Axenic culture and biosynthesis of secondary compounds in lichen symbiotic fungi, the Parmeliaceae

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Abstract

Lichens produce unique secondary metabolites with a rich potential as active bio-compounds. In many cases, the use of these molecules is limited by the low concentration of these compounds in thalli, low growth rate in culture, and changes in chemical patterns between thalli and aposymbiotic culture. Six lichenized fungi (*Arctoparmelia centrifuga*, *Parmelia saxatilis*, *Parmelina tiliacea, Platismatia glauca*, *Xanthoparmelia tinctina* and *Usnea ghattensis*) with biotechnological interest and belonging to Parmeliaceae have been cultured in order to test culture conditions and obtain sufficient biomass for further studies. In addition, we analyzed the compounds synthetized in axenic conditions and they were compared with chemosyndromes identified in complete thalli. *Arctoparmelia centrifuga, P. saxatilis*, *P. tiliacea* and *X. tinctina* were successfully cultivated while for *P. glauca* and *U. ghattensis* we only obtained sporulation and germination of the spores. The chemical pattern of the compounds secreted into the culture media varied significantly from the chemosyndrome of the whole thallus. A protocol is proposed to obtain alectoronic acid in culture.

Keywords: axenic culture, mycobiont, ontogeny.

A lichen is an intimate and long-term symbiosis consisting of a complex interaction between, at least, a fungus (mycobiont), one or more photoautotrophic partners, and associated bacteria (Meessen & Ott 2013). They are able to produce lichen secondary metabolites such us terpenoids and phenolic compounds, which are deposited in either the cortex (external phenols) or the medulla (internal phenols). Although the molecular mechanisms of interaction of bionts are still unclear (Fontaniella et al., 2004; Legaz et al., 2003; Rikkinen, 2012), throughout the interactions (photobiont, mycobiont and bacteria), whole thalli produce secondary metabolites under a multiple interaction of environmental factors (Stocker-Wörgöter & Elix 2006; Brunauer et al., 2007). Secondary metabolites can determine the ecological interactions of lichens (Asplund and Wardle 2017) and have a rich potential as active bio-compounds such as anti-microbial, anti-bacteria, anti-virus and anti-oxidant activity, anti-cancer (e.g. Ristić 2016). Among the most studied secondary compounds are phenols (Boustie & Grube 2007; Chooi 2008; Shukla 2010; Rankovic & Kosanic, 2015; Yamamoto et al., 2015; Xu et al. 2016; Fernández-Moriano et al. 2016; Cimmino et al., 2019; Dieu et al., 2019; Gómez-Serranillo et al., 2014; Nguyen et al., 2019; Zambare and Christopher 2012; Pereira et al., 2017; Sieteiglesias et al., 2019). Terpenoids, molecules of secondary metabolism from mevalonic acid, can also be bioactive molecules, although there is a scarcer body of evidence (Rehman et al., 2018; Bate el al., 2018).

Unfortunately, a key characteristic of lichen-forming fungi is their very low growth rates (Crittenden et al., 1995), with low metabolic turnover (Grube et al., 2013) compared to most other filamentous fungi under natural conditions. In fact, lichen collection in field for biomedical or industrial use has caused serious problems in the conservation of these organisms (Upreti et al., 2005). This aspect has been a constant problem since first attempts of the large-scale production of their unique secondary metabolites.

Different alternatives have been investigated for production of these lichen secondary compounds: chemical synthesis (Elix et al. 1987), biosynthesis from mycobiont or whole thalli culture on laboratory conditions (Valarmathi and Hariharan 2007), transgenic approaches (Chooi et al. 2008), and inoculation of lichen diaspores on cover slips (Anstett et al., 2014). However, Legaz et al. (2011) indicated that the chemical syntheses of lichen compounds are tedious and energy expensive and the polyketide synthesis through transgenic approaches is in its infancy (Miao et al., 2001). Therefore, mycobiont and whole thalli culture in laboratory have been proposed as the

best option to obtain biomass and bio-compounds, conserving the diversity of the populations. However, in many cases, the mycobiont culture in aposymbiotic conditions alters the phenolic pattern synthesized (e.g. Molina et al., 2003) and it is unknown, to what extent, it modifies the ontogenetic development. It is necessary to continue with the improvement of axenic cultivation techniques to increase mycobiont or whole thalli growth and the production of secondary compounds (Shanmugam et al., 2016).

New culture media and protocols for mycobiont growth have been described in aposymbiotic conditions (McDonald and Gaya 2013, Molina et al., 2015, Rafat et al., 2015), increasing the ontogenetic and reproductive knowledge of these organisms. Also, an inverse relationships between growth and the production of secondary metabolites has been stablished modulated by biotic parameters, such as the presence of the photobiont (Shanmugam et al., 2016), and abiotic parameters, such as pH, temperature, nutrients, carbon source (Deduke and Piercey-Normore 2015; Timsina et al., 2013; Shanmugam et al., 2016). In addition, it is known that in culture, mycobionts produce new compounds (phenols and terpenoids) that have not always been molecularly identified but are potentially interesting (Molina et al., 2013).

Parmeliaceae (Ascomycota, Lecanorales) is one of the largest and most diverse families among lichenized fungi, comprising more than 2700 of mainly fruticulose and foliaceous species, grouped in about 80 genera (Thell et al., 2012). This family is a group of choice for the current study given that certain species have demonstrated to produce biologically active compounds in aposymbiotic conditions (Gulluce et al., 2006; Cobanoglu et al., 2010; Manojlović et al. 2012; Mitrovic et al. 2014). In addition, this is one of the most researched families for phylogenetic studies, and providing a set of successful conditions for axenic growth may be of interest to generate enough biomass for DNA isolation and sequencing of the fungal partner (Alors et al., 2017). *Parmelia saxatilis* and *Usnea ghattensis* can be taken as an example of lichens with demonstrated antimicrobial and antioxidant properties (Rauha et al., 2000; Kosanić et al., 2012; Srivastava et al., 2013; Behera et al., 2005), but the lack of known successful *in vitro* conditions for growth and productions of bioactive compounds are hampering their use (Behera et al., 2009). The same phenomenon has been observed with *Xanthoparmelia tinctina*, with high antiviral potential (Karagöz & Aslan, 2005). Antifungal activities have been reported in this family; this is the case of *Platismatia glauca*, which is postulated as a potential natural pesticide (Gulluce et al., 2006). Due to

their phenolic content, many crude extracts have been screened as antioxidant agents (De Paz et al., 2010; Kosanić & Ranković, 2011; Bakir et al., 2013; Fernández-Moriano et al., 2016; Thadhani and Karunaratne 2017), highlighting the case of *Arctoparmelia centrifuga* extracts (Ranković et al., 2010). Anticancer activity of secondary metabolites from Parmeliaceae lichens has also been explored (Yamamoto et al., 1995; Aravind et al., 2014; Kim et al., 2015; Coskun et al., 2015; Stanojković et al., 2015; Ebrahim et al. 2017). For example, usnic acid, a characteristic metabolite of *Usnea* and *Cladonia* (Abdel-Hameed et al. 2016), but also found in the genus *Parmelia*, is the most studied among lichen substances as candidate for novel cancer therapy and other pharmacological uses (Cocchietto et al., 2002). Preliminary analysis of *Xanthoparmelia tinctina* acetone extracts showed the ability to inhibit maturation of biofilms of the opportunistic pathogen *Candida albicans*, where complex phenolic compounds seem to be the main responsible for this activity (Millot et al., 2017).

Considering the ontogenetic, phylogenetic, metabolic and biotechnological interest of this family, we selected *Parmelia saxatilis*, *Usnea ghattensis, Xanthoparmelia tinctina, Platismatia glauca, Parmelina tiliacea*, and *Arctoparmelia centrifuga* in order to: 1) analyse the ontogenetic development, i.e., the ejection and germination of ascospores, and the monitoring of the development and mycobiont growth; 2) molecularly identify the secondary metabolites secreted to the media by the mycobiont in aposymbiotic conditions; and 3) perform a comparative study between phenolic patterns from whole thalli vs. mycobiont in aposymbiotic condition (cultures), on different organic media.

Material and Methods

Sampling

Details of the selected specimens are showed in table 1. Herbarium specimens are deposited in MAF-Lich and S (Thiers, continuously updated). External morphology of herbarium specimens was examined using an Olympus SZX16 or a Leica MZ 7.5 dissecting microscope. Thalli were air-dried and stored in the dark either at 4ºC and no longer than two weeks (*Arctoparmelia centrifuga* and *Parmelia saxatilis*), or at room temperature and from two weeks to up to four months (*Parmelina tiliacea*, *Platismatia glauca*, *Usnea ghattensis*, and *Xanthoparmelia tinctina*).

