Metabolic processes involved with sugar alcohol and secondary metabolite production in the hyperaccumulator lichen Diploschistes muscorum reveal its complex adaptation strategy against heavy-metal stress

Piotr Osyczka, Ewa Latkowska, Kaja Rola, *  

* Corresponding author.  
E-mail address: kaja.skubala@uj.edu.pl (K. Rola).

Article info  
Article history:  
Received 26 March 2021  
Received in revised form  
13 July 2021  
Accepted 16 August 2021  
Available online 17 August 2021  
Corresponding editor: Martin Grube

Keywords:  
Lichen-forming fungi  
Symbiotic association  
Lichen physiology  
Polyols  
Heavy-metal pollution  
Intracellular accumulation

ABSTRACT  
The synthesis of various unique secondary metabolites by lichens is the result of mutualistic symbiotic association between the mycobiont and autotrophic photobiont. The function of these compounds and causal factors for their production are not fully understood. This paper examines the effect of heavy-metal bioaccumulation and physiological parameters related to photosynthesis and carbon metabolism on the production of lichen substances in hyperaccumulator Diploschistes muscorum. The obtained model of secondary metabolite concentrations in the thallus demonstrates that the carbon source provided by the photobiont and associated polyols produced by the mycobiont have positive impact on the production; on the contrary, the increased intracellular load of heavy metals and excessive loss of cell membrane integrity adversely affected secondary metabolite contents. Additionally, the production of secondary metabolites appears to be more dependent on intracellular metal concentrations than on soil pollution level. To compensate for metal stress, both efficient functioning of algal component and sufficient production of secondary metabolites are required. The balanced physiological functioning of mycobiont and photobiont constitutes the complex protective mechanism to alleviate the harmful effects of heavy metal stress on primary and secondary metabolism of lichens.

© 2021 The Authors. Published by Elsevier Ltd on behalf of British Mycological Society. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction
Lichens constitute symbiotic association composed of a fungus and an alga and/or cyanobacterium. Certain species are able to effectively colonise various natural and anthropogenic sites that are strongly enriched with heavy metals (Cuny et al., 2004a; Purvis and Halls, 1996; Rola and Osyczka, 2014, 2018). Some of them show a high tolerance to metal pollution; however, only a limited number of species appears insensitive to extreme metal contamination of the substrate (Osyczka and Rola, 2013a; Purvis, 2014). Those lichens that demonstrate the unique ability for extraordinary accumulation of elements in thallus are considered as hyperaccumulators; there are, for instance, Diploschistes muscorum (Cuny et al., 2004b; Sarret et al., 1998), Acarospora rugulosa (Chisholm et al., 1987), Stereocaulon japonicum (Nakajima et al., 2013) and Lecanora polytropa (Pawlik-Skowronska et al., 2006). The mechanism of heavy-metal accumulation is primarily associated with extracellular ion exchange process, intracellular accumulation and intercellular entrapment of solid particles within the thallus (Purvis and Pawlik-Skowronska, 2008; Richardson, 1995). Heavy metals accumulated intracellularly affect lichen metabolism to the greatest extent (Backor and Loppi, 2005).

Specific lichen secondary metabolites are produced by the mycobiont (Elix, 1996; Huneck, 1999) and deposited in the cortex or in the medullary layer as extracellular tiny crystals on the outer surfaces of the hyphae (Molnár and Farkas, 2010). They are formed from primary metabolites but are not directly involved in the basic metabolism. The production intensity, significance and functions of secondary metabolites in lichens are still poorly recognized. Generally, it is believed that lichen secondary metabolites could influence both biotic and abiotic interactions of lichens with their environment (Deduke et al., 2012). There are active substances and
could provide protection against herbivores, pathogens, competitors and external abiotic factors, such as high UV irradiation (Molnár and Farkas, 2010). Secondary metabolites can play specific ecological roles to allow lichens to adapt or improve tolerance to environmental changes (Culberson and Armaleo, 1992).

Production intensity of lichen secondary metabolites seems to be dependent on heavy-metal stress and may be relevant for host towards alternative secondary pathways, and hence, mycobiont metabolites by the mycobiont. This could be confirmed by the fact that lichens certainly direct carbon out of primary metabolic sequences toward alternative secondary pathways, and hence, mycobiont synthesizes specific secondary metabolites in the form of depsides, deipsidones and dibenzo)furan (Fahselt, 1994).

To put new light into the problem above, specimens of the lichen hyperaccumulator Diploschistes muscorum inhabiting substrates polluted to different degrees were subjected to this study. We intended to identify the factors that regulate the production intensity of secondary metabolites to the greatest extent. To achieve this goal, we applied secondary metabolite content modelling with predictors related to lichen physiological parameters and intracellular accumulation of heavy metals.

2. Materials and methods

2.1. Studied lichen species

*Diploschistes muscorum* (Scop.) R. Sant. is a terricolous crustose lichen ([Fig. S1](#)) well known for its ability to withstand high amount of trace elements present in the surrounding environment (Cuny et al., 2004b; Oszyczka and Rola, 2013a,b). This species is considered a hyperaccumulator, especially of Zn and Pb, due to exceptionally high accumulation capacity for these elements without harming vitality (Maquinay et al., 1961). Secondary metabolites synthesised by this lichen include aryl depsides, i.e. diploschistesic acid and lecanoric acid which are major compounds, while orsellinic acid has been frequently reported as satellite substance (e.g. McCarthy, 2016; Nash et al., 2002).

