

***In Situ* Cellular and Enzymatic Investigations of Saxicolous Lichens Using Correlative Microscopical and Microanalytical Techniques**

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Abstract

Direct *in situ* microscopy visualization is strongly recommended in studies of saxicolous lichen ecology and endolithic microorganisms. With this in mind, lithobiontic microorganisms were detected, localized and determined at the cytological level. A range of microscopy techniques and a number of procedures for preparing lichen colonized rock samples were applied. Scanning Electron Microscopy operating in Back Scattered Electron mode (SEM-BSE), Confocal Laser Scanning Microscopy (CLSM) and occasionally Energy Dispersion Spectroscopy (EDS) were used to study the same sample zones. This observation strategy, called "Correlative Microscopy", allowed us to obtain important information on the physico-chemical state of the mineral substrate, deposition processes of calcium oxalate and the *in situ* ultrastructural determination of lithobiontic microorganisms. Moreover, the observation of the three dimensional organization of living endolithic cells was also possible. On occasions, when cytological discernment was not sufficient, the SEM-BSE signal was used to detect immunolabeled gold particles which had previously been linked to proteins in order

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to indicate the occurrence of ribulose-bisphosphate carboxylase (Rubisco) within endolithic cells of *Trebouxia*. The results of the biodeterioration study of calcareous stone from Jaca Cathedral showed that mycobiont cells in conjunction with a bundle of fungal cells were involved in the decaying of calcium carbonate grains.

Keywords: *Aspicilia intermutans*, calcareous rock, Confocal Laser Scanning Microscopy, *Diploicia canescens*, Energy Dispersive Spectroscopy, granitic rock, *Lecania rabenhorstii*, *Lecidea auriculata*, lithobiontic microorganisms, Scanning Electron Microscopy

1. Introduction

The study of the interface between lichen thallus and lithic substrate began almost an hundred years ago with the work of Bachmann (1890). Fry was the first to study the biogeophysical action of the crustaceous lichens by light microscopy (1922, 1924 and 1927) whereas Doppelbauer (1959) was one of the first investigators to apply light microscopy to the study of endolithic pyrenocarps. Kushnir et al. (1978) were first to investigate endoliths at the cytological level in the Transmission Electron Microscope (TEM). They found structures composed of fatty acids in endolithic lichen fungal cells, after first separating them from the substrate and later processing them for examination by TEM. Friedmann (1971) conducted the first study of endolithic microorganisms using Scanning Electron Microscopy (SEM) operating in the secondary emission mode (SE) which did not involve the removal of biological material from fissures and rock cavities. At that time the SEM-SE technique seemed to be the ideal tool for increasing knowledge about endolithic parts of lichen thalli and it has been applied in many studies on the lichen-rock interface (e.g. Wilson et al., 1981; Gehrman et al., 1988; Gehrman et al., 1992; Gehrman and Krumbein, 1994 and review in: Ascaso and Wierzchos, 1995). However, use of SEM with SE signal detection only allows observation of the external morphology of saxicolous symbionts and other lithobiontic microorganisms but does not provide any information about the ultrastructural characteristics of cells occurring within rock fissures.

Nowadays, microbial ecological studies of the endoliths in their natural habitats using electron microscopy methods are strongly recommended (Brock, 1987). The localization, detection and determination of biological agents by knowledge of their ultrastructural characteristics, on one hand, and of their physico-chemical behavior and interactions with inorganic substrates on the other, could improve our knowledge about endoliths ecology. *In situ* studies of

lithobiontic microorganisms at the cytological level have been one of our targets for the past four years. For this reason we have applied Scanning Electron Microscopy operating in Back-Scattered Electron mode (SEM-BSE) which allow a cytological observation of saxicolous thalli and lithobiontic microorganisms (Ascaso and Wierzchos, 1994). The SEM-BSE technique also allowed us to detect some enzymes in the interior of cells located within stone fissures (Ascaso et al., 1995a).

