

Article

The Temporal Variation of Secondary Metabolites in the Mycobiont Culture and Thallus of *Parmelina carporrhizans* and *Parmelina quercina* Analyzed using High-Performance Liquid Chromatography

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Abstract: Lichens are composite organisms that produce a wide variety of secondary metabolites; many of the compounds have a high potential as bioactive compounds. The major limitations of using bioactive compounds from lichens is their slow growth rate and the damage to environmental populations caused by massive collection. The alternative to the massive collection of lichens in the field is their culture under laboratory conditions. We chose two related lichen species of Parmeliaceae that produce similar metabolites and isolated from spores in cultures placed under axenic conditions for over 550 days. From these cultures, we sampled 35 mg of each species from different culture media at two sampling times. The samples were analyzed using high-performance liquid chromatography (HPLC) to detect and identify major compounds. We found no differences in the metabolites produced within the species in comparisons between different culture media. Our results show that the mycobiont cultures produced different secondary metabolites than those found in natural lichen thalli. Moreover, different secondary metabolites between species and different metabolites over time were observed. We conclude that mycobiont cultures are a promising alternative for determining bioactive compounds and enhancing the efficiency of growth and production. These could be a good option for eco-friendly metabolite production.

Keywords: *Parmelina carporrhizans*; *Parmelina quercina*; mycobiont culture; phenols; polyketides; HPLC



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1. Introduction

Lichens are composite symbiotic organisms, and the lichen body, which is called the thallus (plural: thalli), is formed by the mycobiont, commonly an Ascomycota fungus and one or more photoautotrophic symbionts (photobionts), which may be green algae or cyanobacteria [1]. However, the lichen thallus is more complex, and in recent decades, it has been reported that Basidiomycota yeasts belonging to the Cystobasidiomycetes class [2] and associated bacteria [3] are part of lichen symbiosis. A mycobiont is a symbiont that is responsible for naming lichens and building the complex structure of the lichen thallus, although it can only achieve this in symbiosis with a compatible photobiont [4]. With more than 30,000 species, lichens represent approximately 20% of the currently

known, global fungal biodiversity species. They are widespread organisms and occupy the most extreme ecosystems on Earth, such as Antarctica or the Namib Desert. Lichens produce a wide variety of substances. Some lichen species are well known for their ethnobotanical use and have several traditional applications as wool dyes, food, and health-promoting teas in herbalism, Indian Ayurveda medicine, and traditional Chinese medicine [5–8]. Lichen-forming fungi produce a wide range of secondary metabolites with a rich potential as bioactive compound. Many of them are aromatic polyketides [9] since they are phenols with marked antioxidative properties [10]. They also have allelopathic properties against bacteria [11–14] and fungi [15,16], enabling specific pharmacological applications, such as photoprotection [17,18], cardioprotection [19], neuroprotection [20], antiviral [21], antidiabetic [22], and antitumoral applications [14,23,24] in mammals, by modulating the intracellular concentration of hydrogen peroxide [25].

The excessive collection of lichens, mainly to be used as perfume stabilizers and as dyes, has caused serious problems in the conservation of these organisms [26–29]. For example, *Everniastrum nepalense* is frequently picked up by Indian traders [26] and is on the IUCN red list because of the overexploitation of the species in Nepal [30]. The rare endemic lichen, *Xanthoparmelia beccae*, which is only known from the remote St. Helena Island where there are only four known populations and less than 1000 individuals, is listed as “vulnerable” and is also frequently collected [31]. The limiting factor in use of the secondary metabolites of lichens is often the very slow growth rates [32] of lichen thalli under natural conditions. For these reasons, isolated mycobiont growth in aposymbiotic cultures and whole thalli re-synthesis in the laboratory have been proposed as the best options for the production of secondary lichen metabolites, conserving the diversity of the natural populations of these organisms. They are also sensitive to climate change and the loss or deterioration of habitats [33–36].

