Symbiosis, 38 (2005) 187–201
Balaban, Philadelphia/Rehovot

Culture of Lichen Fungi for Future Production of Biologically Active Compounds

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Received November 26, 2003; Accepted May 20, 2004

Abstract

To obtain large quantities of biologically active lichen substances, high proportions of lichen material are required; naturally grown thalli could be replaced by cultures of aposymbiotically and axenically grown mycobionts. In the present study, the mycobiont of *Xanthoria elegans* (Link) Th. Fr. has been cultured (LB-Medium) and genetically identified by ITS1- and ITS2-sequencing. The cultured mycobiont repeatedly produced typical lichen substances and pigments which partially were also released into the medium. In a second test series, the mycobiont (LB-Medium) and the algal partner (BB-Medium) of the crustose, saxicolous lichen *Lecanora rupicola* (L.) Zahlbr. were isolated and cultured. In this case, it was shown that the production of some of the typical secondary metabolites (e.g. depsides, chromones and xanthones) by the mycobiont was triggered by the presence of the algal partner in a resynthesis experiment.

Keywords: Teloschistaceae, *Xanthoria elegans* (Link) Th. Fr., *Lecanora rupicola* (L.) Zahlbr., mycobiont culture, resynthesis, HPLC-analysis, triggering of secondary metabolite production

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1. Introduction

The culture of lichen mycobionts is an essential tool for investigating the biosynthetic pathways of lichen substances as well as the conditions that are necessary for their production. A considerable number of lichens form substances that exhibit cytotoxic activity against cancer cell lines, such as extracts from *Xanthoria elegans* (Link) Th. Fr. (Ingolfsdottir et al., 2000) or also show antibacterial properties. To date, many species have proved to be a source of important secondary metabolites for food and pharmaceutical industries (e.g. Huneck, 1999). Lichens and lichen products have been used in traditional medicine for centuries and are targets for studies aimed at finding novel natural products with pharmacologically active properties (Ingolfsdottir et al., 1998; Kumar and Muller, 1999; González-Tejero et al., 1995; Agelet and Vallès, 2001). Many lichen substances were found to exhibit antiviral, antibiotic and antitumor activity (Yamamoto, 1990). Under natural conditions lichens are thought to produce most of these compounds as insect-feeding deterrents (Ingolfsdottir et al., 1985; Emmerich et al., 1993), UV-protectants (Fahselt, 1994) and some of them seem to play a role in cell-to-cell communication and signalling (Armaleo, 2000).

Although hitherto attempts to produce lichen polyketides in prokaryotic or eukaryotic host-organisms have proved unsuccessful, genetic engineering could be used to clone lichen polyketide pathway-participating enzymes by means of surrogate hosts in order to produce high yields of polyketides for industrial use as a future alternative to lichen cultures. A precondition for producing polyketides in surrogate hosts is the heterologous expression of the functional polyketide synthases (PKS). However, genes for fungal PKS are very large DNA sequences of usually 6–8 kb (Miao et al., 2001), that often contain intron sequences. Therefore, cloning is very difficult and especially the expression of the protein (PKS) with its original function. For an economic exploitation of lichen substances huge collections of naturally grown thalli are necessary. To prevent the decline of lichen populations in protected areas the only conceivable alternative are bulk quantities of cultured lichens or lichen mycobionts.

A very effective culture method to overcome the slow growth rates of spore-derived lichen mycobionts is the Yamamoto method for culturing lichen fragments (Yamamoto, 1990, 1998). By improving the culture conditions and extending the variety of applied nutrient media, this method allows the separate culturing of mycobionts and photobionts with accelerated growth rates. Axenic cultures are needed for genetic and chemical studies, since natural lichen thalli are often contaminated and colonized by other organisms such as herbivorous invertebrates, lichenicolous and parasitic fungi, bacteria and epiphytic algae.

