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Experimental studies of lichen-forming fungi: formation of depsidones and shikimic-acid derivatives by the cultured mycobionts of three selected species of *Rhizocarpon* (Lecideaceae, lichenized Ascomycota)

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Abstract: Three species of *Rhizocarpon*, *R. oederi*, *R. lecanorinum* and *R. umbilicatum* proved to be fascinating model systems for microbiological and chemical studies. In culture experiments on nutrient rich media, particularly on Murashige Skoog Medium, it was shown that aposymbiotically grown *Rhizocarpon* mycobionts can achieve almost similar growth rates as mycobionts isolated from foliose and fruticose lichens. Furthermore, with high sugar contents in the substrates, the mycobionts did not produce any secondary metabolites. Depsidones (medullary compounds) such as stictic and psoromic acids in two different chemotypes of *R. lecanorinum* were only biosynthesised by mycobionts that were grown on carbohydrate deficient media containing minerals and trace elements in double concentration. Low temperature treatments and stress by drought clearly supported the production of the depsidones. The cultured *Rhizocarpon* fungi (*R. lecanorinum*, *R. umbilicatum*) showed a preference for pH values that are also dominant in the natural habitats of the lichens. In several mycobiont cultures that were exposed for a longer period (4 weeks) to whole spectrum light from a Vitalite fluorescent lamp, rhizocarpic acid and pulvinic acid dilactone were produced. Short-term exposure to UVB irradiation did not have any inductive influence on the production of lichen pigments. Shikimic acid derivatives such as rhizocarpic acid and pulvinic acid dilactone were formed and "expressed" for the first time by aposymbiotically grown *Rhizocarpon* fungi in the absence of algae. The availability of metabolite-producing, axenically grown mycobionts could be a milestone in future investigations of genes that are controlling essential enzymes in shikimic-acid pathways.

Introduction

Recent advances in modern microbiology have afforded considerable progress in experimental lichenology (e.g. AHMADJIAN 1993; YAMAMOTO et al. 1993, YAMAMOTO 1998; BEHERA & MAKHIJA 2001; BEHERA et al. 2009; ADLER et al. 2004; STOCKER-WÖRGÖTTER & ELIX 2002, 2004, 2006; STOCKER-WÖRGÖTTER 2008). Over the past 20 years, culture techniques for

growing mycobionts and photobionts under aposymbiotic and axenic conditions have improved, using modern airflow, clean work benches and electronically adjustable culture chambers.

In the natural environment, saxicolous crustose species such as the *Rhizocarpon geographicum* agg. grow exceptionally slow and are thereby difficult subjects for laboratory manipulations; until recently, species of the genus *Rhizocarpon* have resisted culturing and experimental approaches in the laboratory to elucidate details of ontogenetic processes. However, thallus initiation and development in *R. lecanorinum*, under natural conditions in the field, was reported by CLAYDEN (1998) by combining sets of continuous microscopical observations on the sequence of ascospore germination, mycelial growth, and thallus initiation and differentiation.

Interestingly, the slow growth and longevity of species of the *R. geographicum* agg. proved to be valuable tools for lichenometric studies, the radial growth rate of the thallus (RGR) ranging from 0.02 to 2.0 mm yr^{-1} (HALE 1983; ARMSTRONG 1983; INNES 1985; MATTHEWS 1994; ARMSTRONG 2005). The lichenometric method is often applied by geoscientists to determine the age of moraine deposits of glaciers (e.g. ARMSTRONG 2005; PROCTOR 1983; BRADWELL & ARMSTRONG 2007).

For the present investigation, *R. lecanorinum*, *R. umbilicatum* and *R. oederi* were selected. *R. lecanorinum*, like *R. geographicum*, is a yellow-green species of the *R. geographicum* agg. It is occasionally found in submontane regions, but more commonly in the high mountains, where it grows on siliceous rocks. The thalli of *R. lecanorinum* are areolate, the merging of numerous thalli often forming an extensive cover on rock surfaces. The discrete yellow-green areolae, as for *R. geographicum*, are located on a black prothallus or hypothallus composed of darkly melanised fungal hyphae. The hypothallus extends beyond the areolae to form a marginal ring of varying width (1–2 mm). In the field, typical *R. lecanorinum* with its crescent and orbicular shaped areolae, each segment often partly or entirely surrounding an apothecium, can be easily distinguished from *R. geographicum* s.str., which typically forms angular areoles. Samples of *R. lecanorinum* can differ in their secondary chemistry, the two chemotypes investigated in our present study forming respectively stictic acid and psoromic acid as typical medullary compounds. The psoromic acid chemotype was named as *R. ferax* by MAGNUSSON (1948) and as *R. drepanodes* by FEUERER (1978). The traditional concept of subspecies divisions of the *R. geographicum* agg. could not be confirmed through molecular and phylogenetic studies, because most specimens of the widely distributed and diverse species of this group form a single clade in the phylogenetic tree of on-going studies. For this reason, it was necessary to reject the former hypothesis that a chemical race, e.g. the psoromic acid chemotype of *R. lecanorinum* represents a different taxon or subspecies.

The common yellow-green cortical pigment in all samples of *R. lecanorinum* is rhizocarpic acid, which has been chemically classified as a shikimic and pulvinic acid derivative.

R. umbilicatum differs from the yellow-green *Rhizocarpon* species, as it does not contain rhizocarpic acid in the cortex. It has a chalky white thallus,

which houses the black and mainly orbicular apothecia. As a typical medullary substance, *R. umbilicatum* produces stictic acid. Deviating from the yellow *Rhizocarpon* species which usually grow on silicious rocks, *R. umbilicatum* has one of its major distributions in the northern calcareous Alps, often covering the tops of limestone outcrops.

R. oederi is an ochraceous to rust brown species of *Rhizocarpon*, forming rimose-cracked to areolate patches on silicious rocks with high contents of iron minerals.

Experimental studies of slow growing crustose lichens, particularly *Rhizocarpon* species, have not received much attention, because of the long time required for each study. The present investigations were aimed at finding nutrient media and appropriate culture conditions for the selected *Rhizocarpon* mycobionts, which differ in a number of morphological, chemical and particularly ecological features. Furthermore our culture studies broadened and deepened our understanding of how slow-growing fungi like species of *Rhizocarpon* perceive and respond to internal and external signals under controlled laboratory conditions in order to ensure changes in their cellular metabolism and in their developmental strategies. Since many environmental signals involved are phenomenological, the most important steps of mycelial growth were elucidated. Mycelial growth is dependent upon nutrient supply and particular ecological parameters, including stress situations, that could also be indicative of secondary metabolite production, as found in several of our former studies (BRUNAUER et al. 2007; ELIX & STOCKER-WÖRGÖTTER 2008; HAGER et al. 2007; STOCKER-WÖRGÖTTER & ELIX 2002, 2004, 2006; STOCKER-WÖRGÖTTER et al. 2004; STOCKER-WÖRGÖTTER 2008, 2009) For this reason, an extended test series was initiated to find culture conditions that would trigger the aposymbiotically grown mycobionts to produce polyketides (polymalonyl derivatives), e.g. unique lichen substances such as the depsidones stictic and psoromic acids.

An additional question that resulted from these on-going experiments focussed on finding appropriate "artifical" ecological parameters that could be successfully adopted to induce the production of rhizocarpic acid in cultured *R. lecanorinum* mycobionts.

