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An ultrastructural, anatomical and molecular study of the lichenicolous lichen *Rimularia insularis*

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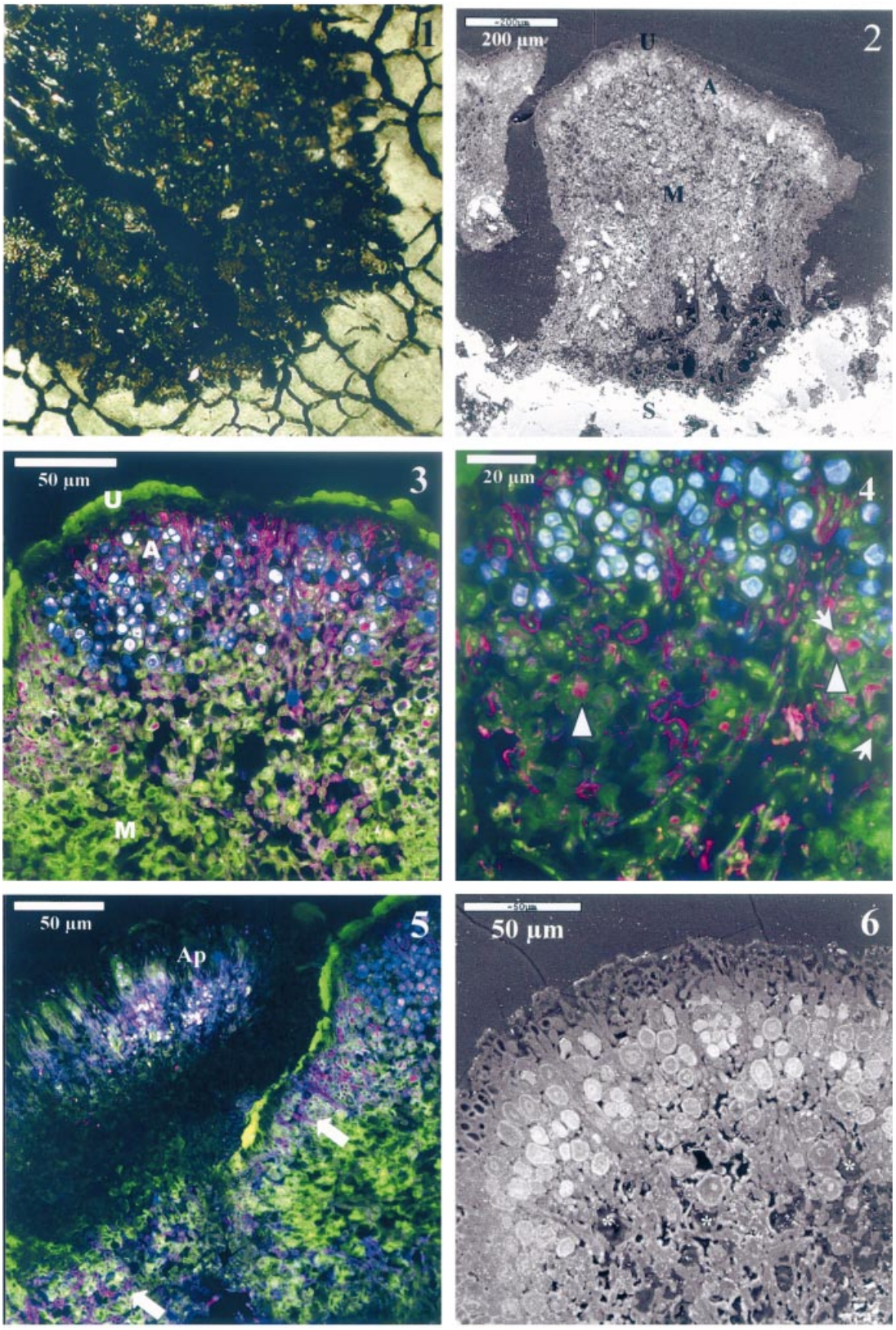
Rimularia insularis forms an photosynthetic thallus on lichens of the *Lecanora rupicola* group. In the thalli of *R. insularis*, some hyphae of the host are detectable, and they are situated mainly in the basal part of the *Rimularia*'s medulla. No *Lecanora* hyphae were present in the *Rimularia*'s algal layer and they were indistinct in the upper parts of its medulla, from where we obtained clean ITS-sequences of *Rimularia*. Direct PCR from various sections of the algal layer detected only one photobiont genotype, although the algal cells could be assigned to two morphologically different types. Calcium deposits were found in the upper parts of the medulla, while abundant rock substrate fragments with different mineral compositions were present in the lower parts. Cavities and fissures in the rock below the infected thalli were filled by fungal cells, PCR analyses of such parts indicated that the *Rimularia* hyphae extend below the host, and into the rock.