The objective of this study was to find optimal culture conditions for the foliose *Xanthoria elegans* (Link) Th. Fr. and the crustose *Lecanora rupicola* (L.) Zahlbr. that allow biosynthesis of secondary metabolites in culture. To trigger the *Lecanora rupicola*-mycobiont to form its typical lichen substances it was resynthesized with its appropriate algal partner.

2. Material and Methods

Lichens

Xanthoria elegans (Link) Th. Fr. (Herbarium: Georg Brunauer 10; University of Salzburg) was collected 20 km from Akureyi, northern Iceland, in September 2002. *Lecanora rupicola* (L.) Zahlbr. (J. Blaha and A. Stallinger 398, GZU; Herbarium, University of Graz) was collected from a siliceous rock at Steirisches Randgebirge, Rennfeld, 500 m south of Pischkalm, Mötschmoaralm, Styria, Austria, N 47°23'34" and E 15°20'34" at an altitude of 1,100 m in May 2002.

Culture media

The mycobiont of *Xanthoria elegans* (Link) Th. Fr. was cultured on Sabouraud-2%-glucose-agar and Lilly & Barnett Medium (Stocker-Wörgötter, 2001b) and for *Lecanora rupicola* (L.) Zahlbr. it was isolated using modified Lilly & Barnett-Medium (Stocker-Wörgötter, 2001b). The photobionts of both lichens were cultivated on Bold's Basal Medium (Deason and Bold, 1960).

Mycobiont isolations

The isolations were undertaken by means of the Yamamoto-method (Yamamoto, 1990, modified). Mycobiont isolates were grown on Lilly & Barnett Medium (LBM) and Sabouraud-2%-glucose-agar (S2%). Small pieces of the thallus were cleaned macroscopically by using forceps and then washed very carefully in sterile double distilled water to which a drop of Tween 80 had been added (Bubrick and Galun, 1986). After a washing procedure of 30 minutes, the thallus fragments were repeatedly rinsed with sterile double distilled water that was changed for several times. The clean pieces were then homogenized in sterile water (1–3 ml), using an autoclaved mortar and a pestle. The resulting suspension contained many small pieces of the cortex, medulla and the algal layer. Finally the suspension was filtered through two sieves of different mesh sizes, 500 µm and 150 µm. Pieces of about 150 µm diameter were picked up under a dissecting microscope with a sterile inoculation needle and/or with sterilized

bamboo sticks and used for inoculation (Yamamoto, 1990). Each tube, containing slanted agar (LBM + 4% glucose) were inoculated with only one fragment.

The original Yamamoto-method used only malt-yeast-extract-medium for all mycobiont isolations. As an alternative we adopted various combinations of nutrient media like Lilly & Barnett Medium (LBM) and Sabouraud-2%-glucose-agar (S2%) as mentioned above. Mycobionts that formed mycelia were subcultured. To prevent algal growth in the mycobiont cultures, the tubes were kept in complete darkness (wrapped in aluminium foil) for 2–3 months. Generally, the isolations were kept in a growth chamber at 20°C, which was regarded as the mean temperature in the natural environment during the summer season. The photobiont isolations obtained from the thallus fragments containing photobionts were transferred to slot agar tubes. For the photobiont isolations Bold's Basal Medium (BBM) was used. The composition of this medium favours algal growth. Photobiont isolations were kept under a light dark regime of 14:10 hours and a light intensity of 50–100 $\mu E\ m^{-2}\ s^{-1}$.

Subculturing

Fungal colonies were homogenized with sterile double distilled water in a sterile mortar and then transferred as a suspension with a pipette to four different nutrient media MS, LBM, S2% and MY (Stocker-Wörgötter, 2001b) in petri dishes with divisions (110 × 15 mm). Afterwards, the culture dishes were sealed with parafilm and kept in a culture chamber. The photobionts were subcultured in petri dishes (110 × 15 mm) containing BBM.

