 doi:10.20944/preprints201703.0219.v1

Short Note

Chemosyndrome Variation in Mycobiont Culture and Thallus of *Parmelina carporrhizans* and *Parmelina quercina*

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Abstract: We cultured *Parmelina carporrhizans* and *P. quercina* in Corn Meal Agar and 0.2% glucose Malt Yeast Agar for 160 days. Chemosyndrome of natural thalli and mycobiont cultures were analyzed by HPLC. Lecanoric acid, atranorin, chloratranorin and ergosterol were detected in *P. carporrhizans* thalli, while lecanoric acid, chloratranorin and aliphates were found in *P. quercina* thalli. The secondary methabolites pattern between thalli and mycobiont culture was completely different in both species. Both species secreted the phenalenone myeloconone C in culture media and was also detected in *P. quercina* mycobiont aggregates. Interestingly, the phenolic compounds produced by the mycobiont culture of *P. carporrhizans* are related to those produced by natural thallus by the same biosynthetic pathway, while the chemosyndrome of *P. quercina* mycobiont implies switch of biosynthetic pathway from acetate-polymalonate pathway to shikimic acid pathway, with pulvinic acid as major compound of mycobiont culture. The role of Myelochonone C, confluentic acid and pulvinic acid produced by mycobiont culture is discussed as possible adaptive vantage in field as photoprotective agent or as byproduct result of stressing artificial culture conditions.

Keywords: *Parmelina*, phenols, HPLC, myeloconone C

1. Introduction

Lichen forming fungus produce a wide range of extrolites (secondary metabolites) and whole lichens have had several folk applications as dyes, food, and health-promoting teas by herbalism, Indian Ayurveda medicine and Tradicional Chinese Medicine [1-4]. Many of the interesting lichen substances are aromatic polyketides [5] with marked antioxidative properties [6] conferring: allelopathy properties against bacteria [7-9] and fungi [10,11] and enabling specific pharmacological applications as photoprotection [12,13] cardioprotection [14] neuroprotection [15] antiviral [16] and antitumoral applications [9,17] in mammals by modulating intracellular concentration of hydrogen peroxide [18]. While lichen substances have interest by the properties exhibited, these substances have had importance in lichen determination by identifying lichen substances as a character in chemotaxonomy. In the second half of the 20th century thin layer chromatography (TLC) has been widely used to identify lichen substances, in the last decades the number of identified lichen substances increased by using more powerful methods [5] as high performance liquid chromatography (HPLC), which have higher sensibility to detect phenolic secondary metabolites [19].

Many of these lichen extrolites are biosynthetically related since they are produced in different steps of the same biosynthetical pathway, consisting in a set of substances called chemosyndrome [20]. Lichen substances are classified in three major classes: i) acetate-polymalonate pathway, ii)

 doi:10.20944/preprints201703.0219.v1

mevalonic acid pathway and iii) shikimic acid pathway. [21,22]. The chemosyndrome varies between and within species, depending on environmental conditions as responses of the lichens to their variable environments [5]. Indeed, the phenolic pattern can vary substantially between the mycobiont growing in aposymbiotic conditions and the mycobiont associated with its photosynthetic partner (lichen symbionts). These variations have been explained as differences in abiotic factors (nutrients, temperature, pH) [19,23,24] and the modulating effect of the photobiont [25].

For this study, we choose two sexually obligated species of a small lichen genus *Parmelina* Hale, characterized by containing lecanoric acid, atranorin and chloratranorin - at a trace level - [26]. *Parmelina carporrhizans* (Taylor) Hale and *Parmelina quercina* (Willd.) Hale are morphochemically similar and have Mediterranean distribution but with different ecological preferences. *P. carporrhizans* grows in more oceanic sites with higher incidence in deciduous phorophytes as *Castanea sativa* while *P. quercina* grows in more continental habitats typically growing on perennial oaks as phorophyte. Although it is reported the coincidence of the two species in the same branch tree, the two species rarely coexist.

Our aims are i) characterize the chemosyndrome composition of these two related species by HPLC and ii) comparing the chemosyndrome variation between species and between natural thallus and mycobiont aposymbiotic culture.

