CARYOLOGIA Vol. 60, no. 1-2: 115-120, 2007

Ultrastructural alterations and HSP 70 induction in *Elodea canadensis* Michx. exposed to heavy metals

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Abstract — The effects of the heavy metals lead (Pb) and cadmium (Cd) on the ultrastructure and the induction of Heat Shock Protein 70 (HSP 70) have been studied in *Elodea canadensis* Michx.
As for the ultrastructural effects, sublethal concentrations of the metals caused alterations of the cell fine ultrastructure. Lead and cadmium induced alterations: chloroplast were swollen with altered thylakoid organization, many cytoplasmic vesicles appeared, cell wall organization changed.
As for HSP, cadmium stress caused the most severe damages, inducing (and/or enhancing) proteins reacting vs HSP70 antibodies, suggesting that these molecular chaperones might be involved in the resistance to toxic effects of cadmium and lead in *E. canadensis*. Therefore, the induction of HSP 70 in *E. canadensis* would confer a higher resistance to pollutants under stressful conditions, lethal for other mosses and higher plant species.

Key words: *Elodea canadensis*, heavy metals, HSP 70, ultrastructure.

INTRODUCTION

Aquatic plants are known to accumulate metals from their environment (Ali and Soltan 1999) and affect metal fluxes through those ecosystems (Jackson *et al*. 1994). Although bodies of water are a major vehicle to heavy metal contamination, works dealing with submerged plants, directly exposed to it, and overall studies on ultrastructural and physiological effects of heavy metals on aquatic plants, are still very scarce. *E. canadensis* is a freshwater microphyte, which, living completely submerged, absorbs mineral elements directly from the aquatic medium through its wide leaf surfaces. Laboratory studies on *E. canadensis* have demonstrated the potential use of this species in removing metals from polluted water (Eugelink 1998). One physiological and ultastructural study on the effects of cadmium on this plant shows that Cd hindered the division and the expansion of chloroplasts also impairing organelle shape and thylakoid system arrangement. In addition Cd greatly disturbed the cell wall organization. Finally leaves showed a decreased photosynthetic activity (Della Vecchia *et al*. 2005). There are no studies on the mechanisms of resistance of aquatic plants to heavy metals. Among the many and different mechanisms of resistance and/or adaptations to heavy metals stress (Bruins *et al.* 2000; Cobbett and Goldsbrough 2002) the induction and/or the increase of the Heat Shock Proteins (HSP) (Boston *et al.* 1996) play a primary role counteracting the toxic effects on proteins and enzymes, protecting them from misfolding and proteolytic pathways (Miernyk 1997).

Heat Shock Proteins (HSP) are a well known group of chaperones expressed in response to stressful conditions. Among HSP families, HSP70 shows interactions with co-chaperonines which coordinate and regulate their activity (Miernyk 1997). The role and function of HSP70 in plant cell are yet to be defined (Wang *et al*. 2004).

The aim of this paper is to investigate the effects of heavy metal stresses (lead and cadmium)

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on cell ultrastructure and HSPs induction in *E. canadensis*.

MATERIALS AND METHODS

Plant material - Field grown *Elodea canadensis* Mich. was collected in the Botanical Gardens of Naples, and used the same day.

In vitro culture - Plants of *E. canadensis* were surface sterilized in ethanol 70% (2 minutes) and in 2% NaClO with the addition of a few drops of Triton X-100 0.8 % (5 minutes). Subsequently, they were washed (10 minutes) with distilled sterile water and cultured in modified Mohr medium (KRUPA, 1964), pH 7.5 (composition: KNO_3 100 mg, $CaCl_2.4H_2O$ 10 mg, $MgSO_4$ 10mg, KH_2PO_4 13,6 mg, $FeSO_4$ 0.4 mg and 1 ml of BMM solution (Nichols, 1973) to 1000 ml distilled water) used as control and in the same medium with the addition of 10^{-4} and 10^{-5} M $Pb(NO_3)_2$ or $CdCl_2$.