Material and Methods

Selected specimens

Rhizocarpon oederi (Weber) Körber (Fig. 1). France, Brittany, Finistère, Comana, in a stone quarry and iron mine, alt. c. 100 m, 28°25'N. 3°55'W, *E. Stocker-Wörgötter* (priv. herb. 12337, Salzburg Univ.)

Rhizocarpon lecanorinum Anders, chemotype 1 (Figs 3, 12–13). Austria, Salzburg, Rauriser Valley, Filzenkar, 47°02'52''N, 13°00'58''E, alt. 1700 m, abundant on siliceous rocks; chemotype 2 (Figs 14–15) Germany, Harz, Brocken, 51°47'51''N, 10°37'11''E, alt. 1100 m, *E. Stocker-Wörgötter* (priv. herb. 12339, Salzburg Univ.)

Rhizocarpon umbilicatum (Ramond) Flagey (Figs 5, 16–17) Germany, Füssen, Tegelberg, 47°33'33''N, 10°46°08''E, alt. 1800 m., *T. Feuerer* (E. Stocker-Wörgötter, priv. herb. 12341, Salzburg Univ.)

The two collections of *R. lecanorinum* differed in their chemical profile. Chemotype 1 contained rhizocarpic acid in the cortex and stictic acid as the typical medullary secondary compound, whereas chemotype 2 also had rhizocarpic acid in the cortex, but psoromic acid instead of stictic acid in the medulla. Stictic acid was confirmed as the major medullary substance in *R. umbilicatum.*

The thalli of *R. oederi* (Fig. 1) were found to be deficient in typical lichen substances; the rust brown thallus pigments are most likely unidentified melanins that are often produced by lichen-forming fungi and other ascomycetous fungi when heavy metals are available in the substrata or when they live in very sun-exposed habitats. Some of the lichen thalli were even found to contain iron particles.

Culturing and culture conditions

Isolation of spores

Species of *Rhizocarpon* form black, lecideine apothecia which contain a large number of colourless, brown, or greenish spores, consisting of 2 or more cells. In most cases the spores are muriform. Although the spores germinate easily, they rarely develop beyond the early germination stages. For this reason, mycobiont isolation from spores was abandoned and the "tissue" (YAMAMOTO et al. 1987, YAMAMOTO 1990) or thallus fragmentation method was preferred.

Isolation of the mycobiont from thallus fragments

The Yamamoto-method (modified, YAMAMOTO 1990) was performed to obtain larger quantities of *Rhizocarpon* mycobionts that would be necessary for subsequently determining the contents of secondary metabolites in the obtained cultures.

Small thallus fragments were dissected from the rock surfaces by the use of tweezers and a scalpel. The obtained thallus pieces were carefully washed in bi-distilled water containing a drop of Tween 80, then homogenized in sterile water with a mortar and pestle. The suspension containing minute thallus particles was filtered through two sieves (500 and 150μm mesh sizes) and pieces with an average size c. 150 µm were picked up under a dissecting microscope using an inoculation needle or bamboo stick. Before transferring the fragments to a nutrient medium, they were examined under higher magnification in order to separate them into particles containing mainly fungal hyphae. Agar slants containing 5–7 ml of an appropriate nutrient medium (for *R. lecanorinum*: Murashige Skoog Medium, 4% glucose 4% mannitol medium, + Murashige Skoog mineral salts, + 18 g agar for 1000 ml of liquid medium, for *R. umbilicatum*: 1,5% Difco-agar (1000 ml), + 2% ribitol, + 0,6 g/l $CaCl_2$, + 5 μg biotin; for *R. oederi*: MS-medium + 4% ribitol + 2% sorbitol + 2% mannitol; + 20ml of 10% $FeCl_3$ solution, for 1000 ml of liquid medium, + 18 g Agar) were inoculated with only one fragment in each tube. The pH values, if necessary, of the media

were adjusted with the aid of a pH meter by adding drops of strong acid (0.1 or 1.0 molar) and base to liquid media that were subsequently sterile filtrated.

The tubes were kept in darkness (covered by aluminium foil) for about 3 months until small fungal colonies had been formed. The fungal colonies were gently homogenized for sub-culturing and plated on nutrient agar in petri-dishes (100 x 20 mm), composed of the same ingredients as described above for the isolations. The isolated mycobionts were kept in a culture chamber with a 14:10 hours, 20°C:10°C day-night regime and a light intensity of 50–100 $\mu E^{-2}s^{-1}$. The contamination rate of the fungal isolates was 20–40%, depending upon the origin of the lichen samples and the sugar contents of the media.

Production of polyketides (depsidones) and pulvinic acid derivatives in the cultures

To induce the production of polyketides such as stictic and psoromic acids, the mycobiont cultures were exposed to a low temperature treatment and to successive desiccation periods. Every 6 weeks the cultures were maintained at -10°C in a freezer for 3 weeks; after each low temperature treatment, the cultures were kept in an exiccator for two weeks. The cold temperature and desiccation treatment was repeated every 4 months for a total time span of one year. Control cultures of the mycobionts were kept in a culture chamber at a stable temperture of 20°C.

Mycobiont cultures of *R. lecanorinum* on nutrient deficient media (BBM Bold's Basal Mineral Agar (STOCKER-WÖRGÖTTER & HAGER 2008) with a double content of trace elements were exposed to permanent light (24h or light dark regimes of 14:10 hours), using a full spectrum Vitalite fluorescent lamp for 4 weeks after an inoculation period of 6 months. Although natural sunlight is known to have much higher UV-B irradiance than most commercial low intensity, full spectrum lamps, the treatment was effective for the mycobionts of *R. lecanorinum* in inducing the production of pulvinic acid derivatives. An additional treatment (5 mycobiont cultures, 6 months old) to UV-B irradiation at 290 nm (289-293 nm, UV absorbance maximum, found for rhizocarpic acid in an UV spectroscopic study, HAUCK et al. 2008) was applied for 20 min every 24 hours for two weeks.

In one case, mycelia of *R. umbilicatum* were extracted in acetone by using a small Shoxlett apparatus to determine the contents of pure substance of stictic acid.

HPLC analyses

Extraction and preparation of the cultured mycobionts

For the chemical analyses, sterile circular plugs of the mycobionts (c. 1.0–1.5 cm diameter) were cut out of the agar plates and freeze-dried at -42°C for at least 12h, using an ice condenser connected to a vacuum pump. The dried discs were then placed in tubes and extracted in methanol for 4 hours; after that the extracts were transferred to HPLC vials and an aliquot of 20 μl from every sample was injected.

Identification of the metabolites

The secondary compounds of the thalli and mycobiont cultures were analysed by high performance liquid chromatography (HPLC) using a Merck-Hitachi system with two pumps, a DAD (photodiode array detector; 190-900 nm wavelength, covering UV and visible spectra) connected to a computer system. Retention time indexes (R_I) were calculated from benzoic acid and solorinic acid controls (ELIX 1996; ELIX et al. 2003; FEIGE et al. 1993). Two solvent systems were used: (A) 1% aqueous orthophosphoric acid, purified water:methanol in the ratio 7:3 and (B) methanol. The run started with 100% A and was raised to 58% B within 15 min, then to 100% B after a further 15 min, followed by isocratic elution in 100% B for a further 10 min. The spectra were identified by means of a spectrum library (comparison with reference substances), and chemical data listed in HUNECK & YOSHIMURA (1996).