INTRODUCTION

Associations between fungi and algae (or cyanobacteria) can be highly complex and may involve multiple symbionts (Hawksworth 1988). Beside the balanced association of photobionts with a single species of fungi as is typical for lichens, additional fungi may occasionally be present. Those that lack a photosynthetic thallus are lichenicolous fungi, while these that form distinct photosynthetic thalli in or on their hosts are termed lichenicolous lichens (Poelt & Doppelbauer 1956, Poelt & Steiner 1971, Hafellner & Poelt 1980, Poelt 1985, Holtan-Hartwig & Timdal 1987). Around 100 species of lichenicolous lichens were listed by Poelt & Doppelbaur (1956), and the number was considerably extended by Rambold & Triebel (1992). They included 205 lichenicolous species in interlecanoralean associations. However, assignments to life styles are sometimes based on small samples sizes, as some species some known only from a few collections or the type. Beside this, some lichens are facultatively lichenicolous, or change with age (Hawksworth *et al.* 1979, Holtan-Hartwig & Timdal 1987). The number of lichenicolous species in other fungal orders is certainly much lower, but this is still poorly studied.

While non-lichenized lichenicolous fungi of larger, predominantly non-lichenized lineages may be associated with the host's mycobiont (de los Ríos & Grube 2000, Grube & de los Ríos 2001, de los Ríos *et al.* 2002), those of lichenized groups rather have an affinity to the hosts algae. The question of algal specificity of lichenicolous lichens has been touched in a few publications. Friedl (1987a, b) found that the original alga of the host (*Cladonia* sp.) is present in young stages of the *Diploschistes muscorum* infection, but it is later replaced by another photobiont. Here, the juvenile parasite changes to a independent lichen by switching to its own photobiont. No indication for such switch is apparent in *Lecanactis grumulosa* which infects *Roccella* thalli (Feige & Lumbsch 1993) or in *Blarneya hibernica* infecting *Enterographa* and *Lecanactis* species (Hawksworth *et al.* 1979). On the other hand, Lücking & Grube (2002) found a switch to a different alga in lichenicolous morphs of the foliicolous species *Chroodiscus coccineus*. In these cases, the manifestation of the lichenicolous lichen thallus apparently occurs by association with the host lichen's algae. It is likely that lichenicolous lichens lost the capacity to form an independent thallus and that they take advantage of the corresponding photobiont of the host, however, this has not been studied by molecular methods.

To understand the interfungal relationships in lichen associations, more ultrastructural and anatomical studies are required. In this study, we focused on the



Figs 1–6. *Rimularia insularis*. **Fig. 1.** General view of thallus colonizing *Lecanora rupicola*. **Fig. 2.** SEM–BSE micrograph of *R. insularis* thallus-granite substrate interface. U, upper cortex; A, algal layer; M, medulla; S, lithic substrate. **Figs 3–5.** Confocal images of a vertical section of *R. insularis* thallus stained with acridine orange. Argon laser (488 nm), Krypton laser (568 nm) and HeNe laser (633 nm). Band pass at 535 nm, band pass at 615 nm and a long pass at 645 nm were used as emission filters. U, upper cortex; A, algal layer; M, medulla. Pink cells correspond to *Rimularia* hyphae. **Fig. 3.** General view. **Fig. 4.** Algal layer and upper part of the medulla. Arrowheads indicate degraded algal cells and arrows penetration of *Lecanora* hyphae into algal cells. **Fig. 5.** Thallus with a *Rimularia* apothecium (Ap). White arrows indicate the accumulation of the *Rimularia* hyphae in the proximity of *R. insularis* apothecium. **Fig. 6.** SEM–BSE micrograph of upper cortex and algal layer of *R. insularis* thallus. Asterisks indicate gaps where the algal cells have died.