The cultures were kept in a climatic room with a temperature ranging from 13°C at night to 20°C by day, 70% constant relative humidity, and 16 hrs light (45 $mEm^{-2}s^{-1}$)/8 hrs dark photoperiod. All the experiments were conducted in triplicate and repeated at least three times.

Electron microscopy - Ultrastructure observation were perfomed on plants treated with 10^{-5} M Pb or Cd for 3 days. After heavy metal treatment, the plants were thoroughly washed in distilled water for 15 min with several changes to eliminate unbound cations.

For conventional transmission electron microscopy (TEM), specimens were fixed in 2% glutaraldehyde in phosphate buffer (0.065 M pH 7.2-7.4) for 1.5 h at room temperature and post-fixed with buffered 2% OsO_4 for 1.5 h, dehydrated with ethanol and propylene oxide and embedded in Spurr's epoxy medium. Ultra-thin (50 nm) sections, stained with uranyl acetate and lead citrate, were mounted on 100-mesh gold grids and observed with a PHILIPS CM12 microscope (STEM).

Extracts for Western blotting - *E. canadensis* leaflets (1 gr FW) were quickly frozen in liquid nitrogen in a mortar and powdered with a pestle. HSP70s were extracted in extraction buffer [32 mM Tris-HCl, pH 7.8, 10% glycerol, 5 mM dithiotreitol, 0.05% Triton X-100]; the homogenate was then filtered through four layers of muslin and centrifuged at 20000 g for 20' min at 4°C (Sorvall RC5C plus – SS34 rotor). The supernatant fraction was designated as the crude extract and used for soluble protein determination, SDS-PAGE analysis and Western blots.

Electrophoresis and Western blotting - HSP70s from *E. canadensis* were resolved by SDS-PAGE performed in 10% gel (4% stacking gel), loaded with 25-30 g protein and run according to Laemmli (1970) for 90 minutes at 40 mA/180 V.

For Western blot analysis, the separated polypeptides were transferred from gels to a nitrocellulose membrane (Hybond ECL^{tm}, Amersham Biosciences) soon after the SDS-PAGE run, then incubated for 2 h at room temperature with antibodies raised against bovine heart HSP 70 (Sigma). After incubating the membrane with secondary antibodies, cross-reacting polypeptides were identified and stained for Horseradish peroxidase activity using the ECL^{tm} method (ECL^{tm} Western blotting Analysis system – ECL^{tm} Hyperfilm^{tm}; Amersham Biosciences) following manufactures' instructions.

Protein determination - Proteins were estimated by the method of BRADFORD (1976) based on the colorimetric assay with Coomassie blue R-250, using the Bio-Rad Protein Assay (Bio-Rad). A standard curve with bovine serum albumin (Sigma) was used to calculate protein concentrations in the extracts.

RESULTS

Ultrastructural organization - Control - The ultrastructural organization of leaf cells of *E. canadensis* consisted of enlarged cylindrical cells surrounded by a typical fibrous wall, with chloroplasts generally distributed in rows beneath the cell walls. They were discoid to spindle-shaped with a well-developed lamellar system, some plastoglobules and prominent starch grains (Fig.1). The cytoplasm also contained some mitochondria with tubular protrusions of the inner membrane, smooth and rough endoplasmic reticulum and dictyosomes.

Effects of Lead on the ultrastructure - No profound alterations were observed in the chloroplast ultrastructure: grana and intergrana arrangement was preserved. Many cytoplasmic vesicles were present, some of which were electrondense (Fig. 2). Profound alterations were observed in the wall structure particularly concerning microfibril orientation in the cell wall. In addition to the areas