Results

In general, all of the isolated spores from the selected *Rhizocarpon* species demonstrated a high rate of germination (c. 80%) on mineral agar. However, after only a few days of incubation on a nutrient-rich substrate such as Murashige Skoog medium, the germ tubes stopped growth completely. The fragment method was more successful, and for this reason was applied for almost all of our following experiments.

In the first test series, the hyphal isolates and mycelia of *R. oederi* were found to grow best on Murashige-Skoog medium with double contents of Murashige Skoog mineral salts, enriched with various carbon sources. A composition of different polyols (as exemplified in the methodology section above), including 20 ml of 10% $FeCl_3$ solution, was found to be particularly favourable for growing this mycobiont. Mycelia with diameters between 10–15 mm were formed after 6–7 months of incubation (Fig. 2).

The aposymbiotically grown mycobionts were screened for contents of lichen substances, similar to that reported for the lichen from the natural site, no typical secondary metabolites were found. The mycelia were composed of brown and reddish-brown hyphae (Fig. 2); interestingly, the colour of the observed mycelia resembled in many respects the colour of the thalli of *R. oederi* from the natural environment. Changes of nutrient media, changes in ecological parameters (lower temperatures and exposure to longer periods of drought) and exposure to full spectrum and selected wavelength of UVB radiation did not have any visible effects on the growth and development of the mycelia. Polyketide production was not detected in any of the test series performed by HPLC analyses of all 20 cultured mycelia.

Both chemotypes of *R. lecanorinum* formed small mycelia (with an overall diameter of 2 mm) after an incubation time of 2 months. The growth rates on the very rich Murashige Skoog medium, containing high quantities of carbon sources (see methods section above) considerably improved growth in the initial stages and also during subsequent months of

cultivation. Our experiments showed that an increase in growth rates can be achieved on agar media with higher nutrient contents. All of the tested *R. lecanorinum* fungi generated mycelia of up to 5–6 mm diameter after 4–6 months of culturing. The *R. lecanorinum* mycobionts under optimized culture conditions were able to achieve the same average mycelia sizes that are accomplished by mycobionts of, for example, foliose and fruticose lichens which are known to grow more rapidly. The growth of *Rhizocarpon* mycobionts was also visibly increased by adding higher contents of mineral salts to the media. Control cultures without mineral salts only generated smaller-sized mycelia with an average diameter of 3–4 mm after 6 months. Cultures without additional supplies of trace minerals grew more slowly. Finally, mycelia grown under mineral deficient conditions ceased growth completely and disintegrated with time.

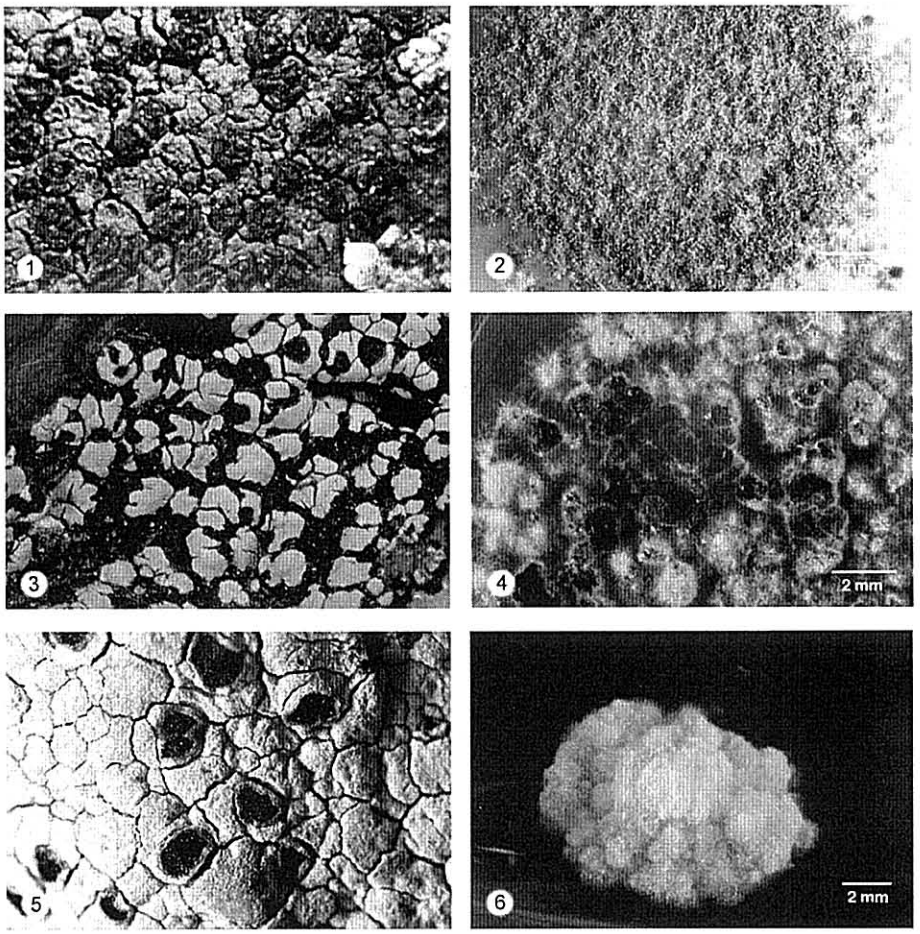
The results obtained in the laboratory clearly indicated that the *Rhizocarpon* fungi are highly adapted to ecological conditions on rock surfaces, where higher mineral contents may be present and accumulate on the uneven rock surfaces in fissures and depressions after every strong rainfall.

Interestingly, the *R. lecanorinum* mycobionts were found to accept a wider range of pH values (slightly acidic, neutral to slightly alkaline) in the nutrient media than the mycelia of *R. umbilicatum* which were found to be adapted to more alkaline substrata (media with pH values of 8-10). The best growth rates of the *R. lecanorinum* mycobionts were observed on Murashige Skoog medium enriched with 4% glucose and 4% mannitol (Fig. 4) and pH values adjusted to between 5 and 7. On such substrata and under such conditions, the mycelia became intensively coloured; at this stage of development the mycobionts were composed of dark brown and also black, strongly melanised hyphae (resembling in many aspects the hypothallus of the intact lichen). After a time period of 6 months, the dark brown and black mycelia stopped horizontal growth and started to form aerial hyphae that gave the surface of the mycelia a velvety appearance. Although these fungal and mycelial colonies were well developed, they did not produce any secondary metabolites.

Depsidones such as stictic acid and psoromic acid were only detected in mycobiont cultures that were grown under nutrient deficient conditions, e.g. on BBM medium with double contents of trace elements.

Mycobiont cultures of *R. lecanorinum* Chemotype 1, grown on mineral agar (Fig. 7) exhibited more moderate growth rates, c. 1 mm/month. By exposing well developed mycelia (6 months old), grown under nutrient deficient conditions, to irradiation from a low intensity full spectrum lamp at frequent intervals for 4 weeks (24h and/or 14h a day) after every month with regular day and night regimes, the cultures started to produce pulvinic acid dilactone and rhizocarpic acid (Figs 7–8). The UV spectrum, typical for pulvinic acid derivatives and rhizocarpic acid is illustrated in Fig. 10. Recrystallised rhizocarpic acid, creating plate-like, prismatic crystals was obtained from cultured mycobionts (Fig. 9).

