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Plant Physiology and Biochemistry 41 (2003) 175–180
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Original article

Differences in the composition of phenolics and fatty acids of cultured mycobiont and thallus of *Physconia distorta*

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Received 14 April 2002; accepted 9 September 2002

Abstract

Germinated ascospores of *Physconia distorta* on an inorganic medium were grown on an enriched medium (Lilly–Barnet medium with 4% glucose), on which they produced swollen hyphae which included numerous lipid droplets. Secondary metabolites extracted from the cultured fungus were analyzed by high performance liquid chromatography and mass spectrometry. Analyses of superficial compounds established the presence of stearic, linoleic and oleic acids, and the corresponding triglycerides. Malonprotocetraric acid, several unknown pigments, and a series of fatty acids were detected as internal compounds. The qualitative and quantitative phenolic and fatty acid composition from the isolated fungal partner and lichenized thalli were different. Fatty acids were the main substances found by liquid chromatography in extracts of axenic cultures, while extracts from intact thalli contain phenolic acids as the main compounds. These results can be explained by metabolic differences as a result of the different available nutrients.

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Keywords: Axenic culture; Fatty acid; Phenolic acid; *Physconia distorta*

1. Introduction

Secondary products of lichens have attracted investigations for over hundred years and information about their structure, biosynthetic origin and phylogenetic significance has accumulated steadily over this period [5,19]. Phenolic substances of lichens probably regulate interactions between mycobionts and photobionts in the lichen thallus. For example, usnic acid produced by the mycobiont can be translocated to the photobiont cells where it behaves as a competitive inhibitor of urease in a range of concentrations similar to those found in lichenized algae [2]. Other enzymatic activities affected include arginase and other enzymes of polyamine metabolism in *Evernia prunastri*, which are regulated by evernic and usnic acids and chloroatranorin, depending on the concentration of these compounds in the thallus [23,32]. Furthermore, lichens are rarely eaten by herbivorous animals since phenolic compounds seem to have an important antiherbivorous effect [22].

Phenolic substances are used in constructing taxonomies [5,27], although intrathalline [11] and intraspecific variations [9] must be considered. In this way, Reyes et al. [34] show a close correlation between environmental factors and lichen metabolism (isozymes and phenolic acids).

Secondary lichen products represent a significant proportion of the thallus dry weight, commonly constituting 0.1–5% (w/w), even more in some cases [12]. Therefore, a large amount of lichen material is needed to extract industrially exploitable amounts of lichen polyphenols. A driving force in the development and maintenance of aposymbiotic lichens has been the collection of bioactive lichen sub-

Abbreviations: DCPIP, 2,6-dichlorophenol-indophenol; HPLC, high performance liquid chromatography; LBM, Lilly and Barnett medium.

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stances, though the growth of the corresponding biont is generally slow under culture conditions. Frequently, phenol production differs both qualitatively and quantitatively from that seen in intact thalli in nature [21,24]. These differences may arise for several reasons: osmotic conditions [16], nutrient supply [17], the physiological state or culture age [38], culture conditions [15], or the presence of a photobiont [14]. However, low resolution detection methods or inadequate extraction methods could also be an artefactual cause of these differences. Sometimes, a mycobiont in axenic culture is able to produce the same phenolic acids as the intact thallus [18] depending on culture conditions.

Physconia distorta is a lichen-forming fungus belonging to the Physciaceae family. Information about the composition of the intact thallus and the cultured fungus in phenolic compounds is limited [4]. Similarly, there are few studies on the development of *Physconia* sp. isolates under axenic conditions [30]. Here, we compared the production of fatty acids and phenolic compounds by the cultured fungal partner and their occurrence in the intact thallus of this lichen species and found quantitative and qualitative differences [34].

2. Results

2.1. Mycobiont development in axenic culture

After 4 months of growth on the culture medium, dark colonies of *P. distorta* mycobiont had developed (2 cm diameter). These colonies showed a zone of aerial growth with a pigmented inner core and an external, unpigmented hyphal zone (Fig. 1A), as well as a growing zone inside the medium. Vertical sections showed that the aerial zone was formed by broadened and globular cells with dark pigments, while the inner medium zone was formed by unpigmented hyphae and longer intersepta (Fig. 1B).

