UNIVERSITY OF PÉCS

Biological Doctoral School

Chemical analyses of depsides and depsidones in populations of the lichen *Hypogymnia physodes* (L.) Nyl. and an analysis of its genetics

PhD Thesis

Katalin Molnár

Supervisor:

Edit Farkas CSc

Inst. Ecology and Botany, HAS, Vácrátót

Consultant:

Éva Salamon-Albert PhD

Dept. Systematic and Ecological Botany and Botanical Garden, Univ. Pécs

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1. Introduction and main objectives

Lichens produce a great variety of secondary metabolites and most of them are unique to lichen-forming fungi. These chemically diverse (aliphatic and aromatic) lichen substances have relatively low molecular weights. They are produced by the mycobiont and accumulate in the cortex (such as atranorin, parietin, usnic acid, fungal melanins) or in the medullary layer (such as physodic acid, physodalic acid, protocetraric acid) as tiny extracellular crystals on the outer surfaces of the hyphae. The photobiont might also have an influence on the secondary metabolism of the mycobiont. Approximately 1050 secondary compounds have been identified to date. Lichens may contain substantial amounts of secondary metabolites, usually between 0.1–10% of the dry weight, but sometimes up to 30%. The distribution patterns of secondary metabolites are usually taxon-specific, and therefore have been widely used in lichen taxonomy and systematics. However, different chemotypes, sometimes with specific geographical distribution, are known in many lichen species (*e.g.*, Calatayud and Rico, 1999; Culberson *et al*., 1984; Filson, 1981; Giralt *et al*., 2010; Hale, 1962; Randlane and Saag, 1989; Sheard, 1974). In addition to their role in lichen chemotaxonomy and systematics, lichen secondary compounds have several possible biological roles, including photoprotection against intense radiation, as well as allelochemical, antiviral, antitumor, antibacterial, antiherbivore, and antioxidant action. These compounds are also important factors in metal homeostasis and pollution tolerance of lichen thalli. Although our knowledge of the contribution of these extracellular products to the success of the lichen symbiosis has increased significantly in the last decades, their biotic and abiotic roles have not been entirely explored.

Several studies have shown that environmental factors, such as light, temperature, pH and contents of the media can influence the secondary metabolism in lichens (*e.g.*, BeGora and Fahselt, 2001; Culberson *et al*., 1977, 1983; Fahselt, 1979; Hamada, 1989, 1991, 1996; Mirando and Fahselt, 1978). These results are based on laboratory experiments and transplantation studies. The relationship between air pollution and lichen compounds was unknown when the current study began, and since then only a few papers have been published (*e.g.*, Białonska and Dayan, 2005).

In this study the chemical diversity of populations from different habitats (particularly from Hungary) of a toxitolerant lichen species were examined, focusing on the possible qualitative and quantitative differences of the production of depsides and depsidones between populations of *Hypogymnia physodes* (L.) Nyl. from naturally different environments, in this

way combining supraindividual and infraindividual research fields. *Hypogymnia physodes* was selected for this purpose, since it is a common foliose lichen throughout the northern hemisphere, and thus it was possible to study herbarium specimens and freshly collected material growing under varying environmental conditions. Furthermore, because this lichen is moderately toxitolerant, specimens from urban areas were also available.

Although many details can be found in the literature about the lichen secondary compounds of this species, Hungarian specimens had not been included. Lichen chemistry assays in Hungary started only in 1998 (Farkas *et al*., 1998, 1999; Farkas, 2007), decades after this type of research began in the rest of the world. It took time, as well, to get the lichen laboratory set up and running. These are the reasons that still, today, the chemical compounds in Hungarian specimens of most lichen species are as yet unexplored. Only limited numbers of quantitative analyses were carried out in the field of lichenology by that time (Białonska and Dayan, 2005; Czeczuga *et al*., 2000).

