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ORIGINAL ARTICLE

# Lichen secondary metabolites affect growth of *Physcomitrella patens* by allelopathy

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**Abstract** Lichen secondary metabolites can function as allelochemicals and affect the development and growth of neighboring bryophytes, fungi, vascular plants, microorganisms, and even other lichens. Lichen overgrowth on bryophytes is frequently observed in nature even though mosses grow faster than lichens, but there is still little information on the interactions between lichens and bryophytes.

In the present study, we used extracts from six lichen thalli containing secondary metabolites like usnic acid, protocetraric acid, atranorin, lecanoric acid, nortistic acid, and thamnolic acid. To observe the influence of these metabolites on bryophytes, the moss *Physcomitrella patens* was cultivated for 5 weeks under laboratory conditions and treated with lichen extracts. Toxicity of natural mixtures of secondary metabolites was tested at three selected doses (0.001, 0.01, and 0.1 %). When the mixture contained substantial amounts of usnic acid, we observed growth inhibition of protonemata and reduced development of gametophores. Significant differences in cell lengths and widths were also noticed. Furthermore, usnic acid had a strong effect on cell division in protonemata suggesting a strong impact on the early stages of bryophyte development by allelochemicals contained in the lichen secondary metabolites.

Biological activities of lichen secondary metabolites were confirmed in several studies such as antiviral, antibacterial, antitumor, antiherbivore, antioxidant, antipyretic, and analgetic action or photoprotection. This work aimed to expand the knowledge on allelopathic effects on bryophyte growth.

**Keywords** Allelopathy · Usnic acid · Bryophytes · Inhibiton ofgrowth

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## Introduction

Allelopathy describes a positive or negative mutual interaction between vascular plants, lichens, and mosses where chemical resources are used (Molisch 1938); nowadays, the term also comprises microorganisms, like microscopic algae, bacteria, and fungi. Compounds which affect other organisms and influence ecosystems are known as allelochemicals. Their production significantly increases when provoked by environmental stress (Reigosa and Sánchez-Moreiras 1999), and allelopathic interactions also alter environmental factors like the concentration of nutrients (Leflaive and Ten-Hage 2007).

Lichens form a symbiotic association between fungi (mycobionts) and algae or cyanobacteria (photobionts, phycobionts), representing nearly one fifth of all known fungal species (Hawksworth and Ainsworth 1995). In spite of their poorly developed morphology, lichens contribute crucially to the vegetation worldwide and even dominate the vegetation of approximately 8 % of terrestrial ecosystems (Inoue et al. 1987). Lichens produce over a thousand different extracellular

secondary metabolites known as "lichen substances/compounds" (Hauck and Huneck 2007). Their functions include but are not restricted to antimicrobial, antiherbivoral, and allelopathic activities (Lawrey 1995; Fahselt 1994), the protection of the photobiont against excessive light, the control of photobiont cell division, or chelation of potentially toxic metals (Hauck et al. 2009; Solhaug et al. 2009; Bačkor et al. 2010). The secondary metabolites are usually located extracellularly on the lichen surface. Thus, many secondary metabolites can be removed from dry thalli by rinsing with acetone (Solhaug and Gauslaa 1996) but preserving lichen viability. Therefore, it is possible to manipulate the content of secondary metabolites in lichen thalli, enabling the investigation of their physiological and ecological functions.

The habitat of lichen and moss populations is very similar resulting in a competition for substrate, water, nutrients, and light (Lawrey 1977). Thus, their secondary metabolites influence the growth and development of surrounding mosses, vascular plants, microorganisms, as well as lichens themselves by allelopathy (Macías et al. 2007). The allelochemical action relies on the influence of cellular processes like respiration, photosynthesis, transpiration, synthesis of nucleic acids and proteins, membrane permeability, as well as membrane transfer of ions (Molnár and Farkas 2010).

Many studies show that lichens can outgrow mosses or liverworts. In the past, it was anticipated that this is caused by chemical interactions (Frahm et al. 2000; During and van Tooren 1990; Sedia and Ehrenfeld 2003; Poelt 1985; Lawrey 1995). Single secondary metabolites that were extracted from different lichen species showed inhibitory effects on germination of *Funaria hygrometrica* and *Ceratodon purpureus* spores (Gardner and Mueller 1981; Frahm et al. 2000). The extraction of secondary metabolites by acetone and by water was used. In both cases, decreased spore germination rate and slower growth of protonemata were reported (Frahm et al. 2000). Here, we used *Physcomitrella patens* as a moss test system because of its status as a model organism and also in hinsight of further intended experiments in metabolomics.

In this study, we tested acetone extracts from six different lichen species to measure the biological activity and influence of secondary metabolites on the growth of protonemata in the moss *P. patens*. Furthermore, we investigated the development of gametophores and changes in cell size in gametophyte and protonema tissue. As morphological studies and investigations on developmental stages other than germination in mosses are rare under the effect of lichen secondary metabolites, we provide these data to broaden our view on the ecological co-existence of lichens and bryophytes.

We tested the following hypotheses:

- Secondary metabolites of lichens are allelochemicals and have influence on growth of neighboring bryophytes.
- Young stages of bryophytes are more affected by lichen allelochemicals than older ones.
- This influence is reflected on the cellular level in protonemata as well as in gametophores

## Material and methods

### Identifications of lichen substances

Extracts of six lichen thalli were used. *Usnea hirta* (L.) F.H. Wigg, (Parmeliaceae) thalli were collected from spruce (*Picea abies*) and pine (*Pinus silvestris*), in parks in Oulu, Finland. Five others were from the herbarium (Complutense University of Madrid, Faculty of Pharmacy, Spain): *Parmotrema robustum* (Degel.) Hale, (Parmeliaceae), (MAF-Lich 10166); *Parmotrema tinctorum* (Delise ex Nyl.) Hale, (Parmeliaceae), (MAF-Lich 10163); *Pleurosticta acetabulum* (Neck.) Elix and Lumbsch, (Parmeliaceae), (MAF-Lich 9914); *Flavoparmelia baltimorensis* (Gyelnik and Foriss) Hale, (Parmeliaceae), (MAF-Lich 6730); *Usnea florida* (L.) F.H.Wigg., (Parmeliaceae), (MAF-Lich 7049).

