BIOLOGIA PLANTARUM 45 (1): 99-104, 2002

Why chloroplasts in apical cell of *Funaria hygrometrica* protonemata treated with lead are distributed in different way than in control

M. KRZESŁOWSKA and A. WOŹNY

Institute of Experimental Biology, Adam Mickiewicz University, Al. Niepodległości 14, PL-61713 Poznań, Poland

Abstract

Two-day-old protonemata of *Funaria hygrometrica* Hedw. growing *in vitro* were treated with 4 μM Pb (given as $PbCl_2$) for 48 h. After this time chloroplasts of apical cell were distributed in different way than in control. In the middle part of the cell they formed one line while in the top one, usually swollen, they were crowded and formed irregular group. The reasons of such chloroplast distribution were: *1*) increase of plastid size, probably the effect of intensive accumulation of starch and appearance of characteristic protuberances, *2*) decrease of cell inner space additionally intensified by thickenings of lateral walls, and *3*) disturbances of microtubule number and array.

Additional key words: chloroplast protuberance, heavy metal, moss, Pb, plastid.

Introduction

Presence of the lead in the medium can cause in *Funaria hygrometrica* protonemata disturbances of spore germination, inhibition of growth (Krupińska 1976, Kardash and Demkiv 1991, Basile *et al.* 1995) and decrease of the number and size of cells building protonema thread (Krupińska 1976). In apical cell Pb induced longitudinal and cross septa formation, lack of both tip body and microtubules and migration of the nucleus to apical, often abnormally swollen, part of the cell (Basile *et al.* 1995). Moreover, wall thickenings built mainly from pectic polysaccharides and containing also callose, cellulose, and lipid substances were formed at the tips (Krzesłowska and Woźny 2000). Chloroplasts were not as numerous as in control (Krupińska 1976) and they formed small groups (Basile *et al.* 1995). Changes in their structure were similar to physiological ageing process. They were characterised by rounded shape, presence of numerous and rather large plastoglobules, distortion of thylakoid system, *etc.* Moreover, in thylakoids running near chloroplast envelope many lead deposits appeared (Krzesłowska and Woźny 1996).

In our previous study on the Pb effects on *Funaria hygrometrica* protonemata we noticed very characteristic distribution of chloroplasts in apical cells, not described earlier. Therefore we tried to find reasons of such chloroplast distribution.

Materials and methods

Protonemata of *Funaria hygrometrica* Hedw. were grown for two days in sterile Petri dishes on the Kofler (1959) medium supplemented with microelements according to Heller (1953). For next 48 h, half of the culture from each dish was treated with aquatic solution of 4 μM $PbCl_2$ (this inhibited the growth of protonema by about 50 %),

Received 2 February 2001, *accepted* 31 May 2001.
Acknowledgements: The cytoskeletal studies were done in the Plant Cell Morphology Department of Wageningen Agriculture University in Holland and supported by the second Tempus Program. The authors thank Prof. M.T.M. Willemse and Dr. A.A.M. van Lammeren for possibility to carry on the studies and for helpful discussion. The cytophotometrical measurements were done in the Plant Cytology and Cytochemistry Department of The University in Lódź, Poland. The authors thank Prof. B. Gabara for enabling them to carry on the study and for helpful advice.
Fax: (+48) 61 8294515, e-mail: adaw@main.amu.edu.pl

another half was grown on distilled water (control).

Morphological observations dealt with distribution, number and shape of chloroplasts. Lengths and widths of apical cells, size of their swollen region and areas of chloroplasts were also measured. In protonemata treated with lead we measured separately the area of chloroplasts present in the apical swollen region of cell and those in its middle part. Measurements of cell length and width were done in light microscope *Reichert* using a micrometric ocular *OK 15* (*Polish Optical Factory, PZO*, Warsaw, Poland). Size of both chloroplasts and swollen regions were measured by the cytophotometer *IMAL 1024* linked with light microscope *Nikon* (Tokyo, Japan)

