main uncertainties concerning the structure and the chemical composition of the Zn-rich deposits. Both inconsistencies concerning the crystalline and non-crystalline structure of deposits with similar element composition and the occurrence of Zn-rich crystals without a significant P content but a relatively high S peak (Fig. **11d**) suggest that there may be several storage forms for Zn in *Thlaspi*. But as the resin used for sample preparation in this study contained S and background values for S in EDAX spectra were high, further investigations are required to confirm the hypothesis that S-containing compounds such as glucosinolates are involved in Zn tolerance in Cruciferae (Mathys, 1977). Moreover, EDAX is not suitable for detecting elements with atomic numbers lower than eleven, so that the proportion of C, O, H and N that may be present in the Zn crystals cannot be verified by this technique. Investigations of these crystals by X-ray microdiffraction techniques would be helpful for further characterizing the Zn storage form in vacuoles of *Thlaspi*.

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J. Barceló

Lab. Fisiologia Vegetal
Facultad de Ciencias
Universidad Autónoma de Barcelona
E-08193 Bellaterra
Spain

Structural Aspects of the Lichen-Rock Interface Using Back-scattered Electron Imaging

C. Ascaso and J. Wierzchos

Centro de Ciencias Medioambientales, Serrano 115, bis., E-28006 Madrid, Spain

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Abstract

The profound knowledge of the structural and chemical characteristics of the interface between lichen thallus and rock, seems to be indispensable for the process of understanding the lichen symbiosis as well as the significance of the weathering action of lichens. One of the most promising techniques to be used in this investigation is the Scanning Electron Microscopy (SEM) in the back-scattered electron (BSE) emission mode. In the present work thalli of *Parmelia conspersa, Aspicilia intermutans* and *Lecidea auriculata* growing in granitic rock were examined by SEM in BSE mode with (Energy Dispersive Spectroscopy) EDS. In the case of the foliose thalli the observation of the interface permits detection of the rhizine/hyphae adherence and determination of the origin of the minerals which adhere to the rhizine/hyphae. In the case of the crustose thalli BSE permits investigation within the ultrastructure of the crustose thallus and crustose lichen-rock contact zone and also allows observations of the penetration and filling of the fissures and cracks of the underlying rock by components of the thallus and other living organisms. The BSE images could contribute to a better knowledge of the cytological state of the rock-inhabiting organisms and also to the understanding of the action of the chemical treatments used in the removal of lichen from building materials.

Key words

Parmelia conspersa, Lecidea auriculata, Aspicilia intermutans, back-scattered electron imaging, lichen, rock, weathering.

Introduction

The study of the lichen thalli-rock substrate interface was initiated many years ago with the application of various techniques. Special emphasis has been placed on the necessity to investigate the type of biogeophysical or biogeochemical action that could occur under a determined lichen species. The technique used for the longest time was Light Microscopy (L. M.) and it provided a view of the thalli-substrate relationship and also knowledge of some minerals present in the rock. However, the low resolution of light microscopy in showing the living elements of the thalli and identifying the microdivided minerals present immediately below the thalli made it a limited technique.

From 1968 scraping techniques were used to obtain material from the lichen-rock interface and the material obtained was studied with the usual techniques of clay mineral research. The first investigations in this field were carried out by Dormaar (1968) and Ascaso et al. (1976). They showed that minerals appeared in the interface zone that were not present in the underlying rock. The nature of interface minerals demonstrated clearly that biogeophysical and/or biogeochemical effects are produced by the thallus on the rock. However, the study of the interface minerals obtained by scraping may not be conclusive because this material can contain unknown minerals transported by weather conditions.

In 1977 Hallbauer and Jahns directly observed the interface zone with new techniques that permitted better resolution than the light microscopical techniques used before. These authors combined, for the first time, Scanning Electron Microscopy (SEM) with Energy Dispersive Spectroscopy (EDS) to distinguish the difference between the lichen and inorganic parts.

Between 1980 and 1982 various works appeared which combined L. M. with scraping techniques. With the latter a mixture of biological material of thallus and rock mixture of biological material of thallus and rock mineral were obtained. This mixture was treated for various times with boiling H_2O_2 which removed the biological material. The remaining inorganic material was investigated with the usual technique of clay mineralogy, such as Infrared Spectroscopy (IR), X-ray diffraction (XRD) and Transmission Electron Microscopy (TEM).

