Cytological Investigations of Lithobiontic Microorganisms in Granitic Rocks

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Abstract

This paper shows the ultrastructure of lithobiontic organisms in a granitic rock from the exterior to the interior, where fissures are found 1–2 mm from the surface. There is clear differentiation at cell level between mycobiont and photobiont cells located on the rock surface which forms part of the lichen thallus and prokaryotic and eukaryotic cells (algae and fungi) found in the fissures. As well as the observations at the ultrastructural level of the microorganisms which live in fissures and cavities, immunolabelling techniques with colloidal gold have been applied to obtain an immunolocalization of Rubisco enzyme in some of the cells. The technique applied here permits Rubisco enzyme to be identified in algae-like cells belonging to fissures where it is difficult to identify the pyrenoid. The mineral environment of the cells situated inside the fissures is investigated by Energy Dispersive Spectrometry (EDS). The biotite particles present in the fissure walls, as well as some lithobiont microorganisms, show a depletion of potassium from interlayer positions.

Key words

Aspicilia, granitic rock, immunogold localization, microprobe, lichen, lithobiontic microorganisms, Rubisco.

Introduction

In 1981, Golubic et al. defined the lithobiontic ecological niche, including in the term "lithobiontic", organisms which live within hard mineral substrates. The physical and chemical characteristics of the substrate are related to the development of microorganisms. The hard rock substrates are very varied and bacteria live in them (including cyanobacteria), algae, fungi, lichens and bryophytes. The organisms that live in the interior of the rock are named chasmoendoliths, cryptoendoliths and

euendoliths, depending on whether they grow in fissures, structural cavities, or actively penetrate the rock forming tunnels.

Various experiments have shown the alteration of certain minerals when they are in contact with bacteria and fungi in culture medium. This alteration can promote the mica-to-vermiculite transition (Weed et al., 1969). Bacteria and fungi are able to concentrate P, Ca, Fe and K which they need for their mineral nutrition (Bell et al., 1994). The capacity of filamentous fungi and yeast for uptake of metal ions has recently been reviewed by Winkelmann and Wringe (1994).

The lichen thalli are products of an association of two or more symbiont organisms, one of which has photosynthetic capacity (photobiont) and the other is a fungus (mycobiont). In some lichen species, particularly in *Xanthoria parietina*, it has been shown that carbonates of Ca and Mg have a positive influence on thallus growth (Armstrong, 1990). Asta (1992) has shown that in some species of terricolous lichens, the mycelium is rich in Ca^{++} , while the algal zone is rich in K^+ and Mg^{++} .

The crustaceous epilithic thalli which present endolithic parts, as well as the lichen endolithic thalli, share positions inside the substrate which are close to other lithobiontic organisms. The ultrastructural investigation of the epilithic part and the adjoining endolithic part permits advances in the knowledge of the symbiosis. The observation at the cytological level of the endolithic microorganisms without the necessity of separation from their substrate permits new knowledge on aspects already investigated by others, as well as the capacity of some organisms to remain in ecological niches of extremely unfavorable environments (Friedmann and Ocampo-Friedmann, 1984), the productivity of cryptoendolithic organisms in certain areas (Friedmann et al., 1993) and the re-establishment of lithobiontic microorganisms in burnt rocks (Garty, 1990 and 1992) and Garty and Binyamini (1990).

The study of the interface between the lichen thallus and the lithic substrate was initiated by Bachmann (1890). This author investigated the relationship between lichen and calcareous (1892 and 1913) and siliceous substrates (1904, 1911 and 1917) making interesting drawings of the interface zone, and at the same

time investigating the action of lithobionts such as algae (Bachmann, 1915) and fungi (Bachmann, 1916). For later observation with the light microscope, he sectioned rocks containing thallus or other lithobionts. A similar method was used in investigations by Baur (1901). Continuing with the light microscope methods, Doppelbaur (1959) presented a new methodology applied to the study of endolithic lichen with pyrenocarps. Basically, this technique consisted of fixing the biological material, eliminating the substrate, embedding and sectioning.

