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Routes of Solute Translocation and the Location of Water in Heteromerous Lichens Visualized with Cryotechniques in Light and Electron Microscopy*,**

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Abstract

Freeze-substitution of cryofixed probes for high resolution light microscopy (LM) and transmission electron microscopy (TEM) was applied to cultured *Trebouxia arboricola* (photobiont of *Xanthoria parietina*) and to the foliose macrolichen *Lobaria virens* (photobiont: *Dictyochloropsis reticulata*) with the aim of achieving a more natural specimen preservation than with conventional preparative procedures and for visualizing drought stress induced cellular alterations in *Lobaria virens* and its photobiont. Considerable loss of water and solutes from both the apoplast and the symplast lead to dramatic shrinkage and deformations of desiccated fungal and algal cells. The cytoplasm was very dense and the membranes were negatively stained in ultrathin sections of dry samples. Low temperature Scanning Electron Microscopy (LTSEM) of frozen-hydrated specimens was used for locating water in the photobiont and medullary layers of fully hydrated, wet *Lobaria virens*, *Peltigera canina*, *Parmelia sulcata* and *Xanthoria parietina*. Water was confined in all of these different taxa to the fully turgescent symplast and to the apoplast. The mycobiont-derived hydrophobic cell wall surface layer served as a "cuticle equivalent" in the thalline interior which remained gas-filled even in wet samples. The significance of these findings is discussed with regard to biochemical and ecophysiological parameters.

* Dedicated to Professor Dr. O.L. Lange, University of Würzburg, who, with his impressive ecophysiological studies on lichens of extreme climates, has triggered our interest in structural aspects of water relations in these fascinating microorganisms

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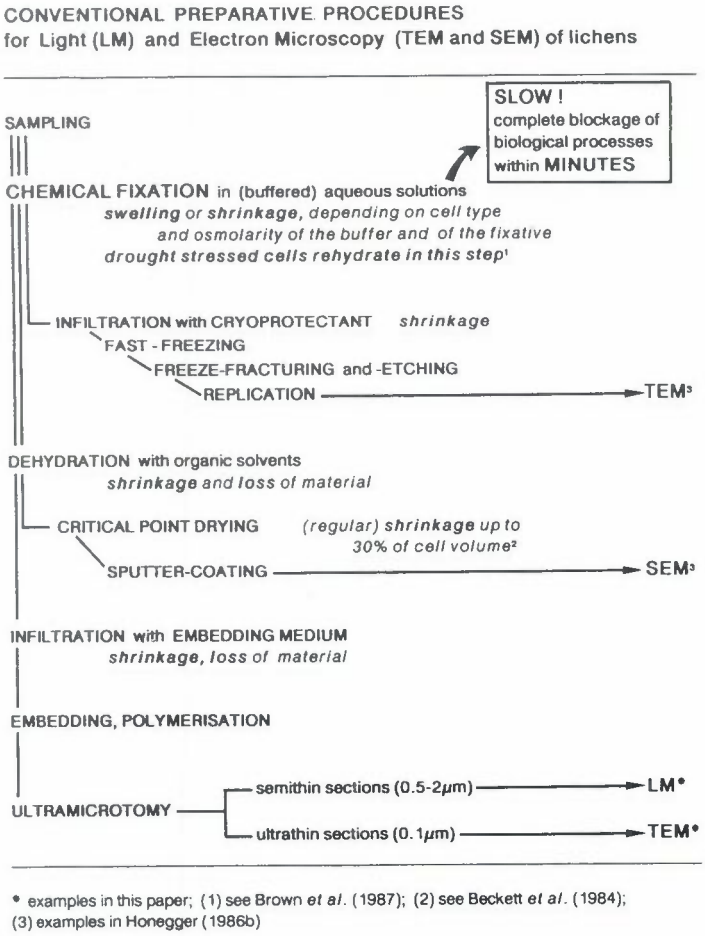
Keywords: lichens, cryofixation, freeze-substitution, freeze-fracturing, LTSEM, TEM, high resolution LM, desiccation, water saturation, solute translocation, *Lobaria virens*, *Peltigera canina*, *Parmelia sulcata*, *Xanthoria parietina*, *Dictyochloropsis reticulata*, *Trebouxia arboricola*

1. Introduction

Conventional preparative techniques for light and electron microscopy (Table 1) have considerably improved our understanding of the functional morphology of lichens in general and of the mycobiont-photobiont interface



* examples in this paper; (1) see Brown *et al.* (1987); (2) see Beckett *et al.* (1984); (3) examples in Honegger (1986b)

Table 1.

in particular. Despite numerous method-dependent artifacts, different types of wall-to-wall appositions and haustoria have been elucidated with these techniques (reviews: Honegger, 1991, 1992). Based on combined ultrastructural and histochemical studies, the most probable routes of solute translocation from the thallus surface to the interior and *vice versa*, especially at the mycobiont-photobiont interface, have been proposed. The wall surfaces of mycobiont and photobiont cells in the gas-filled thalline interior of stratified (heteromerous) lichen thalli were found to be covered by a thin, mycobiont-derived hydrophobic surface layer (Honegger, 1984, 1985, 1986a,b) which is likely to correspond to the hydrophobin layer on the surface of aerial hyphae of non-lichenized fungi (DeVries et al., 1993). Solutes were postulated to be passively translocated within the apoplastic continuum right underneath this thin, water-repellent coat or "cuticle equivalent," which keeps the wall surfaces of the gas-filled thalline interior free of water, a pre-requisite for successful gas exchange (reviews: Honegger, 1991, 1992).