2.2. Study area and sampling

The study was conducted in the Silesia-Cracow Upland which is one of the most polluted regions in Poland from the centuries associated with mining and processing of Zn–Pb ores (Cabala et al., 2008). Altogether six sampling sites were selected. They constitute post-flotation dump (Chorzów town, 50° 20’18.4”N, 18° 56’35.4”E), post-industrial wastes (Bukowno town, 50° 16’18.2”N, 19° 29’34.2”E), post-smelting dump (Piekary Śląskie town, 50° 21’09.5”N, 18° 57’54.2”E), post-smelting dump (Trzebinia town, 50° 09’13.6”N, 19° 27’49.2”E), post-mining wastes (Ujków village, 50° 16’50.8”N, 19° 29’02.0”E) and semi-natural psammophilous grassland (Pazurek village, 50° 20’42.3”N, 19° 37’36.8”E). Sampling was done in early autumn 2017. Ten lichen samples were collected at each site taken to the laboratory right away. Simultaneously, corresponding soil substrates were collected directly from under the lichen thalli. The lichen material was intended for analysis of metal concentrations (see Section 2.4), photosynthetic pigment concentrations (see Section 2.5), photosynthetic efficiency (see Section 2.6), degree of cell membrane damage (see Section 2.7), content of secondary metabolites (see Section 2.8), concentrations of sugar alcohols (see Section 2.9). Part of the material was frozen in liquid nitrogen, dried and stored at −20 °C until analyses. The soil substrate was intended for analysis of metal concentrations (see Section 2.3). Additionally, ten lichen and corresponding soil samples were collected from uncontaminated background site located far from pollution sources (Podlesice village, 50°34’00.0”N, 19°32’27.7”E) for the determination of heavy metal concentrations and calculation of background values (see Section 2.10).

2.3. Metal concentrations in soil samples

The soil samples were dried and passed through a 2-mm sieve. The samples (5 g DW) were digested with 70% HClO₄ (Merck, Suprapur) using a digestor (FOSS, Sweden). Subsequently, flame atomic absorption spectrometry was applied for determination of Zn, Cd, Pb, Cu (Varian 280 Fast Sequential Atomic Absorption Spectrometer, Varian, Australia) and Ni, As (Varian Zeeman 280

---

P. Oszyczka, E. Latkowska and K. Rola Fungal Biology 125 (2021) 999–1008
Atomic Absorption Spectrometer with a 120 Graphite Tube Atomizer, Varian, Australia) concentrations. Certified reference materials, i.e. CRM048–50G (Sigma–Aldrich), BCR-483 (Sigma–Aldrich), and ISE-912 (WEPAL, Wageningen University), were used for quality assurance. Corresponding solutions without samples were used as reagent blanks. The analyses were repeated three times; the mean values were considered to constitute one observation.

2.4. Metal concentrations in lichen samples

Macroscopic foreign materials adhering to thallii surfaces were carefully removed. Lichen samples were divided into two parts: one intended for measurements of total concentrations, the other for intracellular concentrations. For samples in the second part, ca 150 mg of lichen thalli were soaked by shaking for 1 h in 10 ml of a 20-mM Na2EDTA solution and then rinsed in deionised water to remove metals non-specifically bound to cell walls (Brannon and Brown, 1994). The difference between total element content and concentration after EDTA washing was specified as the extra-cellular content treated here as a fraction of metals trapped in the form of solid particles in intercellular spaces and extracellularly bound with exchange sites on the cell walls of symbionts. Then lichen samples were dried at 90 °C for approximately 24 h to a constant weight. Dry and powdered lichen samples were digested in 70% HClO4 (Merck, Suprapur) and 65% HNO3 (Merck, Suprapur) (1:4) and diluted with double-distilled water. Concentrations of particular elements were determined by means of flame atomic absorption spectrometry, using a Varian AA280FS and a Varian AA280Z with a GFA 120. Analyses of elements were repeated at least three times; the mean values were treated as one observation. Additionally, analytical precision was checked against certified reference materials (GPW10015, INCT-OBLT-5); all samples fell within ±10% of the certified value.

2.5. Pigment analysis and measurement of chlorophyll a integrity

Samples of ca 20 mg of lyophilized lichen thalli were washed with CaCO3-saturated 100% acetone (following procedure of Barnes et al., 1992). Then, photosynthetic pigments were extracted from thalli using 3 ml dimethyl sulfoxide (DMSO) with the addition of 2.5 mg/ml polyvinylpyrrolidone (PVPP) twice for 45 min at 65 °C in the dark (shaking at regular intervals). After cooling to room temperature, extracts were centrifuged and diluted 1:1 with fresh DMSO, and the absorbance of the extracts was read at 665.1, 649.1, 480, 435, and 415 nm (Jasco V-650 spectrophotometer, Japan). Concentrations of chlorophyll a, b, and total carotenoids were calculated using Wellburn’s equations (Wellburn, 1994). The degradation of chl a to pheo a (A335/A435) were assessed by determining the ratio between absorbance at 435 and at 415 nm (Garty, 2001). Four replicates were measured, the mean of which was a single observation in further analyses. For a more detailed description of the measurement procedure see Rola et al. (2019).

2.6. Photosynthetic efficiency

The photosynthetic activity of lichen photobiont was measured using an Open Fluor Cam FC 800-O/1010 fluorometer (Photon Systems Instruments, Czech Republic). Prior to fluorescence measurements, fully hydrated lichen thalli were distributed over a surface of petri dishes, covered and dark-adapted for 30 min. The fluorescence was measured using a pulse of saturating white light (2500 nmol photons m−2 s−1). The photosynthetic efficiency was assessed by the maximum quantum yield of PSII in dark-adapted state as inferred from chl a fluorescence emission (Fv/Fm). At least ten individual thalli represented a single sample and the mean value was treated as one observation. For a more detailed description of the measurement procedure see Rola et al. (2019).