lichenicolous lichen *Rimularia insularis* which occurs in species of the *Lecanora rupicola* group (Hertel & Rambold 1990). *R. insularis* (syn. *Lecidea insularis*) is a saxicolous representative of the genus *Rimularia* (*Rimulariaceae*, *Lecanorales*), which comprises species growing on siliceous rocks (both lichenicolous and independent from a host), bryophytes, and bark (Hertel & Rambold 1990). We studied the parasite–host interface by confocal, light and electron microscopy. We also included the substratum in our investigation to see whether this has a role in the infection process. The development of the SEM–BSE technique (Wierzchos & Ascaso 1994) has facilitated such investigations. Previous observations with this technique and confocal microscopy revealed a very close co-occurrence of the some lichen symbionts and other microorganisms in the substrate (Ascaso, Wierzchos & de los Ríos 1995, Wierzchos & Ascaso 1996, Ascaso, Wierzchos & Castelló 1998, de los Ríos *et al*. 2001).

MATERIAL AND METHODS

Material

Material of *Rimularia insularis* collected in Crete and Austria is deposited in the herbarium of the Institute of Botany in Graz (GZU). Investigated specimens: **Austria**: *Steiermark*: Steirisches Randgebirge, Koralpe, Handalpe Nord Weinebene, on siliceous rocks, 1999, *Grube s.n.* – **Crete**: Nomos Chania, Azogires northwest Palaeohora, 1999, *Grube s.n.*

Confocal microscopy

Sections were obtained using a Cryostat (Leitz, Wetzlar; −15 °C knife temperature) microtome and stained with acridine orange (5 mg ml^{-1} aqueous solution) for 5 min. Examination was by a confocal laser scanning microscope (LeicaTCS NT) as described in de los Ríos, Ascaso & Grube (2002) and Grube & de los Ríos (2001). For excitation, we used Argon laser (488 nm), Krypton laser (588 nm) and HeNe laser (633 nm). As emission barrier filters, a band pass at 535 nm, band pass at 615 nm and a long pass at 645 nm were selected. Pictures were recorded by simultaneous scanning of the three channels and were displayed by an overlay technique. Each channel is displayed in a unique colour (green, red or blue). The images were analysed using Leica TCS-NT/SP Software 1.6. The basic dye acridine orange display an orthochromatic (excitation at 502 nm and emission at 536 nm: green) and a metachromatic state (excitation at 460 nm and an emission at 650 nm: red), which allows to distinguish polysaccharides of varying acidity, or DNA from RNA and denatured DNA (Rost 1995).

Transmission electron microscopy

Rimularia insularis and *Lecanora rupicola* thalli were prepared following the protocol described in de los Ríos & Ascaso (2001). Small pieces of lichen thalli were fixed with glutaraldehyde and osmium tetroxide solutions, dehydrated through a series of ethanol solutions, and embedded in Spurr's resin. Ultrathin sections were post-stained with lead citrate (Reynolds 1963) and observed in a Philips 300 transmission electron microscopy.