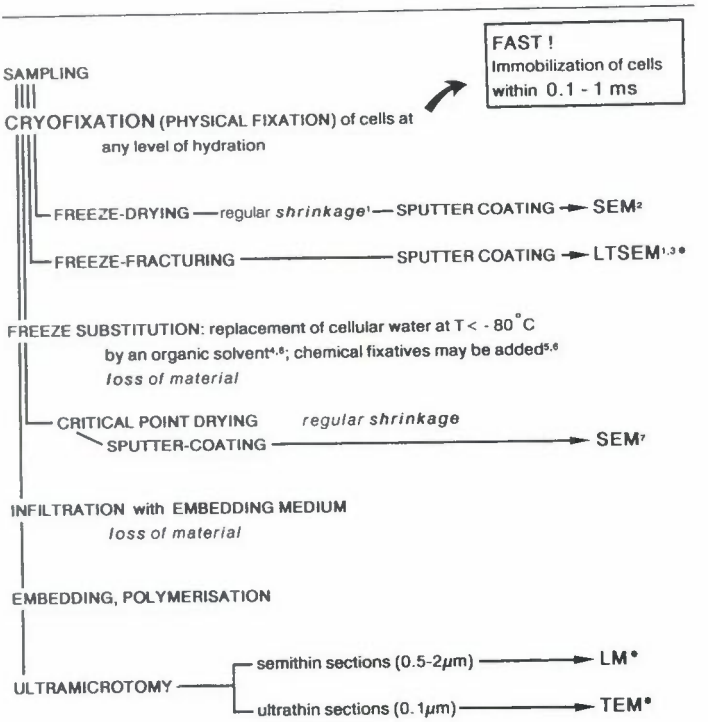
Many physiologists remain doubtful about this view of the location of water in internally stratified lichen thalli. This is based on the observed decline of gas exchange at high thalline water contents in the majority of lichen species investigated (reviews: Lange and Matthes, 1981; Kappen, 1988), usually referred to as a moisture dependent photosynthetic depression. As a result, the intercellular space of the algal and medullary layers was, and still is, assumed to be waterlogged in water-saturated thalli, thus hindering gas exchange (review: Richardson, 1992).

Lichens, as poikilohydrous organisms, are subjected to regular wetting and drying cycles, i.e. to fluctuations of thalline water contents between desiccation ($< 30\%$ water$\cdot$dw^{-1}) and saturation ($> 120\%$ water$\cdot$dw^{-1}). Conventional preparative techniques for light and electron microscopy (Table 1) do not allow the researcher to locate water directly within lichen thalli or to visualize drought stress induced structural alterations at the organismic or cellular level. The fate of cellular membrane systems in desiccated samples cannot be investigated with these procedures as the cells fully rehydrate in the first preparative step, i.e. the chemical fixation in an aqueous fixative (Brown et al., 1987). Attempts to chemically fix desiccated and fully hydrated 'control' cells of the *Trebouxia* photobiont of the lichen *Parmelia sulcata* in the vapour of osmium tetroxide (Brown et al., 1987) gave unsatisfactory results from a cell biological point of view. Only cryotechniques, i.e. physical fixation by ultra-rapid freezing, followed by either freeze-drying, freeze-fracturing or freeze-substitution (Table 2), allow the examination of cells at any level of hydration. Low temperature scanning electron microscopy (LTSEM) of freeze-fractured samples facilitates the location of water within lichen thalli

as has been shown by Beckett in Brown et al. (1987) and Scheidegger (1993). Moreover, cryofixation followed by freeze-substitution applied to plants and microbes gave an overall improved specimen preservation as compared with conventional preparative procedures (reviews: Hoch, 1991; Read, 1991). This type of investigation is missing in lichens.

Single cells, either in suspensions or in monolayers, are relatively easily cryofixed. Excellent results have been achieved with axenically cultured, freeze-substituted lichen photobionts of the genus *Trebouxia* (Sluiman and Lokhorst, 1988; Chida and Ueda, 1991, 1992a,b). Distinctly more difficult to prepare are tissues or tissue-like pseudoparenchyma as only the topmost layer

CRYOTECHNIQUES
for Light (LM) and Electron Microscopy (TEM, SEM and LTSEM)
of Lichens



• examples in this paper; (1) see Beckett *et al.* (1984); (2) examples in Honegger (1986b); (3) examples in Brown *et al.* (1987), Honegger (1993); (4) *e.g.* acetone, methanol; (5) *e.g.* osmium tetroxide, glutaraldehyde, acrolein; (6) see Hoch (1991); (7) as an alternative when LTSEM facilities are not accessible.

Table 2.

of approximately 20 μm depth can be adequately cryofixed by jet or plunge freezing, and not even with high pressure freezing can all problems related to optimal specimen preservation be solved. Additional problems arise in sampled with gas-filled intercellular spaces and in cell types with a high degree of vacuolization (review: Mendgen et al., 1991). Lichen thalli with conglutinate pseudoparenchyma in the cortex and gas-filled algal and medullary layers have been successfully examined in the frozen-hydrated state with LTSEM (Brown et al., 1987; Scheidegger, 1993), but not yet in semi- or ultrathin sections of freeze-substituted samples at either the high resolution light microscopy (LM) or transmission electron microscopy (TEM) level.

The present study aims to explore to what extent cryotechniques might be used in lichenology for (1) achieving a better specimen preservation, (2) visualizing drought stress induced structural alterations at the cellular and organismic levels, and (3) locating water within fully hydrated lichen thalli. The latter two aspects are of central interest in lichen biology.

2. Materials and Methods

Site of collection

Lobaria virens (With.)Laundon (syn. *L. laetevirens* Zahlbr.): on old *Quercus robur* L. above the gouffre (waterfall) in the forêt de Huelgoat in central Brittany (Departement Finistère), France in March, September and November 1992 and in March and July 1993. *Peltigera canina* (L.)Willd.: on moss-covered limestone above Murgtal, 560 m alt. (Kt. St. Gallen, Switzerland) in August 1993. *Parmelia sulcata* Taylor: on *Pyrus communis* L. in a historical garden (Abegg-Garten) in Zürich, November 1992. *Xanthoria parietina* (L.)Th.Fr.: originally from a concrete wall of the Station Biologique de Roscoff (Brittany; Departement Finistère), but now cultured on the flat roof of our institute in Zürich (method see Honegger, 1993a). The photobiont of this species, *Trebouxia arboricola* Puymaly (incl. *T. aggregata* (Archibald) Gärtner; see Friedl, 1989) was isolated and cultured under axenic conditions (isolate No. 83, stored under liquid nitrogen in our laboratory).

