

Structural changes of lamellar cells in leaves of the moss *Polytrichum formosum* Hedw. during winter freezing and thawing processes

NIKOLA LJUBEŠIĆ*, MERCEDES WRISCHER, TATJANA PREBEG, ZVONIMIR DEVIDÉ

Ruđer Bošković Institute, Department of Molecular Biology, P.O. Box 180, HR-10002 Zagreb, Croatia

The lamellar cells of winter leaves of *Polytrichum formosum* were studied by electron microscopy of thin sections, and the results were compared with those for spring and summer plants. Pigment content and photosynthetic activity were also analysed. The lamellar cells of winter plants, when grown at temperatures above zero, had a similar ultrastructure to those of spring and summer plants. Their pigment content and photosynthetic activity were also similar. On the other hand, the lamellar cells of frozen plants (examined at temperatures about $-5\text{ }^{\circ}\text{C}$) were desiccated, i.e. deformed, the cytosol was shrunken, and the ribosomes and mitochondria were aggregated. The thylakoid system of their chloroplasts was irregularly arranged and the grana thylakoids were compressed. The desiccation of the cells was soon annulled when the ambient temperature rose above zero.

Key words: *Polytrichum formosum*, leaves, ultrastructure, pigment, photosynthesis, lamellar cell, freezing, thawing

Introduction

The moss species that grow in temperate climatic zones (at medium geographic latitudes) are well adapted to low winter temperatures, and are, at higher sea levels, even in early spring and late autumn, exposed to daily freezings (at night) and thawings (during the day). During the winter months they may remain at subzero temperatures for days, or even weeks, and are then frequently covered with snow or ice.

Numerous studies on overwintering higher plants have indicated that, during autumn and early winter, these plants undergo acclimation to low temperatures. This process comprises a variety of structural and biochemical adaptations, including changes in the vacuolar system, increase of carbohydrate levels, shifts in the lipid composition of the biomembranes, and *de novo* synthesis of specific proteins (SENER and BACK 1984, SAUTER et al. 1996, UEMURA and STEPONKUS 1997, ROUTABOUL et al. 2000, ATICI and NALBANTOGLU 2003). At present, the expression of the genes responsible for these changes is receiving particular attention (BROWSE and XIN 2001, THOMASHOW 2001, SUNG et al. 2003).

* Corresponding author: Fax +385 1 45 61 177, e-mail: ljubesic@irb.hr

During the freezing process, ice crystals that are formed outside the cells, act as nucleation sites for water, which is thus pulled out of the cells. This eventually results in desiccation of these cells (PEARCE and ASHWORTH 1992, WRISCHER et al. 2000, BALL et al. 2004). We assumed that similar mechanisms may also function in mosses exposed to subzero temperatures.

The anatomy of the leaves of the large moss, *Polytrichum formosum*, is structurally complex. The abaxial part of the leaf consists of large cells with thick walls, the hydroids, which function in the transport of water, and of leptoids, which are efficient in the transport of sugars (THOMAS et al. 1990). The closely arranged longitudinal rows of one cell-layer wide chlorophyll-containing, lamellae on the adaxial side of the leaf have the same function as the mesophyll in higher plants (PAOLILLO and REIGHARD 1967, THOMAS et al. 1996). The lamellar cells are directly exposed to the environment. We studied how low winter temperatures influence the structure and physiology of these cells in *Polytrichum*.