Although with these experiments comprehension about the mineralogy of the interface has improved, there has been a growing interest amongst in-

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vestigators in finding less destructive techniques for investigating the mineralogy of the rock surface under the thallus and also to know the relationship between the symbionts of the thallus and the mineral elements of the substrate. With this in mind, since 1985 there have been many projects which combined LM and SEM techniques (on occasions with EDS) (see Ascaso and Wierzchos, 1994 for a review). Unfortunately the biological and mineral materials are non-conductive substances which require coating with a layer of heavy metal for study with SEM techniques in secondary emission mode. The heavy metal coatings render the specimens unsuitable for EDS analysis. Moreover the topographic images of the contact zone can contain artifacts resulting from the detachment of small particles, or these particles can be obstructed by biological material. The first attempt to apply back-scattered electron images (BSEI) to the lichen-rock interface was reported by Chisholm et al. (1987) and Purvis et al. (1987, 1990). However, in their BSE images a significantly brighter response sufficient for a micromorphological and ultrastructural study was not obtained, probably due to the preparative procedure.

In the past few years a new objective has appeared in this kind of investigation, that is, to work with techniques that provide the capacity to understand the ultrastructure of lichen symbionts lying close to the rock surface. In 1991 ultrathin sections of the lichen-rock contact zone were investigated by Transmission Electron Microscopy (TEM) (Ascaso and Ollacarizqueta, 1991) to achieve a better knowledge of the relationship between the cells of the lichen thalli and the adjacent minerals. These observations also facilitated the investigation of the mycobiont ultrastructure and also the photobiont ultrastructure. In the same research the use of EDS permitted a better understanding of the chemical composition of different bodies of inorganic nature situated inside the thallus near the symbionts. Although this method is very thorough, in the study of the interface of crustaceous lichens it presents some limitations. One of them is the great difficulty in obtaining ultrathin sections of the lichen rock interface, another is that the observation area of the interface is reduced to $1-2\,mm^2$. Taking into account the limitations of the above-mentioned techniques, the SEM with BSE and EDS seems to be a very adequate and complementary tool in the study of the lichen-rock interface. The application of this technique fulfills the following prerequisites: a) a wide scope of observation, preferably of several cm^2 as used in LM, b) high resolution power close to that of TEM, c) possibility to carry out a chemical analysis of inorganic compounds "in situ".

In the present work the application of SEM with BSE and EDS provided knowledge of new aspects of lichen symbiosis and its relationship with the substrate, giving answers to the mineralogical aspects of the interface as well as to the existence or non-existence of cross contamination. It is also possible to clear doubts about the living elements situated in the existing fissures below the thallus and improve the observation of ultrastructural aspects of mycobiont and photobiont.

Materials and Methods

The thallus of *Parmelia conspersa (Ehrht) Ach.*, *Lecidea auriculata* Th. Fr. and *Aspicilia intermutans* (Nyl.) Arn. lichens growing on granitic rock at Bustarviejo de la Sierra, near Madrid (Spain) were collected. A previous petrographic study of this material showed the presence of quartz, orthoclase and plagioclase in granitic rock as essential minerals, and biotite (micaceous mineral), zircon and apatite as accessory minerals (Ascaso, 1985). Hand-cut pieces of rock with corresponding thalli of lichen were processed according to conventional TEM procedures for the preparation of lichen material (Ascaso et al., 1986), applying as the inclusion medium, the LR white low-viscosity resin. After polymerization the block was sawn in the transverse direction. Afterwards, the obtained surfaces were hand polished with abrasive powders of decreasing size. Then the fine polished surfaces (powder diamond grain diameter (0.25 μm in final polishing) were stained with lead citrate (Reynolds, 1963) and, after washing in distilled water and air drying, the surfaces were coated with evaporated carbon. Detailed descriptions of the preparation of transverse sections of the lichen-rock interface is given by Wierzchos and Ascaso (1994). The samples were then examined with SEM Zeiss 960 DSM equipped with a BSE detector and EDS Link ISIS microanalytical system.

