

## Lichen substances and the growth of root associated fungi *Phialocephala fortinii* and *Rhizoscyphus ericae* (Ascomycetes) in pure culture

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Mat-forming lichens are key components of field-layer vegetation of the oligotrophic boreal forests. Hence lichen residues containing secondary substances have a great potential to affect soil microbial processes and both positive and negative impacts on, for example, mycorrhizae have been reported. However, apart from mycorrhizae little is known about the responses of other root-associated fungi to these compounds. Here, we report results from an *in vitro* experiment where impacts of biologically meaningful concentrations of selected lichen secondary metabolites and lichen extracts on the radial growth of two ubiquitous root-associated fungi were under scrutiny. The growth of both fungal species, *Phialocephala fortinii*, a fungus belonging to the dark-septate endophyte complex, and *Rhizoscyphus ericae*, a mycorrhizal associate of Ericaceous plants, responded negatively to the studied concentrations of lichen phenols, whereas lichenan did not affect the growth of either of the species. Extracts from epiphytic *Hypogymnia physodes* had variable effects on the fungi. We conclude that lichen phenols are potentially antibiotic towards root-associated fungi, and lichenan is not readily degradable by the fungal species studied. In order to resolve the dispute about the ecological significance of lichen substances, the degradation processes of these compounds in real microbial community should be traced.

Keywords: allelopathy, dark septate endophytes, ericoid mycorrhiza, facilitation, lichen phenols.

Lichen mats are important vegetation components of oligotrophic boreal forests and subarctic heathlands (Ahti 1977, Ahti & Oksanen 1990). Lichen residues in forest soils generally originate from mat-forming lichens, such as *Cladina* spp., but also to minor extent from those with an epiphytic growth habit (Bandoni & Towers 1967, Esseen 1985). The residues contain phenols and lichen-specific carbohydrates, e.g. lichenan (Henssen & Jahns 1974). Lichen phenols are assumed to contribute to the humus phenols (Dawson *et al.* 1984) and have potential to modify soil processes and microbial species composition in soils; both allelopathic (Brown & Mikola 1974, Tolpysheva 1979 a,b, Goldner *et al.* 1986, Souto *et al.* 2000) and facil-

itative (Crittenden 2000, Stark & Hyvärinen 2003) effects have been reported. The role of lichen specific carbohydrates in soil microbial processes is less studied as such, but laboratory studies indicate that e.g. lichenan provides a direct energy source for cellulolytic microbes (Perlin & Suzuki 1962).

Despite the abundance of lichen phenols in lichen thalli (for example, the concentration of usnic acid in *Cladina* mats being as high as 1–3 %, Huovinen 1985), the presence of the lichen phenols in soils under lichen mats has remained questionable. Dawson *et al.* (1984) reported usnic acid concentrations of 1–4 ppm from arctic soils, but a couple of studies have failed to find any traces of usnic acid under lichen mats (Vainshtein & Ravinskaya 1984, Stark *et al.* 2007). The anomaly may be due to the degradation process of usnic acid. According to Vainshtein and Ravinskaya (1984) the added usnic acid disappeared within 16 days in forest soils and the rate of disappearance was equal in soils under lichen mats and on lichen-free plots. In contrast, in the sterilized soil they found 80% of their addition after 16 days incubation suggesting that microbes in forest soils may rapidly degrade lichen acids.

Ericaceous dwarf shrubs form are common constituents of the oligotrophic boreal forest and subarctic heath vegetation. Roots of the ericaceous plants are colonized by ascomycetous root-associated fungi; both mycorrhiza-forming and dark septate endophytic (DSE) fungi are abundantly involved (Smith & Read 2008, Jumpponen & Trappe 1998). A key feature for both the ericoid mycorrhizal and DSE fungi seem to be their ability to break down complex polymers for their nutrition; ericoid mycorrhizal fungi have been reported to utilize chitin (Kerley & Read 1997), cellulose (Burke & Cairney 1997), polyphenols (Bending & Read 1996a,b), and to some extent also lignin (Bending & Read 1997). According to Caldwell *et al.* (2000), DSE fungi degrade at least cellulose, laminarin, starch and xylan. These observations suggest that ascomycetous root-associated fungi may directly utilize complex lichen-derived substances. Despite the ecological importance of the resource circulation potential related to these fungi, the impacts of lichen substances on the ascomycetous root associates have remained virtually unexplored to date (but see Crittenden 2000).