The study of the endolithic algal habitat by Scanning Electron Microscopy in secondary emission mode (SEM-SE) was initiated by Friedmann (1971). This author carried out observations with reflected light and SEM of endolithic algal cells "in situ". He developed an interesting method combining the observations with the Light Microscope and Scanning Electron Microscope observing the external characteristic surface of the algal cells. In 1976 Le Campion-Alsumard used Transmission Electron Microscopy (TEM) for the study of cyanobacteria at the ultrastructural level in a calcareous substrate, showing internal structures of the cyanobacterial cells such as thylakoids, polyhedral bodies, nucleoplasm, etc. Unfortunately the sample preparation technique based on double inclusion and decalcification of the samples in order to maintain the biological material unaltered by freeing the mineral component, could only be applied to calcareous substrates. On many occasions the biological material has been extracted from the substrate (Kushnir et al., 1978; Friedmann, 1982; Friedmann and Koriem, 1989), and processed later for Transmission Electron Microscopy. Very few investigations have been carried out at the ultrastructural level without extraction of the biological material from the substrate. TEM techniques were applied to the "in situ" observation of the interface between the thallus of *Lecanora albescens* and a calcareous substrate (Ascaso and Ollacarizqueta, 1991). This technique encounters important difficulties even when applied to studies of the lichen thallus-substrate interface in soft rocks. This is why a back-scattered electron imaging technique applied to the study of the polished lichen thallus-substrate interface was introduced recently (Wierzechos and Ascaso, 1994) and may be applied to all types of rock substrates.

In the present work the lithobiontic organisms present in fissures of a granitic rock covered by *Aspicilia* spec. lichen thalli were investigated at the cytological level. In addition to observations at the ultrastructural level of the microorganisms which live in fissures and cavities, immunolabelling techniques with colloidal gold have been applied to obtain an immunolocalization of Rubisco enzyme in some of the cells observed. Difficulties exist for immunolabelling of enzymes situated in the interior of fissures. The mineral environment of the cells located inside the fissures was also investigated by Energy Dispersive Spectrometry (EDS).

Materials and Methods

Granitic rock fragments that show crustaceous lichen thalli of *Aspicilia* on their surface were collected in Bustarviejo de la Sierra, Madrid (Spain).

Once processed and prepared, as described in the three following paragraphs, all the samples were investigated with SEM in back-scattered electron (BSE) emission mode using the DMS 960-Zeiss. The DMS 960-Zeiss was also equipped with an EDSpectrometry analytical system.

Cytological study

Hand-cut pieces with corresponding thalli of lichen were processed according to conventional Transmission Electron Microscopy procedures for the preparation of lichen material (Ascaso et al., 1986) applying the LRWhite resin as the inclusion medium. The blocks were then sawn transversely in a lichen-rock interface section and finely polished. Detailed information about this preparation technique is given by Wierzechos and Ascaso (1994). These lichen-rock transverse sections were prepared for cytological investigations by staining with lead citrate (Reynolds, 1963) for 10 min.

Immunocytochemical study

Some of the hand cut pieces of rock containing lichen thalli on the surface were processed according to TEM procedures in preparation for immunolabelling (Ascaso et al., 1995). Then the lichen-rock transverse sections were prepared as for cytological observations.

Immunolabelling was carried out with anti-Rubisco antiserum of *Euglena gracilis*, provided by Prof. Joaquín Moreno (Biochemistry Department, Valencia University). The pieces were post-stained with uranyl acetate followed by lead citrate (Reynolds, 1963).

Microprobe examinations

The microprobe analysis was carried out in the samples prepared for cytological investigation as well as in the samples prepared for immunolabelling. The microprobe analysis for spatial distribution of elements and microprobe profile was carried out with Energy Dispersive Spectrometry (EDS) Link ISIS microanalytical system.