One hundred milligrams (dw) of lichen samples was extracted in 3-ml dry acetone (Sigma Aldrich) for 5 min according to Lokajová et al. (2014). Each lichen thallus was rinsed three times and the products mixed together to get higher content of metabolites. Acetone was allowed to completely evaporate from the extracts for 24 h at room temperature, and the dry weight of total extractable secondary metabolites was measured. Afterwards, all lichen extracts were dissolved with fresh acetone (Sigma Aldrich, ≥99.5 %) and analyzed by thin layer chromatography (TLC). A standardized TLC method by Orange et al. (2001) was used to identify secondary metabolites. Acetone extracts were applied to pre-coated thin layer plates of silica gel 60F-254 (Merck). For identification of lichen substances by TLC, three solvent systems (A,B,C) were used, and for visualization, they were sprayed with 10 % sulfuric acid and heated for 30 min at 110 °C.

For quantitative identification of lichen substances by high-performance liquid chromatography (HPLC), filtered acetone extracts were analyzed by gradient (Feige et al. 1993) 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 a wavelength of 245 nm (detector Ecom LCD 2084; Ecom, Prague, Czech Republic). Pure usnic acid (Sigma-Aldrich) was used as a standard. Other standards of protocetraric acid, atranorin, lecanoric acid, and nortistic acid were prepared from crystallized acetone extracts from lichens, prior to analyses (internal database at University of Kosice). Each analysis was performed with three independent replicates.

### Preparation of moss material

*P. patens* (Hedw.) [syn.: *Aphanorhegma patens* (Hedw.) Lindb] (Funariaceae) is a model organism for bryophytes. *P. patens* is often used in plant physiology because it has a short cultivation period and reacts fast to changes in the environment (Cove 1993; Lang et al. 2008; Rensing et al. 2008; Sassmann et al. 2015). Plantlets were cultivated under aseptic condition on solid medium at 21 °C on a 14-h/day/10 h night rhythm; the average of artificial irradiance was 48.08 μM $m^{-2}$ $s^{-1}$. The cultivation medium contained 200 mg $l^{-1}$ $NH_4NO_3$, 100 ml $l^{-1}$ $MgSO_4 \cdot 7H_2O$, 400 mg $l^{-1}$ $KH_2PO_4$, and 100 mg $l^{-1}$ $CaCl_2 \cdot 2H_2O$ and was solidified with 0.8 % agar (VWR, Prolab) at a pH of 5.8 according to Gang et al. (2003).

### Application of secondary metabolites

New Petri dishes (5.4 cm in diameter) were prepared with solid medium, and four glass fiber filter disks (Whatman CF/C filters; 2.5 cm in diameter) were placed on the surface of the solid medium (Fig. 1a). Disk pores allow for nutrients to pass through the disk from the medium below (Bačkor et al. 2010). The extracts of secondary metabolites of the six lichen thalli were each dissolved in acetone to a 1 % stock solution. From this stock, the following concentrations were prepared: 0.1 % (1 mg $ml^{-1}$), 0.01 % (100 μg $ml^{-1}$), and 0.001 % (10 μg $ml^{-1}$). Thirty-five microliters of each respective concentration was applied to the glass fiber disks according to Lokajová et al. (2014). Pure acetone of 35 μl was applied as a control on the 4th disk. After evaporation of the acetone for 1 h at room temperature and recrystallization of secondary metabolites, protonemata from *P. patens* were cut into small pieces and planted in the middle of the glass fiber disks. Plantlets were cultivated for 5 weeks; each treatment was repeated seven times.

### Macroscopic and microscopic measurements

To measure the growth parameters over the cultivation period of 5 weeks, each filter disk was photographed every week starting immediately after the cultivation (week 0). Images were taken with a macroscope (Nikon SMZ 1500, objective KH Plan Apo 1.6x, WD 24, Nikon) in combination with a Nikon 1J1 camera. The area on the filter that was occupied by protonemata was quantified using the GSA Image Analysis software (GSA, Rostock).

After the fifth week, gametophore and protonema cells were photographed in the light microscope (Olympus BX41, camera Olympus U-CMAD3), and the length and width of at least 150 cells were measured (Fig. 1b, c). Plantlet parts of each treatment and concentration were selected randomly from different plants of at least two different Petri dishes. The cells in the leaflets were selected in the middle of the leaflet to avoid the cells from the tip (small cells) and the base (elongated cells). Also, the cells of the edge and of the ridge were excluded due to their different shape.

### Statistics

For statistical analysis, the software STATISTICA 7.1 (StatSoft) was used. For the analysis of protonemata growth, a Kruskal-Wallis test was used, followed by a post hoc comparison of the mean ranks over all weeks and treatments. For cell size comparison, a multifactorial ANOVA was chosen followed by a post hoc Tukey HSD test. Due to the high sample sizes (>150), the effect size ($d_{Cohen}$) between the treatment/concentration and the control was calculated. For the significant values, only those were counted with an effect size over 0.5; differences to the control were marked with * (significant, $p < 0.05$), ** (highly significant, $p < 0.01$), or *** (very highly significant, $p < 0.001$).

