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Experimental Studies of the Lichen Symbiosis: DNA-Analyses, Differentiation and Secondary Chemistry of Selected Mycobionts, Artificial Resynthesis of Two- and Tripartite Symbioses

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Abstract

From a culture collection of over 100 mycobionts, isolated from lichens of the neotropical and temperate climatic regions, interesting examples have been selected for DNA-analyses, chemical tests and resynthesis experiments. DNA sequences were used as a means to verify the identity of the mycobiont and the voucher specimens and also to elucidate relationships between the different species within a special lichen family. Species of the *Cladonia verticillaris* complex (e.g. *Cladonia imperialis*, *C. crinita*, *C. verticillaris*) are among the largest known Cladoniaceae in the tropics of Latin America. In their natural environment they have developed a considerable morphological complexity. Their most striking common characteristics are the centrally proliferating scyphi that are organized in storeys up to a height of 15–25 cm. In the cultures, the mycobionts of the *C. verticillaris* complex showed an unusual morphogenetic capacity to form thallus-like structures (small podetia) in the absence of the symbiotic algae. By using a modified Sabouraud-medium, this higher step of differentiation was repeatedly achieved and the mycelia were able to produce the typical secondary compounds found in the natural lichen, fumarprotocetraric acid and its chemical satellites. In this study, the production of the fumarprotocetraric chemosyndrone was achieved for the first time by cultured mycobionts of selected

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species of the *Cladonia verticillaris* complex. Further chemical results were obtained by analysing cultures of species of the genera *Lobaria, Thamnolia* and *Umbilicaria*, by TLC and HPLC. During the experiments it became obvious that beside differentiation processes, ecological factors and stress play a key role for the production of chemical compounds in mycobiont cultures. A short overview is given of the most successful resynthesis experiments conducted during the past 5 years. Well developed thallus structures were formed by *C. calycanthoides* and the tripartite photosymbiodeme *Lobaria fendleri*. In a long-term experiment an artificial resynthesis of the cyanobacterial and also the green photomorph of *L. fendleri* was achieved. As in natural thalli, the typical chemistry was only found in the mycobiont, in the resynthesized green thallus, whereas the cultured cyanobacterial photomorph did not form any secondary compounds.

Keywords: Lichens, lichen mycobiont, culture, resynthesis, *Cladonia, Umbilicaria, Thamnolia*, photosymbiodemes, *Lobaria*, lichen chemistry

1. Introduction

Recently, highly adapted microbiological methods have been explored that allow dissociation and reconstitution experiments of lichens in an artificial environment like culture chambers and phytotrons (e.g. Ahmadjian, 1993; Dibben, 1971; Bubrick, 1988; Armaleo, 1991; Culberson et al., 1992; Yamamoto, 1998; Stocker-Wörgötter, 1995, 1998, 2001a, 2001b; Stocker-Wörgötter and Türk, 1994). Single spore and algal isolates are required to obtain uniform materials. Algal and fungal clones are needed for genetic and chemical studies. However, the development of a mycelium from one germinated spore is generally very slow.

Much of the culture work during the nineties (e.g. Hamada, 1988, 1993; Kinoshita, 1993; Yoshimura et al., 1994; Crittenden et al., 1995; Kon et al., 1997; Stocker-Wörgötter, 1998) was done using the Yamamoto "tissue culture" method (Yamamoto, 1990, 1998). This method uses thallus fragments for the mycobiont isolations. Mycobionts, obtained from fragments, grow more rapidly than single spore isolates. To secure, that the isolated mycobiont is not a contaminant, additional tests are necessary to determine its identity, e.g. a chemical analysis of its secondary compounds and comparison of the DNA of the isolated mycobiont and voucher specimen.

The first part of the presented study uses DNA-analyses as a means to verify the identity of the mycobionts isolated of selected species of the tropical *Cladonia verticillaris* complex and the voucher specimens. The second part of the investigation deals with methods and possibilities to trigger

aposymbiontically grown mycobionts to form the same or a related chemistry as found in the natural lichens. The topic of the last part will be to screen methods for studying developmental processes and differentiation in morphologically very complex ascolichens like species of the *C. verticillaris* complex (e.g. *Cladonia calycanthoides*). A resynthesis is reported that reproduces a fully developed young thallus of *C. calycanthoides* containing the typical chemical compounds (the fumarprotocetraric aggregate) of the intact lichen.

One highlight of the re-establishment experiments is a documentation of how the two different morphotypes of a complex tropical photosymbiodeme like *Lobaria fendleri* form in culture. The juvenile, coralloid (fruticose) cyanobacterial photomorph and the green foliose photomorph develop under very specific culture conditions that may correspond to the seasonal fluctuations of drought and high moisture in a tropical mountain forest.

2. Materials and Methods

Lichens

Selected species of the *Cladonia verticillaris* complex: *Cladonia imperialis* Ahti & Marcelli: Brazil, Itatiaia, endemic to Serra da Mantiquieira. *Cladonia crinita* (Delise ex Pers.) Ahti: Brazil, Minas Gerais, Caraça, endemic to Brazil. *Cladonia calycanthoides* (Vain.) Ahti & Marcelli: Brazil, Minas Gerais, Caraça, endemic to SE-Brazil. *Lobaria fendleri* (Tuck ex Mont) Lindau: Brazil, Campus do Jordão, endemic to SE-Brazil. *Thamnolia vermicularis* (Swartz) Schaerer var. *subuliformis* (Ehr.) Schaerer: Austria, Carinthia, Rottensteiner Valley. *Umbilicaria mammulata* (Ach.) Tuck: USA, Virginia, Shenandoah, Fort Valley.