Results and Discussion

The *Parmelia conspersa* lichen thallus is attached to the substrate by rhizines, as shown in a transverse section of the lichen-rock interface in Figure 1. In the zone where the rhizines are adhered to the rock it is possible to observe a mixture of hyphae belonging to the thallus and minerals (arrow). A more detailed observation of the lower part of the rhizine (Fig. 2) demonstrates how the hyphae detach the rock minerals by mechanical action. These mineral particles demonstrate the same geological nature as the part of the rock belonging to the substrate. Nevertheless the observation of the contact zone also shows mineral particles which were deposited in the biological part by weather factors such as rain and/or wind. From the earliest mineralogical research on the interphase zone (Ascaso et al., 1976) the question of the origin of these minerals had great scientific interest. The arrows note two

Fig. 1 Transverse section of *Parmelia conspersa* interface with granitic rock. Arrow indicates minerals attached to the rhizine. T, thallus; R, rhizine.

Fig. 2 Detail of the lower part of rhizine shown in Figure **1**. The lower arrow indicates a piece of mineral inside the rock. The upper arrow indicates a fragment of the same mineral.

Fig. 3 Detail of Figure **1**. Spherical body with aspect of soredium. A, algae. Arrows, pyrenoids.

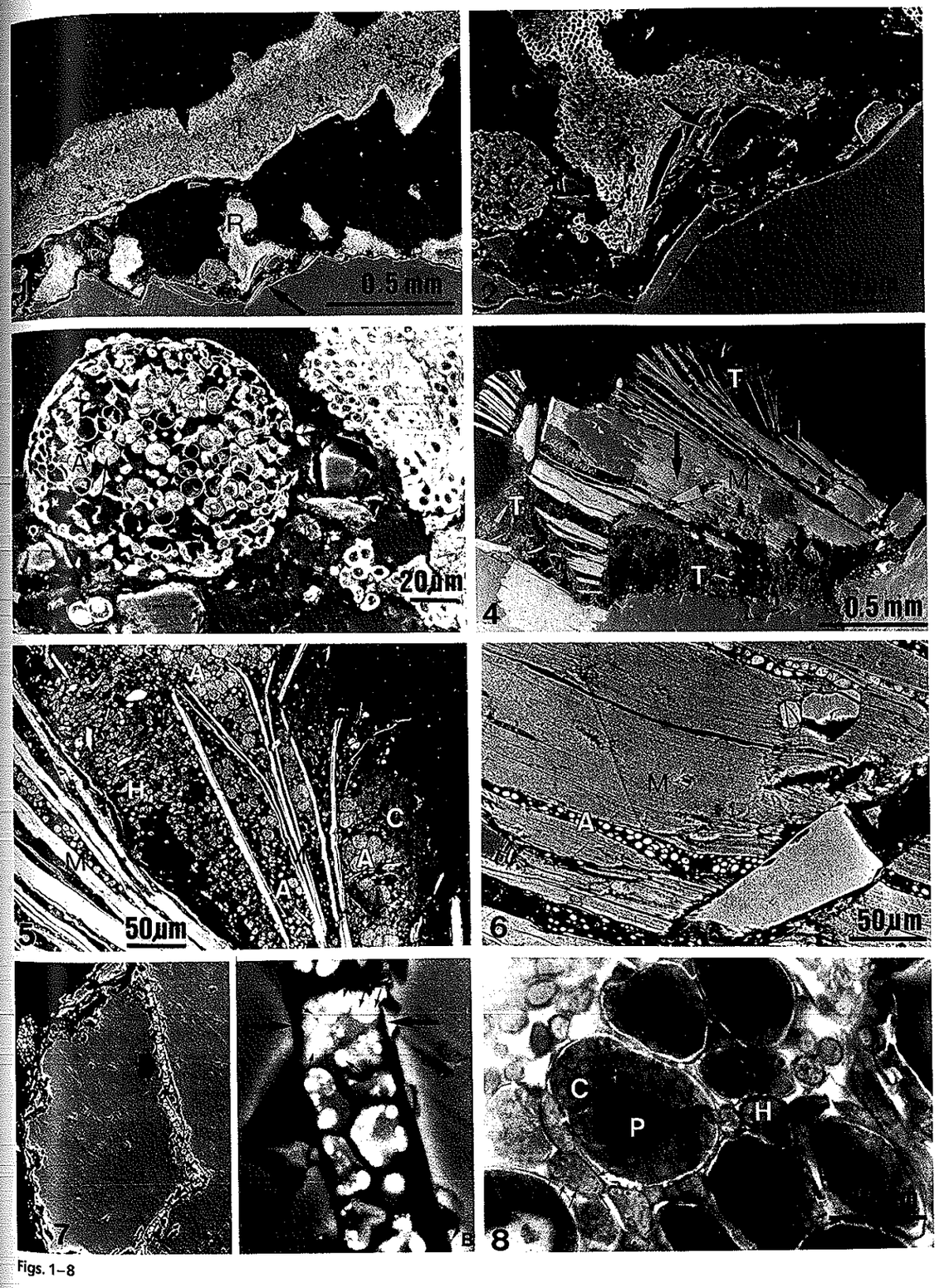
Fig. 4 Transverse section of *Lecidea auriculata* growing on granitic rock. Union of thallus (T) and mica mineral (M).