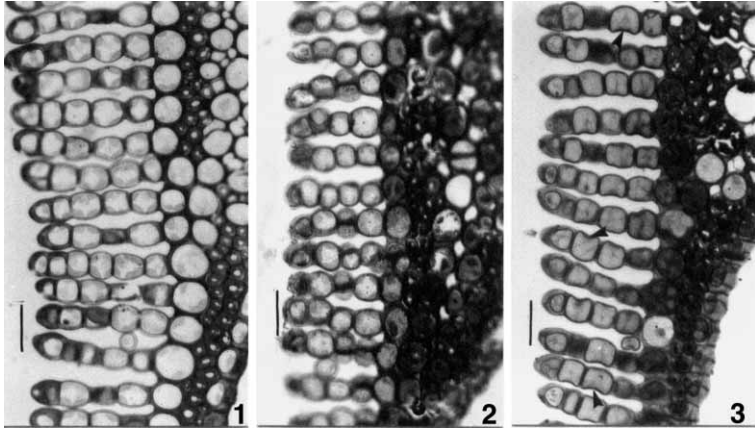
Materials and Methods

Samples of *Polytrichum formosum* Hedw. were collected at its natural habitat, in the Medvednica mountains (near Zagreb), at an altitude of 600–1000 m above sea level. For electron microscopy, the plants were immediately fixed at their habitat; for pigment analyses and measurements of photosynthesis and respiration, they were transferred to the laboratory. The fixation medium (both for light and for electron microscopy) consisted of 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2). The duration of the fixation was 1–2 h, and the temperature of the fixation medium about +2 °C, except when lower temperatures – for the fixation of frozen objects (about –5 °C) were needed. In these cases the vials with the fixation medium were thoroughly cooled by dipping them into the snow/ice that surrounded the moss plants. After rinsing in cold buffer, the material was postfixed in 1% OsO₄, for 2 h. The samples were then dehydrated in a series of ethanol and in acetone, and embedded in Spurr's medium. For light microscopy, semithin sections were stained with toluidine blue and examined under a light microscope (Zeiss Axiovert 35). For electron microscopy, the thin sections were stained with uranyl acetate and lead citrate and examined using a Zeiss EM 10 or a FEI Morgagni 268D microscope.

Pigments were extracted in 80% acetone. Their concentration was calculated according to LICHTENTHALER (1987). Respiration and photosynthetic activity (production of oxygen) were measured with an oxygen electrode (Hansatech Ltd., London) at 20 °C. The reaction mixture contained 0.1 mol/L K-Na-phosphate buffer (pH 7.2) and 0.01 mol/L Na-bicarbonate. The samples for photosynthetic measurements were illuminated with a halogen lamp and, for respiration measurements, they were kept in the dark (WRISCHER et al. 1998, LJUBEŠIĆ et al. 2003).

Results

The arrangement of the cells in the lamellae on the adaxial side of *Polytrichum* leaves was best visible by light microscopy when the leaves were cut perpendicularly to their long axis. In spring leaves, the peripherally arranged chloroplasts were well visible in the roundish lamellar cells (Fig. 1). In winter, these cells remained roundish, but were filled with a



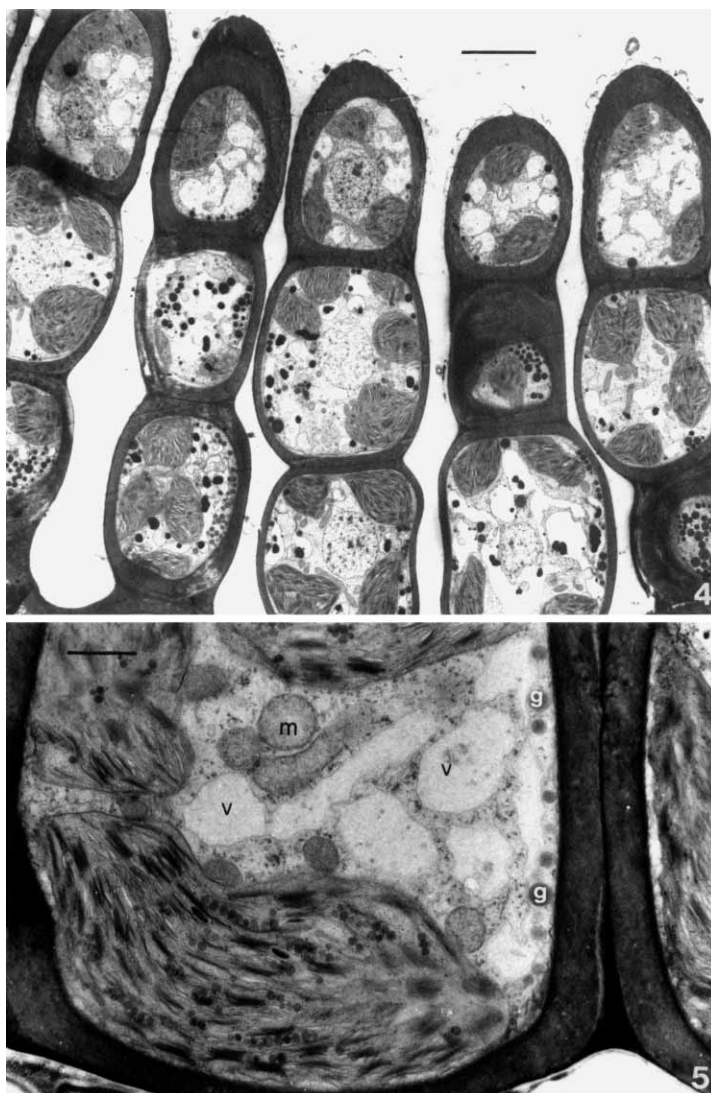
Figs. 1–3. Semithin sections through lamellar cells. Fig. 1. Cells from a spring plant. Fig. 2. Cells from a winter plant collected at a temperature above zero. The marginal cells are intensely stained. Fig. 3. Cells from a frozen winter plant collected at an ambient temperature of -5°C . The cells have indentations (arrowheads). Bars = 20 μm .

