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MICROSCOPICAL INVESTIGATION ON A MARBLE ENCRUSTING LICHEN

P. MODENESI and L. LAJOLO

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Abstract: The wheathering ability of *Aspicilia contorta* thalli with respect to their substrate, a marble tombstone, was studied by conventional, fluorescence, polarized and electron scanning microscopy. Our observations can help to visualize the mechanical fracturing and some product of the lichen metabolism which can affect the mechanical and chemical stability of the substrate.

Introduction

The effects caused by lichen colonization on lithic surfaces often give rise to serious problems of preservation for works holding an artistic and historical interest (Nimis et al. 1987).

This happens whenever, owing to the lichen covering, deterioration, chromatic alterations and loss of detail occur on the stonework.

The biodeterioration of the rock materials achieved by lichens results from physical and chemical processes (Syers & Iskandar 1973, Jones & Wilson 1985, Seaward 1988). The physical ones are erosion and breaking of the superficial layers in relation to hyphal adhesion and penetration between the particles of the substrate. That's because of the expansion and shrinking movements due to the variable hydration conditions of the thallus. The chemical ones, on the contrary, concern the solubilization and chelation processes carried out,in the formation of metallic complexes, by some metabolic products and other substances characteristic of the chemistry of the lichen thallus.

This investigation, based on the use of some methods of microscopical observation in the study of the relationships between an epilitic lichen and its substrate, is the first approach by the authors in the field of biodeterioration by lichens.

The authors hope to contribute to the definition of basic methods to study the physical-chemical changes due to the colonization of lichens on lithic substrates.

Materials and methods

Thalli of *Aspicilia contorta* (Hoffm.) Krempelh. were used in our microscopical investigations.

A few samples are preserved in the author's personal herbaria, a sample is in the lichenological herbarium of the University of Trieste (TSB, Herb. Nimis).

The lichen was growing on a fragment of a white marble of Carrara tombstone found in the graveyard of Semorile (Genoa), colonized mainly by the considered species.

Different procedures of preparation of the material were carried out according to the different kinds of microscopical observation.

1.1 - Small pieces of marble, colonized by the lichen, were obtained by a stone chisel impacting on the unbound block laid on the sand. These fragments were placed in diluted HCl (0.1-0.2 N) according to Fry's (1922) modified method till the $CaCO_3$ was removed (3-4 hours). The material was rinsed in water, then placed in phosphate buffer pH 6.8 0.01 M for 2 hours and subsequently fixed in FAA (formalin-ethanol 60%- acetic acid) (Sass 1958) for 24 hours.

After washing in buffer and after dehydration in an ethanol series, samples were embeded in JB4 water-soluble resin (Polyscience Inc.) (Brinn & Pickett 1979) in BEEM capsules (Polyscience Inc.). Sections 2.5 μm thick were cut with a glass knife on a Reichert OM2 microtome.

The following histochemical reactions were carried out on sections:

1.2 - Toluidine Blue 0 (TBO) 0.5% in water for 2 minutes as a general stain (O'Brien & McCully 1981).

1.3 - Toluidine Blue 0 (TBO) 0.5% in acetate buffer 0.05 M pH 4.4 as a metachromatic stain for polyanions (to demonstrate metachromasia of acid polysaccharides (Pearse 1985).

1.4 Periodic acid - Schiff reaction (PAS) for general localization of insoluble polysaccharides (Pearse 1985).

1.5 Alcian Blue 8GX 1% in acetic acid 3% at pH 2.5 for 30 minutes for the localization of acid polysaccharides (Lev & Spicer 1964).

2.1 - Small pieces of thallus were detached from the substrate by a sharp chisel, then treated according to Yasue method (1969). Samples were placed in acetic acid 5% for 30 min. to remove calcium carbonate and the possible presence of calcium sulphate and phosphate; they were then transferred in $AgNO_3$ 5% for 15 min. and later rinsed in a saturated solution of rubeanic acid (ditio-oxamide) in ethanol 70% including 2 drops of NH_4OH 25% for 1 min.

Through this procedure the crystals of calcium oxalate, insoluble in acetic acid, showed themselves as a dark-brown precipitate when observed with light conventional microscopy.

2.2 - After digestion with acetic acid 5%, some of the samples were dehydrated and embedded in JB4 as described above. Semithin sections, stained with aqueous TBO, were observed by light conventional microscopy supplied with orientative polarization (see further).

Another series of observations were carried out on samples freely sectioned on the substrate. This in order to visualize the interactions between the lichen and its substrate by means of two procedures.

3.1 - Marble sections about 1 mm thick carrying lichen, were obtained by a sharp woodwork chisel, struck by a hammer, impacting on the unbound block. The stroke on the block was thrown perpendiculary to its borders: in this way it detached the small above-mentioned sections, used in the different observations.

3.2 - A parallel series of observations were carried out on sections obtained by the chisel impacting parallely with the surface of the marble and very little below it.

This method allowed to obtain thin marble chips carrying small pieces of lichen, suitable for the superficial view of the thallus.

The samples obtained in this way were employed in the following conditions:
a) observed with orientative polarization
b) observed with fluorescent microscopy
c) observed with scanning electron microscopy

The above cited observations (a) and (b) were carried out by a Leitz Dialux 22 EB microscope.

Particularly in the observations (a) an apparatus for the orientative polarization was added to the microscope, consisting of an analyzer suitable for its interposition between the eye-piece and the lens and of a rotating polarizer placed before che condenser.