Many lichen metabolites are biosynthetically related since they are produced at different stages of the same synthetic pathway. The metabolites produced by lichens are classified in three major classes based on their synthetic pathway: (i) the acetate–polymalonate pathway; (ii) mevalonic acid pathway; and (iii) shikimic acid pathway [37,38]. The secondary metabolites that can be produced by the lichen thalli in natural conditions vary between species and within species depending on environmental conditions, activating different metabolic pathways [9,39]. The major factors affecting the pattern of secondary metabolites produced by lichens are abiotic, including differences in nutrients, temperature and pH [4,40,41]. But also, among the biotic factors affecting the production pattern of secondary metabolites, the modulating effect of the photobiont [39] can be highlighted. Indeed, the phenolic pattern can vary substantially between the mycobiont growing in aposymbiotic conditions and the mycobiont associated with its photosynthetic partner (lichen symbionts).

Of special interest are the genetic and functional characterization of polyketide synthases (PKS) and the regulation of the different metabolic pathways [42]. However, there are some limitations of mycobiont cultures, including the inverse relationships between mycobiont growth and the production of secondary metabolites [4]. This can be resolved, at least in some species, by modulating culture conditions, i.e., changing the carbon source from glucose to sugar alcohols that commonly occur in lichens, such as ribitol, arabitol, and mannitol [43]. Another problem is the loss of the viability of cultures over time; somewhere between 100 and 300 days, the mycobiont culture stops the culture’s growth [4,44,45].

For the present study, we chose two sexually reproducing species belonging to the small lichen genus, *Parmelina* (Hale), representative of the Parmeliaceae family, which is characterized by containing lecanoric acid, atranorin and chloratranorin: *Parmelina carporrhizans* (Taylor) Hale and *Parmelina quercina* (Willd.) [46]. These two foliose and morphochemically similar species have differentiated ecological distributions and different reproductive behavior. The sexual reproduction of *P. quercina* is based on the production of higher and smaller sizes spores with lower germination rates compared with *P. carporrhizans* [47]. It has been suggested that the different reproductive strategies of these

species are related to their distribution. *Parmelina quercina* is mainly distributed in the Mediterranean climate zones of Southern Europe and Morocco, while *Parmelina carporrhizans* grows in more oceanic and humid areas, mainly in the Mediterranean Basin, but also in the Macaronesian region; it is even occasionally found in Great Britain.

Using two related species with different reproductive modes and distributions, we aimed to evaluate the aposymbiotic culture of lichen mycobionts as an alternative source for the production of secondary metabolites instead of natural populations of lichens growing in the field. We also aimed to compare the major compounds produced by mycobiont cultures at different time intervals between and within species.

2. Materials and Methods

2.1. Lichen Sampling

Three healthy and mature fresh thalli of *Parmelina quercina* and *P. carporrhizans* were collected in the summer and autumn of 2012 and placed in paper bags to dry lichen samples and transported to the laboratory. At the end of the experiments, representative samples were deposited in MAF herbarium (MAF-Lich 19,117, MAF-Lich 19,191, and MAF-Lich 19,192) at Farmacy Faculty of the Complutense University of Madrid.

The samples of *Parmelina quercina* were collected on bark of *Quercus ilex* (L.) in Monte de Las Pinedas, La Carlota (Spain), on 20th of July of 2012 (37°42'25.2" N, 4°55'47.9" W) at 182 m and Santa Cruz del Valle, Avila, on October 11th of 2012 (40°14'59.4" N, 5°0'33.8" W) at 689 m. Both localities were representative of Mediterranean forests, woodlands, and scrub biomes. Fresh thalli of *P. carporrhizans* were collected in cultivated chestnuts on bark of *Castanea sativa* (Mill.) in Cuevas del Valle (Spain) on October 11th of 2012 (40°18'28.4" N, 5°00'39.0" W) at 1007 m.