Scanning electron microscopy

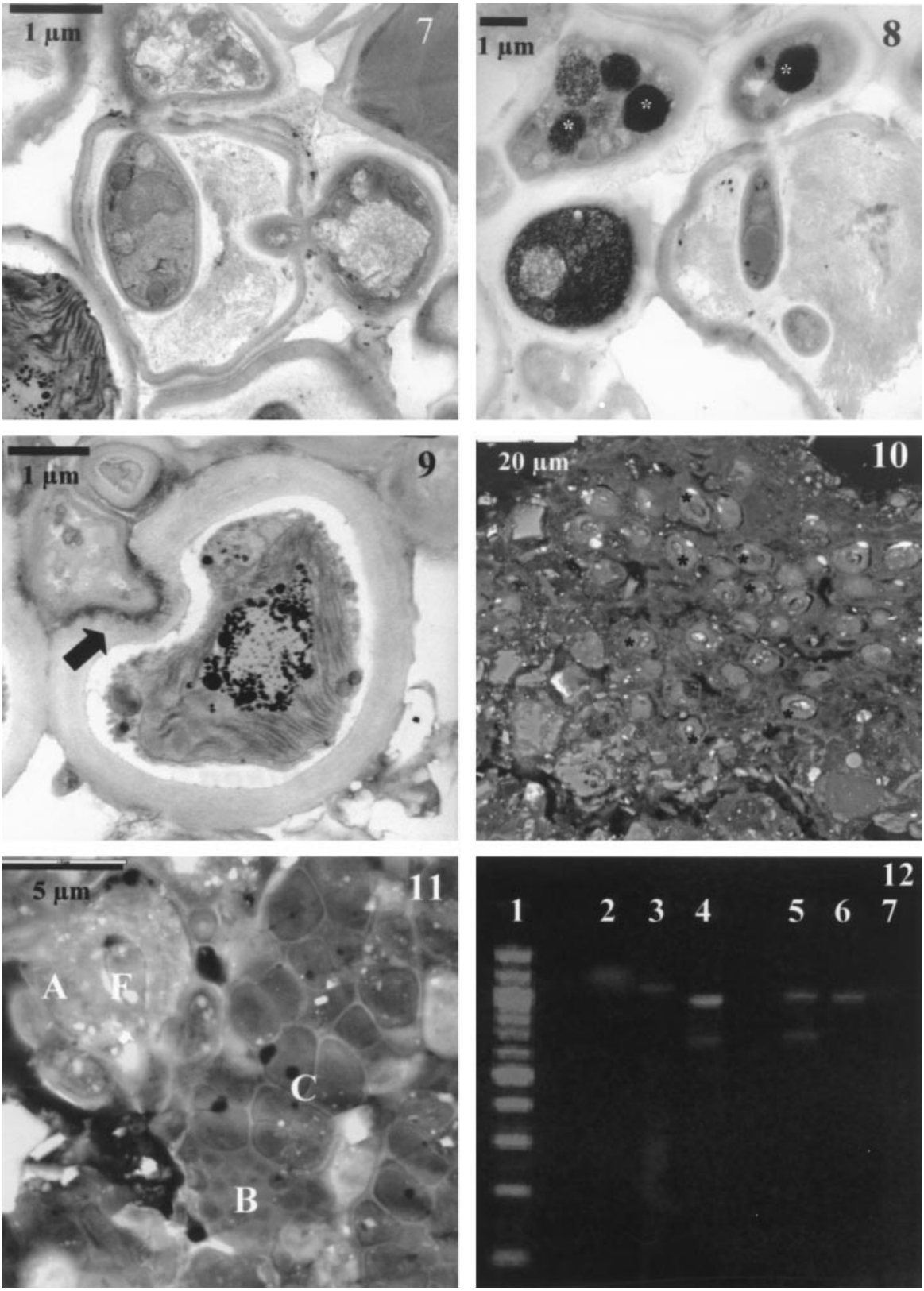
Lichen thalli were processed together with their substrate following Wierzchos & Ascaso (1994) for scanning electron microscopy operating in back-scattered electron mode (SEM–BSE). Hand-cut pieces were fixed with glutaraldehyde and osmium tetroxide solutions, dehydrated through a series of ethanol solutions, and embedded in LR-White acrylic resin. Before observation in a DMS 940-Zeiss microscope, the samples were sawn, finely polished, and carbon-coated. Microprobe analyses were performed using an energy dispersive spectrometry (EDS) Link ISIS micro-analytical system during the SEM observation.

Direct PCR amplification and DNA sequencing

Horizontal sections from different layers of *R. insularis* and *L. bincincta* prepared using a Gilette razorblade and small fragments from lithic substrates were placed in an 0.5 ml Eppendorf tube for direct PCR-amplification of the ITS regions of rDNA from the fungal and the algal partner, according to Wolinski, Grube & Blanz (1999). Primers for amplification of the DNA from the fungal partner were ITS1F and ITS4 (White *et al*. 1990) and from this of the algal partner were ITS1T and ITS4T (Kroken & Taylor 2000). 50 µl PCR mix (10 mM Tris pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/50 µg gelatine) contained 1.25 units of Dynazyme Taq polymerase (Finnzymes, Espoo), 0.2 mM of each of the four dNTPs, 0.5µM of each primer and c. 10–50 ng genomic DNA. Products were either cleaned using QiaQuick Spin kit (Qiagen, Vienna). Both complementary strands were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Vienna) according to the manufacturers instructions. Sequences were run on a ABI310 capillary sequencer (Applied Biosystems,Vienna). The sequences are submitted to GenBank (nos AF534385, AF534386 and AF534387).