Fig. 5 Detail of Figure **4**. A, agae; C, cortex; H, hyphae; M, mica layer.

Fig. 6 Detail of the arrow-noted part in Figure **4**. A, algae; M, mica.

Fig. 7 **a**: general view of rock surface and fissure (arrow). **b**: interior of fissure. Wide arrows: algal cell; little arrows: lipid bodies.

Fig. 8 High magnification of algal cell and hyphae. C, chloroplast; H, hyphae; P, pyrenoid; Arrows, lipid bodies.



Figs. 1–8

parts from the same mineral, one belonging to the rock and another embedded between the hyphae of the thallus. The picture shows the size and distribution of these minerals and also permits observation of the anatomy of the rhizine. On the left side of the figure it is possible to distinguish a spherical body. This spherical body is shown in higher magnification in the next figure (Fig. 3). The algal cells are clearly shown. Part of the fine structure of the algae is also shown, particularly the pyrenoid (arrows). The algal cells are mixed with fungal cells giving a structure with the appearance of a soredium. This structure lies attached to different microdivided minerals.

The observation of the *Lecidea auriculata* interface has elements of great interest. Fig. **4** shows all aspects of the contact between the thallus of *L. auriculata* and the micaceous substrate. The plates of mica are becoming more and more broken. The next BSE image (Fig. **5**) shows that algae, which appear in the center of the figure bonded close to mica sheets, have a central chloroplast with a pyrenoid which is typical for trebouxoid cells. The algal cells seen in Figure **5** are clearly different from the algal cells shown in a deeper part of the contact zone (Fig. **6** proceeds from the place marked by arrow in Fig. **4**). Probably, the observed algal cells in Fig. **6** are free-living algae. More investigations must be done with SEM-BSE to better understand the ultrastructure of these algae. In this part of the micaceous mineral (Fig. **6**) the plates of mica are not yet separated. However, groups of algae are present, even inside fissures as narrow as 10 μm diameter.

Another aspect of the lichen-rock interface, is given in Figure **7**. In Figure **7 a** a general view of the rock surface is presented. The wide arrow indicates the presence of biological material inside a fissure. In Figure **7 b** a highly magnified image of the fissure is presented. It is possible to identify algal cells (arrows) with an abundant presence of lipid bodies inside the cytoplasm (little arrows). The origin of these algal cells is unknown, since they may or may not belong to the lichen thallus. In a high resolution image (Fig. **8**) the contrast has been inverted electronically and this inverted contrast BSE image is comparable to that of the TEM. Note that chloroplast and pyrenoid are distinguishable in algal cells. The fungal cells showed dark zones which correspond to lipid bodies.

Near the *L. auriculata* thallus it is possible to find structures with aspects of cells mixed with inorganic crystals (Fig. **9**). Observation at higher magnification shows that the above-mentioned cells are hyphae (Fig. **10**). The analysis by EDS has shown that the crystals are calcium oxalate. These crystals of calcium oxalate remain intercalated with minerals detached from the rock. The presence of calcium oxalate in lichens in the form of plates of monohydrate calcium oxalate or bipyramids of dihydrated calcium oxalate has been confirmed in numerous publications (Ascaso et al., 1982; Nimis et al., 1992). Recently SEM with EDS has allowed observations of the typical morphology of these oxalates and, at the same time, the analysis of their chemical composition (Adamo et al., 1989; Adamo et al., 1993). Caneva et al. (1991) showed a polished cross-section of lichen-rock interface with the distribution (mapping) of Ca^{++} in the lichen thallus. Edwards et al. (1993) had demonstrated, using FT-Raman Microscopy, that hyphae, calcium oxalate and decomposition products are present up to 20 mm into the stonework.

