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Study of lichens with different state of hydration by the combination of low temperature scanning electron and confocal laser scanning microscopies

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Summary The use of techniques such as low temperature scanning electron microscopy (LTSEM) and confocal laser scanning microscopy (CLSM) allows the study of lichen thalli in different states of hydration and also near the natural state. The spatial organization of desiccated thalli, with reduced, very compact algal layers, is different from that of hydrated ones. Sometimes, the observation with transmission electron microscopy (TEM) of photobiont pyrenoids from desiccated thalli reports pyrenoids with a central part of a weak stained matrix lacking pyrenoglobuli, named “empty zones”. “Empty zones” are not distinguishable with LTSEM and do not present immunolabelling with rubisco antibody in TEM. These zones could be originated by an expansion process during rehydration produced in chemical fixation.

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Introduction

Lichens are the symbiotic phenotype of nutritionally specialized fungi that acquire, in an ecologically obligate mutualistic symbiosis, fixed carbon from a population of green algal or cyanobacterial cells (photobiont) [9]. As poikilohydric microorganisms, lichen-forming thalli and their photobionts have no means of controlling their water relations. So, the water content of the lichen thallus is strongly influenced by the water status of the surrounding environment [9, 12]. Under changing environmental conditions, they adapt to frequent and rapid changes of water content. The responses to the drying of the whole lichen thallus are, of course, a combination of those of the algal and fungal components (mycobiont). To study desiccated-induced changes in lichens, biochemical and ultrastructural studies have to be carried out, with emphasis (where possible) on the effects of

desiccation on the individual components of the lichen association [4].

The comparative study of the photobionts at ultra-structural level, of hydrated and desiccated thallus, presents a lot of problems. The preparation, with conventional techniques, of desiccated thalli for transmission electron microscopy (TEM) involves immediate rehydration of the thalli, which implies that it is impossible to research all of the morphological and ultrastructural features depending on the desiccated state. Our current research focuses in *Trebouxia* photobiont. Within the photobiont, pyrenoids and more specifically, the pyrenoglobuli, have been described to be influenced by hydric status [1, 2, 5, 6]. Besides, pyrenoids are of major physiological importance due to the presence of protein rubisco [3].

Several microscopy techniques have been used to investigate new aspects about the differences among thalli with various hydrated states. TEM has been used to observe

