Lichenological Contributions in Honour of G.B. Feige.
M. Jensen (ed.): Bibliotheca Lichenologica **86**: *215-223.*
J. Cramer in der Gebrüder Borntraeger Verlagsbuchhandlung, Berlin · Stuttgart, 2003

Locating water in the dehydrated thallus of lichens from extreme microhabitats (Antarctica)

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Abstract: Microbial ecology deals with interactions among microorganisms, between microorganisms and their environment, and with water relations in the microhabitat. In the desiccated state, many lichens tolerate long periods of intense stress. The present report describes the use of scanning electron microscopy with backscattered electron imaging (SEM-BSE) to observe – on the spatial nanometer scale – relationships among the epilithic thallus of an Antarctic lichen, microorganisms belonging to epilithic, chasmoendolithic and cryptoendolithic communities and minerals. The main aim of the study was to determine the precise location of minute quantities of water that the thallus may maintain probably obtained from the mineral microenvironment of its rock habitat. Through low temperature scanning electron microscopy (LTSEM), it was possible to observe ice crystals indicative of the presence of water in the dehydrated hyphae of the fungal partner of the lichen when these were cross-fractured. Ice crystals were also detected among the mineral particles of the lithic substrate. Besides implications in the highly controversial topic of water distribution in the lichen thallus, the present findings suggest that under conditions of drought, the presence of small quantities of water in the apoplast may explain the survival of the dehydrated thallus.

Keywords: Antarctica, Lichens, Scanning Electron Microscopy in Back Scattered Mode (SEM-BSE), Low Temperature Scanning Electron Microscopy (LTSEM), Water relations, EPS.

Introduction

The term biocomplexity has recently been used with reference to the study of an ecosystem at the highest of levels. This means that to understand how an ecosystem functions, each of its components needs to be identified and the processes that occur within it need to be deciphered. The appearance of cryptoendolithic antarctic lichen observed *in situ* by SEM-BSE techniques, was reported by ASCASO in 1999. The activity of microorganisms living inside Antarctic rocks and signs of past activity considered biomarkers have recently been described (WIERZCHOS et al. 2002). These authors also explored life and fossilisation processes in the Ross Desert (WIERZCHOS & ASCASO 2001). The part played by the microclimate on

lichens growing on rock surfaces in several regions of the Antarctic and Arctic has also been the subject of research.

Over the past few years, LTSEM has proved to be an indispensable tool for the investigation of ultrastructural differences occurring in thalli containing different amounts of water and to visualize water in hydrated thalli (HONEGGER & PETER 1994, SCHEIDEGGER 1994, HONEGGER 1995, SCHEIDEGGER 1995, HONEGGER et al. 1996, DE LOS RIOS et al. 1999). A relevant line of research has resulted in the detection of water in the thalli of lichens from areas other than Antarctica. This water appears in minimum quantities both within the thallus (extra or intracellular water) and in its microhabitat, which consists of the mineral components of the lithic substrate surrounding the thallus (see review in SCHEIDEGGER et al. 1997). During desiccation, considerable structural changes compensate for the substantial loss of apoplastic and symplastic water (HONEGGER et al. 1996). In an LTSEM study of dehydrated thalli, HONEGGER (1995) demonstrated that the rounded and ovoid cells of green algae shrink and become shrivelled during episodes of water stress, while fungal cell walls are less prone to these deformation phenomena. According to this author and to SCHEIDEGGER et al. (1995), ascomycete lichen mycobionts form a "gas bubble" or large intracellular cavity of unknown ontogeny within the mycobiont hypha. This allows the fungal protoplasm to shrink while the plasma membrane stays in contact with the cell wall. The process seems very similar to that occurring when negative hydrostatic pressure makes xylem hydraulic conductivity vulnerable to cavitation during water transport (ZIMMERMANN & MILBURN 1982), although in hyphae this process takes place inside the living cells. The process of cavitation has also been recently related to the xylem through the use of cryo-SEM (LTSEM) (CANNY 1997).