During the current study the steps taken were:

- to examine the depsides and depsidones of *Hypogymnia physodes* specimens from the largest Hungarian lichen herbaria applying HPTLC with the following aims:
    - to compare the chemical diversity of populations from different geographical sites
    - to explore the patterns of chemical diversity and the correlation with geographical distribution
    - to show if there are any differences between the specimens from Hungary and other countries, as well as between herbarium and freshly collected specimens
    - to determine whether there was any decay of lichen substances during storage
    - to determine if the changes in the environmental/air quality had caused any differences in the secondary chemistry
- to perform quantitative analyses of depsides and depsidones in fresh lichen material by HPLC
- to compare the relative concentrations of lichen substances in specimens from different habitats and to show possible tendencies for further bioindication research

In addition to the chemical diversity in populations of a lichen species from different geographical sites, genetic variability may be shown, as has been reported in the literature for some species (Beard and DePriest, 1996; Zhou *et al.*, 2006; Zoller *et al.*, 1999). Therefore,

the study of genetic diversity among populations of *Hypogymnia physodes* was undertaken as well. The nuclear ribosomal gene complex [comprising nucSSU (18S) rDNS, ITS rDNS (5.8S, ITS1 and ITS2), nucLSU (28S) rDNS], as well as the mitSSU rDNS and the *EF1α* loci are suitable for population studies in lichens.

The AFTOL (Assembling the Fungal Tree of Life) project provided the opportunity to carry out molecular studies on the mycobiont of *H. physodes*.

The steps taken for this portion of the study were:

- to amplify and sequence the gene loci used in AFTOL [nucSSU (18S) rDNS, ITS rDNS (5.8S, ITS1 and ITS2), nucLSU (28S) rDNS, mitSSU rDNS, *RPB1*, *RPB2*, *EF1α* and *mitochondrial ATP synthase protein subunit 6*]
- to compare the genetic variability of populations from different environmental conditions
- to explore the geographical distribution patterns of the possible different genotypes

2. Material

297 specimens from Hungary and 44 specimens from other countries of *Hypogymnia physodes* were screened by HPTLC (high performance thin layer chromatography). Most samples were from dried herbarium specimens; the oldest one was from 1852. The fresh material was collected between 1997–2006. The specimens represented populations from different habitats. In order to study within-population chemical diversity, specimens from the same populations were also analyzed. Additionally, varieties and forms of *H. physodes* were also included in the present study.

13 specimens of the fresh material from locations in natural and urban habitats were analyzed as well by HPLC (high performance liquid chromatography).

A total of 21 *H. physodes* specimens from different populations were used for the DNA analysis.

3. Methods

HPTLC – High Performance Thin Layer Chromatography

6–8 µl acetone extracts of approximately 5 mm × 5 mm thallus fragments were analyzed on 10 cm × 10 cm thin layer chromatographic plates. The standardized method of Arup *et al*.

(1993) was followed. *Platismatia glauca* (atranorin), *Cladonia symphycarpia* (atranorin, norstictic acid), *Pleurosticta acetabulum* (atranorin, norstictic acid), and *Heterodermia leucomelaena* (atranorin, zeorin) were used as control specimens.

HPLC – High Performance Liquid Chromatography

Reversed-phase column (RP-HPLC) and gradient elution were applied. The standardized method of Feige *et al*. (1993) was followed with some modifications [*e.g*., different type of column and bis-(2-ethylhexyl)-phthalate were used]. Approximately 10 mg of air-dried lichen thalli were soaked in 1 ml acetone to extract the lichen substances. Benzoic acid and bis-(2-ethylhexyl)-phthalate were added to the extraction liquid (acetone) as internal standards. 20 μl of acetone extract were injected onto a C18 column. Two solvent systems were used for the gradient elution: 1% orto-phosphoric acid in bidistilled water (solvent system A) and 100% methanol (solvent system B). The elution of the lichen substances was monitored at λ-245 nm.

Two kinds of retention indices were calculated for lichen compounds: R. I. and I value. Relative concentrations (area%) of lichen substances were determined.

