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Calcium ameliorates effects of lead in protonema of *Funaria hygrometrica* Hedw.

M. KRZESŁOWSKA, A. WOŹNY and J. KONIECZNA-KOPERSKA

Institute of Experimental Biology, Adam Mickiewicz University, Collegium Biologicum, Umultowska 89, PL-61614 Poznań, Poland

Abstract

Two-days-old *in vitro* grown protonemata of *Funaria hygrometrica* Hedw. were treated with a mixture $PbCl_2$ (4 μM Pb^{2+}) and $CaCl_2$ (16 μM Ca^{2+}) (Ca+Pb) for 48 h. The results were compared with the control: distilled water (H_2O) and the solution of $PbCl_2$ (4 μM Pb^{2+}) (Pb). Protonemata treated with Ca+Pb were longer and contained more cells than those treated with Pb. Moreover, a lower number of cells showed apical cell deformations typical for lead toxicity: swollen tips and wall thickenings at the apex. If deformations were present they were not as extended as in Pb. In comparison with the control, however, protonemata treated with Ca+Pb were shorter, contained a lower number of cells and some apical cells in this material were altered. It can be concluded that the presence of calcium partially neutralised toxic effects of lead in *Funaria hygrometrica* protonemata cells.

Additional key words: heavy metals, cell wall, tip growth.

Introduction

The content of heavy metals in air, water and soils have been increasing both in urban and petiurban areas during recent years. Pb is one of the main contaminants included both in the European Black List II (EEC 1976) and the North American National Priority List (Rampley and Ogden 1998). Calcium is regarded as the element neutralising toxic effects of various stress factors including heavy metals (Gabara and Gołaszewska 1992, Abdelbasset *et al*. 1995, Mazen and El Maghraby 1997, Tikhaya and Fedorovskaya 2000, Kim *et al*. 2002). The studies carried out so far on the effects of lead in *Funaria hygrometrica* protonema showed that this metal caused first of all growth inhibition (Krupińska 1976, Kardash and Demkiv 1991, Basile *et al*. 1995). Moss protonemata similarly to, *e.g*. pollen tubes, root hairs and fern protonemata elongate thanks to a tip growing apical cell. The process is dependent on calcium (Brewbaker and Kwack 1963). Ca^{2+} is taken up by calcium channels (Pierson *et al*. 1996, Demidchik *et al*. 2002) localized at the tip of the apical cell (Pierson *et al*. 1996). Elevated $[Ca^{2+}]_i$ at the tip is predicted to promote exocytosis and hence cell elongation (Battey *et al*. 1999). Moreover, Ca^{2+} and Ca^{2+}-dependent enzymes regulate assembly and disassembly of microfilaments (for review Battey *et al*. 1999, Franklin-Tong 1999) - crucial structures that tip growth is dependent on (Bibikova *et al*. 1999, Baluška *et al*. 2000, 2003, Vidali *et al*. 2001, Baluška and Volkmann 2002, Čiamporová *et al*. 2003). Microfilaments function in vesicle transport (Taylor and Hepler 1997, Franklin-Tong 1999) and are the target of the Ca-dependent signalling cascade resulting in cell response, *e.g*. growth (Snowman *et al*. 2002).

The studies carried out so far showed that in the apical cell of *Funaria hygrometrica* protonemata (responsible for elongation) lead caused numerous alterations, *e.g.* lack of tip body (Basile *et al*. 1995) swollen tips (Basile *et al*. 1995, Krzesłowska and Woźny 2000) and wall thickenings formation (Krzesłowska and Woźny 2000), lack of microtubules (Basile *et al*. 1995) or a lower number of them and alteration of its array (Krzesłowska and Woźny 2002). Lead deposits were found in the cell wall, endomembrane system, chloroplasts, nucleus and nucleolus (Krzesłowska and Woźny 1996).

It was worthwhile to see if addition of calcium, which can neutralise toxic effects of heavy metals in plant cells, and simultaneously is the main factor which the tip growth is dependent on, to lead solution would also neutralise the toxic effects of the metal observed in moss protonemata.

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Fax: (+48) 61 8294515, e-mail: adaw@main.amu.edu.pl

Materials and methods

Protonemata of *Funaria hygrometrica* Hedw. were growing two days on the Kofler (1959) medium supplemented with microelements according to Heller (1953). For next 48 h two treatments were used: with $PbCl_2$ (Pb) only and with the mixture of $PbCl_2$ + $CaCl_2$ (Ca+Pb) at the final concentration 16 μM Ca^{2+} and 4 μM Pb^{2+}. Control (H_2O) was growing on distilled water. After 48 h the following measurements and observations were carried out: protonema length, the number of cells building it, the number of apical cells in the material with swollen tips and/or wall thickenings. Measurements of protonema length were done in Reichert light microscope using a micrometer ocular. Identification of the compounds building wall thickenings and the method of protonemata preparation for transmission electron microscopy (TEM) observations were identical as described earlier (Krzesłowska and Woźny 1996, 2000). The data are presented as the mean of 3 replicates, $n = 30$.