2.7. Integrity of cell membranes

Ca 100 mg of freshly collected lichen material was placed in a humid chamber (relative humidity, ca 90%) for 24 h to stabilise electrolyte leakage and maintain membrane integrity. Then the samples were rinsed several times in deionised water to remove solid particles. The initial electrical conductivity in μS cm−1 (C) of the deionised water was measured in advance as a blank using a conductivity meter (Seven Go Duo SG23-FKS, Mettler Toledo). The samples were soaked in 50 ml of deionised water in glass weighing bottles, covered tightly with glass plugs, and shaken in a vibration shaker for 1 h (Vibraxmax 100, Heidolph). The conductivity (Cv) of the samples was measured after soaking. Then, the thalli were then boiled for 10 min at 100 °C, causing the total rupture of cell membranes. Conductivity was measured again (Cf) after the samples had cooled to room temperature. Relative electrical conductivity (EC%) indicating loss of membrane integrity was assessed according to the formula:

$$EC\% = \frac{Cv - Cf}{Cf} \times 100\%$$

For a more detailed description of the measurement procedure see Osyczka and Rola (2019).

2.8. Secondary metabolites analysis

2.8.1. Extraction procedure

Prior to lichen secondary metabolite extraction the lyophilized lichen thalli were ground and the samples were weighed in the range of 8.5–10.0 mg. Then, they were extracted with 0.75 ml of acetone in ultrasonic water bath (Sonic-3, Polasonic, Poland) for 15 min at room temperature. Then the extracts were centrifuged at 15,000×g for 10 min. The extraction procedure was performed in triplicate and the supernatants were collected, dried under vacuum and stored at −20 °C until HPLC analyses. The residues were redissolved in 1 ml of HPLC grade methanol and passed through a 0.22 μm PTFE syringe filters (Labex Ltd., Hungary) before injecting 5 μl for HPLC analysis.

2.8.2. HPLC analysis

The analysis was performed on Shimadzu Nexera-i LC-2040C 3D Plus Ultra High-Performance Liquid Chromatograph (Shimadzu, Japan) with a photodiode array detector. The separation of secondary metabolites was achieved using a reverse-phase Kinetex C18 column (150 × 4.6 mm; 2.6 μm) equipped with a Guard column (both from Phenomenex) maintained at 25 °C and the gradient mobile phase consisting of: (A) water and (B) acetonitrile (both acidified with 0.1% formic acid, v/v). A linear gradient elution changed in the following steps: 0 min, 20% B; 3 min, 50% B; 6 min, 55% B; 8 min, 100% B at a flow rate of 0.7 ml min−1. The column was equilibrated for 5 min before the next run. The absorbance was measured in the range of 190–400 nm and the detection was conducted at 254 nm. The identity of lichen compounds was confirmed by mass spectrometry analysis. The quantity of compounds was expressed as relative amounts represented by peak areas.

2.8.3. LC-MS/MS analysis

The qualitative identification of secondary metabolites was confirmed by LC-MS/MS analysis. A Waters (Milford, USA) ACQUITY Ultra Performance Liquid Chromatograph (UPLC) system coupled to
a tandem quadrupole mass spectrometer with an electrospray ionisation source (ESI) was used. The samples of lichen extracts were dissolved in 1 ml 50% acetonitrile and injected, in a volume of 1 μl, into an ACQUITY UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm, Waters), maintained at 40 °C. Chromatographic separations were carried out at a flow rate of 0.3 ml min⁻¹ using a gradient elution program starting from 95% of eluent A (water with 0.1% formic acid, v/v) and changing to 0% A within 10 min. Eluent B was acetonitrile acidified with 0.1% formic acid (v/v). The spectra was measured in the range of 200–700 nm using a Waters eλ PDA detector. The samples were measured in negative ionisation mode and scanned in the range of 50–1000 m/z. The ion spray voltage was set at 3000 V. The MS/MS experiments were run in the scan range from 50 to 600 m/z. The collision energy was set at 50 eV. The compounds were identified by comparing the UV spectra and the values of pseudomolecular ion and fragmentation ions masses with those given in the literature (Huneck and Yoshimura, 1996; Jin et al., 2018; Torres-Benítez et al., 2017).

2.9. Sugar alcohols analysis

2.9.1. Polyls extraction

The lyophilized lichen samples were grounded and weighed in the range of 19.8–20.2 mg and then sugar alcohols were extracted with 1.5 ml of 70% ethanol in ultrasonic water bath (15 min, 75 °C). Then the extracts were centrifuged at 15,000×g for 10 min. The extraction procedure was repeated three times. The supernatants were pooled and the solvent evaporated under vacuum. Until the analyses the samples were kept in a freezer at −20 °C. Next, the dry extracts were redissolved in 1 ml of deionized water (Millipore, USA) for 30 min, with vigorous shaking at room temperature.

2.9.2. Protein elimination

Preparation of samples for HPLC analysis of polyols was performed according to the method of Miyagi et al. (2007) with some modifications. Briefly, deproteinization of water samples was performed by adding 1 ml of 10% trichloroacetic acid solution to 0.4 ml of sample. After centrifugation at 15,000×g for 10 min at 4 °C, the supernatant (0.8 ml) was neutralized by the addition of 0.38 ml of 1.25 M K2CO3. It was centrifuged at 15,000×g for 10 min at 4 °C and then 0.2 ml of the supernatant was used for reaction of sugar derivatization.

2.9.3. Sugar benzylation

For the benzylation 120 μl of 1M KH2PO4 was added to 200 μl of deproteinized samples in a 2-ml polypropylene centrifugation tube, mixed slightly and combined with 25 μl of an undiluted benzoyl chloride solution, and mixed well. Just after, 90 μl of 8 M NaOH was added, the mixture was immediately vortexed for 50 s, and left at room temperature for 10 min. After that 90 μl of 16% H3PO4 were added to stop the reaction and vortexed for 10 s.

2.9.4. Solid phase extraction (SPE)

The formed benzoylated sugars were cleaned by solid-phase extraction (SPE) technique with a Baker bond C18 silica cartridge (J.T. Baker, USA). About 150 mg of the cartridge in a glass column was rinsed by dripping using a laboport vacuum pump (KNF, USA) with 1 ml 100% acetonitrile (three times) followed by 1 ml of water (three times). All the time care was taken not to allow the sorbent bed to dry out. Just after, the aqueous sample was loaded into the conditioned cartridge and each sample tube was rinsed twice with 0.5 ml of deionized water that was also load into the cartridge. The sample was passed through at 5 ml min⁻¹ flow rate. Then, the cartridge was washed with three portion of 1 ml of water followed by 1 ml of 60% acetonitrile (v/v). The cartridge was dried for a few second and finally, the sugar fraction was eluted with three portion of 0.5 ml of 100% acetonitrile. After filtering through a 0.22 μm PTFE membrane filters (Lanford Ltd., Hungary) 20 μl of the sample was subjected to HPLC separation.