Mycobionts: Isolations from *Cladonia imperialis* (Nr. 204), *Cladonia crinita* (Nr. 205), *Cladonia calycanthoides* (Nr. 206), *Lobaria fendleri* (Nr. 301, 302), *Thamnolia vermicularis* var. *subuliformis* (Nr. 401), *Umbilicaria mammulata* (Nr. 507).

Photobionts: *Dictyochloriopsis* sp., *Nostoc* sp. (*Lobaria fendleri*, Nr. 308, 309), *Trebouxia* (*Cladonia calycanthoides*, Nr. 319).

Culture media

Mycobionts: Cladoniaceae: *Cladonia imperialis*: Sabouraud-4%-glucose-agar (Stocker-Wörgötter, 2001b), *Cladonia crinita*: Sabouraud-2%-glucose-agar (Merck Co.). *Cladonia calycanthoides*: Malt-yeast-extract-agar (Yamamoto, 1990). *Thamnolia vermicularis*: Sabouraud-2%-glucose-agar (modified,

Stocker-Wörgötter, 2001b), *Umbilicaria mammulata*: Potato-dextrose-agar (modified, Stocker-Wörgötter 2001b), *Lobaria fendleri* (Sabouraud-2%-glucose agar, Murashige-Skoog-Medium modified, Stocker-Wörgötter 2001a).

Photobionts: 1. BBM (Bold's Basal Medium), Deason and Bold (1960). 2. Malt-Yeast-medium (Yamamoto, 1990).

Mycobiont isolations

Yamamoto-method (after Yamamoto, 1990, modified): The mycobiont isolations were performed on agar slants in tubes containing 5 ml of Sabouraud-2%-glucose-agar or malt-yeast-extract-agar. Small pieces of thallus were washed very carefully in sterile bidistilled water and a drop of Tween 80 (a surface detergent proposed by Bubrick and Galun, 1986) was added. The water was changed for several times. Clean fragments were selected under a dissecting microscope and homogenized in sterile water (1–3 ml) at low speed (5000–10000 rpm for 15 sec) or using a sterile mortar and pestle.

The resulting suspension containing minute fungal-algal fragments was filtered through two sieves of different mesh size (500 and 150 µm). Then, pieces with an average size near 150 µm were picked up using an inoculation needle or bamboo sticks (Yamamoto, 1990) under a dissecting microscope. Agar slants containing nutrient media (listed above) were inoculated with only one fragment in each tube.

The original Yamamoto-method used only malt-yeast-extract-medium for all mycobiont isolations. Meanwhile, several of my experiments have shown that a large number of lichen fungi show higher growth rates in various other media (as listed above).

In many cases the method described can be used to isolate the algal partner, e.g. *Trebouxia, Dictyochloriopsis* and also cyanobacteria like *Nostoc*. When the fragments were examined at a higher magnification, they could be separated into pieces containing mainly algal cells or fungal hyphae. Fragments with a high quantity of algae or cyanobacteria were transferred to tubes containing BBM or malt-yeast-extract agar.

The "tissues" composed of hyphae and only few algal cells were preferentially selected for the mycobiont isolations. To promote the hyphal growth and prevent algal divisions, the tubes were kept in complete darkness (covered by aluminium foil) for 2–3 months. The tropical mycobionts are adapted to higher temperatures (24–30°C) than lichen fungi originating from the temperate samples (e.g. 15°C for *Thamnolia vermicularis*, 20°C for *Umbilicaria mammulata*). The algal dominated isolates were maintained under a light dark regime of 14:10 (alpine) or 12:12 hours (tropical species) and a light intensity of 50–100 µE $m^{-2}s^{-1}$. After 2–3 months the isolates were

checked for growth reactions. Then contamination-free mycelia and algal colonies were transferred to new nutrient media.

For the tropical mycobionts (species of the *C. verticillaris* complex, bionts of *L. fendleri*) the culture chambers were adjusted to 12:12 h, day-night cycles and an alternating temperature of 30:23°C. The algal cultures (green and cyanobacterial photobionts of *L. fendleri*, *Trebouxia* sp. of *C. calycanthoides*) were kept under the same temperature regimes and at a light intensity of 50–100 $\mu Em^{-2}s^{-1}$. The alpine and temperate mycobionts (*Thamnolia vermicularis*, *Umbilicaria mammulata*) were cultivated at a light-dark regime of 14:10 h and an alternating temperature of 20:10°C.

Resynthesis

Cladonia calycanthoides: Sandy soil was collected in an open tropical secondary forest at the natural habitat of the lichen. The substrate was dried and sieved (mesh width of 2 mm). For the resynthesis experiment, 500 g of the substratum was soaked with 300 ml of sterile, double distilled water for 3–4 hours. Then the moist soil was distributed in plexiglass culture dishes (150 × 150 × 100 mm, heat stable materials, Nalgene) up to a height of 30 mm. Dishes, containing the sandy soil were steam sterilized in an autoclave for 3 hours on two successive days with a break of 24 hours.

The substrata were inoculated first with photobiont cells obtained from an axenic culture (*Trebouxia*-isolate N. 319 from *C. calycanthoides*, 14.10.1997), and later with the mycobiont isolates. After about three weeks, the algae had divided and were visible as green spots (with an average diameter of 5 mm).

Axenic grown mycelia, together with 3 drops of sterile water were gently homogenized. Then the hyphal suspension was carefully pipetted on the algal colonies and both partners mixed with a sterile inoculation needle. The resynthesis cultures were incubated in a culture chamber under simulated tropical conditions as described above for the isolated symbionts.

Lobaria fendleri: For the resynthesis experiment, the mycelia were maintained in a non-nutrient medium (without sugars or polyols: e.g. liquid BBM) for 3–4 weeks. To start the re-establishment experiment, fragmented hyphal isolates were transferred to a sterile soil substratum (collected at a habitat of *Peltigera aphthosa*) that had been inoculated with *Nostoc* (axenic *Nostoc* isolate N. 309 from *L. fendleri*, 20.10.1997) and *Dictyochloriopsis* (N. 308) approximately 4 weeks previously.