Results

Morphology of protonema and identification of the compounds building wall thickenings: In comparison with Pb protonema treated with Ca+Pb was longer in about 69 % (Table 1). The number of cells building it was higher in about 92 % (Table. 1). Apical cell in Pb (Fig. 1) showed typical morphological alterations caused by this metal (*e.g.* swollen tips, wall thickenings usually present at the apex and rounded chloroplasts - described in details previously - Krzesłowska and Woźny 2000, 2002). In the material treated with Ca+Pb (Fig. 2) these alterations were also observed, but appeared in a lower number of cells. Moreover they were not so extended. The number of cells with swollen tips was lower in about 33.8 % (Table 1) and swellings were not so wide (Fig. 2). Also lower, in about 20.5 %, was the number of apical cells with thickened wall at the apex (Table 1). In Ca+Pb thickened wall occupied rather small part of swollen tip – only one quarter or less (Fig. 2), while in Pb – about half or more (Fig. 1). Moreover, rarely appeared brown thickenings often observed in Pb (Fig. 1). In Ca+Pb most of them (82 %) was grey (Fig. 2). Cytochemical investigations showed that, similarly to Pb, wall thickenings in the protonemata treated with Ca+Pb were built mainly from pectic polysaccharides and also callose and cellulose. However, they did not contain any lipid substances as it was often found in Pb.

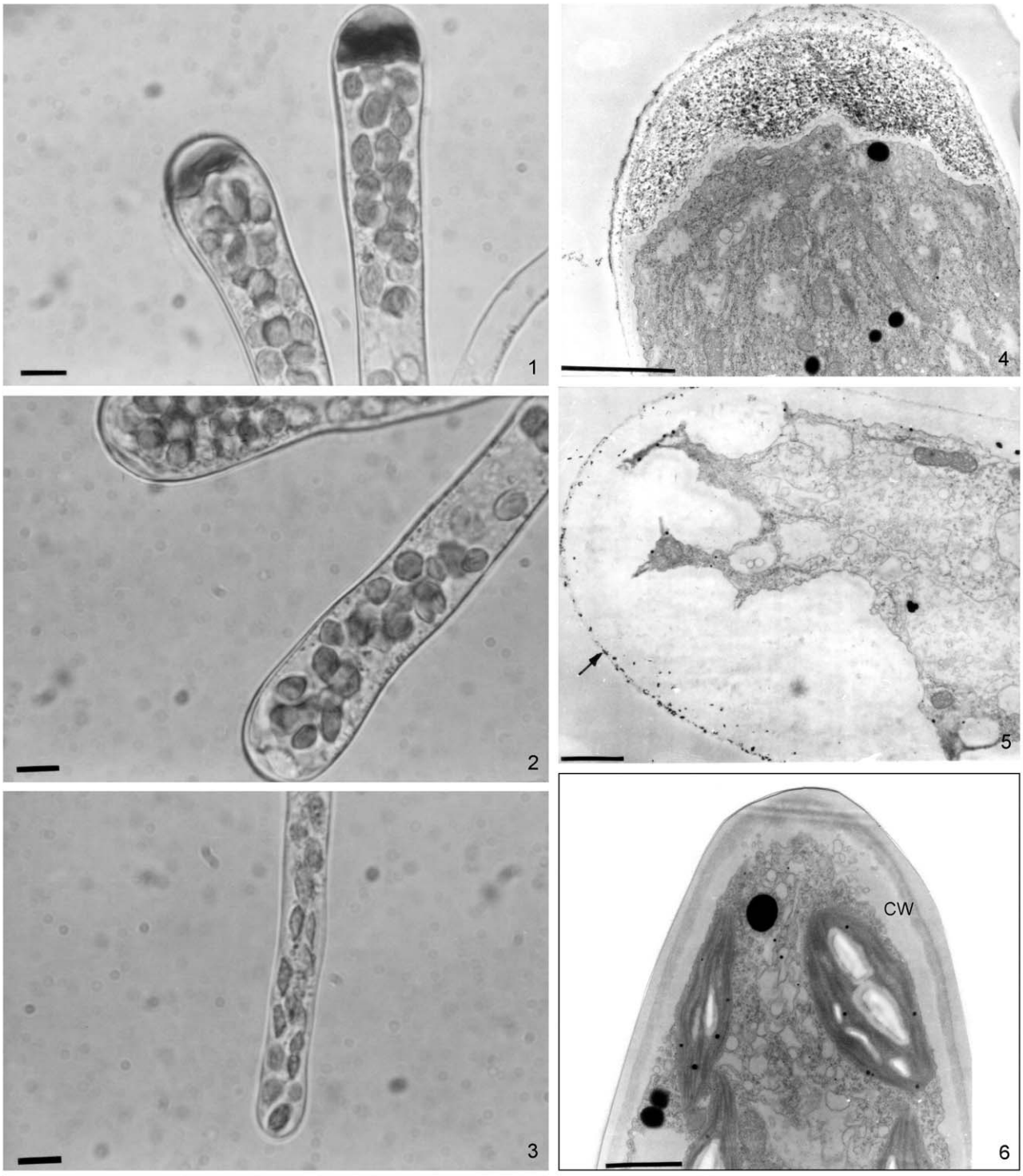
In comparison with the control (Fig. 3), protonemata treated with Ca+Pb were shorter in about 42 % (Table 1). The number of cells building protonema was lower in about 32 % (Table 1). Apical cells in this material showed more often swollen tips (in about 47 % - Table 1). In H_2O thickened wall was not present. Typical wall surrounding the cell was built from pectic polysaccharides and cellulose. The walls between cells of the protonema contained additionally callose, as it was also described earlier (Bopp *et al*. 1991, Krzesłowska and Woźny 2000).