In the observations (b) a Ploem-Opak epi-fluorescent apparatus was added to the microscope together with a Hg HBO vapour lamp (50 W) and with two Leitz filter-systems. The former (H2) determines the blue-violet incident light between 390-490 nm of wavelenght, the latter (A) determines a ultraviolet incident light between 340-380 nm of wavelenght.

In a microscope equipped by epi-illumination the thickness of the examined samples is not very important; this permitted us to employ the marble chips.

For the SEM observations the samples were directly coated 200-220 Å in thickness with gold in a Sputtering Agar Aids and observed with a Cambridge Stereoscan 250 MK2 at an acceleration voltage of 20 Kv.

Results and discussion

Aspicilia contorta is an epilithic areolate lichen which forms a dense covering on the examined marble surface. The tombstone is situated 60 cm above the ground, facing South and having a sub-horizontal lying. Fig. 1, a SEM micrograph obtained with 3-i procedure, shows the substrate underlying the lichen. In SEM observations the marble appears like a rock of granular close structure, made by crystal aggregates of small rhombohedral particles of calcite (grains), here about 200 μm in thickness. They are closely connected without a specific link, owing to interaction forces rising from the typology of formation, which hold them together (Mannoni & Mannoni 1978). In the same illustration some detachments among the

grains are visible, presumably an artefact caused in this case by the cutting technique. According to Manganelli del Fà & Lazzarini (1986) every outer force acting on the rock can affect the stability of the granoblastic structure, producing microfissures. In other words according to the above mentioned authors, already during the excavation of the blocks, the material might undergo stresses becoming still bigger and bigger during the realization of the manufacture. In consequence of this some superficial porosities in the marble may occur. Moreover, because of the anysothropic features in relation to the expansion coefficient of calcite crystals, some tensions inside the rock may take place when directly exposed to the sun light. Such tensions would lead to disaggregation of the grains, together with clear processes of polygonal cracks and superficial crumbling on the exposed areas of the marble (Manganelli del Fà % Lazzarini 1986). In this way the above mentioned events should promote the establishment of various epilithic organisms and their following penetration.

The micrograph 2, a magnification of the foregoing one, shows a bundle of hyphae of *Aspicilia contorta* creeping along the face of a calcite grain, once adhesion surface of another grain.

The lichen hyphae should have penetrated through a pre-existent porosity, reaching about 400 μm in depth, as it's deducible from fig. 1.

We can suppose that the alternate hydration conditions and the lichen growth increase the detachment of the grain.

Hyphal penetration is still visible at 1 mm in depth in fig. 3, another magnificant of fig. 1, showing other interesting effects due to the lichen presence. The central part of the micrograph shows the above examined aspect, the magnification of its upper side (figs. 4, 5, 6) bears evidence of clear and deep incisures on the surface of the calcite crystals, looking like the impression leaved by the lodgings of the hyphal ramifications. Such incisures seem to testify a superficial former corrosion of the grains, appearing as distinctive "furrowings" of the mineral surface.

In our case the SEM observations seem to show that the hyphal ramifications don't penetrate the hard calcite crystals, but eroding them superficially, they go round them, creeping in depth afterwards, exploiting and increasing detachments of the grains.

Fig. 1 - 6: Scanning electron micrographs.

Fig. 1 - Fragment of marble of granoblastic structure carrying *Aspicilia contorta* (arrow). X 70.

Fig. 2 - Along the surface of a calcite grain a bundle of deeply penetrating hyphae, is visible. X 700.

Fig. 3 - At 1 mm in depth (compare with fig. 1), bundles of hyphae and their marks, looking like "furrowings" along the faces of the calcite grains, are visible. X 200.

Fig. 4 - 6: Various aspects of the grain surfaces colonized by the lichen thallus. Clear incisures of the mineral surface and the hyphal ramifications are visible. Fig. 4: X 800; fig. 5: X 500; fig. 6: X 1200.

