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UV-B induces usnic acid in reindeer lichens

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Abstract: Induction of secondary compounds in three reindeer lichens (*Cladonia arbuscula*, *C. rangiferina* and *C. stellaris*) was studied under controlled conditions in a growth chamber. Acetone rinsed (secondary compounds removed) lichen mats were subjected to three light regimes (PAR, PAR+UV-A and PAR+UV-A+UV-B), each combined with simulated herbivory (clipping). After 4 weeks, lichen extracts were analyzed by HPLC for any synthesized secondary compounds. UV-B induced the synthesis of usnic acid in *C. arbuscula* and *C. stellaris* and melanic pigments in *C. rangiferina*. Atranorin, fumarprotocetraric acid and perlatolic acid were not influenced by light quality. None of the identified compounds were significantly influenced by clipping. In conclusion, all three lichen species responded to UV-B radiation by developing cortical UV-B absorbing pigments that might function as protective screens. However, the experiment did not produce evidence for a herbivore-deterrent role of compounds studied.

Key words: atronorin, *Cladonia* spp., fumarprotocetraric acid, herbivory, melanin, perlatolic acid

Introduction

Most lichens contain secondary compounds, often termed lichen products, which are synthesised by the fungal partner of the symbiosis. Lichen compounds generally are phenolics with major absorption bands in the UV-B waveband, and several experiments demonstrate a toxic or repelling effect of lichen compounds for herbivores (e.g. Slansky 1979; Hätscher *et al.* 1991; Emmerich *et al.* 1993; Giez *et al.* 1994; Proksch & Hesbacher 1997; Lawrey 1983; Fröberg *et al.* 1993; Baur *et al.* 1994; Pöykkö & Hyvärinen 2003; Benesperi & Tretiach 2004; Gauslaa 2005; Pöykkö *et al.* 2005). Some lichen compounds are found only in the upper cortex, that is above the photobiont, and probably protect the photobiont from excess/damaging light and grazing. In most lichen species there are also compounds situated underneath the photobiont (in the medulla), where they may function as herbivore deterrents, as well as antibiotics, antioxidants and allelopathic agents.

Chemical protection can be either constitutive (genetically decided), inducive (produced in response to an environmental signal) or a combination of the two. Apart from the un-extractable melanin compounds, a substantial proportion of most lichen compounds can be removed by rinsing dry thalli in acetone (Solhaug & Gauslaa 1996, 2001). The treatment does not seem to harm the physiological properties of the lichen (Solhaug & Gauslaa 1996; Pöykkö *et al.* 2005), and is a useful tool for testing the response of secondary metabolism to an environmental stress.

This study focuses on some common and widespread reindeer lichens (*Cladonia*, subgenus *Cladina*). They often dominate the ground in boreal pine forests and open, low-alpine sites in a wide range of habitats, from humid, open forests, rocks and heaths and form important part of the winter-diet of reindeer (Krog *et al.* 1994). The reindeer lichens lack a well-defined cortex, but a thin layer of loose hyphae covers the photobiont (Krog *et al.* 1994) and in which either usnic

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acid or atranorin are always present. These two compounds rarely occur together in one species (Huovinen & Ahti 1986), possibly because of overlapping ecological roles. In addition, the reindeer lichens usually contain 1 or 2 medullary compounds, of which fumarprotocetraric acid is very common.

We have previously shown that the cortical pigments, parietin and melanin, may be synthesized as a response to UV-B (Solhaug *et al.* 2003; Nybakken *et al.* 2004). These compounds are, however, very different from, for example usnic acid and atranorin, since they absorb significant quantities of visible light in addition to UV-B. As far as we know, no evidence has been found for any effect of environmental factors on concentrations of atranorin, while some studies point towards a UV-B absorbing role for usnic acid (Quilhot *et al.* 1991, 1996; Bjerke *et al.* 2002, 2005). The medullary compound fumarprotocetraric acid has been suggested as an anti-herbivore compound (Giez *et al.* 1994), but we found no studies testing this hypothesis.

We have applied acetone rinsing to test the inductive power of UV-B radiation and herbivory (simulated by clipping) for production of lichen compounds in three species of reindeer lichens with different combinations of cortical and medullary compounds: *Cladonia arbuscula* (usnic acid and fumarprotocetraric acid), *C. rangiferina* (atranorin and fumarprotocetraric acid) and *C. stellaris* (usnic acid and perlatolic acid).