2.2. Mycobiont Isolation and Aposymbiotic Culture

We isolated the mycobionts of *P. carporrhizans* and *P. quercina* from lichen thalli collected less than two weeks prior to the isolation attempt. The aposymbiotic cultures started from ascospores following the inverted Petri dish methods of Ahmadjian [1]. Ten apothecia, five with diameters between 3 and 5 mm and five apothecia with diameters greater than 5 mm, were mechanically cleaned and cut from each lichen thallus. A total of 30 apothecia from each species were washed following the protocols established by Molina and Crespo [48] and attached to the inner side of inverted Petri dish lids with petroleum jelly. The plates for spore germination contained Bold's basal medium (BBM, ref. [49]), and the ascospores discharged upwards onto this inorganic medium, which is suitable for spore germination. The Petri dishes were covered with aluminum foil to ensure the total absence of photobionts, kept at 18–20 °C in the dark, and mycobiont cultures were periodically examined using a Nikon SMZ800 stereomicroscope and an Olympus CX40 microscope. The spores were ejected in groups of eight ascospores, and germination tubes were observed at four days after spore discharge [47]. Twenty-five to thirty days after spore discharge, a total of 90 agar pieces containing plurisporic mycelia of *P. carporrhizans* and a total of 35 agar pieces containing plurisporic mycelia of *P. quercina* were transferred to new plates containing three different types of nutrient-rich culture media: 0.2% glucose malt–yeast extract (0.2G-MY) prepared according to Molina et al. [44], cornmeal agar (CMA) prepared following the manufacturer's instructions (Difco Laboratories, Detroit, MI, USA), and Lilly Barnett medium (LBM, [50]). Petri dishes containing the mycobiont cultures were incubated at constant 20 °C in the dark for 550 days. The mycobiont cultures were checked and pictures were taken periodically under a stereomicroscope.

We cultured both species until we obtained 35 mg of pure mycobiont culture since, with this amount of biomass, we were able to use samples for analysis. Two samplings were realized for each species, at 160 (early) and 550 days (late) for *P. carporrhizans* and at 250 (early) and 550 days (late) for *P. quercina*. We also took a sample of the agar surrounding the lichen mycobionts, which was darkened by the droplets secreted by the aposymbiotic cultures of both species, at 250 days to be analyzed. The mycobiont biomass or agar pieces

of 5 mm² were extracted in 0.5 mL of acetone for 60 min and the extract was freeze-dried until analyses.

2.3. DNA Extraction and Molecular Identification

Prior to DNA extraction, we extracted secondary metabolites using acetone, and then we crushed mycobiont tissue samples with pestles in liquid nitrogen and extracted genomic DNA using the DNeasy Plant Kit (QIAGEN, Redwood City, CA, USA) according to the manufacturer's instructions. To confirm the identity of the mycobiont cultures, we amplified the internal transcribed spacer (ITS) region of the nuclear rDNA from the aposymbiotically grown cultured mycelia, using the fungal-specific forward primer ITS1F and the universal reverse primer ITS4 [51,52]. The amplifications were run using the following parameters: an initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing of primers at 54 °C for 1 min and extension of primers at 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. The ITS sequences were compared with the NCBI nucleotide database to confirm the identity of the mycelia cultured using Blast and deposited in GenBank (accession numbers KM357892 and KM357893). These mycobiont cultures of *P. carporrhizans* were used to sequence the genome using Illumina HiSeq 2000 [53].

2.4. High-Performance Liquid Chromatography

Lichen compounds were identified by HPLC using a protocol based on the study by Feige et al. [54], also described in Benatti et al. [55]. HPLC analyses were performed on an Agilent 1260 quaternary system with an incorporated degasser using a Poroshell 120 EC-C18 column (2.7 µm, 3.0 × 50 mm, Agilent, Palo Alto, CA, USA) for the separation of substances at 30 °C. Two solvents were used at a flow rate of 1.4 mL/min. Solvent A contains aqua bidest, 30% methanol, and 0.0658% trifluoroacetic acid; solvent B is 100% methanol. Thallus samples were soaked in 200 µL of methanol for one hour. Freeze-dried extracts of mycobiont cultures and culture media were resuspended in 200 µL of methanol. Next, 150 µL of these methanol extracts were filtered by centrifuging them for 1 min at 800 rpm through a Pall Acropep Advance filter plate. The filtrate was diluted tenfold with methanol, and 10 µL was automatically injected after equilibrating the HPLC system for two minutes to solvent A. The run continued isocratically for 0.18 min, solvent B was increased to 58% within 5 min, up to 100% within the next 5 min, and then for 0.82 min isocratically in 100% B. The run ended with solvent A being increased back up to 100% within 0.5 min. The column was flushed again for two minutes with 100% solvent before a new run was started. A diode array detector (DAD) detected compounds at 210, 254, 280, and 310 nm. The spectra (λ = 190–650 nm, 2 nm steps) and retention times of detected peaks were compared against a library of authentic metabolites processed under identical conditions using the OpenLAB CDS ChemStation software of Agilent at Imke Schmitt laboratory, located at Senckenberg Biodiversity and Climate Research Centre of Frankfurt am Main (Frankfurt am Main, Germany).