2.2. Metabolite production in axenic culture

In axenic cultures, malonprotocetraric acid (a β-orcinol depsidone) and two pigments, with an absorbance spectrum similar to a coronatoquinone, were the main phenolic acids (Fig. 2). Malonprotocetraric acid was only detected using

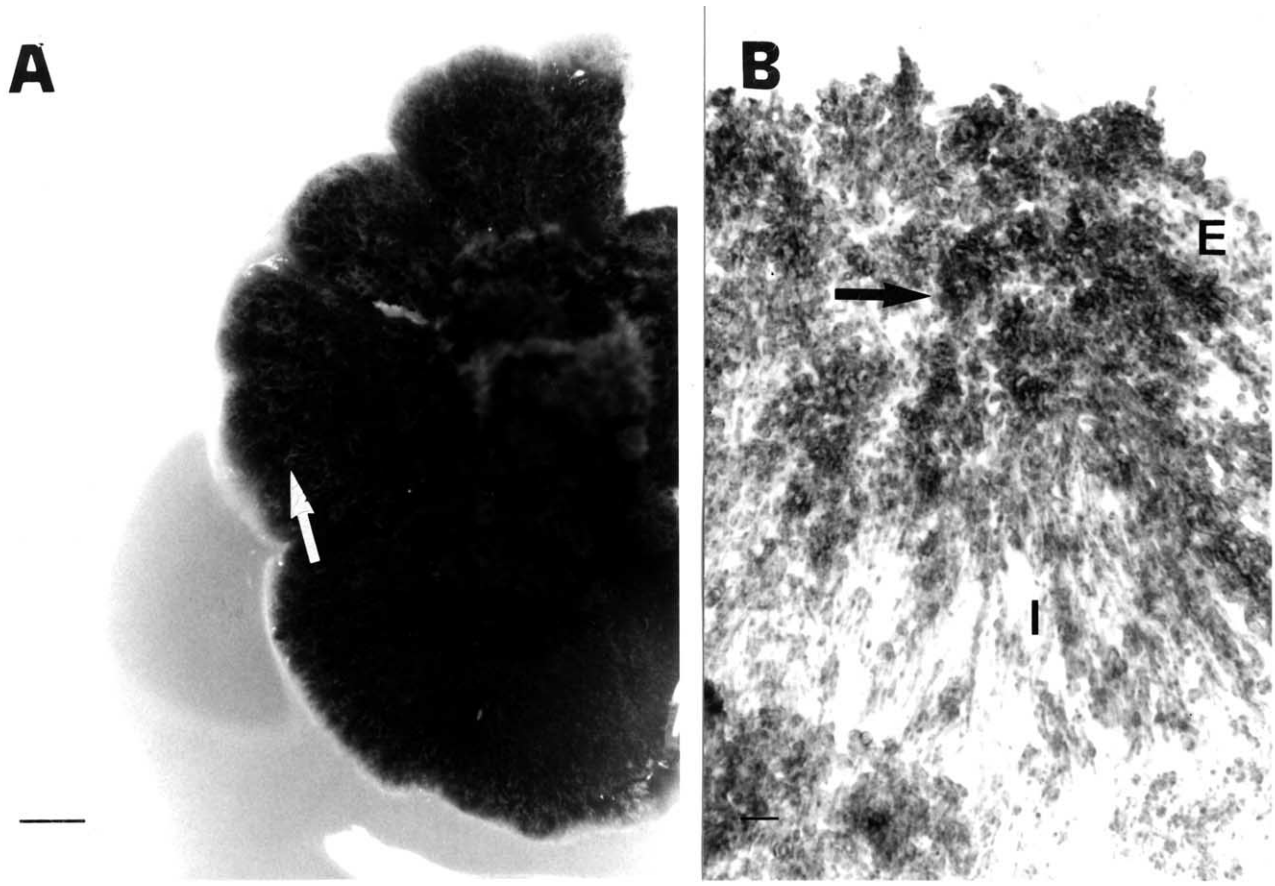

Fig. 1. Development stages of *P. distorta* grown under axenic conditions. (A) Culture after 4 months on LBM 4% glucose. The arrow shows distal hyphae without pigment. Bar = 2 mm. (B) Transverse section of culture after 4 months on LBM 4% glucose; E, external area, I, internal area. Arrow shows accumulation of dark pigments. Bar = 30 μm.