Material and Methods

Plant material

Lichen mats of *Cladonia arbuscula* (Wallr.) Flot., *C. rangiferina* (L.) F. H. Wigg and *C. stellaris* (Opiz) Pouzar & Vězda were collected from open *Pinus sylvestris* forests near Joensuu, Finland (62°36′N, 29°40′E), during August/September 2004. Ten different sites were chosen, situated at least 10 km apart from each other. At each sampling site, all three species were collected from one single area of *c.* 10 m × 10 m. Soil and litter debris were removed, and the lichen mats were cut into 180 small mats of about 7 cm × 7 cm.

Pre-experimental treatment

Ninety of the 7 cm × 7 cm lichen mats were moistened and clipped before acetone rinsing to simulate grazing by reindeer. The rest of the mats were left intact. In the clipped treatment the upper 1 cm (the apices) of the podetia were cut from *c.* 50% of the mat area, equivalent to 5–10% of total dry weight (d. wt). The material removed was dried, weighed, and extracted (see below), to provide a measure of initial content of lichen compounds. Both clipped and unclipped mats were dried for 24 h in a drying room at 10% relative humidity and 30°C, weighed, and finally washed with acetone to remove the secondary compounds according to the method described in Solhaug & Gauslaa (1996, 2001). The mats were immersed in excess acteone (250 ml) for 2 × 20 min (*C. arbuscula* and *C. rangiferina*) or 2 × 10 min (*C. stellaris*) and then left in a fume cupboard overnight to let residual acetone evaporate.

Experimental set-up

Lichen mats were randomly placed into 6 different pot trays each with 30 (three lichen species from each of ten different sites) 7 × 7 cm pots. The bottoms of the pots were filled with bryophytes (mainly *Sphagnum* spp. and *Pleurozium schreberi* collected from one of the sites) to retain water and reduce evaporation. The six treatments were: photosynthetically active radiation (PAR) (later called control), PAR+clipping, PAR+UV-A (later called UV-A), UV-A+clipping, PAR+UV-A+UV-B (later called UV-B) and UV-B+clipping. The PAR, provided by high-pressure sodium lamps (SHP-TS 400W, SYLVANIA, Belgium), was 400 µmol photons m^{-2} s^{-1} measured over the waveband 400–700 nm at the mat surface. The UV-radiation was provided by Q-panel UV-B tubes (UVB-313, Q-Panel Co, Cleveland, OH, USA), using polyester film (0·125 mm, Film Sales Ltd, London, UK) to screen UV-B radiation in the UV-A treatment and cellulose-diacetate (0·115 mm, Film Sales Ltd.) filters to screen any possible UV-C radiation for the UV-B treatment. The light period (PAR) was 18 h, with a UV period in the middle. During the first two weeks the biologically effective UV-B dose given was 6 kJ m^{-2} day^{-1} (2 h 45 min) to let the lichens adapt to the UV-B. The UV-B period was then increased to 3 h 15 min, delivering a UV-B dose of 7·15 kJ m^{-2} day^{-1}. The daily maximum temperature between two lichen mats was 25°C, the minimum temperature (at darkness) was 15°C. The humidity varied from 56% during light exposure to 80% during night. The lichens were sprayed with tap water twice a day; first one hour before the UV-period started, and then 30 min after the end, when PAR was still available, to allow for repair of possible UV-B damage.

Harvesting of lichens

The lichens were harvested after 4 weeks. Whole lichen mats were dried for 24 h in the dark at room temperature, and weighed. From each mat, unclipped podetia from the central part were harvested, and separated into apices (upper 10 mm) and stems (rest of living podetia), and extracted separately. Clipped

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podetia (stems) from all clipped mats were also collected and extracted.

Extraction of lichen compounds

About 50 mg of dry material of either apices or stems was weighed (±0·1 mg), immersed in 2·5 ml of acetone and extracted for 5 (*C. stellaris*) or 10 (*C. arbuscula* and *C. rangiferina*) min on an ice bath. During the first 30 s of the extraction period, the lichen material was crushed and blended with a homogenizer (Ultra-Turrax). The supernatant was collected in a separate vial, while the pellets were re-extracted 3 times, including only a 15 s homogenizing period. After each homogenizing, the blade of the homogenizer was washed in a tube with 2·5 ml clean acetone which was finally added to the combined supernatants. An aliquot of 5 ml of the combined acetone extract (total volume 12·5 ml) was taken out, and the acetone evaporated with a vacuum centrifuge. The dry extract was kept in a refrigerator until HPLC analysis.