Figure **11** shows the interface of the *Aspicilia intermutans* thallus. The algal layer, as well as the cortex and medulla, are easily identifiable (black arrows). The figure also shows, on its left side, a piece of mineral. The lower part of this piece has the same shape as the rock mineral situated immediately under it (white arrows). Between the piece of mineral already mentioned and the rock mineral, there exists a fragment of a well-developed thallus. In a zone situated very close to this (Fig. **12**) several plates of micaceous material (arrows) appear embedded among the hyphae of the thallus. These plates seem to be detached from a micaceous material situated on A in the figure. The application of the EDS microanalytical system to study detached minerals allows the definition of their chemical nature.

Apart from the importance of the study of the thallus anatomy with several details of the fine structure of the symbionts, and the interest in knowing the aspect of the rock surface which is in contact with the thallus, the investigation of the fissures situated below a determinated thallus are also a subject of interest. Figure **13** shows a piece of lichen thallus and also several fissures situated inside the rock (arrows). The aspect of the biological material present inside the fissure is shown in Figure **14**. The algal cells are presented in Trebouxoid aspect with several lipid bodies in the cytoplasm. Going to the interior of the fissures it is possible to observe algal cells situated several mm from the surface. Note that algae in cell division processes were observed (Fig. **15**). In a very close superficial zone there are hyphae whose cellular structures can be appreciated at high magnifications (Fig. **16**).

Although the principal aim of our work was the study of the interaction between lichen thallus and rock, the observation of rock surface with no lichen cover was also done. In this zone the lack of characteristic elements of bioweathering processes was detected.

Fig. 9 Rock surface showing oxalate crystal mixed with different cells. Mi, rock minerals; Ox, oxalate.

Fig. 10 Detail of Figure **9**. H, hyphae; Ox, oxalate.

Fig. 11 Transverse section of *Aspicilia intermutans* growing in granitic rock. The three black arrows indicate the cortex, the algal layer and the medulla, respectively. White arrows indicate the breakage line in mineral.

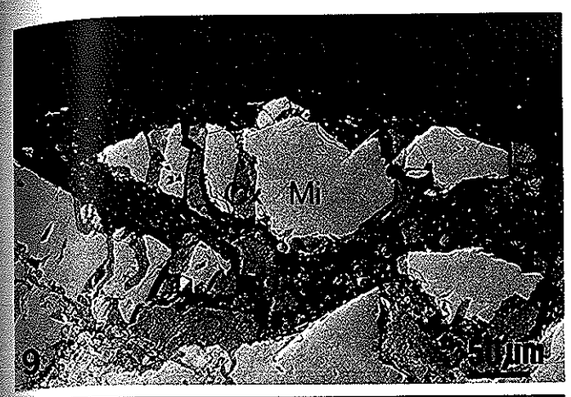
Fig. 12 Thallus of *A. intermutans* with fragments of mica embedded in the mycobiont. A, micaceous material inside the rock. M, mica fragments.

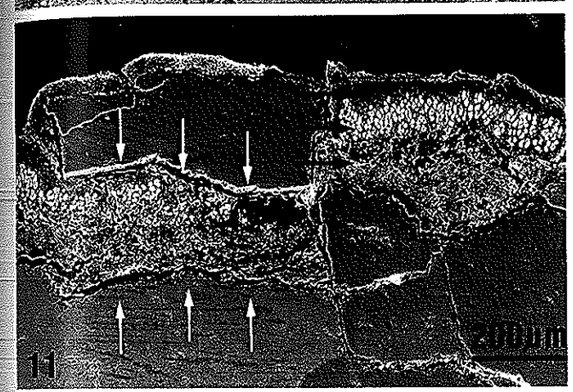
Fig. 13 General view of *A. intermutans* thallus and rock fissures (arrows).