Resynthesis

Lecanora rupicola (L.) Zahlbr. was resynthesized from its symbiotic partners. The mycelia obtained from a subculture were homogenized. Hyphal isolates and algal colonies were mixed and then inoculated onto agar plates (LBM, MY and S2%). A resynthesis of *Xanthoria elegans* (Link) Th. Fr. was performed by a different method because in the method described above, the fast growing algae overgrew the mycobiont and no resynthesis stage was achieved: To circumvent this, the photobiont was suspended in sterile water and then inoculated onto an already growing mycelium of *Xanthoria elegans* (Link) Th. Fr. This resynthesis culture was incubated for 1 month at the same conditions as described for the subcultures. Finally, the chemical profile was examined by HPLC and TLC.

DNA analysis

DNA-extraction: Total DNA was isolated from cultured fungi (Armaleo and

Clerc, 1995) and the region between the 18S and 28S rDNA was sequenced for comparing it to the voucher specimens to assure that the mycobiont of the lichen was cultivated and not any contaminant.

DNA-amplification and purification: ITS 1 and ITS 2-regions, the 5.8S gene and the flanking parts of the 18S and 28S rDNA were amplified using a Gene Amp PCR System thermal cycler. For this PCR, the primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) were used. 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 purified using a Quiaquick PCR product purification kit and resolved in sterile distilled water.

Sequencing of ITS 1 and ITS 2: The purified PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing Kit with AmpliTaq® DNA Polymerase in accordance to the supplier's protocol. The analyses were done by an ABI 310 automated sequencer using again the primers ITS1-F and ITS4.

Microcrystallization-test

To see if cultured fungi produce secondary metabolites under culture conditions, microcrystallization-tests were made routinely, which can also be performed with very low quantities of cultured mycobionts. The mycobiont culture of *Lecanora rupicola* (L.) Zahlbr. was first identified by using this microcrystallization-test because the crystals of the culture and the voucher specimen showed an identical shape (Asahina, 1940; Huneck and Yoshimura, 1996). Microcrystallization-tests can only be applied, if the crystals of the lichen substances are very characteristic. This method can be used very efficiently as a screening tool for minor quantities. In our study, lichen substances from the original thallus of *Lecanora rupicola* (L.) Zahlbr. and its cultured mycobiont were extracted with acetone and recrystallized with glycerine-acetic acid (GA). The cultured mycobiont was freeze-dryed to ensure a better extraction by acetone.

Chromatographic analysis

The lichen substances of the thallus were compared to the previously verified mycobiont by means of High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC). Dry lichen thalli and freeze-dried mycobionts (removed from the medium; 1 cm^2 in diameter) were extracted with methanol for HPLC analysis or with acetone for TLC. Standardized TLC (Culberson and Ammann, 1979; Culberson and Johnson, 1982) was used to identify the complete chemical profile of the cultured mycobiont and resynthesis stages.

Figure 1. *Xanthoria elegans* (Link) Th. Fr. in its natural environment.

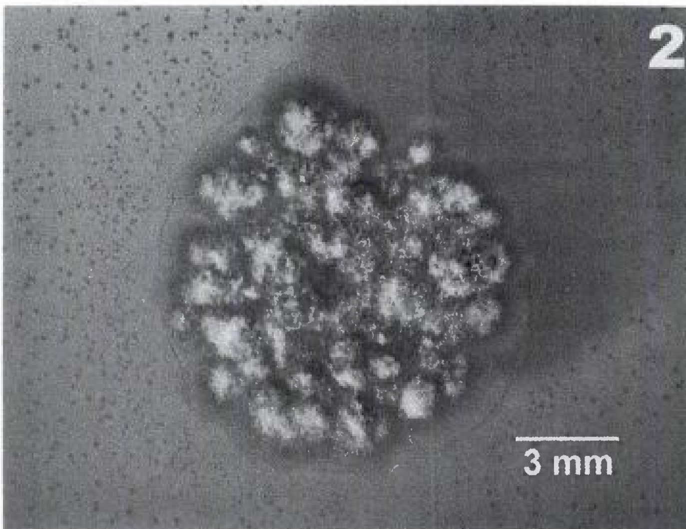


Figure 2. Mycobiont culture of *Xanthoria elegans* (Link) Th. Fr. on LBM surrounded by crystals of lichen substances.

