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Morphogenetic strategies and induction of secondary metabolite biosynthesis in cultured lichen-forming Ascomycota, as exemplified by *Cladia retipora* (Labill.) Nyl. and *Dactylina arctica* (Richards) Nyl.

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Abstract
Over the last ten years a considerable number of lichen fungi (200 voucher specimens and their corresponding mycobionts) have been cultured and screened for the formation of secondary compounds (lichen substances). The production of lichen polyketides was found to be strongly affected by the composition of the nutrient media, dominant environmental parameters in the micro-habitat, and also by morphogenesis and cell differentiation of the mycelia. The present investigation deals with two lichens exhibiting very complex and unusual growth forms. The Australasian lichen *Cladia retipora* is one of the more fascinating fruticose lichens given its capacity to form very complex net-like structures (fenestrations). The North American and Asian lichen *Dactylina arctica* forms finger-like stalks which are well adapted to the harsh climatic conditions of arctic habitats. Cultures of both lichens and their corresponding mycobionts were found to exhibit exceptional morphogenetic strategies and capacities during their development and a well determined pattern (physiological and chronological) for producing typical secondary chemistry. In a re-synthesis and re-lichenization experiment (after culturing for 2 years) complete thalli containing the typical lichen substances were formed *in vitro*.

Keywords: Lichen-forming fungi, Ascomycota, mycobiont culture, resynthesis, re-lichenization, secondary compounds, polyketides, fatty acids, terpenes, *Cladia retipora*, *Dactylina arctica*

1. Introduction

For the lichen-forming Ascomycota, symbiosis with microalgae requires considerable morphogenetic adaptation and nutritional innovation (Sanders, 2001) which has probably evolved independently several times (see Gargas et al., 1995). An amazing diversity of fungal pheno- and genotypes are found within the different lichen families.

If it is true, that lichen forming fungi have their ancestors among fungi which are plant pathogens (Ahmadjian, 1993), they have changed their life-style dramatically, namely from a plant inhabiting mycelium (an endotrophic absorber) into a mycelium that is exotrophic and which accommodates an active photosynthetic partner (algae); a spatial relationship that over time may have transformed the role of the fungus from an inhabitant into an exhabitant.

An alternative hypothesis, that major fungal lineages of the Ascomycota, are derived from symbiotic ancestors has been suggested by Lutzoni et al. (2001). Such findings are inconsistent with that observed in other symbiotic systems, where protists (free-living eukaryotic cells) adopted bacteria, cyanobacteria and/or green algae in vacuols by phagocytosis. After time and increasing interdependence of the two different organisms following gene transfer, a symbiotic state was established. The obligate symbiotic partners are known to have a smaller genome than the originally free-living organisms (e.g. Werner, 1987; 1992)

In a few symbiotic associations where ascomycetous fungi lichenize with filamentous algae, for example with *Trentepohlia* in the tropical lichen genus *Coenogonium*, part of original life strategy may have been preserved. In such cases the fungus produces a few specialised hyphal

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strands that envelop the algal filaments, in order to absorb the polyol, erythritol, from the photobiont. The morphology of the thallus in such *Coenogonium* species is mainly determined by the algal partner (Stocker-Wörgötter, 1997). However, species of the genus *Coenogonium* do not appear to produce any lichen substances. Nevertheless, the marked adaptation in life strategy, morphogenetic capacity and mode of nutrition as found within approximately 20% of the Ascomycota, which have become lichenized (Kirk et al., 2001), could have led to the establishment of thalli with complex morphological features highly adapted to the various habitats encountered in different climatic zones of our planet. It is the morphological diversity of well-adapted lichen thalli that favours the survival of such composite associations (the lichen symbiosis) in pioneer plant societies.

The present study deals with *Cladia retipora*, a representative of the genus *Cladia*, a lichen genus distributed primarily in the Southern Hemisphere. Only one species, *C. aggregata* is known to occur in the Northern Hemisphere; e.g. the Caribbean and in Asia.

Cladia retipora is distinguished by a fruticose thallus which exhibits tree-like branches and subbranches with numerous perforations. In this case, the coral-like morphology is well adapted to house the algal colonies, facilitate gas interchange, moderate the extremes of temperature and maximize their exposure to light and water. These factors are important for the survival of this species in the exposed heath lands encountered in South Eastern Australia, New Caledonia and New Zealand where it occurs.

By contrast, *Dactylina arctica* is a common species in the arctic and alpine regions of the Northern Hemisphere, including Canada, Alaska and Russia. It develops finger-like stalks which protrude from dense pillows of mosses found near long lasting snow banks in alpine and also tundra habitats.

A first objective of this study was to find appropriate nutrient media for the two mycobionts that had resisted culturing in former investigations (Crittenden et al., 1995). Consequently, the study began with extensive screening of alternative nutrient media. A further goal was to obtain insight into the factors which influence mycelium and thallus morphogenesis and to ascertain whether these correlate with the biosynthesis of secondary metabolites in long-term resynthesis (*Cladia retipora*) or re-lichenisation (*Dactylina arctica*) experiments.

2. Material and Methods

At present the systematic position of both *Cladia* and *Dactylina* are not clearly defined and await further intensive molecular studies. Apparently both genera represent side branches of the large family Cladoniaceae (Lecanorales), sharing some characters with species of the genus *Cladonia*, whereas other features (e.g. the reduction of the primary thallus; formation of pseudopodetia) deviate more significantly from representatives of *Cladonia sensu stricto*.

Cladia retipora

The genus *Cladia* is currently considered to comprise 13 species (McCarthy, 2003), all of which occur in Tasmania, and many in mainland Australia and New Zealand. *Cladia aggregata* is the most widely distributed species (it also occurs in the Northern Hemisphere, South Africa and South America) and exhibits the greatest morphological diversity. *Cladia retipora* on the other hand, is restricted to Australia, Tasmania, New Zealand and New Caledonia (Filson, 1981). The specimens of *C. retipora* studied were collected in South Eastern Australia, New South Wales, just south of Morton National Park, 8 km NE of Nerriga, 35°05'S, 150°09'E, 750 m, growing on soil and detritus in clusters up to 20 cm or even meters in diameter.

Dactylina arctica

D. arctica usually forms finger-like stalks that are occasionally branched. Although the stalks are thin-walled and entirely hollow, they survive extended submersion by snow and exposure to strong winds, two climatic features common in arctic and alpine habitats. The specimens of *Dactylina arctica* were collected in Canada, British Columbia, Trophy Mountains (N51°48', W119°55') during a field trip of the IMC5 Meeting (Vancouver) and further samples were provided by Prof. H. Slupetzky, Institute of Geography) from Canada, Yukon Territory, near Fox Lake (80 km N of Whitehorse; N61°14', W135°26') at an elevation of 670 m.

Culture media

Mycobionts: Sabouraud-2% glucose agar; Sabouraud-4% glucose agar, modified (Stocker-Wörgötter, 2002b).

Photobionts: Bold's Basal Medium, modified (Stocker-Wörgötter, 2002a); Malt-yeast extract-Agar (Yamamoto, 1990).

Mycobiont and photobiont isolations and preparations for resynthesis

Modified Yamamoto-Method (after Yamamoto, 1990): The mycobiont isolations were performed in tubes on agar slants containing 5 ml of Sabouraud-2%glucose-agar. Small pieces of thallus were washed very carefully in sterile double distilled water, and a drop of Tween 80 (Bubrick and Galun, 1986) was added. The water was changed several times. Clean fragments were selected under a dissecting microscope and homogenized in sterile water (1–3 ml) at low speed (5,000–10,000 rpm for 15 sec) or by using a sterile mortar and pestle.