3. Results

3.1. Mycobiont Growth and Qualitative Observations

We successfully isolated and grew pure mycobiont cultures from ascospores of both species. The rate of success in *Parmelina carporrhizans* cultures was 100%, and biomass was obtained in all attempts ($n = 90$). Only 11.6% of the attempts resulted in growth ($n = 35$), and more time was needed to reach a high enough level of biomass to analyze the content of secondary metabolites (35 mg), whereas in *P. quercina*, we had less success. In *P. carporrhizans*, the secondary metabolites of the early culture were extracted after 160 days, instead of the 250 days that *P. quercina* needed to reach 35 mg, allowing us to analyze the secondary metabolites produced in the early stage of the mycobiont in the sample biomass.

At 80 days of growth, the cultures of *Parmelina* mycobiont produced striking brownish droplets in both species (Figure 1A,C). After 160 days, in most cases, the culture medium

around the mycobiont culture was stained brown (Figure 1B); in other cases, the dark brown substances crystallized in the contact zone of agar where mycobionts were observed (Figure 1D). After 240 days, the culture media were more intensely stained (Figure 1E) by an unknown mycobiont metabolite.

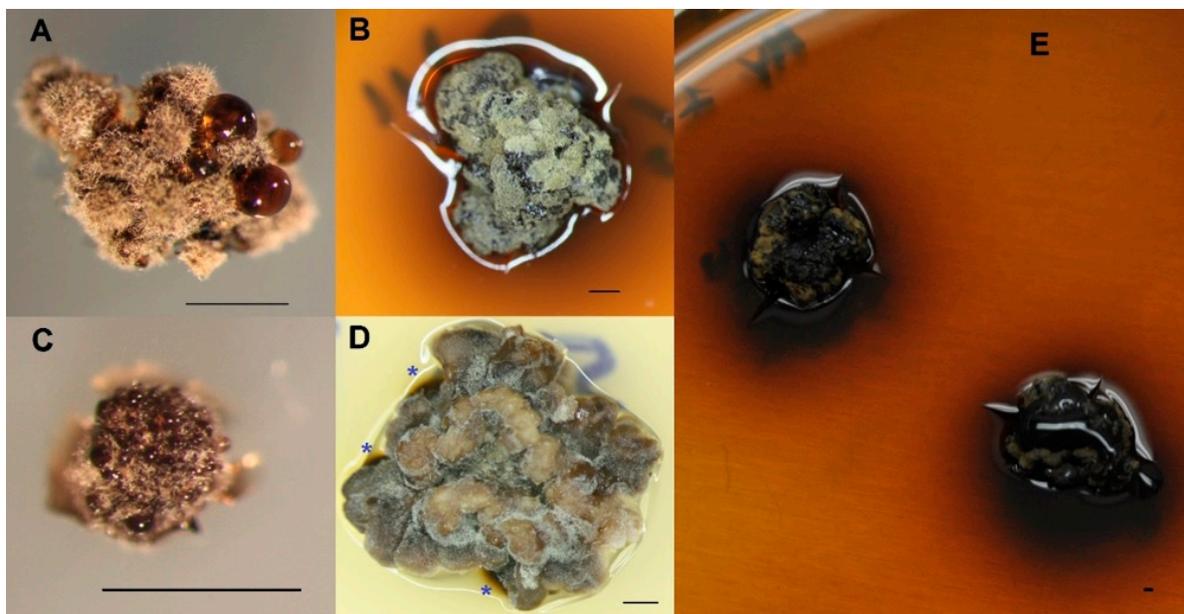


Figure 1. Plurisporic mycobiont culture in axenic conditions. (A) *P. carporrhizans* at 80 days (0.2G-MY) showing drops of a phenolic nature. (B) *P. carporrhizans* at 160 days showing 0.2G-MY agar stained media. (C) *Parmelina quercina* at 80 days showing drops of a phenolic nature growing on LBM. (D) *Parmelina quercina* at 160 days on CMA, showing crystalized brown substance, marked with *. (E) *P. carporrhizans* at 340 days of culture showing intense staining of the 0.2G-MY agar culture media. Scale bar: 1 mm.