## Results

### Secondary metabolites of lichens

The acetone extracts of the extracellular secondary metabolites from six lichen species were analyzed by HPLC and TLC. In the studied lichens, single secondary metabolites and mixtures thereof were detected (Table 1). In the lichen *P. robustum*, the main compounds were protocetraric acid (7.2 %) and atranorin (1.3 %). *P. tinctorum* contained lecanoric acid (15.3 %) and atranorin (0.8 %). *P. acetabulum* contained nortistic acid (7.6 %) and trace amounts of atranorin (0.1 %). In *F. baltimorensis*, we found usnic acid (2.3 %) and protocetraric acid (2.5 %). *U. florida* contained a mixture of the secondary metabolites usnic acid (5.7 %) and thamolic acid (1.3 %), and *U. hirta* contained only usnic acid (5.8 %). The rest represents the biomass of the lichen "body" (photobiont and mycobiont biomass and their respective products) as well as other substances on the surface of lichens like pigments, primary metabolites, or waxes.

### Growth of protonemata

The growth of protonemata on fiber disks containing different lichen metabolites was observed over 5 weeks. Three metabolite extracts had a strong to moderate influence (extracts from *F. baltimorensis*, *U. florida*, and *U. hirta*), and three revealed very little effects (extracts from *P. robustum*, *P. tinctorum*, and *P. acetabulum*).

The extract from *U. hirta* contained only usnic acid and showed the strongest inhibition of all metabolites; 0.1 % concentration was lethal for protonema cells within the first week

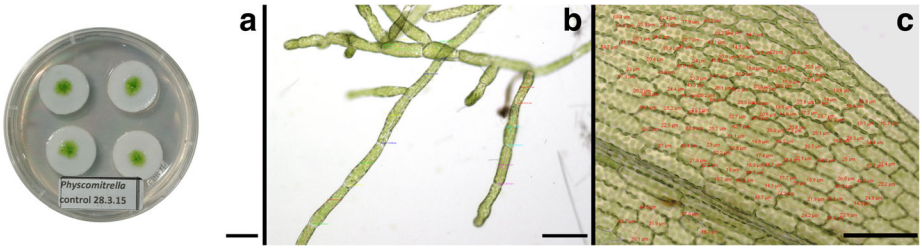


**Fig. 1** **a–c** Macroscopic and microscopic measurements of *Physcomitrella patens*: **a** plantlets in Petri dish on glass fiber paper after 2 weeks, *bar* = 1 cm; **b** cells of protonemata marked by length and width, *bar* = 100 μm; **c** cells of leafy gametophore marked by length and width, *bar* = 100 μm

(Fig. 2a). After 5 weeks, the protonemata on the 0.01 % concentration occupied a smaller area than on the 0.001 % concentration or the control. The metabolites from *F. baltimorensis* inhibited the growth of the protonemata significantly in the highest concentration (Fig. 2b) whereas the 0.01 % concentration had no measurable influence. In general, the area of the protonemata in the two lower concentrations *F. baltimorensis* extracts and the control reached the biggest expansion of all treatments with over 90 $mm^2$. Also, the metabolite treatment from *U. florida* showed a significant reduction of protonema growth in the 0.1 % concentration until week 4 (Fig. 2c); in the fifth week, the difference to the control was still obvious. *U. florida* metabolites of 0.01 % seemed to be a threshold concentration since the effect varied between individual protonemata patches as reflected by the big range of the measured areas (8–88 $mm^2$) in the fifth week. Relevant effect sizes are reported as supplemental data (Supplemental Table 1).

In contrast, the metabolites from *P. acetabulum*, *P. tinctorum*, and *P. robustum* caused no significant differences in all three concentrations compared to the control. The areas of the protonemata treated with *P. acetabulum* extracts showed a very broad variation in area (Fig. 2d). The two metabolite extracts from *P. tinctorum* and *P. robustum* caused very similar trends in all concentrations. After 5 weeks, protonemata area was similar on all concentrations of those two lichen extracts (Fig. 2e, f).

**Table 1** Content (% dw) of secondary metabolites in six lichen species

| Lichen | UA | PCA | A | LA | NA | THA | SUM |
|---|---|---|---|---|---|---|---|
| *U. hirta* | 5.8 | - | - | - | - | - | 5.8 |
| *F. baltimorensis* | 2.3 | 2.5 | - | - | - | - | 4.8 |
| *U. florida* | 5.7 | - | - | - | - | 1.3 | 7.0 |
| *P. acetabulum* | - | - | 0.1 | - | 7.6 | - | 7.7 |
| *P. tinctorum* | - | - | 0.8 | 15.3 | - | - | 16.1 |
| *P. robustum* | - | 7.2 | 1.3 | - | - | - | 8.5 |

*UA* usnic acid, *PCA* protocetraric acid, *A* atranorin, *LA* lecanoric acid, *NA* nortistic acid, *THA* thamnolic acid, *SUM* summary of total secondary metabolites

## Presence of gametophores

In good growth conditions, the protonema cells start to develop buds from which the gametophores develop. We tested the effect of different lichen metabolites on the development of gametophores by determination of their presence or absence after 5 weeks of treatment (Fig. 3a, b). In the controls and in the 0.001 % concentrations of all metabolites, at least one patch out of seven failed to produce gametophores. Similar results were found in the 0.01 % concentrations where at least two out of seven had no gametophores. Contrary, the 0.1 % concentrations showed big differences between the metabolite treatments. On the *U. hirta* and the *F. baltimorensis* extracts, gametophores were absent at all. Also, on the *U. florida* extract, gametophores developed only in two of the seven patches. The amount increased on the *P. acetabulum* extract where gametophores were observed in four cases. In contrast, the *P. tinctorum* and *P. robustum* treatments revealed growing gametophores on all fiber disks.