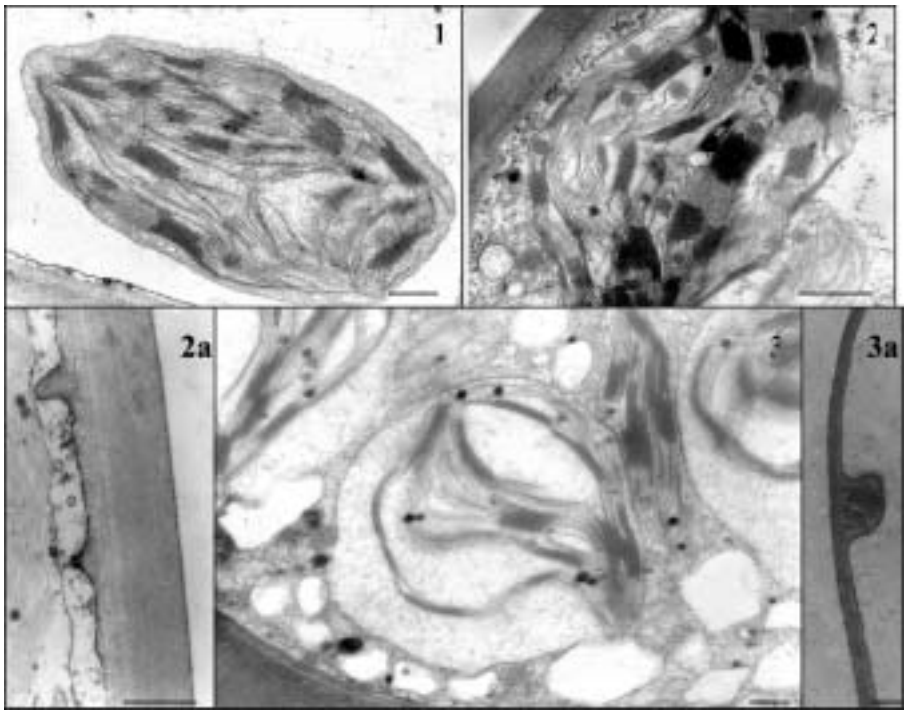

Fig. 1 — TEM micrograph of a leaf upper cell from control *Elodea canadensis* showing a cell with a typical fibrous wall and a chloroplast beneath the cell wall, discoid, with a well-developed lamellar system and some plastoglobules. Scale bar: 300 nm

Fig. 2 — TEM micrograph of a lead-treated sample: the chloroplast is well preserved and shows several grana and stroma thylakoids lined up along main axis. Scale bar: 300 nm. Fig 2a. TEM micrograph of a lead-treated sample showing anomalous bulged cell wall in the outer region of an upper cell. Scale bar: 500 nm

Fig. 3 — TEM micrograph of a cadmium-treated sample: the chloroplast is swollen, cup-shaped with dilated thylakoids. Numerous plastoglobules are also present. The micrograph shows many small vesicles, some of which are electrondense. Scale bar: 300 nm. Fig 3a. TEM micrograph of a cadmium-treated sample showing anomalous bulged cell wall in the outer region of an upper cell. Profound alterations of microfibril orientation in the cell wall are evident near areas with an obvious parallel texture of microfibrils. Scale bar: 500 nm.

with an obvious parallel texture of microfibrils, there were others where the regular arrangement of microfibrils was lost. Here, local wall protuberances in the cytoplasm occurred which occasionally reached considerable dimensions. In these areas, the lamellated wall structure had disintegrated entirely and was replaced by masses of coiled microfibrils which could be clustered to electron dense patches and were embedded in a moderately electron dense matrix. Cross-sectioned cells showed wall abnormalities at low magnification as dense patches which, at higher magnification, proved to be irregularities of microfibril arrangement (Fig. 2a).

Effects of Cd on the ultrastructure - Cadmium treatment caused profound ultrastructural alterations: the chloroplasts were swollen, cup-shaped or irregularly bulged with dilated thylakoids; plastoglobuli increment was present. In addition many cytoplasmic vesicles were present, some of which were electrondense, and altered organization of microfibrils in the cell wall could be seen (Figs. 3 and 3a).

Electrophoresis and western blotting - *E. canadensis* control plants exhibited different polypeptides reacting vs bovine HSP70 antibodies, (Fig. 4).