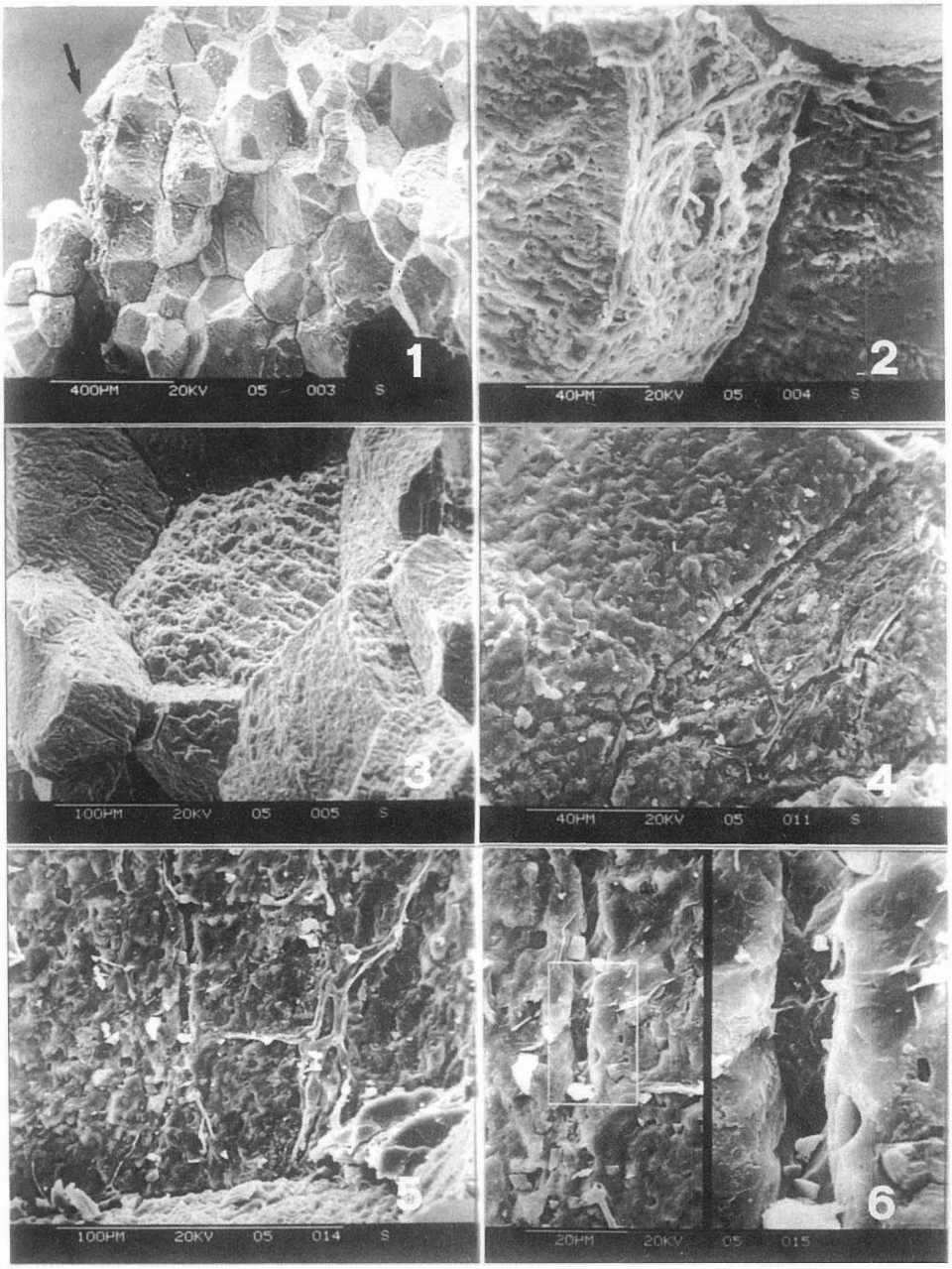
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Fig. 7 shows a semithin section of the upper portion of an areola of *Aspicilia contorta* after removal of the substrate (1.1), stained with TBO (1.3) at pH 4.4.

In this conditions several different polyanions (polycarboxylic acid, polysulphates and polyphospates) carry a negative charge and give a metachromatic reaction, staining magenta-red with TBO.

The remaining portions of the thallus stain orthochromatically (blue); the outer part of the cortex and the medulla stain metachromatically.

The positive PAS (1.4) and Alcian Blue (1.5), here not shown, confirm this fact giving evidence of the mucopolysaccharidic nature (carboxylated polysaccharides) of the substance responsible for the metachromatic staining with TBO.

This type of molecule is capable of linking water in amounts directly related to the intensity of the available negative charges (Modenesi & Vanzo 1986).

Mucopolysaccharidic substances can contribute to thallus hydration, delaying drying and preventing excessive water loss. Fig. 8 shows the lower part of the areola: in direct contact with the substrate the hypothallus in visible. It is made up of a network of hyphal bundles, not penetrating the rock in this area.

On the contrary, two other illustrations (figs. 9, 10) show that thick bundles of hyphae creep in depth, just where this penetration is possible, probably because of the pre-existent superficial disgregations of the grains.

In this case the observations carried out with SEM are confirmed. It's opportune to report the presence, at 400-500 μ in depth, of clusters of algae which have been brought here by the penetrating bundles of hyphae (figs. 10, 11).

This has been reported as a common feature in some epilithic lichens growing on calcareous rocks (Fry 1922). This fact testifies, according to us, the good hydration conditions and the sufficient lighting in depth. Marble is a translucent rock: light passes through a 25 mm in thick section of white marble of Carrara (Mannoni & Mannoni 1978).

Inside the substrate the hyphae constituting the bundles sometimes take a globular appearance (fig. 12), forming dense clusters of spheroidal, thin walled cells.

These formations (Bachmann 1919, Fry 1922, Kushnir et al 1978) in epi- and endolithic lichens mostly on calcareous substrates, are known as oil hyphae containg triacylglycerol as the predominant lipid component (Kushnir et al. 1978).

Following the 1.1 procedure, the dehydration in an ethanol series and the em-

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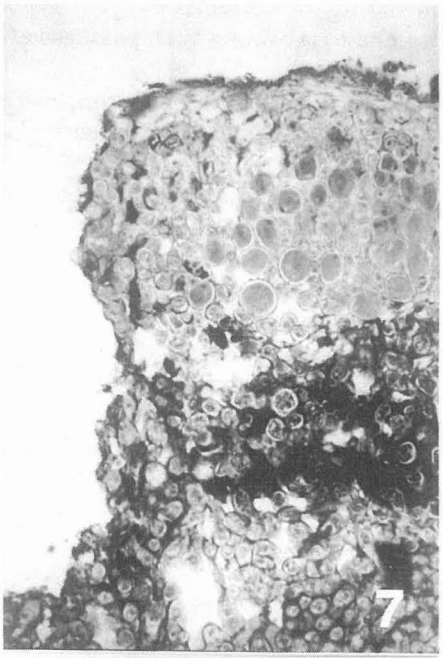
Fig. 7 - 12: Semi-thin cross sections of the thallus of *A. contorta* after removal of calcium carbonate by hydrochloric acid. TBO.

Fig. 7 - Upper portion of an areole. The medulla and the thin outer portion of the cortex stain metachromatically. X 500.

Fig. 8 - Lower part of an areole. Note the network of the bundles of hyphae, constituting the hypothallus, directly in contact with the substrate (here removed). X 500.