The resulting suspension, containing minute fungal-algal

fragments, was filtered through two sieves with different mesh widths (500 and 150 µm). Then pieces with an average size near 150 µm were selected using an inoculation needle or bamboo stick (Yamamoto, 1990) under a dissecting microscope. Agar slants containing nutrient media (listed above) were inoculated with a single fragment in each tube.

In this study, the above methodology was also used to isolate the algal partner, that are *Trebouxia* and *Trebouxia*-like photobionts present in *Cladia retipora* and *Dactylina arctica* (but of unknown systematic position). When the fragments were selected under the dissecting microscope at higher magnification, they could be separated into pieces containing mainly algal cells or fungal hyphae. Fragments containing predominantly green algae were transferred to tubes containing BBM (Bold's Basal Medium or malt-yeast-extract-agar).

Fragments composed mainly of hyphae and only a few algal cells were preferentially selected for the mycobiont cultures. To promote the hyphal growth and prevent algal divisions, the tubes were kept in complete darkness (covered by aluminium foil) for 2–3 months. For the successful culture of the mycobiont derived from the warm temperate species, *Cladia retipora*, it was necessary to maintain it at a higher temperature (23–30°C) than the mycobionts derived from *Dactylina arctica*, which were cultured at 4°C or 10°C for samples collected from arctic and alpine regions, respectively.

The algal dominated isolates were maintained under a light dark regime of 14:10 hours and a light intensity of 50–100 µE $m^{-2}s^{-1}$. After 2–3 months the isolates were checked for growth. Then contamination-free mycelia and algal colonies were transferred to new nutrient media. For the warm temperate mycobionts (*Cladia retipora*) the culture chambers were adjusted to 14:10 h day-night cycles and alternating temperature of 27:10°C. The algal cultures (*Trebouxia* sp. of *C. retipora*) were kept under the same temperature regime at a light intensity of 50–100 µE $m^{-2}s^{-1}$. The mycobionts of *Dactylina arctica* were cultivated at a light-dark regime of 14:10 h and an alternating temperature of 10:4°C.

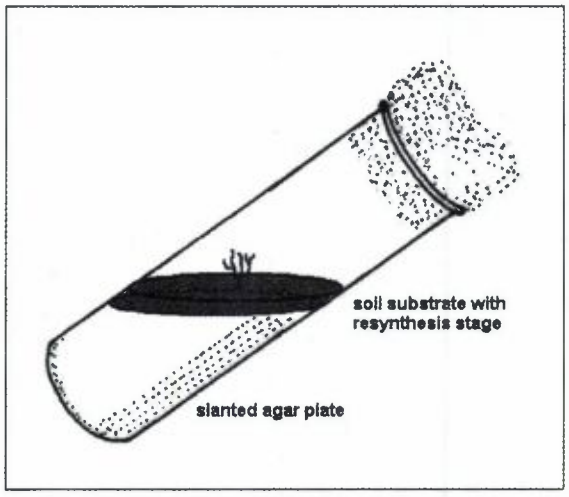


Figure 1. Resynthesis tube, adapted to grow *Cladia retipora*.

Resynthesis

C. retipora: Sandy soil was collected at the natural habitat of the lichen (Nerriga, NSW, Australia) in an open, sclerophyll forest. The substrate (100 g) was dried and sieved (mesh width 2 mm). For the resynthesis experiment, 100 g of the sandy soil was soaked with 300 ml double distilled water (sterile) for 3–4 hours; then it was twice autoclaved for 3 hours on two successive days in order to guarantee axenic conditions. Finally the moist soil was distributed over a slanted agar plate in glass culture tubes (150 × 30 mm) until a 20 mm thick soil cover was achieved (Fig. 1; resynthesis tube). The sterile soil was placed into the tubes on a clean work bench under axenic conditions.

The combined substrates (agar plate with a layer of soil, Fig. 1) were inoculated first with photobiont cells obtained from an axenic culture (*Trebouxia*-isolate N. 133 from *C. retipora*, 14.10.2001). After about three weeks the algal colonies had divided and were visible as green spots (with an average diameter of 5 mm).

Axenically grown mycelia were gently homogenized together with 3 drops of sterile water. Then the hyphal suspension was carefully pipetted onto the algal colonies and both partners mixed with a sterile inoculation needle. Then the tubes were closed with sterile cotton plugs and covered by aluminium foil caps.

The resynthesis cultures were incubated first in a culture chamber under simulated warm temperate conditions (described above) and after incubation for 3–4 months the tubes were moved to a small greenhouse (40 × 30 cm) to expose the cultures to the natural day/night and light cycles for four seasons. In winter, the greenhouse was maintained near a window in the sub-basement, where the temperatures did not drop below –2°C.

Dactylina arctica

In this case, the hyphal colonies were cut into bands with a scalpel and the algal colonies transferred to the upper side of the mycelium bands. For re-lichenization, the mycobiont bands together with the algae were used to inoculate the sterile soil substrate in petri dishes (150 × 30 mm) that had been soaked with (40 ml/500 g) moss extract (prepared by the method of Esser (1982); 500 g of moss was used instead of soil). The pH of the moss extract was adjusted to 5.

Chemical methods

Thin layer chromatography (TLC) was performed by using the standardised method of Culberson and Ammann

(1979) and Elix and Ernst-Russell (1993) for substances like ursolic acid (terpenoid) that could not be detected by HPLC.

Extraction and preparation of the cultures

Circular plugs (ca. 1 cm in diameter) were cut out from each of the agar plates overgrown by the mycobiont. The subcultures tested originated from a single fragment. To prevent contamination, the plugs were prepared in a clean work bench under sterile conditions. The samples were vacuum freeze dried at –42°C for 12 hours. The dried discs were transferred to vials and extracted with methanol for 4 hours. Then the extract was transferred to HPLC vials and an aliquot of 20 µl was injected from each sample.

Identification of the metabolites

Natural compounds were characterized by high-performance liquid chromatography (HPLC) with retention index values (RI) calculated from benzoic acid and solorinic acid controls (Elix, 1996; Elix et al., 2003; Feige et al., 1993). For HPLC analyses two different systems were used: a Hewlett Packard Spectra System (Australia) and a Merck Hitachi Spectra System (Austria), a Phenomex Hypersil 5° C18 column (250 × 4.6 mm) (Australia) and a Beckman 5° C18 column (250 × 4.6 mm) (Austria), and spectrometric detectors operating at 254 nm with a flow rate of 1 ml/min were used. Two solvent systems A and B were used: 1% aqueous orthophosphoric acid and methanol in the ratio 7:3 (A) and methanol (B). The run started with 100% A and was raised to 58% B within 15 min, then to 100% B within a further 15 min, followed by isocratic elution in 100% B for a further 10 min. The HPLCs were coupled to photodiode array detectors for ultraviolet spectroscopic comparisons. By this means the ultraviolet spectra observed for the various components eluting in the HPLC chromatogram were recorded and computer-matched against a library of ultraviolet spectra recorded for authentic metabolites under identical conditions. For each compound the correlation of the ultraviolet spectra of the authentic and natural material was greater than 99.9%.

3. Results

In nature the *Cladia retipora* mycobiont (in contact with *Trebouxia*-cells) forms a very complex thallus with netlike fenestrations and a well developed inner medulla (Fig. 1), internal hyphal strands connecting the perforations with each other by an irregular network. Sometimes, thallus algae or free-living algal cells from the environment (e.g. *Coccomyxa*), become entrapped in this internal network.