In this work we review the possibilities offered by SEM-BSE in the study of saxicolous lichens and lithobiontic microorganisms, including their morphological identification and the *in situ* determination of ultrastructural cell elements leading up to cytological studies. Future perspectives for using the Confocal Laser Scanning Microscopy (CLSM) are also reported. Application of these two complementary microscopy techniques for the examination of the same zone offers great advantages in lichenology and geophysiology. Some examples of the application of correlative microscopy for the study of endolithic microorganisms are also given.

2. Material and Methods

Aspicilia intermutans (Nyl.) Arn. and *Lecidea auriculata* Th. Fr. thalli and some unidentified lichen species were collected from granitic rock in Bustarviejo de la Sierra (Madrid) at 1150 m. above sea level. Previous mineralogical examination of the rock material demonstrated the presence of quartz, orthoclase and plagioclase as the major minerals and biotite, apatite and zircon as accessory minerals. *Diploicia canescens* (Dicks.) Massal. and *Lecania rabenhorstii* (Hepp) Arn. thalli, colonising calcareous litharenite rock were also investigated. These samples were taken from the façade of the Romanic Cathedral of Jaca (Huesca Province, Spain) at a height of 50 cm above ground level. This wall has an easterly orientation but does not receive much direct sunlight. The rock contains quartz and calcite as the main mineral components. Dolomite, plagioclase, biotite, chlorite, muscovite, tourmaline, zircon and opaque were also present as accessory minerals.

The lichen thalli were processed with their substrata following the procedure for observing lichen-rock transverse sections using a Scanning Electron Microscope operating in Back-Scattered Electron mode (SEM-BSE). A more detailed description of this method is given by Wierzchos and Ascaso (1994). Briefly, the thalli with substrate were fixed with glutaraldehyde and osmium tetroxide solutions, and after dehydration, samples were embedded in resin. After polymerization the samples were sawed, finely polished and carbon coated for SEM-BSE and microanalytically examined by Energy Dispersive

Spectroscopy (EDS). Some of the samples were processed by immunogold labelling (30 nm) in order to detect the ribulose-bisphosphate carboxylase enzyme (Rubisco) (Ascaso et al., 1995b) and gold particles were identified using an SEM-BSE signal. Lithobionts colonising the granitic rock that had previously been examined by SEM-BSE and EDS were subsequently observed using Confocal Laser Scanning Microscopy (CLSM). In this manner our data provide a basis for "Correlative Microscopy" studies.

All reported microscopy studies have involved *in situ* examination of lithobiontic microorganisms. The term *in situ* is used because all rock-invading lichens and their accompanying endolithic microorganisms were studied in their natural ecological niches without having previously been submitted to the commonly reported scraping or rasping procedures.

3. Results

Examination of the *A. intermutans* thallus-granitic rock interface zone frequently revealed algal and fungal cells colonies appearing between quartz and feldspar grains. In certain cases endolithic lichen thallus organization is apparent as in Fig. 1A. Fig. 1B is a detailed view of the zone marked with an arrow in Fig. 1A, and Fig. 1C represents detail from the zone marked with arrows in 1B. Fig. 1D shows algal cells of the trebouxoid type (SEM-BSE inverse image) occurring in the zone marked with two arrows in Fig. 1A. All these SEM-BSE micrographs allow *in situ* visualization of algal and fungal cells at an ultrastructural level. Another component of granitic rock which is exposed to invasion by lithobiontic microorganisms is micaceous mineral. Its layers suffer separation and exfoliation as a result of biophysical weathering, as shown in the SEM-BSE micrograph of Fig. 1E. Application of "Correlative Microscopy" by using the CLSM technique for observing the same exfoliated biotite sheet zone revealed an abundance of endolithic fungal cells located between mineral layers (Fig. 1F). SEM-BSE and CLSM were also used to observe algal cells colonising shallow fissure between quartz grains (Figs. 2A and 2B). The SEM-BSE images reveal several ultrastructural elements of the algae, but CLSM projection micrographs give us information about the three dimensional (3D) distribution of these cells. "Correlative Microscopy" has also been applied to examine the contact zone between hyphae and biotite sheets. Fig. 2C (SEM-BSE image) distinctly reveals mineral components of exfoliated biotite layers and other whitish crystals among them. The CLSM image (Fig. 2D), obtained by projection of various confocal slices, revealed 3D reconstruction of hyphal cells interlaced with biotite sheets. A series of figures (2E) shows the elemental spatial distribution of Si, Ca, Fe and K in the same SEM-BSE