2.9.5. Chromatographic analysis of sugar alcohols

The analysis was carried out on Shimadzu Nexera-i LC-2040C 3D Plus UHPLC (Shimadzu, Japan) with a Shimadzu photodiode array detector. Derivatized sugar alcohols were separated on a reverse-phase Kinetex C18 column (150 × 4.6 mm; 2.6 μm) equipped with a Guard column (both from Phenomenex) maintained at 30 °C, using a two-solvent system comprising solvent A (0.05% TFA in water; v/v) and solvent B (0.05% TFA in acetonitrile; v/v) with a linear gradient elution from 60 to 100% B over 10 min and then 100% B for 8 min at a flow rate of 0.8 ml min⁻¹. The total run time was 21.5 min, including a 3 min equilibration time. The wavelength range was 190–400 nm and the detection was performed at 232 nm. The identification of sugar alcohols was performed by comparing their retention times (Rf) and absorption spectra with those obtained for commercial standards: D-arabitol, ribitol (Sigma–Aldrich) and D-mannitol (POCH, Poland).

2.9.6. Quantitation of sugar alcohols

The content of the individual sugar alcohol was expressed as absolute amount per g of DW of thalli estimated on the basis of calibration curve prepared for the commercially available standards. The entire standard sample preparation protocol was performed in the same manner as for the sample. A four point calibration curves were obtained using D-arabitol (from 25 to 200 μg ml⁻¹), ribitol (from 5 to 50 μg ml⁻¹), and D-mannitol (from 25 to 200 μg ml⁻¹).

2.10. Calculations and statistical analyses

In order to take account of the combined effect of several toxic elements on the production of secondary metabolites, we decided to use indices that reflect the overall levels of soil pollution and accumulation of elements in lichen thalli. This approach is justified given the fact that this study is based on the results obtained from lichens naturally occurring in the polluted sites and their metabolic response does not follow directly from experimental treatment with individual elements. The concept was derived from Pollution Load Index (Varol, 2011). Soil Pollution Load Index (SPLI) was calculated based on the following formula:

\[
\text{SPLI} = \sqrt{\text{PIsoilZn} \times \text{PIsoilPb} \times \text{PIsoilCd} \times \text{PIsoilCu} \times \text{PIsoilNi} \times \text{PIsoilAs}}
\]

where \(\text{PIsoil} \) is a calculated value for a single Pollution Index calculated according to the formula

\[
\text{PIsoil} = \frac{\text{C}_{\text{soil}}}{\text{B}_{\text{soil}}}
\]

where \(\text{C}_{\text{soil}}\) is the concentration of element in soil sample and \(\text{B}_{\text{soil}}\) is the background value of mean element concentration in soil samples collected from the background site (\(n = 10\)). The values of \(\text{B}_{\text{soil}}\) are provided in Table S1.

Intracellular Element Load Index (IELI) was applied for the assessment of the total degree of intracellular heavy-metal load in lichen thalli. IELI was calculated according to the following formula:
IELI = \sqrt{\text{PIlichenZn} \times \text{PIlichenPb} \times \text{PIlichenCd} \times \text{PIlichenCu} \times \text{PIlichenNi} \times \text{PIlichenAs}}

where PI\text{lichen} is a value for a single Pollution Index calculated as the quotient:

\[ \text{PIlichen} = \frac{C_{\text{lichen}}}{B_{\text{lichen}}} \]

where \( C_{\text{lichen}} \) is the intracellular concentration of element in lichen thalli and \( B_{\text{lichen}} \) is the background value of mean intracellular concentration of element in lichen thalli collected from the background site (\( n = 10 \)). The same procedure was used with regard to extracellular metal concentrations and values of EELI (Extracellular Element Load Index) were determined for each examined sample. The values of \( B_{\text{lichen}} \) are provided in Table S1.

The bioaccumulation factors were calculated according to the formula: BAF = concentration of element in the lichen/concentration of element in the corresponding substrate.

The correlations between particular parameters were verified by means of Pearson correlation coefficients. Subsequently, the concentrations of sugar alcohols (ribitol, arabitol and mannitol), photosynthetic pigment concentrations (\( chl \text{ a}, chl \text{ b} \) and carotenoids), chlorophyll integrity, \( F_{v}/F_{m} \), EC%, and IELI were subjected to factor analysis (FA) based on principal component analysis (PCA) to obtain uncorrelated factors. The factors with eigenvalues >1 were extracted according to Kaiser criterion and varimax-rotated to facilitate their interpretation. We included to the analysis only IELI assuming that elements accumulated inside the cells have the greatest potential impact on metabolic processes and \( D. \text{ muscorum} \) tends to accumulate elements in amounts much greater than are present in the substrate. Next, we conducted stepwise multiple linear regression analysis using forward variable selection (with a threshold of \( F > 1.00 \) to entry) to investigate the effect of factors derived from factor analysis on the relative concentrations of secondary metabolites. Prior to the analysis, the following assumptions were verified in order to validate the models: distribution normality of residuals were checked using the Kolmogorov–Smirnov test (\( p > 0.05 \)), the potential multicollinearity of the predictors was verified by calculating the variance inflation factors (VIFs), Durbin–Watson statistics were calculated to evaluate the potential presence of a serial correlation of residuals. A detailed residual analysis was performed to detect outliers and influential points. The analysis was applied in relation to relative concentrations of orsellinic acid, diplochinic acid, lecanoric acid and the sum of the relative concentrations of secondary metabolites treated as dependent variables.