Chemistry

The voucher specimens of *C. retipora* contains atranorin and usnic acid as major compounds (HPLC-chromatogram; Fig. 18; UV-spectra, Fig. 18a–c). The triterpene, ursolic acid, and the fatty acid, protolichesterinic acid, were detected by TLC (Fig. 19). Ursolic acid produces a lavender-blue, very characteristic spot (Culberson 1972), whereas protolichesterinic acid is a fatty acid that shows a translucent white spot after spraying with sulfuric acid.

Culture of the mycobionts

For the mycobiont cultures, Sabouraud-2% sucrose agar (Difco Agar; + Murashige mineral salts) was used successfully. The best growth of the mycobionts was obtained in LB-Medium (+ 4% adonitol). This medium increased the growth rates and led to the development of clumpy fungal colonies, but did not induce any differentiation of the mycelium. Surprisingly, the mycobionts exhibited considerable morphogenetic capacity when they were subcultured on solid Murashige medium (soil extract, 1% sucrose, 1.5% Difco-Agar, Fig. 3) and Sabouraud medium (4% sucrose, 2% glucose, + soil extract) (Figs. 4, 5). After an incubation period of 3 months the uppermost, cortical hyphae of the mycelia produced extensive networks of hyphae as can be seen in Figs. 3–5.

Resynthesis

The resynthesis experiments were performed in a "resynthesis tube" that was adapted for this function (Fig. 1). The substrate comprised a combination of a slanted agar plate with a superficial layer of sterile soil, surrounded by an agar ring (3 mm).

Firstly, the photobionts were transferred as a suspension from liquid BBM (in culture flasks) with a pipette (200 µl algal suspension; Fig. 6) into the tube and distributed over the soil substrate.

The algal division stages were of particular interest. As in many cases, one or two vegetative autospores are not released from the mother cell, the cell wall of the original cell being persistent. The young autospore remains inside the original cell wall (Fig. 6 arrow!) and grows within a mother cell, until, finally it has 2 or more cell walls depending on how often this cell cycle takes place. In such cases, the infection by hyphae does not result in penetration of the cell wall, but only in appressorial contacts with the symbiotic alga. This phenomenon explains why in species of *Cladonia* and *Cladia*, contact by both appressorial and intraparietal (penetrating the cell walls, but not the cell membrane) hyphae, is observed. The resynthesis tubes, containing algal colonies as green spots (5 mm in diameter), were incubated in a culture chamber for 3 weeks.

At the same time, gently macerated mycelia (hyphal suspension) were transferred to a liquid mineral solution (e.g. BBM) in Erlenmeyer flasks without any sugar derivatives, in order to stress and starve the mycobionts. After 3 weeks, such pre-treated hyphal colonies were

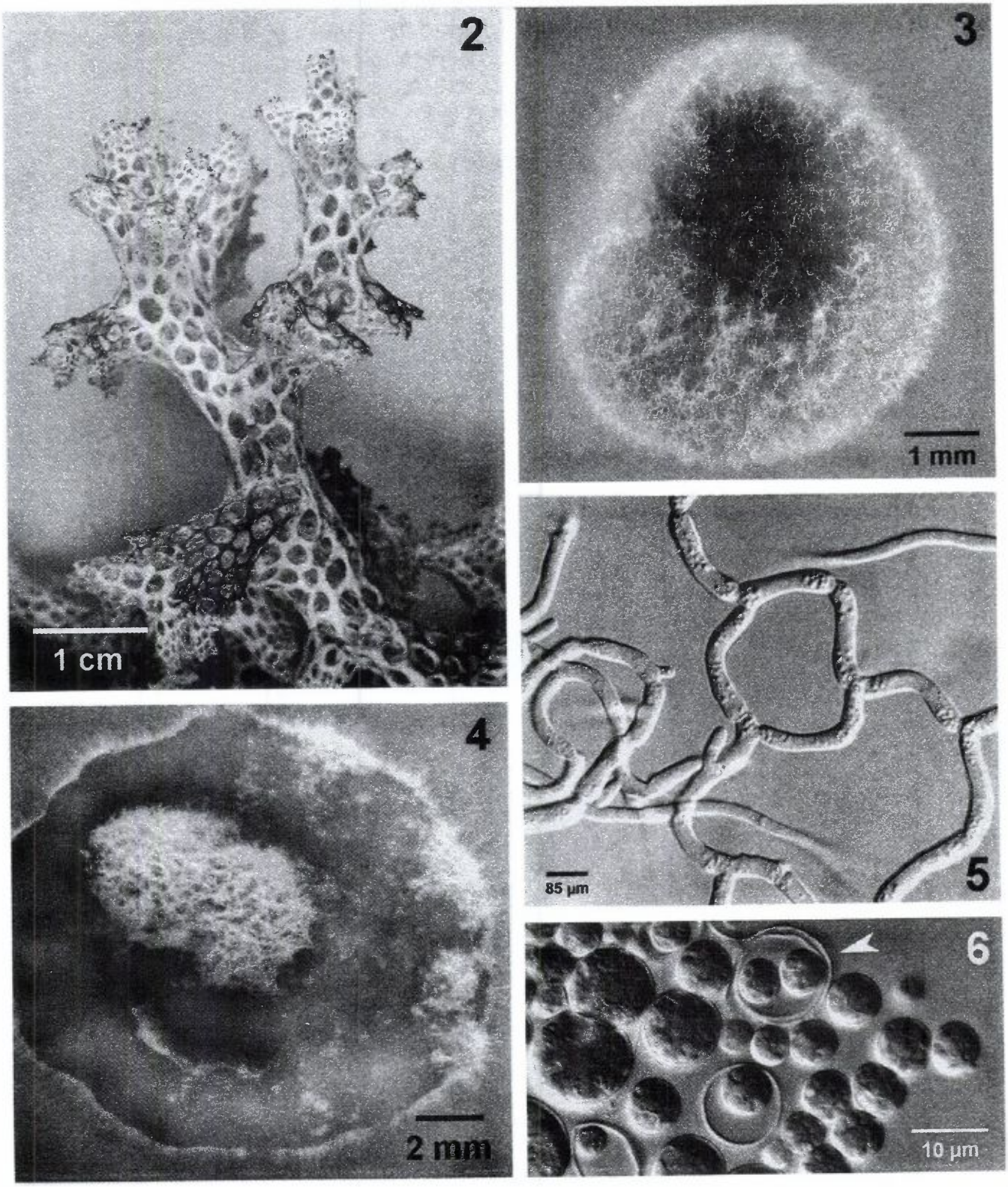


Figure 2. Voucher specimen of *Cladia retipora*, collected in Eastern Australia.
Figure 3. Mycobiont (*C. retipora*) on Murashige Skoog medium (+ soil extract, + 1% sucrose); 3 months.
Figure 4. Mycobiont (*C. retipora*) in Difco Sabouraud 4% sucrose medium (+ 2% glucose, + soil extract); 3 months.
Figure 5. Differentiated mycelium (*C. retipora*) with hyphae forming a network.
Figure 6. *Trebouxia* sp. (with division stages), isolated from *C. retipora*.

