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Content of secondary compounds depends on thallus size in the foliose lichen *Lobaria pulmonaria*

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Abstract: Thalli of *Lobaria pulmonaria* of different sizes were collected from the trunks of two specimens of *Populus tremula*. The secondary lichen compounds, stictic, constictic, norstictic, peristictic, cryptostictic and methyl stictic acid were quantified by HPLC and ranked in order of decreasing concentration. There was a highly significant positive correlation between thallus size and the total concentration of secondary compounds, as well as the total content per unit area for specimens from the two sampled trees. According to hypotheses inferring a herbivore deterrent role of secondary lichen compounds, small, juvenile thalli should be more susceptible to herbivores than larger thalli. Thus herbivory might limit survival of young specimens in habitats rich in lichen-feeding molluscs and thereby reduce reproductive success.

Key words: age-dependency, herbivore defence, lichen compounds, norstictic acid, stictic acid

Introduction

Lichens synthesize a large variety of secondary compounds (Huneck & Yoshimura 1996) and many hypotheses concerning their biological role have been proposed (see reviews by Lawrey 1986; Fahselt 1994; Huneck 1999). For the majority of compounds that are located in the medulla, the hypothesis inferring a functional role in herbivore defence is supported by strong experimental evidence (e.g. Slansky 1979; Lawrey 1983; Pöykkö & Hyvärinen 2003; Pöykkö *et al.* 2005; Gauslaa 2005). However, quantitative information on variation in secondary chemistry is scarce (e.g. Stephenson & Rundel 1979; Golojuch & Lawrey 1988; Hesbacher *et al.* 1996; Nybakken *et al.* 2004; Gauslaa & McEvoy 2005). Furthermore, few physiological and ecological studies of lichens focus on the importance of thallus size (Golojuch & Lawrey 1988; Hestmark *et al.* 1997; Gauslaa & Solhaug 1998), despite the probable importance of thallus size effects in lichen ecophysiology (Larson 1984). Golojuch & Lawrey (1988) showed that in *Vulpicida pinastri*, the concentration of the coloured vulpinic and pinastric acids increases with thallus size, but is not correlated with habitat factors. However, it is not yet known if such a size-dependency is species- and/or compound-specific.

The foliose old forest lichen *Lobaria pulmonaria* is rare or threatened in many parts of the world presumably due to its susceptibility to commercial forestry (Campbell & Fredeen 2004) and air pollution (e.g. Gilbert 1986; Gauslaa 1995). *Lobaria pulmonaria* is probably limited by a low establishment rate (Werth *et al.* 2006). Scheidegger (1995) attributed a recorded loss of juvenile *L. pulmonaria* thalli to grazing by arthropods and slugs and/or overgrowing bryophytes. In broadleaved calcareous deciduous forests in southern Norway, where lichen-feeding molluscs are frequent, a high frequency of grazing marks is common on mature thalli (Gauslaa *et al.* 2006). In such forests juvenile or small *L. pulmonaria* are rare (personal observation), suggesting that juvenile thalli might be poorly defended against snail grazing. Secondary compounds have been

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shown to protect *L. pulmonaria* against snail grazing in laboratory experiments (Gauslaa 2005). The objective of this study was to quantify the secondary compounds in *L. pulmonaria* in relation to thallus size including also juvenile specimens. The main hypothesis being tested was that the total pool of secondary compounds increases with increasing thallus size, a response that would be consistent with the observed lack of juvenile thalli in calcareous forests rich in lichen-feeding molluscs.

Materials and Methods

Lobaria pulmonaria (L.) Hoffm. thalli, ranging from 0·01 to 30 cm^2, were collected on 15 December 2005 from the trunks of two *Populus tremula* trees in an old and open oligotrophic *Picea abies* forest at Kollåsen, Ski, Akershus, SE Norway (59°45′N 10°57′E, 200 m.a.s.l.). The distance between the two trees was 10 m with no clear gradient in canopy cover or light exposure. However tree no. 2 had recently died.

The thalli were air-dried at room temperature in low light and stored at −20°C, the recommended long-term storage method for lichens (Honegger 2003), until the start of the measurements one month later. Thalli with a dry matter weight (d. wt) less than 10 mg were pooled in size-dependent batches. Each pooled batch contained 2–51 thalli with a mean d. wt ranging from 0·08–11·0 mg. Pooled samples were necessary for an accurate determination of the minor compounds and also for increasing the accuracy of size measurements of the very small specimens.