and CLSM visualised zone. This series was obtained using EDS. The detection and localization of calcium within this zone allows us detection of calcium oxalate crystals appearing among biotite layers and hyphal cells.

A special procedure (Wierzchos and Ascaso, 1994) used for studies of the lichen-rock interface zone permits the observation of the microorganisms at the cytological level, situated within fissures, as shown in Fig. 3A. Cytoplasmic storage bodies in fungal and algal endolithic cells were also observed. However, visualization using SEM-SE (Fig. 3B) provided only information about the dimensions and shapes of the cells. Figs. 4A and 4B (detail) show fungal cells occurring within a deep fissure (SEM-BSE image). Ultrastructural details are clearly distinguished. When cytological discernment is not sufficient, the SEM-BSE signal can be used to detect the presence of immunolabelled colloidal gold particles previously linked to proteins. Rubisco enzyme has been marked with 30 nm colloidal gold in the pyrenoid zone of algal cells occurring within rock fissures, and the subsequent detection of these colloidal gold particles was achieved using SEM-BSE. The result of this observation is shown in Fig. 4C, where small black dots reveal the occurrence of Rubisco in the pyrenoid zone of an algal cell. Figs. 4D–F show a transversal section of a lichen colonising a calcareous stone (Jaca Cathedral) which was examined using SEM-BSE. A low magnification image (Fig. 4D) reveals algal layers composing the photobiont zone (asterisk) and a medullary zone with hyphal cells penetrating between mineral grains (arrows). In the zones marked by stars in Fig. 4D, substantially altered calcium carbonate particles were observed. The cells which surround the carbonate nodules have an internal structure which identifies them as fungal: Lipid bodies characteristic for fungal cells (marked by stars) were observed in the SEM-BSE image (Fig. 4E). The left part of Fig. 4D reveals the linear network of structures formed by fungal cells, in detail shown in Fig. 4F. Angular calcium carbonate grains were found between these linear structures. Fungal cells surrounding calcium carbonate nodules, as well as those that form the linear network, demonstrate the spatial and structural continuity of the mycobiont.

4. Discussion and Conclusions

Traditionally, the cytological characteristics of lithobiontic microorganisms have been described by means of Transmission Electron Microscopy or Light Microscopy examinations which were always preceded by *in vitro* plate cultivation of microorganisms. It is very probable that many of these microorganisms do not grow *in vitro* culture. This could lead to misinter-