Results

Figure 1 shows the general aspect of a granitic rock surface covered by the crustaceous lichen thallus of *Aspicilia*. In Figure 2 a detail of the zone marked by the arrow in Figure 1 can be observed. Figure 3 shows the lichen thallus of *Aspicilia* sp and the system of fissures found under the mineral situated beneath the thallus. The thallus displays a heteromorous organization with a cortex, algal zone and an undeveloped medulla. Fungal cells (black arrows) and algal cells (white arrows) can be seen in the fissures under the thallus. Figure 4 is an enlargement of the area indicated by a square in Figure 3. It shows two photobiont cells of *Trebouxia* with their central pyrenoids. Pyrenoglobuli completely filling the inside of the pyrenoid can be seen in the top right algal cell (arrows). The mycobiont cells present in the top left of the figure show numerous lipid bodies in the cytoplasm. The bottom right hand hypha displays a group of concentric bodies. Figure 5 is an enlargement of the area marked with a double white arrow in Figure 3. At the bottom of Figure 5 cells of a microfungus are shown and at the top, rounded algal-like cells. Figure 6 shows another nearby area within the same fragment of granite rock where a more complex system of fissures under the *Aspicilia* thallus can be seen. Lithobiontic cells deeply penetrating into the fissures can be seen, in addition to lichen thallus lobes present at the top of

Fig. 1 General aspect of a granitic rock surface covered by *Aspicilia* s.p.

Fig. 2 Detail of zone marked by an arrow in Figure 1.

the fissure. The top fissure (F) lacks biological elements but the remaining fissures, two on the left of the figure, parallel to each other, and one on the right, are full of lithobiontic cells. Figure 7 is an enlargement of the area indicated by a single arrow in Figure 6. Looking at Figure 6, the reader may well imagine that the cells indicated by a single arrow are similar to the thallus cells or to the bottom fissure cells (shown in Figure 6 by a double arrow). Figure 7 shows that they are more likely to be cells of fungal origin. Figure 8 is an enlargement of these cells showing a large central lipid globule and smaller lipids located in peripheral positions (arrows). Figure 9 is an enlargement of the area marked with two arrows in Figure 6. This image displays structures similar to Figure 7 despite it being in an area located much further inside the rock. Hyphae can be seen at the bottom of the fissure displaying a high lipid content in their cytoplasm whilst the top is formed by packed microfungi. Figure 10 relates to the left hand side of the fissure where Figure 9 is located. Cells can be seen which appear to be algal with peripheral lipids and a clear mass inside, which may relate to a more or less organized chloroplast. Fungal cells are clearly identifiable at the top left of this image.

Despite achievements in the ultrastructural observation of a fissure's biological matter, it would also be advisable to apply immunolabelling techniques which could demonstrate the presence of certain enzymes in cells inside the fissure. Figure 11 shows immunolabelling of Rubisco by colloidal gold of 30 nm indicating the presence of this enzyme in the pyrenoid of an alga-like cell found in a fissure of an area near the lichen thallus location. Figure 12 is an enlargement of an algal pyrenoid. Figure 13 shows a fissure completely occupied by lithobiontic organisms. This fissure cuts through a micaceous mineral. Lithobiontic cells inside enclose sheets of mica. A similar situation is shown in the following figure (Figure 14) where a fissure crosses a micaceous mineral, where lithobiontic organisms and a fragment of the mineral can be seen separated from the top wall. This figure has a line drawn obliquely across it. The

line represents a line scan for different elements as will be shown in Figure 16. Figure 15 shows, in the top left section, the same back-scattered electron image as in Figure 14 and the remaining three sections are EDS X-ray images showing the spatial distribution of Al, K and Fe on the biotite located at the top and bottom of the fissure. The Fe spatial distribution may exactly record the forms of the micaceous material shown in the BSE images. An evident lack of potassium in the biotite above and below the fissure was observed. Figure 16 shows microprobe line profiles across the line drawn in Figure 14 for Al, Si, K and Fe elements. The observation and subtraction of microprobe line profiles for Fe and for K precisely demonstrate sites of K release occurring on the biotite.