HPLC analysis (Stocker-Wörgötter and Elix, 2002) 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

Figure 3. *Lecanora rupicola*. Lichen on silicous rock.

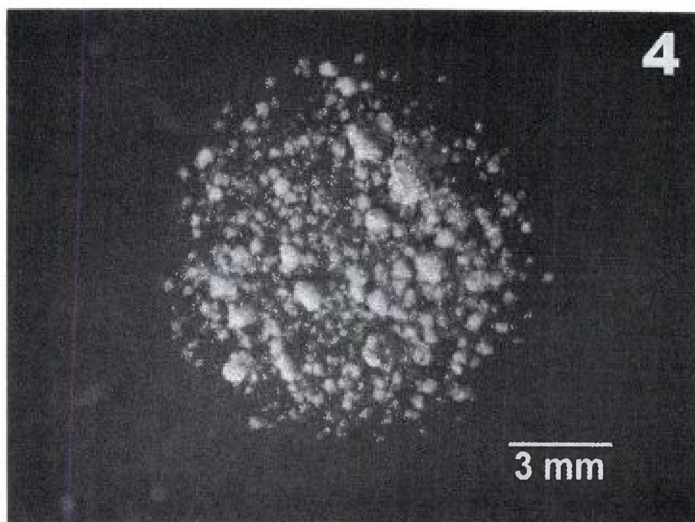


Figure 4. Resynthesized *Lecanora rupicola* growing on LB-Medium.

integration package based on Windows NT. The column used was a Beckman 5C18 (4.6 × 250 mm, 5 μm), flow rate of 1 ml/min. 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. 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 atranorin the correlation of the ultraviolet spectra of the authentic and natural material was greater than 99.9%.

3. Results

Identification of the cultured mycobionts and their parent thalli

Xanthoria elegans (Link) Th. Fr. (Fig. 1) and *Lecanora rupicola* (L.) Zahlbr. (Fig. 3) were identified by anatomy of vegetative thalli and mature spores (Wirth, 1995). The identity of the cultured mycobiont of *Xanthoria elegans* (Link) Th. Fr. (Fig. 2) was confirmed by sequencing the ITS 1 and ITS 2 of its rDNA and an alignment against sequence entries at the NCBI homepage by the free accessible Blast program.

The *Lecanora rupicola* (L.) Zahlbr. mycobiont was chemically identified by comparing the crystals of the secondary compounds in a microcrystallization-test of the voucher specimen and the cultured mycobionts. The obtained HPLC chromatogram of the *Lecanora rupicola* (L.) Zahlbr. mycobiont showed some very small peaks (Fig. 9) but the retention times are not concordant with them in the chromatogram of its voucher specimen. These substances are probably fatty acids as they do not show polyketide characteristic UV-spectra. Due to the fact that during the microcrystallization the extracted lichen substances get highly concentrated on a spot of about 1 cm of diameter, it was possible to get some crystals even for the cultured mycobiont.

The resulting very typical crystals of the lichen substances were found to be identical to some crystals of its voucher specimen and provided a first evidence of the analyzed fungus to be the true mycobiont of *Lecanora rupicola* (L.) Zahlbr. and that the mycobiont indeed produces lichen substances, but in extremely small amounts.

Resynthesis of the cultured mycobiont with its algal partner

The mycobiont of *Lecanora rupicol*a (L.) Zahlbr. exhibited a very weak growth on LBM and no visible growth on S2% and other tested media. In contrast, the resynthesized thallus (Fig. 4) showed a very strong growth on LBM and was therefore used for the further chemical analysis by HPLC.