Conventional preparative techniques for high resolution LM and TEM

Samples were chemically fixed in 1.25% glutaraldehyde and 1.5% acrolein (Serva) on 0.03 M phosphate buffer, pH 7.1, for 2 hr at room temperature. After dehydration in a graded series of ethanol the samples were infiltrated and finally embedded in Historesin (Reichert-Jung); for a detailed description see Honegger (1987). Semithin sections were cut with glass knives, transferred

to a drop of water on a slide, allowed to dry on a hot plate, mounted with Entellan (Merck), and photographed with Nomarski optics on a Reichert Polyvar microscope.

TEM

Chemical fixation in 1.25% glutaraldehyde and 1.5% acrolein (Serva) in 0.033% phosphate buffer, pH 7.1, for 2–4 hr at room temperature was followed by postfixation in 2% buffered osmium tetroxide for 12–16 hr at room temperature. After dehydration in a graded series of acetone the samples were infiltrated and finally embedded in either LR White medium (London Resins) or a 1:1 mixture of Epon and Spurr's epoxy resin. Ultrathin sections were cut with a Diatom diamond knife, stained with uranyl acetate and lead mixture (Sato, 1967), and examined in a Hitachi H7000 transmission electron microscope.

Cryotechniques

LTSEM of frozen-hydrated specimens (method according to Beckett and Read, 1986). Either fully hydrated or dry samples, respectively, were mounted on the specimen holder of a BIO-RAD SP2000A Cryotrans System with commercially available white glue (Konstruvit). Mounted samples were plunge-frozen in subcooled liquid nitrogen, freeze-fractured and, without prior etching, immediately sputter-coated with an alloy of 80% gold and 20% palladium in the fracturing/coating chamber. The non-dedicated Cryotrans System is interfaced in our laboratory with a Hitachi S4000 scanning electron microscope. Samples were examined at an acceleration voltage at 20 kV.

Cryofixation of samples to be freeze-substituted

A BAL-TEC TFD 010 plunge-freezing device (BAL-TEC, Balzers) was used for ultra-rapid cryofixation of cultured *Trebouxia* cells and of either wet or dry thallus fragments of *Lobaria virens*. Fully hydrated samples had been incubated on moist filter paper in a Petri dish for a least 20 hr at 8–10° C. Specimens to be examined in the desiccated state were kept at room temperature (20–24° C) for at lest 4 hr in the laboratory. Fully hydrated thalli (approx. 224% of water·dw^{-1}) were bright green, dry ones (20–26% water·dw^{-1}) greyish-green. Dry weight was determined after 16 hr heating at 100° C. Fully hydrated samples were dissected and mounted in a closed chamber in water-saturated air (to avoid drying out of the specimens), then rapidly transferred to the plunge-freezing device in a tiny plastic box. Fully

hydrated or dry lichen thalli were dissected in stripes as thin as possible under a dissecting microscope which was interlinked with the closed chamber, and mounted on copper platelets of 7×7×0.2 mm. A thin film of water was used for fixing wet fragments to the copper platelet, but dry ones were mounted with a very thin film of hexadecene (Fluka). These copper platelets were inserted into the specimen holder of the plunge freezing device. Propane was used as a cryogen at −182° C; it was liquefied and subcooled by liquid nitrogen. The samples were plunge-frozen by pneumatic injection into the subcooled propane (velocity approx. 10 $m \cdot sec^{-1}$). The copper platelets carrying cryofixed samples were stored under liquid nitrogen until further processing.

Freeze-substitution

A Balzers FSU 010 freeze-substitution apparatus and a deep-freezer running at −85° C were used for the substitution process. Methanol gave better results as a substitution medium than acetone.

High resolution LM: 4% acrolein was added to the methanol. Probes were substituted at −85° C for 2 weeks, at −50° C for 24 hr and at −30° C for 24 hr. The substitution medium was then replaced by pre-cooled methanol and the probes were gradually warmed to room temperature. Infiltration and embedding in Historesin and semithin sectioning were performed as described above.

TEM: 4% acrolein was added to the substitution medium. The samples were substituted for at least 3 weeks at −85° C, then gradually warmed to −60° C. The substitution fluid was then replaced by pre-cooled methanol containing 2% acrolein and 2% osmium tetroxide. In this mixture the samples were kept at −50° C for at least 24 hr, then washed with pre-cooled methanol and incubated in pre-cooled methanol containing 2% osmium tetroxide at −30° C for 24 hr. The substitution fluid was replaced by pre-cooled methanol and the samples gradually warmed to room temperature. Acetone was used as an intermedium for samples to be embedded in a 1:1 mixture of Epon and Spurr's epoxy resin whilst samples to be embedded in LR white were directly infiltrated; both were processed as described above.

3. Results

Specimen preservation with conventional methods vs. cryotechniques

Trebouxia arboricola was used for testing the suitability of our cryofixing system and for establishing a freeze-substitution protocol. Conventionally fixed *Trebouxia* cells revealed the features normally seen with this procedure:

