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Allelopathic effects of lichen secondary metabolites and their naturally occurring mixtures on cultures of aposymbiotically grown lichen photobiont *Trebouxia erici* (Chlorophyta)

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ABSTRACT

In the present work, the phytotoxic effects of secondary metabolites extracted from five lichen species (*Cladonia arbuscula* var. *mitis*, *Cladonia furcata*, *Hypogymnia physodes*, *Evernia prunastri* and *Ramalina farinacea*) on cultures of the aposymbiotically grown lichen photobiont *Trebouxia erici* were evaluated. Toxicity of single secondary metabolites, as well as their naturally occurred mixtures in the lichens was tested at the two selected doses, 0.1 mg/disk and 0.01 mg/disk. Application of all tested metabolites decreased the growth of photobiont cells in a dose dependent manner. However, based on selected physiological parameters, e.g. composition of assimilation pigments, fluorescence of chlorophyll *a*, content of soluble proteins, production of TBARS and ROS, differential phytotoxicity of tested compounds and their mixtures was confirmed. It appears that most of the secondary metabolites tested in the present study may be functioning as allelochemicals which are controlling the cell division of the algal partner inside the thallus. The allelochemicals may play a key role in maintaining the balance between the symbionts forming the lichen thallus.

1. Introduction

Lichens are symbiotic organisms consisting of a fungus (mycobiont) and photosynthetic partner (photobiont), which can be either algae or cyanobacteria. They are the dominant component of vegetation in approximately 8% of terrestrial ecosystems and are able to survive in environments subject to extremes of temperature, desiccation and nutrient status (Ahmadijan, 1993).

Ecological success of symbioses forming lichens may be, in part, explained by production of organic compounds called secondary metabolites. Lichen secondary metabolites are produced by the fungal partner and their presence is mostly restricted to the lichens themselves (Fahselt, 1994; Hauck et al., 2009). Lichen secondary metabolites (mostly depsides and depsidones) are deposited on the surface of hyphae, as well as lichen algae, and typically constituting 0.1% to 5.0% (w/w) of thallus dry weight (Fahselt, 1994). Thus far, more than 1000 secondary metabolites are known to occur in lichens, but only approximately 7% of them have also been discovered in other organisms, for example in non-lichenized fungi or even in higher plants (Bačkor et al., 2013; Beckett and Minibayeva, 2013; Hauck et al., 2009).

Secondary metabolites of lichens are considered to have important biological and ecological roles. The roles thus far confirmed experimentally include mostly antimicrobial activity, allelopathy, antiherbivory, chelating of heavy metals and light screening (Bačkor et al., 2013; Hauck et al., 2009; Latkowska et al., 2006; Lawrey, 1986; Pöykkö et al., 2005; Solhaug et al., 2009).

Some secondary metabolites of lichens appear to have allelopathic effects on vascular plants (Cardarelli et al., 1997; Lechowski et al., 2006) as well as on the algal partner of lichens (Bačkor et al., 2010, 2013). However, the mechanisms of the phytotoxic effects of these compounds on plants, including its own algal partner in the lichen thalli are still not understood sufficiently. It is mainly due to the limited number of chemically defined metabolites tested. Of all the lichen secondary metabolites that have been discovered to date, phytotoxic effects to lichen photobionts have thus far only been confirmed after application of usnic acid and atranorin. Mechanisms of phytotoxicity include growth inhibition, inhibition of chlorophyll *a* fluorescence (F_V/F_M), decrease of plant viability and induction of oxidative stress in the plant cells (Bačkor et al., 2010; Cardarelli et al., 1997; Endo et al., 1998; Han et al., 2004).