dense material, and the marginal cells became intensely stained (Fig. 2). The frozen cells of material collected in winter (at -5°C) had conspicuously irregular outlines (Fig. 3).

Electron microscopy revealed that the lamellar cells were covered by a thick cell wall, which was particularly thick on the marginal cells. In **spring plants**, several lightly stained vacuoles surrounded the centrally arranged nucleus (Fig. 4). The large chloroplasts (3–5 per cell section) contained loosely arranged grana of 3–5 thylakoids, connected to single stroma (intergrana) thylakoids. In the stroma, there were several starch grains and groups of plastoglobules, 0.1 to 0.15 μm in diameter. Small mitochondria, peroxisomes, elements of the endoplasmic reticulum and some dictyosomes were dispersed in the cytoplasm (Fig. 5). Some, about 0.5 μm large, lipid globules were usually located near the cell wall. The lamellar cells of the summer leaves were organized in a similar way. Large chloroplasts in division were often found in these leaves.

In **winter leaves**, the vacuoles of the marginal lamellar cells were filled with a dense granular material (Fig. 6). The other cell organelles were similarly organized as in spring or summer leaves. The chloroplast stroma contained starch grains and plastoglobules with an approximate diameter of 0.1 μm (Fig. 7).

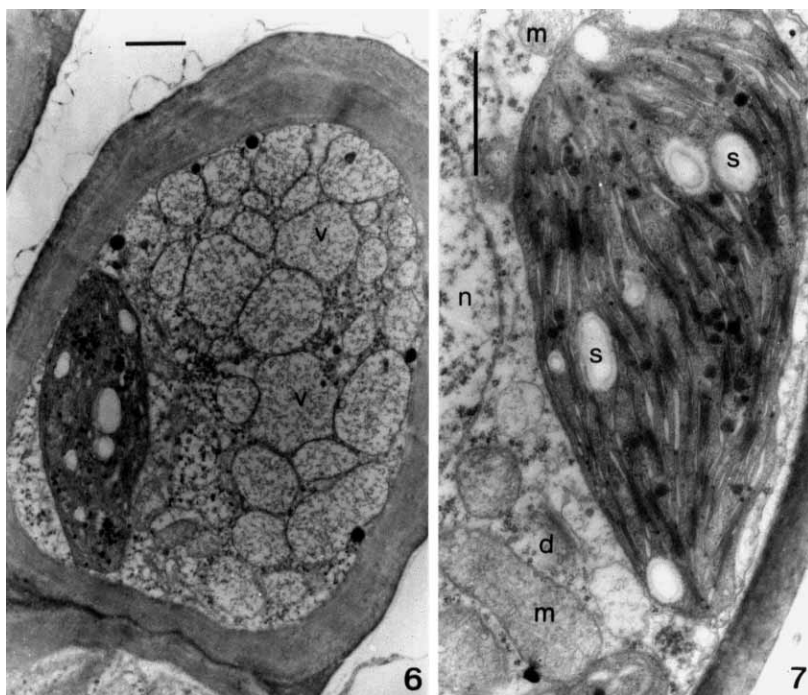
The structure of **frozen winter leaves** differed substantially from that of unfrozen ones. In the lamellar cells, the volume of the cytosol had shrunk and the ribosomes were aggregated and assembled in the areas neighboring the vacuoles. The mitochondria were also packed in between the vacuoles, while the endoplasmic reticulum and the dictyosomes were not discernible (Fig. 9). In the large, irregularly shaped, chloroplasts the thylakoid system was irregularly arranged and its membranes were wavy. The grana thylakoids together with their lumina were compressed (Figs. 8, 10, 11). The peripheral part of the chloroplast stroma was without thylakoids, but numerous straight invaginations of the inner membrane of the envelope became visible in these regions. In the stroma, plastoglobules were numerous and starch grains were still present (Figs. 8, 10).