2. Results

After 80 days on culture, *Parmelina* mycobiont cultures produced striking brownie drops (figure 1A and 1C) and after 160 days in most of the cases mycobiont aggregates stained the culture medium around the mycobiont culture (figure 1B), in other cases dark brown substances crystalized in the contact zone of agar with mycobiont (figure 1D).

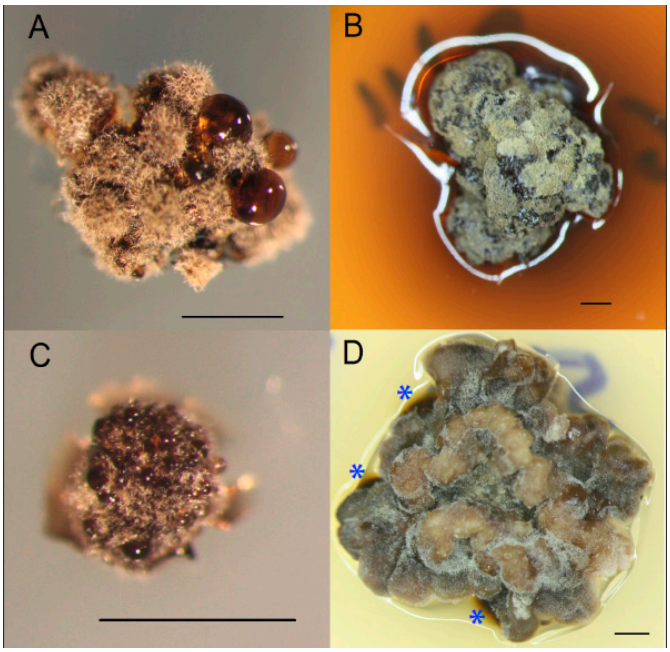

Figure 1. Plurisoporic mycobiont culture in axenic conditions. A) *P. carporrhizans* at 80 days, showing drops of phenolic nature, B) *P. carporrhizans* at 160 days showing agar media stained by myeloconone C, C) *Parmelina quercina* at 80 days showing drops of phenolic nature and D) *Parmelina quercina* at 160 days, * marks crystalized brown substance. Scale bar: 1mm

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The high-performance liquid chromatography (HPLC) showed that the *P. carporrhizans* aggregated on culture (both in CMA and 0.2G-MY) produced as a major compound confluentic acid and others from the acetate-polymalonate pathway (Table 1). However, thallus of *Parmelina carporrhizans* collected from the field contained lecanoric acid, atranorin, chloratranorin, ergosterol and depsides. The *P. quercina* aggregated on culture (0.2G-MY) produced as major compound pulvinic acid (Mevalonic acid pathway) and others substances as 2'-O-Methylphysodic acid and Myelochonone C (Table 1 and Fig. 2). *P. quercina* thallus contained lecanoric acid, chloratranorin and aliphates (Table 1). Both *P. carporrhizans* and *P. quercina* secreted Myelochonone C in culture medium. Unfortunately, we disposed of limited *P. quercina* mycobiont aggregates, and lyophilized samples of *P. quercina* growing on CMA were broken in the travel from Madrid to Frankfurt, and thus we were unable to analyze, for this reason the data from *P. quercina* cultured in CMA is missing in Table 1.