The aim of present work is to study the influence of 5 extracts from the selected lichen species (*Cladonia arbuscula* var. *mitis*, *Cladonia*

Abbreviations: BBM, Bold's basal medium; dw, dry weight; MDA, malondialdehyde; PS II, photosystem II; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

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furcata, *Hypogymnia physodes*, *Evernia prunastri*, *Ramalina farinacea*) on ecophysiological parameters of aposymbiotically grown lichen photobiont *Trebouxia erici*. All selected lichens used in this study are of worldwide distribution; *Cladonia furcata*, *Hypogymnia physodes* and *Ramalina farinacea* are important members of the lichen flora of South Africa. The lichen photobionts are in direct contact with cortical metabolites produced by mycobiont and secondary metabolites may act as allelochemicals, which are also in part responsible for maintaining of balance between bionts inside of lichen thallus. Although photobionts must protect themselves from the toxicity of these compounds, we found in our previous studies (Bačkor et al., 2010, 2013) that lichen secondary metabolites are differentially phytotoxic to the lichen algal partner—photobiont. We hypothesized that lichen photobionts evolved protective mechanisms against phytotoxicity of individual secondary metabolites, as well as naturally occurring mixtures of secondary metabolites in their thalli through hundreds of millions of years of co-evolution with lichen mycobionts. To test this hypothesis, we evaluated the potential phytotoxic effect of individual secondary metabolites and their mixtures on aposymbiotically grown lichen photobiont *Trebouxia erici* using assessment of selected physiological and biochemical parameters.

2. Materials and methods

2.1. Lichen material, extraction of extracellular secondary metabolites and their identification

In the present study, extracellular secondary metabolites from five selected lichen species were used: *Cladonia arbuscula* var. *mitis*, *Cladonia furcata*, *Hypogymnia physodes*, *Evernia prunastri* and *Ramalina farinacea*.

Lichens *Cladonia arbuscula* subsp. *mitis* (Sandst.) Ruoss and *Cladonia furcata* (Hudson) Schrader were collected randomly during October 2012 from the soil surface in the locality Špania Dolina (48°49′N, 19°08′E), central Slovakia, 730 m a.s.l. Lichen *Hypogymnia physodes* (L.) Nyl. was collected at the same time and locality, from the bark of *Picea abies* (L.) Karst. trees, at a height of 1 to 2 m above ground. Lichens *Evernia prunastri* (L.) Ach. and *Ramalina farinacea* (L.) Ach. were collected randomly during September 2012 from the bark of *Quercus* sp. trees, at a height of 1 to 2 m above ground in the locality Košice-Bankov (48°44′42 N, 21°12′28 E), eastern Slovakia, 410 m a.s.l. Macroscopic foreign material adhering to lichen surfaces was removed with forceps. Extractions of secondary metabolites were conducted within 2 weeks after lichen collection.

Cleaned and air-dried lichen samples (approximately 5 g of dry weight) were extracted in cool acetone (150 ml) for 5 min. The extraction was repeated at least three times. Acetone extracts were collected, evaporated and the residues were dissolved with fresh acetone. Acetone extracts were analyzed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). A standardized TLC method for identification of lichen products (Orange et al., 2001) was used. Acetone extracts were applied on the pre-coated thin-layer plates of Merck silica gel 60F-254. Three solvent systems (A, B, C) were used and lichen compounds were visualized by spraying with 10% sulfuric acid and heating for 30 min at 110 °C.

Filtered acetone extracts were also analyzed by gradient HPLC (Pöykkö et al., 2005) under the following conditions: column Tessek SGX C_{18}, flow rate 0.7 ml × min^{-1}. Mobile phase:A = H_2O:acetonitrile:H_3PO_4 (80:19:1) and B = 95% acetonitrile. Gradient program: 0 min 25% B, 5 min 50% B, 20 min 100% B, 25 min 25% B. Detection was performed at the wavelength of 245 nm (detector Ecom LCD 2084). Usnic acid (Aldrich) was used as a standard. Standards of fumarprotocetraric acid, atranorin, protocetraric acid, physodalic acid, physodic acid and evernic acid were prepared from crystallized acetone extracts from lichens, prior to analyses. Each analysis was replicated three times.