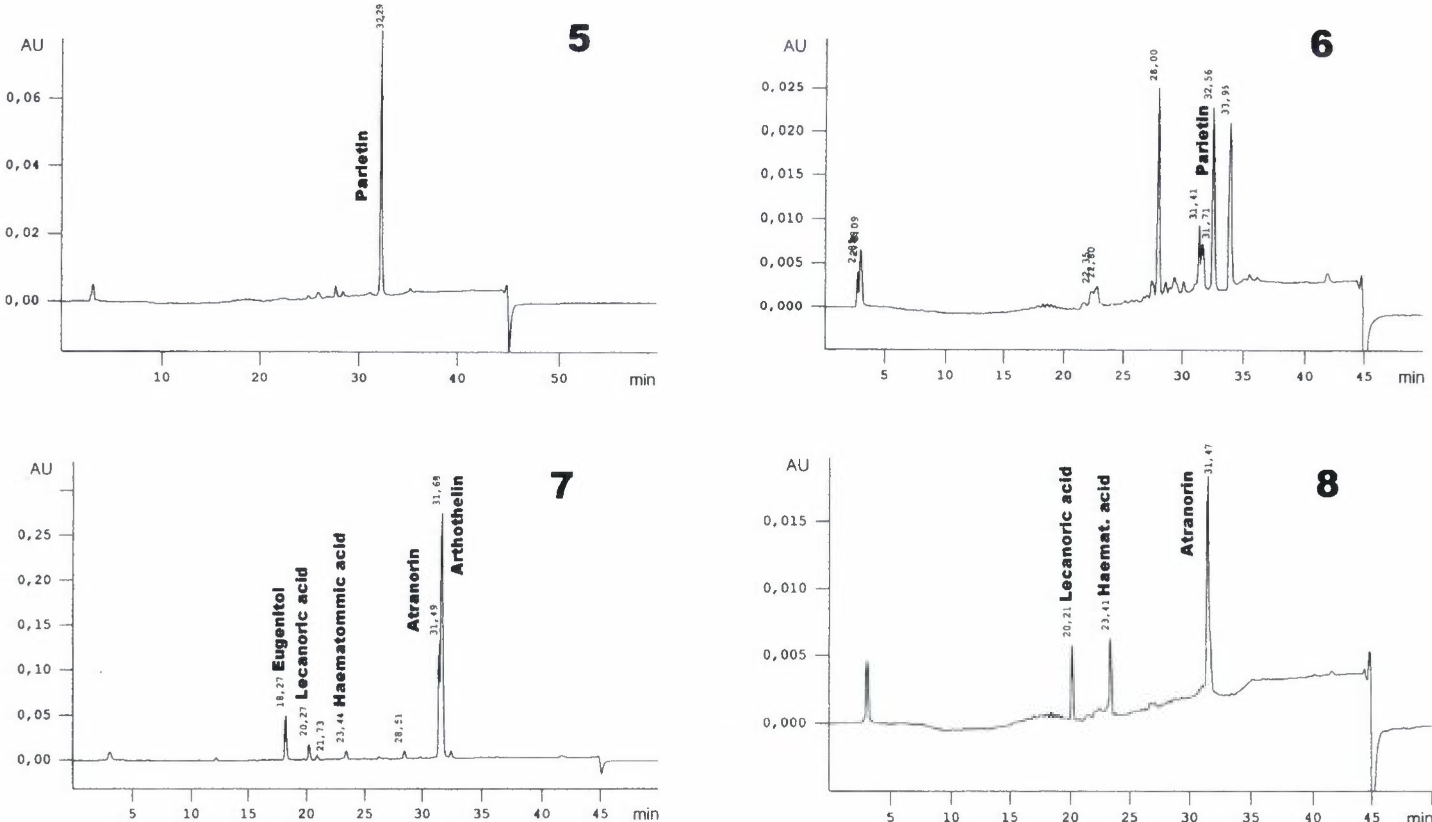
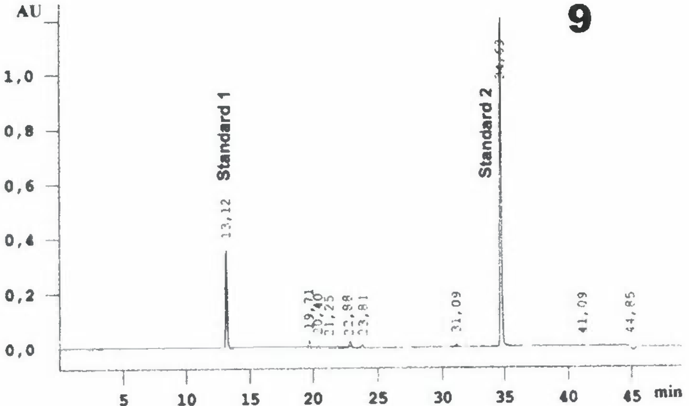