An extract of the biogenetic pathway that leads to the formation of shikimic acid and pulvinic acid derivatives is shown in Fig. 11, Scheme A and B.



Figs 1–6. 1. *Rhizocarpon oederi*; Britanny, France. **2.** Cultured *R. oederi* mycobiont, 6 months old. **3.** *Rhizocarpon lecanorinum*, chemotype1, from Rauriser Valley, Austria. **4.** *R. lecanorinum* mycobiont, grown on on MS 4% glucose 4% mannitol medium; 4 months, no secondary lichen metabolites found. **5.** *Rhizocarpon umbilicatum*, Tegelberg, Germany. **6.** *Rhizocarpon umbilicatum* mycobiont on 1,5% Difco-agar + 2% ribitol + 0,6 g/l CaCl2 + 5 µg biotin, forming stictic acid, 6 months.

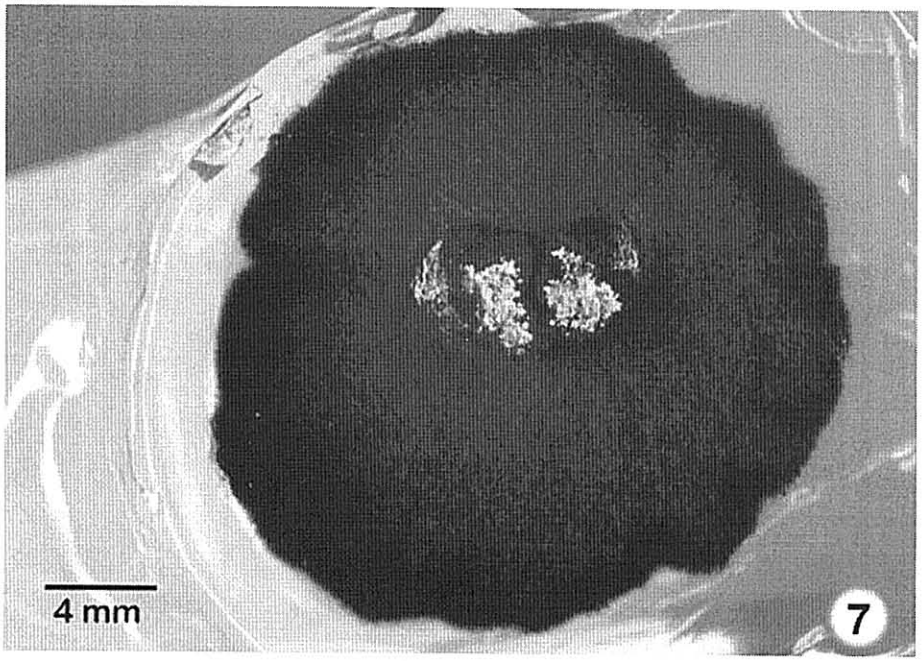


Fig. 7. *Rhizocarpon lecanorinum* culture; aerial hyphae on the top, producing rhizocarpic acid (on BBM-agar with double concentration of trace elements), 7 months.

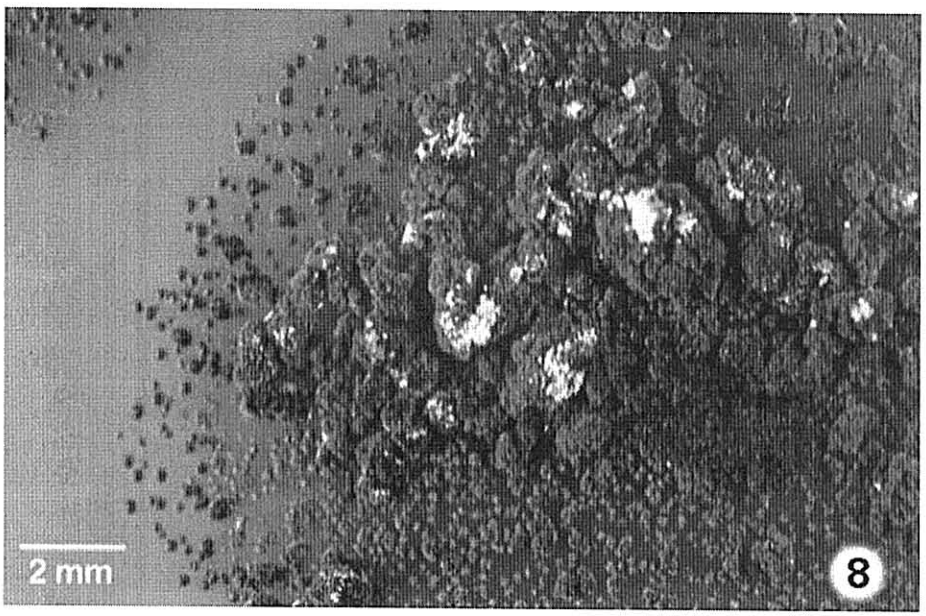


Fig. 8. *R. lecanorinum*-mycobiont produces rhizocarpic acid and a probable precursor after 8 months of incubation.

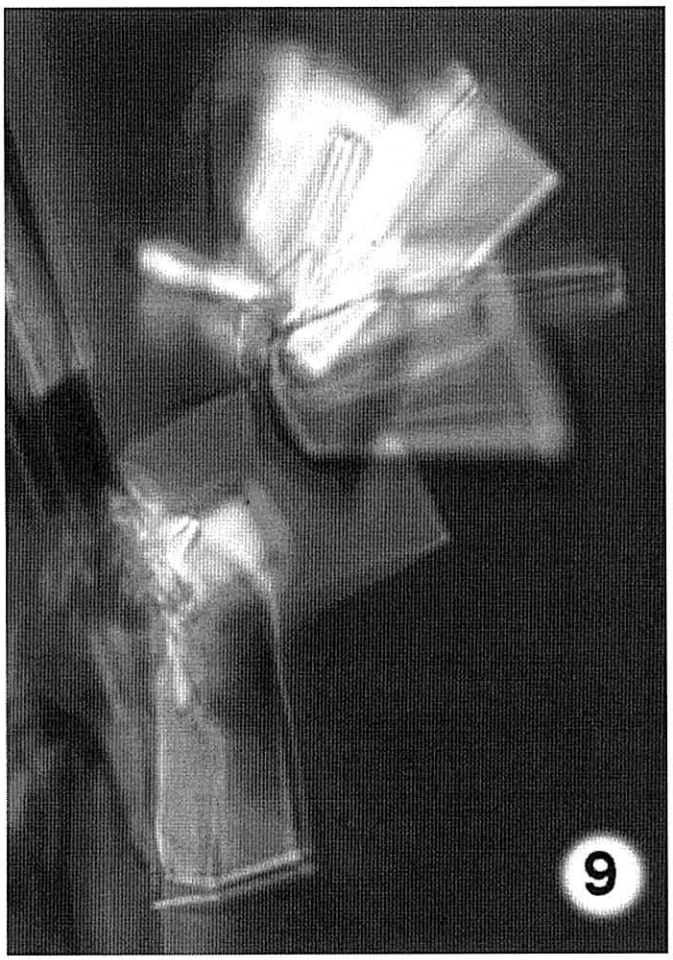


Fig. 9. Re-crystallised rhizocarpic acid from cultured mycobiont (*R. lecanorinum*; chemotype 1).