Isolation and culture

Multi-ascospore isolations were obtained from apothecia of the lichen-forming fungi, according to the discharged spore method of Ahmadjian (1993). The selected apothecia had a size of 2–7 mm to ensure sexual maturity (Molina et al. 1997) and were carefully cleaned following the protocol described by Molina and Crespo (2000). Twenty apothecia per species were used. The clean apothecia were separately attached to the lid of an inverted Petri dish with petroleum jelly. The bottom part of the Petri dishes contained inorganic Basal Bold Medium [BBM; Deason & Bold (1960)]. Inverted Petri dishes were incubated in a growth chamber at 20ºC in the dark. During the first 24–48 h, the apothecia were allowed to discharge ascospores upwards onto the medium; after this time, they were removed to reduce contaminations by bacteria or other fungi, and new sterile lids were placed on. One to two weeks after first germination was noted, we randomly selected 25 isolated and uncontaminated agar pieces containing several germinated ascospores from each species. Ideally, pieces containing a single ascospore or a single group of germinating ascospores were selected, but sometimes several groups of ascospores were carried on a single agar fragment. For long-term growth and measurements, those pieces, were excised, transported and sub-cultured on different organic media. The media chosen were usually successful in other cultures of lichen forming fungi (e.g. Molina et al., 2013; Molina et al., 2015). They were: 0.2% glucose malt-yeast extract (w/v) [0.2%G-MY; according to Molina et al., (2013)], 2% glucose BBM (w/v) [2%G-BBM; Behera & Makhija (2001)]; Lilly and Barnet medium enriched with 3% glucose (w/v) [3%G-LBM; according to Lilly & Barnett (1951) as modified by Lallemant (1985)] and Corn Meal Agar (CMA) following manufacturer's instructions (Difco, Detroit, MI, USA). All procedures were carried out under sterile conditions in a laminar flow chamber. Cultures were incubated at 20 ± 5ºC in the dark and observed and measured periodically until 200 days. Mycobionts were examined using an Olympus CX40 microscope and a magnifier glass (Nikon SMZ800, Tokyo, Japan). For photography, an automatic ring flash system was attached to the camera lens (CANON 450-D, Tokyo, Japan). The observations were carried out using white light and Nomarsky interference contrast.

Different parameters were calculated to assess the mycobiont ontogenetic development: mean apothecia size, ascospore ejection capacity (after 48 h); capacity of ejection and

germination of plurisporic packs, mean by the number of colony-forming units (CFU) per apothecia; growth capacity; and secondary metabolic production. The growth was analyzed with the program ImageJ (http://imagej.nih.gov/ij/) with images taken under binocular (Olimpus SZ30, Japan).

When significant differences were observed between cultures, we quantified the relative culture area growth rate (Evans, 1972) according to the formula:

$$\text{Relative culture area growth rate } (RC_AGR, \text{ mm}^2 \text{ cm}^{-2} \text{ day}^{-1}) = \ln \left(\frac{Aend}{\frac{Astart}{\Delta t}} \right) \times 100$$

Metabolite composition analysis

Phenols and terpenoids extraction

Samples of 300 mg of dried intact thalli and 30 mg of the dried fungal culture were used to extract internal and superficial secondary compounds. First, they were washed off with 15 ml acetone at room temperature for 5 min. The residue was also macerated with 15 ml of acetone for 5 min. Consequently, both solutions were gathered and filtered through a 45 µm Ico plus 3 filter (TeknoKroma, Spain) and then dried in extraction hood. The filtrate was collected in an HPLC vial after the acetone was completely evaporated. Lichen and culture extracts were solubilized in tetrahydrofuran (THF) in order to have a final concentration at 1 mg/mL, solutions were then filtered (0.45 µm) before TLC and HPLC analysis. Seven lichen compounds (purity >95%) described in the studied lichens and referenced in the REN-LICPD library were used as standards.

TLC analysis

Silica Gel G-60 thin layer chromatography (TLC) plates (Merck) were used for TLC analysis. We carried up the chromatography in three different systems as mobile phase: Mobile: C, toluene:acetic acid = 170:30; G, toluene:ethyl acetate:formic acid = 139:83:8 and B', *n*-hexane:methyl tert-butyl ether:formic acid = 140:72:18. The spot volume was 10 µL and usnic acid, atranorin and stictic acid were used as patterns. After revealing the plates using as spray reagent sulfuric anisaldehide (ANS), they were photographed under ultraviolet light at 254 and 365 nm.

HPLC-DAD-MS analysis and Data processing

Analyses were performed on a Prominence Shimadzu LC-20AD system (Marne La Vallée, France) interfaced with a mass spectrometer ADVION expression CMS. A kinetex C18 column (2.6 µm, 100 × 4.6 mm, Phenomenex) was used as a stationary phase at t° = 40 °C. The mobile phase consisted of H_2O + 0.1% HCOOH as solvent A and ACN + 0.1% HCOOH as solvent B with gradient: 20% of B during 5 min, 20%–80% of B during 25 min, 80%–100% of B during 5 min, 100% of B during 7 min, 100%–20% of B during 3 min, 20% of B during 3 min. The flow rate was 0.5 mL/min and 10 µL of each sample were injected. DAD data were recorded at 254 nm and absorption spectra (210–400 nm) were recorded each second. MS data were obtained in the ESI negative mode using the following parameters: capillary temperature 250°C, capillary voltage 180 V, source voltage offset 20 V, source voltage span 20 V, source gas temperature 50 °C, ion spray voltage 3,5 kV. Advion data express software was used for data evaluation. Identification of compounds was done by comparison of their retention time and MS data with standards and the personal database of the lab REN-LICPD under the same chromatographic conditions.

All spectra were analysed using an automatic and standardized identification's method to offer a better comparison between metabolites found in each media for each mycobiont. Each .datx file was exported as *.cdf with ADVION DataExpress software in order to be read by MZmine 2.52 freeware (http://mzmine.sourceforge.net/). Mass data were detected with a noise level of 2E6. Chromatograms were built with ADAP Chromatogram builder with a minimal number of scans of 4 ; group intensity threshold and min highest intensity of 2E6 and m/z tolerance of 0.5 m/z. Chromatograms were deconvoluted with ADAP Wavelets algorithm. Dereplication was then achieved against two in-house .csv database files. The first file had been built with m/z and RT values of standards and led us to identify fairly well these metabolites whereas the other one contains only calculated m/z values of most known lichen metabolites in order to get a possible match. Dereplicated feature lists were exported as .csv files for further treatment.

Statistical analysis

In order to compare if culture growth depends on media and time, and if underlying errors are all uncorrelated with homogeneous variances, we run a General Linear Model (GLM) with culture growth as a response variable and media and time as predictors.

Multiple Comparisons after Post-Hoc Pairwaise test were used for checking differences between culture growth and data were considered significantly different when $p<0.05$. Statistical analyses were performed with software R (R Development Core Team 2016). We used the package 'glm2' for general linear models. The graphics showed the 95% confidence interval, median average and outliers.

Results

Cultivation of mycobionts

The 20 selected apothecia of all the six species ejected muti-sporic packs and spores isolated in the first 24 hours. Ascospores produced septate hyphae with short cells in BBM in the majority of cases (Fig. 1). The number of multi-sporic group per apothecia ejected and able to product CFUs varied considerably between and within the species. The most important differences in ejection and germination were between *P. glauca* and *X. tinctina*, with a CFU average of 13.65 and 1440 in BBM media, respectively (Table 2). When subcultures where transferred to different enrichment media, interspecific and intraspecific variation of development were observed (Fig. 2 and Fig. 3).

Arctoparmelia centrifuga produced a large amount of ellipsoid ascospores with thickened walls (Fig. 1A), which are often in groups of four, but isolated ascospores can be seen on the medium as well. The initial superficial mycelium was observed with intersepta along the hypha. This species showed significant differences ($p \leq 0.001$) among media, with the highest and lowest growth values at 3%G-LBM and 2%G-BBM, respectively (Fig. 2, Table 3). After 40 days, the axenic cultures experienced significant progressive growth ($P \leq 0.001$) until 200 days, but no interaction between media and time ($P \geq 0.001$) was observed (Fig. 2). In 3%G-LBM the RC_AGR for 200 days was 1.406 ± 0.052 mm^2 cm^{-2} day^{-1} (Table 4) and 0.223 ± 0.102 cm^2 of media area, followed by 0.2G-MY (1.37 ± 0.042 mm^2 cm^{-2} day^{-1}) with 0.155 ± 0.08 cm^2 media area and CMA (1.34 ± 0.040 mm^2 cm^{-2} $day^{-1)}$ and media area of 0.130 ± 0.500 cm^2. Morphological appearance was changed along the experiment in media where growth was observed. Coloration of colonies not varied overtime. Mycobionts of CMA media showed orange pink-colored (5YR; 7/8 Munsell colour system). In media 3%G-LBM and 0.2G.MY colonies were pink (2.5YR, 7/4) and rosewood (10YR, 9/4) colour, respectively, with white aerial hyphae on the surfaces (Fig. 4). This fungus did not grow on 2%GBBM.