Principal component analysis (PCA) was performed to show distribution of studied samples according to their characteristics in a form of the data attribute plots (graphic forms) to show differentiation of overall content of secondary metabolites across all studied samples. The analysis was based on the correlation matrix. Since the relative concentrations of secondary metabolites are represented by peak areas at the specific retention times, the sum of relative concentrations was normalized to the range between 0 and 1 for the purpose of this analysis.

The statistical calculations were performed using STATISTICA 13 (StatSoft, Tulsa, OK), STATGRAPHICS CENTURION 18 (StatPoint, Inc), and PAST 3.25 (Hammer et al., 2001).

3. Results

3.1. Content of secondary metabolites and polyols

Three main secondary metabolites were detected in \( D. \text{ muscorum} \) thalli, i.e. orsellinic acid, diplochinic acid and lecanoric acid (Fig. S2, Table S2). All these substances were present in each examined lichen sample. The general proportion in the total amount of secondary metabolites was always the same with lecanoric acid having the highest and orsellinic acid having the lowest relative concentration (Fig. 1a, Table S3).

The relative concentrations of secondary metabolites were significantly and positively correlated with each other (Fig. 2). Three main polyols, i.e. arabitol, ribitol and mannitol were determined; concentrations of ribitol were always the lowest, mannitol as a rule was a predominant polyol, although 12% of the samples contained more arabitol than mannitol (Fig. 1b; Table S3). Ribitol concentrations were strongly positively correlated with the arabitol content, while the mannitol concentrations did not correlate significantly with other polyols (Fig. 2).

3.2. Factors affecting secondary metabolite concentrations

Factor analysis reduced 10 variables to three factors with eigenvalues >1 that jointly explained 83.3% of the total variance (Table 1). Factor 1 was associated with IELI, EC%, photosynthetic efficiency and chlorophyll integrity. Factor 2 was related to arabitol, ribitol and chlorophyll concentrations. Factor 3 was associated with mannitol concentrations.

The results of the multiple stepwise regression analysis are presented in Table 2. A forward stepwise procedure with three Factors (see Table 1) as predictors and secondary metabolites as the dependent variables entered all Factors into the model. Factors 2 and 3 showed significant positive effect, whereas Factor 1 significant negative one. The exception was the model for orsellinic acid where Factor 3 had no significant effect. The strength of effect of Factor 2 was higher than that of Factor 1 in case of each secondary metabolite.

The obtained relationships were also confirmed by PCA results (Fig. S3). The main gradient can be observed along the first axis. Moving from the left to the right side of the diagram, the content of photosynthetic pigments and polyols (ribitol and arabitol) decreases, while intracellular and extracellular contents of heavy-metal elements in the lichen thallus, element concentrations in soil substrate, degree of cell membrane damage and photosynthetic efficiency increase. Lichen samples with the highest relative amount of secondary metabolites were located mainly on the left side of the diagram (Fig. S3).
4. Discussion

4.1. Relationships between polyols and secondary metabolites

Lichen secondary metabolites are produced by the fungal partner, but the role of algal carbohydrates in secondary metabolite biosynthesis remains unclear. On one hand, the photosynthetic partner provides the carbon required for fungal metabolism, hence lichen photobiont is considered crucial for secondary metabolite synthesis as the essential supplier of assimilates in a lichen thallus. For this reason it has been expected that experimental stimulation of mycobiont by algal carbohydrates would increase secondary

---

**Fig. 1.** The relative concentrations of secondary metabolites (a) and contents of polyols (b) in examined lichen samples. Filled squares indicate mean values, boxes indicate standard deviations, whiskers indicate minimum and maximum values.

**Fig. 2.** Heatmap showing Pearson correlation coefficients for studied parameters related to lichen secondary metabolites, polyols, physiological parameters, intracellular concentrations of elements in lichen thallus and element concentrations in the soil substrate. Statistically significant (p < 0.05) correlations are shown. IELI – Intracellular Element Load Index; EELI – Extracellular Element Load Index; SPLI – Soil Pollution Load Index.

**Table 1**
Factors derived from factor analysis. Variables with factor loadings greater than 0.65 are listed; factor loadings are given in parentheses.

| Factor | Variables (factor loadings) | Variance explained (%) |
|--------|-----------------------------|------------------------|
| Factor 1 | Fv/Fm (0.88), IELI (0.80), A435/A415 (0.72), EC% (0.70) | 54.53 |
| Factor 2 | ribitol (0.92), arabitol (0.87), chl a (0.72), chl b (0.67) | 17.75 |
| Factor 3 | mannitol (0.92) | 10.97 |
metabolite production (e.g. Molina et al., 1997). For example, additions of ribitol proved to substantially increase the parietin resynthesis in Xanthoria parietina thalli (Solhaug and Gauslaa 2004). On the other hand, experimental study done by Elshobary et al. (2012) revealed that higher concentrations of carbon sources provided for cultured mycobiont of Cladonia rangiferina decreased secondary metabolite production. Nevertheless, the relationship between concentrations of algal photosynthetic products and secondary metabolite contents has not been studied in lichens growing under natural conditions. In this context the problem become even more complicated because assimilates produced by photobiont are rapidly transferred to mycobiont, irreversibly metabolized into different forms and used for thallus growth, reproduction and other metabolic activities (Deduke et al., 2012).