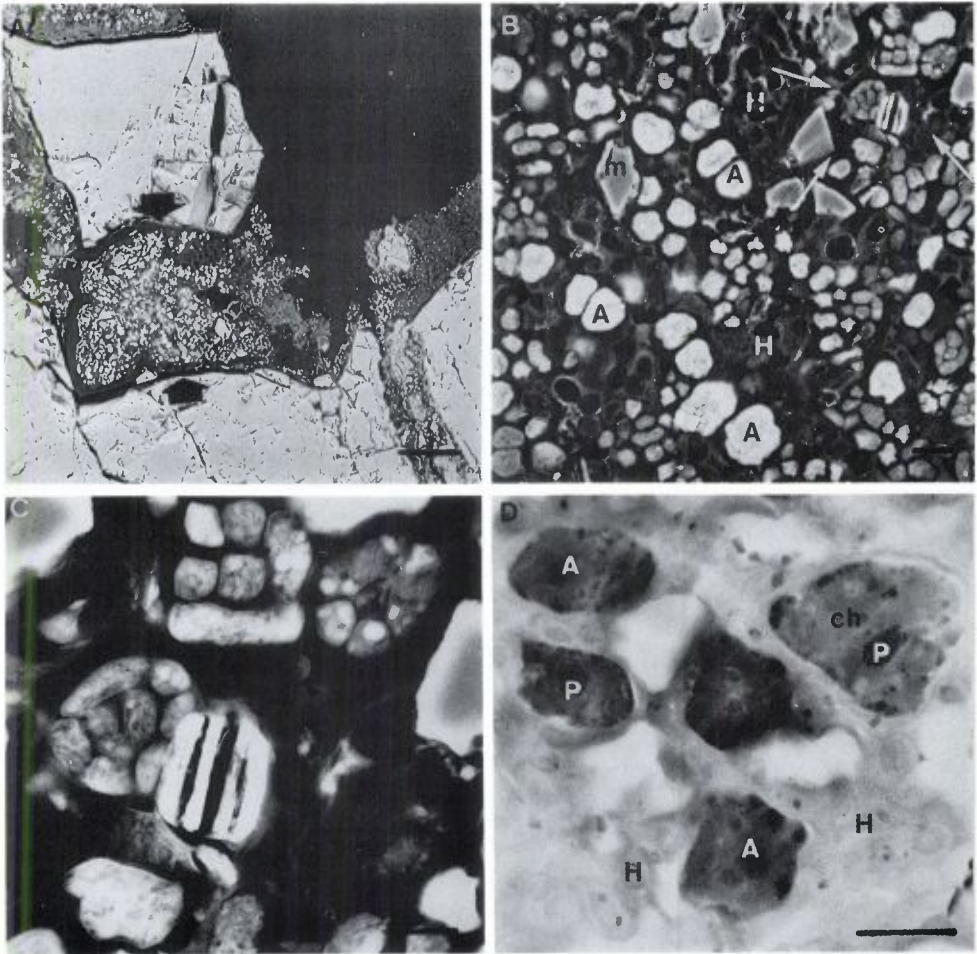


Figure 1. A: Transversal section of the interface between *Aspicilia intermutans* thallus and granitic rock. Polished sample observed using SEM-BSE. Algal and fungal cells between quartz fragments. Bar = 100 μm . B: Detail of zone marked by arrow in Fig. A. Algal (A) and fungal cells (H) mixed with microdivided minerals (m). Bar = 5 μm . C: Detail of zone marked by three arrows in B. Aplanospores. Bar = 2 μm . D: Detail of zone marked by arrow in Fig. A. SEM-BSE inverse image. Algal cells of trebouxoid type. A, algal cells; Ch, chloroplast; P, pyrenoid; H, fungal cells. Bar = 3 μm .

pretations about the nature, population and other ecological characteristics of lithobionts.

The application of Confocal Laser Scanning Microscopy is an important complement to cytological information obtained using the SEM-BSE method.

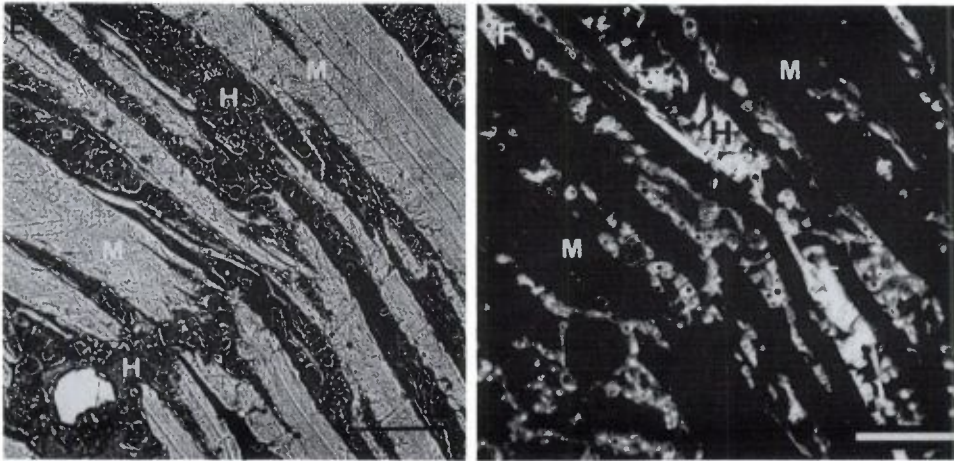


Figure 1. E: SEM-BSE image of fungal cells within mica sheets. M, mica; H, fungal cells. Bar = 18 μm . F: The same zone as that in Fig. 1E but observed using CLSM. M, mica; H, fungal cells. Bar = 18 μm .