HPLC analysis

Extracts were redissolved in 500 µl of acetone (or 400 µl acetone+100 µl solution of the internal standard benzoic acid for every second sample), and analysed on a 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) including a 1040M diodearraydetector. Separation was achieved on an ODS Hypersil 60 × 4·6 mm column. Oven and injector temperatures were 25 and 22°C, respectively. The injection volume was 10 µl, and the flow rate 2 ml min^{-1}. Solvent A consisted of 0·25% orthophosphoric acid and 1·5% tetrahydrofuran in millipore water, while solvent B was 100% methanol (Tamro Medlab Oy, Vantaa, Finland). The run started with 30% B. Within 15 min solvent B was increased to 70% and further to 100%, and then isocratically in 100% B for a further 5 min. At the end of the run, solvent B was reduced to 30% within 1 min, and the column flushed with 30% B for 10 min before the next run started.

The detection wavelength was 245 nm, and the identification of compounds was based on retention times, online UV-spectra, co chromatography of commercial standards (atranorin and fumarprotocetraric acid (Apin Chemicals), usnic acid (Sigma)), and HPLC-MS (Agilent Technologies, Waldbronn, Germany). The conditions for HPLC/API-ES (positive ions) were as follows: a Hypersil RP C18 column with ID 2 mm and 10 cm length was used, the ES fragmentor voltage was 80, the flow rate was 0·4 ml min^{-1}, and injected volume was 5 µl. HPLC/API-ES produced the following fragments of the lichen compounds:

atranorin: 389 (15), 193 (100)
fumarprotocetraric acid: 495 $[M+Mg]^+$ (57), 339 (100)
perlatolic acid: 445 $[M+H]^+$ (15), 221[220 (left bezene ring+CO)+H]$^+$ (100) and 243 $[220+Mg]^+$ (22)
usnic acid: 345 $[M+H]^+$ (100).

The compounds, except perlatolic acid, were quantified against response curves of the above-mentioned standards, and the internal standard, benzoic acid (Sigma). Perlatolic acid is not commercially available, and a response curve of usnic acid was used for quantification of this compound.

Reflectance analysis

UV-B exposed *Cladonia rangiferina* produced a melanic (brown), non-extractable pigment during the experiment. Melanic pigments in the cortex of *Cetraria islandica* absorb strongly in the UV-region (Nybakken *et al.* 2004). Melanic compounds cannot be extracted from lichen thalli with acetone or any other common extraction media. However, the visible, brown pigmentation can be quantified by measuring the reflectance (450–800 nm) from the lichen surface (Solhaug *et al.* 2003).

Lichen mats of *C. rangiferina* were moistened and dried under pressure over the night, to obtain a dense and flatter upper surface of podetia. Reflectance spectra were measured by holding dry and wet lichen thalli against an integrating sphere (model ISP-50-REFL Ocean Optics, NL-6961 LL Eerbeek, The Netherlands) and illuminating them with a halogen lamp (model DH2000, Ocean Optics) through a 600-µm-thick optical fibre connected to the input port of the integrating sphere. Reflectance was detected by a spectrometer (model SD2000, Ocean Optics) connected to the output port of the sphere with a 400 µm-thick fibre. Percent reflection was calculated on the basis of a dark spectrum and a reference spectrum from a white reference tile (WS-2, OceanOptics).

Data analysis

Original contents of secondary compounds were computed from extractions carried out on material collected from the mats before any treatments started (the clipped off material). The effects of the treatments on the content of lichen compounds were tested using a two-way ANOVA with type of light and clipping as factors and concentration of compounds as effects. The effect of different light treatments on the melanic pigmentation in *C. rangiferina* was tested in a one-way ANOVA with mean reflectance ($n=10$) at each wavelength between 450 and 800 nm as input for each treatment. The statistical software applied was SigmaStat 3.1 for Windows (Systat Software Inc.).