Fig. 3 General view of an *Aspicilia* thallus and lithobionts situated in fissures under the lichen. al, algal layer; c, cortex; m, medulla. White arrows, algal cells. Black arrows, fungal cells.

Fig. 4 Detail of algal layer marked in Figure 3. A, algal cell (photobiont); cc, concentric bodies; H, hyphae (mycobiont); L, lipid body; P, pyrenoid. White arrows, pyrenoglobuli.

Fig. 5 Detail of fissure marked by two white arrows in Figure 3. A, algal-like cell; M, microfungus.

Fig. 6 General view of a network of fissures under a lichen thallus (*Aspicilia*). F, empty fissure. Arrows indicate the zones shown in Figures 7–8 and 9–10.

Fig. 7 Detail of the fissure marked by the top arrow shown in Figure 6.

Fig. 8 Enlargement of cells shown in Figure 7. L, lipid body. Arrows, small lipidic bodies.

Bar indicates 1 μ m unless otherwise stated.

Figs. 3–8

Figs. 9-14

Fig. 15 In the top left the same image BSE that in Figure 14. In the top right and in the bottom spatial distribution of Al, K and Fe.

Discussion

In 1987 Brock emphasized that for any study of microbial ecology one should be able to observe the organisms in their natural habitat. In the present investigation the use of SEM with a back-scattered electron detector to investigate polished samples of the thallus/rock interface allowed a clear differentiation at cellular level between mycobiont and photobiont located on the rock surface (Figure 4) and prokaryotic and eukaryotic cells (algae and fungi) found in the fissures. Figures 7 and 8 display cells identified as hyphae. These "in situ" observed hyphae look like the "oil hyphae" described in 1978 by Kushnir et al. who called such structures "oil hyphae" because they contained large amounts of lipids in the form of oil globules. Modenesi and Lajolo (1988) observed that the hyphae forming bundles inside the substrate sometimes

take on a globular appearance. Later, Garty (1992) observed externally some globules in fungal hyphae of an endolithic lichen growing in the burnt rock and also called these hyphae "oil hyphae". Probably the first observation of "oil hyphae" was by Bachmann (1904) who observed these structures beneath the thallus of *Lecidea crustulata* Ach.

Figure 9 of the fissure located 1 mm from the surface shows the same "oil hyphae" and other cells of rock-inhabiting microfungi. The presence of hyphae penetrating 2 mm into the substrate has been documented in Scanning Electron Microscopy working with secondary electrons in *Lecanora muralis* (Salvadori and Lazzarini, 1991). Rock-inhabiting microfungi are present in the enlarged fissure represented in Figure 5, and other types of cells can also be observed in this figure. They are difficult to identify at the moment, which shows that on occasions, certain biological components of the substrate are hard to identify from their ultrastructural appearance. This is why it becomes necessary to apply techniques to localize enzymes. Immunolocalizations, an example of which is presented in this paper, may clearly enhance "in situ" cytological studies of lithobiontic organisms. The technique presented in this work enabled Rubisco enzyme to be identified in algae-like cells belonging to fissures where it was not easy to ultrastructurally identify the pyrenoid of the cells, in order to know whether they were trebouxoid algal cells or not. Locating Rubisco in the photobiont of the thallus of *Parmelia sulcata* has been done in ultrathin section in TEM (Ascaso et al., 1995) and the present work confirms the existence of algae of a trebouxoid nature in