Regression models indicated that the same factors influence the production of three secondary metabolites in D. muscorum. We might have expected such results since production of these substances is probably coupled and can be explained through pathway intermediates. The basic metabolite is orsellinic acid, which is the main intermediate in the biosynthesis of depsides (Goga et al., 2020). The esterification of two orsellinic acid molecules affords lecanoric acid in acetyl-malonate pathway (Goga et al., 2020) and the hydroxylation of lecanoric acid gives diploschistesic acid (Elix and Nash, 1992). The positive effect of ribitol and arabitol concentrations on secondary metabolite production (Tables 1 and 2) is consistent with the fact that the same starting carbohydrates are used for production of each secondary metabolite. The slight positive effect of mannitol on secondary metabolite production was observed only for lecanoric acid. Little or no relationship between the mannitol content and production of metabolites may be associated with the fact that mannitol is frequently regarded as a storage compound in fungi (Cochrane, 1958). For instance, it is found in large amounts in mycelia, fruiting bodies and spores (Lewis and Smith, 1967). Consequently, ribitol produced by photobiont and arabitol which is the result of rapid metabolism of ribitol by mycobiont (Lines et al., 1989) are regarded as a more readily respiring metabolites than mannitol and thus constitute an easy to mobilize reserve (Armstrong and Smith, 1993), especially under stress condition. On the other hand, Brunauer et al. (2007) found that the production of secondary compounds in Xanthoria elegans was strongly induced by the presence of mannitol, whereas ribitol showed negligible effect.

Evidence was provided that the carbon source provided by the photobiont influences the secondary metabolism of the mycobiont (Deduke et al., 2012). The intermediates of primary metabolism that are no longer needed in the quantity in which they are produced can be transferred to another pathway. It is believed that intermediates can be easily forwarded to secondary pathways (Moore, 1998) serving as an alternative sink for extra products of primary metabolism (Shukla et al., 2014). Such a phenomenon is possible for lichens inhabiting polluted habitats. In our previous study, we found that photosynthetic efficiency in D. muscorum specimens collected from polluted sites remained at a very high level (Rola et al., 2019). This indicated that essential photosynthetic process was unaffected by heavy-metal stress and photobionts of this lichen are well adapted to function under adverse conditions. Efficient photosynthetic process probably results in high production of ribitol and thus the formation of a large pool of intermediates that can serve as precursors for lichen secondary metabolite production.

4.2. Effect of intracellular metal accumulation on secondary metabolite production

An increase in metal pollution of the environment in which a given lichen exist does not necessarily imply a proportional increase in element concentrations inside its cells. A large part of the accumulated load can be localized extracellularly and proportion of particular fractions in lichen thalli depends on species and element (e.g. Oszczka and Rola, 2019; Purvis and Pawlik-Skowrońska, 2008). The production of secondary metabolites appears to be more dependent on amount of heavy metals accumulated directly inside the cells than on the level of ambient environment pollution (Fig. 2). The synthesis of metabolites by lichens should therefore be considered in relation to intracellular concentration of toxic elements and not only to ambient environment pollution. Despite the fact that D. muscorum has capability to immobilize metals extracellularly, this lichen demonstrates a clear propensity for hyper-accumulation, to the extent that metal concentrations in thalli are
frequently several times higher than in host substrate (Table S3, see also Osyczka and Rola, 2019; Rola et al., 2019). Consequently, intracellular concentrations are relatively high which, in turn, may result in harmful effects. We invented IELI to account for the cumulative effect of all accumulated elements on lichen secondary metabolism. Nevertheless, the effect of particular elements considered separately may be varied (Fig. 2). The experiments under controlled laboratory conditions would make it possible to verify a direct impact of individual elements on the production of secondary metabolites.

Our study revealed adverse effect of increased heavy-metal intracellular load on the production of secondary metabolites in D. muscorum. Similarly, Nakajima et al. (2019) found significant negative correlations between the relative concentrations of atranorin and stictic acid and Cu concentrations in Stereocaulon japonicum. Such response is probably related to the impaired carbon metabolism of the fungus. Increased level of cell membrane damage, which corresponds to intracellular metal load, additionally indicates a reduced condition of mycobiont (Fig. 2; see also Osyczka and Rola, 2019). On the other hand, Pawlik-Skowronska and Backor (2011) showed that lichens Hypocenomyce scalaris, Cladonia furcata and Lepraria spp. produce more secondary metabolites in response to heavy-metal pollution, whereas Gauslæa et al. (2016) found decreased secondary metabolite production with increasing heavy-metal concentrations in four epiphytic lichens. It certainly depends on metabolite in question. Bialonska and Dayan (2005) observed decrease in the content of physodic acid, hydroxysphysodic acid, and atranorin with simultaneous increase of physodalic acid content in Hypogymnia physodes thalli exposed to pollution, which suggested that the latter compound could act against stress. With regard to the secondary metabolites studied here, Sriviboon et al. (2013) concluded that air pollutants caused the decline of lecanoric acid in Parmotrema tinctorum after transplantation to polluted environment. In the context of tolerant lichens that colonize soil substrate polluted with heavy metals, it was shown that populations of two Cladonia species from metal-rich substrata did not contain significantly higher amount of secondary metabolites than those from control sites (Backor et al., 2011). Similarly, Nakajima et al. (2013) reported that secondary metabolite concentrations in Cladonia humilis are independent of Cu stress.

The results of our study provide possible explanations as to why previous studies found an increased or decreased production of secondary metabolites in lichens exposed to heavy metal stress. Our results suggest that the increased production of secondary metabolites by the investigated hyperaccumulator lichen is not directly induced by heavy metal stress. On the other hand, increased concentrations of secondary metabolites in lichen samples from sites polluted with heavy metals, as for example in Pawlik-Skowronska and Backor (2011), may be associated with several other factors. First of all, we showed that the production of metabolites is related to the concentration of sugar alcohols and the good physiological condition of the photobiont. Additionally, regardless of the extreme pollution, the photosynthetic efficiency remains high in tolerant lichens (see Rola et al., 2019). Secondly, heavy metals accumulated intracellularly negatively affects the production of metabolites to the greatest extent and high level of heavy metal elements in the substrate is not of great importance for lichens that exhibit the ability to immobilize heavy metals extracellularly. Therefore, lichens occurring on polluted substrates, in which photobiont maintain good physiological condition and high level of polyol production is observed, can also produce large quantities of secondary metabolites.