Ultrastructure: Apical cell of material treated with lead (Fig. 4) showed typical ultrastructural alterations caused by this metal described earlier (Krzesłowska and Woźny 2000, 2002), *e.g.* wall thickenings, built from numerous layers, different in structure (often more or less granular). In protonema treated with Ca+Pb (Fig. 5) the alterations of cell ultrastructure were not so strong. Thickened wall observed in TEM showed homogenic structure on the whole area and lower electron density than typical wall surrounding the cell (Fig. 5). Opposite to Pb (Fig. 4) it contained neither numerous layers nor granular regions. Lead deposits were present, but only at the outer part of the cell wall (Fig. 5). Apical cell of control (Fig. 6) showed a typical ultrastructure as earlier described (Krzesłowska and Woźny 1996, 2000, 2002).

Generally, TEM studies supported the morphological observations. Protonema treated with Ca+Pb showed a considerable decrease of lead toxicity symptoms in comparison with Pb. It still showed, however, some alterations in comparison with protonema from the control.

Table 1. Morphological features of protonemata exposed to Ca and Pb (Ca+Pb), only to Pb (Pb), or distilled water (H_2O) for 48 h. Values are means ± SD ($n = 30$).

Features	Ca+Pb	Pb	H_2O
Protonemata length [μm]	458.40 ± 115	271.3 ± 87	793.9 ± 98
Number of cells building protonema	3.03 ± 0.8	1.6 ± 0.6	4.5 ± 0.8
Apical cells with swollen tips [%]	47.50	81.3	7.0
Apical cells with wall thickening [%]	34.70	55.2	0



Figs. 1 - 3. Morphology of apical cells of *Funaria hygrometrica* protonemata, *bar* = 10 μm. Fig. 1. Pb - brown thickenings of cell wall, swollen tips. Fig. 2. Ca+Pb - small grey thickenings of cell wall, little swollen tips. Fig. 3. H_2O - no thickenings of the wall, no swollen tips.
Figs. 4 - 6. Ultrastructure of the *Funaria hygrometrica* protonemata apical cells. Tip regions - longitudinal sections, *bar* = 1 μm. Fig. 4. Pb - thickened wall built from a few regions different in structure. Lead deposits (*arrow*) in thickened wall. Fig. 5. Ca+Pb - homogenic, low electron density thickened wall, lead deposits in the outer part of the thickening (*arrow*). Fig. 6. H_2O - unchanged cell wall surrounded the cell, cw - cell wall.

Discussion

Addition of calcium to lead solution resulted first of all in better growth of protonemata. They were longer and contained more cells. Some other symptoms of lead effects, *e.g*., swollen tips and thickened wall were visible, but they were present in a lower number of cells and were not so extended. It suggests that calcium protected *Funaria hygrometrica* protonemata from lead toxicity.