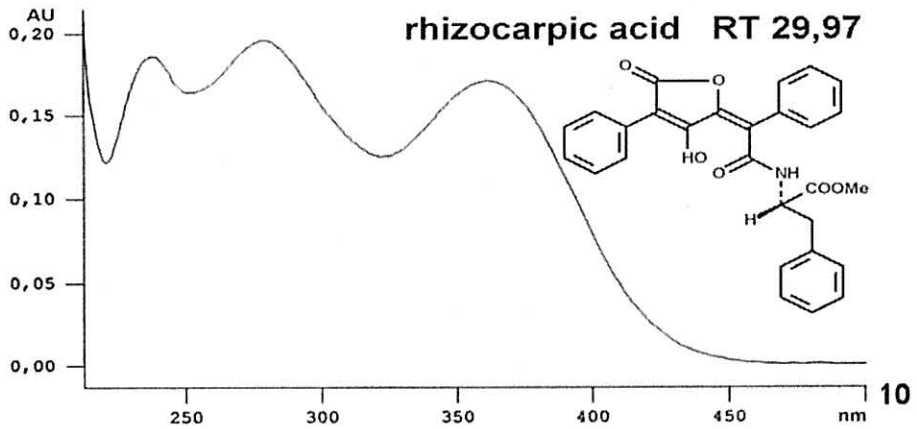
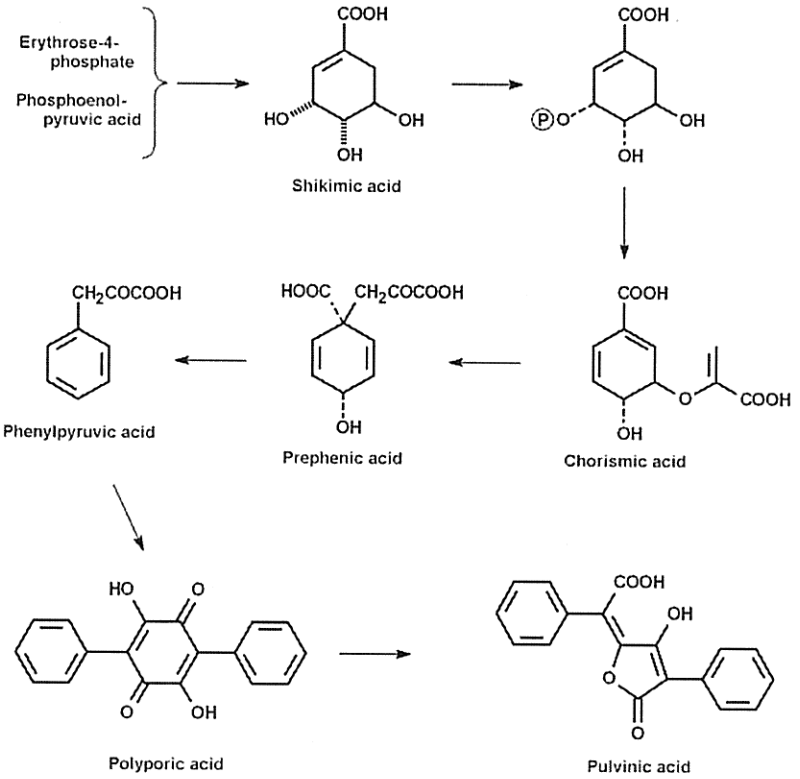
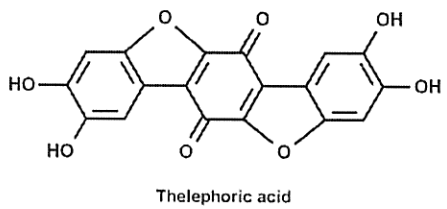


Fig. 10. UV spectrum of rhizocarpic acid.

Scheme A



Scheme B



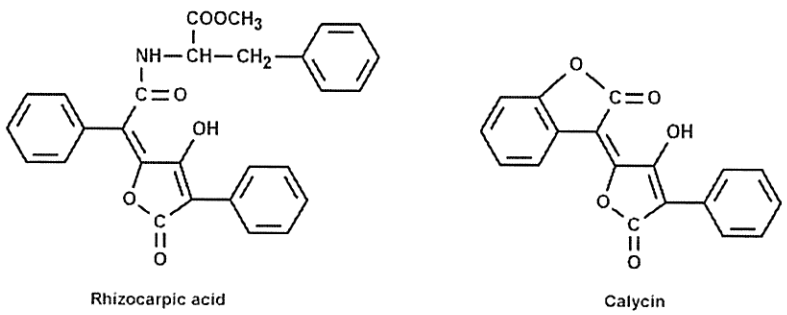


Fig. 11. Scheme A. Extract of biogenetic pathway leading to the biosynthesis of pulvinic acid, and **Scheme B.** Typical pulvinic acid derivatives, like rhizocarpic acid, found in lichens.

In contrast to the cultured lichen fungi that were grown under full spectrum light, mycobiont cultures that were only exposed to UV-B irradiation at 290 nm did not biosynthesize rhizocarpic acid; even when the pH values in the nutrient media were altered in a range of 5 to 10. The formation of rhizocarpic acid in the mycobiont cultures of *R. lecanorinum* chemotypes was not found to be pH dependent.

By performing low temperature treatments and by exposure to slow desiccation by evaporation, medullary compounds such as stictic and psoromic acids were formed in low concentrations in 20 mycobiont cultures. Even when these cultures were exposed to the irradiation of full spectrum lamps for a longer time (3 months), pulvinic acid derivatives and rhizocarpic acid were not biosynthesised.

The highest growth rates of *R. umbilicatum* mycobionts were found on Difco Agar (with 2% of ribitol, + 0,6 g/l $CaCl_2$, + 5 μg biotin). On this medium composition, the lichen fungus started to produce the depsidone, stictic acid, after 6 months of incubation (Fig. 6), forming a stictic acid chemosyndrome (constictic, hypostictic, stictic) initially. However older cultures (about 6 months) contained only stictic acid, suggesting that the former chemically related compounds were probably metabolised into stictic acid. Finally, the highest concentrations of stictic acid were produced by cultures of *R. umbilicatum*. The extraction of 10 pre-dried mycelia (with an average diameter of 6–7 mm, c. 1 g of dry weight) in a Soxhlett apparatus, produced a quantity of 0.222 mg pure stictic acid crystals.

In contrast to *R. umbilicatum* and *R. lecanorinum*, the mycobionts of *R. oederi* failed to grow on the nutrient deficient media or Difco agar, which has been tested to be an excellent basal culture medium to optimize culture conditions for many lichen fungi.

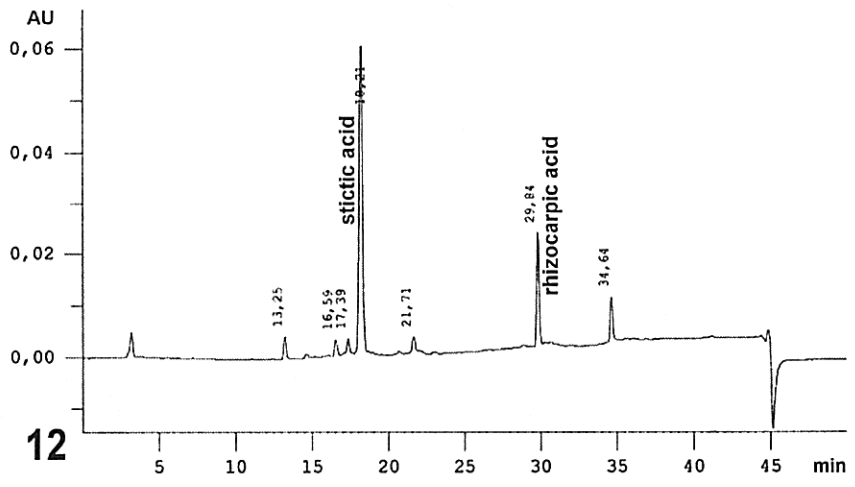


Fig. 12. HPLC chromatogram of *Rhizocarpon lecanorinum* (chemotype 1).