Microtubules were visualised by a standard double immunofluorescence method which was in some stages adopted for the *F. hygrometrica* protonemata. Material was fixed in the mixture of microtubule stabilising buffer: MSB (50 μM PIPES, 5 mM EDTA, 5 mM $MgSO_4$) and 4 % paraformaldehyde. Chlorophyll was removed in an ethanol series in increasing concentration. For easier penetration of antibodies, cell walls were partly digested by a mixture of enzymes according to Quader *et al.* (1986). The perforations of plasma membrane were done by treating the material with 0.05 % *Triton-100*. First antibody was rabbit polyclonal antitubulin (R229) dissolved in protein stabilising buffer (PBS) in the ratio 1:150. The material was then rinsed in 0.1 % solution of bovine serum albumine in MSB. Then the second antibody, GAR/Ig/FITC (*Nordic*, Tilburg, Holland) dissolved in 1 % PBS in the ratio 1:150, was used. Studies were done in epifluorescent microscope (*Nikon Microphot FXA*) using filter *FITC EX 470-490 (DN 510) BA 515 EF* and in confocal laser scanning microscope *Bio-Rad MRC 600*. For studies in transmission electron microscope the material was prepared as described in Krzesłowska and Woźny (1996).

Statistical evaluation was done by the programme *Statistica 4.2*.

Results

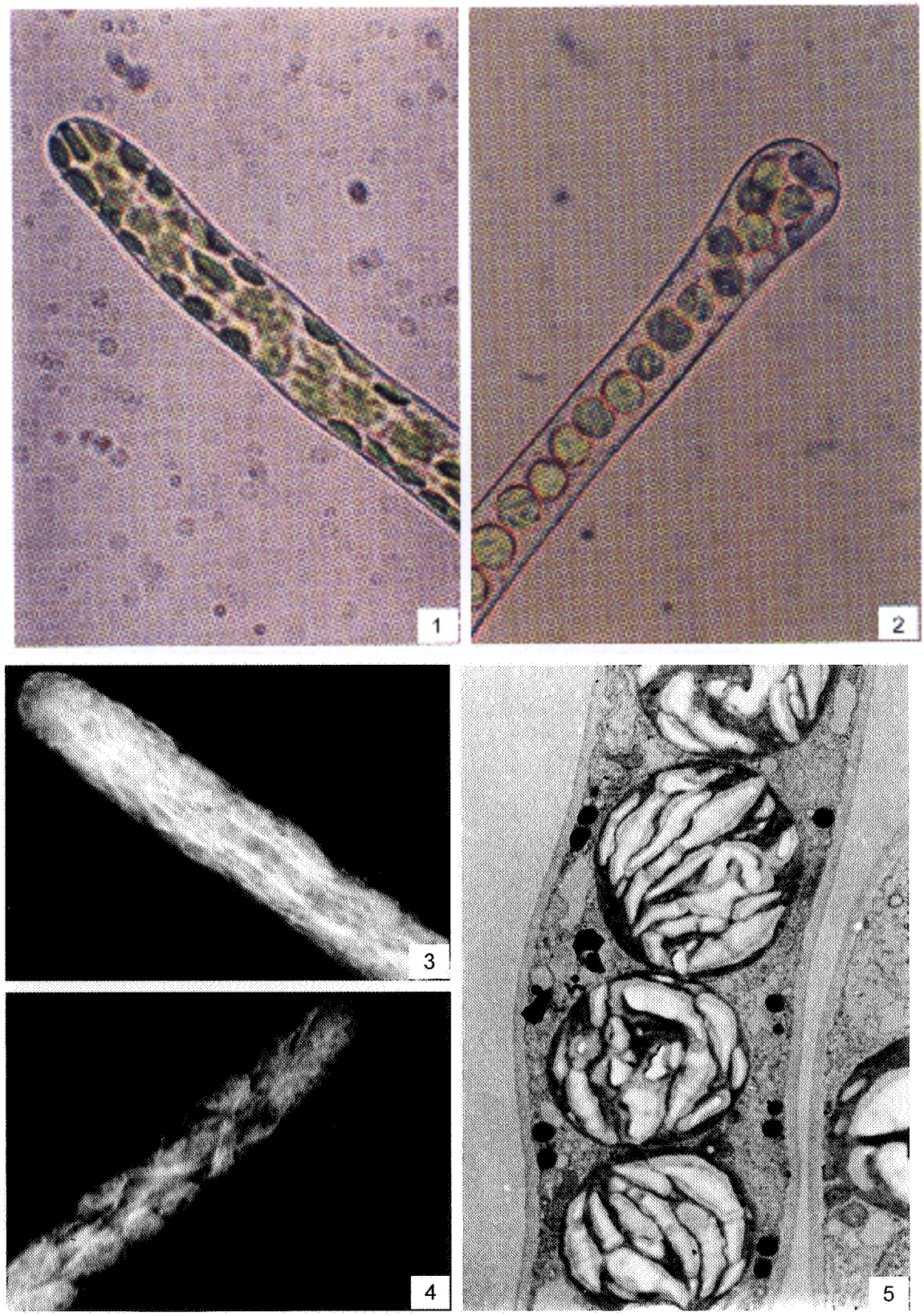
Distribution of chloroplasts: In control protonemata chloroplasts were evenly distributed along lateral walls (Fig. 1) of the apical cell. Three plastids were usually present in the cross section of the cell. Different distribution of chloroplast was in protonemata treated with lead. In the middle part of the cell, chloroplasts formed one line only (Figs. 2, 5) while in the top part, which was often swollen, they were crowded and formed an irregular group (Fig. 2). Thus in the cross section of apical cell in its middle part only one plastid was present, while in the top region several chloroplasts were found.