Parmelina tiliacea ejected naked and bipolar ascospores (Fig.1B), generally in groups of eight, and we detected different growth depending on media. The mycelial colony was significantly larger ($P \leq 0.001$) in 2%G-BBM and 3%G-LBM than that observed for 0.2G-MY and CMA media (Fig. 2 E,F). The cultures presented a significant increase in their growth from 2 months, then subsequently stabilized from 100 days until analysis had been completed. An interaction between media and time has not been observed in none of the cases ($P \leq 0.001$, Table 3). *Parmelina tiliacea* showed the largest macroscopic development after six months of growth, with 3.05 ± 0.11 mm^2 cm^{-2} day^{-1} RC_AGR on 3%G-LBM (Table 4) and 0.834 ± 0.294 cm^2 media area. Compact growth was observed in every media analysed with brownish coloration (Fig. 4) after 60 days, less 2%G-BBM colonies with orange pink colour (5YR: 7/8). Development on 0.2G-MY media gave rise to brown-coloured (7.5YR: 4/2) cultures with white aerial hyphae with numerous dark brown (7.5YR 2/2) tips and brown (7.5YR 4/2) liquid released by the colonies. Same coloration and morphological structure was observed in mycobiont grew on CMA media but without presence of liquid secretion. A brown (5YR: 3/2) three-dimensional growth was observed on 3%G-LBM cultures with many folds and withe aerial hyphae on the surface

Parmelia saxatilis had naked, globular and thin-walled ascospores, packed together in groups of four or eight (Fig.1C). In 3%G-LBM, it was observed a significant increase ($P \leq 0.001$) in their growth after 100 days (Fig. 2C, D). The interaction between media and time on the growth of the mycobionts was not found in none of the analysed cases ($P \geq 0.001$, Table 3) and the RC_AGR on 3%G-LBM for 200 days was 1.45 ± 0.05 mm^2 cm^{-2} day^{-1} (Table 4) with 0.364 ± 0.172 cm^2 media area. Morphological aspect (Fig. 4E, F, G and H) of CMA and 0.2%G-MY colonies was very similar along the experiment including presence of white aerial hyphae, but colonies growing on CMA (2.5YR: 8/2) not presented the brown coloration showed by colonies growing on 0.2G-MY (7.5YR 4/2) or their light brown (10YR: 5/10) liquid secretion. Development on 2%G-BBM media showed brown-colored colonies (10YR: 4/4) with abundant white aerial hyphae, with presence of a light brown (10YR: 5/10) liquid secretion tip. On the other hand, a compact growth was observed when *P. saxatilis* colonies grew on 3%G-LBM (5YR: 4/2) with very limited presence of white aerial hyphae.

Platismatia glauca ejected naked and bipolar ascospores generally in groups of eight (Fig. 1D). The growth success for this species was the lowest over the 200 days of experiment, scarcely developing in every cultured media, no more than a few cells

divisions in any case analysed (Fig. 3 A, B). There were no significant differences ($P \leq 0.001$, Table 3) on media, time or the interaction between media and tie on the growth of the mycobionts colonies. The development on all media used was poor and no change in coloration was observed (data not shown).

Xanthoparmelia tinctina dispersed naked and bipolar ascospores in groups of eight, where the first hyphae sometimes appeared on both sides of the ascospores (Fig. 1E). The cultured showed significant differences among treatments ($P \leq 0.001$, Fig. 4 C, D), with up to 0.164 ± 0.075 cm^2 generated in 0.2G-MY media. The growth has increased significantly ($P \leq 0.001$) from the beginning to achieve the maximum at 200 days. Morphological appearance of the colonies showed were similar and not varied with the type of media it had grown on (Fig. 4N, P, Q). Every mycelium analysed showed brown pigmentation (5yr: 4/2) since early states of growth with white and long aerial hyphae forming a dense layer on the surfaces.

Usnea ghattensis produced naked and globular ascospores with thickened walls (Fig. 1F) and frequently dispersed as single or double-packed ascospores. These cultures achieved a maximum area of 0.009 cm^2 after 6 months of incubation, reaching the maximum of growing at 20 days. There were significant differences among media ($P \leq 0.001$, Fig. 3E, F), with no development of germinated spores in 2%G-BBM media. During this time, any colonies showed visible coloration for different media (data not shown).

Chemical analysis by HPLC-DAD-MS

The chemical analysis was based on detected ionisable compounds in negative mode ESI. Important differences in chemosyndrome were observed between mycobiont and thalli.

In *A. centrifuga,* seven compounds including α-alectoronic acid, α-collatolic acid atranorin and usnic acid were found to correspond to the compounds detected in natural thalli (Figure X1 ; Table 4 supplementary data). Mycobionts grown in 3% G-LBM synthetized five compounds in this most inductive medium followed by the 0.2% G-MY (three compounds) and the media 2% G-BBM and CMA. The depsidone α-alectoronic acid but also its corresponding diphenylether β-alectoronic acid (Figure X2) were detected in 0.2% G-MY, 3% G-LBM media. One putative compound (m/z 265.1 / Rt

24.9 min) was shared for CMA and 2% G-BBM while another one (m/z 279.9 / Rt 19.5 min) was shared between 0.2% G-MY and 2% G-BBM (Table 4, supplementary data).

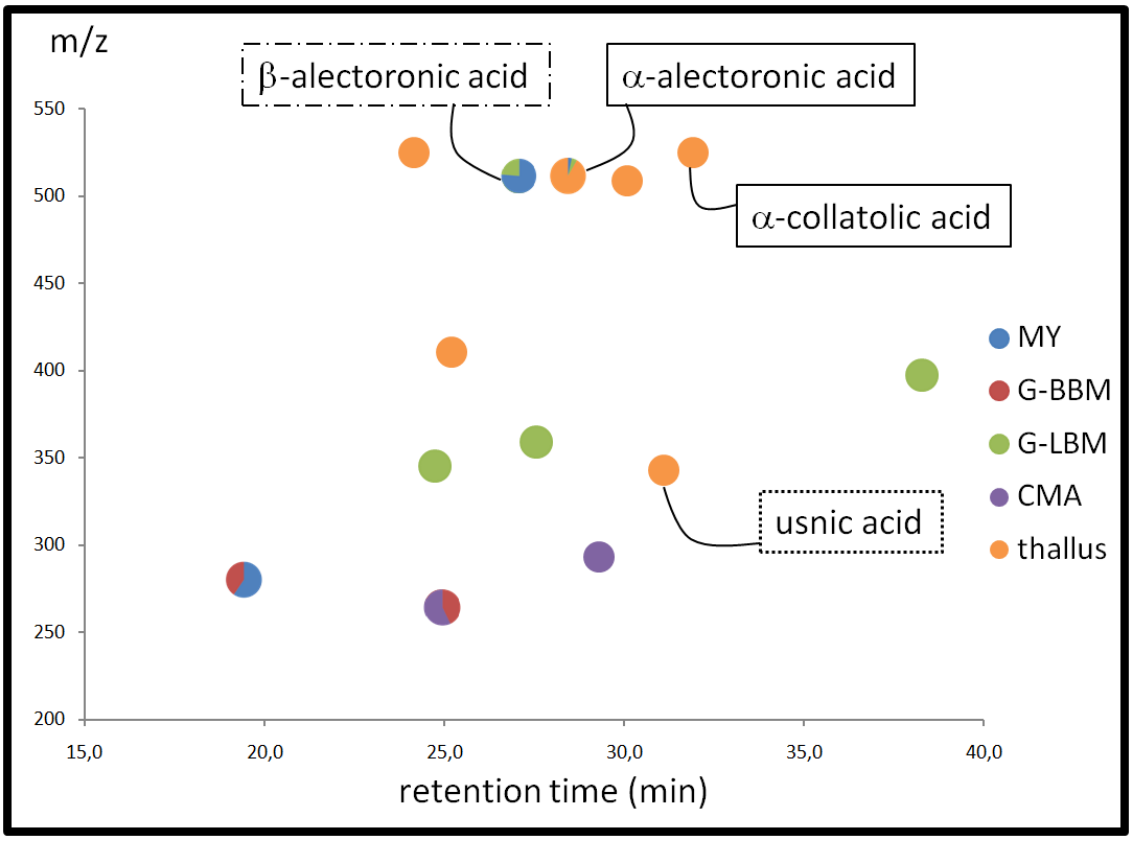
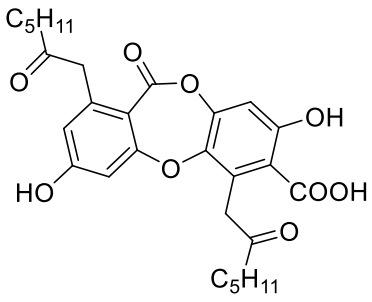
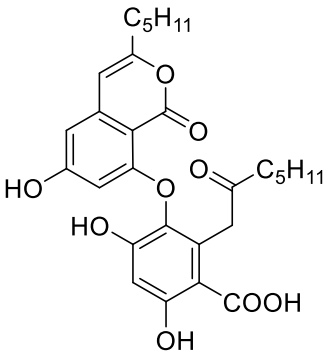


Figure X1. Identification of the metabolites by their retention time (Rt) and their molecular mass in negative mode ESI (reported m/z) for the mycobionts of *A. centrifuga* cultivated in the four medium (CMA, 0.2% G-MY, 3% G-LBM, 2% G-BBM) and the natural thalli. Each frame which surrounds the identity of the metabolites corresponds to a family ▪▪▪▪▪▪▪▪▪▪ : dibenzofuran, ——— : depsidone, · — · – : diphenylether, · · — · · : depside; – – – – : cycloaliphatic compound



α-alectoronic acid



β-alectoronic acid

Figure X2. Chemical structures of the depsidone α-alectoronic acid and its corresponding diphenylether β-alectoronic acid

The depsidones salazinic and lobaric acids and the depsides atranorin and chloroatranorin (only detectable by UV detection) are found in *P. saxatilis* thallus as the major compounds along with nordivaricatic acid (Figure X3; Table 5, supplementary data). Twelve and nine compounds were detected in 3% G-LBM and 2% G-BBM media respectively while five compounds were detected in 0.2% G-MY and only one in CMA. Three compounds were common between the most productive media (0.2% G-MY, 3% G-LBM, 2% G-BBM). Two are suspected to be aromatic compounds belonging to quinone family (solorinic acid), monoaromatic phenol's (2,4-di-O-methylolivetonic acid) and one is unknown from our database. The most productive medium 3% G-LBM revealed the presence of the cycloaliphatic compound aspicilin, the aromatic compounds physodic acid and some compounds like stictic, protocetraric acid derivatives, chromone (roccellin) and steroids are also suspected.

