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Infection mechanisms of lichenicolous fungi studied by various microscopic techniques

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Abstract: Infections of lichenicolous fungi are studied at the cellular level using different microscopic techniques. Confocal laser scanning microscopy of samples stained with Acridine Orange allows the differentiation of hyphae pertaining to a lichenicolous fungus versus hyphae of the host mycobiont. This is similarly possible by hybridizing rRNA in hyphae of a particular symbiont with a specific, labelled DNA fragment. Transmission electron microscopy was used to study details of the interactions among symbionts including the structure of haustoria or ultrastructural features of parasitized cells. The investigations reveal a diversity of infection mechanisms of lichenicolous fungi.

Introduction

As immobile heterotrophs, fungi evolved various strategies to interact with their environment and to form associations with other organisms. About 1000 fungi, which is almost 2% of all described fungi, specialized to live as non-lichenized lichenicolous fungi on lichen symbioses. Lichenicolous fungi and their hosts belong to diverse orders of fungi, and similarly diverse are the biological relationships displayed by lichenicolous fungi. A distinction has been made between saprotrophic lichenicolous fungi developing on decayed lichen thalli, commensals with a stable relationship lacking symptoms and parasites, which are recognized by their effect to growth and development of the thalli (see also JEFFRIES & YOUNG 1994). However, there is a continuum between these main types, and only a few studies were carried out at the microscopic level to investigate cellular interactions of lichenicolous fungi with their hosts.

Various types of cellular interactions are possible: Fungal nutrient uptake may be accomplished by the whole surface of the cells or more specifically by haustoria, appressoria and others. Additionally, the lichenicolous fungus is usually specialized to one of the host symbionts. For example, *Zwackhiomyces coepulonus* shows preference for the algal cells and infects these with haustoria (GRUBE & HAFELLNER 1990), whereas lichenicolous heterobasidiomycetes with tremelloid haustoria contact the fungal symbiont of the host (DIEDERICH 1996). In several lichenicolous fungi, parts of the host plectenchyma are degraded and

anatomical changes are observed. The contact with any of the symbionts is not clear in such cases (FEIGE et al. 1993, DE LOS RÍOS & GRUBE 2000). The infecting organs and ranges of specificity to the hosts are to some extend specific for the systematic groups of lichenicolous fungi.

Details of lichenicolous infections and the interactions of lichenicolous fungi at the cellular level are often difficult to observe using only transmission light microscopy because the hyphae of host and lichenicolous fungus can be anatomically similar and are then hardly discerned. Therefore the application of other microscopical techniques combined with staining procedures can be useful. In this work we discuss the possibilities of Confocal Laser Scanning Microscopy (CLSM) and Transmission Electron Microscopy (TEM) to study infection structures of lichenicolous fungi.

Material and methods

Sections were prepared using a Cryostat (Leitz). Studies with epifluorescence were carried out with a Zeiss Axioskop. The sections were stained with a 1% aqueous solution of the optical brightener Calcofluor White. For this dye with a high affinity to linear polysaccharides, we have used filter set 01 from Zeiss. All dyes were applied for 5 min. Afterwards, excessive dye was washed using tap water or 5% KOH.

For examination with a Confocal Laser Scanning Microscope we used the system LeicaTCS NT. Sections were stained with the fluorescent dye Acridine Orange, applied as 1% aquous solution. In situ hybridization was carried out as described in GRUBE & DE LOS RÍOS (2001). For excitation, Argon, Krypton and HeNe laser were selected and excitation filter TP 488/568/633 was used. Various emission barrier filters were tested which are individually adjustable with the microscope used. The images were analyzed using Leica TCS-NT/SP Software 1.6. Three-dimensional (3D) images were made up of several 16-20 confocal sections using computer assisted methodology at 0,5-1 µm increments through the sample.