The water relations of the lichen thallus are linked to the lichen's microhabitat and to its morphological and anatomical features. These features enable the thallus to exploit different water sources (NASH 1996). However, when the endolithic microorganism is a symbiont of a lichen thallus or is a free living alga, cyanobacterium or fungus, it is only the microhabitat that plays an important role in its hydric strategy. The microhabitat of lithobiontic microorganisms and lichens is provided by the pores, fissures and cavities of the rocks where these organisms live. The mineral components of the lithic substrate around a particular microhabitat, and the microclimate (light, temperature and relative humidity) make up the environment where the lithobiont will develop. Water in the form of vapour, rain, dew or ice is an essential element of the microclimate and may also be a component of the mineral substrate, since water in several forms may become trapped among mineral particles. Organic polymers of microbial origin (extracellular polymeric substances, EPS) bind cells and other particulate materials together (cohesion) and to the substrate (adhesion) (WINGENDER et al. 1999). These substances might be involved in the absorption and storage of water during dehydration processes.

The present study was designed to locate the small amounts of water that the natural dehydrated thallus of a crustose lichen could obtain from the environment or substrate in the dry conditions of Antarctica. Possible sites in the mineral substrate that may be able to harbour water were also identified.

Materials and methods

Materials

Lichen samples were collected from Antarctic rocks at the Ross Sea Scott Coast, Granite Harbour (77° 00′ S, 162° 34′ E), where there is no, or infrequent, rainfall and species such as *Lecidea cancriformis* Dodge & Baker are able to survive long periods of dehydration.

Mean air temperature ranges from –3.1°C (warmest month) to –26.1°C (coldest month) and relative humidity is 20 to 80% during the austral summer season (1999/2000). Mean annual precipitation, falling as snow, is 130 mm (rainfall equivalent). Mineralogical examination of this material demonstrated the presence of quartz, orthoclase and plagioclase as the main minerals, and biotite, zircon and apatite as accessory minerals.

Samples dehydrated in natural conditions were collected from the Ross Sea Scott Coast by Dr. Leopoldo Garcia Sancho in January 2000 and kept at –20°C until processing for microscopy and microanalytical procedures.

SEM-BSE and EDS

Samples were processed for *in situ* visualisation of the lichen symbionts and the rock-microorganism interface by scanning electron microscopy (SEM) operating in back-scattered electron (BSE) emission mode. Details of this preparative procedure are given in WIERZCHOS & ASCASO (1994). Briefly, the SEM-BSE method involves two stages. The first is the sample preparation procedure which includes fixing the rock-specimen sample in glutaraldehyde, staining with osmium tetroxide and/or uranyl acetate and preparing finely polished blocks containing the resin-embedded rock samples. The BSE signal is strongly dependent on the mean atomic number of the target (JOY 1991). Thus, the SEM-BSE procedure not only enables samples with different inorganic features to be visualised, but also allows the identification of ultrastructural elements of the microorganisms by staining with heavy metals. In the second stage, carbon coated biological-mineralogical sections are observed using the BSE detector.

LTSEM

Small fragments of granite grains with the thallus adhered to the surface were placed in the adjustable slit of the sample holder of the CT1500 Cryotrans instrument (Oxford). The fragments were fixed to the holder using OCT compound (Gurr BDH, UK). Specimens were immediately plunge-frozen in supercooled nitrogen and transferred to the pre-chamber of the microscope precooled to –180°C. This step was performed using a cryotransfer capsule to transfer specimens under vacuum from the nitrogen slush to the microscope's prechamber. Closing the capsule while still in the slush avoids the introduction of water during specimen transfer. This was followed by fracturing in the plane perpendicular to the lichen-rock interface with the help of a cooled metal blade. Specimens were subsequently sputter coated with gold (with no etching) and transferred to the chamber of a Zeiss 960 scanning electron microscope precooled to –160°C. Photographs were also taken of the specimens before fracturing, to establish whether the appearance of ice over the lichen sample could be attributed to condensation during sample processing.