Figure 5. HPLC chromatogram of *Xanthoria elegans* (Link) Th. Fr. lichen. Showing a single peak of parietin. Figure 6. HPLC chromatogram of *Xanthoria elegans* (Link) Th. Fr. mycobont showing parietin production and additional secondary metabolites. Figure 7. Chromatogram of *Lecanora rupicola* lichen. Figure 8. HPLC analysis of resynthesized *Lecanora rupicola* (L.) Zahlbr. under culture conditions.



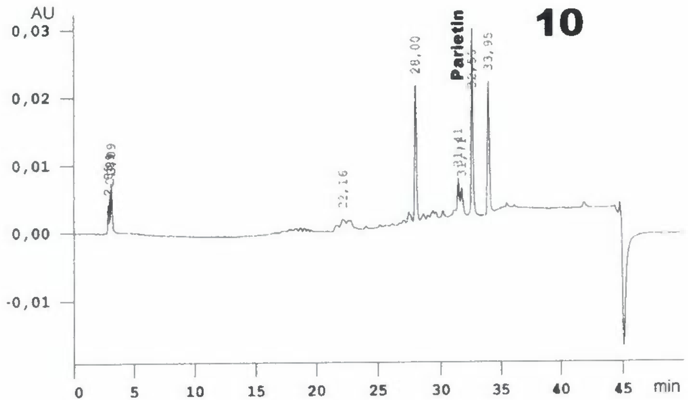
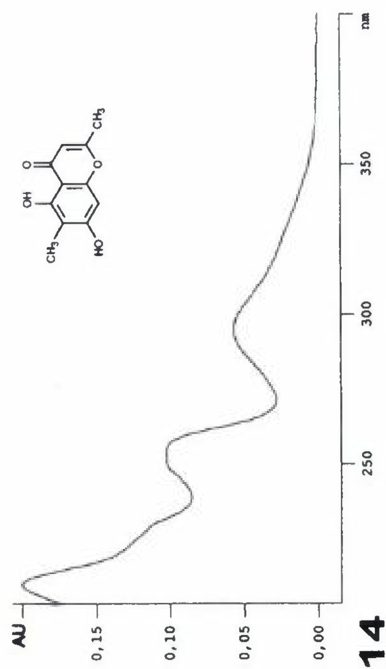
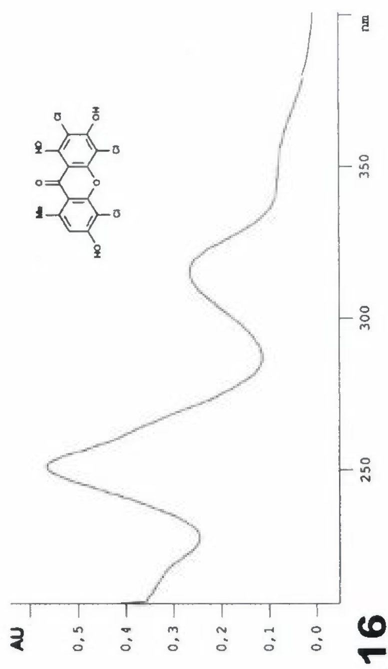
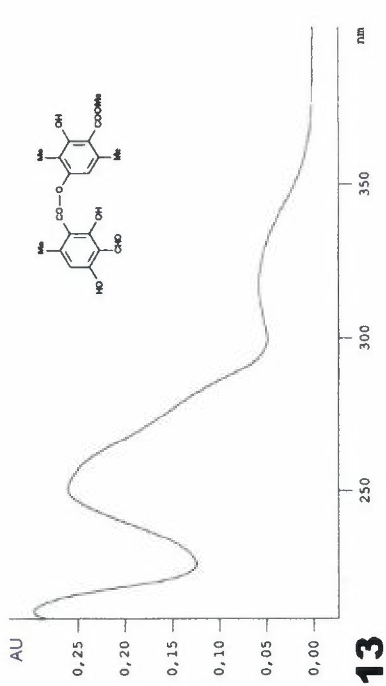