Cadmium stress caused an increase in proteins reacting vs HSP70 antibodies with respect to control samples. A densitometric analysis (Image J software – NIH) demonstrates a 25 % increase of Hsp70 in Cd 10^{-5} stressed plants, and a 70 % increment in Cadmium 10^{-4} stressed plants, suggesting that this increase was concentration dependent. Lead stress provoked a 60% increase in a 100 KDa protein reacting vs bovine HSP70 antibodies with respect to the control, whereas the 70 kDa band remained essentially unchanged. It should be noted that in the control samples a 85 kDa band was present; this band remained unchanged

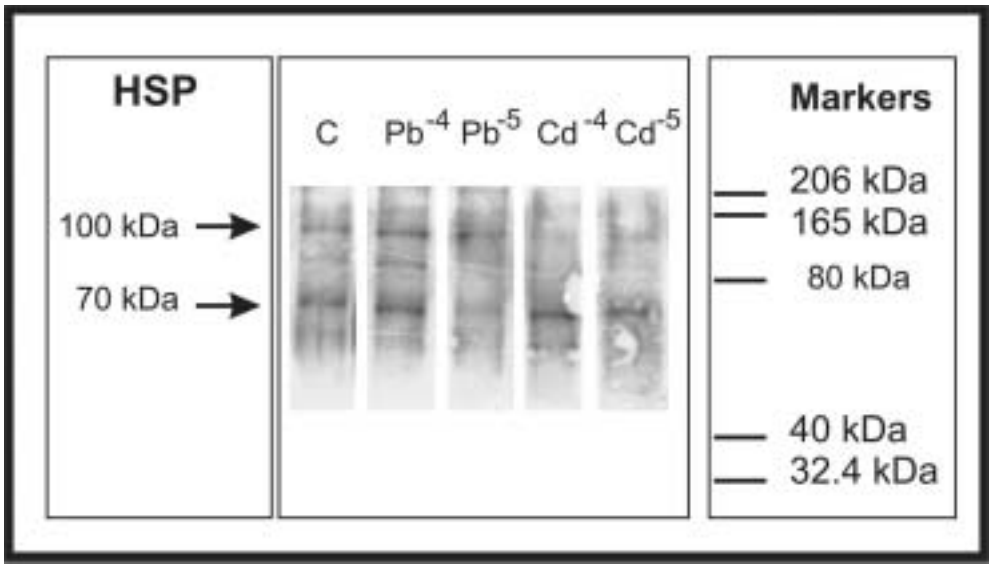

Fig. 4 — Western blots of crude extracts of Elodea densa exposed for 6 days at different concentrations of Cadmium (Cd) and Lead (Pb). Lane C, crude extract of control (not stressed) Elodea; Pb-4 lane, extract from 6 days stressed Elodea by 10-4M Lead; Pb-5 lane, extract from 6 days stressed Elodea by 10-5M Lead; Cd-4 lane, extract from 6 days stressed Elodea by 10-4M Cadmium; Cd-5 lane, extract from 6 days stressed Elodea by 10-5M Cadmium. "HSP" box: molecular weight of stained bands, based on the relative migration factor established using molecular weight markers ("Markers" box).
Western blotting was made utilising antibodies raised against bovine HSP70 (Sigma). Other details in the text.

in lead stressed plants and disappeared in Cd stressed plants.