To conclude, it is pertinent to ask whether the lichen phenols; some of them highly toxic towards organisms in general (Elix 1996, Cocchietto *et al.* 2002), but also potential to act as carbon sources (Bandoni & Towers 1967, Kutney *et al.* 1977, Stark & Hyvärinen 2003), have either allelopathic or facilitative impact on the ascomycetous root-associated fungi. The objective of this study was to investigate the impacts of lichen substances on the radial growth of two well-documented representatives of ascomycetous root associ-

ates: first, *Phialocephala fortinii*, a fungus belonging to DSE complex, and, second, *Rhizoscyphus ericae*, a mycorrhizal symbiont of ericaceous plants. Crude extracts were obtained both from mat-forming lichens and from an epiphytic lichen species to see whether their impacts on these fungi would differ. In addition, we aimed to compare the impacts of pure substances vs crude extracts, and finally, to test whether lichen-derived carbohydrate lichenan would have growth impacts on these fungal strains.

## Materials and Methods

### Lichen substances

Extraction of lichen acids (crude extracts) from *Cladina mitis* (Sandst.) Hustich, *C. stellaris* (Opiz) Pouzar & Vezda and *Hypogymnia physodes* (L.) Nyl. were carried out by repeated Soxhlet extraction (see Elix, 1975) of air-dried thalli collected three days earlier. *C. mitis* and *C. stellaris* were extracted in acetone and *H. physodes* both in methanol and ethyl acetate. After the extraction, the solvents were left to evaporate into dryness in a fume hood. The quality of the extracts was checked by thin layer chromatography and mass spectrometry. In the crude extracts the ratios (wt/wt) were as follows: *C. mitis* usnic acid : fumaprotocetraric acid 10 : 1, *C. stellaris* usnic acid : perlatolic acid 10 : 1, *H. physodes* ethyl acetate extract: atranorin : physodic acids 1 : 2, and *H. physodes* methanol extract: only physodic acids. Pure substances (atranorin, lichenan and usnic acid) were purchased from Sigma (St Louis, USA).

### Fungal media

All substances were added in dry form to the autoclaved and cooled fungal media ( $\frac{1}{2}$  MMN, modified Melin-Norkrans-medium, Marx 1969) to yield the following concentrations on the fungal plates (mg mL<sup>-1</sup>): atranorin 0.026, lichenan 0.047, usnic acid 0.034, *Cladina mitis* extract 0.033, *C. stellaris* extract 0.042, *Hypogymnia physodes* methanol extract 0.044 and *H. physodes* ethyl acetate extract 0.166. Concentrations of *Cladina* extracts and usnic acid in the media were thousand times lower compared to those found in *Cladina* mats (1–3% of dry weight, Huovinen 1985). In the case of *H. physodes* the concentrations were comparable to those used by Vainshtein & Tolpysheva (1992). Thus, the applied concentrations can be considered biologically relevant in forest soils in association with lichen residues (see also Dawson *et al.* 1984).

## Fungal isolates

We used two fungal isolates in the experiment: a North American strain of *Phialocephala fortinii* Wilcox & Wang (strain SE 24), originally isolated from *Lupinus latifolius* Agardh. by O'Dell et al. (1993), and a British *Rhizoscyphus ericae* (D.J. Read) W.Y. Zhuang & Korf (strain Read 100; the same as UAMH 6563, Egger & Sigler 1993), original host plant unknown. However, the *R. ericae* strain is tested for ability to form mycorrhiza with *Calluna vulgaris* (Pearson & Read 1973) and *Gaultheria shallon* (Xiao & Berch 1995). Both fungal species inhabit a wide range of ecosystems worldwide being especially common in stressful conditions, such as oligotrophic heaths and arcto-alpine environments (Smith & Read 2008; Jumpponen & Trappe 1998). We chose these specific strains because they have been used in numerous controlled experiments being thus well-documented (Pearson & Read 1973, Xiao & Berch 1995, Jumpponen & Trappe 1998, Jumpponen et al. 1998, Sharples et al. 1999, Caldwell et al. 2000, Ruotsalainen & Kytöviita 2004). The sequences for these strains are available in GenBank: <http://www.ncbi.nlm.nih.gov/Genbank/> with accession numbers AF055885 (*P. fortinii*, Jumpponen & Trappe 1998) and AF096505 (*R. ericae*, Chambers et al. 1999)].

## Fungal cultures

Plugs of 10 mm in diameter of young fungal plate culture were placed on agar plates (90 mm in diameter) containing growth media (25 mL) and lichen substances, two replicates per treatment. Pure 1/2 MMN plates with no additions were used as controls. We used agar plates and measurement of colony diameter because this approach had been used in similar kind of studies earlier (Brown & Mikola 1974, Goldner et al. 1986; Vainshtein & Tolpysheva 1992, Kaitera et al. 1996, Halama & Haluwyn 1997). Plates were incubated for 28 days in room temperature in dark conditions. The relative growth rates (RGR) of the isolates were calculated on the basis of the radial growth according to Hunt (1990):

$$RGR = [\ln(end) - \ln(start)] / [t(end) - t(start)]$$

where  $t(end) - t(start)$  was 28 days.