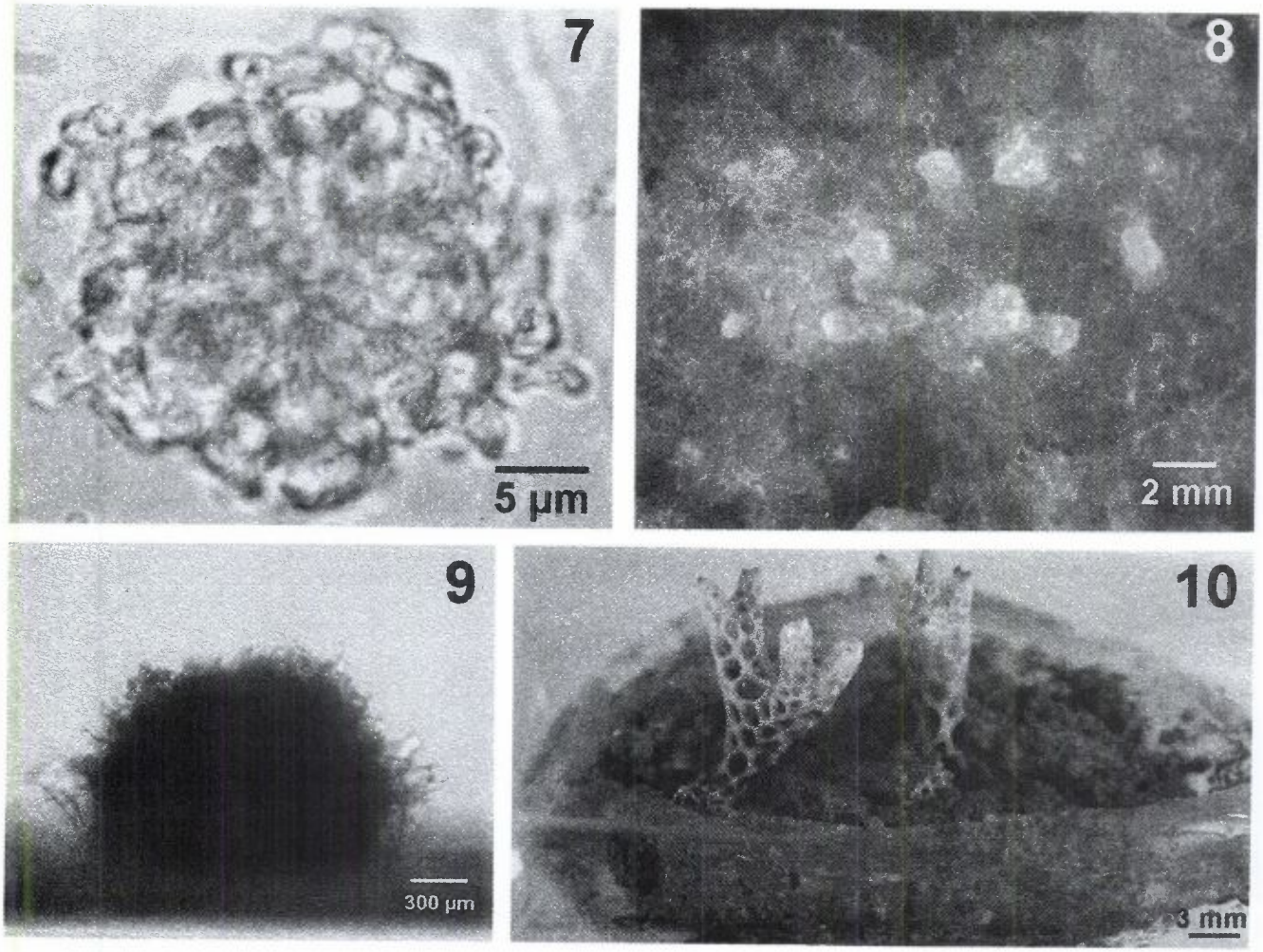


Figure 7. Early resynthesis stage (*Cladia retipora*) on soil substrate; 1 month.
Figure 8. Resynthesis stage (*C. retipora*) with a rim of outgrowing hyphae on soil; 1 month.
Figure 9. Juvenile stages (*C. retipora*) on soil; 4 months. Arrangement of scales occurs in circles.
Figure 10. Juvenile stages of *C. retipora* grown in a resynthesis tube in a green house; 2 years.

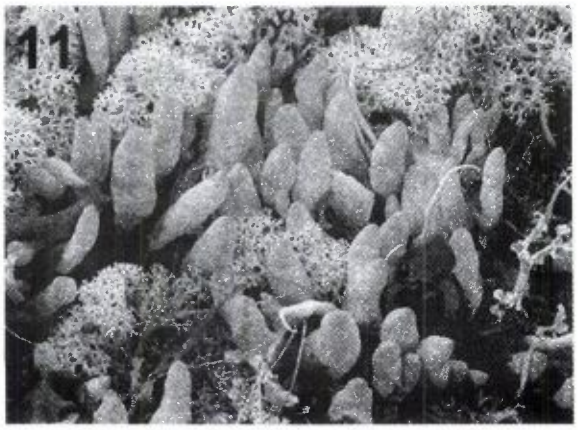
inoculated on to the algal colonies in the resynthesis tubes and gently mixed.

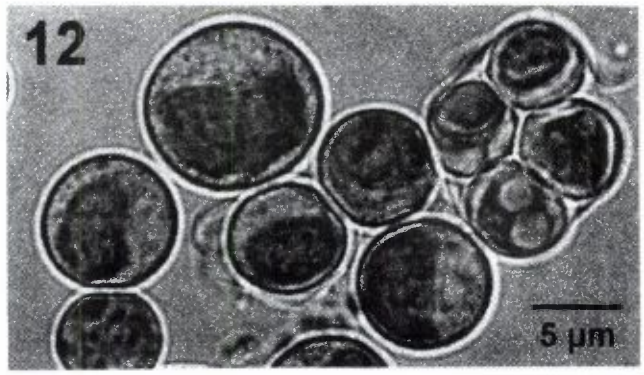
Minute resynthesis stages, lichen primordia composed of mycelia and enclosed algae, were present in 1-month old cultures (Fig. 7). Several *Trebouxia*-cells (+ their autospores) were infected by fungal hyphae. In many cases, these early algal-fungal associations already showed hyphal outgrowths at their margin (Fig. 9).

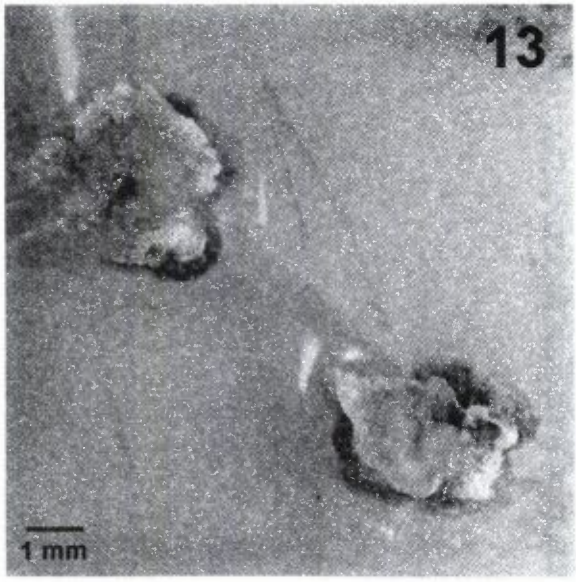
After 4 months, the resynthesis cultures formed recognizable scales which appeared to form circles (Fig. 8). In each circle, approximately 20 minute scales developed. These were subsequently found to be the starting units for the complex network of hyphae that are responsible for the thallus of *Cladia retipora*. However, most of these cultures did not develop beyond this stage.