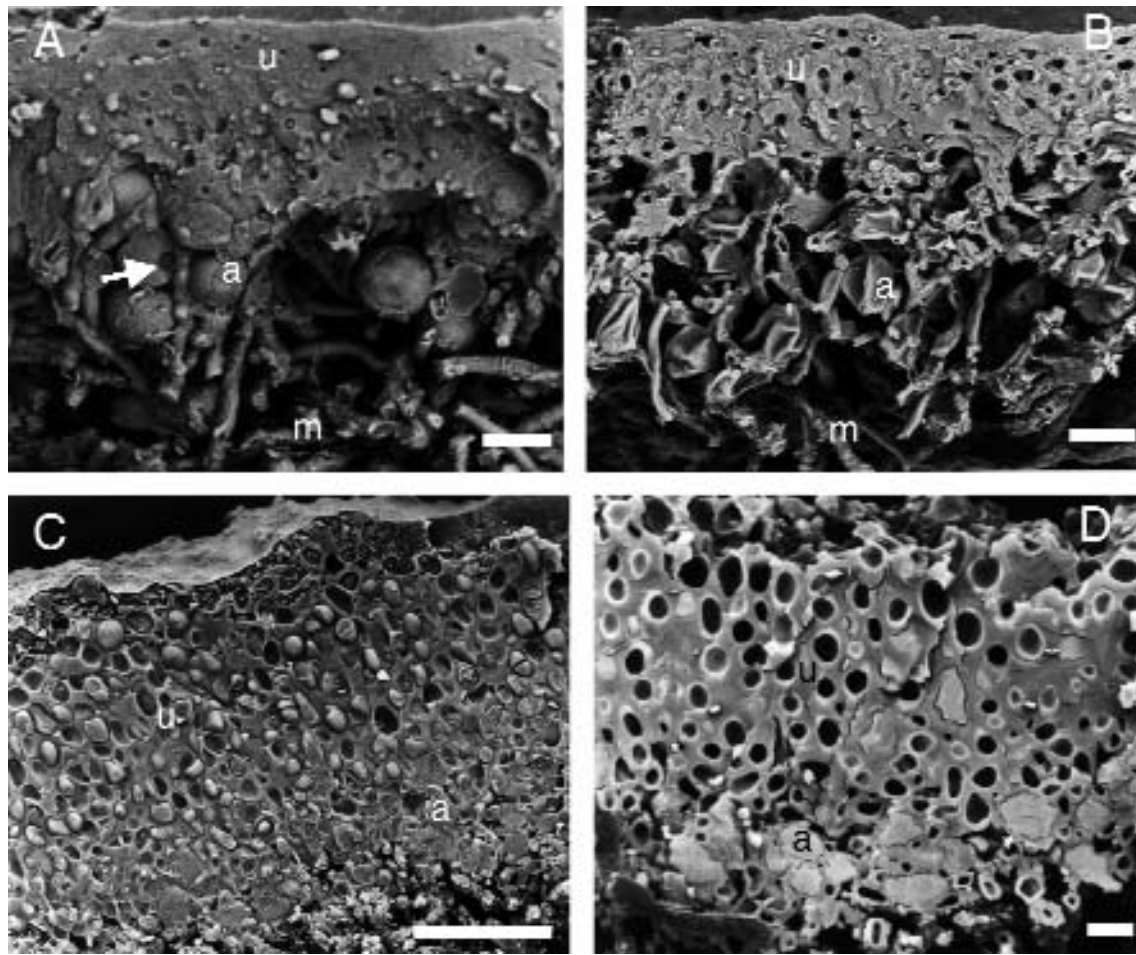
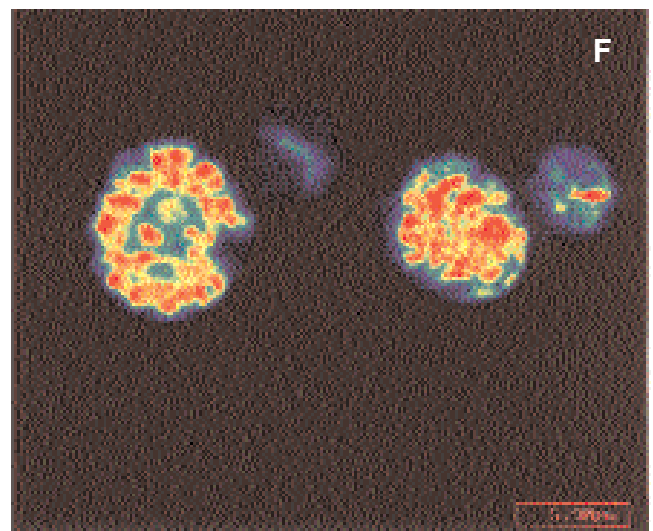
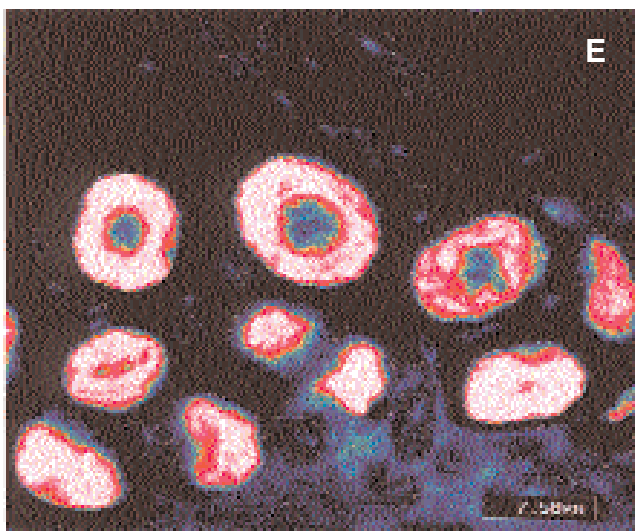
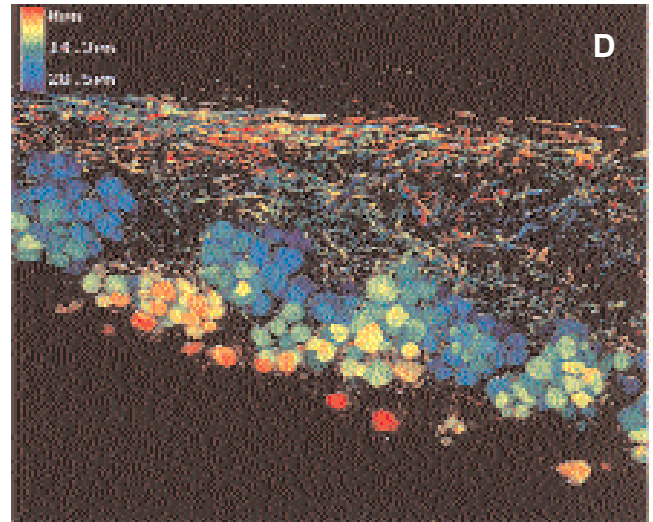