2.2. Organisms and culture conditions

Aposymbiotically grown lichen photobiont *Trebouxia erici* Ahmadjian (UTEX 911) was used in the present study. This species belong to Chlorophyta, a division of green algae. Algae were cultivated on agar medium (1.5%) previously developed for cultivation of lichen algae by Ahmadijan (1993). This was Bold's Basal Medium (BBM 3 N) plus 10 g casein acid hydrolysate and 20 g glucose per liter with the pH adjusted to 6.5 (Bačkor et al., 2010). Cultures were maintained at 22 °C under a 16-h photoperiod and 30 μmol m^{-2} s^{-1} artificial irradiance ("cool white" tubes).

2.3. Allelopathic assay

Photobionts were cultivated on the surface of Whatman CF/C filters (glass fiber filter disks, 25 mm in diameter). Glass fiber filter disks may be directly used for disruption of algal cells grown on their surfaces in the mortar (Bačkor et al., 2010). Crystals of lichen secondary metabolites on the surface of fibers of the disk are reminiscent of the situation in natural lichens, where extracellular secondary metabolites located on the surface of hyphae are in the contact with algal cells in the photobiont layer. Briefly, cells from *Trebouxia erici* (approximately two inoculation loops) grown on stock solid *Trebouxia* medium were transferred into 50 ml of liquid *Trebouxia* media in an Erlenmeyer flask. Cells were suspended by gentle stirring on a magnetic stirrer for 1 h. Cultures were maintained for the week in a cultivation room under conditions described previously, with daily stirring on magnetic stirrer for about 1 h. The homogeneity of algal suspension was verified microscopically and the number of cells calculated using a standard hemocytometer. Cell density of cultures was adjusted to approximately 10^6 cells ml^{-1} of medium before quantitative cultivation of photobionts.

For cultivation of *Trebouxia erici*, sterilized 25 mm (in diameter) Whatman CF/C filters were subjected to three different pretreatments. Isolated secondary metabolites, as well as their naturally occurring mixtures in lichens, final content 0.01 and 0.1 mg/disk, were dissolved in acetone (volume 50 μl) and applied by automatic pipette on the surface of disks. The same volume of pure acetone was used for control disks. After evaporation of acetone for 4 h, 4 disks were transferred to the surface of solid *Trebouxia* medium (1.5%) in a separate Petri dish, 10 cm in diameter. Finally, 30 μl of algal suspension was inoculated into the centre of each disk. Disk pores allowed supplemental nutrient media to pass through the disk and permit growth to be easily determined from changes in biomass (Bačkor et al., 2010). The total mass of cultures was calculated by subtracting the mean fresh weight (fw) of a Whatman CF/C disk saturated by identical medium, from the fw of a disk supporting algal cultures after 14 days of cultivation. Each treatment was replicated eighteen times.

2.4. Pigment analysis and measurement of chlorophyll a integrity

Weighed Whatman CF/C disks with grown photobiont cells were directly extracted in the dark for 1 h at 65 °C in 5 ml of dimethyl sulfoxide (DMSO). To maximize chlorophyll extraction, cell aggregates were homogenized using mortar; glass fibers of disks facilitated disruption of cell walls of algae. After cooling to ambient temperature, the absorbance of the extract, as a reflection of turbidity, was determined at 750 nm with a spectrophotometer to be certain that it was always less than 0.01. The absorbance of extracts was then read at 665, 649, 435 and 415 nm to assess chlorophyll content and the possibility of chlorophyll *a* degradation (Barnes et al., 1992; Wellburn, 1994). To utilize the linear portion of the response curve, extracts from disks with very high cell densities (absorbance at 665 nm higher than 0.8) were diluted with fresh DMSO to fall into the absorbance range 0.2–0.8. To determine the content of "total" carotenoids, absorbance was read at 480 nm. Chlorophyll *a*, chlorophyll *b*, chlorophyll $a + b$ and total carotenoids were calculated using equations derived from specific absorption coefficients