## Cell size

Measurements of the cell length and width revealed strong changes in the protonemata and minor changes in the gametophores cells; details are given in Fig. 4.

Significant changes in the length of the protonema cells occured in all metabolite extracts except for *U. hirta* (Fig. 4a). On the 0.001 % and 0.01 % extract concentrations of *F. baltimorensis*, *P. acetabulum*, *P. tinctorum*, and *P. robustum*, protonemata showed shorter cells. At extract concentrations of 0.1 % of *P. acetabulum* and *P. robustum*, significantly shorter cells were observed. In all concentrations of the *F. baltimorensis* and *U. florida* extracts, the cell width was significantly smaller than in the control (Fig. 4b). Cells treated with low concentration of *P. acetabulum* extracts were thinner, whereas the metabolites from *P. tinctorum* led to thinner cells only in 0.1 % concentration. In *U. hirta* 0.1 % concentration, no living protonemata cells were found.

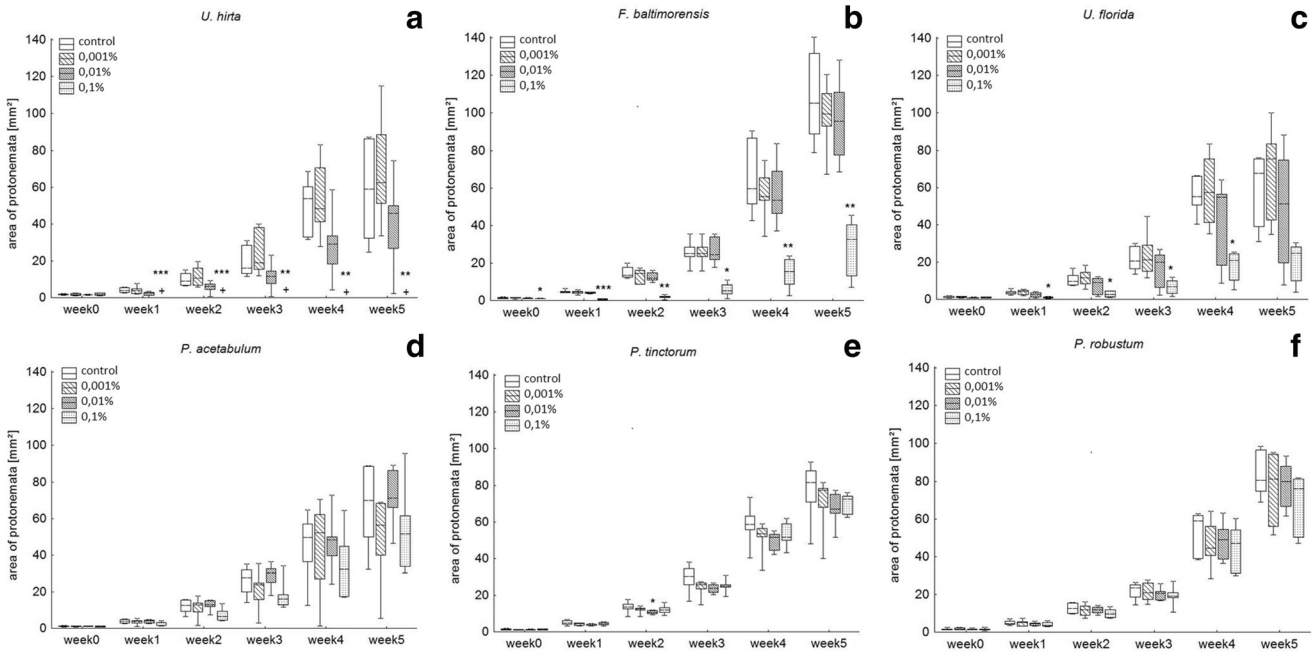


**Fig. 2** **a–f** Graphic description of moss protonemata cultivated on different metabolite concentrations (control, 0.001, 0.01, and 0.1 %) over a period of 5 weeks, each figure shows the *box whisker plots* of one metabolite treatment, **a** extract of *U. hirta*; **b** extract of *F. baltimorensis*; **c** extract of *U. florida*; **d** extract of *P. acetabulum*; **e** extract of *P. tinctorum*; **f** extract of *P. robustum*. *Asterisk* indicates significantly different medians compared with the control, *plus sign* indicates treatments where no living protonema was found

The length of the gametophore cells was less influenced by the metabolites than in the protonemata (Fig. 4a, c). After *U. hirta*, *F. baltimorensis*, and *U. florida* extract treatment, a trend toward longer cells was apparent. For the *P. tinctorum* extract, no data were available because of too little leafy material for measurements. At highest metabolite concentrations, significantly longer cells were found after treatment with *F. baltimorensis* and *U. florida* extracts. The 0.01 % concentration of *U. hirta* and *F. baltimorensis* extracts caused wider cells than the treatment with the other metabolites. The same pattern was observed in the 0.1 % concentration. As for protonema, no living gametophore was found in this concentration of the *U. hirta* extract.

## Discussion

In the present study, we analyzed the effect of six different lichen extracts containing secondary metabolites on the growth of the moss *P. patens* in vitro.