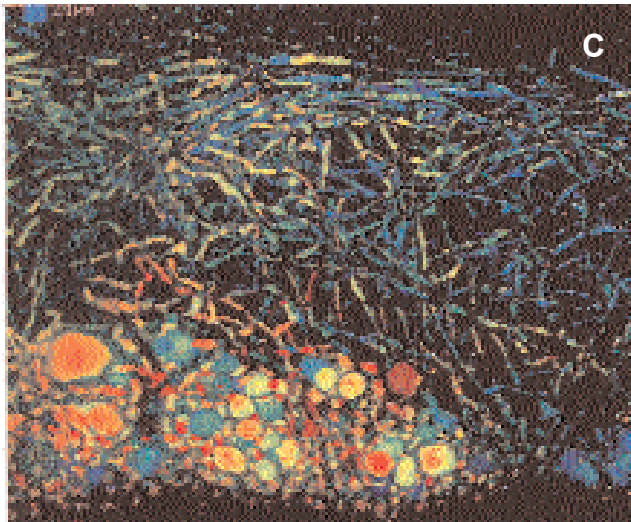
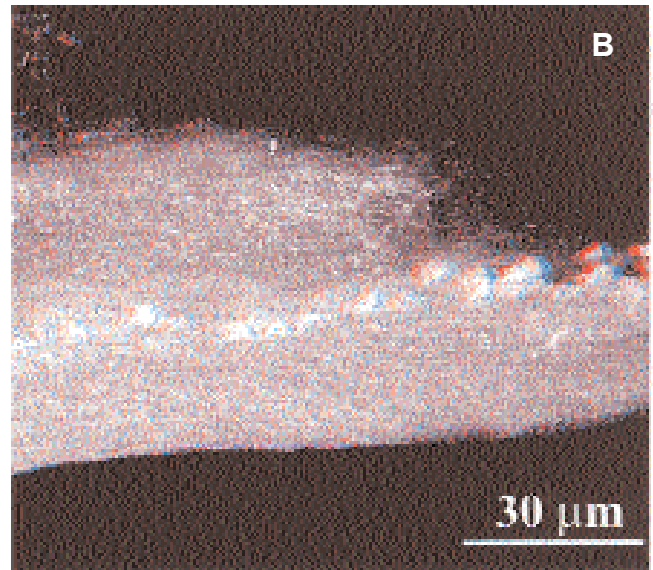
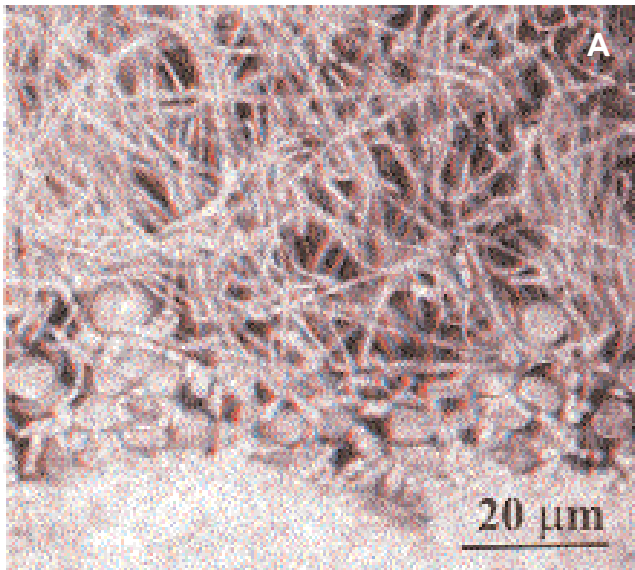