Results

Resynthesis of lichen compounds

UV-B induced the resynthesis of usnic acid in apices of both clipped and non-clipped mats of *C. arbuscula* and *C. stellaris*. After 4 weeks, the UV-B treated mats contained twice as much usnic acid as those that

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TABLE 1. *One- (clipped material) and two-way (apices and stems) tests of effect of light (PAR, PAR+UV-A and PAR+UV-A+UV-B) and herbivory (clipping, no clipping) ns=P>0·05*

	Light			Herbivory			Light × Herbivory		
	df	F	*P*	df	F	*P*	df	F	*P*
C. arbuscula									
Apices									
Usnic acid	2, 54	11·727	<0·001	1, 54	0·001	ns	2, 54	0·596	ns
Fumarprotocetraric acid	2, 54	1·414	ns	1, 54	0·157	ns	2, 54	0·326	ns
Stems									
Usnic acid	2, 54	2·598	ns	1, 54	0·179	ns	2, 54	0·088	ns
Fumarprotocetraric acid	2, 54	0·138	ns	1, 54	0·138	ns	2, 54	0·651	ns
Clipped stems									
Usnic acid	2, 27	5·066	0·014						
Fumarprotocetraric acid	2, 27	0·441	ns						
C. rangiferina									
Apices									
Atranorin	2, 54	1·925	ns	1, 54	0·267	ns	2, 54	0·423	ns
Fumarprotocetraric acid	2, 54	0·387	ns	1, 54	0·512	ns	2, 54	0·590	ns
Stems									
Atranorin	2, 54	1·165	ns	1, 54	2·745	ns	2, 54	0·097	ns
Fumarprotocetraric acid	2, 54	0·120	ns	1, 54	0·407	ns	2, 54	0·731	ns
Clipped stems									
Atranorin	2, 27	0·284	ns						
Fumarprotocetraric acid	2, 27	0·131	ns						
C. stellaris									
Apices									
Usnic acid	2, 54	10·651	<0·001	1, 54	1·069	ns	2, 54	0·004	ns
Perlatolic acid	2, 54	0·129	ns	1, 54	0·090	ns	2, 54	2·439	ns
Stems									
Usnic acid	2, 54	2·295	ns	1, 54	1·191	ns	2, 54	3·437	0·039
Perlatolic acid	2, 54	0·434	ns	1, 54	0·035	ns	2, 54	1·375	ns
Clipped stems									
Usnic acid	2, 27	2·370	ns						
Perlatolic acid	2, 27	1·215	ns						

were PAR treated (Table 1, Fig. 1). In clipped mats of *C. arbuscula*, the same induction effect of UV-B was found in stems of clipped podetia. In stems of *C. stellaris* there was a statistically significant interaction between type of light and clipping. The Holm-Sidak test showed that within clipped mats there is a tendency for UV-B treated mats to have higher content of usnic acid than the PAR-treated. There was no direct effect of clipping on the synthesis of usnic acid in any of the species. The other lichen compounds, atranorin, fumarprotocetraric acid and perlatolic acid, were not affected by UV-radiation or by clipping (Fig. 1, Table 1).

The melanic pigmentation in tips of *C. rangiferina*, quantified by reflectance measurements (Fig. 2), was significantly lower ($P<0·001$), that is the thalli were darker, in UV-B treated lichens than in controls and UV-A treated ones, which indicates that UV-B induced melanic pigments in the lichens. The effect could be seen with the naked eye.

Pre-treatment levels of lichen compounds

The dibenzofuran usnic acid is the major compound in *C. arbuscula* and *C. stellaris*, representing 2·1 and 2·4% of the d. wt of intact apices (all sites pooled, before acetone rinsing) (Fig. 3). In *C. arbuscula* the depsidone fumarprotocetraric acid constitutes