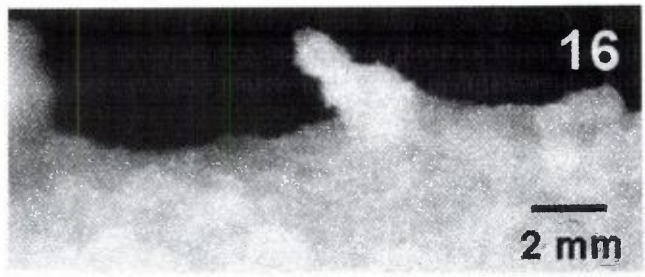
In an additional experiment, the cultures were moved to a green house in order to expose them to the daily and seasonal changes of natural light/dark regimes and temperature changes.

After an incubation time of 2 years, only 4 tubes from 100 inoculated cultures, produced complete young stages of *Cladia retipora*, as is shown in Fig. 10. In these starting units, the scales first differentiated into columns that grew vertically together, forming a relatively thin network. Subsequently, more hyphae appeared growing parallel and fusing together, strengthening and stabilising the network. After time, the algal cells colonized the network, and in the final step (18 months - and after moisturising the cultures), the inner medulla was formed by more hyphae, integrating further algal cells and connecting the fenestrations to one another and filling out the originally hollow tree-like structures. A detailed study of these processes is in preparation.









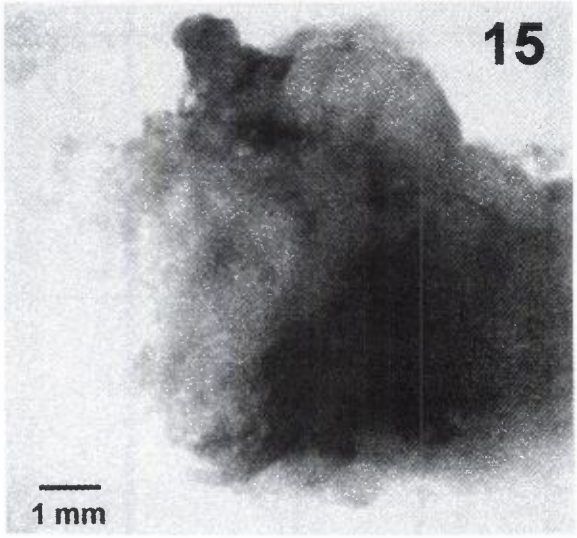
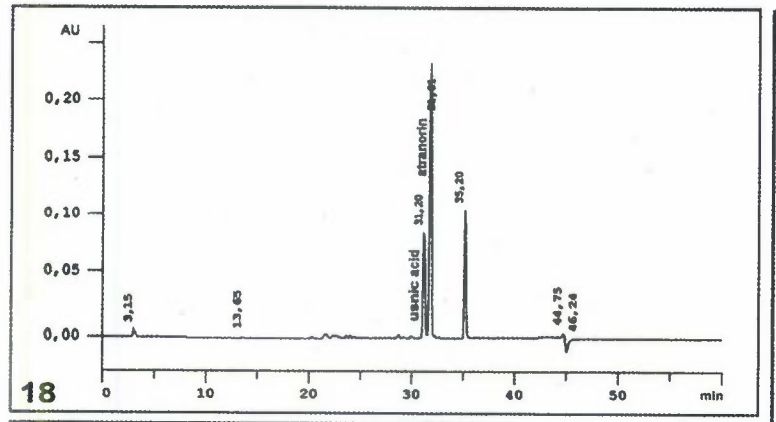
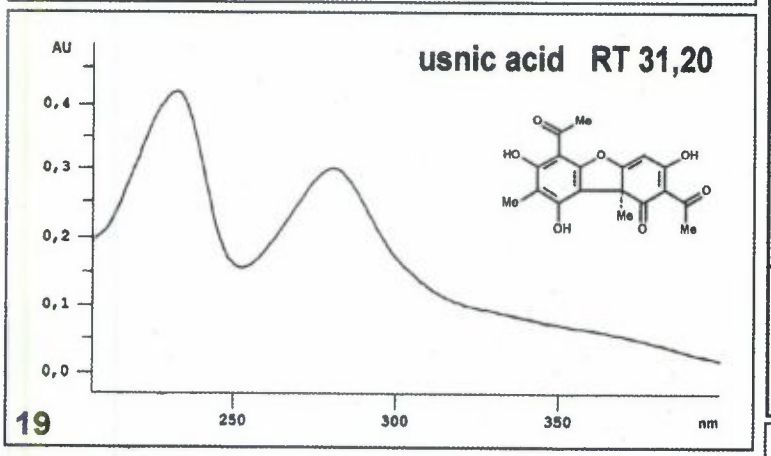
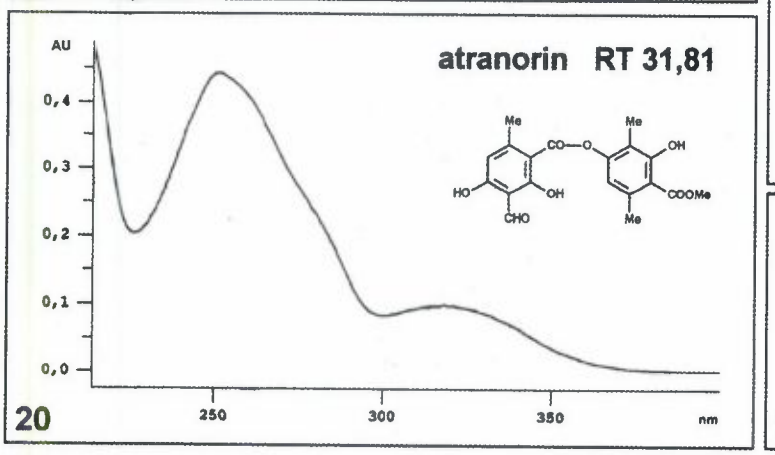
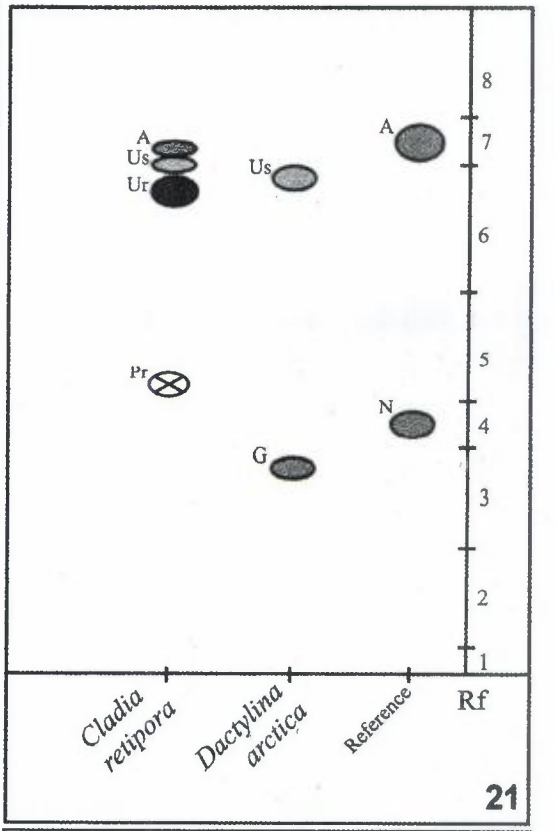


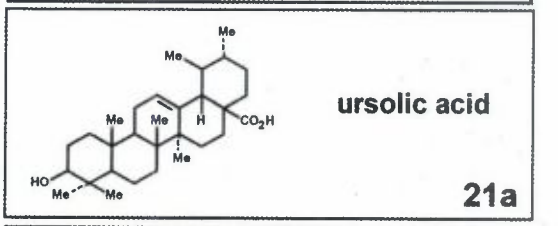
Figure 11. *Dactylina arctica* in the natural environment growing with Cladoniaceae and species of the genus *Thamnolia*. (modified, from http://home.hiroshima-u.ac.jp/lichen/jimam/dactylina.htm).
Figure 12. Isolated *Trebouxia*-like photobiont of *Dactylina arctica*.
Figure 13. Mycobiont (*D. arctica*) on Sabouraud 4% glucose medium; 5 weeks.
Figure 14. Mycobiont (*D. arctica*) on Sabouraud 2% glucose medium; 6 months.
Figure 15. Mycobiont (*D. arctica*) initiating cell differentiation (stalk development in LB 4% glucose medium); 6 months.
Figure 16. Development of juvenile stalk (*D. arctica*), growing from a hyphal band; 7 months.
Figure 17. Juvenile stages of *D. arctica*, grown in a cool temperature chamber; 2 years.