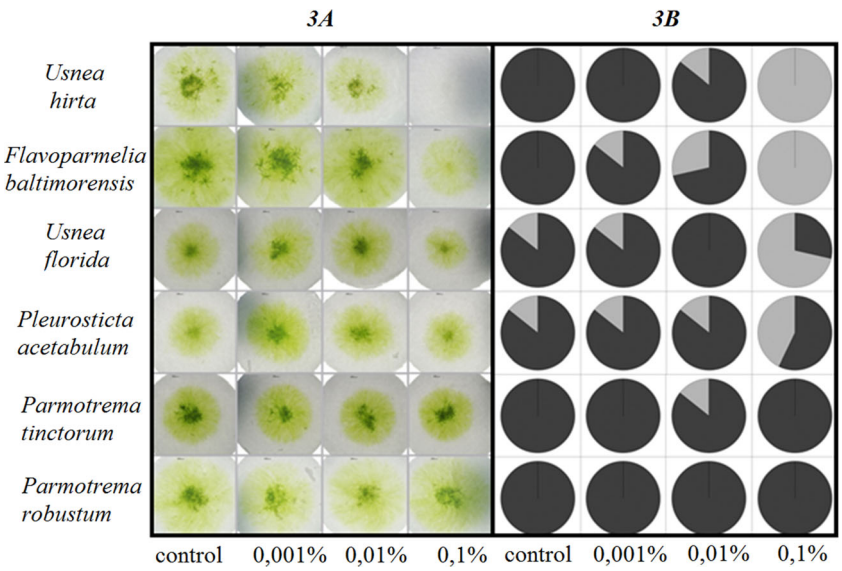


**Fig. 3** **a–b** Typical growth and gametophore development on glass fiber disks treated by different metabolites. **a** Area with protonemata and gametophores after 5 weeks. **b** Cake graphs show presence (*black*) or absence (*gray*) of gametophores, $n = 7$

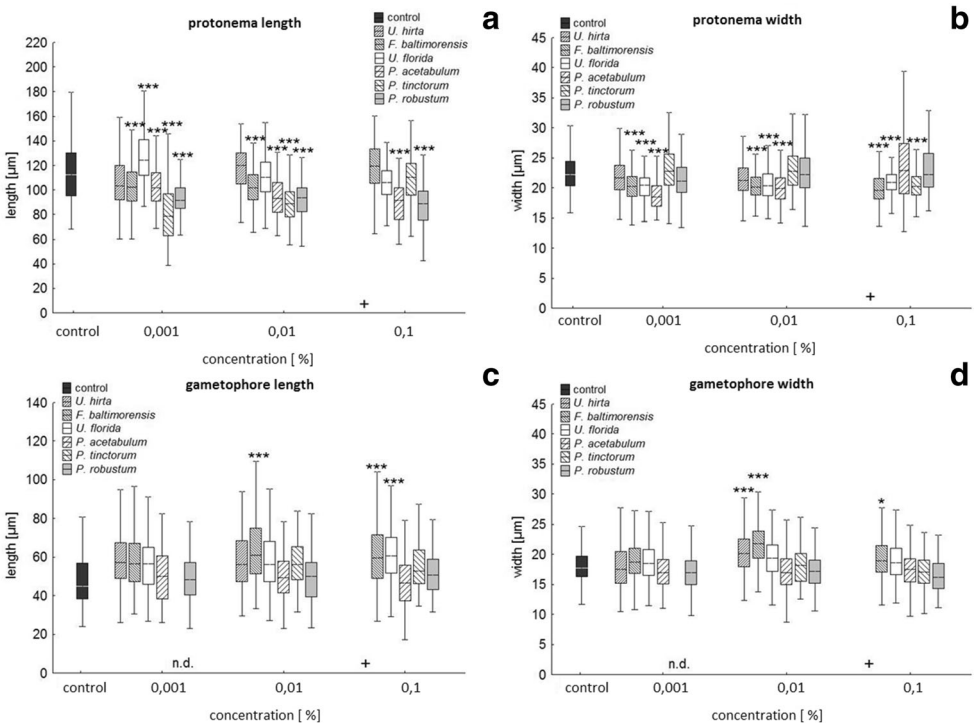


**Fig. 4** **a–d** Length and width of protonema and gametophore cells after different metabolite treatments (control, 0.001, 0.01, and 0,1 %) after 5 weeks of cultivation, **a** length of protonema cells, **b** width of protonema cells, **c** length of gametophore cells, **d** width of gametophore cells. *Asterisk* indicates significant medians compared with the control which have an effect size over 0.5, *plus sign* indicates treatments where no living protonema was found, *n.d.* = no data. Effective numbers can be found in supplemental material. The *whiskers* represent 1.5× interquartile range (IQR) of data, outliers are not shown

Burkin et al. (2012) showed that lichens have an effetive system of conservation of secondary metabolites, which can be used for several studies and preserved for several decades and stored in herbarium. This unique ability allows lichen secondary metabolites to be used for their biological and ecological activities. Histologically, lichen secondary metabolites are stored either in the cortex or the medulla (Ranković 2015). The cortical compounds are mostly usnic acid and atranorin, but anthraquinones, derivates of vulpinic acid, and xanthones can also be present. All of them exhibit a typical pigmentation except for atranorin. On the other hand, lichen secondary metabolites are classified by their biological function; while secondary metabolites of the medulla are potential regulators of algal symbionts (Bačkor et al. 2010), cortical compounds can act as a kind of light filter (Marques 2013).

Most lichen substances will appear in benzene and ether extracts, but certain compounds (erythrin, thamnolic acid, beta-orcinol, depsidones) can be extracted only in acetone. Polyols (arabinitol, mannitol, etc.) are mostly found in acetone extracts, but on the other hand, saccharides are extracted with alcoholic solvents or water after prior removal of other substituents (Ahmadjian and Hale 1973). Solubility of usnic acid as a one of the secondary metabolite of lichens was tested by Jin et al. (2012). Usnic acid is almost impossible to dissolve in water. In nature, even rain is never a pure water because of a lot of admixtures.