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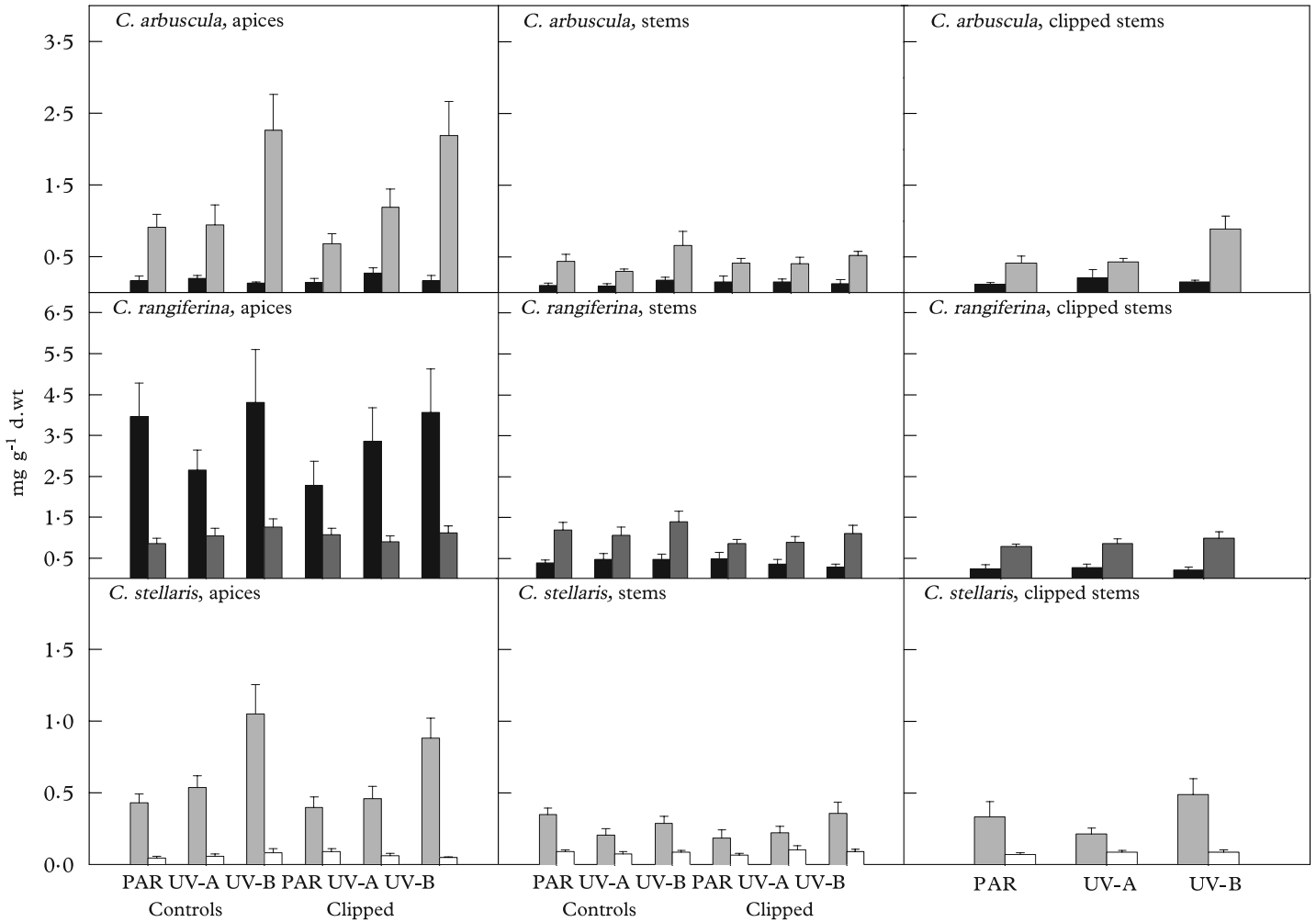

FIG. 1. Concentrations of lichen compounds in apices, stems and clipped stems of *Cladonia arbuscula*, *C. rangiferina* and *C. stellaris*. ■ =fumarprotocetraric acid, ▒=usnic acid, ▒=atranorin, □=perlatolic acid. Mean values are plotted ± 1 SEM (n=10).