DISCUSSION

Ultrastructural damage appears to have distinct features according to the metal used. The main toxic effect of lead and cadmium influenced the chloroplast membrane arrangement and the cell wall organization. Cd was more toxic than Pb, this is in agreement with other studies on the toxicity of heavy metals (for a review, see RAO 1982; BROWN 1984; TYLER 1990) It is known that the heavy metals exert their high toxicity by binding to structural proteins or enzymatic electron donor groups. Furthermore, cadmium and organic lead are known to damage the chloroplast ultrastructure, causing alterations to the grana (HEUMANN 1987; DELLA VECCHIA *et al*. 2005). Lead, but also Cd, induces alterations especially in cell-wall three-dimensional organization. The wall alterations found in *E. canadensis* after exposure to inorganic lead and cadmium have also been described in the internodal cells of the filamentous alga *Chara vulgaris* (HEUMANN 1987), in maize coleoptile cells (KUTSCHERA *et al.* 1987), in lily pollen tubes (RODERER and REISS 1988) and in Cd exposed *E. canadensis* transfer cells (DELLA VECCHIA *et al.* 2005). It was hypothesized that plant cell growth might be inhibited by the interaction of certain divalent cations (such as lead or cadmium) with the anionic contents of the secretory vesicles and formation of a gel with the acidic groups (TEPFER and TAYLOR 1981). However, it was subsequently suggested that cell wall alterations induced by inorganic lead, but also by cadmium, do not result from a direct attack of metals to wall components but are the consequence of disturbances of various metabolic processes involved in the synthesis, translocation and/or deposition of cell wall material (HEUMANN 1987). On the other hand, the toxic effects of lead on microtubule assembly and three-dimensional organization (BASILE *et al.* 1995) can disturb the delicate and co-ordinated mechanism of wall assembly. The alteration of chloroplast shape could be due to the toxic effect of lead and cadmium on cytoskeletal proteins or to membrane damage or even to competition with other cations that have a physiological role.

Pb and Cd-treated samples of *E. canadensis* show cells with numerous cytoplasmic vesicles. It was shown that the presence of heavy metals such as zinc may induce high vacuolization in cereal roots and hence triggers a mechanism which ultimately leads to compartmentalization of metal in the vacuole (DAVIES *et al.* 1992). Such an altera-

tion acts as a sequestration mechanism allowing growth, even at fairly high metal concentrations. The sequestration of the metals in the vesicles has a primary role in determining tolerance, as also showed for lead and cadmium (BASILE *et al.* 2001; CARGINALE *et al.* 2004): sequestration of the heavy metals inside the vacuole reduces the amount of metal which is free and thus able to exert toxic effects in the cytoplasm. Further studies should be performed to assess the presence of lead or cadmium in the cytoplasmic vesicles found in the heavy metal treated specimens.

One of the most evident metabolic alterations due to heavy metals is the induction of mRNAs for Heat Shock Proteins and the synthesis of these proteins in plants growing on heavy metals-polluted soils (NEUMANN *et al.* 1994). Moreover, the induction of HSP 70 in *Raphidocelis subcapitata* has been described following exposure to different environmental pollutants (BIERKEN *et al.* 1998).

In this view, a crucial point of the whole process is played by HSP 70, involved in the recovery and re-folding of mis-folded or denaturated proteins (BOSTON *et al.* 1996), often together with HSP100s and other chaperones (SUN *et al.* 2002). The occurrence and the functions of HSP 70 proteins upon heavy metals stress observed in *E. canadensis* depend on the type of pollutant. Cadmium exhibited a cumulative effect which appears to be effective until 10^{-4} M and three days of exposition. Lead stress caused a noticeable effect only at 10^{-4} M, suggesting a lighter effect than Cd. The occurrence of a 100kDa peptide reacting vs Hsp70 antibody could be explained either by a heavy weight Hsp70s (MIERNYK 1999) or by a complex formed together with HSP 40, the chaperonin HSP 100 (MIERNYK 1997).

It is essential, under stressful conditions, to maintain proteins in their functional conformation, and possibly to recover the unfolded proteins to restore their function properly and possibly to induce a tolerance. Under this light the presence of 100 kDa proteins reacting vs HSP70 antibodies would suggest the formation of HSP100 complexes, involved in protection from unfolding of proteins (MIERNYK 1997).

Further studies are necessary to clarify the physiological (and possibly molecular) basis for this low sensitivity to pollutants which involves, at least in part, the induction of HSPs.

Aknowledgenments — This work was supported by a grant from MIUR, PROGETTO TIDe, Obiettivo realizzativo: 1.9; Bioaccumulo.

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Received November 30th 2006; accepted January 30th 2007