

Research article

Alterations in secondary metabolism of aposymbiotically grown mycobionts of *Xanthoria elegans* and cultured resynthesis stages

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Abstract

HPLC analyses of *Xanthoria elegans* cultivated on different media and either aposymbiotically or with its photobiont revealed that the carbon source and the presence of the algal partner have an impact on the secondary metabolism of the mycobiont. The aposymbiotically (without photobiont) grown mycobiont contained up to 70% more of the main compounds in its thallus than in resynthesis stage. Although this is speculative, the induction of the polyketide pathway may be a feedback mechanism to the absence of the photobiont. All cultures produce a variety of substances which were not detectable in the voucher specimen. Besides physcion (the major substance), we were able to identify emodin as well as physcion-bisanthrone, teloschistin monoacetate and derivatives. A strong inducible effect on the production of physcion, physcion-bisanthrone and on their precursors and derivatives was found for mannitol. By contrast, supplementation of ribitol had negligible effects, if any, on polyketide quantities although it is the main carbon source for the mycobiont in free-living lichens with *Trebouxia* photobiont.

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1. Introduction

The impact of the nutrient situation and the climate on the metabolism and in particular on the secondary chemistry of lichens is often discussed. The cosmopolitan lichen *Xanthoria elegans* is very adaptable which allows this lichen to colonize places all over the world, in temperate climates as well as in extreme habitats independently of the altitudinal belt. It is

poorly investigated how the different environments influence this lichen and how the lichen reacts on them. Secondary metabolites can give a competitive edge to the organism [11], although they are not essential for reproduction or survival [2]. Therefore, a flexible secondary metabolism – as many lichen seem to have [17] – would be an advantage to prepare for extreme climate conditions, since the secondary metabolism provides inter alia pigments to protect the lichen from UV damage [7]. Further, different competition situations or the presence of herbivores might result in the production of allopathic compounds or feeding deterrents in some lichens [4]. For *X. elegans* physcion and emodin might be compounds with protective features since they exhibit numerous biological activities which make these anthraquinones also candidates for further pharmacological investigations and applications [11,19]. Especially emodin exhibits a great variety of biological activities and many experimental data exists about molecular targets of this substance and its mode of action [4,5,13,16]. Such multi-functionality is not unusual for

Abbreviations: BBM, Bold's Basal medium; BLAST, basic logical alignment research tool; DAD, diode array detector; DMF, dimethylformamide; gDNA, genomic DNA; HPLC, high performance liquid chromatography; ITS, internal transcribed spacer; LBM, Lilly and Barnett medium; MS, Murashige and Skoog medium; MY, mycobiont cultures; M-Y, Malt-Yeast medium; PDA, Potato-Dextrose-Agar; RES, resynthesis cultures; S2%, Sabouraud-2%-Glucose-Agar.

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secondary metabolites and a common phenomenon in plants and it even seems that it is an essential fitness factor [28].

Anthraquinones are not only restricted to lichens or even fungi, they are also widely spread in higher plants [10,23–25] where they are involved in interactions between the plants and their environment. In this case, emodin again plays an important role in protecting the immature fruit against seed predators and additionally accelerates the passage of seeds through the digestive tract of fruit-feeding animals which influences seed viability. The group of anthraquinones contains a large number of natural quinones [10,23,24] and many of these compounds are lichen substances. Most of the typical lichen substances are products of the polyketide biosynthetic pathway (acetyl-polymalonyl pathway) which as are anthraquinones [4]. In higher plants the acetyl-polymalonyl pathway is not the only source for anthraquinone production. Only Polygonaceae and Rhamnaceae use this pathway to synthesise anthraquinones, whereas it is produced via *O*-succinylbenzoic acid in the Bignoniaceae and Verbenaceae [11]. Another difference between anthraquinones of higher plants and lichens is that they are often sugar-conjugated (glycosides) in higher plants [10,23,24].

Lichens are also known to modify the basic structures of its secondary metabolites to generate analogues and derivatives with often very heterogeneous activities [4]. Moreover, up to now some enzyme reactions were discovered, that transform one anthraquinone into another. In plants for example chryso-phanol is synthesised by dehydroxylation of emodin and also physcion is supposed to be an emodin derivative [15,24].