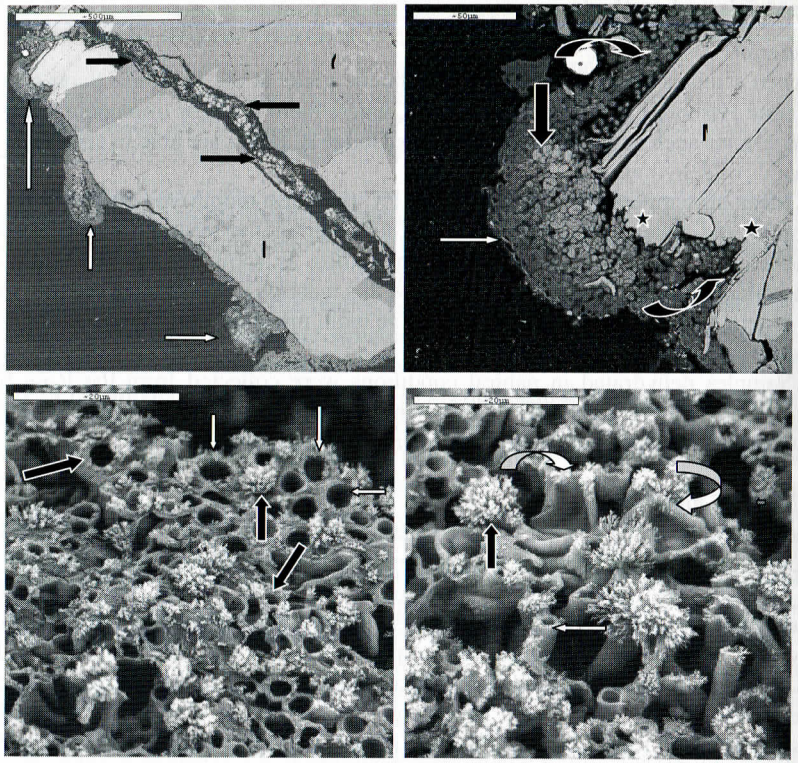

Fig. 1 (upper left): SEM-BSE picture of the transverse section of the *Lecidea cancriformis* Granite Harbour rock interface. White arrows indicate saxicolous lichen thallus and black arrows indicated groups of algae and fungi occupying an endolithic position in a fissure. F: Feldspat; Q, Quarz.
Fig. 2 (upper right): Detailed view of the area marked by a long white arrow in Fig. 1. White arrow marked the fungal cells of the thallus upper cortex. Black arrow indicates the photobiont. Curved arrows mark free-living fungi. Mica (M) layers are open (asterisk) and mica edges appear disintegrated (stars).
Fig. 3 (lower left): Transverse section of the dehydrated thallus of *Lecidea cancriformis*. Image of the upper cortex after fracturing the sample inside the microscope's prechamber. Hyphae apparently devoid of cytoplasm (white arrows). Note the presence of ice "tufts" over and around some of the transversally cut hyphae (black arrows).
Fig. 4 (lower right): Algal layer containing collapsed photobiont cells (curved arrows). Some of the transversally fractured hyphae (white arrows) showed ice "tufts" over their fractured surface (black arrows).

Results

The SEM-BSE images in Fig. 1 and 2 show how it is possible to observe the interface zone between living microorganisms and their mineral substrate, and thus get to know the microecosystem's components and the relationships between microorganisms and minerals.

Fig. 1 shows a cross section of a piece of rock containing a saxicolous lichen thallus (white arrows) and several algae and fungi (black arrows) occupying an endolithic position. Fig. 2 is a detailed view of an area of Fig. 1 (marked by the long white arrow), showing the lichen thallus over a mica mineral. The white arrow points to fungal cells of the lichen's upper cortex. The black arrow indicates the photobiont, and the curved arrow marks free-living microorganisms (fungi) adhered to the mica. The actions of the lichen mycobiont and free-living fungi gives rise to physical and probably chemical bioweathering of the micaceous mineral, such that the layers of mica come apart (asterisk) and the mica edges disintegrate or "crumble" (stars).