A similar procedure was performed with the green photobiont (*Dictyochloriopsis*-isolate N. 308 from *L. fendleri*, 22.10.1997). In this case, the hyphal suspension was gently mixed with divided stages of algal colonies growing on a soil substratum that had been soaked in a sterile bark extract

instead of water. This extract was prepared from bark collected in the habitat of the lichen following the recipe given for soil extracts, as described in Esser (1976). The high moisture content of the soil was reduced by maintaining the dishes opened in a hood for 48 hours under axenic conditions.

DNA analysis

DNA-extraction, purification and sequencing: Total DNA was extracted from cultured mycobionts for the comparisons with the voucher specimens (Armaleo and Clerc, 1995).

DNA- amplification and purification: The ITS-regions, the 5.8 region and the flanking parts of the small and large subunits (SSU 18S and LSU 28S) of the rDNA were amplified using a Gene Amp PCR System thermal cycler. Primers for the PCR were ITS1, ITS2, ITS3 and ITS4 (Gardes and Bruns, 1993; White et al., 1990). The PCR mix contained 1.25 units of Dynazyme Taq polymerase (Finnzymes), 0.2 mM of each of the four dNTPs, 0.5 µM of each primer and 10–50 ng genomic DNA. The PCR products were cleaned by polyethylene glycol (PEG) precipitation and the complementary strands of the DNA were sequenced using a Dye Terminator Ready reaction Kit (Perkin Elmer) following the instructions of the manufacturer. The second purification was performed using Promega Wizard Preparation or later on, by a Quiaquick PCR product purification kit. Sequences were run on a long range gel for a stretch sequencer or on a ABI 310 automated sequencer. For the alignments of the sequences a Pile-up Programme was used and the adjustments were done manually.

Chemical methods

Extraction and chemical analysis of the cultures: Circular plugs (c. 1.5 cm diameter) were cut out from each of the agarplates overgrown by the mycobiont. The samples were dried overnight in a hood (12–15 hours) under sterile conditions and afterwards stored in a vacuum desiccator over P_2O_5. Dried discs with attached mycelia were extracted 5 times with acetone at 40°C. Finally, the extracts were evaporated using a mini-vaporator (Supelco/Sigma) under a stream of nitrogen at reduced pressure (modified from Culberson et al., 1992). For chemical analysis (HPLC) the extracts were redissolved in acetone and briefly centrifuged. An aliquot of 5–20 µl from every sample was injected.

Chemical analysis: HPLC analysis was performed using a Hitachi/Merck HPLC system including two pumps, a DAD (Photodiode array detector; 190–800 nm wavelength range) and a computer system with an integration package on the basis of windows NT. The column was a Beckman C8 (4.6 × 250 mm, 5 µm). Solvent A was composed of a mixture of 70% H_2O/methanol/1% phosphoric

acid and solvent B containing pure methanol. A 40 min gradient from 80 to 15% was run for 20 min and then followed by 15% solvent A. Then the column was washed and re-equilibrated for 10 min at 80% solvent A. Peaks were detected at 270 nm and the best chromatogram selected for identification.

The HPLC results of the cultured mycobionts were compared with TLC (Thin layer chromatography) identifications of the voucher specimens (extracts of the original lichens). For fumarprotocetraric acid control of the tested tropical Cladoniaceae served a pure sample, received from Prof. Leuckert (University of Berlin). For the TLC analyses the standardized method of Culberson and Ammann (1979), working with 3 solvent systems (A, B, C) was used.

3. Results

DNA-analyses of the cultured mycobionts and the voucher specimens

The original thalli of the lichens investigated only rarely form fruiting bodies and mature spores. In particular, *Thamnolia vermicularis* has never been found producing apothecia and it is thought to be a lichen formed by an imperfect ascomyceteous fungus. Most of the mycobiont cultures, used in this investigation were obtained from fragments. One advantage of the fragment method is that basically any lichen material can be used for the isolations of the symbionts and that it needs only a very small quantity of lichen materials.

However, the fragment method includes one serious problem. The fragments can be contaminated by epibionts, endobionts or parasites.

In this study, most of the cultured mycobionts of the *Cladonia verticillaris* complex have been successfully identified by DNA-analyses. By using cultures for genetic studies, I was able to get considerable higher amounts of DNA than was found using the voucher specimens. Identical sequences of voucher and mycobiont culture were obtained for *Cladonia imperialis*, *C. verticillaris*, *C. crinita* and *C. calycanthoides*.

Cultured mycobionts and their secondary chemistry

Cladonia imperialis: *C. imperialis* is one of the most spectacular lichens among tropical Cladoniaceae in Brazil. In the natural environment (Fig. 1) it forms very robust and tall podetia with many tiers, with an astonishing height of 15–25 cm. In culture, the *C. imperialis*-mycobiont, grown on Sabouraud-4%-glucose-agar, shows the unusual capacity to form small podetia (Fig. 2). Mycobiont cultures differentiating podetia contained large amounts of the typical secondary compounds (Fig. 17) fumarprotocetraric acid (major),