The allelopathic potential of lichen substances is confirmed in several studies. Whiton and Lawrey (1984) showed the inhibition by two secondary metabolites (vulpinic acid, evernic acid) on spore germination of the lichens *Graphis scripta* and *Caloplaca citrina*. Even fungal symbionts can be strongly inhibited by allelopathic substances produced from lichens. An example is the phenol protection by lecanoric acid, which controls the growth of the mycobiont *Nectria parmeliae* (Lawrey 2000). Lichenized fungi show better tolerance toward the inhibitory effect of secondary lichen substances than non-lichenized fungi (Armstrong and Welch 2007). The epiphytic lichen *Hypogymnia physodes* affects wood-consuming fungi, thereby protecting the own substrate from decomposition (Henningsson and Lundstrom 1970). Furthermore, lichen secondary metabolites can inhibit mycorrhiza species as well as their plant partners (Lawrey 1995).

Mosses can be affected by lichen metabolites too. Heilman and Sharp (1963) observed an inhibition and outgrowth of *Frullania eboracensis* on the bark of *Aesculus octandra* by the lichen *Thelotrema petractoides*. Other lichens like *Lecidea albocaerulescens* inhibited moss growth on rocks in the same environment (Molnár and Farkas 2010). Our tests on protonemata development revealed the most negative effect by treatments with extracts of *U. hirta*, *F. baltimorensis*, and *U. florida*, all containing usnic acid.

Growth in plants is affected by a combination of factors such as photoperiod, nutrients, or substrate conditions. The toxicity of lichen secondary substances depends mostly on their concentration and pH (Frahm et al. 2000). Gardner and Mueller (1981) mentioned that usnic acid has a pH-dependent toxicity effect to spore germination on *F. hygrometrica*. This suggests that the allelopathic effect

on surrounding organisms of usnic acid is caused by acidification as natural concentrations of humic acid, also reducing spore germination and retarded bud formation in *Funaria hygrometrica* (Glime 2007).

Usnic acid not only caused a decrease in pH but also showed antimitotic effects as demonstrated in taxonomically diverse organisms (Cardarelli et al. 1997). Cell size of *Scenedesmus* sp. significantly increased in *Scenedesmus* sp. cultures which may be a result of the effect of usnic acid on the spindle apparatus during mitosis (Al Bekairi et al. 1991). Our measurements of the cell size of the protonemata and the gametophores revealed bigger changes in the protonemata than in the gametophores. Considering the fact that the protonemata are growing in and on the media, these cells interact directly with lichen metabolites. Except for the *U. hirta* treatment, protonema cell sizes decreased independently of the concentration or the type of metabolites. Extracts from *U. hirta* affected protonema growth in the lower concentrations but lead to cell death in the highest concentration, as confirmed in the area of measurements. We suppose that usnic acid has a stronger effect on cell division than on cell size (development). Similar effects were reported for protonemata treated with thalli from *Cladonia foliacea*: the containing usnic acid leads to a growth reduction of existing protonema filaments and a decreased development of new ones (Giordano et al. 1999). The authors reported intracellular changes in the cytoplasm of the filaments like the formation of many granules and microvesicles but no changes in the cytoskeleton. Our results show reduced cell sizes also in the other extract treatments, indicating a slight impact on the cell metabolism by metabolites other than usnic acid too. Similarly, evernic acid showed an inhibiting effect on spore germination and growth of protonemata of three mosses (*Ceratodon purpures*, *Funaria hygrometrica*, *Mnium cuspidatum*) which grow in the vicinity of lichens (Molnár and Farkas 2010).

Gametophores reacted differently to the protonemata. The cells exhibited similar or bigger sizes than the control. Especially, the two higher concentrations (10 and 100 μg) of *U. hirta*, *F. baltimorensis*, and *U. florida* metabolites caused a significant increase in cell length and width although these treatments affected protonema growth as well as gametophore development. The increased cell size could be due to an adaption of the survived gametophore. Mosses which were first inhibited by metabolites grew faster after the third week (Giordano et al. 1999). Since our measurements are from the fifth week, the gametophore cells could have recovered from the initial negative effects of the lichen metabolites and started to follow this pattern.

Phytotoxic activity on chloroplasts were first described by Inoue et al. (1983). The site of usnic acid inhibition lies at the oxidation side of photosystem II in chloroplasts isolated from spinach (Inoue et al. 1987). The (-) form of usnic acid is an irreversible inhibitor of the key plant enzyme 4-hydroxyphenylpyruvate dioxygenase leading to the inhibition of carotenoid synthesis and bleaching (Romagni et al. 2000; Cocchietto et al. 2002). Usnic acid inhibits oxygen exchanges of mesophyll cell protoplasts of *Commelina communis* (Vavasseur et al. 1991) supposedly by acting on key enzymes (Cocchietto et al. 2002). Usnic acid showed also an antitranspiration activity on sunflowers, where a decrease of water loss without affecting $CO_2$ uptake was reported (Lascève and Gaugain 1990). Besides the effect on the photosystem, usnic acid also interacts with phytohormone regulation by decreasing the amount of free auxin (Legaz et al. 2004). This effect, although it was found on trees, may lead to a decrease in protonemata growth and bud formation thereby explaining the reduced number of developed gametophores after 5 weeks. Studies on chloroplast ultrastructure of leaves from *Quercus* spp. occupied by lichen thalli showed a smaller number of thylakoids forming grana and a decreased width of grana (Ascaso and Rapsch 1985). Also, a lower amount of chlorophyll *a* and *b* was found after the treatment of evernic acid from *Evernia prunastri* on leaves of *Quercus rotundifolia* (Ascaso and Rapsch 1985) and on leaves of *Spinacia oleracea* (Ascaso and Rapsch 1985). If such effects occur in leaves, which are protected by a cuticle, then the same process should happen faster in mosses because they are lacking such a protection layer. Therefore, the reduced photosynthesis rate and the production of detoxing granules and mini vesicles could be an adaption of the moss to the lichen metabolites, finally leading to the observed decrease in cell development.