This study aimed to identify stimulating factors for the mycobionts polyketide synthesis in the widespread foliose lichen *Xanthoria elegans*. Therefore we have now investigated how the secondary metabolism of cultured *Xanthoria elegans* reacts to supply of lichen typical polyols by quantifying variations within the secondary metabolite content. The mycobiont was analysed on several different media aposymbiotically and in resynthesis stage. Targeting such factors that enhance the metabolite production of lichens is an essential step on the way to a successful biochemical and genetic investigation of lichens secondary metabolism and to produce larger amounts of certain biologically active compounds by lichen cultures without endangering natural lichen populations.

2. Results

Xanthoria elegans turned out to be easily cultivable. Within the first week after taking the mycobiont into culture, hyphal growth was observed under the microscope and after 1 month of culturing the mycobiont was forming colonies of about 1–2 mm in diameter. Another month later the mycobiont more than doubled its biomass and 46 out of 50 slanted agar tubes stayed free of any fungal or bacterial contamination, which is very unusual and argues for an allelopathic effect of the mycobiont against potential contaminants.

Subsequently on G-LBM subcultured mycobiont also grew fast until it reached after about 3 month a nearly stationary phase where no more growth could be observed. Albeit, it

exhibited a still active secondary metabolism: the colour of the thallus turned from light pink to a dark orange on both media within the next 3 month. Only on G-LB medium crystals of lichen substances appeared on and even inside the culture medium. HPLC analyses of the cultured mycobiont showed the expression of a variety of anthraquinones (Fig. 1B). The chromatogram of the voucher specimen just showed physcion as the only strongly expressed anthraquinone while other polyketides were only present in traces too little for spectral identification (Fig. 1A).

Qualitative analysis of the expressed polyketides showed the production of substances biogenetically related to physcion, as emodin, physcion-bisanthrone, as well as derivatives of these compounds. Moreover, we detected a variety of unknown anthrones (Fig. 1B).

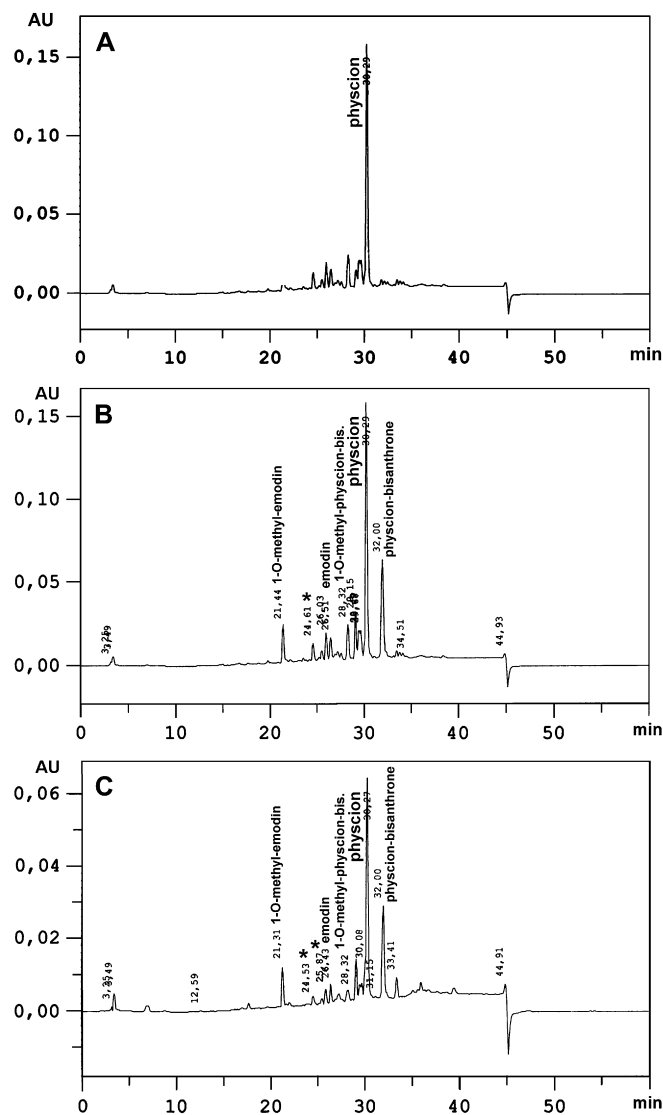


Fig. 1. (A) *Xanthoria elegans* HPLC chromatogram of the voucher specimen; (B) *X. elegans* chromatogram of the cultured mycobiont showing a variety of anthraquinones; and (C) *X. elegans* chromatogram of the resynthesis; (*) unknown substances.