Although the spatial resolution of confocal microscope images is not the same as with SEM-BSE, this technique is unique for visualising endolithic microorganisms in their natural undisturbed condition. When autofluorescence is detected, even living biological material can be visualised. It is commonly accepted that the biophysical deterioration of rocks is caused by hyphae and thallus penetration and by the expansion and contraction of biological material. These frequently occurring processes are caused by alternate drying and moistening cycles which lead to the separation of mineral grains. The CLSM technique allows us to investigate these processes in real time (unpublished results) and 3D reconstructed CLSM images reveal information about the spatial organization of lithobiontic microorganisms (Fig. 2B). Data about the spatial distribution of microorganism cells can also give us information about the number of microorganisms occupying a determined volume of the fissure. A knowledge of the microorganisms number per unit volume of a fissure and/or per unit volume of rock is important for geomicrobiology studies. These data could yield a more precise indication of CO_2 exchange behavior in cryptoendolithic microbial communities.

In this work two different microscopy and microanalytical techniques were combined. When data were collected from the same sample zone using different microscopy techniques the "Correlative Microscopy" strategy was performed.

"Correlative Microscopy" examination of the calcium oxalate crystals found among hyphae and biotite sheets in a granitic rock from an unpolluted area

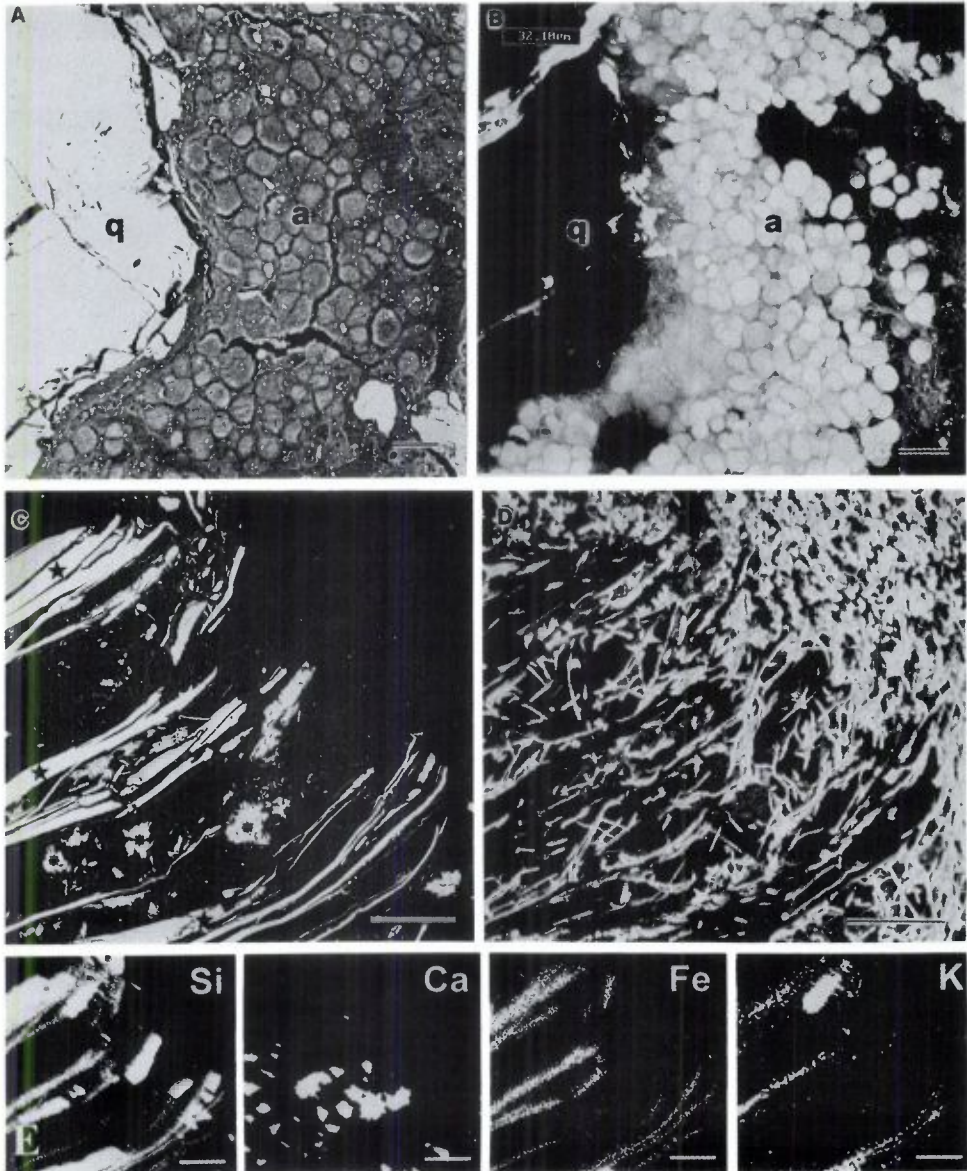


Figure 2. A and B: Correlative microscopy from *Lecidea auriculata*-granitic rock contact zone. A - SEM-BSE image and B - CLSM image of the same lichen-rock interface zone. Left part of the figures is quartz grain (q) and right part are algal cells (a). Bar = 10 μ m. C, D and E. Correlative microscopy of zone occupied by mica and fungal cells. C - SEM-BSE image, D - CLSM image and E - EDS spatial distribution of elements (Si, Ca, Fe and K). Stars - mica, sheets and asterisk - calcium oxalate crystals. Bar = 20 μ m.

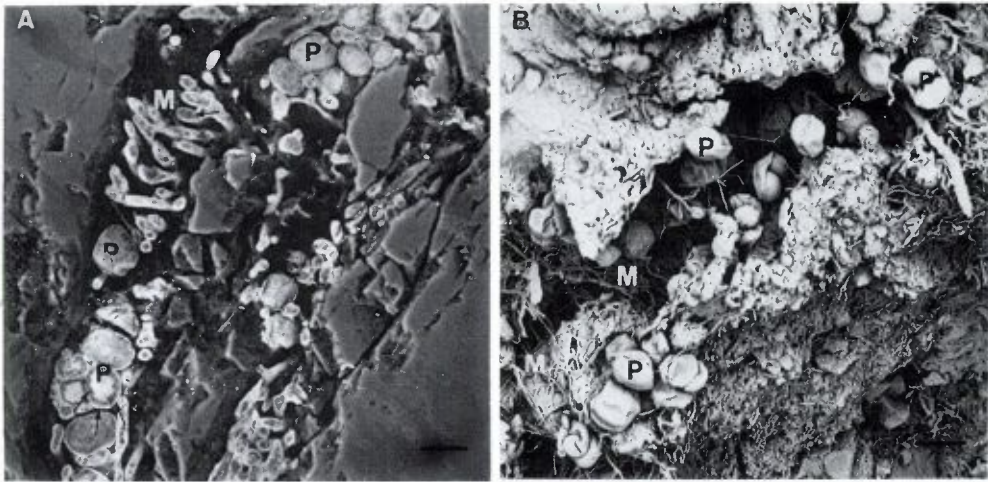


Figure 3. A: Fissure occupied by endolithic microorganisms. Polished transversal section observed using SEM-BSE. This mode of visualization allows detection of microorganism ultrastructural details: P, photobionts; M, mycobiont; arrows, cytoplasmic storage bodies. Bar = 10 μm . B: Fissure occupied by endolithic microorganisms. Fractured transversal section observed using SEM-SE. P, photobiont; M, mycobiont. No ultrastructural details observed. Bar = 10 μm .