Figs. 4–5. Thin sections through lamellar cells in spring (April–May). Fig. 4. The marginal cells have thick cell walls. Bar = 5 μ m. Fig. 5. A lamellar cell with chloroplasts, mitochondria (m), and several vacuoles (v). g = lipid globules. Bar = 1 μ m.

Only a short temperature increase above zero was sufficient to reverse the effect of freezing. The cells became roundish again, the cytosol reassumed its normal structure, and in the chloroplasts, the grana thylakoids returned to their regular, loosely arranged, pattern (Fig. 12).

Pigments and photosynthetic activity were measured several times during spring, summer, autumn and winter. The chlorophyll and carotenoid levels did not change much, throughout the year. The values found for the photosynthetic activity of winter leaves were only slightly lower than those found in spring and summer leaves (Tab. 1).

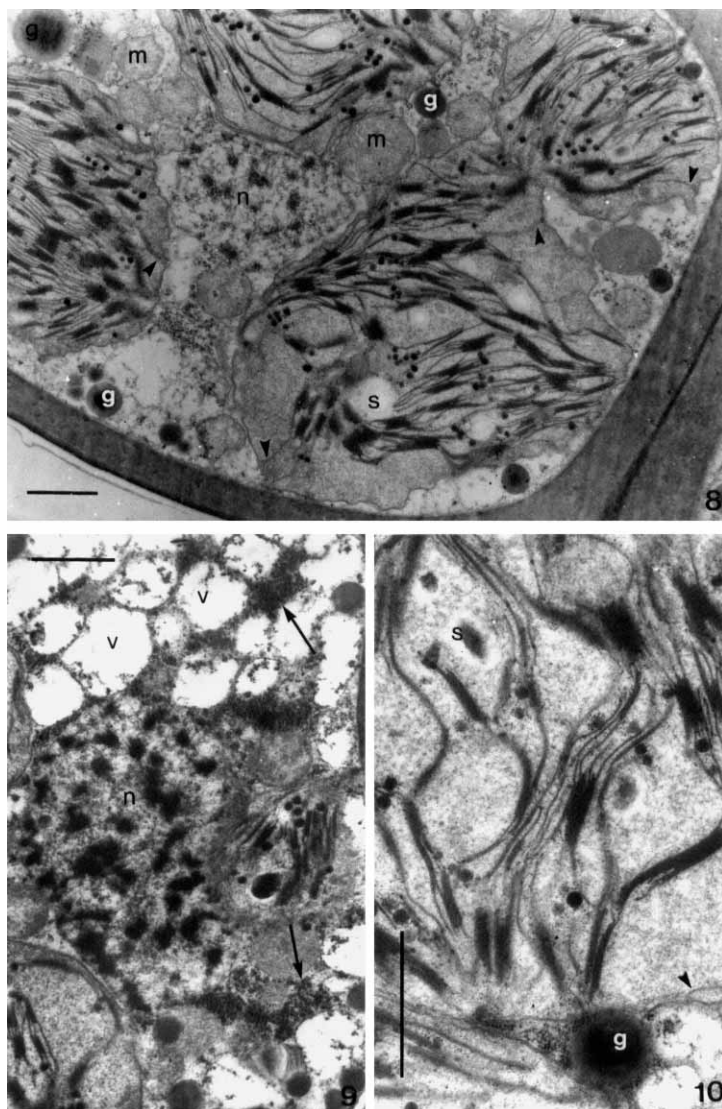


Figs. 6–7. Thin sections through lamellar cells of material collected in winter at ambient temperatures above zero. v = vacuoles, m = mitochondria, n = nucleus, s = starch, d = dictyosome. Fig. 6. The vacuoles of the marginal cell are filled with a granular material. Fig. 7. The thylakoid system of the chloroplasts has a normal ultrastructure. There are starch grains and plastoglobules in the chloroplast stroma. Bars = 1 μ m.