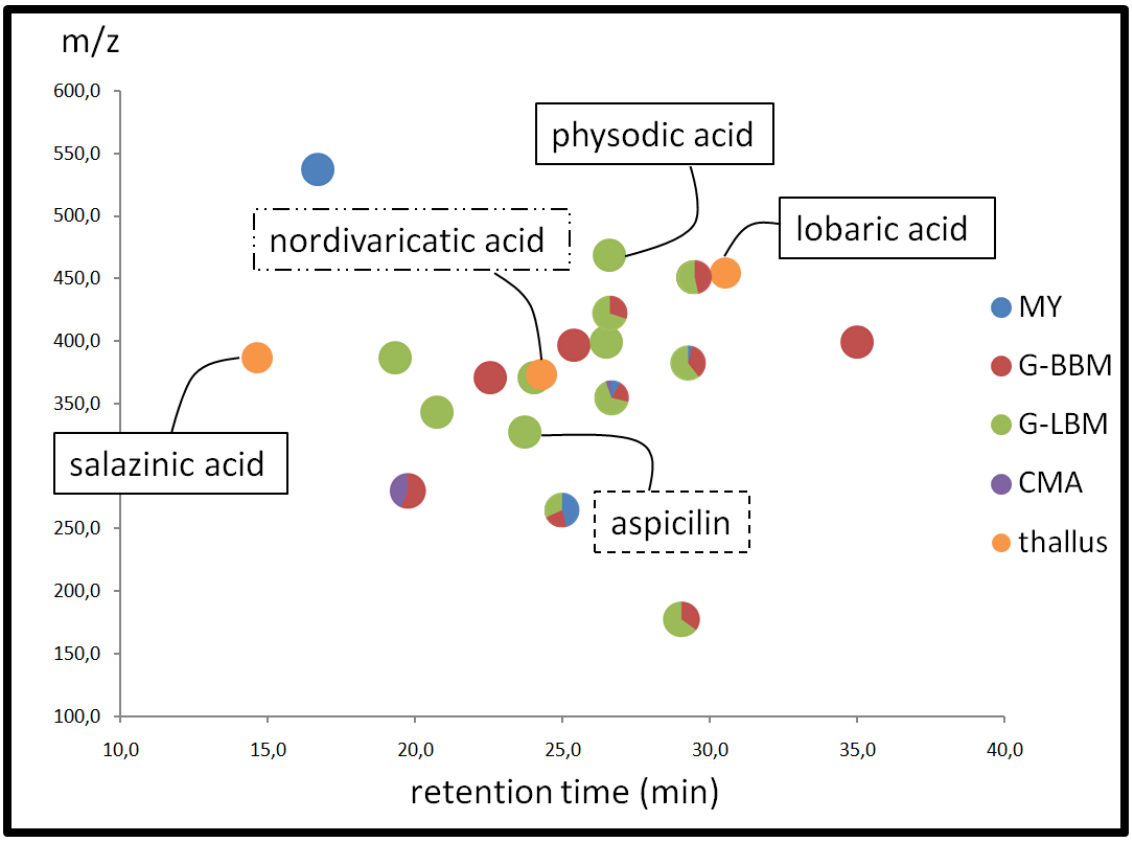


Figure X3. Identification of the metabolites by their retention time (Rt) and their molecular mass in negative mode ESI (reported m/z) for the mycobionts of *P. saxatilis* cultivated in the four medium (CMA, 0.2% G-MY, 3% G-LBM, 2% G-BBM) and the natural thalli. Each frame

which surrounds the identity of the metabolites corresponds to a family▪▪▪▪▪▪▪▪▪ : dibenzofurans, ——— : depsidones, · — · — : diphenylether, · · — · · : depside; – – – – : cycloaliphatic compound

In *P. tiliacea* thalli, four compounds were detected and the depsides lecanoric acid, atranorin and chloroatranorin (only detectable by UV detection) were found to be the major compounds produced (Figure X4 ; Table 6, supplementary data). Cultivation of the mycobionts in 2% G-BBM medium allowed the detection of eight compounds while 0.2% G-MY, 3% G-LBM, CMA media produced respectively six, five and two detectable compounds. Interestingly, α- and β–alectoronic acids and usnic acid were produced in the 3% G-LBM medium. One compound was shared between CMA, 3% G-LBM, 2%G-BBM and 0.2% G-MY media (m/z 265.2/ Rt = 25.0 min). In the productive 2% G-BBM, several putative compounds were detected such as stictic acid derivatives but also nitrogen compounds (scabrosin derivate), the cycloaliphatic aspicilin, the quinone solorinic acid and a pulvinic acid derivative epanorin.

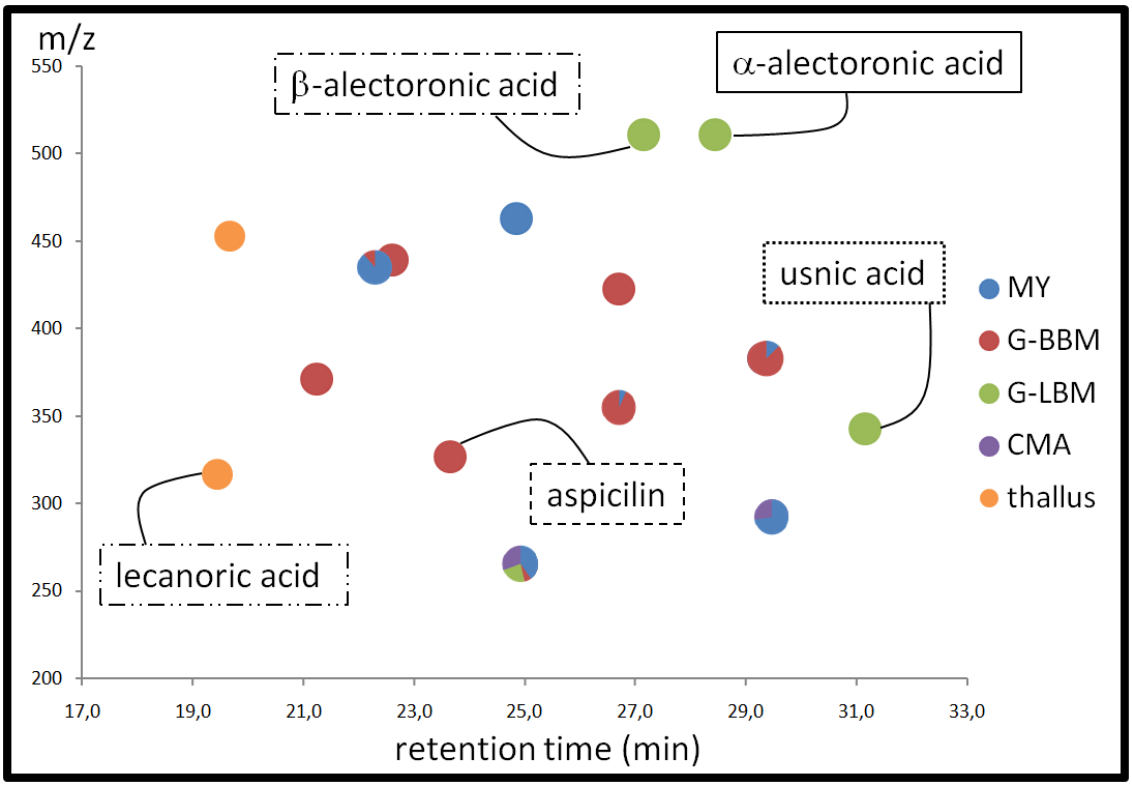


Figure X4. Identification of the metabolites by their retention time (Rt) and their molecular mass in negative mode ESI (reported m/z) for the mycobionts of *P. tiliacea* cultivated in the four medium (CMA, 0.2% G-MY, 3% G-LBM, 2% G-BBM) and the natural thalli. Each frame which surrounds the identity of the metabolites corresponds to a family: ▪▪▪▪▪▪▪▪▪ :

dibenzofuran, ——— : depsidone, ·—·– : diphenylether, ··—·· : depside;
– – – – : cycloaliphatic compound

Three depsidones (protocetraric, norstictic, psoromic acids) and three putative stictic acid derivatives were detected as major compounds in *X. tinctina* thalli (Figure X5 ; Table 7, supplementary data). Cultivation of the mycobionts in 2% G-BBM media allowed the detection of seventeen compounds while in 3% G-LBM eleven compounds were detected, and seven in 0.2% G-MY and CMA. The depside α-alectoronic acid was also detected in the four media while its related diphenylether β-alectoronic acid was detected in three media except in the less productive CMA medium. The dibenzofuran usnic acid was synthesized in 0.2%-MY and CMA media though it was its isomer which was likely identified in the crude thalli. One other aromatic depsidone, the long chain physodic acid was also produced in the 3% G-LBM medium. Aspicilin was also detected in 3% G-LBM medium like for *P saxatilis*. All extracts of mycobionts grown in the four media exhibited the same compound suspected to be 2,4-di-O-methylolivetonic acid (m/z 265.1 / Rt 25.7 min). Several terpenoids were also suspected to be formed in 2% G-BBM medium along with several monoaromatic phenols.