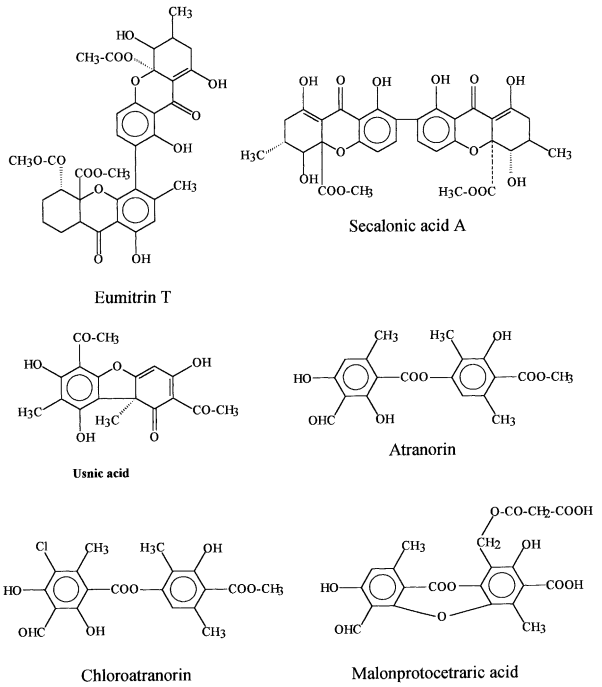

Fig. 2. Structural formulae of main phenolics naturally occurring in *P. distorta* thalli.

mixtures of several organic solvents as extraction solvent, as indicated above. When phenolic compounds were removed using only ethanol, malonprotocetraric was not detected. Oleic and linoleic acids were the main substances detected by high performance liquid chromatography (HPLC) in extracts of axenic cultures, and both the compounds were removed with acetone alone. Stearic acid was detected by ^{1}H-NMR spectroscopy.

Organic extracts obtained from thalli and analysed by HPLC contained two *bis*-anthraquinones (eumitrin T and secalonic acid A), usnic acid, and two depsides (atranorin and chloroatranorin) as the main inner phenolic compounds (Fig. 2). These latter two compounds were also found as the main superficial phenols.

2.3. Effect of a reducing environment on metabolite production by intact thalli

When thalli were incubated with a reducing agent, such as 100 mM ascorbic acid, the concentration of phenolic acids diminished over time, whereas that of the fatty acids increased (Fig. 3, Table 1). The concentration of ascorbic acid in the medium diminished from the initial value (100 mM) to 54 mM after 6 h, and decreased to 32 mM after 12 h. Thalli absorbed a small quantity of ascorbic acid, increasing its content in control thalli from 0.5 μg mg^{-1} dry weight (1.13 μM) up to 2.7 μg mg^{-1} dry weight (6.10 μM) after 6 h and to 3.6 μg mg^{-1} dry weight (8.14 μM) 12 h later. Concentration values of ascorbic acid in rehydrated thallus were calculated by assuming a thallus density of 0.4 mg ml^{-1} [36].

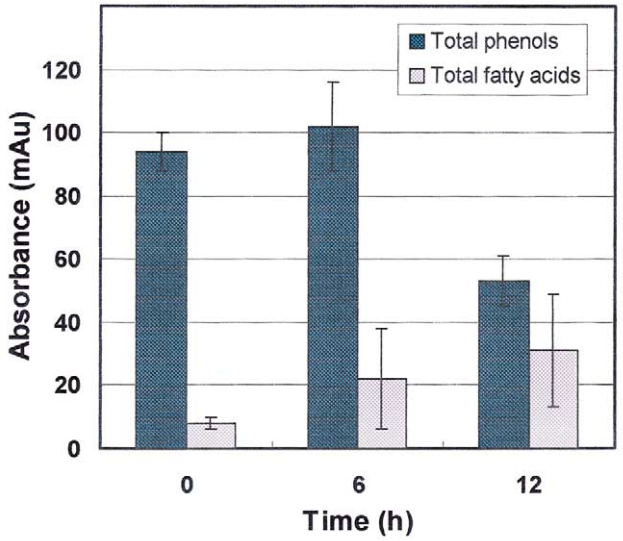

Fig. 3. Total phenols and fatty acids from *P. distorta* natural thalli after incubation on 100 mM ascorbic acid for 0, 6 and 12 h.

3. Discussion

Our results reveal differences between the cultured mycobiont and the intact thallus. Intact thalli contained several phenolic acids, such as atranorin and chloroatranorin, which are structurally related and have methyl-3-orsellinic acid as a common precursor [28], usnic acid, biosynthesized from methylphloroacetophenone [35] and two *bis*-anthraquinone derivatives, eumitrin T (Elix and Wardlaw, unpublished) and secalonic acid A. Yang et al. [37] showed that eumitrins were homologous compounds, structurally related to secalonic acid A. The biosynthesis of secalonic acid and eumitrins is discussed by Elix and Whitton [6]; both series of compounds are yellow pigments with mycotoxic activity. Secalonic acid A is not one of the more common metabolites present in lichen-forming fungi, and has been used as a systematic character supporting the establishment of the genus *Flavoparmelia* [13], and also for species separation [26].