DNA analysis

Genomic DNA was extracted from dried lichen thalli using a modified protocol. 2% sodium dodecil sulphate (SDS) was used as a lysis buffer. The following loci were amplified and sequenced with fungus- and locus-specific primers: internal transcribed spacer (ITS rDNA), nuclear small subunit ribosomal DNA (nucSSU rDNA), nuclear large subunit ribosomal DNA (nucLSU rDNA), mitochondrial small subunit ribosomal DNA (mitSSU rDNA), RNA polymerase II largest subunit (*RPB1*), RNA polymerase II second largest subunit (*RPB2*), and elongation factor 1 alpha (*EF1α*). Sequences were assembled and edited using the software package Sequencher 4.5 (Gene Codes Corporation), and aligned manually in MacClade 4.06. In order to determine the similarities of the populations, Maximum Parsimony (MP) quick tests were run for ITS and *EF1α* conducted in PAUP* 4.0b10.

4. Results

Literature studies and theoretical background

1. The biological role of lichen substances was summarized based on 179 literature sources.
2. Research on secondary lichen substances for bioindication problems is a new field, which resulted in 10 papers so far internationally (including the current research). (Further details are mentioned in Chapter 5, under *Basis for continuing research in this area*).

HPTLC

1. Five lichen substances were identified by HPTLC analysis of 341 *Hypogymnia physodes* specimens: protocetraric acid, 3-hydroxyphysodic acid, physodalic acid, physodic acid, and atranorin.
2. No differences were detected between specimens from different habitats.
3. No differences were detected between specimens collected at different times (1852–2006).
4. There were no differences in lichen compounds between the younger (marginal) and the older (submarginal and central) part of the thallus.
5. There were no significant chemical differences between the varieties and forms of this species. In some cases, the patches of protocetraric acid and atranorin were absent or very pale due to their very low concentrations. While one of these substances may have been absent in one specimen of a certain variety or form, it was detected in other specimens of the same variety or form.
6. The cortical location of atranorin and chloroatranorin was identified by analyzing different parts of the thallus (intact thallus, medullary layer, soralia). The other lichen substances were located in the medulla.

HPLC

1. Seven lichen substances were identified in all specimens: the β-orcinol para-depsides atranorin and chloroatranorin, the β-orcinol depsidones physodalic and protocetraric acids, as well as the orcinol depsidones physodic, 3-hydroxyphysodic, and 2'-*O*-methylphysodic acids (Fig. 1).
2. The locations of the lichen compounds were determined based on the results of this study and on the literature: atranorin and chloroatranorin are located in the cortex, physodalic,

protocetraric, physodic, 3-hydroxyphysodic, and 2'-*O*-methylphysodic acids are located in the medullary layer of the thallus.

3. According to peak areas on the HPLC chromatograms, medullary depsidones represented the major secondary compound pool (41.66–77.56 area%), while cortical depsides occurred in substantially lower concentrations (minor compounds, 2.61–16.88 area%).
4. The quantity of depsides and depsidones was variable between specimens (atranorin: 1.71–15.18 area%, chloroatranorin: 0.79–1.93 area%, physodalic acid: 7,95–23,39 area%, protocetraric acid: 0,26–1,87 area%, physodic acid: 0,007–5,14 area%, 3-hydroxyphysodic acid: 22,22–50,75 area% and 2'-*O*-methylphysodic acid: 4,44–15,00 area%).
5. Since our specimens originated from sites of different environmental conditions and HPLC is suitable for quantitative analysis, we confirmed that such studies are appropriate for bioindication and monitoring assays.