(Figs. 2C–E) contributed complementary information about the precipitation of calcium oxalates within the lichen thallus. In the present work calcium oxalate crystals were detected near Ca-free mica layers. The importance of the oxalate presence in the lichen thallus is very well represented in the literature about lichens and biodeterioration of stoneworks collected by Piervitori et al. (1994; 1996).

The results concerning the Jaca Cathedral showed that mycobionts from the crustaceous epilithic thallus together with a bundle of fungal cells form continuous structures which penetrate deep within the decaying calcareous stone. These masses of fungal cells occasionally surround the carbonate nodules and provoke angular dissolution of calcite grains which locally form a linear pore network, occupied by fungal cells. The biodeterioration of carbonate rocks induced by endolithic lichens and fungi was also mentioned by Krumbein (1969). Recent SEM examinations of calcareous rocks reported by Gehrman and Krumbein (1994) confirm that at least in lichen colonization, oxalic acid is an agent of carbonate rock biodeterioration. However, in the case of Jaca Cathedral a lack of calcium oxalates was noted, although calcium oxalate crystals were expected as the result of the reaction between oxalic acid excreted by the lichen and calcium carbonate. Apart from oxalic acid, a variety of other

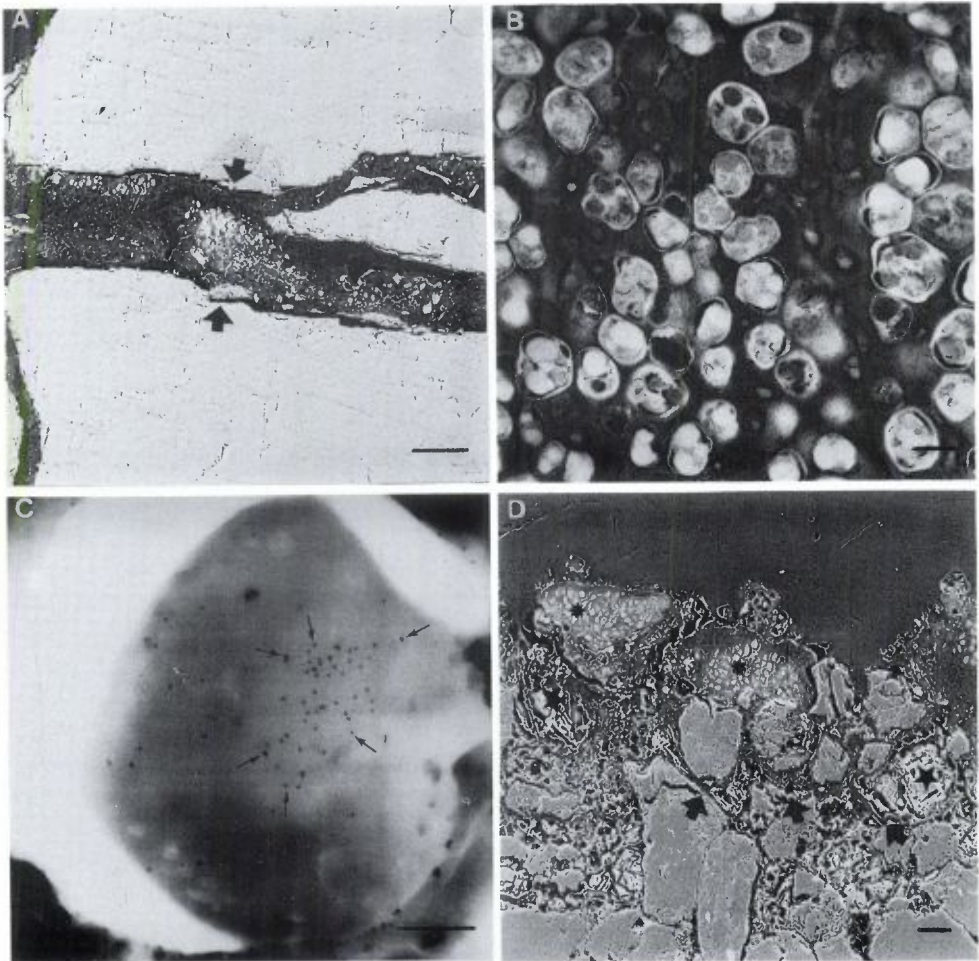


Figure 4. A. Fissure localised close to *Lecidea auriculata* thallus. Arrows: cryptoendolithic microorganisms. SEM-BSE image. Bar = 50 μm . B. Detail of zone marked by arrow in Fig. A. Fungal cells with lipid bodies. SEM-BSE image. Bar = 2 μm . C. Photobiont cell present within fissure. Arrows indicate the immunogold localization of Rubisco. Gold particle size: 30 