Morphological features of chloroplasts: We found considerable differences in shape, size, and number of chloroplasts between control and Pb-treated protonemata. In control the chloroplasts were elliptic (Figs. 1, 6) and their area was 14.70 ± 7.39 μm^2. The size of chloroplasts was similar in different parts of the cell. Lead-treated protonemata contained 25 % less chloroplasts. They were rounded (Figs. 2, 5). In apical cell middle region chloroplasts were almost two times larger (33.46 ± 19.45 μm^2) than in the top region (15.91 ± 6.66 μm^2). The lengths of apical cells were similar in both control and Pb-treated material. However, they differed in width (Table 1). Apical cells of protonemata treated with lead were narrower than the control ones, and in many places they contained lateral thickenings of cell walls. Tip part of the cell was swollen. This region was about 50 % wider than its middle part (Table 1). The differences in area of plastids from middle part of apical cell and width of cell were statistically significant between lead treated protonemata and control ones.

Table 1. Morphological features of protonema apical cell. Protonemata were exposed to water containing 0 (control) or 4 μM Pb for 48 h. Values are means ± SD ($n = 30$).

Treatment	Feature	
Control	length [μm]	110.90 ± 32.65
	width [μm]	10.12 ± 1.52
	number of plastids	33.87 ± 9.67
	area of plastids [μm^2]	14.70 ± 7.39
Pb-treated	length [μm]	115.33 ± 51.69
	width [μm]	9.31 ± 1.32
	number of plastids	25.53 ± 8.50
	area of plastids in swollen tip [μm^2]	15.91 ± 6.66
	area of plastids in middle part [μm^2]	33.46 ± 19.45
	width of the swollen tip [μm]	14.06 ± 2.56

Microtubules: In apical cells of control protonemata (Fig. 3) numerous cortical microtubules were aligned parallel to its longer axis. Their distribution was regular in the whole cell and they ran straightforward. We did not observe any disturbances of the array and number of them. In the lead treated material microtubule bundles were less numerous and their array in the cell was out of order. Most of them was strongly waved. They surrounded chloroplasts which were distributed in different way than in control. Only rarely microtubule bundles running straightforward were found (Fig. 4).



Figs. 1 - 2. Chloroplast distribution and shape in control (Fig. 1) and lead-treated (Fig. 2) protonemata (*bar* = 10 μm).
Figs. 3 - 4. Array and number of microtubules: in control (Fig. 3) and lead-treated (Fig. 4) protonemata (*bar* = 10 μm).
Fig. 5. Chloroplast ultrastructure, distribution and shape in lead treated protonemata (*bar* = 1 μm).