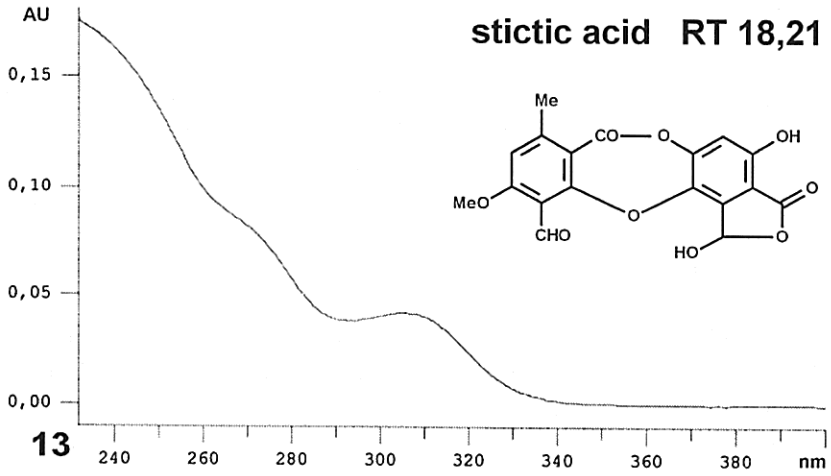


Fig. 13. UV spectrum of stictic acid.

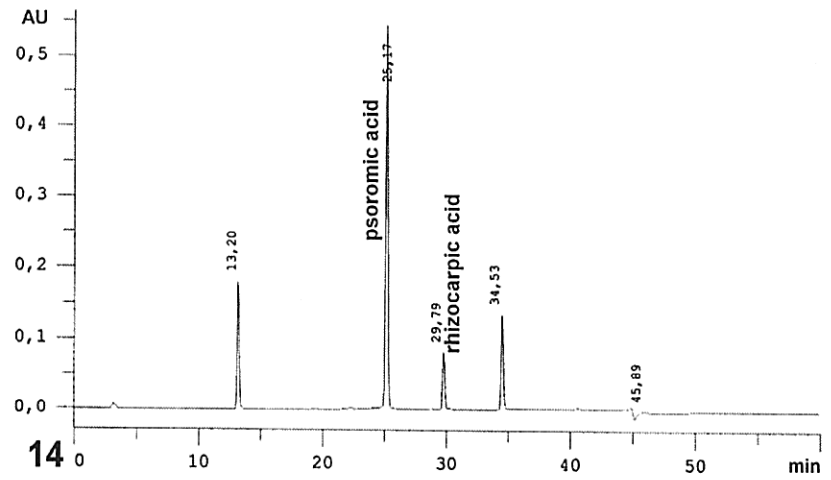


Fig. 14. HPLC chromatogram of *Rhizocarpon lecanorinum* (chemotype 2).

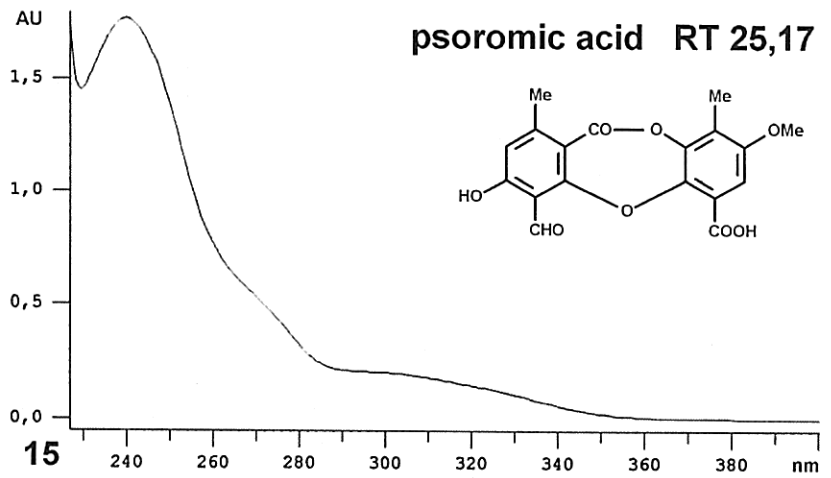


Fig. 15. HPLC chromatogram of psoromic acid.

Conclusions

The results demonstrated that mycobionts of selected species of *Rhizocarpon* can be grown aposymbiotically under laboratory conditions. Surprisingly, under culture conditions that were based on preliminary field observations, it was possible to obtain mycelia with growth rates that did not deviate from growth rates achieved by mycobionts of foliose and fruticose lichens. Exposure to nutrient media with high mineral contents and carbon sources that are known to facilitate the transfer of metabolites between photo- and mycobionts, allowed us to mimic the major needs of *Rhizocarpon* lichens from their natural habitat into the culture chamber.

Our efforts to screen growth and development of *Rhizocarpon* mycobionts on several media with different pH values clearly indicated that the *Rhizocarpon* fungi are highly adapted to ecological conditions on rock surfaces, where increased superficial mineral contents may be present and accumulate in the uneven rock fissures and depressions after every strong rainfall.

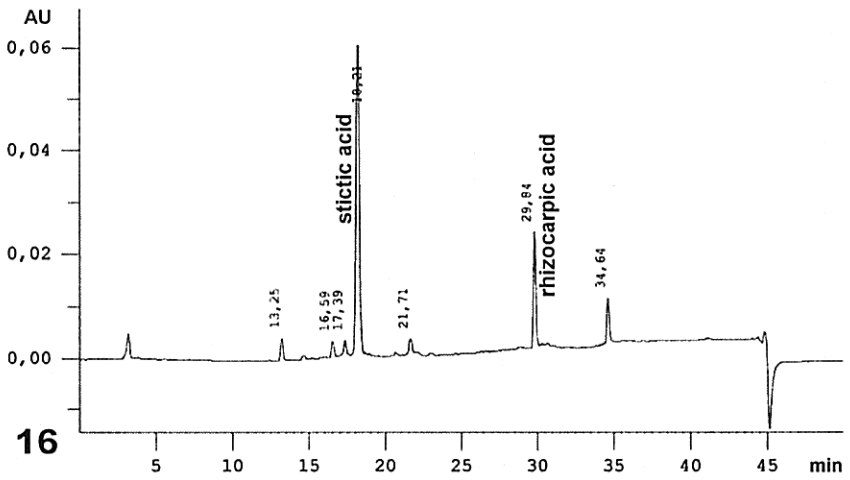


Fig. 16. HPLC chromatogram of *R. umbilicatum*.