Figure 9. HPLC analysis of aposymbiontically grown *Lecanora rupicola* (L.) Zahlbr. mycobiont. Figure 10. HPLC analysis of resynthesized *Xanthoria elegans* (Link) Th. Fr. under culture conditions. Figure 11. UV absorption spectrum of parietin derived from *Xanthoria elegans* (Link) Th. Fr. Figure 12. UV absorption spectrum of lecanoric acid.







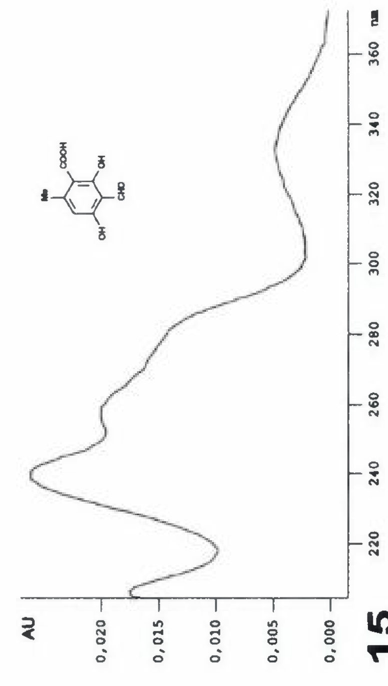


Figure 13. UV absorption spectrum of atranorin. Figure 14. UV absorption spectrum of eugenitol. Figure 15. UV absorption spectrum of haematommic acid. Figure 16. Spectrum of arthothelin, the major component of this specimen of *Lecanora rupicola* (L.) Zahlbr.

HPLC analyses of the secondary metabolite pattern

The microcrystallization-test of the *Lecanora rupicola* (L.) Zahlbr. fungal partner has already revealed that the mycobiont is able to form very small amounts of secondary compounds and the TLC analysis of the *Xanthoria elegans* (Link) Th. Fr. mycobiont indicated the production of parietin. HPLC of *X. elegans* (Link) Th. Fr. revealed a more complex chemical signature: Although both, the voucher specimen and the mycobiont of *X. elegans* (Link) Th. Fr. produce parietin (Fig. 11), the pattern of satellite substances differed considerably. The chromatogram of the cultured mycobiont of *X. elegans* (Link) Th. Fr. showed peaks of unknown substances, which have not been detected in the natural lichen thallus (Figs. 5 and 6). In the further test series it was shown that the algal partner had hardly any influence on the production of secondary compounds by the mycobiont of *X. elegans* (Link) Th. Fr. (Fig. 10), whereas the chromatogram of the *Lecanora rupicola* (L.) Zahlbr. resynthesis starts to produce lichen substances in higher amounts. To screen alterations in the chemical pattern, the resynthesis stages of *X. elegans* (Link) Th. Fr. were also tested by HPLC. Surprisingly the early fungal-algal association showed nearly the same chemical profile as the aposymbiotically grown mycobiont.

The chemical profiles of the *L. rupicola* (L.) Zahlbr. resynthesis cultures and the original thallus did not deviate very much from each other (Figs. 7 and 8). Both chromatograms show peaks of lecanoric acid (Fig. 12), atranorin (Fig. 13) and a trace of haematommic acid (Fig. 15), which could also be a degradation product of atranorin, generated during extraction in methanol. The voucher specimen produces two more lichen substances, the chromone Eugenitol (Fig. 14) and the xanthone arthothelin (Fig. 16), that could not be recognized in the cultures. In contrast, the aposymbiotically (the same medium as for the resynthesis) grown mycobiont of *L. rupicola* (L.) Zahlbr., on LBM, did not produce any detectable lichen substances.