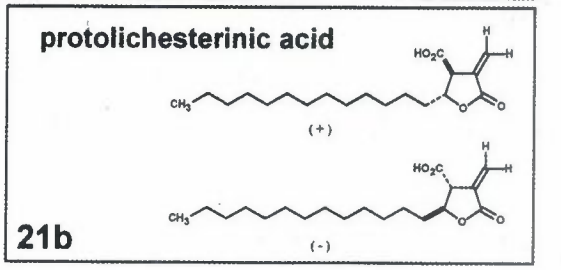


Figure 18. HPLC chromatogramme (*Cladia retipora*).
Figure 19. UV-spectrum of usnic acid (*C. retipora*).
Figure 20. UV-spectrum of atranorin (major compound in cultured *C. retipora*).
Figure 21. TLC-plate of *Cladia retipora* and *Dactylina arctica.*
Figure 21a/b. Chemical structures of the triterpene ursolic acid and protolichesterinic acid.

Chemistry and temporal pattern of occurrence of lichen substances in the cultured mycobiont of Cladia retipora *and in its* in vitro *resynthesis products*

The aposymbiotically grown mycobionts (clumpy growth forms) did not produce any depsides in the LB-medium. However, in modified Murashige and Sabouraud medium, the mycelia differentiated superficially to form net-like structures and after about 3 months (as soon as the hyphal network became visible), atranorin was produced. The concentration of atranorin increased, provided the moisture content of the cultures was relatively high. Usnic acid was only found in long term-cultures that were grown in a culture chamber under similar light-dark regimes as the photobionts. Light was obviously necessary to induce the production of usnic acid in the 12 months-old cultures, since usnic acid was absent in the control cultures growing in the dark.

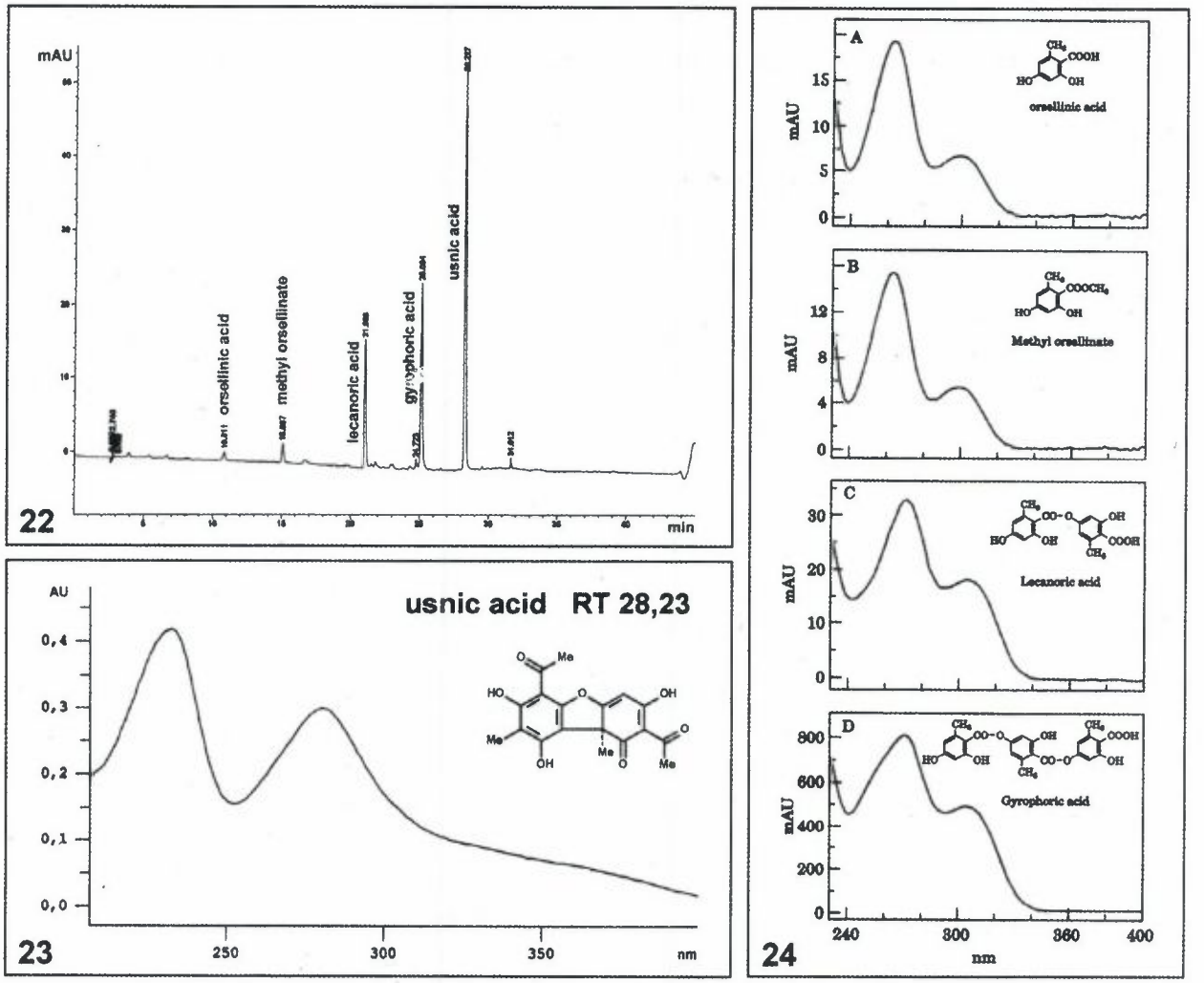


Figure 22. HPLC chromatogramme of *Dactylina arctica*, showing the lecanoric/gyrophoric chemosyndrome.
Figure 23. UV-spectrum of usnic acid (the major compound in *D. arctica*).
Figure 24. UV-spectra of the lecanoric/gyrophoric acid chemosyndrome.

Interestingly, the triterpene ursolic acid and the fatty acid protolichesterinic acid were not produced by any of the well developed mycobionts. By contrast, the less differentiated, clumpy cultures biosynthesised protolichesterinic acid in significant quantities, that could be readily detected by HPLC and TLC analyses.

Ursolic acid, on the other hand, was only detected in resynthesized primordia after an incubation period of 4 months, when about 80% of the algal colonies were enclosed by fungal hyphae. As the quantities of ursolic acid were too low to be detected by TLC, its presence was verified by a microcrystallization tests showing the typical crystals. A mixture of atranorin and ursolic acid was found in the scales and the columns (hyphal strands) building up the net-like structures of the thallus. All three compounds were only found in the thalli that had been in culture for 2 years, where atranorin was the major and usnic acid the minor component.