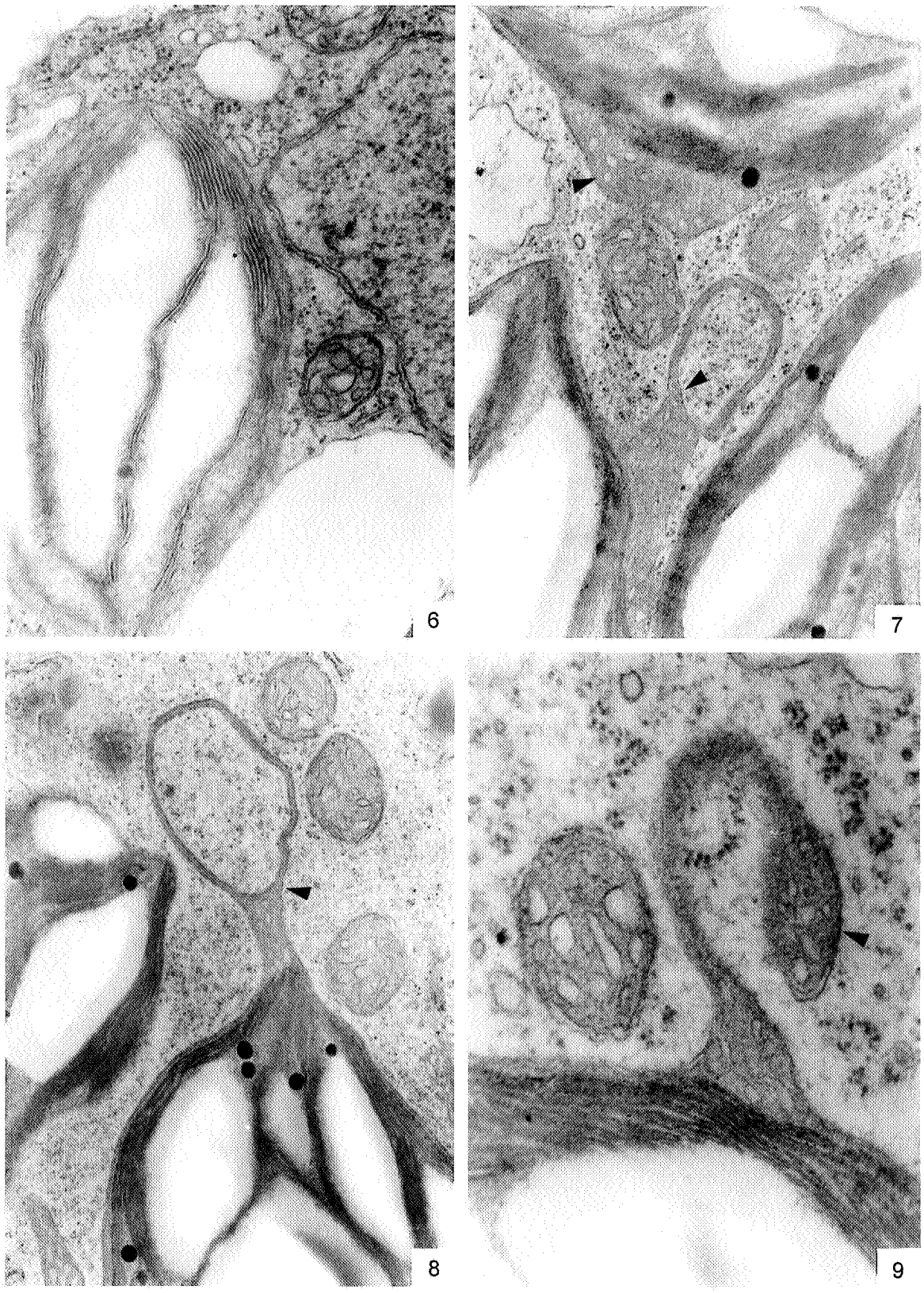


Fig. 6. Ultrastructure of control chloroplasts (*bar* = 200 nm).
Figs. 7 - 9. Various types of chloroplast protuberances (*arrowheads*) in protonemata treated with lead (Figs. 7, 8: *bar* = 500 nm, Fig. 9: *bar* = 200 nm).

Ultrastructure: Chloroplasts in apical cell of control protonemata were elliptical (Fig. 6). Their thylakoid system was placed in parallel to the longer axis of the organelle. Inside it was possible to find stacks containing 3 - 6 thylakoids (Fig. 6) similar to grana of flowering plants and stroma unstacked thylakoids. We often found not very large plastoglobules and starch grains in stroma (Fig. 6).

Opposite to this, chloroplasts of lead treated protonema were strongly rounded and formed one line in the middle part of the cell. In many cases they were full of starch grains and their thylakoids were often swollen and contained many plastoglobules (Figs. 5, 8). In many cases the periphery region of chloroplasts was enlarged and some kind of protuberances, not observed in chloroplasts of the control, were formed (Figs. 7 - 9). Some of them were very similar to the section of mitochondrion (Fig. 9). They contained numerous vesicles similar to mitochondrion cristae (Figs. 7, 9). Other ones were built from two structurally different regions: one placed near the proper plastid contained some vesicles and the further one formed from several membranes (Figs. 7, 8).