In summary, we could confirm the negative influence of secondary metabolites on the bryophyte *P. patens* mainly by affecting the early stages of growth.

## Conclusion

The growth inhibition of both, protonemata and gametophores, in presence of usnic acid was confirmed. Secondary metabolites of lichens led to changes in length and width of protonemata but had less influence on the gametophore. This supports our hypothesis that the early stages of plant growth are influenced most, especially those which are in direct contact with lichen secondary metabolites.

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**Compliance with ethical standards**

**Conflicts of interest** The authors declare no conflict of interest.

## References


Ahmadjian V, Hale ME (1973) The Lichens. Academic Press, New York, p 649, ISBN 0-12-044950-1

Al Bekairi AM, Qureshi S, Shah AH, Krishna DR, Chaudhry MA (1991) Effect of dextro usnic acid on testicular nucleic acids and epididymal spermatozoa in mice. Fitoterapia 62(3):258–260

Armstrong RA, Welch AR (2007) Competition in lichen communities. In. Symbiosis 43:1–12

Ascaso C, Rapsch S (1985) Chloroplast-ultrastructure and chlorophyll content in leaves from Quercus branches with and without epiphytic lichen thalli. Plant Cell Environ 8(9):651–656. doi:10.1111/1365-3040.ep11611691

Bačkor M, Klemová K, Bačkorová M, Ivanova V (2010) Comparison of the Phytotoxic Effects of Usnic Acid on Cultures of Free-Living Alga Scenedesmus quadricauda and Aposymbiotically Grown Lichen Photobiont Trebouxia erici. J Chem Ecol 36(4):405–411. doi:10.1007/s10886-010-9776-4

Burkin AA, Tolpysheva TYu, Kononenko GP (2012) Preservation of secondary fungal metabolites in herbarium lichen specimens. Moscow University Biological Sciences Bulletin 67(3):121-125, doi:10.3103/S0096392512030030

Cardarelli M, Serino G, Campanella L, Ercole P, de Cicco Nardone F, Alesiani O, Rossiello F (1997) Antimitotic effects of usnic acid on different biological systems. Cell Mol Life Sci 53(8):667–672. doi:10.1007/s000180050086

Cocchietto M, Skert N, Nimis P, Sava G (2002) A review on usnic acid, an interesting natural compound. Naturwissenschaften 89(4):137–146. doi:10.1007/s00114-002-0305-3

Cove DJ (1993) The Moss Physcomitrella patens, a Model System with Potential for the Study of Plant Reproduction. Plant Cell Online 5(10):1483–1488. doi:10.1105/tpc.5.10.1483

During HJ, van Tooren BF (1990) Bryophyte interactions with other plants. In. Bot J Linn Soc 104(1-3):79–98. doi:10.1111/j.1095-8339.1990.tb02212.x

Fahselt D (1994) Secondary biochemistry of lichens. In. Symbiosis 16: 117–165

Feige GB, Lumbsch HT, Huneck S, Elix JA (1993) Identification of lichen substances by a standardized high-performance liquid chromatographic method. J Chromatogr A 646(2):417–427. doi:10.1016/0021-9673(93)83356-W

Frahm JP, Specht A, Reifenrath K, Vargas YL (2000) Allelopathic effects of crustose lichens, epiphytic bryophytes and vascular plants. In. Nova Edwigia 70:245–254

Gang Y-Y, Du G-S, Shi D-J, Wang M-Z, Li X-D, Hua Z-L (2003) Establishment of *in vitro* regeneration system of the *Atrichum* mosses. Acta Bot. Sin. 45:1475-1480

Gardner CHR, Mueller DMJ (1981) Factors Affecting the Toxicity of Several Lichen Acids. Effect of pH and Lichen Acid Concentration. In. Am J Bot 68(1):87. doi:10.2307/2442995

Giordano S, Alfano F, Basile A, Cobianchi R (1999) Toxic effects of the thallus of the lichen on the growth and morphogenesis of bryophytes. Cryptogam Bryol 20(1):35–41. doi:10.1016/S1290-0796(99)80005-4

Glime JM (2007) Bryophyte Ecology, vol 1, Physiological Ecology

Hauck M, Huneck S (2007) The putative role of fumarprotocetraric acid in the manganese tolerance of the lichen Lecanora conizaeoides. In. LIC 39(03):301. doi:10.1017/S0024282907006664

Hauck M, Jürgens SR, Huneck S, Leuschner CH (2009) High acidity tolerance in lichens with fumarprotocetraric, perlatolic or thamnolic acids is correlated with low pKa1 values of these lichen substances. Environ Pollut 157(10):2776–2780. doi:10.1016/j.envpol.2009.04.022

Hawksworth DL, Ainsworth GC (1995) Ainsworth and Bisby's dictionary of the fungi, 8th edn. CAB International, Wallingford

Heilman AS, Sharp AJ (1963) A probable antibioticeffect of some lichens on bryophytes. In. Rev Bryol Lichénol 32:215

Henningsson B, Lundstrom H (1970) The influence of lichens, lichen extracts and usnic acid on wood destroying fungi. Material und Organismen 5(1):19–31