Discussion

Polytrichum formosum tolerates low winter temperatures excellently. The ultrastructure, the chlorophyll content and the photosynthetic activity of the lamellar cells, in winter, remained similar to those in spring and summer plants. Low night temperatures, during autumn, may already stimulate acclimation to low temperatures. In mid winter, the plants had already passed through repeated freezings and thawings and were well-acclimated to freezing temperatures. Our ultrastructural analyses showed that freezing the cells resulted in desiccation (dehydration). By applying cryo-scanning electron microscopy some authors detected desiccation and ice crystals in the intercellular spaces of frozen higher-plant leaves (PEARCE and ASHWORTH 1992, BALL et al. 2004). With our method of sample preparation, the deformed shape of the frozen moss cells and the collapse of the cell lumen unequivocally indicated their desiccation. There appears to be a genetically defined overlapping relationship between cold acclimation and dehydration (BROWSE and XIN 2001). It is assumed that the natural desiccation tolerance of mosses also explains the excellent results achieved with cryopreservation (BURCH 2003).

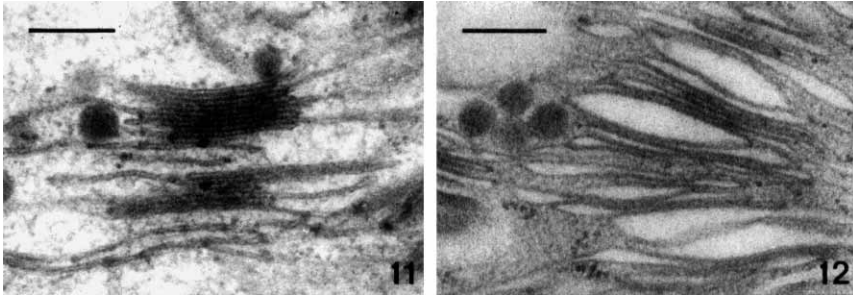
The desiccation in frozen plants of *Polytrichum* could, within a surprisingly short period of time, be annulled by exposure to above-zero temperatures. This could even happen



Figs. 8–10. Thin sections through the lamellar cells of frozen material collected during winter, at an ambient temperature of -5°C . n = nucleus, m = mitochondrion, v = vacuoles, g = lipid globule, arrowheads = invaginations of the inner chloroplast membrane, arrows = aggregated ribosomes. Fig. 8. Irregularly shaped chloroplasts with enlarged stroma. Fig. 9. Part of a cell with ribosomes compressed between the vacuoles. Fig. 10. Part of a chloroplast with compressed grana thylakoids and starch grains in the stroma. Bars = $1\mu\text{m}$.

during the preparation (fixation) of the tissue, when the temperature of the fixation medium was slightly higher (i.e. slightly above zero) than that of the frozen plant.

The unusual membranous invaginations of the inner chloroplast membrane have been found repeatedly in frozen chloroplasts, during the winter months (SENER and BECK 1984,



Figs. 11–12. Chloroplast grana of winter leaves at high magnification. Fig. 11. Compressed grana thylakoids from a frozen leaf ($-5\text{ }^{\circ}\text{C}$). Fig. 12. Grana thylakoids from thawed / defrosted leaf (above zero). Bars = $0.2\text{ }\mu\text{m}$.

Tab. 1. Total chlorophylls, total carotenoids, photosynthetic activity and respiration of *Polytrichum* leaves.

	Total chlorophylls ($\mu\text{g/g fr. wt.}$)	Total carotenoids ($\mu\text{g/g fr. wt.}$)	Photosynthetic activity ($\mu\text{mol O}_2/\text{g fr.wt./h}$)	Respiration ($\mu\text{mol O}_2/\text{g fr.wt./h}$)
spring	2.53 ± 0.11	0.55 ± 0.05	125.12 ± 4.88	40.28 ± 5
winter	2.17 ± 0.02	0.47 ± 0.05	115.13 ± 14.2	45.83 ± 4.17

WRISCHER et al. 2000, LJUBEŠIĆ et al. 2003). The increased thylakoid-free stroma regions in frozen *Polytrichum* lamellar cells facilitated the detection of these invaginations. What causes these structures is still unknown. A possible explanation would be a different lipid composition of the inner and the outer chloroplast membranes causing an unequal response to low temperatures (BROWSE and XIN 2001).