RESULTS AND DISCUSSION

Externally, the thalli of *Rimularia insularis* colonizing lichens of the *Lecanora rupicola* group can easily be recognized by their olivaceous brown colour and marginate ascomata (Fig. 1). The species differs from *Rhizocarpon inimicum* on the same host, which has blackish grey areoles and emarginate ascomata (Hertel



Figs 7–12. *Rimularia insularis*. **and** *Lecanora rupicola* **Figs 7–9.** TEM micrographs of *R. insularis* and *L. rupicola* thalli. **Fig. 7.** *Rimularia*'s algal layer, showing fungal penetration of algal cells. **Fig. 8.** Fungal and degraded algal cells from *Rimularia*'s algal layer. Asterisks indicate electro–dense vacuolar content in fungal cells. **Fig. 9.** *Lecanora*'s algal layer. Arrow indicates an intraparietal haustorium. **Figs 10–11.** SEM–BSE micrographs of old *R. insularis* infections showing multiple algal cells penetrated by fungi (**Fig. 10**, asterisks) and bacterial (B) and cyanobacterial (C) colonies in the proximity of algal cells (A) penetrated by fungi (F) (**Fig. 11**). **Fig. 12.** Direct PCR products of horizontal sections of *Lecanora* and *Rimularia* thalli, using fungal-specific primers of ITS region. Lane 1, size markers; Lane 2–3, products obtained from *Lecanora*'s medulla; Lane 4: products obtained from *Rimularia*'s algal layer; Lane 5–6, products obtained from upper part of *Rimularia*'s medulla; Lane 7, products obtained from the lower part of *Rimularia*'s medulla.