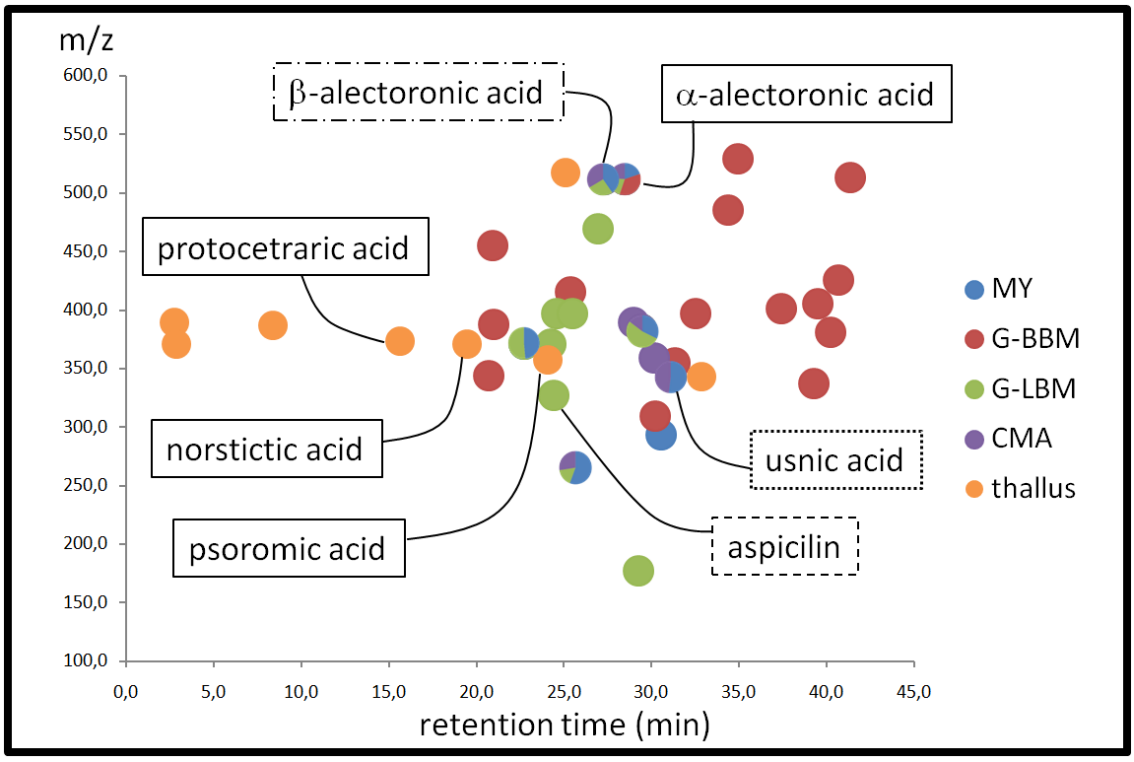


Figure X5. Identification of the metabolites by their retention time (Rt) and their molecular mass in negative mode ESI (reported m/z) for the mycobionts of *X. tinctina* cultivated in the four medium (CMA, 0.2%G-MY, 3% G-LBM, 2%G-BBM) and the natural thalli. Each frame which

surrounds the identity of the metabolites corresponds to a family: ▪▪▪▪▪▪▪▪▪ : dibenzofuran, ——— : depsidone, ·—·– : diphenylether, ··—·· : depside; – – – – : cycloaliphatic compound

Chromatographic analyses showed that atranorin, divaricatic and caperatic acids were detected in *P. glauca* thalli while usnic (as major compound), psoromic and evernic acids were the only metabolites detected in *U. ghattensis* thalli (data not shown).

Discussion

All species analysed showed ability to produce and eject ascospores. The dispersion of ascospores were as groups containing 4–8 ascospores, less in *U. gatthensis* where they were attached to the media as single or double packed. The temporal viability of meiotically produced fungal spores is not well documented (Leavitt & Lumbsch, 2016). However, results showed that, at least in *Parmelia saxatilis, Parmelia tiliacea,* and *Xanthoparmelia tinctina,* most of the spores from asci germinate and develop mycelium. This reproductive strategy in which the spores remain united to generate a new mycelium could assure a greater genetic variability in the future thallus if compared with those models of dispersion of individual spores, for example *Usnea ghattensis*. This hypothesis should be tested with new experimental designs. Ascospores ejected from the ascomata were discharged properly with considerable variation between the species. In most of the cases, more than 300 spores were discharged by apothecia and even reach 1440 ± 968 per apothecia of X. *tinctina*. However, with 13 ± 15 ejected spores, the lichen *P. glauca* presented the lowest number between the studied species. Besides intraspecific variation, this result could be explained by the thallus sampling period, a very important factor for the ascospores isolation in temperate regions of Northern Hemisphere (Yamamoto et al., 2002, Sangvichien et al., 2011). Indeed, samples collected during wet periods give significant better results under laboratory conditions than those collected along dry season (Ahmadjian, 1993; Kranner et al., 2008). The degree of freshness of the specimens can also be a limiting factor. In fact, the fungi that produced less spores were also the ones that had been stored for longer periods. These might be some of the underlying reasons for the unsuccessful attempts to culture some lichen forming fungi (Crittenden et al., 1995; Sangvichien et al., 2011). The germination time is considerable wide and can last anywhere from hours

to weeks, with more time for foliose and fruticulose lichens than crustose forms (Yoshimura et al., 2002). In the species studied, after contact with the surface media, discharged spores germinated between four and five days after being ejected, which agrees with what has been described for other parmelioids (Molina & Crespo 2000; Alors 2017). In relation to development after germination, the sizes of the analysed mycelial colonies were considerably different, both within and among species along the development (Fig. 3 and Fig. 5). Variations were detected depending on media (Molina et al., 2015; Shanmugam et al., 2016) and time (Molina et al., 2015), but no interaction between them in any of the analyzed cases (Table 3), with a notable exception of *P. glauca* with no more than a few cells divisions. The yield in the production of mycelium (Fig. 2 and 3, table 4) for *A. centrifuga*, *P. saxatilis*, *P. tiliace* and *X. tinctina* was similar to that obtained for other lichenized fungi in culture, such as *Physconia distorta* (Molina et al., 2013) or *Anaptichia ciliaris* (Molina et al., 2015) for the same culture media.

High discrepancies in chemical profiles for a given species cultured in axenic conditions appear to be correlated to the culture medium (Behera & Makhija, 2001; Molina et al., 2003). In fact, chemosyndrome variations between mycobiont culture and natural thalli where observed in all species. This can be attributed to change in environmental factors such as nutrients, temperature, pH substrate, day–night cycles, etc. between both conditions (Stocker-Wörgötten, 2008) and the absence of carbon sources provided by the photobiont (Brunahuer et al. 2007). Thus, Shanmugam et al (2016) report that the best condition to obtain the own compounds of natural thallus in axenic cultures is to grow the mycobiont with the photobiont and in the absence of an additional carbon source, something that is an added difficulty. Hager et al. (2008) propose as an alternative to cultivate the mycobiont under temperature and UV-C stress conditions.
A wide range of metabolites belonging to several families (depsides, depsidones, dibenzofurans, diphenylethers, quinones, pulvinic acid derivatives...) were detected in the media whatever the mycobionts. A high number of compounds were detected after cultivation in G-xBM media. One of them α-alectoronic acid (Culberson, 1969; Brodo et al., 2001) was found in several cultivated thalli with or without its relative diphenylether: 3% G-LBM for *A. centrifuga* and *P.tiliacea*, 0.2% G-MY for *A. centrifuga* and all media for *P. tinctina*. The presence of the diphenylether is usually reported as an artefact formed during extraction or by degradation but it could be also a

pathway for its biogenetic synthesis. The presence of α-alectoronic acid is interesting given that this secondary compound has cytotoxic activity (Gordana et al., 2012) and original antibacterial activity acting as an enzyme inhibitor on polo-like kinase1 *(Williams, pharm biol, 2011*). It is interesting to underline that several monoaromatic phenols such as the suspected 2-O-methylolivetonic acid, 2,4-di-O-methylolivetonic acid could be biogenetic precursors of the long-chain depsidones such as physodic, α collatolic and α-alectoronic acids. Moreover, isousnic acid was detected in thalli of *P. tinctina* and its mycobiont can produce usnic acid when cultivated on 0.2% G-MY. Interestingly, the cycloaliphatic compound aspicilin was identified for mycobionts (*P. saxatilis* and *P. tinctina*) cultivated in the same medium 3% G-LBM.