Even so, the concentration of usnic acid was much lower in the juvenile resynthesis stages than in thalli collected from the natural environment. Furthermore, in the natural thalli usnic acid was the major component rather than atranorin as observed in the resynthesized thalli.

In vitro *relichenization of* Dactylina arctica *(Figs. 11–17)*

Myco- and photobiont cultures and chemistry: The mycobiont of *Dactylina arctica* was grown in two different nutrient media (Figs. 13, 14). The highest growth rates were obtained when using Sabouraud-4% glucose medium. Such mycelia showed very little cell differentiation (Fig. 14). More interesting results were obtained with Sabouraud 2% glucose medium (enriched with moss extract, as described above), where slightly elevated mycelia with a white, marginal rim were formed (Fig. 13). After a culturing period of 6 weeks, orsellinic and lecanoric acids could be detected by HPLC analyses. The tridepsides, methyl gyrophorate and gyrophoric acid were detected in 6-

month old cultures (Fig. 15) that had started to form very tiny stalks after being stressed by cold temperature treatment for 3 weeks at –23°C.

Re-lichenisation: The *Trebouxia*-like photobionts (of unknown systematic position) were grown in BBM (+ moss extract), where they formed a significant number of division stages (Fig. 12). For re-lichenisation they were transferred to "hyphal bands" (see Methods). The hyphal strands which contained the algal colonies were inoculated on the sterile soil substrate (enriched with moss extract). In a first stage (which needed about 4 months), the algae were incorporated into the fungal mass and overgrown by cortical hyphae. As soon as this process had been effected (the algal cells are only visible as very faint spots; Fig. 16), a juvenile stalk began to protrude at the margin of the hyphal band. Over time, the algal colonies expanded the stalks from the inside and were transported along by "moving" hyphae (in German: "Schiebehyphen") to their final positions.

Fully developed, stalked thalli were formed in a long-term culture after an incubation period of 2 years. These stalks developed in a single line along the hyphal bands (Fig. 17). Similar growth patterns have also been observed in nature, where the thalli often appear in lines, connected by strong hyphal bands hidden in the moss cushions.

The complete lecanoric acid/gyrophoric acid chemosyndrome was first detected in the very young stalks that developed at the margin of the hyphal bands (ca. 5-month-old cultures).

The most highly developed thalli (with well differentiated stalks) were obtained when the cultures were kept in a cool temperature chamber at 4°C during the night and 10°C during the day (Fig. 17).

4. Discussion and Conclusions

In general, our experimental knowledge of the growth conditions and morphogenetic strategies of lichen-forming fungi is very limited, and the nutritional requirements of specific bionts conjoined in a lichen symbiosis have not been studied extensively.

Our recent investigation, combining an experimental study of the complex morphogenetic capacity of the mycobiont from *Cladia retipora* and the uncommon growth form engendered by the mycobiont of *Dacylina arctica*, showed that the basic developmental capacity of thallus primordia (i.e. soredia-like fragments with algal cells enclosed by hyphae) or even much larger units (hyphal bands with a large number of algal colonies) can be used as effective nuclei for producing complex, morphologically diverse thalline structures.

Such studies certainly complement the original concept that a new lichen thallus derives from a single fungal spore encountering an algal cell, as has been proposed by former investigators (e.g. Ahmadjian, 1966). Given our recent results it seems likely that some lichen thalli are certainly derived from multiple individuals (e.g. soredia) which are not necessarily genetically uniform.

The present study and former reports (Kinoshita, 1993; Kinoshita et al., 1993; Stocker-Wörgötter, 2005; Stocker-Wörgötter and Elix, 2002; 2004; Stenroos et al., 2003) have shown that the production of secondary lichen compounds is dependent upon specific nutrient requirements as well as being associated with morphogenetic differentiation processes.

Our investigation demonstrated that in the case of the *Cladia retipora* mycobiont the formation of the p-depside atranorin was induced by a complex interplay of physiological, morphological and environmental factors, including a specific nutrient medium which favoured low growth rates, the differentiation of a complex hyphal network (morphogenesis) and a high water content to maximize the accumulation of atranorin.

Klee and Steubing (1977) have previously reported that the production of atranorin in *Hypogymnia physodes*, *Parmelia saxatilis* and *Pseudevernia furfuracea* increased when the substrate had a high moisture content.

Interestingly, 3-methylorsellinic and haematommic acid, precursors of atranorin, were not detected in the cultures.

Exposure of the mycobionts to light and slow dehydration resulted in a biosynthetic shift to usnic acid production, a dibenzofuran derived from the alternative C9 precursor, methylphoroacetophenone.

A large cluster of genes is known to be responsible for the PKS (polyketide synthases) function (Turner, 2000) both for controlling the alternative pathways products and effecting various chemical modifications of the initial secondary metabolites.

In the case of the *Dactylina arctica* mycobiont, only the lecanoric acid/gyrophoric acid chemosyndrome was observed when it was grown on a medium containing reduced sugars which slowed the growth rates and promoted differentiation. The biosynthesis of the secondary products occurred sequentially with initial production of orsellinic acid and lecanoric acid and subsequent formation of methyl gyrophorate and gyrophoric acid.

In the resynthesis cultures the complete chemosyndrome was only formed in well developed thalli (juvenile stalks) after an incubation period of 5 months.

In both *Cladia retipora* and *Dactylina* there was an obvious correlation between the morphological development of these fruticose lichens and the induction/production of secondary lichen compounds. However, such a correlation is not always found in foliose lichens (e.g. selected species of the genus *Xanthoparmelia*), as was shown in a recent study by Zocher and Stocker-Wörgötter (2005).

In the present culture experiments, only the less differentiated mycobionts of *Cladia retipora* produced the fatty acid protolichesterinic acid. In this case, fatty acid biosynthesis predominated over polyketide production. As protolichestinic acid is also present in the natural thalli,

which indicates that in the symbiosis both pathways (fatty acid and polyketide biosynthesis) are functional.

At present, we have no explanation why ursolic acid (a triterpene) was only detected in the resynthesis stages. The occurrence of the triperpenes ursolic acid and friedelin is not restricted to lichens as they also occur in fungi, mosses and higher plants and are biosynthesised via the mevalonic acid pathway (Huneck and Yoshimura, 1996).

Several previous investigations (e.g. Yamamoto et al., 1987, 1998) have used very stable culture conditions which can be readily maintained in the laboratory to produce large quantities of metabolic products. Under such conditions, the mycobionts preferentially produce fatty acids (stearic, linoleic, oleic acids) and triglycerides rather than typical lichen polyketides (Molina et al., 2003; Adler et al., 2003).

A more successful programme for the production of lichen polyketides has been established in our laboratory which facilitate the variation of culture conditions, employs improved media, simulates particular microclimatic parameters and provides a very flexible means for changing them.

The present study involved extensive field work in Australia and Canada in order to obtain some insight into the climatic and environmental conditions required by the selected species.

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REFERENCES

Adler, M.T., Fazio, A., Bertoni, M.D., Rosso, M.L., Maier, M.S., and Thell, A. 2003. Culture experiments and DNA verification of a mycobiont isolated from *Punctelia praesignis* (Parmeliaceae, lichenized Ascomycotina). *Bibliotheca Lichenologica* **88**: 1–8.

Ahmadjian, V. 1966. Artificial reestablishment of the lichen *Cladonia cristatella*. *Science* **151**: 199–201.