Acknowledgement

The authors would like to thank Dr. Volker Magnus for the critical reading of the manuscript.

References

- ATICI, Ö., NALBANTOĞLU, B., 2003: Antifreeze proteins in higher plants. *Phytochemistry* 64, 1187–1196.
- BALL, M.C., CANNY, M. J., HUANG, C. X., HEADY, R. D., 2004: Structural changes in acclimated and unacclimated leaves during freezing and thawing. *Funct. Plant Biol.* 31, 29–40.
- BROWSE, J., XIN Z., 2001: Temperature sensing and cold acclimation. *Curr. Opin. Plant Biol.* 4, 241–146.
- BURCH, J., 2003: Some mosses survive cryopreservation without prior pretreatment. *Bryologist* 106, 270–277.

- LICHTENTHALER, H. K., 1987: Chlorophylls and carotenoids. Pigments of photosynthetic membranes. *Meth. Enzymol.* 148, 350–382.
- LJUBEŠIĆ, N., WRISCHER, M., PREBEG, T., DEVIDÉ, Z., 2003: Chloroplast structure and function in wild-type and *aurea*-type leaves of the Japanese spindle-tree over their life span. *Acta Bot. Croat.* 62, 1–10.
- PAOLILLO, D. J. JR., REIGHARD, J. A., 1967: Ultrastructural features of some polytrichaceous moss leaves. *Bryologist* 70, 61–69.
- PEARCE, R. S., ASHWORTH, E. N., 1992: Cell shape and localization of ice in leaves of overwintering wheat during frost stress in the field. *Planta* 188, 324–331.
- ROUTABOUL, J.-M., FISCHER, S. F., BROWSE, J., 2000: Trienoic fatty acids are required to maintain chloroplast function at low temperatures. *Plant Physiol.* 124, 1697–1705.
- SAUTER, J. J., WISNIEWSKI, M., WITT, W., 1996: Interrelationships between ultrastructure, sugar levels, and frost hardness of ray parenchyma cells during frost acclimation and deacclimation in poplar (*Populus x canadensis* Moench "robusta") wood. *J. Plant Physiol.* 149, 451–461.
- SENER, M., BECK, E., 1984: Correlation of chloroplast ultrastructure and membrane lipid composition to the different degrees of frost resistance achieved in leaves of spinach, ivy, and spruce. *J. Plant Physiol.* 117, 41–55.
- SUNG, D.-Y., KAPLAN, F., LEE, K.-J., GUY, C. L., 2003: Acquired tolerance to temperature extremes. *Trends Plant Sci.* 8, 179–187.
- THOMAS, R. J., GRETHLEIN, A. J., PEROU, C. M., SCHEIRER, D. C., 1990: Translocation in *Polytrichum commune* (Bryophyta). III. Loading of sugars in source leaves. *Amer. J. Bot.* 77, 1574–1581.
- THOMAS, R. J., RYDER, S. H., GARDNER, M. I., SHEETZ, J. P., NICHIPOR, S. D., 1996: Photosynthetic function of leaf lamellae in *Polytrichum commune*. *Bryologist* 99, 6–11.
- THOMASHOW, M. F., 2001: So what's new in the field of plant cold acclimation? Lots! *Plant Physiol.* 125, 89–93.
- UEMURA, M., STEPONKUS, P. L., 1997: Effect of cold acclimation on the lipid composition of the inner and outer membrane of the chloroplast envelope isolated from rye leaves. *Plant Physiol.* 114, 1493–1500.
- WRISCHER, M., LJUBEŠIĆ, N., SALOPEK, B., 1998: The role of carotenoids in the structural and functional stability of thylakoids in plastids of dark-grown spruce seedlings. *J. Plant Physiol.* 153, 46–52.
- WRISCHER, M., LJUBEŠIĆ, N., MAGNUS, V., DEVIDÉ, Z., 2000: Structural and functional characteristics of overwintering blackberry leaves. *Acta Bot. Croat.* 59, 5–16.