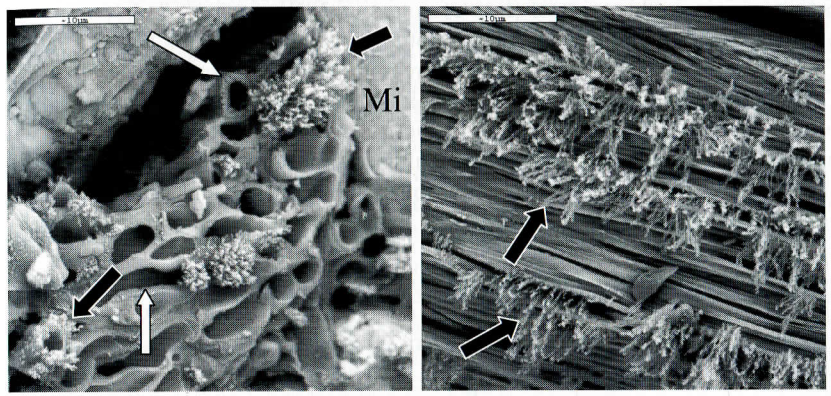

Fig. 5 (left): The lichen-substrate interface showing longitudinal and transversally sectioned hyphae (white arrows) of the lower part of the medulla adhered to mineral particles via the cell walls and extracellular matrix. Black arrows indicate ice "tufts" in the hyphae.
Fig. 6 (right): Ice "tufts" between the mica layers (black arrows).

To locate the water in the epilithic saxicolous thallus and mineral substrate, the samples were also plunge-frozen and the rock-lichen thallus specimens were fractured in the LTSEM instrument (thus avoiding contamination during fracturing) to expose an inner plane. Fig. 3 shows the appearance of a transversely fractured section of the dehydrated lichen thallus, in which hyphae, apparently devoid of cytoplasm (white arrows), may be observed in the upper cortex. The bright "tufts" (black arrows) are zones where ice crystals formed after sample fracturing indicating the previous presence of liquid water or ice in the lichen mycobiont. Fig. 4 is an image of the collapsed algal layer and provides a detailed view of the totally collapsed algal cells (curved arrows) among the hyphae (white arrow) and "tufts" of ice crystals (black arrows). This collapsing includes the cell wall in the case of the photobiont. Fig. 5 shows images of the lower medulla, with hyphae (white arrows) adhered to microdivided minerals (Mi). In this area, ice tufts may also be seen on the hyphae (black arrow). The cell walls of the hyphae and substances from the matrix are responsible for binding cells and particulate grains together. Fig. 5 shows a detailed image of these hyphae and several unevenly distributed ice crystals. These tufts are absent on the rock surface and are also lacking in most

of the cross-fractured hyphae.

When we examined the substrate under the lichen thallus (Fig. 6) the laminated minerals (shown by EDS to be mica) showed these ice tufts between bundles of mica (black arrows). This suggests that the exposed mica layers (SEM-BSE image in Fig. 2 marked by an asterisk) may act as a water reservoir.

Discussion

It was observed by SEM-BSE that the lichen thallus and associated microorganisms produced mechanical and chemical changes in the mica of the rock from Granite Harbour. The present authors previously detected mechanical and chemical alterations in this mineral (loss of potassium) (WIERZCHOS & ASCASO 1996) and the transformation of biotite to biotite-vermiculite (WIERZCHOS & ASCASO 1998) in granite rock from a temperate region (Madrid, Spain). In the mica from Granite Harbour (Antarctica), ASCASO & WIERZCHOS (2002) were also able to observe exfoliation and undulation of biotite layers owing to direct contact with hyphal cells. Even after the lichen thallus disappears, these undulated biotite sheets may reflect this physical action of epilithic hyphae and may therefore be considered biomarkers. The SEM-BSE image in Fig. 2 shows hyphal cells in close proximity to the biotite layers of the substrate. In this case, the physical action of hyphal cells did not give rise to undulated biotite layers, but it was possible to observe separated biotite sheets of a "crumbled" appearance (marked with an asterisk). These crumbled biotite layers act as water reservoirs, as shown in the image in Fig. 6 obtained through LTSEM. This technique may thus be used to detect water or ice in the lichen thallus, as well as in the microsites of the minerals that make up the microhabitat. We therefore consider it an essential tool for gaining insight into the processes that take place when the microhabitat's abiotic and biotic components become dehydrated. Although EPS are known to maintain a high degree of hydration in the immediate vicinity of the microbial cells and thus assist in their survival under conditions of desiccation (SUTHERLAND 1999), other mechanisms might help maintain a certain degree of moisture in the rock microhabitat, such as water storage in the microsites of laminar minerals. It is also likely that the presence of water among the mica layers aids the chemical action of hyphal cells on the mineral giving rise to Fe-rich diagenetic minerals. This type of mineral has been observed in these zones and may also be considered a biomarker of the activity of hyphal cells (ASCASO & WIERZCHOS 2002).