Dark pigments were observed in axenic cultures of *P. distorta* mycobiont after 4 months of growth (Fig. 1A). These pigments were located in the core of the colony. The external area showed distal hyphae without pigment (Fig. 1B).

In contrast, the main phenolic metabolite extracted from axenic cultures of *P. distorta* was malonprotocetraric acid, a β-orcinol depsidone not closely related biosynthetically to the other phenols extracted from intact thalli. Elix and Yu [7] suggested that hypoprotocetraric acid is a precursor of malonprotocetraric acid.

Two pigments of unknown structure were also separated by HPLC. Their absorbance spectra are similar to that of coronatoquinone, the structure of which has been recently elucidated [10].

The more obvious metabolic difference between intact thalli and axenic cultures in *P. distorta* was the presence of fatty acids (oleic, linoleic and stearic acids) and their triglyc-

Table 1
Phenolic acids and fatty acids (absorbance units) from *P. distorta* thalli incubated on 100 mM ascorbic acid for 0, 6 and 12 h

Phenols	Retention time (min)	Incubation time (h)		
		0	6	12
Phenolic acids				
Eumitrin T	17.61	2.25 ± 0.18	_	_
Methyl β-orsellinate	19.50	_	7.25 ± 0.64	9.50 ± 0.83
Secalonic acid A	23.25	9.25 ± 1.05	1.5 ± 0.12	1.0 ± 0.09
Usnic acid	27.11	6.00 ± 0.58	1.0 ± 0.12	4.5 ± 0.34
Atranorin	27.87	65.25 ± 5.42	80 ± 10.42	28.75 ± 2.43
Chloratranorin	28.66	10.50 ± 1.10	12.0 ± 1.05	6.0 ± 0.54
Fatty acids				
Fatty acid A	23.79	3.0 ± 0.28	4.0 ± 0.33	7.0 ± 0.57
Fatty acid B	24.13	2.0 ± 0.16	5.25 ± 0.48	6.75 ± 0.55
Oleic acid	30.62	_	2.0 ± 0.34	2.0 ± 0.37
Linoleic acid	30.96	2.75 ± 0.30	11.25 ±1.06	11.25 ± 1.12

eride derivatives, in agreement with the large lipid drops observed on culture [30]. The presence of remarkable amounts of fatty acids in the axenic culture, which was not observed in the intact thallus, suggests a different metabolic regulation in each case, as a consequence of the available nutrients, as proposed by Hamada et al. [17] and Kon et al. [21]. These authors described qualitative differences and an increase in the amount of secondary products when lichen fungi grow on enriched media. Molina et al. [30] showed the occurrence of lipidic drops when *Xanthoria parietina* cultures were grown on 4% glucose. At least, a physiological hypothesis that explains these results has been put forward.

One of the main differences between intact thalli and aposimbiotic cultures is the carbon source. Lichen-forming fungi with green algae as photobiont use ribitol, while aposymbiotic cultures were grown on 4% glucose. In a glucose-enriched medium, the amount of NAD(P)H in thalli would be high as a consequence of the oxidative metabolism of glucose through glycolysis and the Krebs cycle. Under these conditions, the enzyme fatty acid synthase is favored. However, in the intact thalli, the reducing power would be expected to be significantly less since the available nutrients are much more reduced than those in the case of axenic cultures where a glucose supplement was provided. In this case, when an aromatic synthase is working to produce lichen phenols, an enhanced production of NADPH is not essential [5].

It can be then expected that a reducing environment, artificially produced by supplying a powerful reducing compound, such as ascorbic acid, to lichen thalli, must then enhance fatty acids' production. Caviglia and Modenesi [3] reported the occurrence of ascorbic acid in lichens, that could be metabolized by different ways: (a) as an antioxidant by rapidly removing different active oxygen species generated during aerobic metabolism and during exposure to pollutant and herbicides; (b) as a direct electron donor in photosynthetic (for example, to de-epoxydase enzyme) and mitochondrial electrons transport and (c) as a cofactor in several enzymatic reactions and in the ascorbate–glutathione cycle. In order to determine the cause of the low amount of fatty acids in the lichen thallus, ascorbic acid was used to produce a reductive internal environment in the lichen thallus.