Fig. 9 - 10: Some strong bundles of hyphae leave the hypothallus, they deeply creep into the substrate exploiting its porosities. X 500.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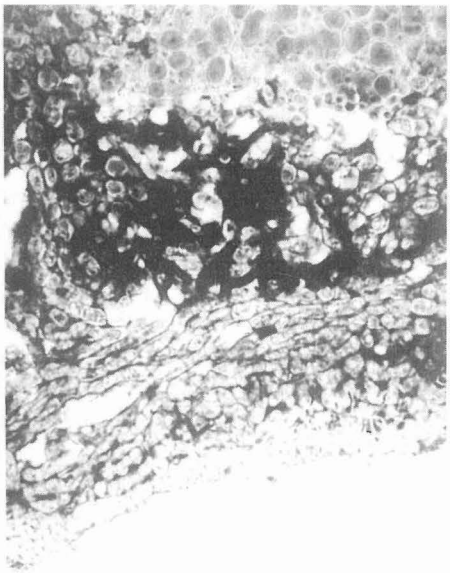

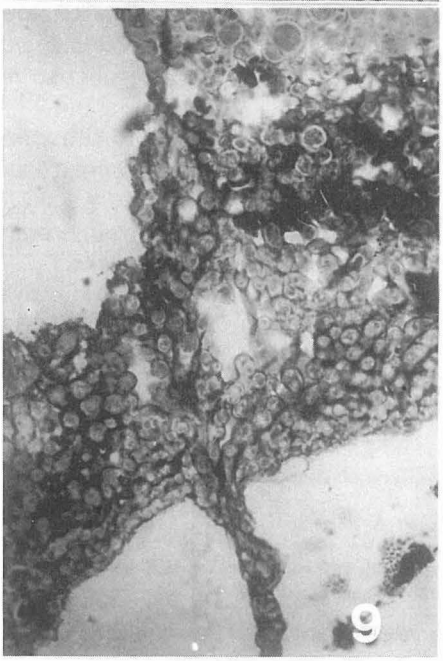
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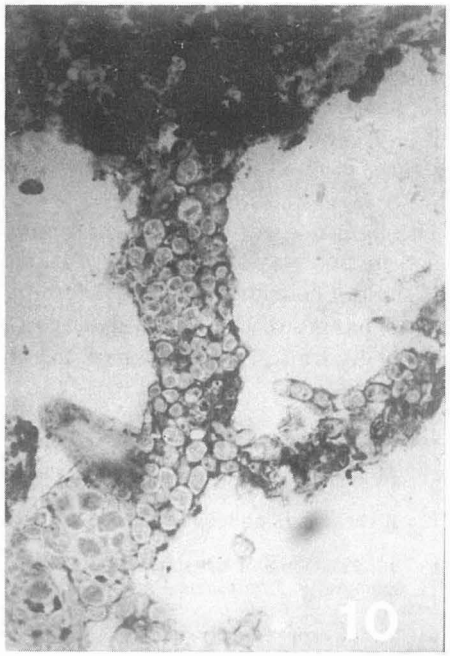
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bedding in resin have completely removed the lipidic contents.

The histochemical evidence may be obtained by avoiding such passages after digestion with HCl (1.6).

The content, little oil drops, is displayed by staining with benzopyren, specific and very sensitive to lipids which stain bluish in fluorescence when observed under UV light (A filter) (fig. 13).

Using such stain the formations of oil hyphae appear to be plentiful and diffused in the whole soft hyphal network penetrating marble through its several porosities (fig. 14). Beside the apothecia (fig. 15), in accordance with the observations of Fry (1922), a lot of such formations are visible; this may be in relation to the zones of high metabolism.

According to other authors, Fry (1922) suggested that the lipid formed a storage of waste product in relation the adverse environment conditions, such as the scarcity of nitrogen and the excess of calcium carbonate.

Kushnir et al. (1978), on the basis of the observation that isolated mycobionts of endolithic lichens retained their ability to produce and accumulate unusually large quantities of lipids under optimal growth conditions, think this feature as to be due to the genetics of the fungus, but they consider still enigmatic its display in epi- endolithic lichens. Other authors (Dertien et al 1977) suggest that oil is a possible source of endogenous water or that it represents a storage material and a metabolic fuel (Sorokin 1967).

Apart from the functional meaning of the oil hyphae, it seems reasonable to point out their massive presence in the suprficial zone of the marble colonized by *Aspicilia contorta*. The role played by the lipidic material with respect to rock biodeterioration is unknown.

Fig. 16 shows the thalline surface of *A. contorta* still binded to the marble substrate, viewed with light microscopy equipped with orientative polarization (3.2).

The light doesn't pass the thick areolate thallus, but it does through marble cracks, in this way drawing the outlines of the thalline compartiments.

It's under consideration the possibility of using this type of observations to follow the changes of the size and of the borderline of the areoles in relation to the different hydration conditions of the thallus.

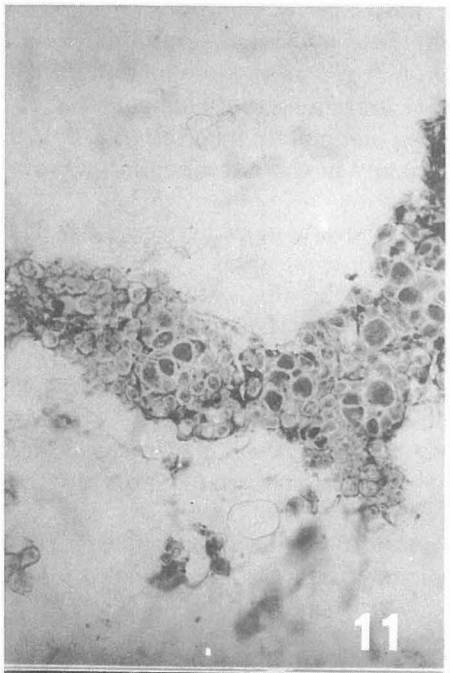
Fig. 11 - At 400-500 μm in depth inside the substrate some clusters of algae are visible. X 500.