The LTSEM technique also allows the investigator to fracture frozen, hydrated microorganisms inside the rock such that their ultrastructure may be observed and also permits observation of EPS in the frozen, hydrated state. Under laboratory conditions, the presence or absence of water in the extracellular spaces of a particular lichen thallus may depend on the hydration process, the lichen substances present, thallus anatomy (SOUZA-EGIPSY et al. 2000, HONEGGER & HUGELSHOFER 2000), and also on the way in which the excess water is removed from the thallus before it is subjected to freezing. It is also likely that certain lichen thalli never present water in extracellular spaces, regardless of the hydration method used, due to the presence or to the efficiency of the hydrophobic cell wall layer described by SCHERRER et al. (2000).

During the present LTSEM examination of algal and fungal cells, the structure

of their cytoplasm or appearance of wall polysaccharides could not be observed (this would be possible if they were hydrated). Nevertheless, it should be highlighted that locating the exact position of minute quantities of water in these dehydrated fungal cells and adjacent minerals was possible because the sublimation step normally conducted in LTSEM procedures was omitted. When dehydrated lichen specimens from Antarctica are subjected to the routine process of subliming the ice for a couple of minutes after fracturing, only highly collapsed algal cells and discretely collapsed fungal cells may be observed. Further, the fungal cells show an empty space in the form of a gas bubble or cavity as described by HONEGGER in 1995. Any possible trace of water in the dehydrated thallus sublimes and thus vanishes. The main contribution of the present study is the recommendation that when processing dehydrated specimens such as these from Antarctica for microscopy, the sublimation stage should be omitted. In this manner, it is possible to observe tufts of ice crystals in different zones of the thallus (upper cortex and medulla) that are always associated with transverse or longitudinal fractures of hyphae. These areas showing tufts of ice crystals indicative of the presence of water, are usually seen around the free space (gas bubble or large cavity according to HONEGGER 1995) that dehydrated hyphae show when transversally fractured. At present it is difficult to discern whether the intracellular water that emerges from the hypha when cold-fractured is derived from the cell cytoplasm (reduced to a band around the hyphal central space) or emerges from the space that could exist between the fungal wall and the plasmalema, i.e., via the apoplastic continuum. It is highly possible that the latter is the route of water translocation since, according to HONEGGER (1995, 1997), one of the functions of the hydrophobic cell surface layer is to push the water towards the apoplast where it may move by capillary action. This apoplastic water can also be produced by cavitation, since it is thought the phenomenon could induce an apoplastic water pulse (SCHEIDEGGER et al. 1995). The method of visualising intracellular water in dehydrated thalli proposed here, may be applied to dehydrated thalli from different sources. It is quite possible that the mycobiont translocates fungal metabolites to the photobiont via this apoplastic route by capillarity (HONEGGER & PETER 1994), but this is the first report of the detection of water at this site in a dehydrated lichen. The chemical composition of apoplastic fluid is unknown, but through the analysis of fluids leaked after dehydration stress episodes, it was shown to contains a wide range of carbonates (MACFARLANE & KERSHAW 1985; DUDLEY & LECHOWICZ 1987).

Some authors have reported the possibility of protection mechanisms established via the activation of genes (CLOSE et al. 1993, SCHNEIDER et al. 1993) in so-called "resurrection" plants. In lichens, there may also be a molecular basis for water stress resistance mechanisms, but the present results indicate the presence of a certain amount of water both in the dehydrated thallus and mica layers close to the thallus. The water in the mica might therefore act as a reservoir and contribute towards this resistance to water stress induced by adverse climatic conditions. It should be noted that it was not possible to detect water within the algal cells, since their total collapsing prevents their fracturing. This possibility should not, however, be ruled out. It may be concluded that the presence of water in the apoplast of the naturally dehydrated thallus may explain its survival in conditions of extreme drought.

Acknowledgements

The authors gratefully acknowledge Ana Burton for the English revision and Fernando Pinto for skilful technical assistance. This study was financed by grants BOS2000-1121 and ANT99-0680-CO2-02 from the DGICYT.

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