## Statistical analyses

The relative growth rate during the course of the experiment was first examined with a linear model (function *lm*, analysis being identical to ANOVA procedure, Crawley 2007) using fungal species (*P. fortinii* and *R. ericae*) and lichen substance (seven substances and the control) as explanatory variables. As the interaction (spe-

cies\*substance) was statistically significant, we continued by testing the impact of substance separately on both fungal species with a linear model. *A priori* contrasts were applied to determine which treatments differed from the control. Residual plots were used to control the assumptions on normality and homoscedasticity. Analyses were carried out by using R, version 2.4.0 (R Development Core Team, 2007).

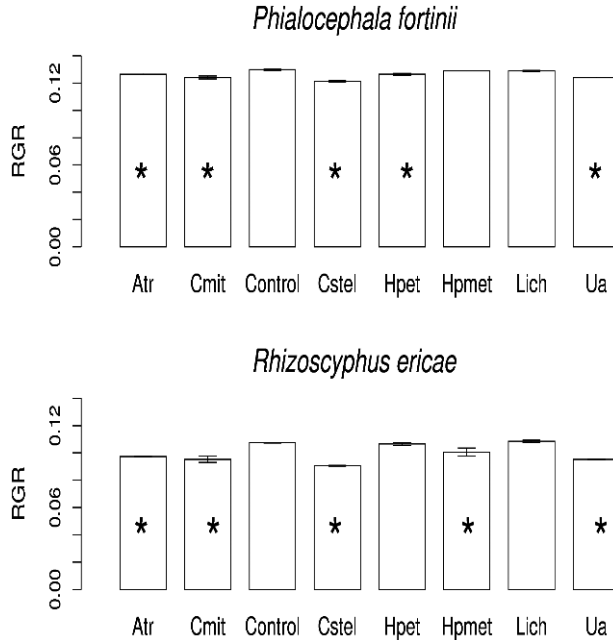
## Results

The lichen substances had an impact on fungal growth and these effects differed among the studied fungal species (the main effects for species  $F_{1,16}$ ,  $P < 0.001$  and lichen substance  $F_{7,16}$ ,  $P < 0.001$  with a statistically significant interaction,  $F_{7,16}$ ,  $P < 0.001$ ; Fig. 1). When the species were studied separately, lichen substances still had a statistically significant impact on the RGRs of both fungal species (*P. fortinii*:  $F_{7,8}$ ,  $P < 0.001$ , *R. ericae*:  $F_{7,8}$ ,  $P < 0.001$ , Fig. 1). The average RGR of *R. ericae* was lower than that of *P. fortinii* (Fig. 1).

The contrast analysis indicated that all the phenolic substances except the crude extracts of *Hypogymnia physodes* decreased the growth rates of both fungal species (Fig. 1). The fungal species responded differently to *H. physodes* extracts: the ethyl acetate extract including both atranorin and physodic acids suppressed the growth of *P. fortinii*, but not that of *R. ericae* (Fig. 1). Opposite to that, the growth of *R. ericae* was suppressed by the methanol extract, which only included physodic acids, but not by the ethyl acetate extract (Fig. 1). Lichenan did not affect the growth rates of either of the fungal species (Fig. 1).

## Discussion

We found both the crude extracts and pure phenols, atranorin and usnic acid to inhibit the growth of *P. fortinii* and *R. ericae*. The negative impact is in agreement with a number of previous studies on the effects of lichen phenols on fungi (Henningson & Lundström 1970, Brown & Mikola 1974, Tolpysheva 1979a,b, Goldner *et al.* 1986, Vainshtein & Tolpysheva 1992, Lawrey *et al.* 1994, Torzilli & Lawrey 1995, Halama & Van Haluwyn 1997, Lawrey 2000). However, it must be emphasized that our experiment was carried out by using solid substances in relatively low concentrations (26–47 ppm, *H. physodes* crude extract in 166 ppm) and, for example, Halama and Haluwyn's (1997) experiment was carried out with concentrations as high as 10 000 ppm. Therefore, our results suggest that these compounds are potentially toxic against some root associated fungi in relatively low concentrations.



**Figure 1.** Relative growth rates (RGR  $\text{mm}^{-1} \text{mm}^{-1} \text{day}^{-1}$ )  $\pm$  SE of the radial growth of *Phialocephala fortinii* and *Rhizoscyphus ericae* grown for 28 days in agar plates containing lichen substances. Atr = pure atranorin, Cmit = crude extract of *Cladonia mitis*, Control = control treatment, Cstel = crude extract of *C. stellaris*, Hpet = ethyl acetate extract of *Hypogymnia physodes*, Hpmet = methanol extract of *H. physodes*, Lich = lichenan, Ua= pure usnic acid. Absence of the error bar indicates the same RGR values (N=2). Asterisks denote significant difference from the control (P < 0.05).