Fig. 12 - A cluster of "oil hyphae" inside the substrate. X 500.

Fig. 13 - 15: Fragments of the thallus after digestion of calcium carbonate by HCl stained with benzophyrene. Fluorescence microscopy, UV light.

Fig. 13 - The oil drops contained in the oil hyphae are visible on account of their bluish UV fluorescence after staining with benzophyrene. X 500.

Fig. 14 - All the fine hyphal weaving, penetrating the marble, are rich in oil storages. X 100.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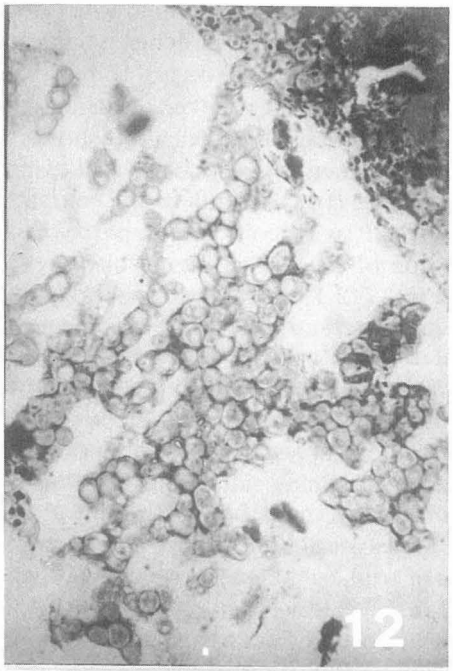
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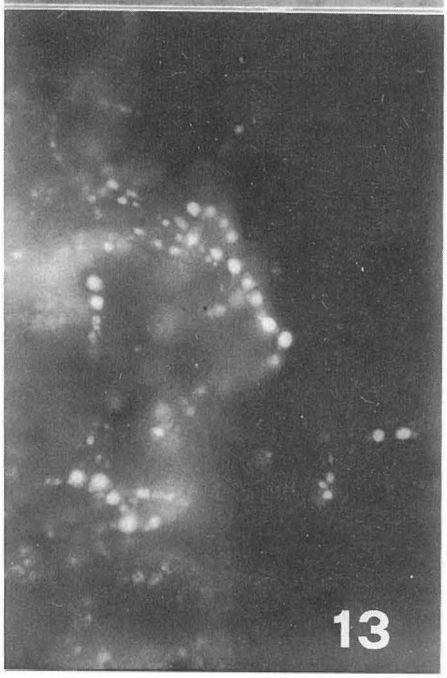
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In fact according to Malinowski (1911) the areolate structure is an adaptation peculiar to the epilithic lichens against alternate hydration situations.

The figs. 18, 19, 20 regard thin sections (2.2) of material in which the calcium carbonate has been removed by acetic acid 5%, and stained with aqueous TBO (1.2).

The orientative polarization shows, when completely inserted (fig. 20), that some birefractive crystals are still visible mainly in the lateral epilithic parts of the areoles (figs. 18 and 19 for comparison).

Fig. 21, with polarization partly inserted, shows a more general view of the location of crystals in the areoles.

Fig. 17 (2.1) shows the appearance of a thalline areole treated with acetic acid 5% and then stained with the Yasue method (1969) for the localization of calcium oxalate. This appears as a brown-black precipitate.

The upper view of the areole with the oxalate precipitate placed as a ring, confirms the observations carried out with orientative polarization.

Oxalic acid seems to be an important agent of weathering of rocks (Jones et al. 1980), forming salts whose cations depend on the nature of the lithic matrix colonized by the lichen (Wilson et al. 1980).

Ascaso et al.(1882) were able to point out that dissolution of calcite by secretion of oxalic acid led to precipitation of calcium oxalate in *Caloplaca callopisma* and other calcicolous lichens.

Our observations visualize this phenomenon in *A. contorta* showing the localization of the oxalate.

Sections obtained with the 3.1 procedure have been observed by fluorescence microscopy.

Fig. 22 shows one of these samples observed under violet-blue light (H2 filter). In the picture are visible the cross sections of an areole and of a lecanorine apothecium, in which the algae, fluorescent in red because of the chlorophyll a, climb up the thalline edge.

The medullary hyphae, beneath the apothecium and in the near areole, penetrate into the marble and have a yellowish fluorescence.

The cortex is not visible by means of this type of observation, but when the sections were observed under ultraviolet light (A filter) the algal layer is hardly visible, the medulla and the cortex appear bluish fluorescent (fig. 23). Fig. 24 shows such bluish fluorescence by a greater magnification, this picture may be

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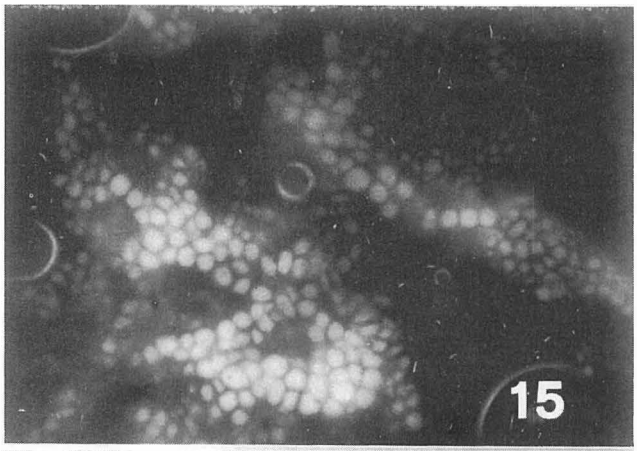
Fig. 15 - The oil storages are mainly placed beneath the apothecia. X 500.