For transmission electron microscopy (TEM), samples were fixed, dehydrated and embedded in Spur's resin (DE LOS RÍOS & ASCASO 2001). Previously to TEM preparation, samples were carefully checked under the light microscope to avoid degraded tissue. Ultrathin sections were deposited in fomvar covered copper grids, post-stained with lead citrate (REYNOLDS 1963) and observed in a Philips 300 transmission electron microscope.

Specimens studied: *Arthonia cinnabarinula*: Costa Rica, Cordillera de Talamanca: trail from village San Gerardo de Rivas to Cerro Chirripó, on *Trichothelium epiphyllum*, Matzer 1523 & Pelzmann (GZU); *Biatoropsis usnearum*: Austria, Styria: Joglland, Rabenwald oberhalb Anger, on *Usnea rigida*, 2000, A. de los Rios (GZU); *Dacampia engeliana*: Austria, Styria: Eisenerzer Alpen, Stadelstein, on *Solorina saccata*, Hafellner 43289 (GZU); *Plectocarpon lichenum*: Slovenia, Pohorje: on *Lobaria pulmonaria*, Mayrhofer 12689 (GZU); *Polycoccum pulvinatum*: Spain, Islas Canarias, Gran Canaria: Risco prieto, on *Physcia caesia*, Hafellner 32920 (GZU); *Pyrenidium actinellum*: Austria, Styria: Koralpe, Weinebene, on *Peltigera venosa*, Grube (GZU); *Tremella cetrariicola*:

Austria, Tirol: Pfundser Tscheybach, Schluchtwald, on *Tuckermannopsis chlorophylla*, Obermayer 2297 (GZU); *Zwackhiomyces coepulonus*: Austria, Upper Austria: Nördliche Kalkalpen, Dachsteingruppe, Lackenmoosalm, on *Xanthoria elegans*, Grube (GZU).

Results and discussion

The application of CLSM differenciates lichenicolous and host mycobiont hyphae when cell walls are stained with Acridine Orange. This is possible due to their different affinities to the dye and to differences in autofluorescence. Acridine Orange is characterized by dichroism with an orthochromatic (excitation at 502 nm and emission at 536 nm: green) and a metachromatic state (excitation at 460 nm and emission at 650 nm: red, see ROST 1995). The latter is characteristic for acid polysaccharides. To visualize the differential staining, various channels - as defined by particular combination of diverse excitation wavelengths and emission filters - are simultaneously observed. The signal intensity from a single channel is represented in a particular colour (green, red or blue), thus the overlay of these channels optimally resolves differences in fluorescence patterns. The laser excitation and filter conditions are not the same in all the lichenicolous associations and must be optimized individually. Three excitation lasers, Argon laser (488 nm), Krypton laser (568 nm) and HeNe laser (633 nm), were used in this study. The use of the three laser and three emission barrier filters (band pass at 535 nm, band pass at 615 nm and a long pass at 645 nm) is found useful to study interactions of hyphae with the photobionts. The signal collected by the long pass 645 nm filter can be assigned mainly to the autofluorescence of the chlorophylls. In infection zones, where algal cells are not present, the excitation with Argon laser (488 nm), the use of a band pass filter at 535 nm and a long pass filter at 615 nm discriminates the different fungal hyphae in lichenicolous associations. Occasionally, if certain autofluorescent substances exist in the thalli, e.g. of cellulose-like hyphal wall componenents, the excitation with Argon and Krypton lasers and the combination of the images obtained with band pass filters at 535 nm and 615 nm is advisable.

By differential visualization of the lichenicolous and host mycobiont hyphae using the presented technique it is possible to study the distribution and the interactions of both fungal symbionts, either with the algal partner or with each other. Various kinds of interactions among symbionts can be studied in greater detail. These include haustorial and appressorial interactions with the photobiont and various types of interactions with the host mycobiont such as differently shaped haustoria and intrahyphal growth.