Ahmadjian, V. 1993. *The Lichen Symbiosis*. John Wiley and Sons, New York.

Bubrick, P. and Galun, M. 1986. Spore to spore resynthesis of *Xanthoria parietina*. *Lichenologist* **18**: 47–49.

Crittenden, P.D., David, J.C., Hawksworth, D.L., and Campbell, F.S. 1995. Attempted isolation and success in the culturing of a broad spectrum of lichen-forming and lichenicolous fungi. *New Phytologist* **130**: 267–297.

Culberson, C.F. 1972. Improved conditions and new data for the identification of lichen products by a standardised thin layer chromatographic method. *Journal of Chromatography* **72**: 113–125.

Culberson, C.F. and Ammann, K. 1979. Standardmethode zur Dünnschichtchromatographie von Flechtensubstanzen. *Herzogia* **5**: 1–24.

Elix, J.A. 1996. Biochemistry and secondary metabolites. In: *Lichen Biology*. Chapter 9. Nash III, T.H., ed. Cambridge University Press, Cambridge, pp. 154–180.

Elix, J.A. and Ernst-Russell, K.D. 1993. A catalogue of standardized thin layer chromatographic data and biosynthetic relationships for lichen substances, 2nd edition.

Elix, J.A., Giralt, M., and Wardlaw, J.H. 2003. New chlorodepsides from the lichen *Dimelaena radiata*. *Bibliotheca Lichenologica* **86**: 1–7.

Elix, J.A. and Wardlaw, J.H. 2000. Lusitanic acid, peristictic and verrucigeric acid. Three new beta-orcinol depsidones from the lichen *Relicina sydneyensis* and *Xanthoparmelia verrucigera*. *Australian Journal of Chemistry* **53**: 815–818.

Esser, K. 1982. Kryptogamen: Cyanobakterien, Algen, Pilze, Flechten. Praktium und Lehrbuch, 2nd edition. Springer Verlag, Berlin, Heidelberg, New York, Tokyo. pp. 23.

Feige, G.B., Lumbsch, H.T., Huneck, S., and Elix, J.A. 1993. The identification of lichen substances by a standardized high-performance liquid chromatographic method. *Journal of Chromathography* **646**: 417–427.

Filson, R.B. 1981. A revision of the genus *Cladia* Nyl. *Journal Hattori Botanical Laboratory* **49**: 1–75.

Gargas, A., DePriest, P., Grube, M., and Tehler, A. 1995. Multiple origins of the lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science* **268**: 1492–1495.

Huneck, S. and Yoshimura, I. 1996. *Identification of Lichen Substances*. Springer Verlag, Berlin, Heidelberg, New York, Tokyo.

Kantvilas, G. and Jarman, S.J. 1999. Lichens of rainforests in Tasmania and South-Eastern Australia. *Flora of Australia*, Supplementary Series No. 9, Canberra.

Kinoshita, Y. 1993. *The Production of Lichen Substances for Pharmaceutical Use by Lichen Tissue Culture*. Nippon Paint Publications, Osaka, 77 pp.

Kinoshita, Y., Hayase, S., Yamamoto, Y., Yoshimura, I., Kurokawa, T., Ahti, T., and Yamada, Y. 1993. Morphogenetic capacity of the mycobiont in *Usnea* (lichenised ascomycete). *Proceedings of the Japan Academy* **69**: 18–21.

Kirk, P.M., Cannon P.F., David, J.C., and Stalpers, J.A. 2001. *Ainsworth and Bisby's Dictionary of the Fungi*, 9th ed., CAB International, Wallingford, UK.

Klee, R. and Steubing, L. 1977. Die Veränderung der Atranorinkonzentration in Laubflechten durch Licht, Wasserversorgung und SO_2 Begasung. *Bryologist* **88**: 171–174.

McCarthy, P.M. 2003. *Catalogue of Australian Lichens. Flora of Australia*. Supplementary Series No. 19. Australian Biological Resources Study, Canberra.

Lutzoni, F., Pagel, M., and Reeb, V. 2001. Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* **411**: 937–940.

Molina, M.C., Crespo, A., Vicente, C., and Elix, J.A. 2003. Differences in the composition of phenolics and fatty acids of cultured mycobionts and thallus of *Physconia distorta*. *Plant Physiology and Biochemistry* **41**: 175–180.

Sanders, W.S. 2001. Lichens: The interface between mycology and plant morphology. *Bioscience* **51**: 1025–1035.

Stenroos, S., Stocker-Wörgötter, E., Yoshimura, I., Myllis, L., Thell, A., and Hyvonen, J. 2003. Culture experiments and DNA sequence data confirm the identity of *Lobaria* photomorphs. *Canadian Journal of Botany* **81**: 232–247.

Stocker-Wörgötter, E. 2002a. Resynthesis of photosymbiodemes. In: *Protocols in Lichenology*. Springer Lab Manual. Kranner, I., Beckett, R., and Varma, A., eds. Springer Verlag, Heidelberg, New York, pp. 47–60.

Stocker-Wörgötter, E. 2002b. Analysis of secondary compounds in cultured lichen mycobionts. In: *Protocols in Lichenology*. Springer Lab Manual. Kranner, I., Beckett, R., and Varma, A., eds. Springer Verlag, Heidelberg, New York, pp. 296–306.

Stocker-Wörgötter, E. 1997. Investigation on the photobiont and resynthesis of the tropical lichen *Coenogonium leprieurii* (Mont.) Nvel. from the NE coast of Brazil in culture. *Symbiosis* **23**: 117–124.

Stocker-Wörgötter, E. and Elix, J.A. 2002. Secondary chemistry of cultured mycobionts: formation of a complete chemosyndrome by the lichen fungus of *Lobaria spathulata*. *Lichenologist* **34**: 351–359.

Stocker-Wörgötter, E. and Elix, J.A. 2004. Experimental studies of lichenized fungi: Formation of rare depsides and dibenzofurans by the cultured mycobiont of *Bunodophoron patagonicum* (Sphaerophoraceae, lichenised Ascomycota). *Bibliotheca Lichenologica* **88**: 659–669.

Stocker-Wörgötter, E. 2005. Approaches to a biotechnology of lichen forming fungi: Induction of polyketide pathways and the formation of chemosyndromes in axenically cultured mycobionts. *Recent Research in Developments of Phytochemistry* **9**: 115-131.

Turner, G. 2000. Exploitation of fungal secondary metabolites old and new. *Microbiology Today* **27**: 118–120.

Werner, D. 1987. *Pflanzliche und mikrobielle Symbiosen*. Georg Thieme Verlag, Stuttgart.

Werner, D. 1992. *Symbiosis of Plants and Microbes*. Chapman and Hall, London.

Yamamoto, Y., Mizuguchi, R., Takayama, S., and Yamada, Y. 1987. Effects of culture conditions on the growth of Usneaceae lichen tissue cultures. *Plant Cell Physiology* **28**: 1421–1426.

Yamamoto, Y. 1990. Studies of cell aggregates and the production of natural pigments in plant cell culture. Nippon Paint Publications, Osaka, 119 pp.

Yamamoto, Y. 1998. Screening of biological activities and isolation of biological active compounds from lichens. *Recent Research in Developments of Phytochemistry* **2**: 23–34.

Zocher, B. and Stocker-Wörgötter, E. 2005. Secondary chemistry of lichen forming ascomycetes: culture experiments with isolates of *Xanthoparmelia* species from Australia. *Folia Cryptogamia Estonica* **41**: 123–134.