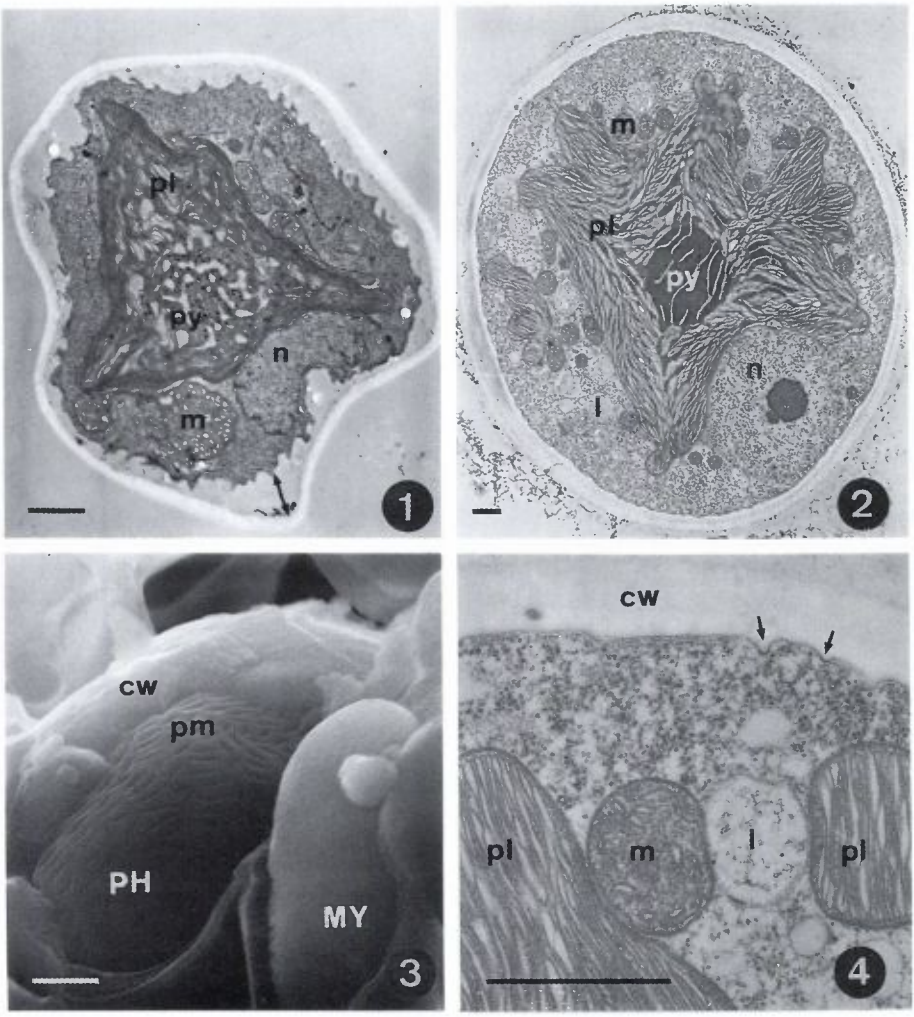
plasmolysis created an irregular gap between the cell wall and the protoplast; the cell organelles and especially the thylakoids showed more or less severe shrinkage artifacts due to hypoosmotic chemical fixation and dehydration (Fig. 1). Cryofixed, freeze-substituted cells and their organelles were preserved in the fully turgescent state (Figs. 2, 4). Particularly interesting were the tiny depressions which occur at irregular intervals in the otherwise tight plasmamembrane (Figs. 2, 4). It could be that they correspond to the rod-shaped depression that can be seen in the plasmamembrane of freeze-fractured symbiotic *Trebouxia* cells (Fig. 3). This substructure seems to be a peculiarity of *Trebouxia* spp. (Honegger, 1986b; Brown et al., 1987; Fiechter and Honegger, 1988). The function of these grooves remains unknown, but it is at least imaginable that they are involved in cell wall synthesis.

The foliose macrolichen *Lobaria virens* was chosen as a test organism as its photobiont, *Dictyochloropsis reticulata* (Tsch.-W.)Tsch.-W. (formerly described ast *Myrmecia reticulata* Tsch.-W.; Chlorococcales, Chlorophyceae) is, in the symbiotic state, one of the most difficult cell types in lichenology to be adequately preserved with conventional preparative procedures. Severe shrinkage artifacts invariably occur (e.g. Brunner and Honegger, 1985). These are even more dramatic in autosporangia and autospores than in normal vegetative cells (Figs. 5, 8).

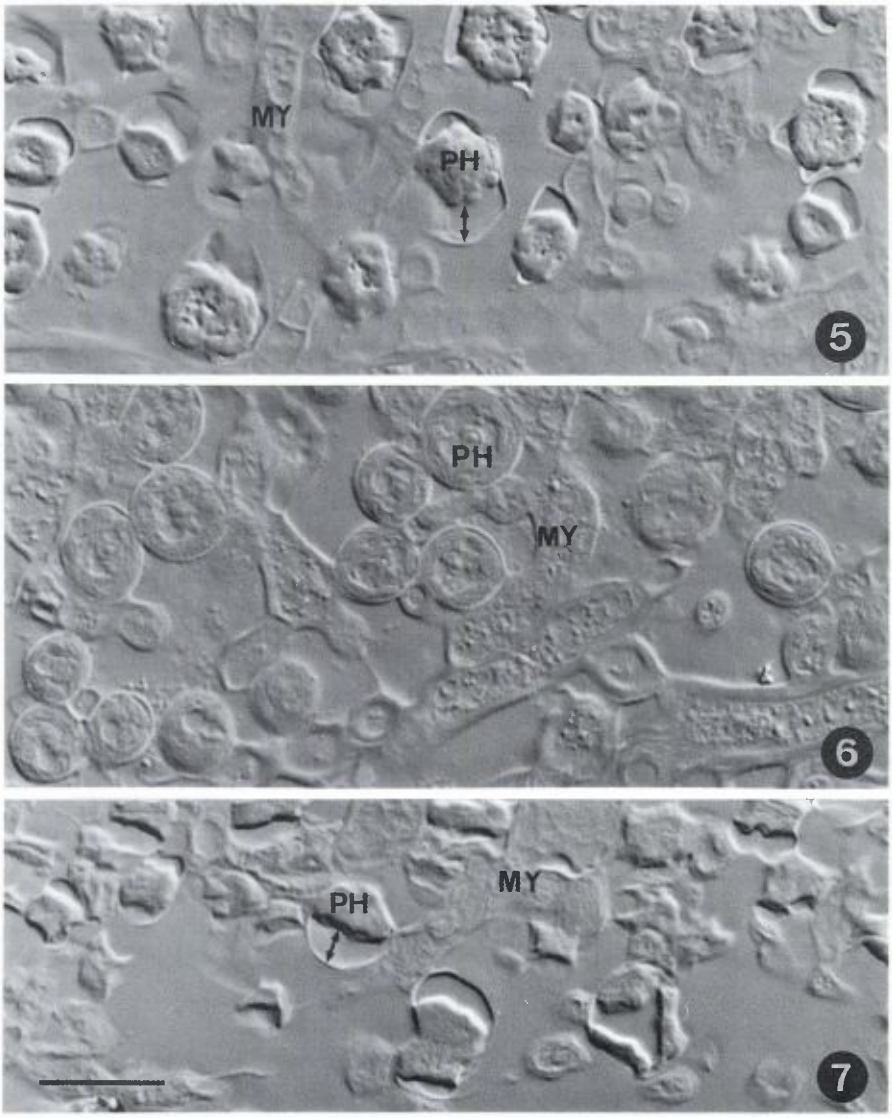
Considerable differences with regard to specimen preservation were noted between conventionally prepared (Figs. 5,8–9) and cryofixed, freeze-substituted samples (Figs. 6, 10–11). Chemically fixed, dehydrated cells appeared distinctly less turgescent than cryofixed ones. The cell organelles of the mycobiont and photobiont shrank considerably during conventional preparative procedures but retained their fully turgescent state and tight outlines after cryofixation and freeze-substitution (Figs. 5–6, 8–11).