4. Discussion

Xanthoria elegans (Link) Th. Fr. has a high physiological and genetic diversity and its metabolism also seems to be adapted to regional climatic conditions (Murtagh et al., 2002). Furthermore, Ingolfsdottir et al. (2000) reported that extracts of naturally grown *X. elegans* (Link) Th. Fr. thalli showed significant cytotoxic properties against various cancer cell lines. This very common lichen is a very interesting object to study chemical pathways in lichens and fungi, because parietin is an anthraquinon-pigment produced by polyketide synthases that can be easily recognized by its bright orange color and studied during its step by step production.

Comparing the chemical pattern of the parent lichen and the cultured mycobiont grown under variable culture conditions provided some interesting results. HPLC analyses of cultured *X. elegans* (Link) Th. Fr.-mycobionts demonstrated that the standard culture condition promoted the production of fungal substances (e.g. anthraquinones like parietin) but also yet unidentifiable compounds that may be accumulated precursors or degradation products of polyketides as a consequence of an altered biosynthesis pathway.

Likewise Adler et al. (2003) identified substances that were produced by the mycobiont of *Punctelia subpraesignis* under stable culture conditions as fatty acids (polyketide related biosynthesis pathway) and triglycerides. The production of these substances was the result of an also altered metabolism of the mycobiont under culture conditions.

On the other hand, the aposymbiontically grown *Lecanora rupicola* (L.) Zahlbr. mycobiont exhibited an extremely slow growth and did not produce lichen substances in quantities identifiable by HPLC. Only after resynthesis typical substances, such as atranorin and lecanoric acid, were produced in larger amounts. Other metabolites that appeared in the HPLC-chromatogram of the voucher specimen, such as eugenitol and arthothelin, were not present in the resynthesized thallus.

Referring to our recent knowledge, in this case, the biosynthetic pathways of the three lichen substances, shown in the HPLC-chromatogram (Fig. 8) of the resynthesis of *L. rupicola* (L.) Zahlbr., were probably triggered by the presence of the algae. From a biotechnological point of view, atranorin and lecanoric acid are potentially important for industrial production; for example atranorin shows antinociceptive activity (inhibiting the pain transmission-process) (Maia et al., 2002) and lecanoric acid exhibits growth inhibitory effects on lettuce seedlings (Nishitoba et al., 1987) and also influences mitosis (cytostatic effects) in roots of *Allium cepa* (Sucharita et al., 1983). Thus, triggering secondary metabolism in cultures of *L. rupicola* (L.) Zahlbr. to enhance production of its lichen substances may have practical applications.

Further experiments to enhance secondary metabolism of mycobiont cultures involve exposure to stress factors, such as osmotic stress, temperature shifts and various light intensities. Increased UV-radiation on mycobionts, in general, has already been found (e.g. Honegger and Kutasi, 1990; Solhaug et al., 2003) to induce anthraquinone formation.

Acknowledgements

We would like to thank Prof. Olafur Andrésson (Institute for Experimental Pathology, University of Iceland, Reijkjavik) for participation in the Project "Euketides" No. QLK3-CT-2002-01940. Julia Blaha (Institute of Botany,

University of Graz, Austria) who kindly provided *Lecanora rupicola* (L.) Zahlbr., Martin Grube (Institute of Botany, University of Graz) for help with the DNA analyses and to Prof. J.A. Elix (Department of Chemistry, Australian National University, Canberra) for his support in the chemical analyses. We greatly appreciate the help of colleagues Alfredo Passo, Alexandra Uhl, Armin Hager and Bettina Zocher. The culturing and HPLC-analyses were generously supported by the Austrian Science Foundation Project No. 15378-B06.

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