4.3. Complex strategy related to heavy-metal stress

To understand the heavy metal tolerance of the examined lichen hyperaccumulator, apart from the simple fact of the positive effect of polyols and negative impact of intracellular metal accumulation on the production of secondary metabolites, it is worth taking a closer look at the action of metabolic processes that may be responsible for the phenomenon. Some lichen secondary metabolites accumulated on the outer surface of fungal hyphae are supposed to play an important role in the extracellular immobilization of heavy metals. Metal complexation by lichen substances, commonly termed as chelation, is another way to control the absorption and neutralization of toxic elements in thalli (Backor and Loppi, 2009). Pawlik-Skowronska and Backor (2011) found strong positive correlation between Zn and Pb accumulation and content of secondary metabolites in the thalli of three lichen species. Simultaneously, lower amount of metal-complexing phytochelatins were found in individuals from polluted site than in those sampled from the control site. Presumably fewer harmful Zn and Pb ions reach the photobiont cells in lichens containing higher amounts of secondary metabolites. It seems reasonably in the context of our results since protection of algal cells is necessary to maintain optimal efficiency of the photosynthetic process and sufficient production of ribitol by photobiont. Consequently, this will ensure efficient synthesis of secondary metabolites. Nevertheless, extensive intracellular metal load ultimately leads to cell membrane damage of fungal hyphae and impairment of metabolism (Fig. 2). To compensate this negative effect both efficient functioning of algal component and adequate production of secondary metabolites are required. Secondary metabolites could reduce intracellular accumulation of metals through their increased extracellular immobilization, while the photosynthesis products of photobiont can provide the basis for the production of secondary metabolites. A strategy of immobilization metals outside the cells is highly probable for D. muscorum since stable complexes of Zn with diphloschistesic and lecanoric acids were previously observed in specimens growing on calamine-rich soils in Belgium (Cuny et al., 1995). Additionally, immobilisation of Zn and Pb by extracellular oxalates on mycobiont hyphae may inhibits intracellular uptake of toxic elements (Sarret et al., 1998).

There are other functional reasons for maintaining an adequate production of secondary metabolites as well. Recently it was hypothesized that some of them play a role in protection against oxidative stress in fungi (Kosanić and Ranković 2019; Luo et al., 2009). Therefore, extensive production of metabolites has the potential to compensate for heavy-metal stress in lichens. Both lecanoric and orsellinic acids produced by D. muscorum proved to demonstrate antioxidant activity (Lopes et al., 2008; Luo et al., 2009), whereby the last showed more than 8.5 times higher antioxidant potential than lecanoric acid (Lopes et al., 2008).

5. Conclusions

Our study shows how intracellularly accumulated heavy metals and various physiological parameters influence the production of secondary metabolites in hyperaccumulator D. muscorum. We observed that the carbon source provided by the photobiont and polyols processed by the mycobiont had a positive effect on the secondary metabolites content in lichen thallus. On the other hand, the increased intracellular heavy-metal load reduced the concentrations of secondary metabolites. Such response is probably related to the impaired carbon metabolism of the fungus due to...
heavy-metal stress, which ultimately leads to cell membrane damage of fungal hyphae and thereby decrease in secondary metabolite production. We also found that regardless of soil pollution level, the production of secondary metabolites in D. muscorum directly depend on the metal load inside cells. Consequently, the synthesis of metabolites by lichens should be considered in relation to intracellular concentration of toxic elements and not only to ambient environment pollution.

To compensate the negative effect of metal stress both efficient functioning of algal component and relevant production of secondary metabolites are required. The protection of algal cells is necessary for lichens inhabiting polluted sites in order to maintain optimal efficiency of the photosynthetic process and production of rhibitol which provide the precursors for the production of secondary metabolites. This may be of great importance since secondary metabolites reduce intracellular accumulation of metals through their extracellular immobilization. All of this constitutes the complex protective mechanism against excessive negative effects of toxic trace elements on both primary and secondary metabolism of lichens.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The study was financially supported by the National Science Centre, Poland [grant number 2016/23/B/NZ8/00759].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2021.08.002.