Fig. 1 Low temperature scanning electron microscopy (LTSEM) micrographs of *Parmelia omphalodes* and *Lasallia hispanica* thalli. Bar 10 μm . (A) Upper cortex (u), photobiont layer (a) and medulla (m) of *P. omphalodes*, hydrated thallus. Arrow indicates haustorium. (B) Upper cortex (u) and photobiont layer (a) of *P. omphalodes*, desiccated thallus. (C) Upper cortex (u) and algal layer (a) of *L. hispanica*, hydrated thallus, with some photobiont cells in the transversal section. (D) Upper cortex (u) and algal layer (a) of desiccated *L. hispanica*

both ultrastructural features which differ from one state to another and the presence of rubisco in the pyrenoid by immunolabelling. Low temperature scanning electron microscopy (LTSEM) and confocal laser scanning microscopy (CLSM) have been used to observe the thallus and the photobiont without having to carry out special processes similar to those needed for TEM. In the past few

years LTSEM has been found to be a valuable method to investigate structural changes of thalli with different water contents [8, 10, 11, 14–17]. CLSM has been employed for the first time in this kind of lichen study. The combination of high power resolution of laser and the possibility of obtaining three-dimensional images makes it possible to analyze spatial relationships among symbionts.

Fig. 2 [Page 253](A), (B). Three-dimensional projection images of acridine orange stained *Parmelia omphalodes* thallus, obtained by processing 20 confocal sections (at 0.6 μm intervals). Argon laser (488 nm with Band pass 515–545 nm filter). Upper cortex (u), algal layer (a), medulla (m). (A) Hydrated thallus. (B) Desiccated thallus. (C) and (D) Three-dimensional images of acridine orange stained *Lasallia hispanica* thallus, presented as deep colour coding (red: 0 μm , green: 12 μm , and blue: 24 μm). These images were obtained processing 24 and 28 confocal sections respectively (at 1 μm intervals). Laser conditions as in (A) and (B). (C) Hydrated thallus. (D) Desiccated thallus. (E) Single confocal section (false colour) of algal layer from hydrated *Parmelia omphalodes* thallus stained with acridine orange. Arrows indicate pyrenoid. (F) Single confocal section of photobiont cells of hydrated *P. omphalodes* thallus obtained with autofluorescence using Argon (488 nm) laser and long pass 515 nm filter (false colour). Arrows indicate tubules



Materials and methods

Plant material *Lasallia hispanica* (Frey) Sancho & Crespo and *Parmelia omphalodes* (L.) Ach. were collected from Sierra de Guadarrama (Madrid, Spain), in a dry state.

Low temperature scanning electron microscopy We hydrated the specimens which were to be investigated in the fully hydrated state with distilled water and maintained them in an exsiccator with 100% relative air humidity at 20°C, over a period of 24 h in continuous light. We fully hydrated and allowed to dry at room temperature samples which were to be studied in the desiccated state. Water content of hydrated samples was 130–140% their dry weight and 20–40% their dry weight in the dehydrated state. We removed superficial water films from fully hydrated samples by shaking them.

We mounted specimens with O.C.T. compound (Gurr) and fixed them mechanically on specimen holder of Oxford CT1500 Cryotransfer System. We plunge-froze the specimens in subcooled liquid nitrogen and then transferred them to the preparation unit via an air-lock transfer device. We fractured the sample in the preparation unit and transferred it directly via a second air lock to the microscope cold stage where we warmed the sample at –90°C and etched for 2 min. Then we transferred it to a sample preparation unit and carried out a gold sputter-coating after raising the pressure to 4×10^{-1} torr. After coating the specimens were transferred again to the Zeiss scanning electron microscopy cold stage. We investigated specimens on the microscopy cold stage at –135°C and with an accelerating voltage of 15 kV.