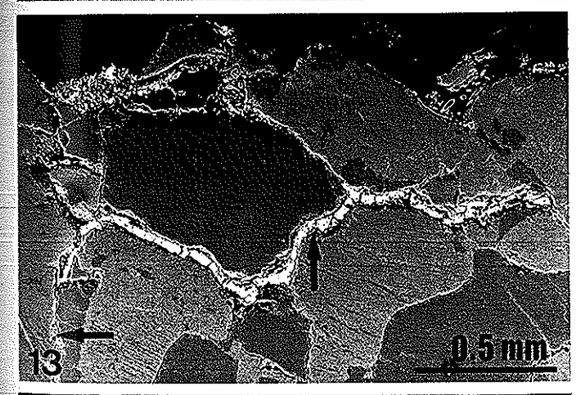
Fig. 14 Detail of the fissure shown in Figure **13**. Algal cells. L, lipid bodies.

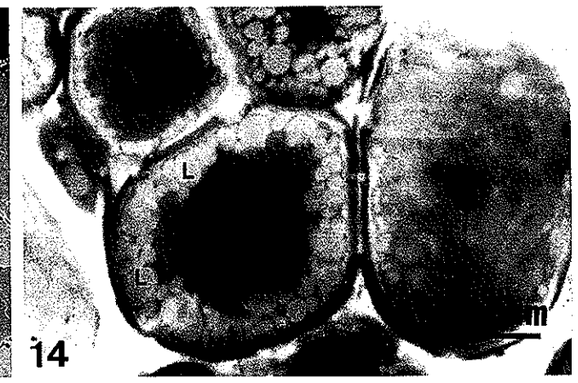
Fig. 15 Detail of a group of algal cells in a deep fissure.

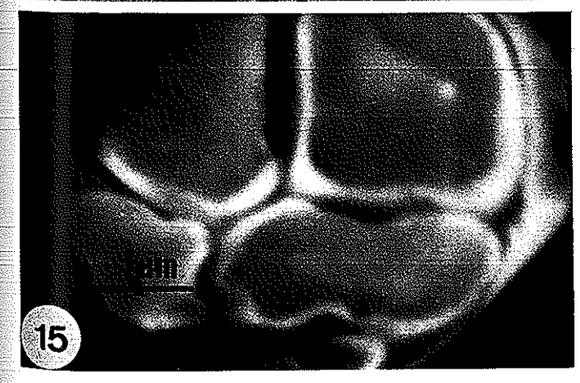
Fig. 16 High magnification of hyphae.

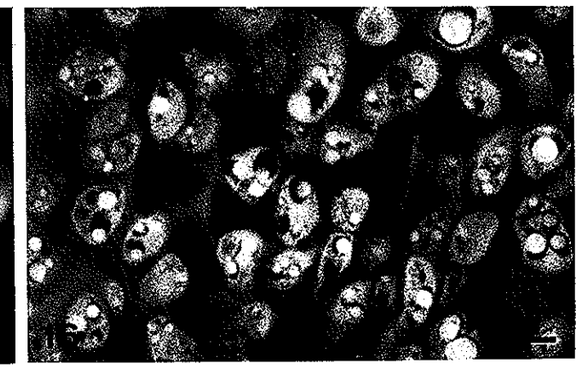












Figs. 9–16

Conclusions

The scanning electron microscopy operating in back-scattered electron emission mode permits investigation of the ultrastructure of the crustose thallus and crustose lichen-rock contact zone, until now unimaginable by applying other known techniques.

The application of the SEM-BSE technique with EDS system for the study of specially processed samples not only permits the chemical analysis of the minerals and embedded inorganic compounds, but also allows a detailed observation e.g. of the relationship between the oxalate crystals and the living elements which surround them, allowing the biological identification of the latter.

In the case where SEM in secondary electron mode have been used to investigate aspects such as the post fire recovery of rock-inhabiting algae, microfungi and lichens (Garty, 1992) the SEM-BSE images could contribute to a better knowledge of the cytological state of the rock-inhabiting organisms.

Study of the lichen-rock interface with the SEM-BSE technique also has other aspects. One of the primary applications of the observation at cellular level in the lichen-rock interface has interest for all types of research which are related to the study of biodeterioration in monuments. Chemical treatment is an alternative to the mechanical removal of lichens from building materials (Martin et al., 1992). The application of chemical should be based on the knowledge of the degree to which they affect the structural integrity, and also the physiology of the living elements which have to be removed from rock surfaces.