Fig. 16 - Upper view of the thallus of *A. contorta* still binded to the marble, observed with orientative polarization. The light doesn't pass through the thick thalline areoles, looking dark, while it does through the fissures where the thin hypothallus occurs. X 100.

Fig. 17 - Upper view of a thalline areole after digestion of calcium carbonate by acetic acid and then treated according to Yasue (1969) method. The calcium oxalate precipitates stain dark-brown. X 100.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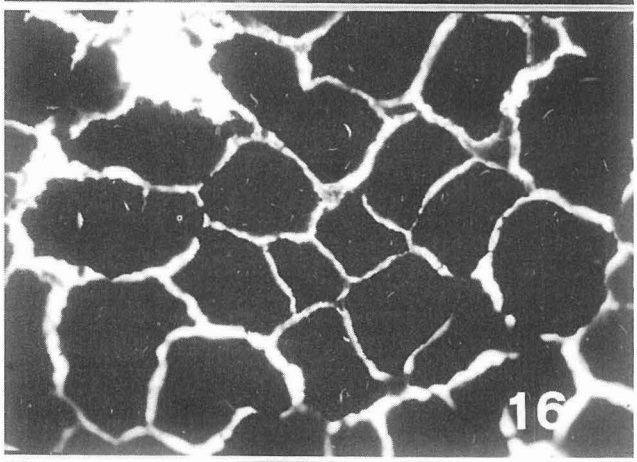
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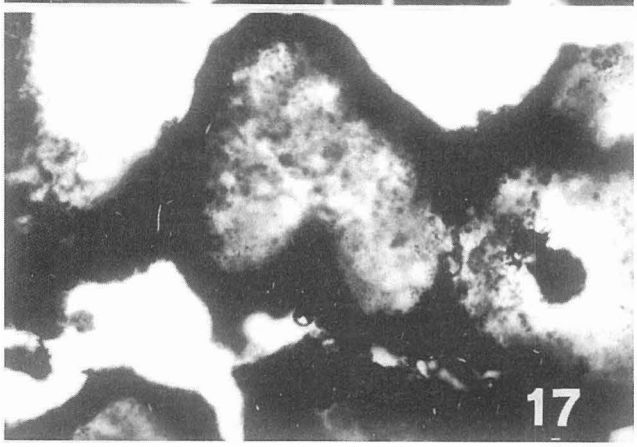
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compared with a section obtained by the same procedure (3.1), but observed in orientative polarization (fig. 25). In the cortex a deposit of birefractive crystalline material is clearly visible, corresponding to the material responsible of the bluish fluorescence of the cortex in UV.

The localization of this UV fluorescent material may be observed one more time following the 3.2 procedure. Examining the thallus with fluorescence microscopy, a bluish fluorescent deposit becomes visible, widespread on the cortical surface of the areoles and absent in the crack. Such deposit can be removed by a previous immersion in acetone for a few minutes.

Some investigations (TLC analysis, data not reported) carried out about the nature of this substance and informations from the literature (Culberson 1969, 1970; Culberson et al. 1977) led us to exclude any connection between the substance observed by fluorescence microscopy and the lichen substances known in *A. contorta*. This substance, moreover, though is soluble in acetone as the lichen substances, unlike these ones appears to be strongly water soluble.

In fact observing figs. 27 and 28 we can note, as well as it's visible with fluorescence microscopy, the immediate removal of the UV fluorescent cortical substance due to a water drop (fig. 26) added to a section initially examined in dry conditions (fig. 27).