Confocal microscopy Lichen samples were mounted in agar blocks, and vibratome sections 30–40 µm thick were cut and examined using confocal laser scanning microscope model LSM310-Zeiss. An excitation wavelength of Argon (488 nm) laser was used and the emission was collected with a long pass filter of >515 nm. The three-dimensional (3D) images were made up of several (16–28) confocal sections at 0.5–1 µm increments through the sample, by the computer-assisted microscopy. Three-dimensional reconstruction and animation software with multiple rendering models was applied for the visualization of lichen thallus structures. Almost all samples were stained with acridine orange (5 min, 50 mg/ml

aqueous solution) and emission fluorescence was detected passing light with a 515–545 nm band-pass filter.

TEM and immunocytochemical study For conventional observation, samples of both species in a desiccated state were fixed, dehydrated, and embedded in Spurr's resin as in Ascaso et al. [1]. In immunocytochemical studies samples in a desiccated state were fixed in 2.5% (v/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2.5 h at 4°C. Afterwards they were dehydrated at 4°C and embedded in LR-White according to Ascaso et al. [3]. Immunolabelling was carried out with antirubisco antiserum of *Euglena gracilis*, provided by Dr. Joaquín Moreno (Biochemistry Department, Valencia University), according to Ascaso et al. [3]. Ultrathin sections were poststained with uranyl acetate followed by lead citrate [13] and observed in Phillips 300 transmission electron microscope.