Resynthesis cultures (RES) appeared less intensely coloured than the mycobiont (MY) and with only small and few lichen compound crystals on the medium. This visual differences between MY and RES became apparent in the HPLC analyses of the cultures (Figs. 1B,C). Although both cultures (aprosymbiotically grown and resynthesis) expressed nearly the same pattern of substances, the quantitative analysis revealed that MY (Fig. 2A) contained up to 70% more of its major compounds than the resynthesis stage (Fig. 2B, Table 1). The total polyketide content differed remarkably between MY and RES and moreover one – yet unknown – compound was only found in resynthesis cultures. MY expressed the double polyketide amount of the corresponding resynthesis cultures while the total content-ratios between cultures from the three different media (+M/–R, –M/+R, –M/–R) are the same in MY and RES (Fig. 3). Further, the metabolism of cultured *X. elegans* directly reacts to supplementation of typical lichen polyols such as ribitol and mannitol. The response becomes apparent in the total amount of produced secondary metabolites as well as in an altered ratio amongst particular substances. A strong activating effect on the production of physcion, physcion-bisanthrone and on their precursors and derivatives was found by application of mannitol. In reaction to mannitol another unknown substance was produced (peaks 24.61 in Fig. 1B and 24.53 in Fig. 1C), which could not be detected in cultures on media –M/–R or –M/+R. However, supplementation of ribitol had negligible effects, if any, on polyketide quantities.

3. Discussion

Up to now it was assumed that different morphology and chemical phenotype are a consequence of a different genetical background but as shown in this study, *X. elegans* is able to produce many different (but related) compounds in culture while the voucher specimen just produced physcion in higher quantities. The factors that make the mycobiont produce this broad spectrum of related quinones and anthrones are not

known yet but many biotic (e.g. herbivores) and abiotic (light intensity, temperature, osmotic stress, soil minerals and seasonality) factors must be considered [9,26]. Concerning emodin, at least in higher plants the light intensity and especially seasonality governs the emodin status of the plant organs [18]. Our experiments on cultures of the lichen *X. elegans* showed that the carbon source has also an effect on the activity of the secondary metabolism. Even substances were produced that could not be detected in the voucher specimen. This corresponds with experiments of Solhaug and Gauslaa [19] who tested several carbohydrates on naturally grown lichen thalli for their potential to activate polyketide production. They concluded that supplementation of photosynthates by the photobiont up-regulates the physcion production. Since the voucher specimen does not produce emodin in identifiable amounts, the induction of emodin production by the cultivated *X. elegans*-mycobiont may have other reasons. To further assess the influence of the contact between the mycobiont and the photobiont on the secondary metabolism, lichen compound contents of aposymbiotically grown (MY) cultures were compared to them of resynthesis stages (RES). In a *Trebouxia*-lichen ribitol is dispensed in large part by the photobiont, taken over by the mycobiont and converted into mannitol as source of energy in its metabolism [22]. Evaluation and comparison of total polyketide contents and quantities of single anthraquinones of MY and RES incubated on 3 different nutrient media (+M/–R; –M/+R; –M/–R) showed that mannitol had a strong activating effect on the polyketide expression of MY and RES while ribitol had negligible effects. In accordance with the negligible effect of ribitol on the cultured mycobiont we observed a low anthraquinone production in the RES cultures. This might be a culture-effect as Solhaug & Gauslaa [19] had shown in a study on the naturally grown lichen thallus of closely related *Xanthoria parietina* that physcion biosynthesis is more strongly activated by supplementation of ribitol than due to mannitol.

Why the RES-cultures express much lower anthraquinone-levels than MY-cultures remains unclear. Although this is