The air-dry weights measured in the laboratory were calibrated to d. wt by using the ratio between air-dry and oven-dry (at 70°C) weights obtained from surplus thalli. Subsequently, all thalli were hydrated and photographed. Thallus area was calculated from the digital images using the UTHSCSA ImageTool for Windows, ver. 3 software (University of Texas Health Science Center, San Antonio, Texas, USA).

Chlorophylls and secondary metabolites were extracted from hydrated thalli for three days in a refrigerator with 1–10 ml N,N-dimethylformamide (DMF) depending on batch weight. Compounds extracted with DMF were quantified by HPLC using an ODS Hypersil column, 60 × 4·6 mm using 0·25% orthophosphoric acid and 1·5% tetrahydrofuran (A) and 100% methanol (B) as mobile phases at 2 ml min^{-1}, and UV detection at 245 nm. The run started with 30% B which was increased to 70% after 15 min. After a further 15 min 100% B was reached and held for 5 min after which the amount decreased to 30% in 1 minute. A 10 min post-run of 30% B was performed. Compound identification was based on retention times, online UV-spectra, co-chromatography of commercial standard norstictic acid (Gaia Chemical Corporation, Gaylordsville, USA) and a stictic acid standard kindly given by Prof. Harrie Sipman (Botanical Museum Berlin). The compounds were quantified against response curves of the above-mentioned standards. The constictic, peristictic, cryptostictic and methyl stictic acids were quantified using the response curve of norstictic acid. Absorbance was measured at 647 nm and 664 nm with a spectrophotometer and chlorophylls *a* and *b* concentrations were calculated (Porra *et al.* 1989).

Linear regression was computed to quantify relationships between thallus size and the quantity of total compounds. Spearman's rank correlation was used for Chl *a* concentration in tree no. 2, for which distribution deviated from normal. Analysis of covariance (ANCOVA) was used to detect differences between groups when taking thallus size into account.

Results

All size-dependent batches studied contained stictic (60% of the total extractable secondary compound pool), constictic (27%), norstictic (5·5%), peristictic (4%), cryptostictic (2·5%) and methyl stictic acid (1%). The mean total concentration was 2·3% of dry weight with a variation for individual thalli ranging from 0·7% to 3·6% (n=67). The concentration as well as the content per unit area of each secondary compound, except methyl stictic acid, differed between the two sampled trees (P<0·01, ANCOVA: data not shown). The living tree hosted the thalli with the highest content of secondary compounds.

Five compounds, methyl stictic acid excluded, and total quantities were intercorrelated both when measured per unit area as well as per unit weight (P<0·05, Pearson correlation: data not shown). Methyl stictic acid correlated (P<0·01) with most compounds, but not with norstictic acid when measured per unit weight. The size-dependent relationships of the six compounds were similar (data not shown) and, therefore, we present only the total content of secondary compounds in the following analysis.

The content of compounds per unit thallus area (Fig. 1A) and their concentration (Fig. 1B) increased with increasing thallus area. Because of the tree-dependent difference (see above), the size-dependency was most significant when samples from the two trees were treated separately. Small thalli

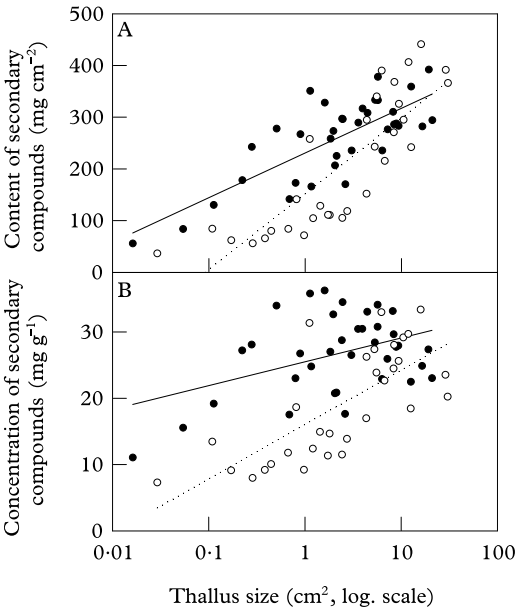

FIG. 1. Relationships in *Lobaria pulmonaria* between the total pool of secondary compounds and thallus size. A, lichen compounds measured as content per unit area (tree no. 1: r^2=0·593, P<0·0001; tree no. 2: r^2=0·701, P<0·0001); B, concentration of lichen compounds (tree no. 1: r^2=0·181, P=0·01; tree no. 2: r^2=0·524, P<0·001). Tree no. 1: ●, solid line, n=36; tree no. 2: ○, dotted line, n=31. Linear regression lines indicated.