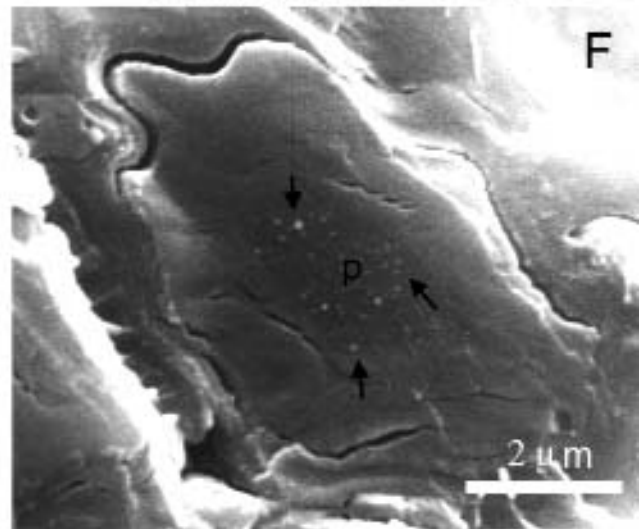
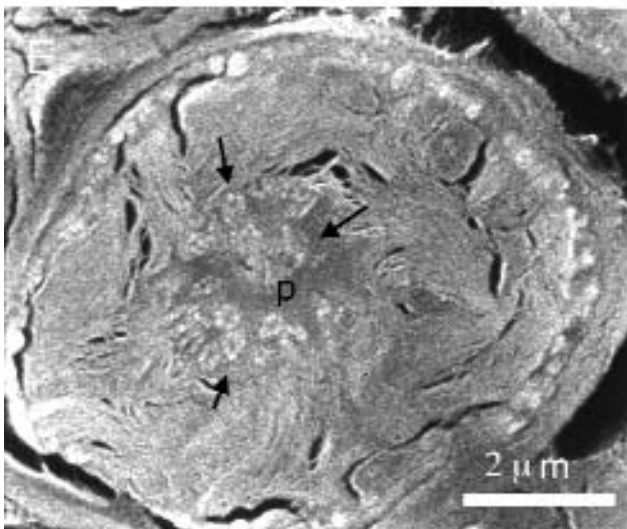
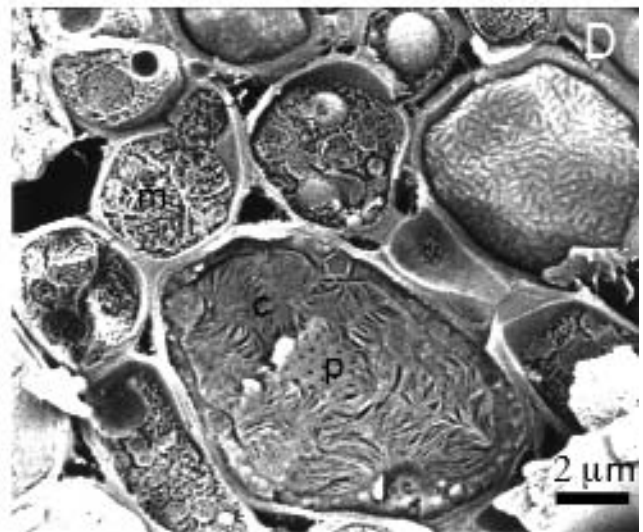
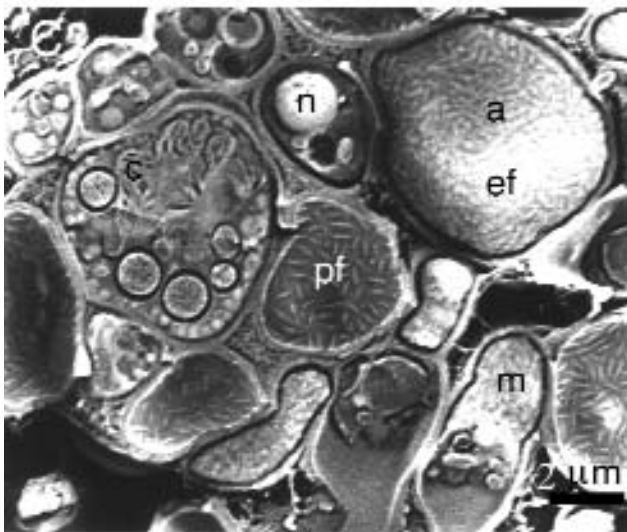
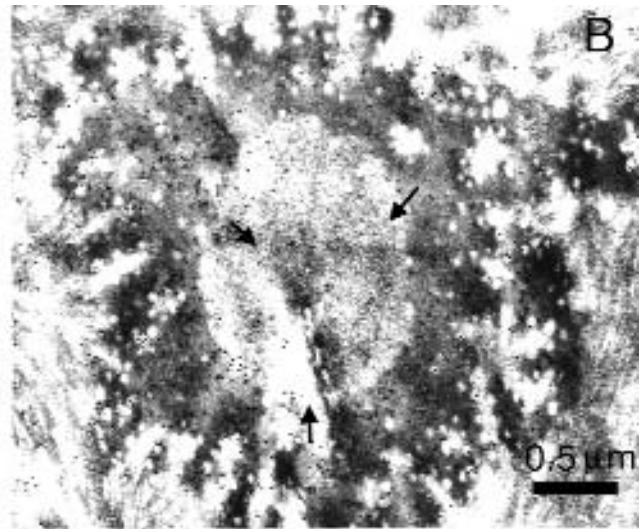
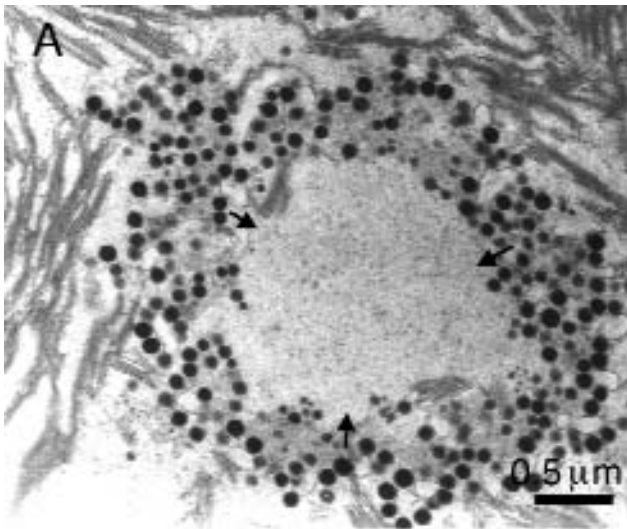
Results

General aspect of the thalli A research in hydrated and desiccated thallus with LTSEM has been done in both species. This microscopy shows the different organization of the thallus in each state. In the hydrated state, photobiont cells from *P. omphalodes* are roundish- or globular-shaped and separated from the rest of the cells (Fig. 1A). In the desiccated state (Fig. 1B), collapsed photobiont cells are partially hidden among the mycobiont cells.