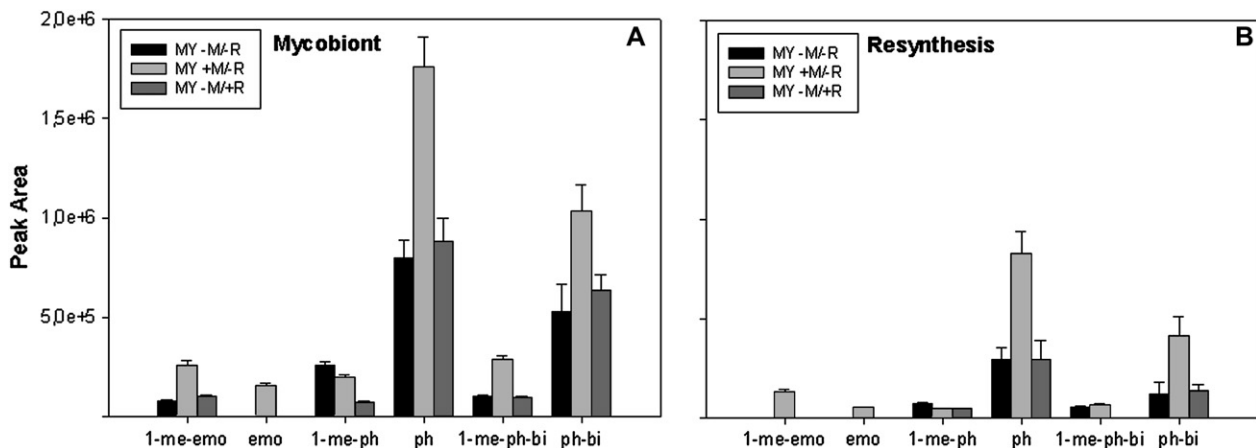


Fig. 2. (A) Changes in anthraquinone production due to different polyol supply in the cultured mycobiont; and (B) changes in anthraquinone production due to different polyol supply in the resynthesis stage; 1-me-emo (1-O-methyl-emodin), emo (emodin), 1-me-ph (1-O-methyl-physcion), ph (physcion), 1-me-ph-bi (1-O-methyl-physcion-bisanthrone), ph-bi (physcion-bisanthrone).

Table 1
Content of selected anthraquinones, displayed by HPLC substance-peak areas

Sample	Peak Integral (Area)					
	1-O-me-emodin	Emodin	1-O-me-physcion	Physcion	1-O-me-physcion-bisanthrone	Physcion-bisanthrone
MY –M/–R	77703	0	259244	796545	99707	529612
RES –M/–R	0	0	73188	294577	56207	120336
MY +M/–R	259979	156889	197385	1762632	287715	1033636
RES +M/–R	134297	52523	47561	827751	68484	412013
MY –M/+R	102172	0	70431	880847	93591	636814
RES –M/+R	0	0	48433	293730	0	137353

speculative, the upregulation of the anthraquinone biosynthesis may represent a (stress) response of this lichen to the absence of the photobiont. Another possibility would be that the photobiont supplies some antioxidative substances like ascorbic acid which is known to strongly influence the secondary metabolism and could also lead to a reduced polyketide-expression [17].

To explain why the metabolism of *X. elegans* reacts like this under culture conditions, more information is necessary about the mechanisms responsible for a modulation of the polyketide pathway in lichens and on which level regulations take place. It is conceivable that the mycobiont adapts its biosynthesis pathway by itself in reaction to the artificial culture environment but it is also possible that the activity of participating enzymes is modulated by a lack of certain trace elements or an unusual pH of the nutrient medium.

4. Methods

4.1. Lichen material

Xanthoria elegans (Link) Th. Fr. (Herbarium: Georg Brunauer 10; University of Salzburg) was collected 20 km from Akureyi, Northern Iceland, in September 2002 and identified by anatomical characters [29].

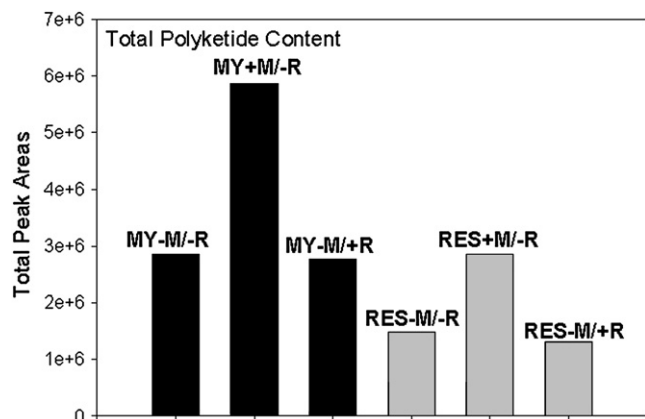


Fig. 3. Total polyketide content of the mycobiont and the resynthesis stages.

4.2. Mycobiont & photobiont isolation

The isolations were performed after a modified Yamamoto method [30] on agar slants in tubes containing 5 ml of solid medium as described by Brunauer and Stocker-Wörgötter [3].

4.3. Subculturing

Fungal colonies were homogenised in sterile double distilled water using a sterile mortar and pestle. Five spots of the suspension were then transferred with a pipette to petri dishes (110 × 15 mm), containing G-LBM nutrient medium (the same as the isolation grew on). Culture dishes were sealed with parafilm and kept in a culture chamber under a 16:8 light/dark regime at approximately 50–100 $\mu\text{E m}^{-2} \text{s}^{-1}$. Microscopically not contaminated photobionts were transferred and spread out on petri dishes (110 × 15 mm) containing BBM.