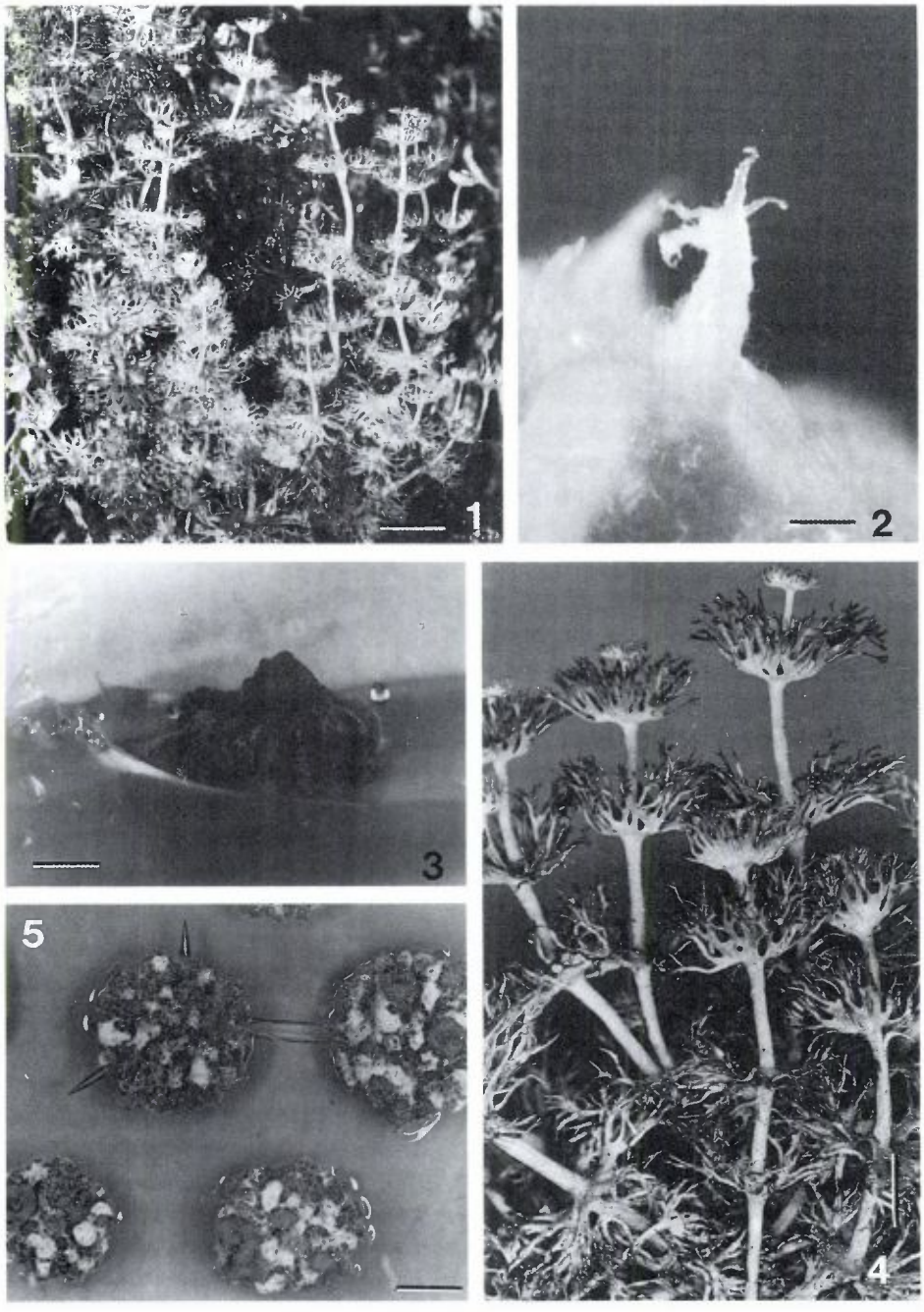

Figure 1. *Cladonia imperialis* in the natural environment (forms thalli with a height of 20–25 cm). Bar = 4 cm.

confumarprotocetraric (minor) and protocetraric acids (minor) known from the natural lichen. Mycobionts, grown on MY-extract-agar (Fig. 3) that were only poorly differentiated, did not form any of the depsidones.

Cladonia crinita: *C. crinita* (Fig. 4) is a very robust Brazilian relative of *C. imperialis*. In the natural environment it grows up to a height of 15 cm. The *C. crinita* mycobiont was successfully cultured on Sabouraud-2%-glucose-agar (Fig. 5). Although the medium contained only 2% less glucose, it did not form any podetia or secondary compounds. This result indicates that lichen mycobionts are very sensitive to slightly deviating contents of sugars in the nutrient medium. In a further experiment, on Sabouraud-4%-glucose agar, podetia formation was also initiated by this mycobiont. In cultures of the *C. crinita* fungus with well developed podetia, the typical lichen substances, fumarprotocetraric acid and its satellite substances, were detected.

Thamnolia vermicularis var. *subuliformis*: Varieties of *T. vermicularis* usually differ in their chemistry. Two chemotypes are known. The voucher specimens of *T. vermicularis* var. *subuliformis*, used in this study, contained baeomycesic and squamatic acids. During the growth phase of 7–12 months, the mycobiont cultures of *T. vermicularis* var. *subuliformis* (Fig. 9) were exposed to high light intensities (10–12 klx), but the temperatures were kept low at 15°C. Finally, the lichen substances formed on aerial hyphae, just before the cultures began to dry out. The exposure to strong light together with drought stimulated the fungus to synthesize baeomycesic and squamatic acids (Fig. 11; HPLC-chromatogram). In this case, the growth form, did not influence the synthesis of the substances.

Umbilicaria mammulata: *U. mammulata*, a North American species, often grows together with *Lassalia papulosa* on outcrops of silicious rocks. The mycobionts of *U. mammulata* (Fig. 11) and also other species of the genera *Umbilicaria* (*U. virginis*) and *Lassalia* (*L. pustulata*) grow very well on Potato-dextrose-agar (recommended by C. Culberson, pers. comm.). In culture, the *Umbilicaria*-mammulata mycobiont produced the tridepsides gyrophoric and

Figures 2–5 on opposite page:

Figure 2. Cultured mycobiont of *Cladonia imperialis* on modified Sabouraud-4%-glucose agar, producing secondary compounds. Bar = 2 mm.