Discussion

Chloroplasts of apical cells in correctly developed chloronemal protonemata are elliptic with well developed thylakoid system similar to grana of flowering plants (Woźny 1978). Stroma contains small amounts of starch, some plastoglobules and ribosomes (Młodzianowski and Woźny 1972). In the lumen of the cell 2 - 3 chloroplasts are present. Why are chloroplasts in apical cell of *F. hygrometrica* Hedw. protonemata treated with lead distributed in different way? The study showed at least some reasons. First of them can be the increase of chloroplast size. Simultaneously, cells in lead treated material were narrower than the control ones. Their lateral walls were often thickened what made the lumen of the cell additionally smaller. Therefore plastids almost two times larger than in control, filled with starch granules (in the middle part of the cell), could not be placed in another way than lined-up single-file.

The increase in chloroplast size was observed also as the effect of other heavy metals on plant, *e.g.,* in cadmium treated *Phaeodactylum tricornutum* Bohlin (Torres *et al.* 2000). Distribution of plastids in one line was found in protonemata grown in the dark where full of starch, large amyloplasts occupied the whole cell lumen (Młodzianowski and Woźny 1972). This may suggest accumulation of starch as one of the reasons why plastids in lead treated protonemata were considerable larger than in the control ones. Additionally, their size was increased because of the enlargement of the periphery region where some kind of protuberances were formed. Structurally similar protuberances were observed also in chloroplasts of colchicine treated material (they were described as deep invaginations of cytoplasm (Schnepf *et al.* 1982) and in chloroplasts of brachycytes (Schnepf and Reinhard 1997). The presence of protuberances in chloroplast is often regarded normal (Heitz 1936, Spencer and Unt 1965 and references therein, Vesk *et al.* 1965, Kohler *et al.* 1997). Normally present protuberances were stroma filled and containing some infoldings of chloroplast inner envelope (Bourett *et al.* 1999) were, however, ultrastructurally different from chloroplast protuberances of lead treated protonemata (full of vesicles or containing concentric system of thylakoids). Presence of electron transparent vesicles in the periphery of organelle is regarded as sign of degenerating process (vesicles can be degenerated thylakoids - Młodzianowski *et al.* 1970, Pakhlavouni *et al.* 2000). In lead treated protonemata we observed some other symptoms characteristic for degeneration of the organelle, *e.g.,* their rounded shape or swollen thylakoids. This supports the point of view that described protuberances in lead treated protonemata do not normally appear and are rather the symptom of degeneration.

Chloroplasts present in tip of the apical cell were smaller (the size was similar to control) than those present in the middle part. They were dislocated into the wide swollen tip region by abnormally large chloroplasts under the tip. Moreover, we supposed that characteristic distribution of plastids in lead treated protonemata might be not only the result of increase of their size and a decrease of volume of the cell lumen. In growing tip cells such as protonema apical cell the distribution of organelles depends on the microtubule array (Brown and Lemmon 1980, Kiermayer 1981, Schnepf *et al.* 1982). Interphase apical cell of the control characterised parallel array of microtubules to longer axis of the cell (Abel *et al.* 1989). In lead treated material serious disturbances of both microtubule array and number could influence the distribution of chloroplasts. Changes of chloroplast distribution in cells of lead treated protonemata were observed also by Basile *et al.* 1995: they formed small groups distributed regularly along the longer axis of the thread, explained as the result of microtubule lack in cells treated with the metal.

We found that the presence of Pb strongly influenced apical cells of protonemata and caused the increase of plastid size (probably because of starch accumulation and

appearance of protuberances), decrease of cell light additionally intensified by thickenings of its lateral walls, and disturbances in number and array of mictotubules. All the reasons caused probably characteristic distribution of chloroplast, different than in the control.

References

Abel, W.O., Knebel, W., Koop, H.U., Marienfeld, J.R., Quader, H., Reski, R., Schnepf, E., Sporlein, B.: A cytokinin-sensitive mutant of the moss. *Physcomitrella patens,* defective in chloroplast division. - Protoplasma **152**: 1-13, 1989.

Basile, A., Giordano, S., Spagnuolo, V., Alfano, F., Castaldo-Cobianchi, R.: Effect of lead and colchicine on morphogenesis in protonemata of moss *Funaria hygrometrica.* - Ann. Bot. **76**: 597-606, 1995.

Bourett, T.M., Czymmek, K.J., Howard, R.J.: Ultrastructure of chloroplast protuberances in rice leaves preserved by high-pressure freezing. - Planta **208**: 472-479, 1999.

Brown, D.H., Lemmon, B.E.: Ultrastructure of sporogenesis in a moss, *Ditrichum pallidum* III. Spore wall formation. - Amer. J. Bot. **67**: 918-934, 1980.