Protective effect of Ca^{2+} on Pb^{2+} toxicity may be the result of competition at the entry level. It has been shown lately that non essential elements, *e.g*., Pb, Cd use channels and transporters of the plasma membrane, normally functioning in uptake of other, essential for the cell ions, *e.g.*, Ca, Mg (for review Clemens 2001). For Pb^{2+} it is probably calmodulin-binding cyclic nucleotide-gated channel (Arazi *et al*. 1999). Ca^{2+} and Pb^{2+} are similar in ionic radius, oxidation state and electric charge. They may compete in entering the cells (Garland and Wilkins 1981). Thus presence of Ca^{2+} in the medium may limit Pb^{2+} uptake and transport causing in this way its lower concentration in protoplast (Kawasaki and Moritsugu 1987). It has been found lately that Pb^{2+} uptake was completely inhibited by presence of Ca^{2+} into *Chlorella vulgaris* cells (Slaveykova and Wilkinson 2002). Ca^{2+} blocked also Pb^{2+} transport into the *Oryza sativa* roots and Pb^{2+} toxicity on root growth (Kim *et al*. 2002). Competition between Ca^{2+} and Pb^{2+} in entering cell could cause lower concentration of lead in protoplast of *Funaria hygrometrica* protonemata and simultaneously closer to optimal cytosolic concentration of calcium. It could result in better elongation growth of protonemata because as it was mentioned above calcium is a crucial element for tip growing cells (reviewed in Steer and Steer 1989, Schiefelbein *et al*. 1992, Franklin-Tong 1999). In moss protonemata, similarly to pollen tubes and root hairs, Ca^{2+} controls cytoskeleton function and exocytosis and it is one of the main factor which their growth is dependent on (Reiss and Herth 1979b, Schnepf 1986).

Better elongation growth of protonemata resulted probably in both lower number of apical cells with thickened wall at the apex and smaller size of thickenings. Pollen tubes transferred to supra-optimal concentrations of calcium caused growth inhibition and alteration of cytoskeleton similar to protonemata treated with lead, produced very thick walls over the immediate tube apex (Reiss and Herth 1979a, Picton and Steer 1983). The authors of these publications predicted that in spite of the slower growth, vesicle continued fusion in the same intensity what caused the formation of thick cap, built from wall compounds at the apex. Thickened wall in the apex was observed also in *Funaria hygrometrica* protonemata treated with Ca^{2+} at concentration (32 μM) strongly inhibiting its growth (Konieczna *et al*., unpublished data).

Presence of calcium caused not only better elongation growth. Protonemata contained more cells in comparison with material treated with lead only. It suggests that more frequent cell divisions occurred.

Lead causes a decrease in mitotic activity (Woźny 1995 and literature cited therein, Samardakiewicz and Woźny 2004). In meristematic cells, Pb causes alterations of the mitotic process (Wierzbicka 1989, Samardakiewicz and Woźny in press) and inhibition of cytokinesis (Wierzbicka 1989, Woźny 1995). Calcium ions can neutralise toxic effects of this metal on cells divisions as it was shown, *e.g*., in pea cells (Gabara and Gołaszewska 1992). Presence of Ca^{2+} together with Pb^{2+} in the medium caused an increase of the mitotic index, decrease of number of cells with alterations, *e.g*., chromosome fragmentation or chromatin bridges (Gabara and Gołaszewska 1992). Moreover, calcium cancelled the alterations in structure of mitotic spindle, chromatin and chromosomes (Marme 1985). In our opinion also in *Funaria hygrometrica* protonema presence of calcium together with lead caused less altered cell divisions which resulted in a higher number of cells building it.

Swollen tips similar to this observed in lead treated protonemata were found also in pollen tubes under sub-optimal calcium concentration. Similar effect caused cytochalasin, so it was concluded that actin filament alterations allowed swelling to occur (Picton and Steer 1983). In contrast, the tips of apical caulonemal cells of the moss *Physcomitrella* swelled when they were treated with an anti-microtubule drug - cremart (Doonan *et al*. 1988) and apical cells of fern *Adiantum* protonemata treated with colchicine (Murata and Wada 1989). Swollen tips appeared also in *Picea abies* pollen tube treated with colchicine (Anderhag *et al*. 2000). The reason of it was probably disruption of the microtubules in the tip. It caused also the inhibition of tube elongation. The authors concluded that microtubules and microfilaments coordinate to drive tip extension in conifer pollen tubes in a model that differs from angiosperms (Anderhag *et al*. 2000), where microtubule are required rather to limit growth to a single point (Bibikova *et al*. 1999). Alterations in microtubule cytoskeleton were observed earlier in *Funaria* protonemata treated with lead (Basile *et al*. 1995, Krzesłowska and Woźny 2002). It could be the reason of swollen tips appearance. Microtubular cytoskeleton is sensitive to calcium concentration (Hepler 1980, Kiehart 1981). Therefore addition of the element to lead solution could influence microtubule stability and cause a decrease of swollen tips occurrence in protonemata.

Presence of swollen tips and thickened wall in protonemata cells treated with lead suggests that lead altered both microtubular and microfilament cytoskeleton. Such a point of view supports the fact that addition of calcium neutralised both the effects. We think that better cytoskeleton function could be one of the main intercellular reason why addition of calcium to lead solution resulted in partial neutralisation of lead toxic effects in protonemata cells.

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