3.2. Identification of secondary metabolites by High-Performance Liquid Chromatography

We identified lecanoric acid as a major compound and atranorin and chloratranorin as minor substances in the thalli of *P. carporrhizans* and *P. quercina* by HPLC. (Table 1). In addition, we identified evernic acid in *P. carporrhizans* at the trace level. In the early mycobiont culture of *P. carporrhizans*, confluent acid was identified as a major compound, whereas in the early mycobiont culture of *P. quercina*, we detected an unknown substance with a retention time (RT) of 6.97 min and identified pulvinic acid as a minor substance. This unknown substance was secreted by both species, *P. carporrhizans* and *P. quercina*, in the culture medium at 240 days. Norlobaridone was identified in late mycobiont cultures of *P. carporrhizans* as a major substance, while in late mycobiont cultures of *P. quercina*, different unknown substances with retention times of 5.79 and 6.97 min (Table 1) were detected as major substances. Notably, we did not find differences in the lichen substances produced in different culture media. The UV spectra of the unknown substances reported in Table 1 are provided in Figure 2.

Table 1. Lichen substances and retention times (RTs) of *Parmelina carporrhizans* and *Parmelina quercina* found in natural thalli and mycobiont cultures at different time intervals. Early time interval is 160 days for *P. carporrhizans* and 250 days for *P. quercina*. Late time intervals are 550 days for both species. Secretion was analyzed from agar pieces at 250 days in both species. The quantity of lichen substances is categorized according to if they were present as major or minor components of the extract, or if they were only present at trace levels as Ma, mi, or tra.

Substances	RT	Thallus	<i>P. carporrhizans</i>			<i>P. quercina</i>			
			Early	Late	Secreted	Thallus	Early	Late	Secreted
Lecanoric acid	4.66	Ma	-	-	-	Ma	-	-	-
Atranorin acid	7.68	mi	-	-	-	tra	-	-	-
Chloratranorin	8.12	mi	-	-	-	tra	-	-	-
Evernic acid	6.11	tra	-	-	-	-	-	-	-
Confluentin acid	6.32	-	Ma	-	-	-	-	-	-
Pulvinic acid	4.15	-	-	-	-	-	mi	-	-
Norlobaridone	6.26	-	-	Ma	-	-	-	-	-
Unknown 1	5.79	-	-	-	-	-	-	Ma	-
Unknown 2	6.26	-	-	-	-	-	-	Ma	-
Unknown 3	6.97	-	-	-	Ma	-	Ma	-	Ma