Heitz, E.: Untersuchungen über den Bau der Plastiden. I. Die gerichteten Chlorophyllscheiben der Chloroplasten. - Planta **26**: 134-163, 1936.

Heller, R.: Recherches sur la nutrition minerale des tissus végétaux cultivés *in vitro.* - Ann. Sci. nat. Biol. vég. **14**: 1-223, 1953.

Kardash, O.R., Demkiv, L.O.: Effect of lead on the growth and development of the gametophyte *Funaria hygrometrica* Hedw. - Ukr. bot. Zh. **48**: 31-33 , 1991.

Kiermayer, O.: Cytoplasmic basis of morphogenesis in *Micrasterias.* - In: Kiermayer, O. (ed.): Cytomorphogenesis in Plants (Cell Biology Monographs 8). Pp. 147-190. Springer-Verlag, Wien - New York 1981.

Kofler, L.: Contribution à l'ètude biologique des mousses cultivées *in vitro.* Germination des spores, croissance et dévelopment du protonéma chez *Funaria hygrometrica* .- Rev. Bryol. Lichenol. **24**: 1-199, 1959.

Kohler, R.H., Cao, J., Zipfel, W.R., Webb, W.W., Hanson, M.R.: Exchange of protein molecules through connections between higher plant plastids. - Science **276**: 2039-2042, 1997.

Krupińska, I.: Influence of lead tetraethyl on the growth of *Funaria hygrometrica* L. and *Marchantia polymorpha* L. - Acta Soc. Bot. Pol. **45**: 421-432, 1976.

Krzesłowska, M., Woźny, A.: Lead uptake, localization and changes in cell ultrastructure of *Funaria hygrometrica* protonemata. - Biol. Plant. **38**: 253-259, 1996.

Krzesłowska, M., Woźny, A.: Wall thickenings - moss protonema apical cell reaction to lead. - Biol. Plant. **43**: 93-98, 2000.

Młodzianowski, F., Szweykowska, A., Schneider, J.: The effect of chloramphenicol on the ultrastructure of chloroplast in the protonema in *Funaria hygrometrica.* - Acta Soc. Bot. Pol. **39**: 38-43, 1970.

Młodzianowski, F., Woźny, A.: The variability of plastid structure in *Funaria hygrometrica* as affected by environmental conditions. - Folia histochem. cytochem. **10**: 51-56, 1972.

Pakhlavouni, I.K., Khaibullina, L.A., Serebryakova, V.N.: [Carotenoid composition and chloroplast ultrastructure in two cell lines of *Mandragora turcomanica (Solanaceae)*.] - Fiziol. Rast. **47**: 38-47, 2000. [In Russ.]

Quader, H., Deichgraber, G., Schnepf, E.: The cytoskeleton of *Cobaea* seed hairs: pattering during cell wall differentiation. - Planta **168**: 1-10, 1986.

Schnepf, E., Hrdina, B., Lehne, A.: Spore germination, development of the microtubule system and protonema cell morphogenesis in the moss *Funaria hygrometrica*: effects of inhibitors and growth substances. - Biochem. Physiol. Pflanz. **177**: 461-482, 1982.

Schnepf, E., Reinhard, C.: Brachycytes in *Funaria* protonemata: Induction by abscisic acid and fine structure. - J. Plant Physiol. **151**: 166-175, 1997.

Spencer, D., Unt, H.: Biochemical and structural correlations in isolated spinach chloroplasts of *Portulaca oleracea* L. - Z. Pflanzenphysiol. **87**: 37-53, 1965.

Torres, E., Cid, A., Herrero, C., Abalde, J.: Effect of cadmium on growth, ATP content, carbon fixation and ultrastructure in the marine diatom *Phaeodactylum tricornutum* Bohlin. - Water Air Soil Pollut. **117**: 1-14, 2000.

Vesk, M., Mercer, F.V., Possingham, J.V.: Observations on the origin of chloroplasts and mitochondria in the leaf cells of higher plants. - Aust. J. Bot. **13**: 161-169, 1965.

Woźny, A.: Wpływ Cytokinin na Rozwój Plastydów w Splątku *Ceratodon purpureus* Brid. oraz Liścieniach Ogórka (*Cucumis sativus*). [The Influence of Cytokinin on the Plastids Development in *Ceratodon purpureus* Brid. Protonemata and Cucumber Cotyledons (*Cucumis sativus*).] - Wyd. Nauk. Uniw. Im. Adama Mickiewicza, Poznań 1978. [In Polish].