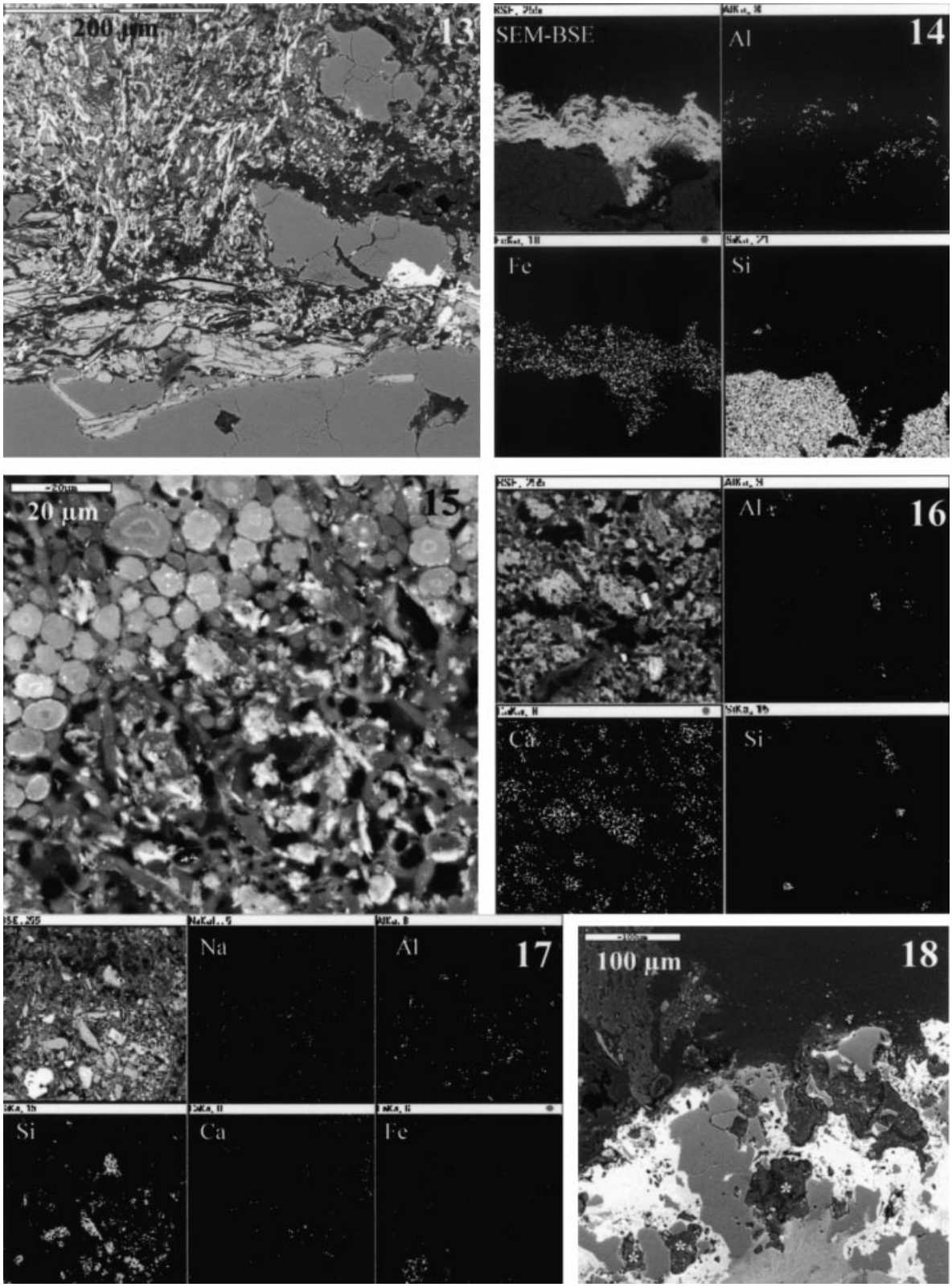
1970, Hertel & Rambold 1990, Poelt & Vězda 1984). In transverse sections of *R. insularis* thalli, an upper cortex, a heterogeneous algal layer and a thick medulla are distinguished (Fig. 2). The upper cortex of *R. insularis* thalli is thinner than that of the host and lacks crystals (data not shown), its uppermost hyphae have amorphous brown wall pigments (Atra-brown; Meyer & Printzen 2000). In confocal microscopy, cords of pink and green hyphae are distinguishable in the lichen thalli (Fig. 3) after staining with acridine orange and visualizing with the overlay technique; the *Rimularia* and *Lecanora* hyphae evidently differ in their affinity for acridine orange. The pink cells occur under the upper thallus surface in the algal layer, where they surround the algal cells (Fig. 4). Some of the pink hyphae are also found in the medulla (Fig. 3). When *R. insularis* apothecia are present, the pink hyphae are concentrated in their proximity (Fig. 5, arrows). This pattern and the fact that such coloured hyphae are absent in uninfected *L. rupicola* thalli, suggests that the pink cells belonging to *R. insularis*, whereas the green cells are typically from *L. rupicola*.

Two different parts can be distinguished in the algal layer (Fig. 6). The part closer to the upper cortex has a denser composition with algal and fungal cells. The algal cells in this region have two distinct morphologies, which differ from that of photobionts in the uninfected host thalli: some are small (approx. 4–8 μm diam) with strongly lobulate chloroplasts, and others are larger (approx. 10–14 μm diam) and lack chloroplasts with lobules. The density of algal cells in the lower part of the algal layer is reduced. The algal cells there were usually of the larger type and gaps, where the algal cells have died, are present (Fig. 6, asterisks). Although we observed two morphologically different kind of algae, marked ultrastructural differences in pyrenoid ultrastructure, important features in the classification of *Trebouxia* species (Friedl 1989), were not found (data not shown). In addition, direct PCR amplifications and sequencing of the photobiont ITS region from sections of the algal layer indicate the presence of only one species of *Trebouxia* in the host and in the lichenicolous lichen. This was also suggested by A. Beck (unpubl.), and rejects an alternative earlier hypothesis, assuming the presence of a separate photobiont in the parasite (e.g. Hawksworth 1988).