Visualization of drought-stress induced structural alterations in Lobaria virens

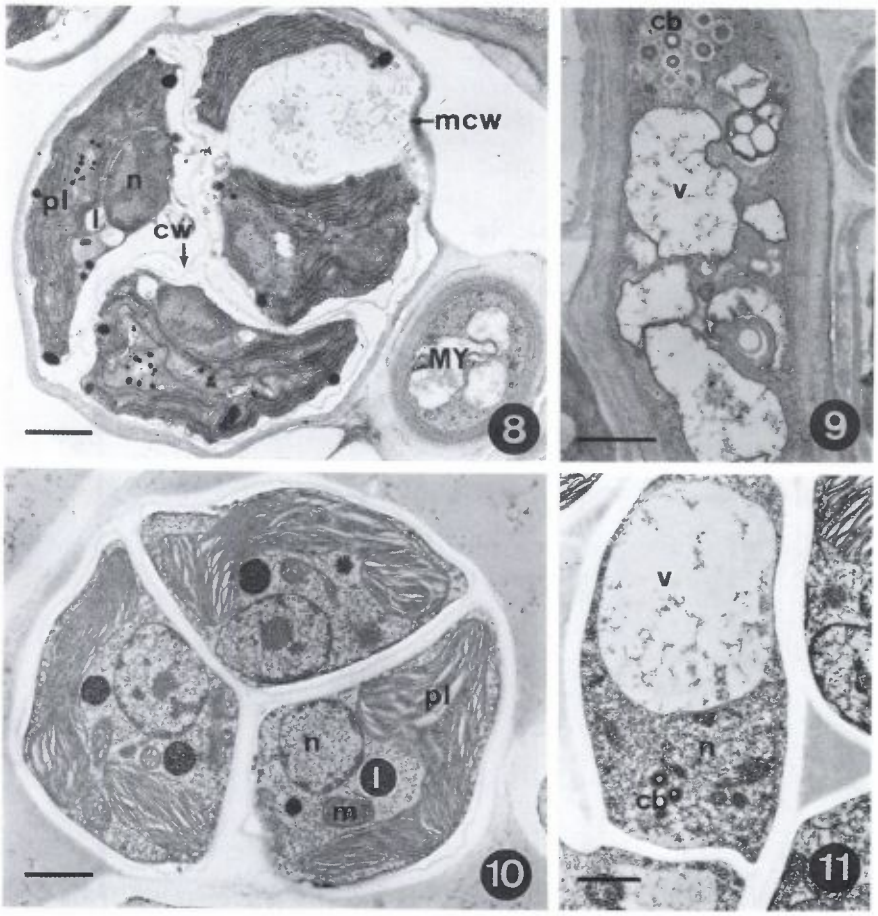
LTSEM of freeze-fractured samples in the frozen-hydrated state is certainly the most easy way to visualize the external morphology of fungal and algal cells in lichens at different levels of thalline hydration (Figs. 12, 14). In combination with high resolution LM of semithin and TEM of ultrathin sections of freeze-substituted probes (Figs. 6–7, 13, 15) the cellular and subcellular levels can be explored. Fungal and algal cells were fully turgescent in wet thalli (Figs. 6, 12–13, 20–21), but dramatically deformed in dry ones (Figs. 7, 14–15). The cytoplasm of fungal and algal cells was very dense in desiccated probes (20–26% water·dw^{-1}), and most of the membranes revealed negative contrast (best visible in the chloroplast). The thylakoids were very densely packed, but



Figures 1–4. Conventionally prepared vs. cryofixed *Trebouxia arboricola*.
(1) TEM of a conventionally prepared cell with severe shrinkage artifacts, best visible at the cell periphery (arrows).
(2) TEM of a cryofixed, freeze-substituted cell.
(3) LTSEM of a freeze-fractured, symbiotic *Trebouxia arboricola* cell (PH) in the thallus of *Xanthoria parietina* with characteristic depressions in the plasma membrane.
(4) Detail of the periphery of a freeze-substituted cell. Arrows point to depressions in the plasmamembrane. Abbreviations: (cw) cell wall; (l) lysosome; (m) mitochondrium; (n) nucleus; (pl) chloroplast; (pm) plasmamembrane; (py) pyrenoid. Bar = 1 μm.



Figures 5–7. LM (differential interference contrast) of semithin sections of the algal layer of *Lobaria virens*; photobiont (PH): *Dictyochloropsis reticulata*.
(5) Conventionally prepared specimen with dramatic shrinkage artifacts in the photobiont, best visible at the cell periphery (arrows).
(6) Fully hydrated sample (approx. 224% water·dw^{-1}) after cryofixation and freeze-substitution;
(7) Dry sample (approx. 20–26% water·dw^{-1}) after cryofixation and freeze-substitution. Arrows point to gaps between the cell wall and the plasma membrane, a method-dependent artifact (MY) mycobiont. Bar = 10 μm, same magnification throughout.



Figures 8–11. TEM of conventionally prepared and cryofixed, freeze-substituted *Lobaria virens*/*Dictyochloropsis reticulata*.