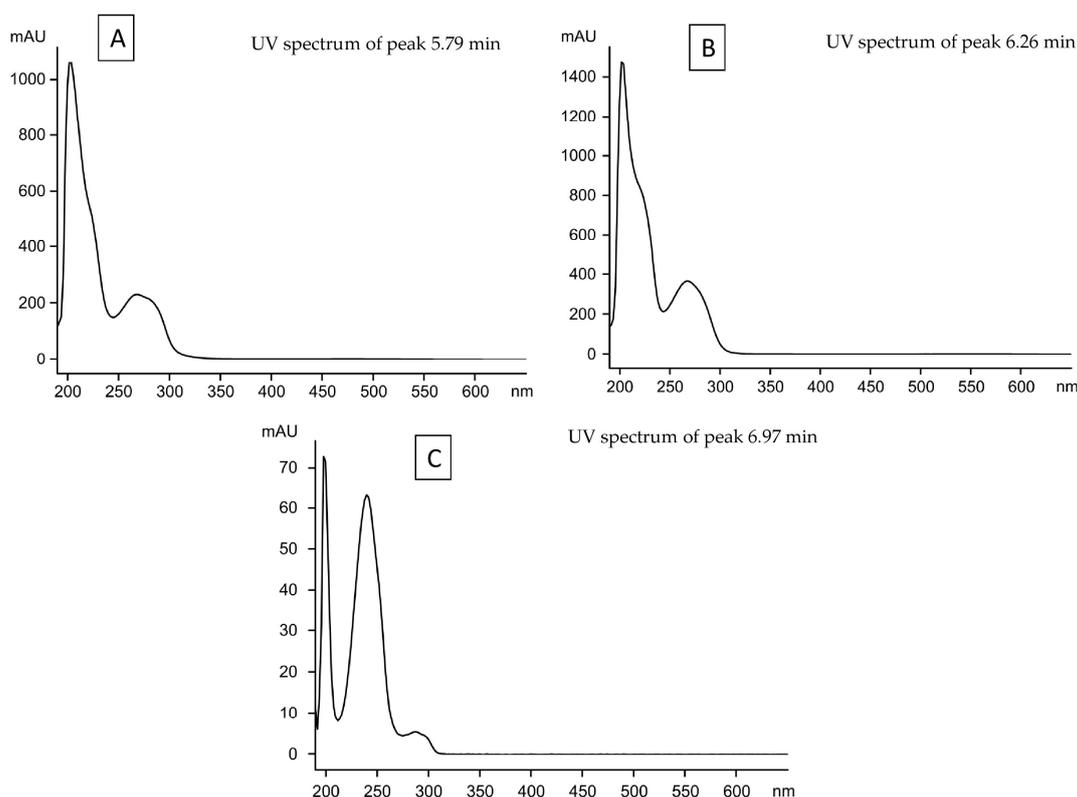


Figure 2. UV spectra of the unknown substances detected as major substances. (A) Unknown substance with retention time of 5.79 (B); unknown substance with retention time of 6.26; and (C) unknown substance with retention time of 6.97.

4. Discussion

Although the thalli of *Parmelina carporrhizans* and *Parmelina quercina* produce similar secondary metabolites in the field under different ecological conditions, we found that their mycobionts in aposymbiotic cultures produce different secondary metabolites under the same conditions. We found in natural thalli almost the same secondary metabolites as those described above. In addition to lecanoric acid, atranorin, and chloratranorin produced by thalli of both species [46], we found evernic acid in *P. carporrhizans* at a trace level. Notably,

the presence of evernic acid in the natural thallus of *P. carporrhizans* thallus is not reported in the literature. Contrarily, we did not find gyrophoric acid in *P. quercina*, which was reported at a trace level only once by Clerc and Truong [56].

Comparing the secondary metabolites produced by the *Parmelina* lichen thalli and the aposymbiotic cultures of *Parmelina*, we found that completely different metabolites are produced by the isolated mycobiont. In mycobiont cultures, we found confluent acid, norlobaridone as major substances produced in the early and late stages of *P. carporrhizans* and one unknown substance secreted by mycobionts of *P. carporrhizans*, whereas pulvinic acid and unknown substances were detected in *P. quercina*. In *P. carporrhizans*, the metabolites that we found in thalli (lecanoric acid, atranorin acid and chloratranorin) and the metabolites found in the mycobiont in aposymbiotic culture (confluent acid in early cultures and norlobaridone in late cultures) are produced at different stages in the same pathway. Contrarily, the metabolites found in *P. quercina* mycobiont cultures (pulvinic acid), with respect of the metabolites found in natural thalli (lecanoric acid, atranorin acid and chloratranorin), implied a change of the metabolic pathway [57] from the acetate–mevalonate pathway to the shikimic acid pathway via the expressions of different polyketide synthases [25]. Similar results for the variation between secondary metabolites produced by thalli and mycobiont cultures were reported [4,39,44], arguing that the change of biosynthetic pathway also changed metabolite production [57] via the expression of different polyketide synthases [4]. This is attributed to the absence of carbon sources provided by the photobionts [39] or suboptimal environmental conditions that caused stress [4].

