.1 M Tris–HCl buffer (pH 8.5) containing 2 mM mercaptoethanol and 1 mM EDTA, and centrifuged at 20 000 × *g* for 15 min at 4 °C. The reaction mixture contained 0.1 M Tris–HCl buffer (pH 8.5), catalase (25 μg) and 0.01 M *cis*-1,4-diamino-2-butene. The reaction was started by addition of 0.2 ml of supernatant, incubated for 1 h at 37 °C and stopped by adding 1 ml of Ehrlich's reagent. The mixture with stopped enzymatic reac-

tion was incubated at 50 °C for 5 min, and then chilled on an ice bath before reading the absorbance of produced pyrrol at 563 nm. Enzymatic activity is expressed in pkat g^{-1} FW of viable cells.

4.8. Phenylalanine ammonia-lyase assay

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was extracted and its specific activity determined by the modified method of Jangaard [15]. The amount of cinnamic acid produced in the assay mixture was determined by monitoring the absorbance at 275 nm. Enzymatic activity is expressed in pkat g^{-1} FW of viable cells.

Acknowledgements

We are grateful to Dr. M. Kamínek for critical reading of the manuscript. We thank N. Hatašová for excellent technical assistance. This work was supported by the grant of Ministry of Education LN00 A081.

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