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Zn/Pb-tolerant lichens with higher content of secondary metabolites produce less phytochelatins than specimens living in unpolluted habitats

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ABSTRACT

Many lichens can cope with heavy-metal stress, however, the mechanisms of lichen tolerance are still not fully understood. Some lichen secondary metabolites (depsides and depsidones), produced in lichens by the fungal symbiont and accumulated on the outer surface of its hyphae, are supposed to play an important role in the extracellular immoblilization of heavy metals. Lichen photobionts (algal partners in the symbiosis), although surrounded by the mycobiont hyphae, may also accumulate high amounts of trace metals. This can lead to physiological disruptions and morphological damage in algal cells and hence affect the lichen physiological status. We hypothesized that lichen species/specimens living in heavily polluted sites and showing HM tolerance possess a higher content of secondary metabolites than those living in unpolluted sites. Hence, their photobionts can be better protected from the excess of metal ions and need to produce less metal-complexing phytochelatins (PCn) to combat metal toxicity. Specimens of *Hypocenomyce scalaris*, *Cladonia furcata* and *Lepraria* spp. sampled from Zn/Pb-polluted and control sites were compared for the accumulation of Zn/Pb and secondary metabolites, as well as for their production of phytochelatins and glutathione in response to experimental Zn or Pb exposure. Generally, the lichen specimens sampled from the HM-polluted site contained higher amounts of Zn and Pb as well as lichen substances (different depsides and depsidones) than those from the control site. A strong positive correlation was found between the accumulation of secondary metabolites and Zn/Pb accumulation (R^2 = 0.98 and 0.63, respectively). For the first time, production of phytochelatins (PC_{2-3}) in response to Zn and Pb (50–200 μM) exposure was found in *H. scalaris*, *L. elobata*, *L. incana* and *C. furcata*. In both species of *Lepraria* also cysteine, a substrate for GSH and PCs synthesis was detected. The lichens from the polluted site produced under the same exposure conditions, or in response to higher metal concentrations, lower amounts of PCn than those sampled from the control site. It strongly suggests that less Zn and Pb ions reached the photobiont cells of the lichens containing higher amounts of secondary metabolites (lecanoric, fumarprotocetraric, stictic, constictic acids, antranorin). The results obtained support the putative role of some metabolites in heavy-metal tolerance of the lichens inhabiting metal-polluted habitats.

1. Introduction

Lichens can cope with heavy-metal stress; however, the mechanisms of lichen tolerance are still not fully understood (Purvis and Pawlik-Skowrońska, 2008; Bačkor and Loppi, 2009). Involvement of organic acids, e.g. oxalic, in extracellular binding of various metal cations was reported in heavy-metal tolerant lichen species (Purvis, 1984; Sarret et al., 1998). Some lichen secondary metabolites (depsides and depsidones), produced in lichens by the fungal symbiont and accumulated on the outer surface of its hyphae, are also supposed to play an important role in the extracellular immoblilization of metals (Purvis and Pawlik-Skowrońska, 2008 and references therein). Copper complexation by norstictic, psoromic and usnic acids (Purvis et al., 1987, 1990; Takani et al., 2002) as well as the putative role of fumarprotocetraric and physodalic acids in Mn tolerance were reported (Hauck and Huneck, 2007a,b). An increasing amount of physodalic acid was also found in *Hypogymnia physodes* transplanted to a heavy-metal-polluted area (Bialońska and Dayan, 2005). Extracts of secondary metabolites from some species of epiphytic *Parmelia* and *Lobaria* (e.g. *L. pulmonaria*) containing stictic acid, constictic acid and atranorin, possesed higher complexing capacity for Cu *in vitro* than those obtained from *L. virens* which did not contain these substances (Cabral, 2002, 2003).

Lichen photobionts (the algal partners in the symbiosis), despite being surrounded by the mycobiont hyphae, may accumulate large quantities of trace metals (Goyal and Seward, 1982) and their

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excess may cause physiological disruptions and morphological damage in algal cells and hence affect the lichen physiological status (Sanita di Toppi et al., 2005; Bačkor and Fahselt, 2008; Pawlik-Skowrońska et al., 2008). For instance, Zn, Cd and Cu were found to be growth inhibitors of *Trebouxia* algae (Bačkor and Fahselt, 2008). However, similarly to free-living microalgae (Pawlik-Skowrońska, 2000; Morelli and Scarano, 2004; Pawlik-Skowrońska et al., 2004, 2007) lichen photobionts of the genus *Trebouxia* defend against the excess of accumulated metal(loid)s like Cd, Pb, Zn, Cu, As by prompt biosynthesis of thiol oligopeptides-phytochelatins, involved in metal detoxification by intracellular complexation with –SH groups of cysteine (Pawlik-Skowrońska et al., 2002; Bačkor et al., 2007; Sanita di Toppi et al., 2008; Mrak et al., 2010). Phytochelatins of the general structure (γGluCys)*n*Gly, *n* = 2–7, synthesized from glutathione possess higher metal-complexing capacity than their precursors (Zenk, 1996). Glutathione, the main non-enzymatic antioxidant in lichens (Kranner, 2002) is a direct substrate for the PC synthesis in these organisms (Pawlik-Skowrońska et al., 2002). Mycobionts (despite the high glutathione pool) do not produce phytochelatins in response to heavy-metal exposure (Pawlik-Skowrońska et al., 2002; Bačkor et al., 2006).