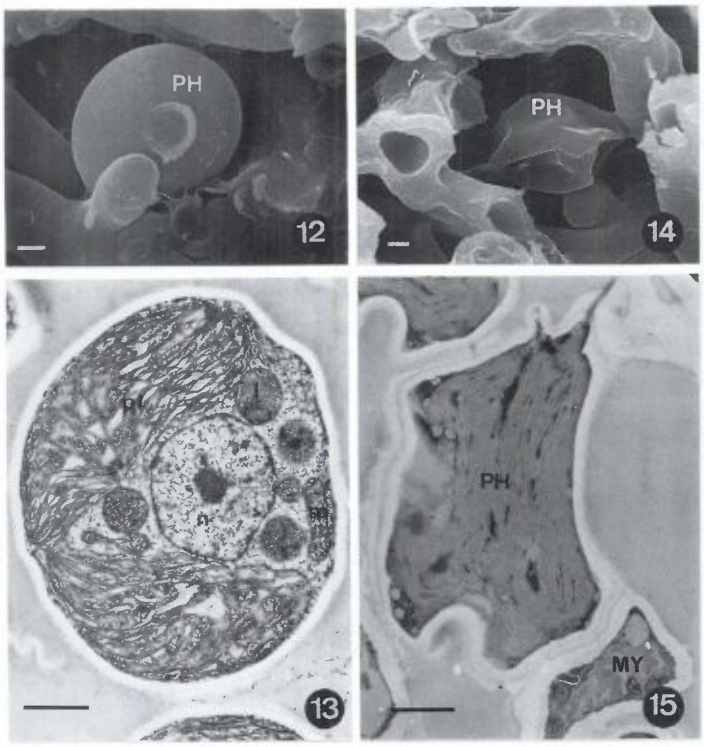
(8) Conventionally prepared autosporangium of the photobiont with severe shrinkage artifacts, best visible at the cell periphery.

(9) Conventionally fixed mycobiont hypha in the algal layer with irregularly deformed vacuoles.

(10) Freeze-substituted autosporangium of the photobiont with fully turgescent autospores.

(11) Freeze-substituted mycobiont hypha in the algal layer with a fully turgescent vacuole.

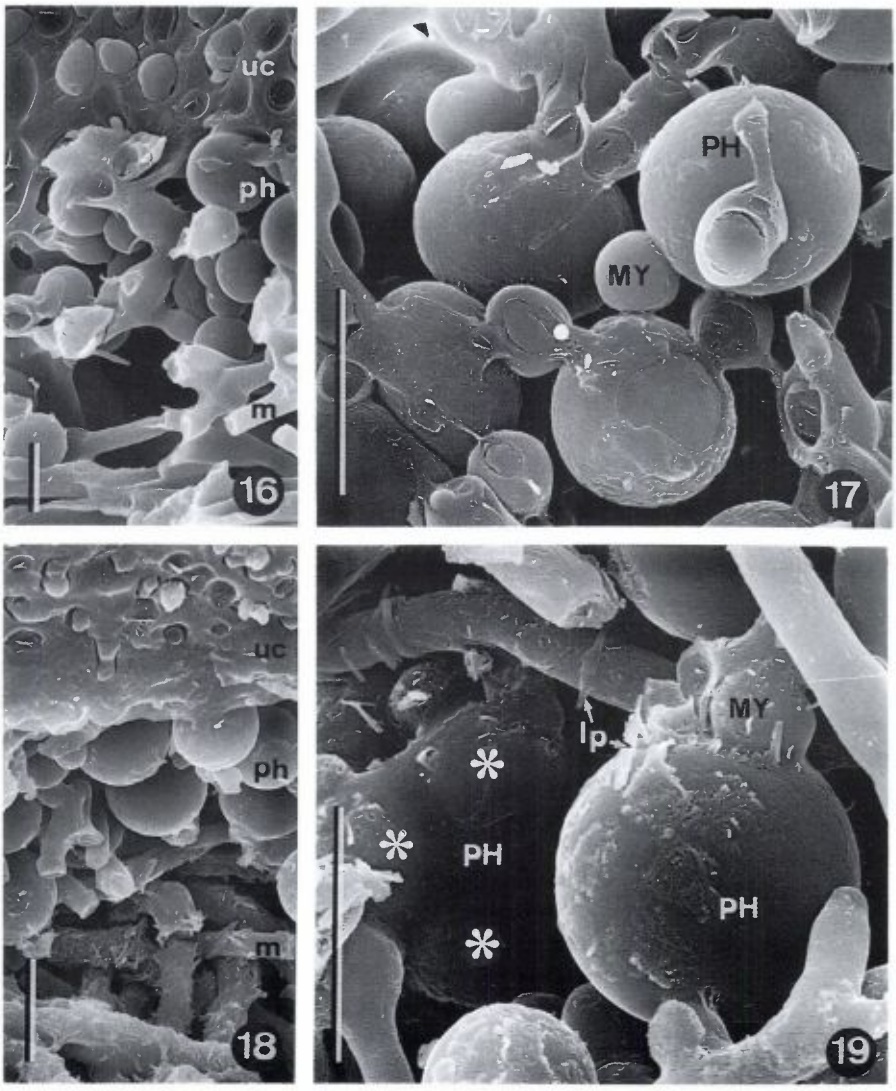
Abbreviations: (MY) mycobiont; (cb) concentric bodies; (cw) cell wall; (l) lysosome; (m) mitochondrium; (mcw) mother cell wall; (n) nucleus; (pl), chloroplast; (v) vacuole. Bar = 1 μm.