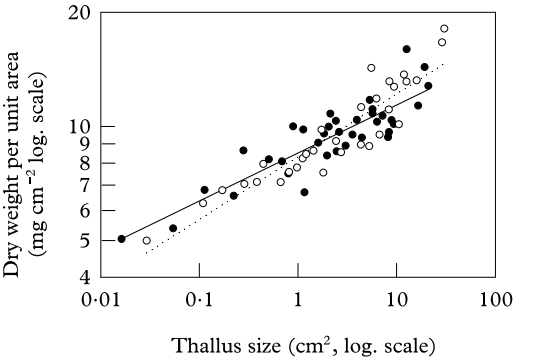

FIG. 2. Relationship between thallus dry weight per unit area and thallus size in *Lobaria pulmonaria* from tree no. 1 (●, r^2=0·767, P<0·0001, n=36) and tree no. 2 (○, r^2=0·847, P<0·0001, n=31). Linear regression lines indicated.

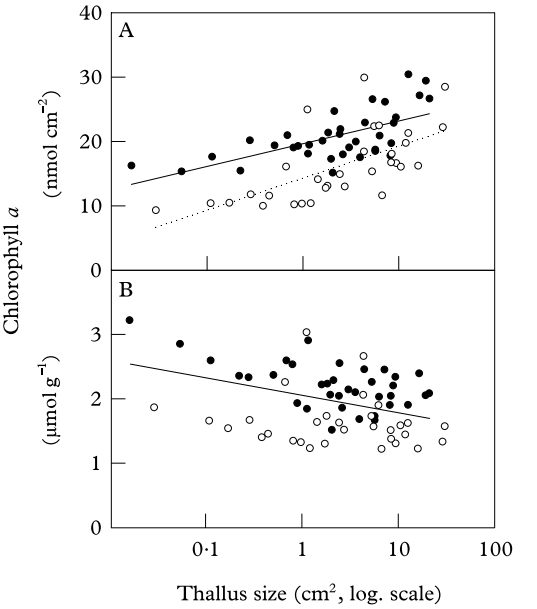

FIG. 3. The relationship between thallus size and chlorophyll *a* in *Lobaria pulmonaria*. A, content per area unit (tree no. 1: r^2=0·413, P<0·001; tree no. 2: r^2=0·423, P<0·001); B; concentration (tree no. 1: r^2=0·348, P<0·001; tree no. 2: not significant (Spearman's rank correlation)). Tree no. 1: ●, solid line, n=36; tree no. 2: ○, dotted line, n=31. Linear regression lines indicated.

were thin with low dry weight per area (5 mg cm^{-2}), and this variable increased linearly with thallus area in a log-log plot to 18 mg cm^{-2} for the largest thalli (Fig. 2).

Chlorophyll *a* per unit area increased with thallus size for both trees (Fig. 3A) but to a lesser extent than the increase in dry weight per unit area (Fig. 2). As a result the Chl *a* concentration decreased slightly with increasing thallus size for tree no. 1 (Fig. 3B). The tree with the lowest Chl *a* concentrations in *L. pulmonaria* was the tree with the lowest content of secondary compounds. The Chl *a*/*b*-ratio showed no significant correlation with thallus size. The content of lichen compounds and Chl a per unit area covaried (tree no. 1: r^2=0·258, P<0·01; tree no. 2: r^2=0·534, P<0·0001; linear regression), whereas, in contrast, lichen compound concentration decreased with increasing Chl *a* concentration in *L. pulmonaria* from tree no. 1 (r^2=0·183, P<0·01, linear regression) while no significant correlation was found in specimens from tree no. 2.

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Discussion

Small, juvenile thalli of *L. pulmonaria* had a lower content of lichen compounds than large thalli (Fig. 1). Our results are similar to the reported age-dependence for atranorin, but not for vulpinic acid, two cortical pigments studied in *Letharia vulpina*-branches differing in age (Stephenson & Rundel 1979). Vulpinic and pinastric acids in the foliose lichen *Vulpicida pinastri* decreased with increasing thallus size (Golojuch & Lawrey 1988). Multiple functional roles (e.g. Lawrey 1986; Fahselt 1994; Huneck 1999) may cause the size-dependency to be compound-specific and different sampled size ranges may produce contrasting relationships, implying a possible lack of a common general trend for all species and/or compounds.