Fig. 1C and 1D are images with LTSEM, of the hydrated and desiccated *L. hispanica* thalli with a lot of cells in the transversal section. Note that, in the algal layer in the desiccated thallus, the cells are densely packed with several photobiont cells in the cortex.

The use of CLSM in the study of thalli *Lasallia hispanica* and *P. omphalodes* makes it possible to obtain three-dimensional images of the relationship between the photobiont and the mycobiont in the thallus. Figure 2A is a three dimensional reconstruction of *P. omphalodes* hydrated thallus, stained with acridine orange; it is a stereo image which can be seen with three-dimensional color glasses. Note how, in this hydrated thallus, the cells of the algal layer are apparently independent as they are widely spaced. In the desiccated thallus (Fig. 2B), the spatial organization of the photobiont in

Fig. 3 [Page 255] (A) Transmission electron microscopy (TEM) of photobiont pyrenoid of *Lasallia hispanica* with “empty zones” (arrows). (B) Immunogold-TEM localization of rubisco in the pyrenoid of *Trebouxia* photobiont of *Parmelia omphalodes*. Arrows indicate “empty zones”. (C)–(F) Micrographs obtained by LTSEM of freeze-fractured *Lasallia hispanica* thalli. Algal cell (a), chloroplast (c), exoplasmic fracture (ef), protoplasmic fracture (pf), mycobiont cell (m), nucleus (n), pyrenoid (p). (C), (D) Transversal section and plasmalemma faces of photobiont and mycobiont cells from algal layer of the hydrated thallus. White arrows indicate lipidic globules. Black arrow indicates concentric body. (E) Photobiont cell of hydrated thallus fixed with 3% glutaraldehyde and osmium tetroxide. Arrows indicate pyrenoglobuli. (F) Photobiont cell of desiccated thallus fixed with osmium tetroxide vapours. Arrows indicate pyrenoglobuli



the algal layer is different from that in the hydrated thallus. Photobiont cells appear much closer to the cortex than in the hydrated state. Cells in the algal layer do not seem widely spaced as are in the hydrated state. Figures 2C and 2D are three-dimensional images of hydrated and desiccated *L. hispanica* with the deep coding mode of image presentation. The different depths are shown with different colors that are described on the scale of the left corner of the figure. In this species which have the algal layer looser, in the desiccated state photobiont cells tend to be more compact.

Photobiont cell We focused our work on the ultrastructural observation of the photobiont by using a variety of techniques. Figure 2E is an image of photobiont cells with CSLM; the use of false color makes the pyrenoid in the center of the chloroplast very distinguishable. The autofluorescence of photobiont cells in hydrated thalli has also been studied and this reveals a chloroplast by the autofluorescence of the chlorophylls with a central pyrenoid, distinguishable because it lacks autofluorescence (Fig. 2F). Black arrows signal several penetrations of fluorescence in the pyrenoid.

The study of desiccated thallus by TEM shows, in some occasions, photobiont cells with pyrenoids that in their central part appear not to be so dark, and without pyrenoglobuli (Fig. 3A). We have named these pyrenoglobuli-free areas of weakly-stained matrix "empty zones".

To know the nature of these "empty zones", we immunolabelled the rubisco in the photobiont cells that present them. Empty zones lack immunolabelling, which is however present in the rest of the pyrenoid (Fig. 3B).

To clarify if the origin of the "empty zones" is related to the conventional preparation for TEM, we carried out the study of the photobiont with LTSEM in the hydrated and desiccated state. With this microscopy we obtained images that show several ultrastructural details of the photobiont cells, which can be compared with images of TEM. In Fig. 3C there are photobiont and mycobiont cells in the algal layer of the hydrated thallus. In the photobiont cells it is possible to see specific plasmalemma invaginations (plasmic fracture face, exoplasmic fracture face), chloroplast thylakoids, lipidic globules with marginal distribution and other cytoplasmic organules. Plasmolysis is not appreciable. In Fig. 3D, pyrenoid is distinguishable in the center of the chloroplast as a differentiated structure with a series of holes that could correspond with the tubules observable by TEM (Fig. 3A). Besides, it is possible to observe by LTSEM pyrenoglobuli in samples fixed with osmium tetroxide (Fig. 3E). Images of pyrenoid and pyrenoglobuli are very similar to their images obtained by TEM. Pyrenoglobuli appear with periferical localization in the pyrenoid.