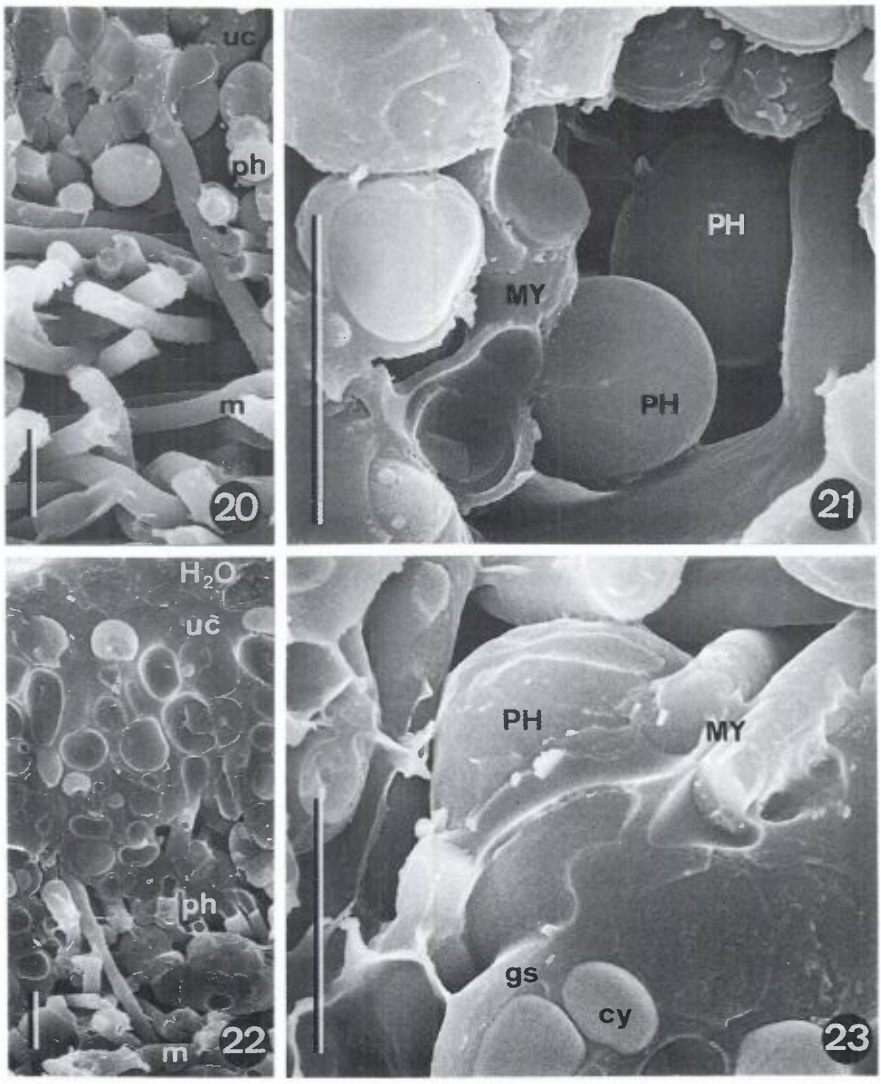
Figures 12–15. Visualization of drought stress induced structural alterations with cryotechniques applied to *Lobaria virens* and its photobiont, *Dictyochloropsis reticulata*.
(12), (14) LTSEM of cryofixed, freeze-fractured probes in the frozen-hydrated state.
(13), (15) TEM of cryofixed, freeze-substituted specimens.
(14), (15) dry probes ($\sim$ 20–26% water·dw^{-1})
Abbreviations: (l) lysosome; (m) mitochondrium; (MY) mycobiont; (n) nucleus; (PH) photobiont; (pl) chloroplast. Bar = 1 μm.

not deformed. In samples which had been embedded in epoxy resins (Fig. 15) the deformed cell wall, especially of the photobiont, was in close contact with the plasmamembrane, but a gap was observed between the inflated wall and the plasmamembrane in methacrylate-embedded material (Fig. 7). Considering the outlines of desiccated *Dictyochloropsis* cells in LTSEM (Fig. 14) one can conclude that the situation observed in methacrylate embedded material is an artifact related to this particular embedding medium.

The present findings are in accordance with published LTSEM data on



Figures 16–19. Location of water in fully hydrated macrolichens. LTSEM of frozen-hydrated, freeze-fractured thalli. (16), (17) *Xanthoria parietina*; photobiont: *Trebouxia arboricola*. No water is found on the cell wall surfaces of either mycobiont, or photobiont: (18), (19) *Parmelia sulcata*; photobiont; *Trebouxia impressa*. Asterisks on 3 autospores which are ensheathed by the degrading mother cell wall. Crystalline secondary metabolites are seen on medullary hyphae and on the fungal and algal partners in the photobiont layer. No water is found on these hydrophobic cell wall surfaces within the thalline interior. Bar = 10 μm.



Figures 20–23. Location of water in fully hydrated macrolichens. LTSEM of frozen-hydrated, freeze-fractured thalli. (20), (21) *Lobaria virens*; photobiont: *Dictyochloropsis reticulata*. No water is seen on the cell water surfaces of either partner. (22), (23) *Peltigera canina*; photobiont: *Nostoc punctiforme*. Colonies of cyanobacterial cells are embedded in their fully hydrated gelatinous sheath. The superficial water film (H_2O) is seen as ice in (22), but no water is found on the surfaces of either mycobiont, or photobiont in the thalline interior. Abbreviations: (cy) cyanobiont; (gs) gelatinous sheath) (lp) crystalline secondary lichen products; (m) medullary layer; (MY) mycobiont; (PH) photobiont; (ph) photobiont layer; (uc) conglutinate upper cortex. Bar = 10 μm.

drought-stress induced structural alterations in *Parmelia sulcata* (Brown et al., 1987) and *Xanthoria parietina* (Honegger, 1993b) and their photobionts of the genus *Trebouxia*. During the drying cycles water and solutes are lost from the symplast, thus leading to dramatic deformations of the cells. Part of the lost solutes have been recovered from the apoplast in the rewetting phase after drought stress events (MacFarlane and Kershaw, 1985; Dudley and Lechowicz, 1987).

Location of water in water-saturated thalli

Fully hydrated, wet thalli of foliose macrolichens with a superficial water film were mounted on the specimen holder and examined with LTSEM after plunge-freezing, freeze-fracturing and sputter-coating. Four taxa belonging to three orders of ascomycetes were investigated with special regard to the location of water in the thalline interior. *Parmelia sulcata* (Lecanorales; photobiont: *Trebouxia impressa*; Figs. 18–19), already studied by Brown et al. (1987), was re-examined and compared with *Xanthoria parietina* (Teloschistales; photobiont: *Trebouxia arboricola*; Figs. 16–17), *Lobaria virens* (photobiont: *Dictyochloropsis reticulata*; Figs. 20–21) and *Peltigera canina* (both Peltigerales; photobiont: *Nostoc punctiforme*; Figs. 22–23). In all of these distinctly different species, the medullary and photobiont layers were gas-filled even in the oversaturated state of hydration. Water was clearly confined to the symplasts and to the apoplastic continuum of the fully hydrated aerial hyphae and photobiont cells of the thalline interior, the hydrophobic cell wall surfaces remaining free of water (Figs. 16–23). The intercellular space of the photobiont and medullary layers does not serve as a water reservoir. These findings are in accordance with the observations of Brown et al. (1987) in *Parmelia sulcata*.

4. Discussion

Methodological aspects

Cryotechniques provide excellent tools for structural investigations of the poikilohydrous water relations in lichens; such studies have the potential of bridging ecophysiological and biochemical data with ultrastructural observations. In the present study only the two extremes of thalline hydration (the water saturated and the dry state, respectively) were examined in *Lobaria virens*, but all intermediate levels could be explored with these preparative procedures.