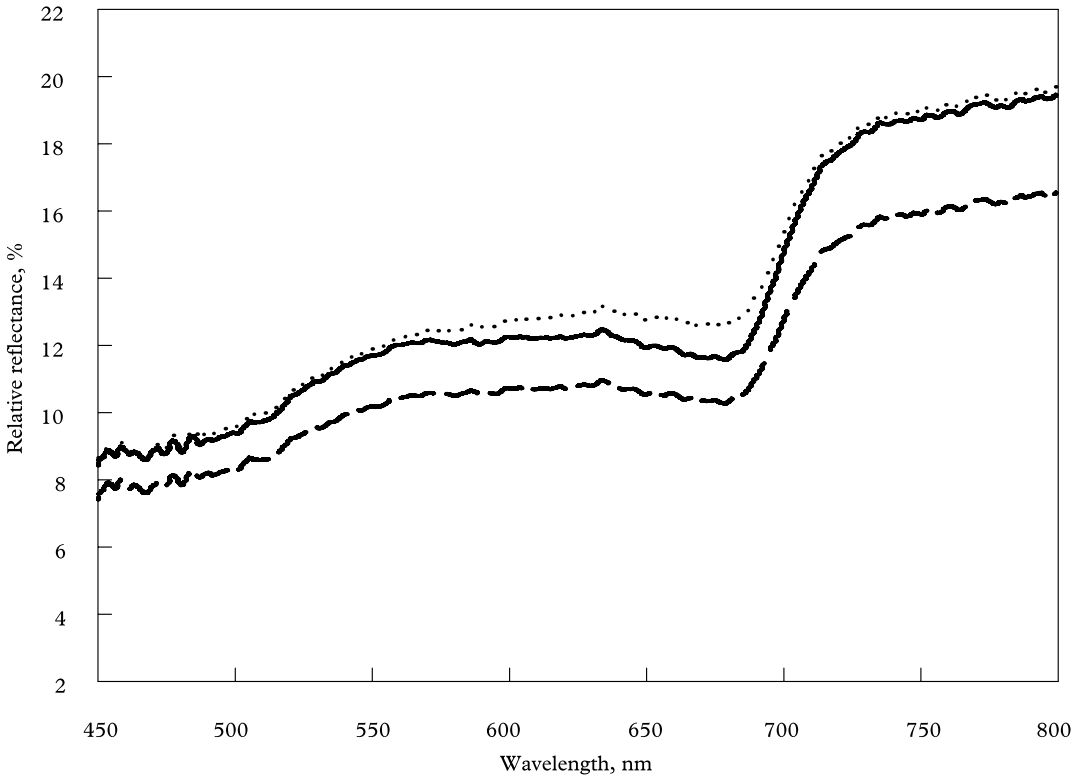

FIG. 2. Reflectance (%) from the surface of dry *Cladonia rangiferina* podetia after 28 days-exposure to three different radiation treatments (—— controls, · · · · UV-A, – – – – UV-B).

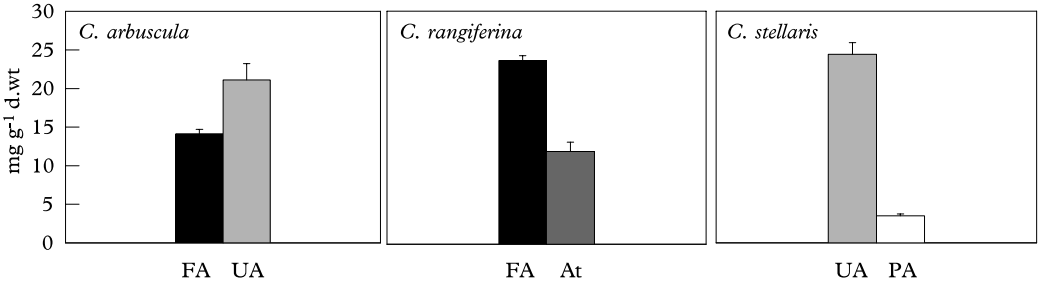

FIG. 3. Concentrations of lichen compounds in tips of *Cladonia arbuscula*, *C. rangiferina* and *C. stellaris* before acetone rinsing and treatment. Mean values are plotted ± 1 SEM (n=30) for all 10 sites pooled. For key see Fig. 1.

1·4% of the d. wt, while *C. stellaris* contains 0·4% of the para-depside perlatolic acid. In *C. rangiferina*, fumarprotocetraric acid is the dominant compound, 2·4% of d. wt, while 1·2% of the d. wt is the para-depside atranorin. The total content of extracted secondary compounds represented 3·5% of the dry weight of *C. arbuscula* and *C. rangiferina* and 2·8% in *C. stellaris*.

The concentration of usnic acid in both *C. arbuscula* and *C. stellaris* was up to 75% higher in apical areas compared to lower parts of the podetia, (compare apices and stems in Fig. 1) after acetone washing and

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4 weeks treatment. Atranorin in *C. rangiferina*, on the other hand, did not vary with depth within mats. Concentration of fumarprotocetraric acid in *C. rangiferina* was up to 90% higher in apices than in stems, while the corresponding difference in *C. arbuscula* was somewhat lower, up to 50%. The content of perlatolic acid in *C. stellaris* was slightly higher in stems than in apices.