When ascorbic acid in thalli is increased from 1.13 to 6.10 μM, an increase in the amount of fatty acids occurred. In parallel, a decrease in the phenolic acid total was observed (Fig. 3, Table 1). Ascorbic acid is a potent reductor and antioxidant [3,20] which could be used as a reducing agent to produce NADPH via glutathione reductase and so to increase the NAD(P)H pool in the thalli [3,33], leading to an activation of fatty acid synthase [5]. The results of this enhanced activity are shown in Fig. 3. The marked decrease in the amount of ascorbic acid in the medium when lichen thalli were incubated on it can not only be due to an uptake of the compound by the lichen thallus but also to an auto-oxidation regulated by cations [33].

Our results show differences in both the phenolic products and the fatty acids produced by *P. distorta* when grown alone in axenic culture as compared with the intact lichen thallus. The production of these two classes of compounds seems to be interconnected since both biosynthetic pathways use the same precursor, acetyl-CoA. In the intact thallus, the concentration of NADPH is expected to be lower, and then acetyl-CoA is mainly used in the biosynthesis of phenolic compounds, while fatty acid biosynthesis by pure cultures of the fungus is enhanced by increased levels of NADPH.

4. Methods

4.1. Lichen material

P. distorta (Witk.) J.R. Laundon thalli growing on *Castanea sativa* L. were collected from the Sierra de Gredos (Ávila, Spain) in 1998. Thalli were air-dried and stored at room temperature in the dark, not longer than 2 weeks. Voucher specimens are preserved in MAF 6875.

4.2. Isolation and culture of lichen mycobionts

Isolated multispores (i.e. groups of ascospores discharged together from a single ascus) were obtained from apothecia

of *P. distorta*. Fungi were grown from discharged spores following Ahmadjian [1]. After germination, uncontaminated multispore isolates were transferred to 4% (w/v) glucose LBM, pH 5.30 [25]. Inoculated plates were incubated at 18 ± 2 °C for 4 months, using the method described by Molina and Crespo [29]. Thalli and cultures that had previously been weighed were cut into vertical sections 15 μm thick with a Slee MTC cryostat at the times indicated, and then stained with lactophenol cotton blue. Periodically, mycobionts were examined using a Wild M8 Leica magnifying glass and a Leitz DMR light microscope. An automatic ring flash fixed to the camera lens was used to obtain micrographs.

4.3. Phenols and fatty acid extraction

Samples of 300 mg of dried intact thalli and 30 mg of the dried fungal culture were used for internal and superficial phenolic acid extractions by the method of Pedrosa and Legaz [31]. Superficial fatty acids and phenols were first washed off with 15 ml acetone at room temperature for 5 min. The acetone extract was dried, and the dry residue redissolved in 1 ml of methanol and analysed by HPLC. The residue was macerated with 15 ml diethyl ether/ethyl acetate (65/35, v/v) for 15 min, filtered through Whatman No. 2 filter paper, and then harvested. Solid residues were then macerated twice with 15 ml chloroform/acetonitrile (60/40, v/v) for 15 min. Both ether and chloroform phases containing inner phenols were collected in the same tube and the solvents evaporated. The dry residues were redissolved in 1 ml of methanol and then analysed by HPLC.

4.4. Metabolite composition analysis

HPLC analysis was carried out by the method of Elix et al. [8]. A Hewlett Packard series 1050 system, a phenomenex Hypersil 5C18 column (250 × 4.6 mm), and a photodiode array detector operating at 220 and 254 nm were used. A flow rate of 1 ml min^{-1} has been used. Two solvent systems were employed: 1% aqueous orthophosphoric acid and methanol (L) in the ratio 3/7 (v/v) and methanol (M). The run started with 100% L and was raised to 58% M within 15 min, then to 100 M for a further 15 min, followed by isocratic elution in 100% M for a further 10 min. Fatty acids were determined by their corresponding high resolution mass spectra. The chemical structures were deduced from the corresponding ^{1}H NMR and UV spectral data [8].

4.5. Thalli floated on ascorbic acid

Samples of 300 mg of intact lichen thalli were floated on 100 mM ascorbic acid for 0, 6 and 12 h at 25 °C. To test reproducibility, three different assays were carried out. Superficial and inner metabolites were extracted as indicated above. The total phenolic compounds and total fatty acids were obtained by adding the superficial and inner extraction values together. Ascorbic acid amounts on the incubated medium and in lichen samples were estimated by DCPIP-coupled redox reaction according to the method of Relimpio et al. [33].

Acknowledgements

We wish to thank David L. Hawksworth for critically reading the manuscript. This project has been supported by the Spanish Ministerio de Ciencia y Tecnología project PB-98-0774 and by a grant for a postdoctoral fellowship (M.C.M.) from the Comunidad Autónoma de Madrid (BOCM 18.03.98, no. 65).

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