For many, but certainly not for all types of investigations, LTSEM of frozen-hydrated specimens will replace conventional preparative procedures for SEM, not only because this technique is less time-consuming from a preparative point of view, but mainly because it yields superior results with regard to the preservation of cells in a near-natural state (Beckett et al., 1984; Read, 1991). In contrast to LTSEM freeze-substitution of cryofixed specimens for high resolution LM and for TEM is not yet routinely applicable in multicellular botanical or mycological specimens. Problems arise partly with the poor reproducibility of the results and also with the long time required for the substitution process (several weeks in lichens). The present ultrastructural data of freeze-substituted mycobiont and photobiont cells of *Lobaria virens* are far from optimal, but they show that it is certainly worth the efforts of optimizing the technique for lichen samples. For a critical analysis of conventionally prepared specimens the comparison with freeze-substituted probes is very useful. However, even shrinkage artifacts of conventionally prepared samples may provide interesting information. The distinctly greater shrinkage in one partner of a symbiotic system than in the other, observed, e.g., in the *Dictyochloropsis* photobiont of *Lobaria virens*, may reflect different osmolarities in comparison with the associated mycobiont.

Location of water in fully hydrated thalli

The present LTSEM data on the location of water in the photobiont and medullary layers of taxonomically diverse macrolichens support the view that water does not accumulate in the intercellular space of fully hydrated thalli, but is confined to the cells and to the apoplast as proposed in earlier investigations (reviews: Honegger, 1991, 1992). A decline of CO_2 exchange at high levels of thalline hydration as reported for *Parmelia sulcata* and *Peltigera canina* (review: Lange and Matthes, 1981) is therefore unlikely to be due to waterlogged intercellular spaces. It remains to be investigated whether this decline in CO_2 uptake really reflects a depression of photosynthetic activity. Light transmission through the cortical layer was found to be highest in fully hydrated thalli (Ertl, 1951). Laboratory and field studies on chlorophyll fluorescence in a range of lichen species clearly show that the highest energy transfer between the light-harvesting pigment complex and photosystem II occurs at high thalline water contents (Bilger et al., 1989; Schröter and Kappen, 1991). Experimental data are missing, but it is at least imaginable that the respiration rates of the quantitatively predominant fungal partner of macrolichens are also highest in the fully hydrated state. If so, there should be enough CO_2 in the thalline interior to allow maximum photosynthetic

activity without supply from the thalline exterior. Recycling of respiratory CO_2 within the thallus could be the reason for the observed decline in CO_2 uptake at high levels of hydration in taxa which have no conglutinate lower cortex such as *Peltigera* spp. or *Teloschistes lacunosus* (Palmer and Friedman, 1991). At least in fully hydrated *Peltigera canina*, the medullary layer was found not be be waterlogged and thus is unlikely to act as a diffusion barrier. It could well be that water-saturated lichen thalli with upper and lower cortical layers resemble "bottle gardens," the fully hydrated gelatinous matrix of the cortical layers being more or less gas tight. If so it would be impossible to measure from outside what is actually going on in the thalline interior as it is the quantitatively predominant heterotrophic fungal partner of the lichen symbiosis which creates the leaf-like morphology and forms the conglutinate cortical layers at the thalline periphery. The photoautotroph is hidden within this morphologically complex fungal structure and may be similar to algal endosymbionts of invertebrates where it is impossible to estimate the photosynthetic activity by measuring the CO_2 level outside the C-heterotrophic host.

Various investigators pointed to the difficulties in defining water saturation in lichens (reviews: Kershaw, 1985; Kappen, 1988). The present data indicate that the thalline water storage capacity is relatively well defined, i.e. restricted to the cells and cell walls, but the determination of the fresh weight (with/without superficial water film) is a difficult task. Quantitative determinations of the intercellular space, e.g. by porosimetric measurements, are unlikely to provide information on the water storage capacity as assumed by Valladares and Ascaso (1993) in *Umbilicaria* spp., but rather on the dimensions of the gas-filled space of the thalline interior.

Drought-stress induced structural alterations and the flux of solutes between the mycobiont and photobiont

Investigations of drought stress induced structural alterations at the mycobiont-photobiont interface give a valuable insight into translocation processes between the partners of the lichen symbiosis. The dramatic loss of water and solutes from the apoplast and symplast of both partners as observed with LTSEM, high resolution LM and TEM techniques of desiccated samples support the view that the regularly occuring wetting and drying cycles are the main driving forces of fluxes of solutes not only between the mycobiont and photobiont, but also from the thalline surface to the interior and *vice versa* (reviews: Honegger, 1991, 1992). The fungal partner does not protect the photobiont from desiccation; both cell types are obviously

fully adapted to extreme fluctuations of their water contents. All of these major fluxes are spatially confined to the apoplast, i.e. the space between the plasmamembrane and the hydrophobic cell wall surface layer in the medullary and photobiont layers, and the cell wall and gelatinous matrix in the conglutinate pseudoparenchyma of the cortical layers. The chemical composition of apoplastic fluids remains to be investigated. Analysis of leachage fluids after drought stress events reveal a broad spectrum of soluble carbohydrates of photobiont and mycobiont origin (MacFarlane and Kershaw, 1985; Dudley and Lechowicz, 1987). Evidently there is not only a one way translocation of soluble carbohydrates from the photobiont to the mycobiont, but also a passive transport, driven by capillary forces, of mycobiont-derived fungal metabolites (soluble carbohydrates and possibly also soluble forms of secondary metabolites) to the photobiont in the rewetting phase after drought stress events. It could be that these drought stress induced, passive fluxes of solutes back and forth in the apoplastic continuum between the partners are essential for the functioning of the lichen symbiosis. The majority of lichens of temperate climates die off or disintegrate when being kept under continuously moist conditions.

The detailed analysis of metabolic interactions in taxonomically diverse lichen mycobionts and photobionts with combined ecophysiological, biochemical and ultrastructural methods will be a fascinating task for future investigations. Cryotechniques in light and electron microscopy, now applicable to lichens, provide some of the tools which have long been waited for.

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