- ◀ Fig. 9 Detail of the fissure marked by two black arrows shown in Figure 6. L, lipid body; M, microfungus.
 Fig. 10 Algal cell-like and fungal cells located in the left side of the lithobionts shown in Figure 9. H, fungal cell; L, lipid body.
 Fig. 11 Immunolabelling of Rubisco with colloidal gold in an algal cell located inside a fissure. Arrows indicated particles of gold of 30 nm.
 Fig. 12 Detail of an algal pyrenoid marked with anti-Rubisco antibodies (gold particles 30 nm).
 Fig. 13 Fissure in micaceous minerals with lithobionts inside and mica layers embedded in the lithobionts cells.
 Fig. 14 Fissure in micaceous mineral showing lithobionts. A line scan obliquely cross the fissure.
 Bar indicates 1 μ m unless otherwise stated.

Fig. 16 Microprobe line profiles across the line drawn in Figure 14 for Al, Si, K and Fe elements.

certain areas of surface fissures. In some cases, green algae colonizing burnt surfaces of the rock have been observed (Garty, 1990; Garty and Binyamini, 1990) and it was thought that these were probably *Trebouxia*. Immunological evidence for the existence of free-living *Trebouxia* has been reported by Mukhtar et al. (1994), after scraping rock surface and incubating the algal cells in culture medium. In the future it will be possible to localize "in situ" (as well as the Rubisco presented here) other *Trebouxia* proteins without the necessity to remove the cells from the surface of the rock. This will provide new data about the existence of free-living *Trebouxia*.

Figures 13 and 14 shows a biotite mineral containing lithobiont cells. Danin and Caneva (1990) observed that lithobionts develop among the calcareous rock crystals decreasing the coherence of the crystal to the rock surface. Lithobiontic niches (Golubic et al., 1981) are called euendolithic when microorganisms actively penetrate calcareous substrates. In the case of the microorganisms shown in Figures 13 and 14, the cells pass through previously well packed micas which currently appear poor in K. It can be clearly seen that of the five types of granite decay cited by Bell et al. (1994), several took place in the microsite shown in the figures: physical disruption of minerals or uptake of mineral elements, in this case potassium, but we cannot discard other elements. This loss of potassium from the micas shown in the mapping of Figure 15 demonstrates an alteration of the mineral substrate exerted by biological elements. These organisms first appear to be chasmoendolithic, i.e., organisms which colonize fissures and cracks in the rock, but their substrate altering capacity infers that they actively penetrate some areas of the substrate and therefore could be called euendolithic. The loss of potassium from the mica may be attributed to

oxalic acid being secreted by hyphae, since Eckard (1985) experimentally demonstrated a heavy extraction of K over a period of several weeks due to the action of 0.1 M oxalic acid. Solubilization processes, in which bacteria and fungi are involved, with dissolution being performed by acids or complexation phenomena, have been well documented by Robert and Berthelin (1986). Ascaso and Wierzechos (1994) showed algal and fungal cells closely packed with mica sheets occupying a surface position in the rock. Surface located micas could also be seen to undergo losses of K when in contact with lichen thallus cells (Wierzechos and Ascaso, 1995). The possibly low pH in the thallus matter facilitates K⁺ protonation and washing away from interlaminar areas. Although some papers on the influence of calcium and magnesium on the growth of some lichen species on slates exist (Armstrong, 1990). However, the destination of K⁺ and its role, if this element is indeed taken up by the thallus, is practically unknown. The few research papers existing on the subject state that this element is preferably located in the algal area (Asta, 1992) whilst Ca⁺⁺ is located in the mycelium tissue.

The evidence of K⁺ losses in micas belonging to fissures, as observed "in situ" in this paper, and the evidence of K⁺ losses in surface micas where rhizins of *Parmelia conspersa* are attached (Wierzechos and Ascaso, 1995) provide support to some of the phenomena occurring in microsites. The capacity of lithobiontic cells to act on the mineral material nearest to their walls could clarify aspects relating to the depth of biodeterioration caused by epilithic and endolithic organisms.

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