Moreover, it is possible to illustrate interactions in three dimensions. In Figure 1, we show a 'gallery' of confocal images of *Zwackhiomyces coepulonus* haustoria, which penetrate a *Xanthoria* photobiont cell. This gallery, derived from a series of images at different optical planes, confirms the observations by GRUBE & HAFELLNER (1990) made with transmission light microscope and Methylen Blue staining. Another kind of interaction with the photobiont is also well illustrated by the presence of appressorial structures. Figure 2 shows a confocal image of hyphae of *Arthonia cinnabarinula* which are densely attached to the

Phycopeltis algae of the host.

The heterobasidiomycete *Biatoropsis usnearum* produces gall-like malformations in *Usnea* thalli (DIEDERICH & CHRISTIANSEN 1994). In the CLSM image (Figure 3), cells with tremelloid haustoria are clearly distinguished by their orangish colour from the greenish host mycobiont cells. The galls are internally structured, basidia are situated at the surface of the gall whereas infective hyphae with haustoria are abundant in the central zone and to a lesser extend in the basal layers (GRUBE & DE LOS RÍOS 2001). The distribution of the parasite hyphae can be compared with other heterobasidiomycetes species which also form galls. Figure 4 shows a detail of a gall formed by *Tremella cetrariicola* in *Tuckermannopsis chlorophylla*. In contrast to *Biatoropsis*, the hyphae of the parasite are here homogeneously distributed.

Fluorescence microscopy (both epifluorescence and CLSM) is particularly useful if dark pigmented layers are present in the infections. The infection of the ascomycete *Plectocarpon lichenum* on *Lobaria pulmonaria* produces an infection in which the ascomata of the parasite are situated on top of a gall. The excipular plectenchyma and the hypothecium contains dark brown intercellular pigments. The hypothecium is subtended by a necrotic layer, which becomes lighter coloured at the base. Confocal microscopy shows that this necrotic layer includes lichenicolous hyphae and degraded hyphae or abundant remnants of cell walls, apparently of the host (Figure 5).

Differential staining of cell walls and the localization of different hyphae can be confirmed by specific labelling of nucleic acids using in situ hybridization. This is also suitable in cases, where the cell walls of the associated fungal

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Figure 1: Gallery of confocal images of *Xanthoria* thalli infected by *Zwackhiomyces coepulonus*, showing the penetration of *Z. coepulonus* haustoria in *Xanthoria* photobiont cell. Staining with Acridine Orange. Argon laser (488nm), Krypton laser (568nm) 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. Bar = 15 µm.