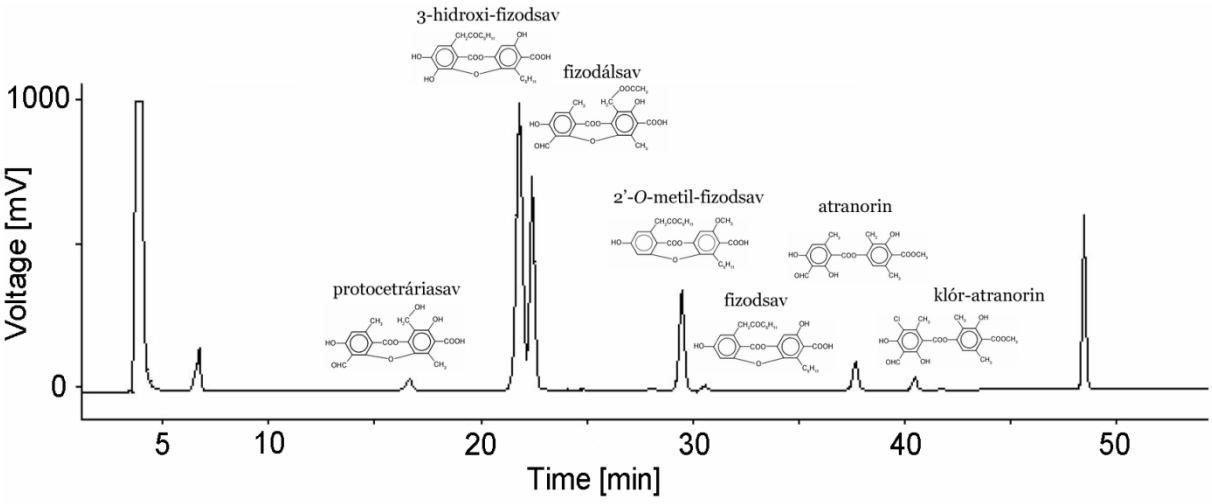

Fig. 1. HPLC chromatogram of the acetone extract of *Hypogymnia physodes* (collected on the mountain Látó-hegy, Budapest, Hungary, collection no. 208/a) at 245 nm.

DNA analysis

1. DNA amplification and sequencing yielded 16 ITS (6 kb), 18 nucLSU (1.4 kb), 3 nucSSU (1.6 kb), 21 mitSSU (8 kb), 1 *RPB1* (A–D region 1.2 kb; D–G region 1.8 kb), 10 *RPB2* (5–7 region 1.2 kb; 7–11 region 9 kb), and 15 *EF1α* (1.4 kb) sequences.
2. ITS, nucLSU, nucSSU, mitSSU, and *EF1α* can be informative at the population level in lichens. We found no differentiation at all among the mitSSU sequences. The length of

the nucLSU sequences were variable, 14 specimens contained an intron (400 bps). The nucSSU sequences varied in the number of introns (with 200 bps length in the positions 342 and 1367). We detected only small differentiation among the *ITS* and *EF1α* sequences of populations, confirmed by the MP analysis.

Methodological and other results

1. Applying HPTLC in order to analyze the secondary compounds in the entire Hungarian population of a lichen species is unique and novel.
2. The following details can be stated based on the HPTLC analysis of the lichen *Hypogymnia physodes*:

   a) Solvent systems A and B were more effective than solvent system C for this species.

   b) Solvent system B was most appropriate for the detection of atranorin/chloroatranorin.

   c) Protocetraric acid was detected only in solvent system B.

   d) Solvent system A was most appropriate for the separation of 3-hydroxyphysodic and physodalic acids.

   e) There is no HPTLC R_F data in the literature for chloroatranorin and 2'-*O*-methylphysodic acid, and no standard compounds were available for this study. However, R_F classes (which are based on TLC data) are suitable for comparisons. Thus according to R_F classes, in solvent system B chloroatranorin has the same R_F value as atranorin, and 2'-*O*-methylphysodic as 3-hydroxyphysodic acid. (Both can be detected by HPLC.)
3. In Hungary this study was the first to apply HPLC for qualitative and quantitative purposes in lichenological investigations. The quantitative application of HPLC is new in lichenology at international level as well. The standardized method was modified as it was described in Chapter 3.
4. The Hungarian distribution map of *Hypogymnia physodes* has been created based on the collections from the present study and all Hungarian herbaria (Fig. 2).
5. Ten new Hungarian chemical names for lichen substances have been created following the instructions of Farkas (2007): diffraktasav, divarikatinasav, giroforasav, orszellinsav, perlatolsav, pinasztrisav, pszoromasav, szferoforin, 2'-*O*-metil-fizodsav, 3-hidroxi-fizodsav.
6. The DNA analysis on lichens in this study is unique in Hungary (Farkas *et al.*, 2008) and only a few international studies have been carried out in order to determine the relation between genetic diversity and environmental conditions.