References

Armstrong, R.A., Smith, S.N., 1993. Radial growth and carbohydrate-levels in the lichen Parmelia conspersa on north and south facing rock surfaces. Symbiosis 15, 23–38.
Backor, M., Loppi, S., 2009. Interactions of lichens with heavy metals. Biol. Plant. 53, 214–222.
Backor, M., Péli, E.R., Vántov, I., 2011. Copper tolerance in the macrolichens Cladonia furcata and Cladina arbuscula subsp. mitsi is constitutive rather than inducible. Chemosphere 85, 106–113.
Barnes, J.D., Balague, L., Manrique, E., Elvira, S., Davison, A.W., 1992. A reappraisal of the use of DMSO for the extraction and determination of chlorophylls a and b in lichens and higher plants. Environ. Exp. Bot. 32, 83–100.
Bialońka, D., Dayan, F.E., 2005. Chemistry of the lichen Hypogymnia physodes transplanted to an industrial region. J. Chem. Ecol. 31, 2975–2991.
Branquinho, C., Brown, D.H., 1994. A method for studying the cellular location of lead in lichens. Lichenologist 26, 83–90.
Brunauer, G., Hager, A., Grube, M., Turk, R., Stocker-Wächtler, E., 2007. Alterations in secondary metabolism of apio symbiotically grown mycobionts of Xanthoria elegans and cultured reisynthesis stages. Plant Physiol. Biochem. 45, 146–151.
Cabala, J., Zgolis, B., Dubiel, R., 2008. Geochemical and geophysical study of historical Zn-Pb ore processing waste dump areas (southern Poland). Pol. J. Environ. Stud. 17 (5), 693–700.
Chisholm, J.E., Jones, C.G., Purvis, O.W., 1987. Hydrated copper oxalate, moolooite, in lichens. Stud. Mycol. 17 (5), 693–700.
Cuny, D., Denayer, F.O., de Foucault, B., Schumacker, R., Coleen, P., van Haluwyn, C., 2004a. Patterns of metal soil contamination and changes in terrestrial crypto-game communities. Environ. Pollut. 129, 289–297.
and Stereocaulon japonicum growing in Cu-polluted sites in Japan. Ecotoxicol. Environ. Saf. 97, 154–159.
Nakajima, H., Fujimoto, N., Yamamoto, Y., Anemiya, T., Itoh, K., 2019. Response of secondary metabolites to Cu in the Cu-hyperaccumulator lichen Stereocaulon japonicum. Environ. Sci. Pollut. Res. 26, 905–912.
Nash III, T.H., Ryan, B.D., Gries, C., Bungartz, F., 2002. Lichen Flora of the Greater Sonoran Desert Region, vol. 1. Arizona State University, Tempe.
Osyczka, P., Rola, K., 2013a. Cladonia lichens as the most effective and essential pioneers in strongly contaminated slag dumps. Cent. Eur. J. Biol. 8 (9), 876–887.
Osyczka, P., Rola, K., 2013b. Response of the lichen Cladonia rei Schaet. to strong heavy metal contamination of the substrate. Environ. Sci. Pollut. Res. 20, 5076–5084.
Osyczka, P., Rola, K., 2019. Integrity of lichen cell membranes as an indicator of heavy-metal pollution levels in soil. Ecotoxicol. Environ. Saf. 174, 26–34.
Pawlak-Skowronska, B., Backor, M., 2011. Zn/Pb-tolerant lichens with higher content of secondary metabolites produce less phytochelatins than specimens living in unpolluted habitats. Environ. Exp. Bot. 72, 64–70.
Pawlak-Skowronska, B., Purvis, O.W., Drzewiecki, J., Skowronska, T., 2006. Cellular mechanisms of Cu-tolerance in the epilithic lichen Lecanora polytropa growing at a copper mine. Lichenologist 38, 267–275.
Purvis, O.W., 2014. Adaptation and interaction of saxicolous crustose lichens with metals. Bot. Stud. 55, 23.
Purvis, O.W., Halls, C., 1996. A review of lichens in metal-enriched environments. Lichenologist 28, 71–601.
Purvis, O.W., Pawlik-Skowronska, B., 2008. Chapter 12. Lichens and metals. In: Avery, S.V., Stratford, M., van West, P. (Eds.), Stress in Yeasts and Filamentous Fungi. Elsevier, Amsterdam, pp. 175–200.
Purvis, O.W., Elix, J.A., Broomhead, J.A., Jones, G.C., 1987. The occurrence of copper norstictic acid in lichens from cupriferous substrata. Lichenologist 19, 193–203.
Purvis, O.W., Elix, J.A., Gaul, K.L., 1990. The occurrence of copper-psoromic acid in lichens from cupriferous substrata. Lichenologist 22, 345–354.
Rola, K., Osyczka, P., 2014. Cryptogamic community structure as a biondicator of soil condition along a pollution gradient. Environ. Monit. Assess. 186, 5897–5910.
Rola, K., Osyczka, P., 2018. Cryptogamic communities as a useful biondication tool for estimating the degree of soil pollution with heavy metals. Ecol. Indicat. 88, 454–464.
Richardson, D.H.S., 1995. Metal uptake in lichens. Symbiosis 18, 119–127.
Rola, K., Osyczka, E., Myśliwa-Kurdziel, B., Osyczka, P., 2019. Heavy-metal tolerance of photobiont in pioneer lichens inhabiting heavily polluted sites. Sci. Total Environ. 679, 260–269.
Rosier, D.J., Melick, D.R., Seppelt, R.D., 1992. Reductions in the polyhydric alcohol content of lichens as an indicator of environmental pollution. Antarct. Sci. 4, 185–189.
Sarret, G., Manceau, A., Cuny, D., van Halowyn, C., Deruelle, S., Hazemann, J.-L., SOLD, Y., Eybert-Béard, L., Menthonnex, J.-J., 1998. Mechanisms of lichen resistance to metallic pollution. Environ. Sci. Technol. 32, 3325–3330.
Shukla, V., Upreti, D.K., Raij, R., 2014. Lichen to Biomonitor the Environment. Springer Verlag, Netherland.
Solhaug, K.A., Gauld, Y., 2004. Photosynthates stimulate the UV-B induced fungal anthraquinone synthesis in the foliose lichen Xanthoria parietina. Plant Cell Environ. 27, 167–176.
Sriviboon, C., Sriviboon, T., Boonpeng, C., Khongsatra, P., Boonpragob, K., 2013. Quantities of secondary metabolites from the lichen Parmotrema tinctorum before and after transplanted to polluted areas in Bangkok, Thailand. In: Pure and Applied Chemistry International Conference. PACCON 2013.
Takami, M., Yajima, T., Masuda, H., Yanauchi, O., 2002. Spectroscopic and structural characterization of copper (II) and palladium (II) complexes of a lichen sub-stance usnic acid and its derivatives. Possible forms of environmental metals retained in lichens. J. Inorg. Biochem. 91, 139–150.
Torres-Benitez, A., Rivero-Montalvo, M., Sepúlveda, B., Castro, O.N., Nogles, E., Simurgiotis, M.J., Garcia-Beltrán, O., Areche, C., 2017. Metabolomic analysis of two Parmotrema lichens: P. robustum (Degel.) Hale and P. ordinum (Matt. Arg.) Hale using UHPLC-ESI-OT-MS-MS. Molecules 22, 1861.
Vantová, I., Backor, M., Kledus, B., Backorová, M., Kováčik, J., 2013. Copper uptake and copper-induced physiological changes in the epiphytic lichen Evernia prunastri. Plant Growth Regul. 69, 1–9.
Varolf, M., 2011. Assessment of heavy metal contamination in sediments of the Tigris River (Turkey) using pollution indices and multivariate statistical techniques. J. Hazard Mater. 195, 355–364.
Welch, A.R., 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolutions. J. Plant Physiol. 144, 307–313.