Arctoparmelia centrifuga mycobiont showed compact appearance and fourteen compounds where detected by HPLC-DAD-MS considering all the media analysed. Nevertheless, neither atranorin nor usnic acid, phenolic compounds present in natural thallus, were synthetized on aposymbiotic conditions. The same difference could be observed between the thalli and the mycobionts of *P. saxatilis*, *P. tiliacea* and *P. tinctina*. Deduke et al. (2012) found a significant positive relationship between quantity of usnic acid and atranorin for this species, suggesting that similar environmental characteristics could affect both compounds, even though being produced by different pathways. It is known that usnic acid and atranorin are photoprotective compounds for photobionts in lichen symbiosis (Fernandez et al., 1998; Solhaug et al., 2003).

The fastest RC_AGR was in *P. saxatilis* in 0.2%G-MY and then in *P. tiliacea* in 3%G-LBM. Both stopped growing after 200 and 100 days, respectively. These results are consistent with the theory that the mycobiont would use the carbon source to grow until one or several fundamentals nutrients were missing in the media. From then on, the carbon source could be used to massively synthesize phenolic compounds (Bu'lock 1961, Timsina et al., 2013). Both fungi growing on CMA show a shortage of secondary compounds that could be explained by the absence of an additional carbon source. Corn meal infusion provides nitrogen, vitamins, minerals and amino acids essential for growth, without glucose, while in the other glucose enrich media, a greater number of secondary compounds were detected. On the other hand, it seems that higher sugar concentrations increase osmotic pressure on the mycobiont cells, activating the polyketide pathway (Hamada et al., 1993; Timsina et al., 2013). However, in the case of *X. tinctina* the number and variety of secondary compounds do not appear to be

determined by the culture medium. The results from other authors are also controversial. While Valarmathi et al. (2009) have reported the downregulation of the PKS gene and lower number of compounds produced upon increasing sucrose concentration, Shanmugam et al., (2016) proposed a range of optimal low sucrose concentrations for growth and a range of high concentrations for the synthesis of phenols.

Conclusions

In the present study, we report successful protocols for the growth in axenic conditions of four lichen-forming fungi (*A. centrifuga, P. saxatilis, P. tiliacea* and *X. tinctina*), which will allow the obtaining of useful biomass for biotechnology, phylogenetic studies and other disciplines. We also present a useful method to obtain α-alectoronic acid, a molecule with cytotoxic activity. A significant number of unknown secondary compounds have been detected in these cultures. New research will be necessary to identify them and determine their potential as active biomolecules.

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Table 1 Lichen-forming fungi used to isolate and cultivate the mycobiont, under axenic conditions, and its herbarium code

Species	Herbarium code	Location
Arctoparmelia centrifuga (L.) Hale 1986	S-9639	Sweden, Hälsingland (61°56′25”N, 15°36′36″E)
Parmelia saxatilis (L.) Ach. 1803	S-9551	Sweden, Hälsingland (61°27′47,8”N, 17°05′40,3″E)
Parmelina tiliacea (Hoff.) Hale 1974	MAF-Lich	Spain, Ávila, (N 40 18′ 28.4” W 5 00′ 39.0″)
Platismatia glauca (L.) W.L. Culb. & C.F. Culb. 1968	S-9565	Sweden, Jämtland (62°19′26,0”N, 14°25′46,3″E)
Usnea ghattensis G. Awashti 1986	MAF-Lich	India, Maharashtra (N17 55.19″ E 73 44,04″)
Xanthoparmelia tinctina (Maheu & A. Gillet) Hale 1974	MAF-Lich	Spain, Madrid, San Lorenzo de El Escorial, Silla de Felipe II

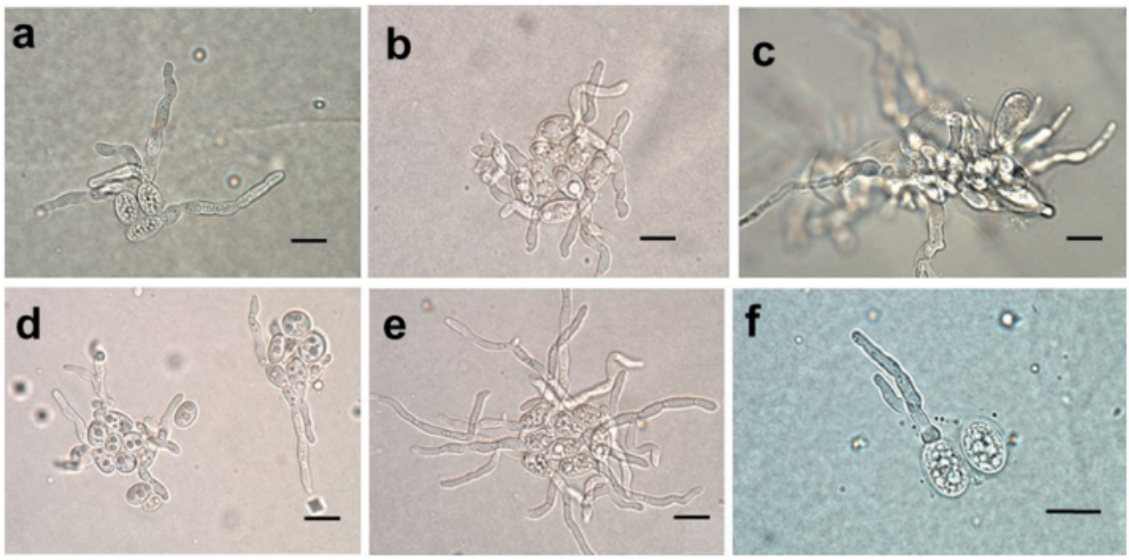

Fig. 1 Ascospores germination after 15 days on BBM from **a** *Arctoparmelia centrifuga*, **b** *Parmelina tiliacea*, **c** *Parmelia saxatilis*, **d** *Platismatia glauca*, **e** *Xanthoparmelia tinctina*, **f** *Usnea ghattensis*. Scale = 10 μm

Table 2 Apothecia diameter size of twenty sample (three thalli or samples by species), spore ejection in inorganic Basal Bold Medium (BBM) and productivity per species. (CFU = colony-forming units in BBM)

Species	Apothecia size (mm)	Spore ejection	Productivity (CFU)
Arctoparmelia centrifuga	5.42 ± 1.44	after 24 h	834.62 ± 539.24
Parmelia saxatilis	3.07 ± 1.81	after 24 h	534.71 ± 453.50
Parmelina tiliacea	6.15 ± 2.18	after 24 h	383.53 ± 420.10
Platismatia glauca	3.85 ± 0.89	after 24 h	13.65 ± 15.58
Usnea ghattensis	3.45 ± 0.48	after 24 h	676.85 ± 718.23
Xanthoparmelia tinctina	3.33 ± 0.61	after 24 h	1440 ± 968.11

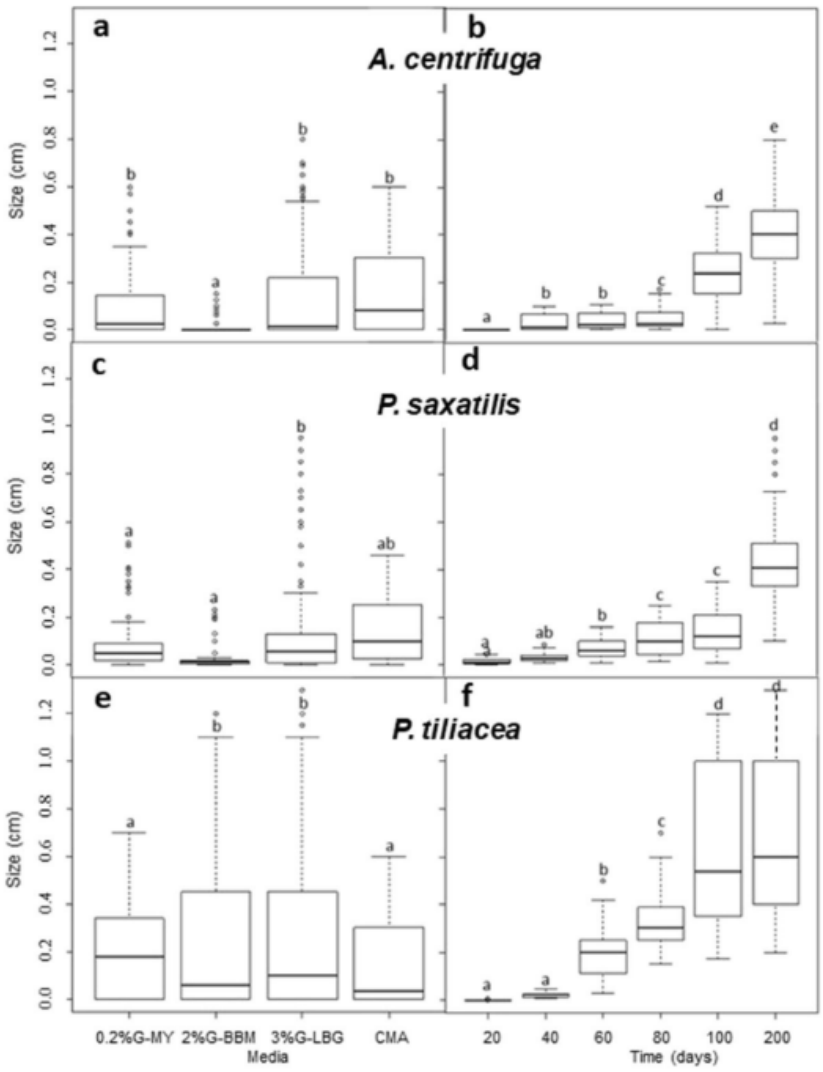

Fig. 2 Mycobiont size (larger diameter) of **a**, **b** *Arctoparmelia centrifuga*, **c**, **d** *Parmelia saxatilis* and **e**, **f** *Parmelina tiliacea*, considering organic media (0.2-MY, 2%G-BBM, 3%G-LBG, CMA), incubation time (0 to 200 days) and the interaction between the two factors. Lower case letter denotes significantly different mean values ($p < 0.05$; Multiple Comparisons after Post-Hoc Pairwaise test). Box extents indicate 95% confidence interval, center is mean average. All assays were done for twenty sub-cultured samples