Figure 3. Cultured mycobiont (*Cladonia imperialis*) on malt yeast agar, forming clumpy colonies without lichen substances. Bar = 1.5 mm.

Figure 4. Original thalli of *Cladonia crinita* (*Cladonia verticillaris* complex). Bar = 1.5 cm.

Figure 5. Cultured mycobiont of *Cladonia crinita*, after 4 months of incubation on Sabouraud-2%-glucose agar. Bar = 2 mm.

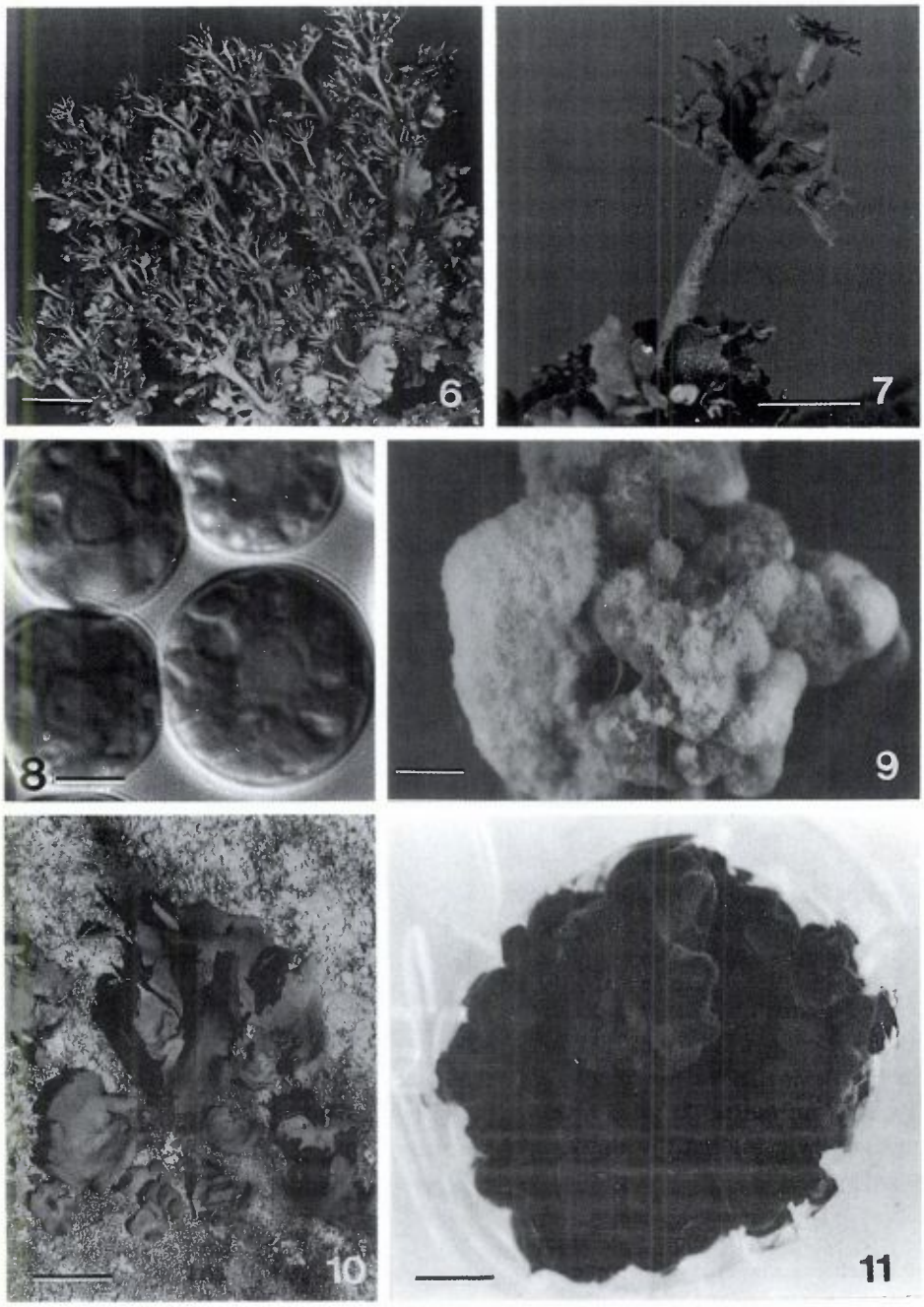

Figure 6. *Cladonia calycanthoides* in the natural habitat. Bar = 2.5 cm.
Figure 7. Resynthesized thallus (*Cladonia calycanthoides*), obtained in a long term experiment after 2 years. Bar = 3 mm.

umbilicaric acids. The secondary compounds were detected by HPLC analyses (Fig. 18; notice higher peak of umbilicaric than of gyrophoric acid) and TLC controls. In the voucher specimens gyrophoric, lecanoric and a trace of hiascic acid, but no umbilicaric acid were found. The results show that the mycobiont of *U. mammulata* has the potential to form umbilicaric acid as the major tridepside, whereas lecanoric and hiascic acids were absent.

The secondary compounds were only formed in very dry cultures that had transformed into lobe-like structures. Drought as a stress factor was found to be absolutely necessary to trigger the fungus to produce the two tridepsides.

Resynthesis studies

Cladonia calycanthoides: *C. calycanthoides* (Fig. 6) was the first species of the *C. verticillaris* complex that was resynthesized from its components. Both symbionts were isolated by the Yamamoto-method (as described above). For the resynthesis, hyphal segments were mixed with axenically cultivated *Trebouxia*-isolates (Fig. 8). Tiny primordia developed after 3 months. The anatomical structure of the primordia was comparable to soredia; the *Trebouxia*-cells were contacted by intraparietal hyphae. Only few of the hyphae penetrated the cells and behaved like haustoria.