LTSEM allows a better observation of the photobiont ultrastructure when it is applied to a hydrated thallus than when applied to a desiccated thallus. The collapsed state produced by the dehydration makes the cell more fragile, and obtaining sectioned photobiont cells becomes difficult.

We made an effort, however, to obtain maximum details, at a cytological level. Comparing the photobiont in both states, we could observe that cells in the desiccated state were very collapsed, star-like shaped and not round shaped as in the hydrated state. There are also differences in their internal appearance, with very reduced cytoplasm, and with chloroplasts with thylakoids densely packed. We observed the pyrenoid in a dehydrated state (Fig. 3F); it appeared full of pyrenoglobuli and its volume was smaller than the pyrenoid volume in hydrated cells.

Discussion

The use of techniques such as CLSM and LTSEM makes it possible to observe the general appearance of the thalli at any level of hydration, and enables discerning the artifacts that might result from the conventional preparation of the sample for TEM.

In LTSEM, there is a total specimen preservation near to the natural state because samples are ultra-rapid cryofixed, which implies a quick, good immobilization of cells [11]. In CLSM, samples are observed in their natural state; it could become a mainstream approach to determine spatial relationships between symbionts to high resolution in lichens.

The different appearance of the desiccated state and the hydrated state with a reduced algal layer and photobiont cells partially hidden between mycobiont cells is a result of the collapse of photobiont cells for cytorrhysis. It had been observed that photobiont and mycobiont cells responded differently to dehydration. Photobiont cells have a wall less resistant to negative turgor pressure because of cytorrhysis; however, mycobiont cells that are usually very thick-walled are resistant to high negative turgor pressures [10, 11, 14, 15]. The algal layer in desiccated state has much compacted cells. In fact, although mycobiont cells collapse in lower grades, they are pulled when the photobiont cells collapse. In the process of dehydration there is a decrease in the space around the cells; this is clearly revealed in the thalli observation by CLSM. In *P. omphalodes*, the above described effects are more evident than in *L. hispanica* because the algal layer is narrow and very close to the upper cortex.

The ultrastructural study of symbionts at various hydration levels of the thallus is of great importance to know how they adapt morphologically and physiologically to the frequent, quick changes of the hydric status, and this knowledge could help to better understand the response of the whole thallus. The ultrastructural study of the *Trebouxia* photobiont has revealed that the pyrenoid is an organelle influenced by the hydric status of the cells. Pyrenoids of the *Trebouxia* photobiont of *L. hispanica* and *P. omphalodes* are *Impressa*-type sensu Friedl [7]. Its visualization by CSLM shows a zone without fluorescence except for some tubular inclusions. These inclusions with fluorescence

must be thylakoidal tubules that cross the pyrenoid of this type of pyrenoid and are of thylakoidal origin [3].

Damage to the pyrenoid had been previously observed under experimental drying regime [1, 2, 5, 6]. Authors related the damage more to dispersion than to substantial degradation of the protein of the pyrenoid matrix. The presence of “empty zones” could be related to the processes of desiccation-rehydration. The fact that chemical fixation in aqueous solution, which is the first step in the conventional preparation of the sample for TEM, caused the rehydration of the desiccated cells, led us to think that the collapsed pyrenoid in the dehydrated state expands with the rehydration and creates the “empty zones”. Immunolabelling of rubisco in pyrenoids with “empty zones” reveals a lack of rubisco in these zones and therefore a different nature of the pyrenoidal matrix.

In LTSEM study of our material, we have not seen “empty zones” in pyrenoid from photobionts of desiccated thalli. The pyrenoid in dehydrated photobiont cells, by LTSEM, has a volume smaller than that of the pyrenoid of hydrated photobiont cells, but the ratio of pyrenoid volume to chloroplast volume is similar in both states. Therefore, the hypothesis that the “empty zones” can originate by expansion in the rehydration process is confirmed. To clarify the above noted aspects, however, more studies combining several research techniques are necessary.

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