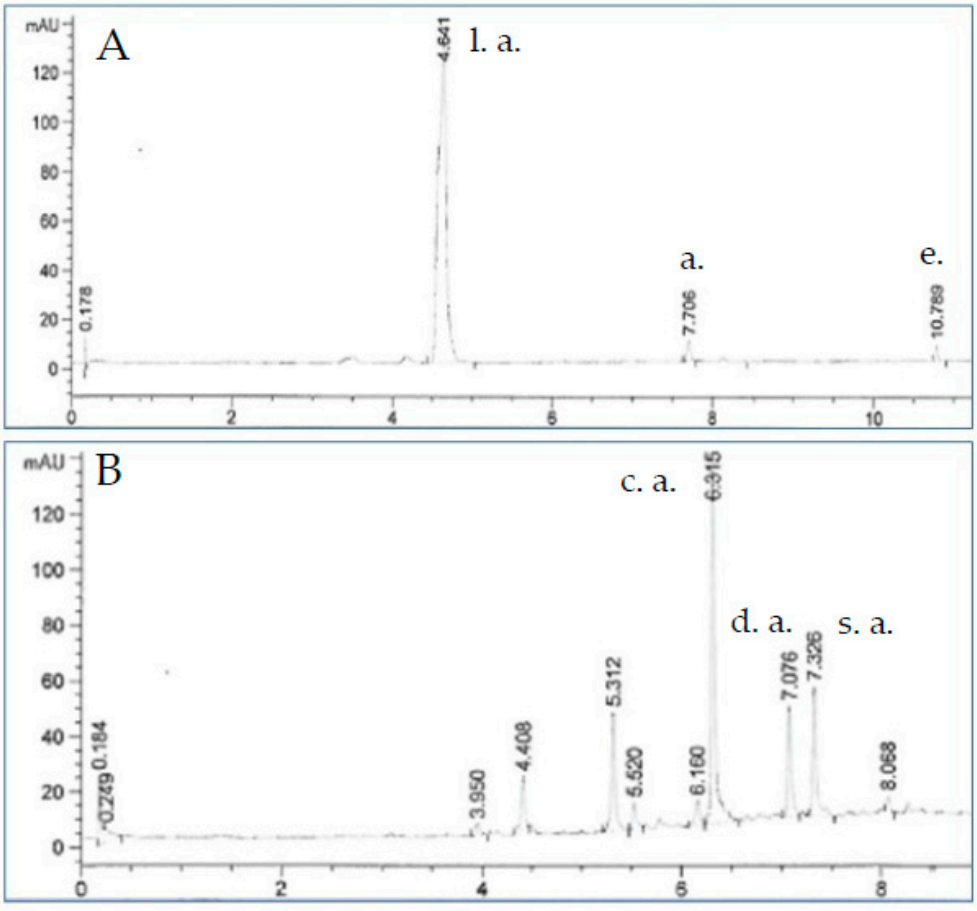

Figure 2. Chemosyndrome of *Parmelina carporrhizans*. A) Chemosyndrome of natural thalli. B) Chemosyndrome of aposymbiotic mycobiont culture. l. a.: lecanoric acid, a.: atranorin, e.: ergosterol, c. a.: confluentic acid, d. a.: decarboxyanziaic acid, and s. a.: stenosporic acid.

3. Discussion

We found similar, almost the same chemosyndrome in *Parmelina carporrhizans* and *Parmelina quercina* in natural thalli as previously described. In addition to lecanoric acid, atranorin and chloratranorin, we found ergosterol in natural thallus of *P. carporrhizans*, which is part of fungal membrane and have antitumoral activity [27]. However, we did not found gyrophoric acid that was reported at trace level by Clerc and Truong [28]. In *P. quercina* thallus, in addition to the lecanoric

 doi:10.20944/preprints201703.0219.v1

acid and chloratranorin we found aliphates. While chemosyndrome was completely different between species in mycobiont cultures, both mycobiont cultures secreted myeloconone C to the media (see Table 1). Myeloconone C was described for first time in thallus of *Myeloconis parva* with the deduced formula (C14H10O7) [29]. The myeloconones are phenalenones [30,31] found also in other lichen species as medullary pigments (Myeloconis [29]; Peltula [32] and Buellia [33]) but we reported here for first time the production and secretion of a myeloconone in mycobiont aposymbiotic culture.

Table 1. Lichen substances and retention time of *Parmelina carporrhizans* and *Parmelina quercina* found in natural thalli and mycobiont culture.