A further challenge in this investigation was that the numerous spores produced by most *Rhizocarpon* species could not be used. Contamination-free mycobiont cultures from thallus fragments were preferred, but thallus fragments of the three investigated species of *Rhizocarpon* were quite difficult to handle. Almost all of the tested thalli were strongly fixed to the rock surfaces. An additional problem was to exclude *Rhizocarpon* thalli that are regularly colonised by lichen associated fungi, including parasites and lichenicolous symptoms.

In this context, a noteworthy statement in the study of CLAYDEN (1998) should be mentioned, namely 'that the life history of *R. lecanorinum* focuses not so much on a complex thallus development and morphogenesis, but gears towards a very high investment of ascospore production and sexual reproduction'. Considering this statement, the effective use of spores for our

experiments would have facilitated our investigations immensely, considering the possibility to obtain genetically more uniform materials. Although the high germination rate of *R. leconorinum* spores was also recorded in the natural environment by CLAYDEN (1998) and also their peculiarity to stop hyphal growth shortly after germination, in our opinion and in agreement with Clayden, this fact can be hardly interpreted as a successful reproductive strategy.

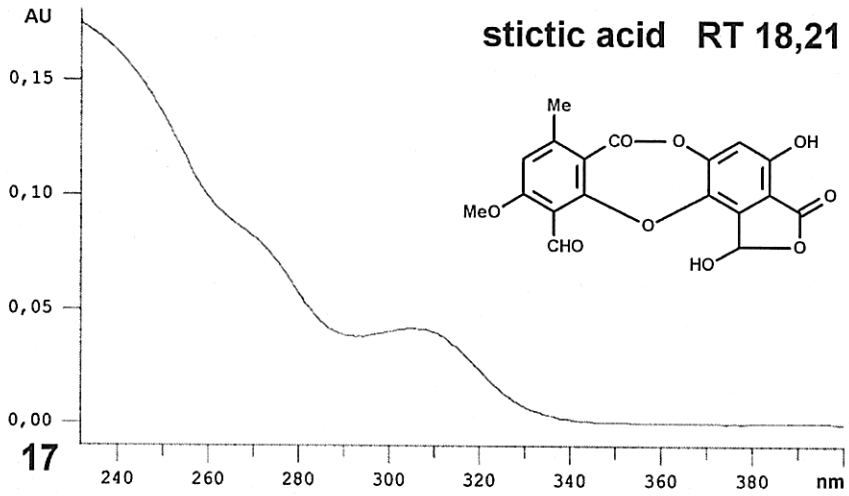


Fig. 17. UV spectrum of stictic acid (*R. umbilicatum*; mycobiont).

It seems to be a necessity for the lichen fungus to find an appropriate photobiont partner for a "resynthesis under natural conditions" within the close neighbourhood of the "parent" thallus. The quick termination of fungal growth would most probably prevent the propagation of species of the genus *Rhizocarpon*. This is very unlikely, as the yellow-green species of *Rhizocarpon* form extensive populations on siliceous rocks. From this point of view, one might think of the possibility that something in the life history of *Rhizocarpon* is missing, such as mites or other tiny animals that transport and may eat germinated spores/algae. By surviving digestion (which has been recorded for many reproduction units and also algae of lichens) and using digested remains as valuable nitrogenous sources, such an animal mediated reproductive strategy could favour improved conditions for survival and re-lichenisation of the mycobiont at distances from the "parent thalli". This might explain why yellow-green *Rhizocarpon* species start their symbiosis as a soredium-like cell aggregate composed of hyphae and algae (CLAYDEN 1998).

Another interpretation of our culture experiments is that the fungus must enter very rapidly into an interaction with a reliable photobiont to guarantee the survival of a new functional fungal relationship, This is supported by the finding that hyphal isolates of *R. umbilicatum* show increased growth rates on mineral-enriched media that contain larger quantities of polyols (transfer metabolites between photobionts and mycobionts).

Exceptions for the requirement of a high nutrient supply were the two mycobionts originating from the different chemotypes of *R. lecanorinum*. These mycobionts were able to invest into resources for secondary metabolic processes even when they were grown on agar plates without additional carbon sources. The fungus seems to use available polyols or glucose first for growth and production of aerial hyphae (as a pre-stage of cortex formation), and as soon as growth becomes limited after a decrease of nutrient supplies in the media, excess carbohydrates, probably present in the agar gels themselves, could then be used for the production of polyketides. The idea that excess sugars produced by the photosynthesis of the algae could be used by the mycobionts to produce polyketides was already hypothesised by MOSBACH (1973). Only very limited nutrient supplies in form of sugars were necessary to induce the production of depsidones (stictic and psoromic acids) for the two *R. lecanorinum* mycobionts tested. This may be concurrent with the situation on rock surfaces where the availability of nutrients can be very low. The low concentrations of stictic and psoromic acids that were detected by the HPLC analyses could then be explained by the fact that the fungi were grown aposymbiotically and under dry conditions. The first occurrence of pulvinic acid dilactone and rhizocarpic acid in the aposymbiotically grown mycobiont cultures (*R. lecanorinum*) that were exposed to irradiation from full spectrum lamps, highlighted the investigations. It was very exciting to observe the appearance of the yellow crystals that were continuously deposited on the surfaces of the hyphal cell walls over a time span of three weeks or more.

A further interesting finding was that light covering the entire spectrum was required to induce rhizocarpic acid production. Irradiation from a lamp with only one selected UV light wave length (290 nm, maximum UV absorbance of rhizocarpic acid) did not have the same effect. Rhizocarpic acid and pulvinic acid dilactone (yellow-green pigments) have been interpreted as having the properties of an effective sunscreen, filtering the high intensity of incident UV-A and UV-B light of the solar spectrum (e.g. HIDALGO et al. 2002). Lichens may use these compounds for similar reasons since significantly higher contents of rhizocarpic acid concentrations were reported in lichens, e.g. *Acarospora schleicheri*, that were collected in several areas located at higher altitudes (RUBIO et al. 2002). These findings may partly apply to *Rhizocarpon lecanorinum* which is not restricted in its distribution to high altitudes but could also be found on rocks of lower elevations and even at sea level. The present study indicates that the production of the yellowish-green pigments by the *R. lecanorinum* mycobionts is not dependent upon the presence of incident UV-A and UV-B irradiation to be biosynthesised. This fact has to be interpreted as a long-term effect that may refer to long-term exposure to "mixed" light, which may be also induced by natural daylight or in our laboratory by whole spectrum light. The accumulated pigments can certainly act as excellent UV filters and are able to prevent damage to the photosynthetic apparatus of the green photobionts. However, UV light does not seem to be required to stimulate the production of rhizocarpic acid and pulvinic acid derivatives.

Rhizocarpic acid-producing, cultured mycobionts of *R. lecanorinum* could serve in the future as excellent model systems for molecular approaches studying transcription of genes controlling shikimic acid metabolism, involved also in chalcone synthesis (MANN 1995) and for ultra-structural investigations by elucidating export of pulvinic acid derivatives out of hyphal cell membranes and walls. In general, it remains obscure as to how secondary metabolites are formed within the protoplasmata of fungal cells, the type of "vesicles" in which they are exported from the fungal cells, and finally how they are crystallised and deposited on the cell wall surfaces.

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References

ADLER, M. D. , FAZIO, A. BERTONI, M. D., ROSSO, M. L., MAIER, M. S. & THELL, A. (2004): Culture experiments and DNA verification of a mycobiont isolated from *Punctelia praesignis* (Parmeliaceae, lichenized Ascomycetina). – *Bibliotheca Lichenologica* **88:** 1–8.