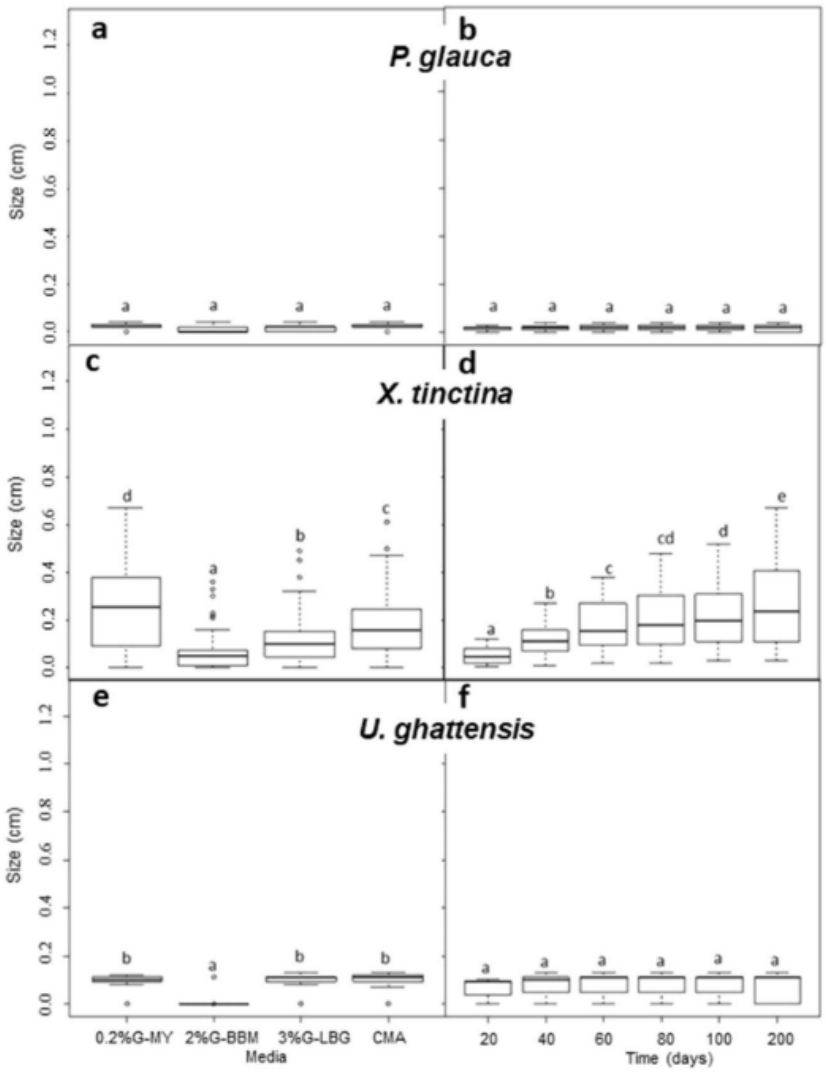

Fig. 3 Mybobiont size of **a**, **b** *Platismatia glauca*, **c**, **d** *Xanthoparmelia tinctina y* **e**, **f** *Usnea ghattensis* considering organic media (0.2-MY, 2%G-BBM, 3%G-LBG, CMA), incubation time (200 days) and the interaction between the two factors. Lower case letter denotes significantly different mean values ($p \leq 0.05$; Multiple Comparisons after Post-Hoc Pairwaise test). Box extents indicate 95% confidence interval, center is mean average. All assays were done for twenty sub-cultured samples

Table 3 Summary of statistic parameters for growth rate of *Arctoparmelia centrifuga, Parmelia saxatilis, Parmelina tiliacea, Platismatia glauca, Xanthoparmelia tinctina, Usnea ghattensis* (considering organic media (0.2%G-MY, 2%G-BBM, 3%G-LBG, CMA), incubation time (0 to 200 days) and the interaction between the two factors

	Arctoparmelia centrifuga			*Parmelia saxatilis*			*Parmelina tiliacea*		
	df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Media	1	79.253	<0.001	1	73.205	<0.001	1	124.313	<0.001
Time	3	6.098	<0.001	3	36.012	0.013	3	3.330	<0.001
Media*Time	3	1.345	0.275	3	0.751	0.522	3	0.538	0.656
D^2	76			87			57		
	Platismatia glauca			*Usnea ghattensis*			*Xanthoparmelia tinctina*		
	df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Media	1	0.555	0.357	1	2.313	0.121	1	25.850	<0.001
Time	3	0.533	0.659	3	8.809	<0.001	3	7.977	<0.001
Media*Time	3	0.469	0.704	3	0.061	0.980	3	0.011	0.998
D^2	37			68			57		

Table 4 Relative culture area growth rate (RCAGR) of *Arctoparmelia centrifuga*, *Parmelia saxatilis*, *Parmelina tiliacea*, *Usnea ghattensis* and *Xanthoparmelia tinctina*, at different media

Species	Experiment		Growth rate ($mm^2\ cm^{-1}\ day^{-1}$)
	Media	Time (days)	
Arctoparmelia centrifuga	0.2%G-MY	0–200	1.369 ± 0.042
	3%-LBG	0–200	1.406 ± 0.052
	2%G-BBM	0–200	0.988 ± 0.082
	CMA	0–200	1.345 ± 0.040
Parmelia saxatilis	0.2%G-MY	0–200	3.140 ± 0.072
	3%-LBG	0–200	1.453 ± 0.054
	2%G-BBM	0–200	1.168 ± 0.063
	CMA	0–200	1.350 ± 0.016
Parmelina tiliacea	0.2%G-MY	0–100	1.380 ± 0.050
	3%-LBG	0–100	3.054 ± 0.107
	2%G-BBM	0–100	2.996 ± 0.089
	CMA	0–100	1.340 ± 0.190
Usnea ghattensis	0.2%G-MY	0–200	1.060 ± 0.017
	3%-LBG	0–200	1.074 ± 0.071
	2%G-BBM	0–200	0
	CMA	0–200	1.079 ± 0.190
Xanthoparmelia tinctina	0.2%G-MY	0–200	1.363 ± 0.067
	3%-LBG	0–200	1.194 ± 0.114
	2%G-BBM	0–200	1.495 ± 0.136
	CMA	0–200	1.264 ± 0.098

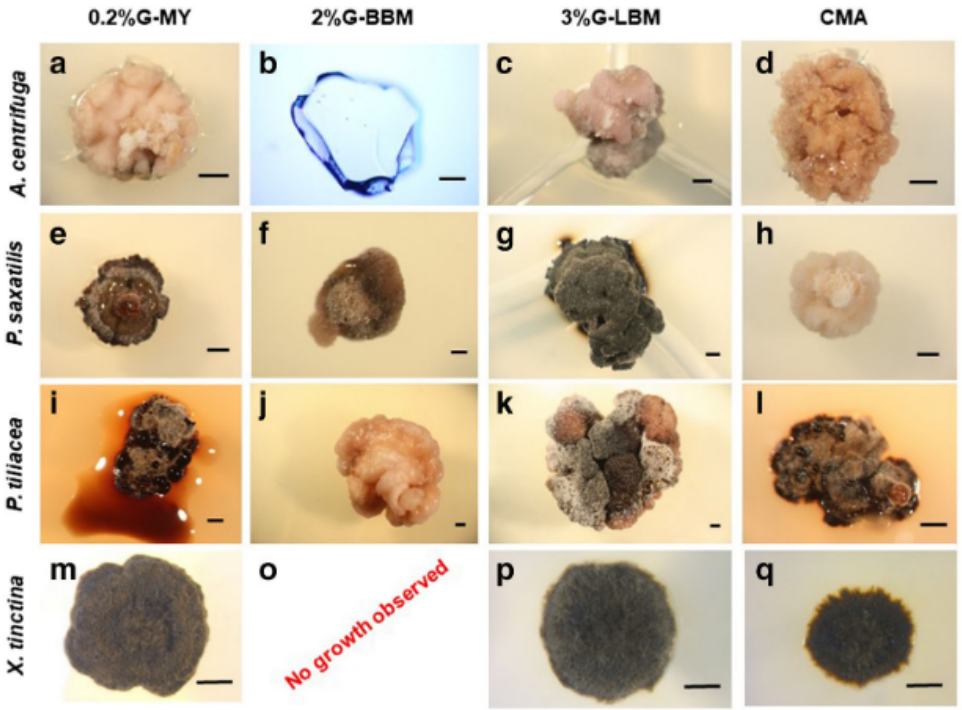

Fig 4 Ontogenetic development of mycobiont cultures on four enriched media after 200 days. **a**, **b**, **c**, **d** *Arctoparmelia centrifuga*. **e**, **f**, **g**, **h** *Parmelia saxatilis* **i**, **j**, **k**, **l** *Parmelina tiliacea*, **m**, **o**, **p**, **q** *Xanthoparmelia tinctina*. Scale = 6 mm