Usnic acid, typically found in mat-forming *Cladonia* species (Huovinen & Ahti 1986) as well as from epiphytic genera *Alectoria* and *Usnea* (e.g. Bandoni & Towers 1967), is one of the most effective antibiotics found in lichens (Lawrey 1986, Elix 1996, Cocchiello *et al.* 2002). The growth of both fungal species was greatly reduced on pure usnic acid containing media indicating thus a strong physiological inhibition at the studied concentration (34 ppm, which is thousand times lower than average usnic acid concentration found in *Cladonia* mats, Huovinen *et al.* 1985). Additionally, in our experiment, usnic acid caused a clear reduction in the fungal growth added in a solid form. According to Lechowski *et al.* (2006), concentrations as low as 1-5 ppm were enough to cause a marked growth reduction in a hydroponic tomato culture, however, in their study usnic acid was solved by using natrium hydroxide and the soluble form could be more growth inhibitory. On the other hand, both bacteria (Kutney 1977b) and fungi (Bandoni & Towers 1967, Kutney *et al.* 1977a) have

been shown to be able to directly degrade usnic acid. The discrepancy between these results – either the inhibition or the facilitation of the fungal growth due to usnic acid – is probably concentration-dependent and may also be related to the differences in the solubility of the usnic acid in the growth media.

Lichen phenols originating from mat-forming lichens had a negative impact on both fungi, both as pure substances and as crude extracts. In contrast, we found the crude extract from the epiphytic *Hypogymnia physodes* to have variable effects on fungal growth. Despite this, pure atranorin (present in the ethyl-acetate crude extract of *H. physodes*) clearly inhibited the growth of both fungal strains. The methanol extract from *H. physodes* containing mainly physodic acids inhibited the growth of *R. ericae*, but not the growth of *P. fortinii*. In contrast, the ethyl acetate extract containing both atranorin and physodic acids (1:2, wt:wt) was inhibitory towards *P. fortinii*, but not towards *R. ericae*. These results suggest that *P. fortinii* is more sensitive to atranorin than *R. ericae*. Tolpysheva (1985) and Halama & Van Haluwyn (1997) have also reported atranorin to have variable effects on fungi. In the crude extracts, there was also a minor fraction of unidentified phenols. In addition to them, the methanol extract contained sugars and the ethyl acetate extract contained sterols. In combinations with the lichen acids, these substances may also have played some role.

We did not detect any statistically significant difference in growth rate in the lichenan plates compared to the control plates. However, the actual growth rate on one *R. ericae* plate was higher compared to control. To our knowledge, we are not aware of any study on the impacts of pure lichenan on the growth of root associated fungi; the only existing information we have found is an enzymological study which shows that ascomycetous fungi should be able to degrade lichenan (Chen *et al.* 2006). It may also be that the media we used did not favour usage of complex polymers, or that the carbon assimilated from lichenan was respired and did not accumulate as radial growth (as the carbon derived from complex polymers may not be allocated into biomass, Paul & Clark 1989). In addition, the nutrient rich fungal media may decrease the production of fungal enzymes (Cohen 1972; Nehls *et al.* 2001). Therefore, we conclude that lichenan forms a potential carbon source for both *R. ericae* and *P. fortinii*, and that this aspect requires further research.

In general, *P. fortinii* had a higher growth rate than *R. ericae*. Higher growth rate of dark septate endophyte *P. fortinii* compared to the growth of mycorrhiza-forming *R. ericae* on artificial media suggests that *P. fortinii* may be more efficient in the utilization of external resources than *R. ericae* (see also Ruotsalainen & Kytöviita 2004). This suggests that *P. fortinii* may be more adapted to a sapro-

bic life in soil than *R. ericae*. Although *P. fortinii* is frequently associated with healthy roots of various plant taxa, (Jumpponen & Trappe 1998, Addy et al. 2005), the data this far suggest a multi-functional symbiosis with a continuum of responses from mutualism to parasitism in the host plants (Mandyam & Jumpponen 2005). In contrast, the slower growth rate in the case of *R. ericae* may be related to an avoidance of the induction of plant defence mechanisms, which is found to be typical to 'non-harmful' endophytic fungi (Hadacek & Kraus, 2002). To conclude, this result may reflect the difference between these two fungal species: that *R. ericae* is a documented mycorrhizal fungus (Pearson & Read 1973) and, despite the root-association, *P. fortinii* may be more saprobic by nature.

To conclude, our results show that *Phialocephala fortinii* and *Rhizoscyphus ericae* may be harmfully affected by lichen phenols when these substances are found in studied concentrations in their natural environment. We did not find any marked impact of lichenan on the growth of these fungi. We suggest that more tracer studies on the fate of lichen-derived phenols under the lichen-mats are needed and assume that the balance between phenol degradation and inhibition is both concentration- and condition-dependent.

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