Species	Thallus	Cultured in CMA	Cultured in MY	Secreted to media (MY)
P.carporrhizans	**lecanoric acid[a]** **RT: 4.641**	**confluentic acid[a]** **RT: 6.315**	**confluentic acid[a]** **RT: 6.315**	myelochonone C
	atranorin[a] RT: 7.706	decarboxyanziaic acid[a] RT: 7.076	decarboxyanziaic acid[a] RT: 7.076	
	chloratranorin[a] RT: 8.091	stenosporic acid[a] RT: 7.320	stenosporic acid[a] RT: 7.320	
	ergosterol[c] RT: 10.789	unknown substances[a] RT: 4.394, 5.301, 6.309, 7.073	unknown substances[a] RT:4.394, 5.301, 6.155, 6.309, 7.073	
	unknown depsides[a]			
P. quercina	**lecanoric acid[a]** **RT: 4.641**	missing	**pulvinic acid[b]**	myelochonone C
	chloratranorin[a] RT: 8.091		2'-O-methylphysodic acid[a]	
	aliphates[c]		myelochonone C	
			unknown substances	
			(quinones, depsides, depsidones)[a]	

a: acetate-polymalonate pathway; b: shikimic acid pathway: c: mevalonic acid pathway. Bolded: major substances of the chemosyndrome

Cultures of both species produced and secreted the phenalenone myeloconone C to the culture media, which probably have allelopathic properties similar to those exhibited by plant phenalenones [34]. Moreover, by the photosensitizer effect against oxygen singlet which has been produced several patents (US4803161A [35] US7253206B2, [36] ES 2357926B2 [37]).The secretion of myeloconone C by *Parmelina* mycobiont culture make more easy obtaining the substance. Further research can establish *Parmelina* mycobiont cultures as a myeloconone obtaining system.

Further, the chemosyndrome within species and between the aposymbiotic culture and thallus were different, being different and more numerous the secondary metabolites of mycobiont culture than those produced in natural thalli (Table 1, Fig 2). Similar results of variation of chemosyndrome between thallus and culture were reported [19,25,38] arguing that the switch of biosynthetic pathway changed the chemosyndrome [38] by expression of different polyketide synthases [19]. The chemosyndrome variation between thallus and mycobiont culture can be attributed to the absence of carbon sources provided by the photobiont or suboptimal environmental conditions producing stress. In resynthesis studies, the decreasing amount of substances in culture after photobiont contact was registered [25]. Moreover, the importance of environmental conditions in

 doi:10.20944/preprints201703.0219.v1

chemosyndrome variation in culture was revealed by carbon source in culture media and simulated environmental stress [39] changing the expression of the polyketide synthase genes and chemosyndrome [19]. However, the natural thallus and mycobiont culture can produce the same chemosyndrome [40] at least under certain culture conditions such as at variable temperatures [23,24,39]. It has been seen that the age of the culture can change the chemosyndrome and can allow mycobiont to produce common substances present in lichen thallus for example atranorin, which was produced in culture only after five months and desiccation treatment in *Parmotrema reticulatum* [41].

Attending to major substances, *Parmelina carporrhizans* thalli produced lecanoric acid in natural condition while in aposymbiotic stage it produced confluentic acid. Both substances are related by the acetate-mevalonate pathway being a minor chemosyndrome variation. These are produced in the two culture media accompanied by almost the same chemosyndrome according to Stocker-Wörgötter et al. [40] but contrasting with other previous reports [41]. Conversely, in *Parmelina quercina* the biosynthetic pathway of the major substance produced changes from the acetate-mevalonate pathway in natural thallus (Lecanoric acid) to shikimic acid pathway in aposymbiotic stage (pulvinic acid).

We do don't have a clear explanation of why two close species which produce the same chemosyndrome in natural thallus produced different chemosyndrome in culture. We can speculate that the mycobiont culture chemosyndrome could be very similar to the chemosyndrome of aposymbiotic pre-thallus stage in nature being related to adaptation to different habitats and different ecological valence. In this case make sense that *Parmelina carporrhizans* inhabiting more oceanic habitats is producing confluentic acid with biocide properties [42] very useful in humid places while *P. quercina* inhabiting continental drier habitat is producing pulvininic acid as major compound, which confers vantage by its photoprotective properties and DNA protection against oxidative stress [43] as an adaptation to drought and insolation.