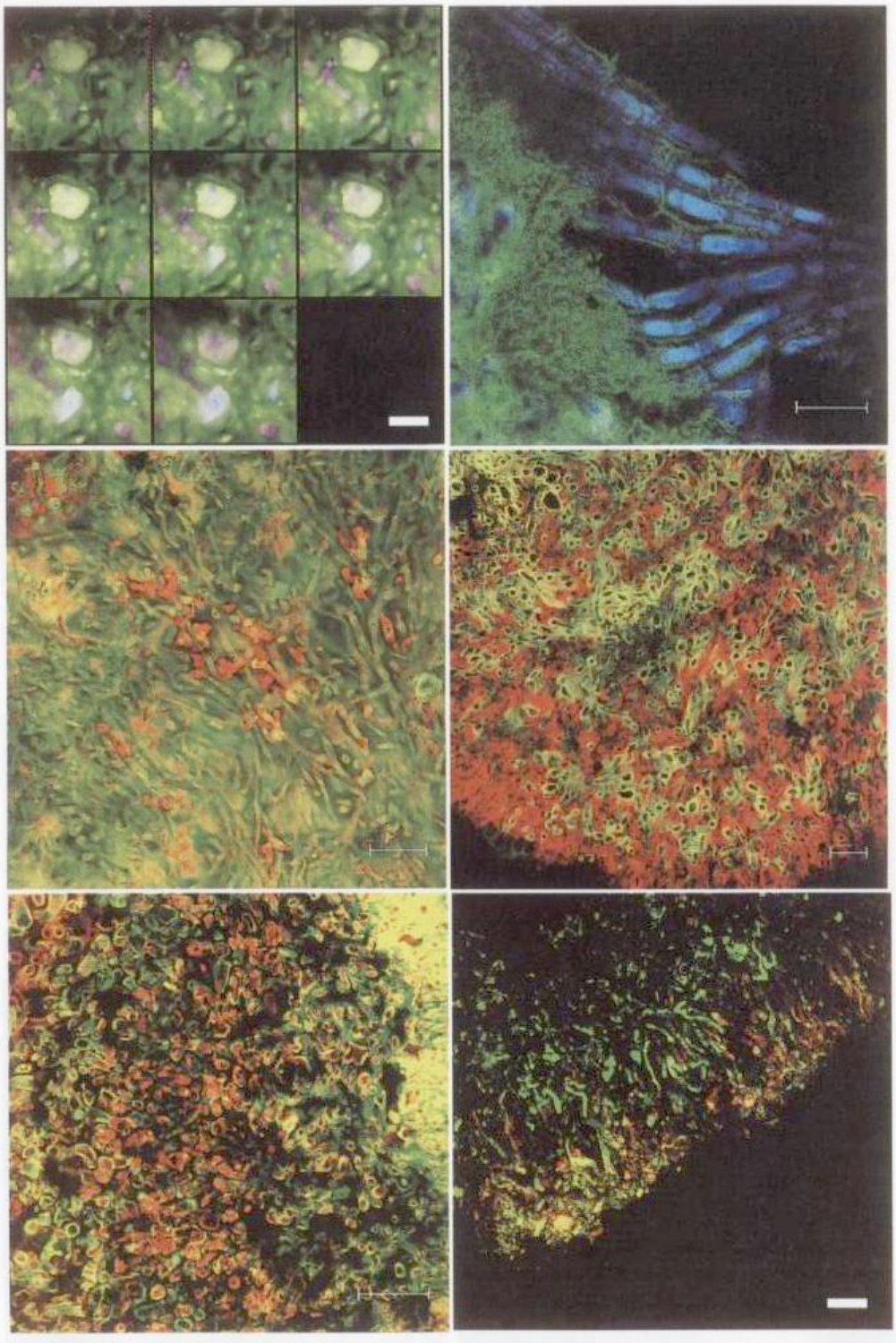
Figure 2: Confocal image of an infection of *Trichothelium* sp. by *Arthonia cinnabarinula*. Staining with Acridine Orange. Argon laser (488nm), Krypton laser (568nm) and HeNe laser (633 nm). Band pass at 535 nm and a long pass at 645 nm were used as emission filters.