Different *Trebouxia* ITS sequences were obtained from *R. insularis* thalli collected in the Mediterranean and Alps. While the Mediterranean *Trebouxia* sequence represents an unnamed lineage, termed clade 2 in Helms *et al*. (2001), which could not be assigned to a known species, the sequence of the alpine specimen belonged to the *T. jamesii* complex, and was also present in adjacent thalli of unrelated, crustose *Lecanorales* (yet distinct from the *T. jamesii* sequences found in *Letharia* by Kroken & Taylor 2000, data not shown). In both cases, the sequences were identical to those obtained from the algal layer in the adjacent uninfected zones of the host *L. rupicola*. This suggests that different *Trebouxia* species may be present in *L. rupicola*, but all that can be accepted by *R. insularis* upon its establishment on the host. The observed differences in algal morphologies could reflect physiological stages of the photobionts, induced by this infection. Different conditions for the photobionts may be present due to the thinner and more pigmented thallus cortex type of the parasite. However, changes in cell size and morphology of their chloroplast have been reported in algal cells when these become associated with the fungus during lichenization, or in lichens where the same species but morphologically different algae are present in hymenia (Ahmadjian 1974).

In the upper part of the algal layer, contacts between *Rimularia* hyphae and algal cells are apparent, but definite penetrations of the photobiont cell walls are not observed (Fig. 6). However, in the lower part of the algal layer, algal cells penetrated by fungal haustorial are frequently observed (Figs 6–8). Occasionally, these algal cells appeared degraded and completely invaded by fungal cells. Confocal microscopy shows that the penetrated algal cells were pink (Fig. 4, arrowheads), in contrast to the blue healthy algae (Figs 3–4). Although the algae of the lower part of the algal layer appear in the proximity of *R. insularis* hyphae, penetration by *Lecanora* hyphae can be visualized by confocal microscopy (Fig. 4, arrows) and intraparietal haustoria are normally observed inside *Lecanora* thalli (Fig. 9). *R. insularis* apparently penetrates algal cells which are already penetrated by *Lecanora* hyphae (Figs 7–8), and apparently kills them in such cases. Fungal cells of this area appear full of dense vacuolar contents (Fig. 8), which could indicate a high degradative activity (Boissière 1982). In old infections, a more complex degradation is observed. Some zones of the algal layer appeared completely altered with a majority of algal cells invaded by fungi (Fig. 10, asterisks). In these degraded zones, cyanobacterial and other bacterial colonies are occasionally observed (Fig. 11), which possibly exploit released nutrients from the lichen or benefit otherwise from the ecological conditions of degradation. This is apparently different from the phenomenon of cyanophily, which is characteristically observed in healthy thalli (Poelt & Mayrhofer 1987).

Below the algal layer, there is a thick medulla, where *Rimularia* and host hyphae are intermixed (Figs 3–4). A higher frequency of *R. insularis* hyphae occurs in the upper part of the medulla, but some of its hyphae extend into the lower part of the medulla. The direct PCR of horizontal sections of the algal layer and the upper part of the medulla from *Rimularia* thalli, using fungal-specific primers (Fig. 12), and subsequent sequencing corroborate the dominance of the *Rimularia* mycobiont in these layers. The basal part of the medulla, which is in contact with the lithic substrate, is a mixture of diverse living and dead fungal cells (Fig. 13). This is in accordance with the observations of Rambold & Triebel (1992) who reported that in the development of *R. insularis* thalli, the basal parts of the



Figs 13–18. SEM–BSE and EDS analysis images of *Rimularia insularis* thallus-granite substrate interface from Crete. **Fig. 13.** SEM–BSE image of the part of the medulla which is in contact with the substrate. **Fig. 14.** EDS spatial distribution of aluminium (Al), iron (Fe) and silicon (Si) in the substrate zone under the thallus in Fig. 2. **Fig. 15.** SEM–BSE image of the algal layer and upper part of the medulla. **Fig. 16.** SEM–BSE micrograph and EDS spatial distribution of aluminium (Al), calcium (Ca) and silicon (Si) in the *Rimularia*'s medulla. **Fig. 17.** SEM–BSE micrograph and EDS spatial distribution of sodium (Na), aluminium (Al), calcium (Ca), iron (Fe) and silicon (Si) in a medullar zone with multiple mineral fragments. **Fig. 18.** SEM–BSE micrograph of *R. insularis*–granite interface, showing cavities with presence of fungal cells in the lithic substrate (asterisks).

host thallus become damaged over time and the host areoles tend to erode, together with the thallus of the lichenicolous lichen. In this zone, the recognition of both fungal symbionts is difficult. Confocal microscopy observations suggest a higher abundance of *L. rupicola* fungal cells (Fig. 3). However, amplification of DNA from the fungal cells present in this zone was not possible with our direct PCR technique (Fig. 12, Line 7); the prevalence of dead hyphae as well as calcium oxalates as PCR inhibitors in this zone probably impairs direct DNA amplification.