The observations at the ultrastructural level that can be carried out on the living organisms on the rock surface, as well as in the fissures, lead to the identification of algae, fungi, bacteria, etc. and also to knowledge of the relationship between them. Therefore one can achieve a better understanding of the biological weathering processes, which, according to Robert and Berthelin (1986), included biochemical weathering (result of the secretions of living organisms) but also other processes associated more specifically with the presence and activity of the organisms (solubilization and insolubilization of elements).

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C. Ascaso and *J. Wierzchos*

Centro de Ciencias Medioambientales
Serrano 115, bis.
E-28006 Madrid
Spain

"Pollen Buds" in *Ophiorrhiza* (Rubiaceae) and their Role in Pollenkitt Release

Martina Weber and A. Igersheim
Institut für Botanik, Universität Wien, Rennweg 14, A-1030 Wien/Austria

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Abstract

Pollen buds are apertural protrusions formed by the ectintine. In their wall unesterified and methyl-esterified pectins are detected by immunogold labeling. During pollenkitt formation pollen buds become adpressed and connected to the tapetum wall. The following expansion of the endothecium causes the mechanical rupture of the tapetum at the binding sites of the pollen buds. These events are accompanied by the release of the pollenkitt.

Key words

Apertural protrusions, ectintine, endothecium expansion, pectins, pollenkitt transfer, tapetum.

Abbreviations and Symbols

pb:	pollen bud
el:	endothecial layer
ml:	middle layer
pk:	pollenkitt
t:	tapetum
TEM:	transmission electron microscopy
SEM:	scanning electron microscopy
LM:	light microscopy

Introduction

Pollen buds[1] are defined as apertural intine protrusions and were first reported by Philip and Mathew (1975). In the following, the formation of pollen buds and their detachment from the pollen grain have been recorded from various taxa of Rubiaceae (Chennaveeraiah and Shivakumar, 1983; Mathew and Philip, 1987; Igersheim and Weber, 1993). The essential point in the results of Philip and Mathew (1975) and Mathew and Philip (1987) was that the vegetative nucleus is eliminated from the pollen grain via pollen bud detachment. Igersheim and Weber (1993) refute the published data in their ultrastructural reinvestigation: the vegetative nucleus remains in the pollen grain even after the pollen buds are detached.

The present paper is now focussed on the functional aspects of pollen bud formation and their detachment, and explains a possible role in pollenkitt release.

Materials and Methods

Anthers of the heterogameous *Ophiorrhiza longiflora* Bl. from Java, Puff 920916-1/4 (Figs. **1–2, 14–17**) and of an autogamous *Ophiorrhiza*[2] species from Sumatra, cultivated in the greenhouses of the Botanical Garden of the University of Vienna [cult. HBV sub RR 89-6; coll. M. Frimmel s. n. (Figs. **3–13**)] were investigated.

For LM and SEM, anthers were fixed in FPA. Microtome techniques used for material embedded in KULZER's Technovit 7100 follows Igersheim (1993). Pollenkitt lipids were stained with Sudan black (0.08 %) for 5 min, rinsed with glycerine and distilled water (1 : 1), and embedded in glycerine jelly. Pectins were localized with Ruthenium red staining (Gerlach, 1984). For contrast enhancement Ruthenium red staining was post-stained by toluidine blue (Figs. **1–2**).

For SEM investigations anthers were dehydrated in an alcohol series and FDA (Gerstenberger and Leins, 1978) and subsequently critical-point dried. The specimens were mounted on aluminium stubs with nail polish and sputter coated with gold.

The TEM embedding and staining processes follow Weber (1992).

For immunolabeling OsO_4 post fixation was omitted. After dehydration in a graded ethanol series samples were embedded in LR White (London Resin Co., Ltd.). Ultrathin sections were collected on gold grids and treated as follows: Preincubation in 2 % buffered BSA-solution (0.01 M PBS, pH 7.4 and 0.1 % Tween 20) for 90 min at room temperature; 2 h incubation with the primary antibody JIM5 or JIM7 (produced in rat) which recognizes unesterified (JIM5) and methyl-esterified (JIM7) pectins (Knox et al., 1990). After washing in PBS, sections were floated on a 1 : 30 dilution of goat antirat-IgG coupled to 10 nm col-

[1] "Pollen bud" will be used throughout the text, although the term is not appropriate and fortunate.

[2] As there is no recent revision of the genus, it is not possible at present to correctly identify the species.

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