ARMSTRONG, R. A. (1983): Growth curve of the lichen *Rhizocarpon geographicum*. – *New Phytologist* **94:** 619–622.

ARMSTRONG, R. A. (2005): Radial growth of *Rhizocarpon* Section *Rhizocarpon* lichen thalli over six years at Snoqualmie Pass in the Cascade Range, Washington State. – *Arctic, Antarctic, and Alpine Research* **37:** 411–415.

BEHERA, B. C. & MAKHIJA, U. (2001): Effect of various culture conditions on growth and production of salaziic acid in *Bulbothrix setschwanensis* (lichenized ascomycetes) in vitro. – *Current Science* **80:** 1424–1427.

BEHERA, B.C. VERMA, N., SONONE, A. & MAKHIJA, U. (2009): Opimization of culture conditions for lichen *Usnea ghattensis* G. Awasthi to increase biomass and antioxidant metabolite production. – *Food Technology and Biotechnology* **47:** 7–12.

BRADWELL, T. & ARMSTRONG, R. A. (2007): Growth rates of *Rhizocarpon geographicum* lichens: a review with new data from Iceland. – *Journal of Quaternary Science* **22:** 311–320.

BRUNAUER, G., HAGER, A., TÜRK, R. & STOCKER-WÖRGÖTTER, E. (2007): Alterations in the secondary metabolism of aposymbiotically grown *Xanthoria elegans* mycobionts and resynthesis stages. – *Plant Physiology and Biochemistry* **45:** 146–151.

CLAYDEN, S. (1998): Thallus initiation and development in the lichen *Rhizocarpon lecanorinum*. – *New Phytologist* **139:** 685–695.

ELIX, J. A. (1996): Biochemistry and Secondary metabolites. *In*: Nash III, T. H. (ed.). *Lichen Biology*. Cambridge University Press, Cambridge, pp. 154–180.

ELIX, J.A., GIRALT, M. & WARDLAW, J.H. (2003) New chloro-depsides from the lichen *Dimelaena radiata*. – *Bibliotheca Lichenologica* **86:** 1–7.

ELIX, J. A. & STOCKER-WÖRGÖTTER, E. (2008): Biochemistry and secondary metabolites. *In*: Nash III, T.H. (ed.). *Lichen Biology*, Cambridge University Press, Cambridge, pp. 103–133.

FEIGE, G. B., LUMBSCH, H. T., HUNECK, S. & ELIX, J. A. (1993): The identification of lichen substances by a standardized high-performance liquid chromatographic method. – *Journal of Chromatography* **646:** 417–427.

FEUERER, T. (1978): Zur Kenntnis der Flechtengattung *Rhizocarpon* in Bayern. – *Berichte der Bayerischen Botanischen Gesellschaft* **49:** 59–135.

HAGER, A., BRUNAUER, G., TÜRK, R. & STOCKER-WÖRGÖTTER, E. (2008): Investigations on production, biological functions and bioactivity of common lichen metabolites as exemplified by the Australian lichen *Heterodea mülleri*. – *Journal of Chemical Ecology* **34:** 113–120.

HALE, M. (1983) *The Biology of Lichens*. 3rd ed., Edward Arnold, London, 176 pp.

HAUCK, M., JÜRGENS, S. R., WILLENBRUCH, K., HUNECK, S. & LEUSCHNER, C. (2009): Dissociation and metal binding characteristics of yellow lichen substances suggest a relationship with site preferences of lichens. – *Annals of Botany* 2009, **103:** 13–22.

HIDALGO, M. E., FERNÁNDEZ, E., PONCE, M., RUBIO, C. & QUILHOT, W. (2002): Photophysical, photochemical, and thermodynamic properties of shikimic acid derivates: calycin and rhizocarpic acid (lichens). – *Journal of Photochemistry and Photobiology* **66:** 213–217.

HUNECK, S. & YOSHIMURA, I. (1996): *Identification of Lichen Substances*. Springer Verlag, Berlin, 493 pp.

INNES, J. L. (1985): Lichenometry. – *Progress in Physical Geography* **9:** 187–254.

MAGNUSSON, A. H. (1948): Lichens from More Fylke in Western Norway. – *Arkiv för Botanik* **33A:** 1–36.

MANN, J. (1995) *Chemical Aspects of Biosynthesis*. Oxford University Press, Oxford, 96 pp.

MOSBACH, K. (1973): Biosynthesis of Lichen Substances. *In*: Ahmadjian, V. & Hale, M. (eds). *The Lichens*. Academic Press, New York, pp. 543–546.

MATTHEWS, J. A. (1994): Lichenometric dating. A review with particular reference to 'Little Ice Age' moraines in Southern Norway. *In*: Beck, C. (ed.). *Dating in Surface Contexts*. New Mexico University Press, Albuquerque, pp. 185–212.

PROCTOR, M. C. F. (1983): Sizes and growth rates of thalli of the lichen *Rhizocarpon geographicum* on the moraines of the glacier de Valsorey, Valais, Switzerland. – *Lichenologist* **15:** 249–261.

RUBIO, C., FERNÁNDEZ, M. E., HIDALGO, W. & QUILHOT, W. (2002): Effects of solar UVB radiation in the accumulation of rhizocarpic acid in a lichen from alpine zones of Chile. – *Boletín De La Sociedad Chilena De Química* **47:** 67–72.

STOCKER-WÖRGÖTTER, E. (2008): Metabolic diversity of lichen forming, ascomycetous fungi: culturing, production of polyketides and shikimic acid derivatives, and PKS genes. – *Natural Product Reports* [Cambridge] **25:** 188–200.

STOCKER-WÖRGÖTTER, E. & 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. & ELIX, J. A. (2004): Experimental studies of lichenized fungi: formation of rare depsides and dibenzofurans by the cultured mycobiont of *Bunodophoron patagonicum* (Sphaerophoraceae, lichenized Ascomycota). – *Bibliotheca Lichenologica* **88:** 659–669.

STOCKER-WÖRGÖTTER, E. & ELIX, J. A. (2006): 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. – *Symbiosis* **41:** 9–20.

STOCKER-WÖRGÖTTER, E. & ELIX, J. A. & GRUBE, M. (2004): Secondary chemistry of lichen-forming fungi: chemosyndromic variation and DNA-analyses of cultures and chemotypes in the *Ramalina farinacea* complex. – *Bryologist* **107:** 152–162.

STOCKER-WÖRGÖTTER, E. & HAGER, A. (2008): Culture Methods for lichens and lichen symbionts. *In*: T. H. Nash III, (ed.). *Lichen Biology*. Cambridge University Press, Cambridge, pp. 353–363.

YAMAMOTO, Y. (1990): *Studies of Cell Aggregates and the Production of Natural Pigments in Plant Cell Culture*. Nippon Paint Publications, 119 pp.

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

YAMAMOTO, Y., MIZUGUCHI, R., TAKAYAMA, S. & YAMADA, Y. (1987): Effects of culture conditions on the growth of Usneaceae lichen tissue cultures. – *Plant Cell Physiology* **28:** 1421–1426.

YAMAMOTO, Y., MIURA, Y., HIGUCHI, M., KINOSHITA, Y & YOSHIMURA, I. (1993): Using lichen tissue cultures in Modern Biology. – *Bryologist* **96**: 384–393.