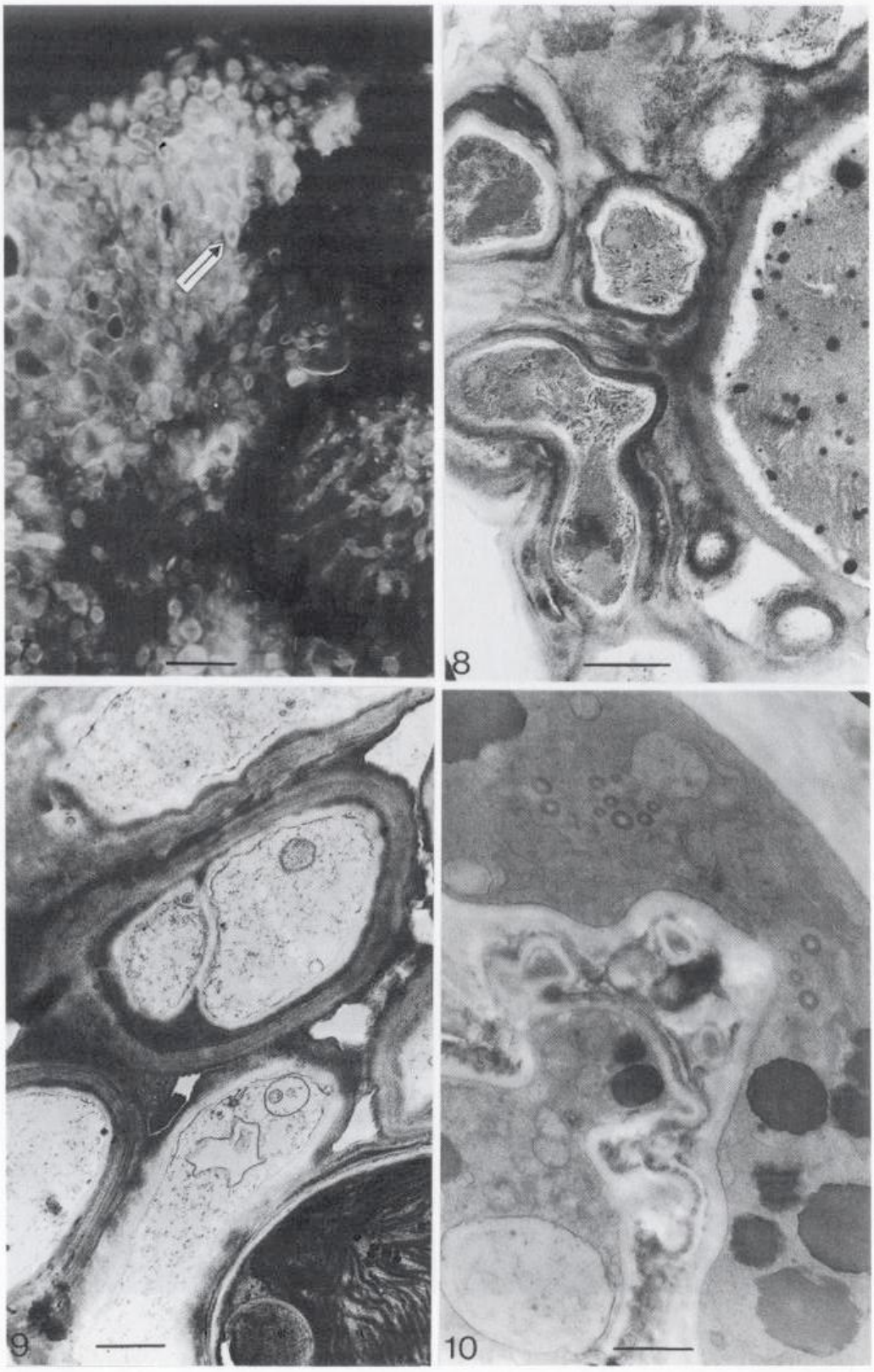
Figure 3: Confocal microscope images of a section through the gall formed in *Usnea* sp. by *Biatoropsis usnearum*. Staining with Acridine Orange. Argon laser (488 nm) was selected for excitation and as emission filters a band pass at 535 nm and a long pass at 615 nm were chosen. Orange cells correspond to *Biatoropsis* cells.

Figure 4: Confocal microscope images of a section through the gall formed in *Tuckermannopsis chlorophylla* by *Tremella cetrariicola*. Staining with Acridine Orange. Red cells correspond to *Tremella*. Laser and filter conditions as Figure 3.

Figure 5: Confocal microscope image of a section through a gall formed in *Lobaria pulmonaria* by *Plectocarpon lichenum*, detail from the necrotic layer below the hypothecium. Staining with Acridine Orange. Laser and filter conditions as Figure 3.

Figure 6: Confocal laser scanning image of sections through the gall formed in *Usnea* sp. by *Biatoropsis usnearum*. *In situ* hybridization using a probe specific for LSU rRNA of *Usnea*. Hybridized hyphal contents of *Usnea* are in green, weak reddish autofluorescence is present in *Biatoropsis* cells, but no green signal. Bar = 20 µm.