Most plants respond contrary to our results (as reviewed by Boege & Marquis 2005), whereas some increase the chemical defence with age (e.g. Fritz *et al.* 2001). A low content in younger plants is often explained by the growth-differentiation balance hypothesis (Herms & Mattson 1992), which suggests that early allocation of energy is insufficient for both growth and secondary metabolites. Such considerations may be valid for lichens as well.

The concentrations of vulpinic acid and physodic acid were higher in sorediate parts than in non-sorediate parts of *V. pinastri* and *Hypogymnia physodes*, respectively, in accordance to the optimal defence theory (Hyvärinen *et al.* 2000). Soredia were practically absent in our thalli apart from a few of the very largest batches. In fact, the largest sampled batches had apparently slightly lower concentrations of secondary compounds (Fig. 1B). Therefore a significant part of the size-dependency shown in Fig. 1 cannot be explained by amounts of soralia.

We cannot explain the difference in total content between the two trees studied. Canopy cover, aspect and light conditions seemed to be similar around the two trees. Besides, in *L. pulmonaria*, the medullary compounds studied do not seem to function as a protection against excessive solar radiation (McEvoy 2006). The sun-screening function in *L. pulmonaria* is handled by brown, cortical melanic pigments induced by UV-B and insoluble in acetone (Gauslaa & Solhaug 2001). The tree with the smallest quantity of secondary compounds died approximately one year prior to the collection. This might have caused enrichment in cations leaking from the tree (Gauslaa 1995) which possibly could have allowed fixed carbon to be allocated to new lichen growth rather than to secondary compounds. However, such nutrient enrichment might be expected to increase the chlorophyll pool (Palmqvist & Dahlman 2006), which was actually not the case.

Regression models gave stronger relationships when size was correlated with content per unit area rather than with concentration. The syntheses of lichen compounds is based upon photosynthates delivered from the photobiont (Solhaug & Gauslaa 2004; McEvoy *et al.* 2006). Therefore, one would expect a positive correlation between pools of Chl and secondary compounds which indeed was observed in our dataset with content per unit thallus area. Thallus thickness, measured as dry weight per unit area, increases more than Chl *a* with increasing thallus size (Figs 2 & 3). As a result, the Chl *a* concentration decreases with thallus size despite the fact that Chl *a* per unit area clearly increases.

The compounds are unevenly distributed within the thallus. Most medullary secondary compounds are located in the photobiont layer in the upper part of the medulla (Fahselt & Alstrup 1997). Furthermore, secondary compounds function as protection against stresses such as excess solar radiation and grazing, which both often hit the upper side. Lichen-feeding molluscs graze the upper cortex and photobiont level in the upper part of the medulla as documented by Gauslaa *et al.* (2006). Therefore, content per unit area may be a more functionally relevant variable than concentration.

The forest lichen *L. pulmonaria* is red-listed in several countries and shows declining populations mainly due to industrial forestry. Many recent studies propose

dispersal and/or reproduction to be a highly limiting factor for a number of old forest lichens (e.g. Scheidegger 1995; Sillett *et al.* 2000; Dettki *et al.* 2000; Gu *et al.* 2001; Walser *et al.* 2001; Öckinger *et al.* 2005). Werth *et al.* (2006) argue that establishment rather than dispersal of *L. pulmonaria* is limiting.

An antiherbivore function has recently been demonstrated for the secondary compounds in this particular lichen, as well as for compounds in a number of other lichens (Gauslaa 2005). The concentrations of lichen compounds in the smallest thalli of *L. pulmonaria* in this study are equal to the residual concentration in acetone-rinsed intact thalli, since acetone-rinsing cannot extract all secondary compounds in this species (unpublished data). Residual concentrations are insufficient to give *L. pulmonaria* a strong herbivore defence (Gauslaa 2005). Loss of juvenile thalli due to slugs and arthropods has been described in a Swiss transplantation study (Scheidegger *et al.* 1995; Scheidegger 1995). In conclusion, due to low contents of secondary compounds, herbivory might limit survival of young specimens in habitats rich in lichen-feeding molluscs and thereby reduce this species' reproductive success.

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