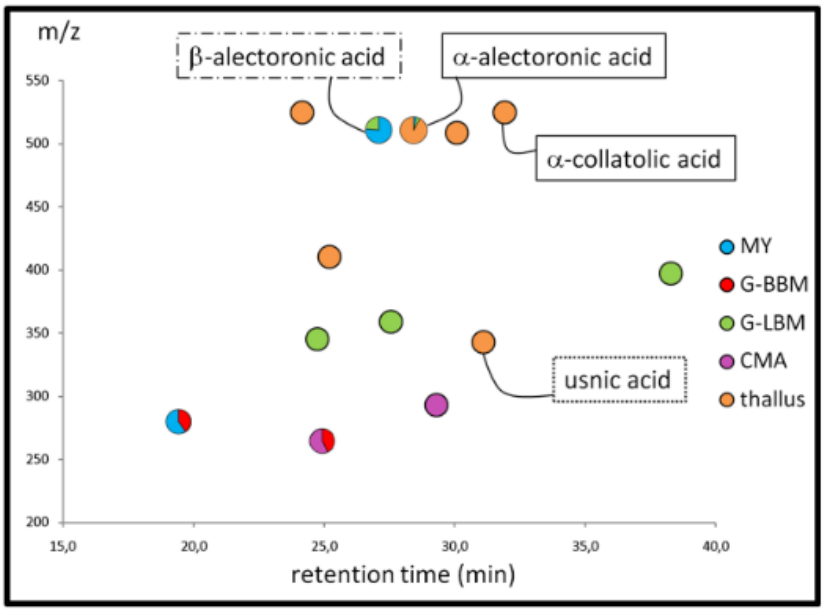

Fig. 5 HPLC-MS detected metabolites and identification of metabolites according to their retention time (Rt) combined to their molecular mass in negative mode ESI (reported m/z) for the mycobionts of *A. centrifuga* cultivated in the four media (CMA, 0.2%G-MY, 3% G-LBM, 2%G-BBM) and the natural thalli. Part size in pie-charts is according to the intensity of peak detection for shared compounds. Each frame which surrounds the identity of the metabolites corresponds to a structural family ▪▪▪▪▪▪▪▪▪: dibenzofuran, ——— : depsidone, · — · — : diphenylether

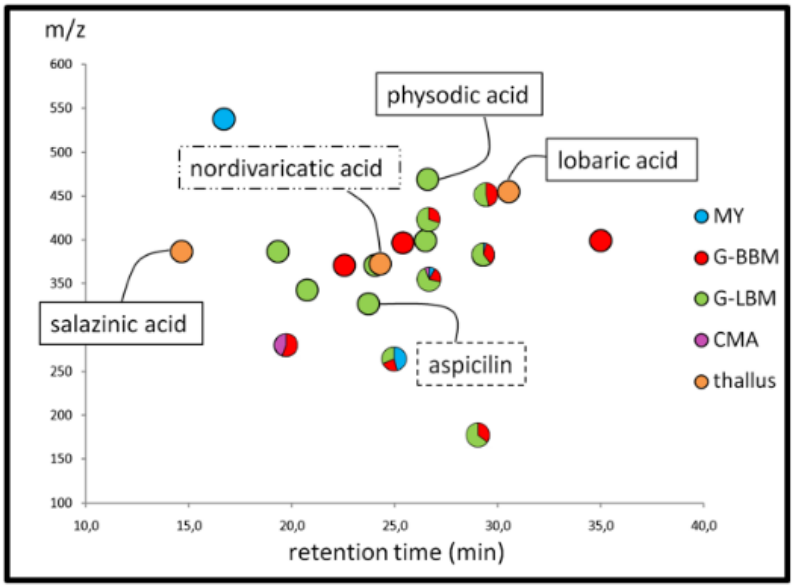

Fig. 6 HPLC-MS detected metabolites and identification of metabolites according to their retention time (Rt) combined to their molecular mass in negative mode ESI (reported m/z) for the mycobionts of *P.saxatilis* cultivated in the four media (CMA, 0.2%G-MY, 3% G-LBM, 2%G-BBM) and the natural thalli. Part size in pie-charts is according to the intensity of peak detection for shared compounds. Each frame which surrounds the identity of the metabolites corresponds to a structural family ——— : depsidone, · · — · · : depside, — — — — : cycloaliphatic compound

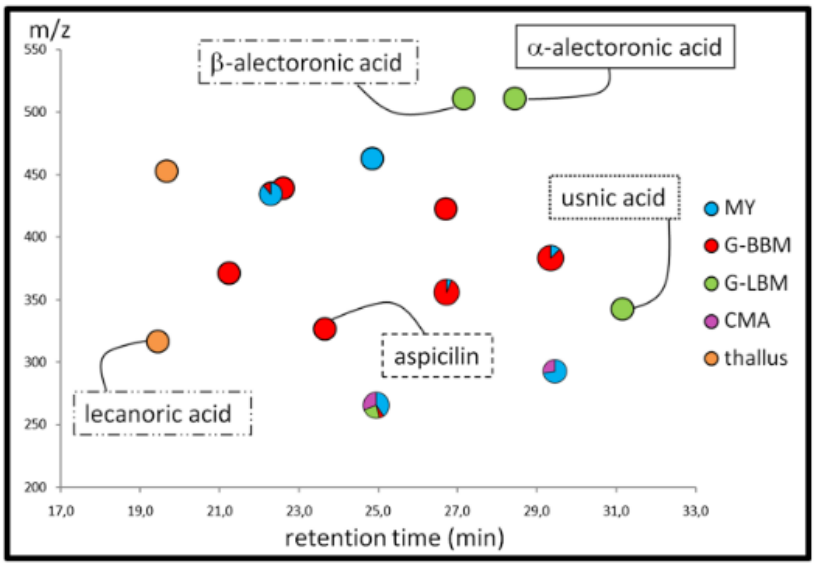

Fig. 7 HPLC-MS detected metabolites and identification of metabolites according to their retention time (Rt) combined to their molecular mass in negative mode ESI (reported m/z) for the mycobionts of *P. tiliacea* cultivated in the four media (CMA, 0.2%G-MY, 3% G-LBM, 2%G-BBM) and the natural thalli. Part size in pie-charts is according to the intensity of peak detection for shared compounds. Each frame which surrounds the identity of the metabolites corresponds to a structural family: ▪▪▪▪▪▪▪▪▪ : dibenzofuran, ——— : depsidone, · — · —: diphenylether, · · — · · : depside; — — — — : cycloaliphatic compound

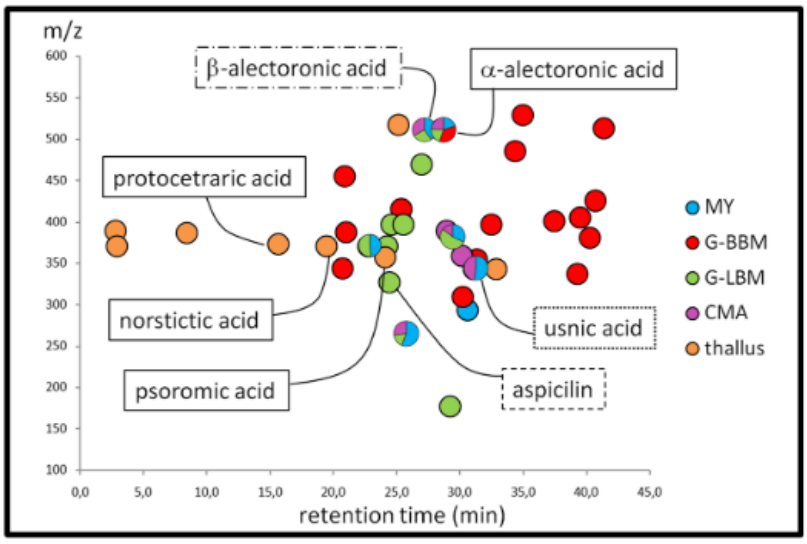

Fig. 8 HPLC-MS detected metabolites and identification of metabolites according to their retention time (Rt) combined to their molecular mass in negative mode ESI (reported m/z) for the mycobionts of *X. tinctina* cultivated in the four media (CMA, 0.2%G-MY, 3% G-LBM, 2%G-BBM) and the natural thalli. Part size in pie-charts is according to the intensity of peak detection for shared compounds. Each frame which surrounds the identity of the metabolites corresponds to a structural family: ▪▪▪▪▪▪▪▪▪: dibenzofuran, ——— : depsidone, · — · — : diphenylether, · · — · ·: depside; — — — — : cycloaliphatic compound