After 5–6 months, most of the primordia had differentiated into well developed squamules. As also observed in former studies with alpine Cladoniaceae, the podetia arose from the center or the margin of corticated squamules. In the case of *C. calycanthoides*, most of the podetia grew up from the margin of the squamule. During early development the juvenile podetia were club-shaped and only fungal. Gradually the algae "colonized" the fungal structure (prepodetium). The algal colonies were released from the squamules fixed to the bottom of the developing podetium. After one year, fully developed scyphi with a well arranged algal layer were observed. The deeply branched margin of the central cavity formed by hyphal strands proliferating from an undivided scyphal plate. A further year was necessary to develop the second scyphus that grew up from the middle of the central cavity of the first. About 5 of 100 squamules were found to differentiate a vertical thallus – a two-storeyed verticillate podetium within two years (Fig. 7).

Figures 8–11 on opposite page:

Figure 8. *Trebouxia* sp., isolated from *Cladonia calycanthoides*. Bar = 4 µm.

Figure 9. Cultured mycobiont of *Thamnolia vermicularis* var. *subuliformis* on Sabouraud-2%-glucose agar. Bar = 1.2 mm.

Figure 10. *Umbilicaria mammulata* in the natural environment. Bar = 1 cm.

Figure 11. Cultured mycobiont of *Umbilicaria mammulata*, grown on Potato-dextrose agar. Bar = 1.3 mm.


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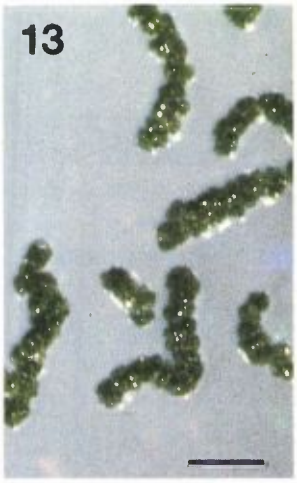
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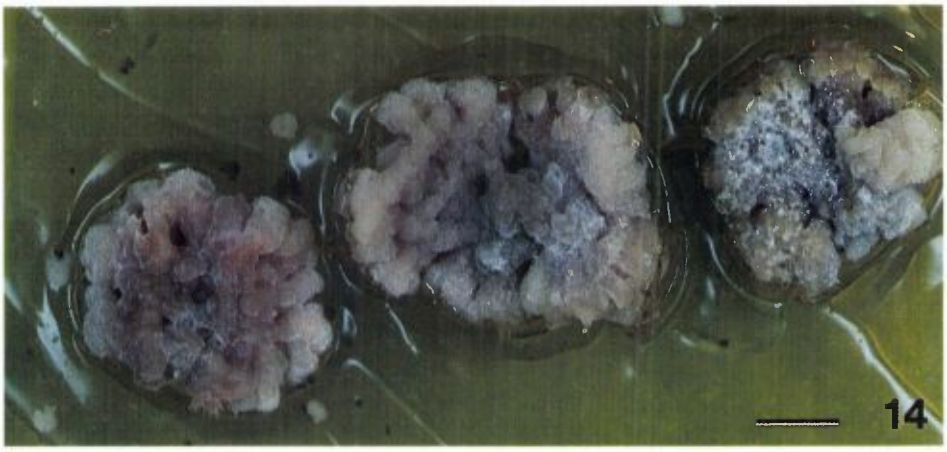
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If the development under natural tropical conditions occurs as slow as under laboratory conditions, a relatively high age for this type of fruticose lichen has to be predicted. Five to twenty five scyphi (arranged like in a tower) of one podetium of *C. imperialis* and 10 tiers formed by *C. calycanthoides* in the natural environment may correspond to 5 or at least 10 years of age.

Secondary compounds like fumarprotocetraric acid, confumarprotocetraric and protocetraric acids (also typical for *C. calycanthoides*) were formed in primordia, squamules and podetia. Some of the squamules that did not differentiate podetia also produced small HPLC-peaks that were identified as virensic acid.

Lobaria fendleri: *L. fendleri* (Fig. 12) is a dimorphic photosymbiodeme forming a tripartite symbiosis. In nature, the green photomorph is foliose, whereas the cyanobacterial photomorph is fruticose. The latter grows tightly fixed to the green lobes. The tropical *L. fendleri*-symbiosis is comparable and in many aspects similar to the *L. amplissima*-photosymbiodeme known from Scandinavia. The mycobionts of *L. fendleri* (isolated from both types of photomorphs) grew very slowly, however, an increase of the growth rates was obtained using modified Murashige-Skoog-medium.

In the first resynthesis experiment, the fragmented mycobiont was inoculated on division stages of axenically grown *Dictyochloriopsis* and *Nostoc* cells (Fig. 13). After 4 months the substrate was covered by minute globose primodia of the cyanobacterial photomorph. The green algal colonies had been completely overgrown by *Nostoc*. After a further period of two months the cyanomorph primordia had differentiated into branched structures of 2–3 mm height. Their structures were comparable to juvenile stages of the cyanobacterial photomorph normally situated on the green lobes of the photosymbiodeme. In culture, it was possible to grow them apart from the green photomorphs. Gradually, the cyanobacterial photomorphs (Fig. 15) differentiated thick bundles of root-like hyphae that dipped into the soil and kept them in an upright position like "tiny trees".

Figures 12–16 on opposite page:

Figure 12. Thallus of *Lobaria fendleri* (photosymbiodeme) with its coralloid cyanobacterial photomorph. Bar = 3 mm.

Figure 13. *Nostoc* sp., isolate of the cyanobacterial photomorph in axenic culture (*Lobaria fendleri*). Bar = 600 µm.