Discussion

This study shows that the lichen compound usnic acid is induced by UV-B in the two reindeer lichens *Cladonia arbuscula* and *C. stellaris*. This supports the hypothesis inferring a UV-B screening role for this compound (e.g. Rundel 1978), with two absorbance peaks, one at 232 nm and one at 282 nm. Usnic acid absorbs very little PAR, and can hardly play a role in screening of visible light. The literature on screening *in situ* by a UV-B absorber such as usnic acid under natural conditions, however, is scarce. Buffoni-Hall *et al.* (2003) showed that *C. arbuscula* ssp. *mitis* is sensitive to UV-B radiation in the air-dry state because DNA damage caused by UV-B was not repaired. At low temperatures (2°C), there was no repair even in fully hydrated lichens.

In intact mats, the UV-B induction of usnic acid was seen only in the exposed upper and youngest parts of the podetia (the apices, see Fig. 1). This spatial distribution is in accordance with a UV-B protecting role, as very little solar radiation will penetrate through the dense canopy and down to lower parts of the podetia. It is also in line with the optimal defence theory (Rhoades 1979), which predicts that plant parts with high fitness value will be highly defended. In untreated thalli of *C. stellaris* and *C. rangiferina* the apical 10 mm regions accounts for *c.* 50% of their photosynthetic capacity (Moser *et al.* 1983). A gradual reduction in photosynthetic capabilities and respiratory rates (Carstairs & Oechel 1978; Nash 1980), as well as phenolic concentrations (Mirando & Fahselt 1978; Fahselt 1984; Golojuch & Lawrey 1988) has also been measured from the tips to the bases of lichen podetia. In *C. arbuscula*, usnic acid was also UV-B induced in clipped stems. This shows that older parts of the lichen are also able to respond to increased solar radiation when they become exposed. The other cortical compound involved in this study, atranorin, did not respond to UV-B (Fig. 2). To our knowledge, there are no reports of the direct induction of atranorin by radiation in any part of the solar spectrum. However, the concentration of atranorin increases along a natural light gradient in *Hypogymnia physodes* (K. A. Solhaug *et al.*, pers comm.), which may imply a light-driven constitutive pool of atranorin.

None of the acetone-extractable compounds in *C. rangiferina* were induced by UV-B. However, UV-B and PAR absorbing melanic pigments accumulated in the apices of the acetone rinsed lichen mats during the experimental period in the same way as in the upper cortex of other fruticose lichens (Nybakken *et al.* 2004) and also in some foliose lichens (Gauslaa & Solhaug 2001). Quantification of the pigmentation by reflectance measurements showed that UV-B treated mats were significantly darker than those treated with PAR and UV-A. Alpine, exposed populations of *C. rangiferina* are often dark brown, contrasting with the grey colours at more shaded sites. *Cladonia rangiferina* is presumably well protected against high solar radiation by these inducible melanic compounds, and has additionally a high constitutive pool of phenolic compounds.

To our knowledge, this is the only published study in which the impact of simulated herbivory on induction of lichen compounds has been tested. None of the compounds included were influenced by the simulated herbivory (clipping). However, simulated herbivory and/or laboratory conditions may not necessarily be ecologically relevant. Scissors do not leave saliva or faeces on the lichens, and do not even technically mimic the way the podetia are torn by animal teeth/tongues, and not at all how lichens on the ground are trampled down by big animals. Such factors have been proved important for higher plants'

responses to herbivores (Baldwin 1990). Abundant evidence exsists, however, that lichen compounds may function in defence (e.g. Hesbacher *et al.* 1995; Backor *et al.* 2003; Gauslaa 2005; Pöykkö *et al.* 2005).

In conclusion, our results show that UV-B induces accumulation of usnic acid and melanic compounds, but there is still no evidence for any of the lichen compounds included in this study being produced as a response to herbivory. Effects of natural herbivory on the content of lichen compounds should be tested in future studies, and also more feeding experiments should be done to test related lichen species with partly overlapping, but not identical, composition of lichen compounds, to determine which compounds are effective feeding deterrents in nature.

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