For carbon source experiments 3 month old mycobiont culture spots of one petri dish were harvested, homogenised in sterile water and split in two equal volumes. For resynthesis cultures algae were taken from the nutrient medium with a sterile pipette tip and mixed thoroughly with one aliquot of the mycobiont homogenate. Both homogenates (mycobiont with and without photobiont) were then spotted on the test media containing 5 g mannitol (+M/–R) or 5 g ribitol (–M/+R) and on G-LBM without polyol content.

4.4. Culture media

For the mycobiont-isolation from the lichen the following media were used:

G-LBM (a self-modified Lilly & Barnett-medium [14]; 1 L G-LBM (pH 5,5) contains: 35 g Glucose; 1,6 g L-Asparagine; 0,4 g $\text{MgSO}_4 \times 7\text{H}_2\text{O}$; 0,8 g KH_2PO_4 ; 4 mg NaCl; 200 mg CaCl; 200 mg Cu-Acetate; 0,2 mg $\text{Fe}(\text{NO}_3)_3 \times 9\text{H}_2\text{O}$; 0,2 mg $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$; 0,1 mg $\text{MnSO}_4 \times \text{H}_2\text{O}$; 100 μg Thiamine-HCl; 5 μg Biotin; 14 g Agar-Agar.

M-Y [30]

MS [20]

PDA (Sigma P-2182)

S2% (Fluka 84086)

The photobiont was isolated and cultivated on BBM [6].

4.5. Chromatographic analysis

For HPLC analysis, dry lichen thalli and vacuum-dried mycobionts (removed from the medium; c. 1 cm² in diameter) were extracted for 4 h with methanol. HPLC analysis [21] was performed using a Hitachi/Merck HPLC system including two pumps, a DAD (190–800 nm wavelength range) and a computer system with integration package based on Windows NT. The column used was a Beckman 5C18 (4.6 × 250 mm, 5 μm). 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, thereafter to 100% B within a further 15 min, followed by isocratic elution in 100% B for another 10 min, the flow rate was consistently 1 ml min⁻¹. 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, for comparing them with authentic metabolites under identical analysis conditions. The mycobiont was either aposymbiotically or in resynthesis stage incubated on nutrient media where mannitol (+M) or ribitol (+R) were added. The contents of selected lichen substances of the cultures on the different media (containing mannitol, ribitol or medium without polyol supplement) were compared to each others as well as the polyketide content of the aposymbiotically grown mycobiont versus the resynthesis stage.

To allow quantifications of single substances (mg g⁻¹ dry weight), a dilution series with pure phycion was made to allow conversion of integrated peak areas into indications of weight.

4.6. DNA isolation, amplification, sequencing and sequence alignment

Genomic DNA (gDNA) of the cultured photobiont was obtained by heating an algal colony diluted in 50 μl ddH₂O for 5 min at 97 °C. The suspension was then spun down (16,000 g; 5 min) and 2 μl of the supernatant were taken as template for the PCR. Total mycobiont DNA was isolated using CTAB procedure [Ref. [1], modified] and 10–20 ng of OD₂₆₀ quantified gDNA was used for PCR.

PCR-reactions were performed with primers ITS1-F [8] and ITS4 [27] to specifically amplify the fungal ITS 1 and ITS 2 and the 5.8S rDNA. Primers ITS1T and ITS4T [12] were taken for the algal ITS regions. The PCR mix contained 1 unit of Dynazyme II Taq polymerase (Finnzymes), 0.2 mM of each of the four dNTPs, 0.5 μM of each primer. PCR-conditions: 95 °C 1 min as initial denaturation step; 30 cycles: 94 °C (denaturation) 30 s, 56 °C (annealing) 30 s and 72 °C (extension) 50 s.

The PCR products were purified with QIAquick PCR purification Kit (Qiagen) and eluted with sterile water. Purified PCR-products were quantified by measuring absorbance at 260 nm. About 10 ng DNA was taken for sequencing reaction using Big Dye Terminator reaction kit (ABI PRISM, Perkin-

Elmer) according to the suppliers manual. Primer ITS1-F and ITS1T were used again for cycle sequencing.

Reaction products were ethanol precipitated as recommended in the suppliers manual before they were resolved in 20 μl of deionised DMF and loaded on ABI 377 (Perkin-Elmer, Applied Biosystems) automated sequencer.

Each sequence fragment was subjected to BLAST searches to verify their identity.

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