Figure 14. Mycobiont, isolated from the green photomorph, grown on Murashige-Skoog medium for 8 months (*Lobaria fendleri*). Bar = 1 mm.

Figure 15. Resynthesized, cyanobacterial photomorph (*Lobaria fendleri*), 6 month in culture. Bar = 1 mm.

Figure 16. Resynthesized green photomorph (*Lobaria fendleri*), growing on soil with bark extract. Bar = 1 mm.

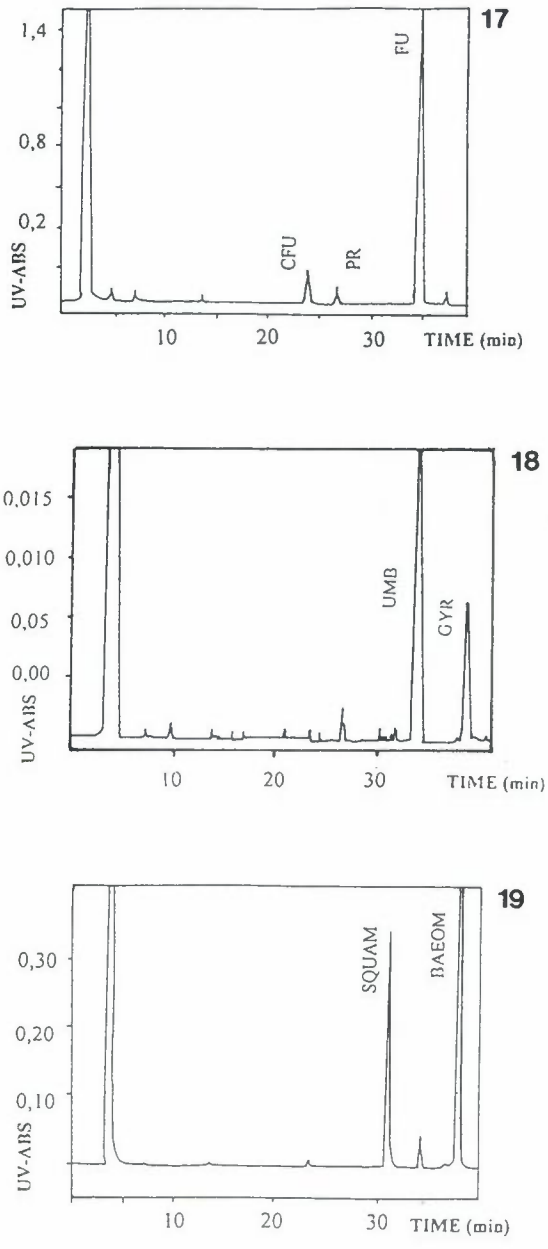

Figure 17. HPLC: Chromatogram of the cultured mycobiont of *Cladonia imperialis*.
Figure 18. HPLC: Chromatogram of the cultured mycobiont of *Umbilicaria mammulata*. Notice: additional peak of umbilicaric acid.
Figure 19. HPLC: Chromatogram of the cultured mycobiont of *Thamnolia vermicularis* var. *subuliformis*.

In a second experiment, the green photomorph was recombined from its partners. Young green thallus lobes (Fig. 16) only developed on sterile soil containing a bark extract from the tropical habitat of the lichens. Differentiation of the green lobes, at least in the early stage of development did not depend on the presence of cyanobacteria or the cyanobacterial photomorph. However, after one year of culture, the green stages stopped growing. This fact may indicate, that the cyanobacteria may affect the production of increased biomass by the green photomorph at an advanced stage of development.

The typical chemistry of *L. fendleri* (gyrophoric and 4-O-methylgyrophoric acid) was found in the cultured mycobiont and the resynthesized green photomorph. The mycobiont produced the secondary compounds only in highly differentiated lobe-like structures (Fig. 14) in a long-term experiment after one year.

In the cyanobacterial photomorphs no secondary compounds were detected, although some of the cyanobacterial photomorphs were resynthesized from mycelia isolated of the green photomorphs. The genetic similarity of the mycobiont of the green and the cyanobacterial photomorphs of *L. fendleri* was proven by DNA analyses performed by Stenroos (pers. comm.).

4. Discussion

DNA-analyses of the cultured mycobionts and the voucher specimens

It has been shown that mycobionts isolated from thallus fragments show considerable higher growth rates than mycobionts grown from polyspore or single spore isolates. In the present study, the DNA-analyses revealed that most of the mycobionts isolated by the Yamamoto method (Yamamoto, 1990) were identical with the voucher samples. In future, DNA-analyses are planned to be a routine for checking the identity of the cultured mycobionts.

Furthermore, the test series demonstrated that higher amounts of DNA could be extracted for sequencing from the cultures than was available from all the voucher samples tested. It was found that the majority of natural lichen thalli gave only weak bands of DNA; in such cases, cultures could serve to increase template material for DNA sequencing. DNA from cultures may also be used to design new primers with a high degree of specificity.

Until recent times, DNA-analyses were mainly applied to answer questions of phylogenetic relationships between lichen genera and species (e.g. Arup and Grube, 1998; De Priest, 1993) within the ascomycetous fungi. Experimental investigations of the lichen symbiosis (photosymbiodemes) using molecular methods have only been performed by a few investigators (Armaleo and Clerc,

1991; Armaleo and Clerc, 1995; Armaleo and Miao, 1998; Goffinet and Bayer, 1997; Stenroos, pers. comm.).

Cultured mycobionts and their secondary chemistry

In the natural environment *C. imperialis* and *C. crinita* produce the secondary compounds (fumarprotocetraric acid and its chemical satellites) typical for all the representatives of the *C. verticillaris* complex. Although the morphology of the different species is very complex and highly variable, the chemistry was found to be very uniform (Ahti and Marcelli, 1995).