nm. SEM-BSE image. Bar = 1 μm . D. Transversal section of the interface between *Lecania rabenhorstii* and calcareous stone from Jaca Cathedral (Spain). Asterisk – lichen thallus algal layer; stars – calcium carbonate nodules; arrows – fungal cells. SEM-BSE image. Bar = 20 μm .

acids may be involved in the biodeterioration of carbonate rocks. Much more work is still needed to fully understand these phenomena, including *in situ*

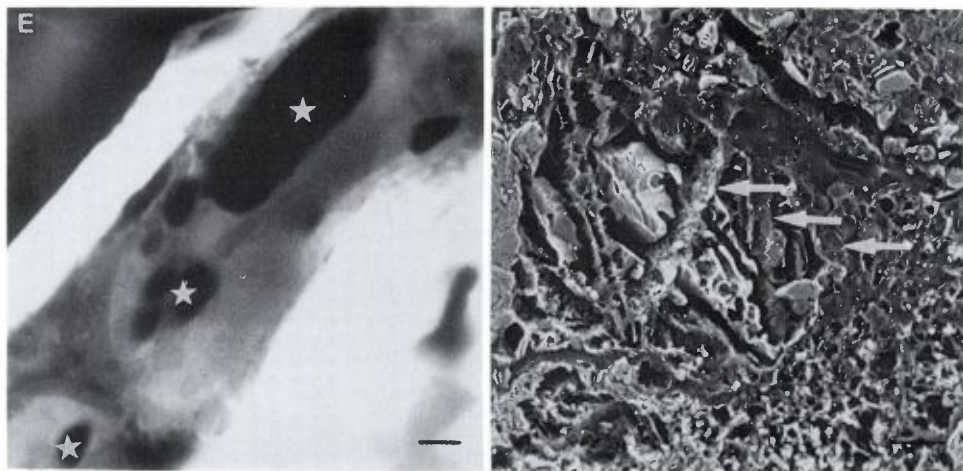


Figure 4. E. Detail of the fungal cell. Stars, lipid bodies. SEM-BSE image. Bar = 1 μm . F. Calcium carbonate nodule (C) surround by hyphal cells (arrows). SEM-BSE image. Bar = 10 μm .

studies of the deposition processes and the role of calcium oxalates forming within saxicolous lichens thalli.

Application of Electron Microscopy techniques and Confocal Laser Scanning Microscopy in investigations of saxicolous lichens and lithobiontic microorganisms opens new and important possibilities for visualization and examination of lithobiontic lichens and their accompanying epilithic and endolithic microorganisms. In particular cases "Correlative Microscopy" should also improve our knowledge about the ecology of endoliths and the mechanisms by which they interact with mineral substrate. Observation of ultrastructural cell features of saxicolous thalli by SEM-BSE and observation of other lithobiontic microorganisms at the cytological level may in the near future permit the establishment of a relationship between cytological characteristics and the function of the microorganisms occupying lithobiontic niches. This *in situ* visualization strategy could also provide further information about interactions between lichen thallus components and other lithobiontic microorganisms.

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