The spatial element distribution maps obtained by EDS analysis in the zone of the substrate below *Rimularia* thalli (from Crete) has revealed a lithic substrate rich in iron and silicon but without calcium. Fig. 14 shows the distribution of Fe, Al and Si in the zone under the thalli in Fig. 2; a zone of rich in iron minerals with some inclusions of micaceous minerals can be seen directly in contact with the lichen, and a zone with quartz is found beneath it. In the medullary zone immediately under the algal layer, abundant mineral deposits are mixed with *Rimularia* hyphae (Fig. 15). The EDS analysis demonstrates that these deposits are composed mainly of calcium (Fig. 16). Calcium oxalates are one of the biominerals most often described occurring in lichens (Ascaso, Sancho & Rodriguez-Pascual 1990, Ascaso & Wierzchos 1995, Modenesi *et al*. 2000, Wadsten & Moberg 1985) and could also be present inside thalli infected with *R. insularis*. Numerous rock fragments are also included between hyphae of the basal part of the medulla. Heterogeneity in the mineral composition has been observed by EDS analysis through these rock fragments (Fig. 17). The majority of the fragments could originate directly from the substrate. The presence of calcium oxalate and mineral fragments in the lichen thalli indicates a combined biogeophysical and biogeochemical action on the substrate.

The fungal mixture observed in the lower part of the medulla extends into the rock. Multiple fissures and cavities in the substrate occur below these hyphae (Figs 2, 18). Some cavities colonized by fungal cells occur mainly in zones rich in silicon. However, zones of the lithic substrate rich in iron appeared without cavities, which are again observed deeper in the substrate (50–100 μm) where iron is not present (Fig. 14). This suggests that iron minerals are more resistant to lichen action than the other present minerals. The biogeophysical and biogeochemical processes induced by lichen thalli frequently generate fissures and cavities in lithic substrates (Adamo & Violante 2000, Ascaso & Wierzchos 1995, Ascaso *et al*. 1998, Chen, Blume & Beyer 2000, de los Ríos *et al*. 2001). In the interface between *R. insularis*-infected thalli and the rock, *Rimularia* and *Lecanora* hyphae are intricately intermixed and both seem to grow into the substrate. The presence of *Rimularia* hyphae inside the rock was confirmed by the direct PCR amplification using small fragments from lithic substrate (data not shown). This indicates that fungal hyphae of *R. insularis* can be present in the substrate, even when the infected host thallus is eroded. It leaves us to speculate that these regions may subsequently be recolonized by new areoles of *L. rupicola* and possibly be re-infected by *Rimularia* hyphae from within the substrate and not necessarily always by spores.

Our findings suggest new parameters for the distribution and frequency of lichenicolous lichens on saxicolous hosts. It will be interesting to investigate further, whether *Rimularia insularis* infections or those of other lichenicolous fungi are more common over lithic substrates showing abundant fissures or cavities in the weathering rind. These evidently may harbor hyphae of lichens, but apparently also of their lichenicolous fungi and adjacent fungi or microorganisms (Ascaso *et al*. 1995, Wierzchos & Ascaso 1996, Ascaso *et al*. 1998, Bjelland & Ekman 2000, de los Ríos *et al*. 2001). The formation of these fissures or cavities is dependent on the physico-chemical features of the substrate, the lichen species, and the presence of other associated microorganisms (Ascaso *et al*. 1998, Bjelland & Thorseth 2002, de los Ríos *et al*. 2001). Moreover, the presence of different photobionts, in eco- or geographically distinct samples of a host species, could be another aspect of infection frequency, which still needs to be studied in more detail. If the lichenicolous lichen(-fungus) has a certain preference for a particular photobiont species, it might be more commonly found where the host contains this photobiont.

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