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symbionts have similar chemistry. Using in situ hybridization with a fluorescent probe specific to the rRNA of a particular symbiont, it is possible to selectively stain the cytoplasm of hyphae, i.e. the distribution of ribosomes in the cells. The staining pattern can be analyzed using confocal laser scanning microscopy. Figure 6 shows hyphae of *Usnea* in the *Biatoropsis* infection, which are labelled (green) by a probe specific to a fragment of LSU rRNA, obtained from an uninfected *Usnea* thallus. The hyphae of the parasite *Biatoropsis usnearum* are not labelled, but show a weak autofluorescence (red). As the *Usnea* hyphae are strongly intermingled with those of *Biatoropsis* in infections, it is obvious that a specific probe for *Biatoropsis* cannot be generated directly; axenic cultures were not available. Probes for particular mycobionts could in principle be designed at different taxonomic levels (e.g. a probe for Lecanorales, Tremellales, etc.), however, the differentiation of mycobionts can technically be difficult, if the fungi are closely related. The applications of in situ hybridization in fungi are still limited (STERFLINGER et al. 1998, TREMBLEY et al. 2000), except in yeasts. When protocols are further refined, this could also be a promising technique to investigate the biology of interfungal relationships.

CLSM is ideally suited to visualize the distribution of lichenicolous fungi in their hosts, especially in combination with various staining techniques. However, investigations of ultrastructural details in cellular interactions, TEM is more appropriate.

Epifluorescence microscopy may in some instance also be a very useful and comparatively simple technique, e.g. to detect intrahyphal growth of a lichenicolous fungus in sections stained with Calcofluor White (DE LOS RÍOS & GRUBE 2000). In the infection of *Physcia caesia* by *Polycoccum pulvinatum,* hyphae of *Polycoccum* appear within the host hyphae, but the hyphae of the parasite are so tightly attached to the inner host wall that this phenomenon is difficult to illustrate with this technique (Figure 7). TEM confirms the intrahyphal growth (Figure 8). The invasion of host mycobiont cells is similar to that observed in *Dacampia engeliana* infecting *Solorina* thalli. Figure 9 shows *Dacampia* hyphae which invade some host mycobiont cells in the algal layer of *Solorina.* TEM can also be used to study the structure of haustoria, e.g. in *Pyrenidium actinellum* on *Peltigera venosa.* Figure 10 shows multiple small finger-like outgrowths of a haustorium, which are developed prior to penetrations of the host cells (DE LOS RÍOS & GRUBE 2000).

Studies with different microscopic techniques show not only a great diversity of biological strategies of lichenicolous fungi at the cellular level (see also DE LOS RÍOS & GRUBE 2000), but are suitable to achieve a better understanding

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Figure 7: Epifluorescence image of *Physcia caesia* thalli infected by *Polycoccum pulvinatum.* Calcofluor White staining. The Arrow points on an exemplary sectioned cell which is infected with a *Polycoccum* hypha. Bar = 10 µm.

Figure 8: TEM micrograph of algal layer of *Physcia caesia* thalli infected by *Polycoccum pulvinatum*. Bar = 1µm.

Figure 9: TEM micrograph of algal layer of *Solorina saccata* infected by *Dacampia engeliana*. Bar = 1µm.

Figure 10: TEM micrograph of the haustorial apparatus of *Pyrenidium actinellum* on *Peltigera venosa* prior to penetration of the cell wall. Bar = 1µm.

of their biology in general. More interaction types than those presented here may still be discovered after investigations in further groups of lichenicolous fungi. However, our studies suggest that a general trend might be present in patterns of coevolution (see DIEDERICH 2000). Lichenicolous fungi that are phylogenetically related to lichens seem to have a general preference for the algal symbionts in lichen associations, whereas those lichen parasites of primarily non-lichenized groups are possibly better adapted to parasitism on the lichen mycobiont.

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