Frequently the environmental conditions as carbon source, PH levels, temperature variation and stress in general modifies the chemosyndrome in mycobiont culture [19,23,24] however *P. carporrhizans* produced confluentic acid as major compound and practically the same chemosyndrome in both media assayed. How much is the relative weight of genetics and environment in determining the chemosyndrome substances of aposymbiotic mycobiont in laboratory culture and natural pre-thallus stage? Further studies are needed to unravel if we can use chemosyndrome variation in mycobiont culture of *Parmelina* to deduce adaptive strategies of aposymbiotic phase in nature or if we can produce in culture a set of different chemosyndromes by changing culture conditions and activating different polyketide synthases.

4. Materials and Methods

4.1 Lichen Material

Fresh thallus of *Parmelina quercina* was collected on *Quercus ilex* in Monte de Las Pinedas, La Carlota (Spain) in 20th of July of 2012 [37º 42' 25.2" N, 4º 55' 47.9" W] at 182m. And fresh thalli of *P. carporrhizans* were collected on *Castanea sativa* phorophyte in Cuevas del Valle (Spain) October 11th of 2012 [40º 18' 28.4" N, 5º 00' 39.0" W] at 1007m.

Vouchers are deposited in the herbarium of the Faculty of Pharmacy at Complutense University of Madrid (MAF-Lich 19117, MAF-Lich 19191, and MAF-Lich 19192).

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4.2 Isolation and culture

We isolated *P. carporrhizans* mycobiont from ascospores following the inverted Petri dish methods of Ahmadjian [44] Apothecia were mechanically cleaned and washed following the protocols established by Molina and Crespo [45] and attached to the inner side of inverted Petri dish lids with petroleum jelly. The plates contained Bold's basal medium (BBM, [46]) for spore germination [47]. Twenty-five to thirty days after spore germination plurisporic mycelia were transferred to new plates on two different culture media: 0.2% glucose malt-yeast extract (0.2G-MY) according to Molina *et al.* [48] and cornmeal agar (CMA) following the manufacturer's instructions (Difco Laboratories, Detroit, MI, USA). Cultures were incubated at constant 20ºC in the dark [44] for 160 days.

4.3 DNA extraction and molecular identification

Prior to DNA extraction, we extracted secondary metabolites with acetone, and then crushed the samples with pestles in liquid nitrogen and extracted genomic DNA with the DNeasy Plant Kit (QIAGEN, Redwood City, California, 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 axenic cultured tissues. The identity of the sequences and specimens were confirmed using the MegaBLAST search function in GenBank. ITS sequences were deposited in GenBank (accession numbers KM357892 and KM357893), and *Parmelina carporrhizans* genome was sequenced using Illumina from these samples [49].

4.4 High-performance liquid chromatography

Approximately 35 mg of pure mycobiont culture and agar pieces of 5 mm^2 were extracted in 0.5 ml of acetone for 60 min and the extract was freeze-dried and sent from Madrid to Frankfurt to be analyzed. Extracts were resuspended with 200 µl methanol at room temperature for 1 hour prior analysis. For HPLC, an Agilent Technologies 1260 quaternary system with incorporated degasser and a DAD was used. The detection of compounds was done using OpenLAB CDS ChemStation software (Agilent Technologies) by comparing spectra and retention time against a library of spectra from authentic metabolites. For more details see [50]

Acknowledgments: This work was supported by the Spanish Ministerio de Economia y Competitividad (CGL2013-42498-P), the research funding program Landes-Offensive zur Entwicklung Wissenschaftlich-Oekonomischer Exzellenz (LOEWE) of Hesse's Ministry of Higher Education, Research, and the Arts through the Senckenberg Biodiversity and Climate Research Centre (BiK-F).

Author Contributions: M.C.M., D.A. and A.C. conceived and designed the experiments; A.M and I.S. performed the experiments; M.C.M., D.A. and P.K.D. analyzed the data; D.A. and M.C.M. contributed reagents/materials/analysis tools; D.A. wrote the paper. All authors discussed the results and revised the manuscript."

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

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