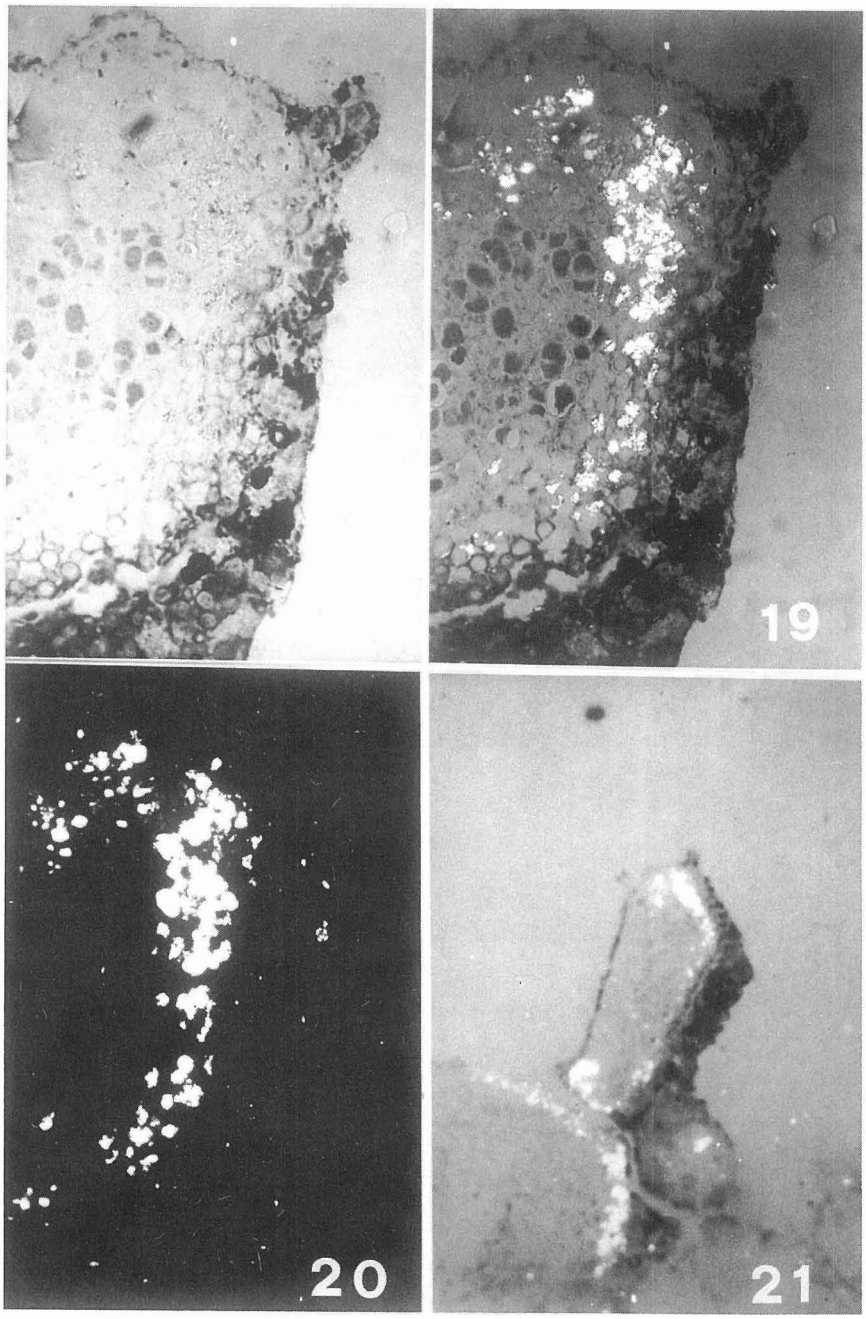
Some lichen substances show a very low water solubility, about 5 to 57 mg/l (Iskandar & Syers 1972); however this is sufficient, owing to the presence of polar groups, to justify their capability to form metallic complexes with the cations constituting the mineral surfaces (Syers & Iskandar 1973).

At present we are investigating the nature of the UV fluorescent substance, trying to characterize it and to determine its possible capability to complex metal cations of the substrate.

Fig. 18 - 21: Cross sections of a thalline areole after removal of calcium carbonate by acetic acid. Observations with the orientative polarization.

Fig. 18 - 20: Series of micrographs showing the appearance of the birefractive crystals of calcium oxalate along the lateral portions of an areole. Polarization excluded (fig. 18), partly inserted (fig. 19), entirely inserted (fig. 20). X 500.

Fig. 21 - General view of the thalline areoles containing the birefractive crystals of calcium oxalate. Polarization partly inserted. X 100.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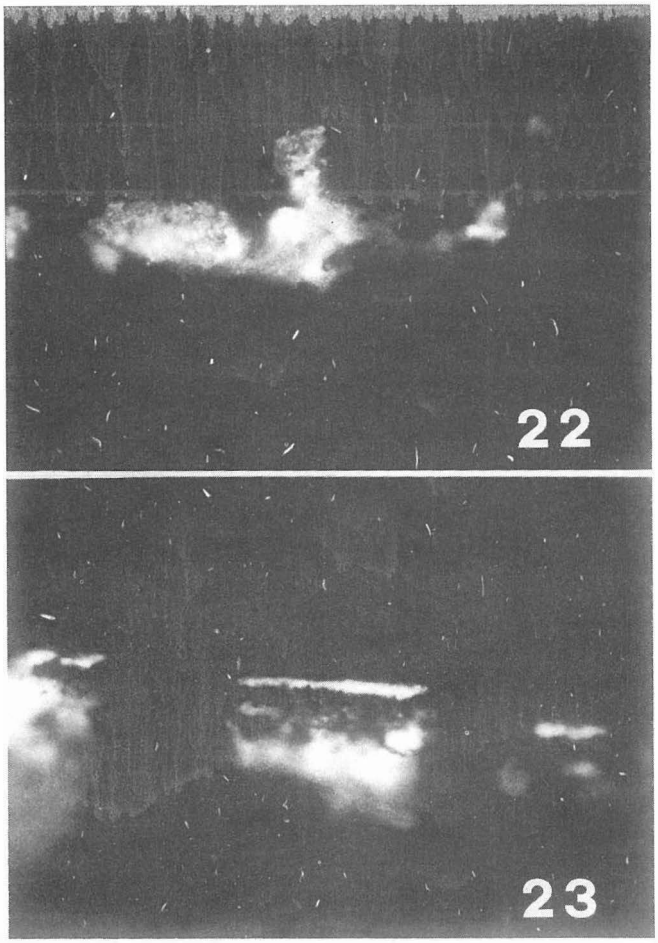


Fig. 22 - 24, 26 - 28: "Free-hand" sections of *A. contorta* thalli still binded to the marble. Fluorescence microscopy.