In heavy-metal-polluted areas, lichens which accumulate high amounts of heavy metals (e.g. Cu, Zn, Pb, Cd, Mn) were found to be metal tolerant (Sarret et al., 1998; Pawlik-Skowrońska et al., 2006, 2008; Hauck and Huneck, 2007b; Dzubaj et al., 2008). As reported recently, in the native populations of *Hypocenomyce scalaris, Lepraria* spp. and *Cladonia furcata* inhabiting Zn/Pb/Cd-polluted habitats, the photosynthetic pigments of their photobionts were less affected by high amounts of those metals than in populations of the same or related species living in unpolluted sites (Pawlik-Skowrońska et al., 2008). It suggests that one of the mechanisms of the higher metal tolerance of some common lichens may be a more effective protection of their photobionts from the toxic excess of metal ions by means of a higher production of specific secondary metabolites in the surrounding mycobiont hyphae. Those substances are responsible not only for the high hydrophobicity of lichen surfaces (protecting against absorption of metal-containing solutes, Hauck et al., 2008), but also for the extracellular immobilization of metals (Hauck and Huneck, 2007a,b). Various metal affinities to different lichen substances suggest that some of them may play a general role in metal homeostasis in lichens (Hauck, 2008).

We hypothesized that HM-tolerant lichen species/specimens living in heavily polluted sites may posses a higher content of secondary metabolites in the thallus, providing their photobionts with better protection from the excess of metal ions than those living in unpolluted sites. Hence, photobiont cells in HM-tolerant lichens need to produce smaller amounts of metal-complexing phytochelatins to combat metal toxicity. As reported recently (Pawlik-Skowrońska, 2000; Pawlik-Skowrońska et al., 2002, 2007; Bačkor et al., 2007), in free-living algae, apo-symbiotically grown photobionts and entire lichens, increasing phytochelatin content and length of the peptide chains positively correlated with increasing metal concentrations in the environment and served as a good biomarker of metal bioavailability to algal cells

The aim of this work was to compare the content of secondary metabolites in lichens sampled from Zn/Pb-polluted and unpolluted habitats and their phytochelatin production in response to exposure to high zinc/lead concentrations.

2. Materials and methods

2.1. Lichen sampling

Samples of *Cladonia furcata* (Huds.) Schrad., *Hypocenomyce scalaris* (Ach.) Choisy, and *Lepraria incana* (L.) Ach. were collected in October 2003 together with their substrata in forest on a reclaimed dolomite-containing mine tailing dump (Bukowno, Silesian Upland, S. Poland – B/B*) polluted mainly with zinc and lead. Soils and pine tree bark contained, respectively: Zn: 8624 and 281 μg/g DW; Pb: 936 and 342 μg/g DW (Pawlik-Skowrońska et al., 2008). Samples of *C. furcata* and *H. scalaris* as well as *Lepraria elobata* Tønsberg were collected also in a background control site in Augustowska Forest (NE Poland, A/A*). They were stored in clean plastic boxes in a climate chamber (10 °C, 25 μmol m^{-2} s^{-1}) before analysis and experiments.

2.2. Lichen taxonomic identification

The collected lichens were identified using a stereomicroscope and/or microscope and *Lepraria* spp. also by thin-layer chromatography (Orange et al., 2001) as reported previously (Pawlik-Skowrońska et al., 2008).

2.3. Analysis of lichen secondary metabolites

Secondary metabolites were analyzed using thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). Cleaned lichen samples (15–25 mg of dry weight) were extracted in cool acetone for 60 min (Feige et al., 1993). The extraction was repeated at least three times. Acetone extracts were collected, evaporated and the residues were dissolved with fresh 1.5 ml of acetone. 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 60 F-254. Three solvent systems were used and 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 (Feige et al., 1993; Lumbsch, 2002) 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 254 nm (detector Ecom LCD 2084). Authentic samples of lichen substances were used when available: atranorin (Sigma, compound was a mixture of atranorin and chloroatranorin), usnic acid (Aldrich). Other lichen metabolites were determined qualitatively against microextracts of herbarium specimens with TLC as well as HPLC-determined chemistry, and relative concentrations were calculated from separated single compound area. Microextracts of herbarium compounds were as follows: *Parmelia subrudecta* (lecanoric acid), *Evernia mesomorpha* (divaricatic acid), *Parmelia perlata* and *P. conspersa* (stictic and constictic acids), *Cladonia deformis* (zeorin) and *C. rangiferina* (fumarprotocetraric acid and atranorin). Three replicates were used for each analysis.

2.4. Determination of zinc and lead contents in lichens samples

5–20 mg (FW) of lichen samples collected from the polluted and unpolluted sites were separated from the substratum, washed with deionized water, dried and digested with 64% HNO_3 (*p.a.*) by ultrasonication under high pressure and temperature. Then 30% H_2O_2 was added (2:1, v/v) and the mineralized samples were diluted with deionized water before analysis by means of flame atomic absorption spectrometry (AAS). The determinations were made in triplicates and data are expressed as means ± SE.

2.5. Experimental heavy-metal treatments

Fresh lichen thalli (20 mg) were soaked for 24 h in 5 mM Hepes (N-2-hydroxyethylpiperazine-N′-2ethanesulfonic acid) buffer, pH 6.8 containing 50, 100, 200 μM Zn or Pb (added as $Zn(NO_3)_2 \times 6H_2O$ or $Pb(NO_3)_2$) under light (photosynthetic photon