Until recent times, fumarprotocetraric acid and its satellite substances had not been isolated from cultured mycobionts, but had been reported from natural diaspores (soredia) of other species of the genus *Cladonia* and their primordia produced by resynthesis (Zorer et al., 1996). Based on these and other data it was suggested that algae or their transfer products (polyols like ribitol) may play an essential role for the production of characteristic depsidones produced by many species of the Cladoniaceae.

The first report that the special depsidone, grayanic acid, was formed in a mycobiont culture (in the absence of algae) was published by Culberson and Armaleo (1992). However, depsidones belonging to the fumarprotocetraric acid chemosyndrone were not isolated from any of the cultured *Cladonia*-mycobionts reported by various authors and how they were formed remained unresolved to until recent times.

The results presented indicate that the production of depsidones like fumarprotocetraric acid and its satellite substances can be induced by culture conditions that promote a higher order of differentiation in the lichen fungi, e.g. the formation of resynthesis stages (a hyphal network to house the algal partner) or podetia (highly differentiated structures serving for reproduction).

It is possible that mycobiont strains of the tropical *C. verticillaris* complex have evolved a different genetic control mechanism to switch on or off the pathway for polyketide (depsidone) production than is found in *Cladonia* populations derived from temperate and arctic climatic regions.

Kon et al. (1997) found that although the mycobionts of *Usnea orientalis* produced only very low quantities of the characteristic lichen compounds salazinic and usnic acid in culture, they biosynthesized dibenzofurans like hypostrepsilic and isostrepsilic acids. "Tissues" with the natural photobionts contained higher amounts of the lichen substances, whereas the pathway to produce the dibenzofurans was obviously inhibited.

Ernst-Russell et al. (1999) found higher amounts of a naphthazarine (hybocarpone) in spore-derived mycobiont cultures of *Lecanora hybocarpa*. This substance was shown to be cytotoxic and a new bioactive lichen compound.

Most of the recently described secondary substances, biosynthesized by mycobiont cultures, were produced under osmotically stressed conditions (e.g. Yamamoto et al., 1985; Hamada, 1988; Hamada, 1993; Miyagawa et al., 1993). To some extent, the dry conditions in the cultures are comparable to osmotic stress caused by drought rather than by high quantities of carbohydrates.

In the present study the *Thamnolia*-mycobionts needed both high light intensities and drought to produce the typical chemistry in culture. This mycobiont was unique in synthesizing the lichen compounds without any recognizable higher differentiation.

Species of the lichen genera *Umbilicaria* and *Lassalia* were successfully grown on modifications of potato-dextrose-agar. The formation of the typical tridepsides, gyrophoric acid and umbilicaric acid, in mycobiont cultures was also stimulated by a transformation of the growth forms (lobe like structures forming aerial hyphae) and stress factors like desiccation and low temperature treatments. As shown in another study, the *U. virginis*-mycobiont needed low temperature treatment to induce the production of related lichen substances in culture (Stocker-Wörgötter, 2001b).

A detailed study of the chemistry of natural thalli of the lichen family Umbilicariaceae was reported by Narui et al. (1987).

To some extent, these results reflect the ecological niches in which these lichen fungi have adapted to survive. The *Umbilicaria* and *Lassalia* species generally grow in very exposed habitats on rock outcrops, where they can dry out very rapidly. As yet the question of whether the tridepsides have any function in water transport or in controlling the water content of the thalli has not been established by experimental studies.

Species of the genus *Thamnolia* live in open, windy and sun-exposed environments. The hypothesis that thamnolic, baeomycesic and squamatic acids (medullary substances) are involved in the protection of the algae by filtering high light intensities has not been tested. Only cortical substances, like atranorin and usnic acids have been considered to perform this function during active phases of the lichen photosynthesis (e.g. Rundel, 1978; Lawrey, 1984).

The resynthesis studies showed that tropical Cladoniaceae are excellent subjects to study complex differentiation processes in the laboratory. These preliminary results gave some indication of how storeyed podetia may form in nature. Indeed, it was demonstrated that one fully developed scyphus needed approximately one year for differentiation. If these results were extrapolated to calculate developmental processes in the natural environment, it indicates that one podetium of *C. imperialis* composed of up to 20 tiers may have an age of 15–20 years. Of course, this could only be verified by field studies.

The resynthesis studies of *L. fendleri* demonstrated that it is much easier to re-establish the cyanobacterial photomorph in a culture chamber than the corresponding green photomorph.

As shown in earlier resynthesis studies with *Peltigera leucophlebia* and *P. britannica* (Stocker-Wörgötter, 1995, 2000a) the development of the different photomorphs is also regulated by ecological factors. This is in agreement with field studies performed by Brodo and Richardson (1978), Ott (1988), Poelt (1986) and Poelt and Mayrhofer (1988). An ultrastructural study of the triple symbiosis of *Peltigera aphthosa* was reported by Honegger (1982).

The moist conditions at the beginning of the experiment and the formation of hormogonia and hormocysts favoured the development of the cyanobacterial photomorph in the presented study. The results showed that the lobes of the green photomorph developed only under very specific culture conditions in a highly adapted culture chamber. It was also demonstrated that the cyanobacterial photomorph is not necessary for early development and growth of the green lobes, but it does have an important function in maintaining and stabilizing the symbiosis in the mature thalli.

As was the case with the tropical Cladoniaceae, the characteristic secondary lichen compounds of *L. fendleri* were only formed by a highly differentiated mycobiont and these in the lobes of the green photomorph. The genetic similarity of the mycobiont of the green and the cyanobacterial photomorphs of *L. fendleri* has been established by DNA analyses performed by Stenroos (pers. comm.).

In order to simulate the tropical climate more effectively with periods of drought and high rain falls, the use of a phytotron (e.g. Dibben, 1971) would be advantageous in future studies.

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