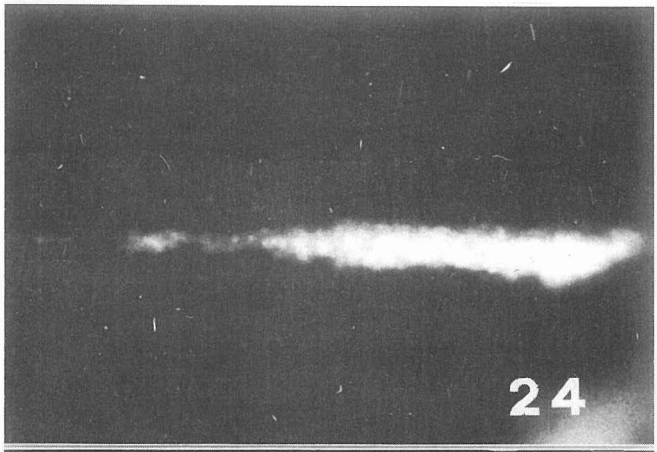
Fig. 22 - Cross section of an areole and an apothecium. Observation under violet-blue exciting light. X 100.

Fig. 23 - Cross sections of an areole observed under UV exciting light. Note the intensely fluorescent (bluish) strip at the cortical level. X 100.

Fig. 24 - Magnification of the preceding one showing the fluorescent strip at the cortical level. X 500.

Fig. 25 - Cross sections of a thallilne areole observed in polarized light. In correspondance of the cortex a shining band is visible due to the occurrence of birefractive crystals. X 100.

Fig. 26 - Upper view of the thallus observed under UV exciting light. A bluish fluorescence occurs upon every areole. X 100.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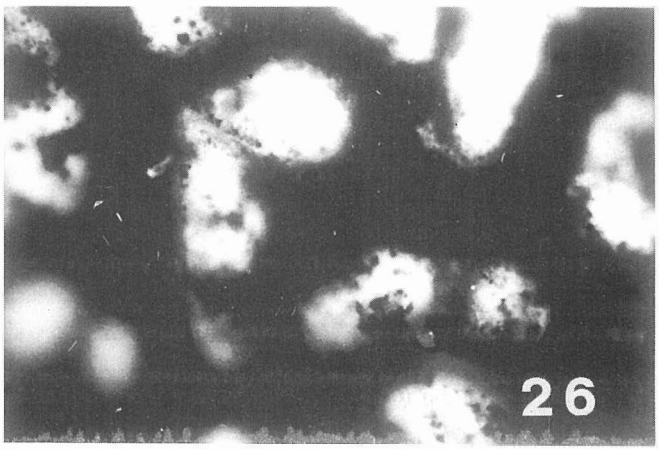
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Conclusion

The techniques of observation with the optical microscopy provide a valuable tool of investigation because of their versatility and suitabillity to the different conditions of study.

The use of resins for the preparation of samples and the use of modern methods of histochemical observations make easier the possibility of carrying out careful structural and clear morphofunctional studies, integrated by parallel observations under polarized light and with epifluorescence microscopy, as it's shown by our work.

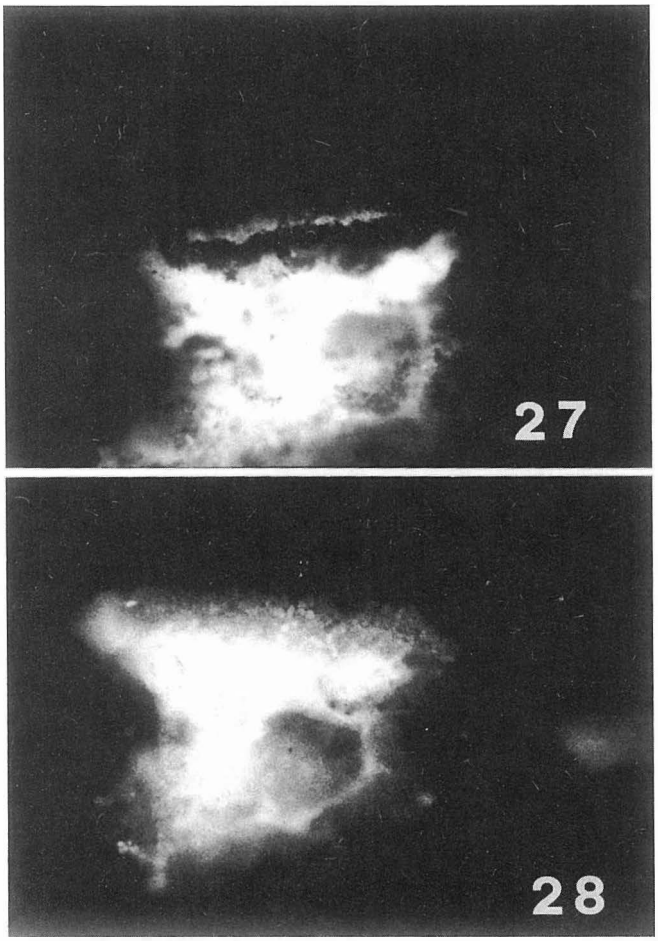
The methods of microscopical investigation constitute a useful background which can contribute, together with other methods of study, to the knowledge of the complex relationships between a lichen and its substrate, through the direct visualization of the effects.

When the lithic matrix colonized by the lichen holds such interest that it warrants its preservation the direct visualization of physical and chemical alterations may contribute to the determination of precise preservative actions.

This in accordance with the type of the relations observed, depending on the nature of the substrate and on the lichen species.

Fig. 27 - Cross section of a thalline areole observed under UV exciting light. The sample is laid on the slide in dry conditions. X 100.

Fig. 28 - The same preceding picture observed after the sample was wetted directly under the lens. Note that the bluish fluorescent strip once distinguishable in the cortex, now it's no longer visible. X 100.



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Authors' address:
Dr. Paolo Modenesi
Dr. Lorenzo Lajolo
Istituto Botanico "Hanbury"
Università di Genova
Corso Dogali 1/c
16136 Genova