The secondary metabolites that we found in the mycobiont of *Parmelina* species in aposymbiotic cultures are reported to be bioactive molecules. Norlobaridone and confluent acid, both produced by *P. carporrhizans* mycobionts are antioxidants and have antimicrobial activity [14,58]. Additionally, the mycobiont of *P. quercina* produces pulvinic acid, which has marked antioxidant properties, and showed photoprotection against UV rays [59]. We can speculate that if these metabolites are also synthesized by mycobionts under natural conditions, they may have an adaptive advantage related to their habitat. The photoprotection offered by pulvinic acid could be useful in the exposed bark of the Mediterranean forests of evergreen *Quercus* species, which *P. quercina* inhabits. Furthermore, the antimicrobial activity of confluent acid could be suitable for *P. carporrhizans*, which adapts to shade and humid places, where fast growing organisms such as bacteria can proliferate. The metabolites produced by the mycobionts of *P. carporrhizans* and the mycobionts of *P. quercina* are different, but interestingly, the secreted substance present in the agar was the same in both species, and this was also the major metabolite found in late cultures of *P. quercina*. Following the same reasoning applied to metabolites found in the mycobiont cultures, the substance secreted to agar by both mycobionts, if it is produced by mycobionts in natural conditions, should be adaptive for both species in their respective habitats. We failed to identify this metabolite, which, based on our observations of the droplets, looks like a phenol; however, we know with certainty that they have a retention time of 6.97 min (Table 1). Further studies are needed to identify the substance secreted by *Parmelina* mycobionts, and if it is confirmed that this substance has bioactive properties, using it would be advantageous since it does not need to be extracted. Although the change in metabolites produced by mycobionts as a result of growth in different culture media has previously been reported [21,60], we observed no differences in the secondary metabolites produced by *Parmelina* mycobionts in aposymbiotic growth between the tested culture media (0.2G-MY, CMA, and Lilly Barnett medium). This result is unexpected. A possible explanation could be that the culture media are not sufficiently different in terms of pH, carbon source, nucleotides and other ingredients that could lead to a change in the secondary metabolism of lichen mycobionts.

We found that different secondary metabolites were produced at different times. Confluent acid was produced as a major substance by the early mycobiont cultures of *P. carporrhizans*, while norlobaridone acid was the major substance produced by the late mycobiont cultures. Similarly, early mycobiont cultures of *P. quercina* produced pulvinic

acid as minor secondary metabolites and a substance with 6.97 min of retention time as a major substance, while the late mycobiont culture produced substances with 5.79 and 6.26 min retention [21] times, both as major substances. As far as we know, this is the first study on the change in secondary metabolites of mycobiont cultures over time. The mycobiont cultures often did not survive for the long time period of 550 days (this study), showing a loss of viability between 100 and 300 days [4,44,45]. Moreover, only few studies analyzed the variation of metabolites in “late cultures”. For example, Cordeiro et al. [60] compared early stages of 2 months with late cultures of 6 months for aposymbiotically grown mycobionts of *Ramalina* species. In this study, we did not find any depsides at the early stage but did find sekikaic acid after 6 months. On the other hand, Fazio et al. [21] studied changes in the profiles of metabolites in mycobionts of the Parmeliaceae lichen (*Parmotrema reticulatum*) for up to 10 months, combining the growth on different culture media with stress to desiccation (which simulates senescence). However, they did not find differences in metabolites between 5 and 10 months.

Our findings suggest that mycobiont cultures could be an alternative source of bioactive secondary metabolites of potential interest instead of natural populations of lichens. Furthermore, the study of the changes in secondary metabolites over time enriches the catalogue of substances produced by mycobionts. Our study also shows that it is possible to maintain cultures for long periods of time (up to 550 days), and current research is solving problems such as pH variation over time in cultures by improving growth [43].

5. Conclusions

We conclude that the aposymbiotic cultures of lichen mycobionts have a high potential as a source of bioactive compounds since they increase the catalogue of substances produced by the lichen-forming fungus, most importantly, without harming natural lichen populations or the environment. Our study shows a longevity superior to that commonly achieved by mycobiont cultures and points to the importance of time (“early” vs. “late” stages) in characterizing the metabolites produced by mycobionts. We not only found additional secondary metabolites in aposymbiotically grown mycobionts but also different secondary metabolites over time. Future studies in mycobiont cultures are needed to better characterize the secondary metabolites produced by more species, under different conditions and at different times. Additional research is needed for the modulation of the cultures to improve the growth rates.

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Conflicts of Interest: The authors declare no conflict of interest.

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