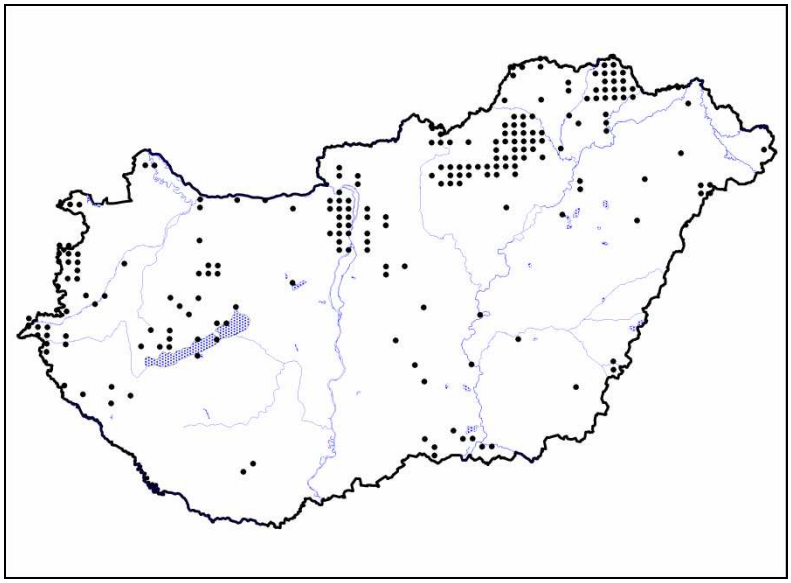

Fig. 2. Distribution map of *Hypogymnia physodes* in Hungary.

5. Discussion and future directions

According to the current study *Hypogymnia physodes* specimens from sites of different environmental conditions do not show any qualitative differences in terms of secondary lichen compounds, even though much data can be found in the literature about the influences of environmental factors on lichen substances. In this study, however, there were no extreme differences between natural habitats of the studied specimens as was in experimental studies known from the literature. Furthermore, none of the environmental factors entirely inhibited the biosynthesis of any lichen compounds, although this effect can occur with lichen substances in other species.

Chemical contents of old and freshly collected specimens were similar, which means that herbarium specimens are suitable for taxonomic and monitoring studies after even a long period of storage.

All seven lichen substances were identified by HPLC in all specimens, including varieties and forms, thus confirming the HPTLC results, *i.e.*, the samples were uniform qualitatively, which means that this lichen species has only one chemotype. Quantitative chemical differences were detected between the samples without showing any geographical pattern, but there were differences between these samples and those investigated in Turkish assays, justifying the present study.

Small differences were revealed among the ITS, nucLSU, nucSSU, and *EF1α* sequences in populations from different habitats (15 Hungarian and three Swedish), indicating high gene

flow, but no geographical patterns were detected. The results need to be confirmed by investigation of further specimens and other markers used for population genetics of lichenized fungi, for instance SSRs (Simple Sequence Repeats, *i.e.*, microsatellites and mini-satellites). The PKS genes should be investigated as well, since they are responsible for the biosynthesis of polyketides.

Basis for continuing research in this area

From the publications related to the dissertation two papers are especially important to be mentioned, which set the stage for future work in this area of lichen research. The first paper (Molnár and Farkas, 2010) is a review of the biological role of lichen substances, including the most recent results, and recognized internationally. The second paper (Molnár and Farkas, 2011) is a qualitative and quantitative study of lichen substances as bioindicators, which is a new idea in lichenology, and confirmed by the relatively few papers published since the beginning of this study.

Many details about the secondary metabolism of this widespread toxitolerant lichen species are still not entirely understood, and further studies are needed to determine its usefulness in bioindication research. Moreover, the study of other more sensitive and more rare lichen species are necessary as well, in order to determine whether natural environmental factors influence the biosynthesis of lichen compounds, and to identify those substances. Statistical sampling and experimental conditions might also be used for more detailed studies.

6. Publications related to the dissertation

Scientific papers

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