Jin J, Rao Y, Bian X, Zeng A, Yang G (2013) Solubility of (+)-Usnic Acid in Water, Ethanol, Acetone, Ethyl Acetate and n-Hexane. Journal of Solution Chemistry 45(5):1018–1027

Inoue Y, Crofts AR, Govindjee, Murata N, Renger G, Satoh K (1983) The oxygen evolving system of photosynthesis. Academic Press, Tokyo, pp 351–356

Inoue H, Noguchi M, Kubo K (1987) Site of inhibition of usnic acid at oxidizing side of photosystem 2 of spinach chloroplasts. Photosynthetica 21:88–90

Lang D, Zimmer AD, Rensing SA, Reski R (2008) Exploring plant biodiversity. The Physcomitrella genome and beyond. In. Trends Plant Sci 13(10):542–549. doi:10.1016/j.tplants.2008.07.002

Lascève G, Gaugain F (1990) Effects of Usnic Acid on Sunflower and Maize Plantlets. J Plant Physiol 136(6):723–727. doi:10.1016/S0176-1617(11)81352-0

Lawrey JD (1977) Inhibition of Moss Spore Germination by Acetone Extracts of Terricolous Cladonia Species. In. Bull Torrey Botanical Club 104(1):49. doi:10.2307/2484664

Lawrey JD (1995) The chemical ecology of lichen mycoparasites. A review. In. Can J Bot 73(S1):603–608. doi:10.1139/b95-301

Lawrey JD (2000) Chemical Interactions Between Two Lichen-degrading Fungi. J Chem Ecol 26(8):1821–1831. doi:10.1023/A:1005540622612

Leflaive J, Ten-Hage L (2007) Algal and cyanobacterial secondary metabolites in freshwaters. A comparison of allelopathic compounds and toxins. In. Freshwater Biol 52(2):199–214. doi:10.1111/j.1365-2427.2006.01689.x

Legaz ME, Monsó MA, Vicente C (2004) Harmful effects of epiphytic lichens on trees. In. Recent Res Dev Agronomy Horticulture 1:1–10

Lokajová V, Bačkorová M, Bačkor M (2014) Allelopathic effects of lichen secondary metabolites and their naturally occurring mixtures on cultures of aposymbiotically grown lichen photobiont Trebouxia erici (Chlorophyta). In. S Afr J Bot 93:86–91. doi:10.1016/j.sajb.2014.03.015

Macías FA, Molinillo JMG, Varela RM, Galindo JCG (2007) Allelopathy—a natural alternative for weed control. In. Pest Manag Sci 63(4):327–348. doi:10.1002/ps.1342

Marques J (2013) A framework for assessing the vulnerability of schist surfaces to lichen-induced weathering in the Upper Douro region (NE Portugal). Rubim Almeida y GracielaPaz, Directores

Molisch H (1938) Der Einfluss einer Pflanze auf die Andere, Allelopathie. In. Nature 141(3568):493. doi:10.1038/141493a0

Molnár K, Farkas E (2010) Current Results on Biological Activities of Lichen Secondary Metabolites. A Review. In. Zeitschrift für Naturforschung C 65:3–4. doi:10.1515/znc-2010-3-401

Orange A, James PW, White FJ (2001) Microchemical methods for the identification of lichens. British Lichen Society, London

Poelt J (1985) Über auf Moosen parasitierende Flechten. In. Sydowia, Annales Mycologici SerII 38:241–254

Ranković B (2015) Lichen Secondary Metabolites. Springer International Publishing, Cham

Reigosa MJ, Sánchez-Moreiras AGL (1999) Ecophysiological Approach in Allelopathy. In. Crit Rev Plant Sci 5:577–608. doi:10.1080/07352689991309405

Rensing SA, Lang D, Zimmer AD, Terry A, Salamov A, Shapiro H et al (2008) The Physcomitrella Genome Reveals Evolutionary Insights

into the Conquest of Land by Plants. Science 319(5859):64–69. doi:10.1126/science.1150646

Romagni JG, Meazza G, Nanayakkara NPD, Dayan FE (2000) The phytotoxic lichen metabolite, usnic acid, is a potent inhibitor of plant p - hydroxyphenylpyruvate dioxygenase. FEBS Lett 480(2-3):301–305. doi:10.1016/S0014-5793(00)01907-4

Sassmann S, Adlassnig W, Puschenreiter M, Cadenas EJP, Leyvas M, Lichtscheidl I, Lang I (2015) Free metal ion availability is a major factor for tolerance and growth in Physcomitrella patens. In. Environ Exp Bot 110:1–10. doi:10.1016/j.envexpbot.2014.08.010

Sedia EG, Ehrenfeld JG (2003) Lichens and mosses promote alternate stable plant communities in the New Jersey Pinelands. Oikos 100(3):447–458. doi:10.1034/j.1600-0706.2003.12058.x

Solhaug KA, Gauslaa Y (1996) Parietin, a photoprotective secondary product of the lichen Xanthoria parietina. Oecologia 108(3):412–418. doi:10.1007/BF00333715

Solhaug KA, Lind M, Nybakken L, Gauslaa Y (2009) Possible functional roles of cortical depsides and medullary depsidones in the foliose lichen Hypogymnia physodes. In. Flora - Morphol Distrib Funct Ecol Plants 204(1):40–48. doi:10.1016/j.flora.2007.12.002

Vavasseur A, Gautier H, Thibaud MCH, Lascève G (1991) Effects of Usnic Acid on the Oxygen Exchange Properties of Mesophyll Cell Protoplasts from Commelina communis L. J Plant Physiol 139(1): 90–94. doi:10.1016/S0176-1617(11)80171-9

Whiton JC, Lawrey JD (1984) Inhibition of crustose lichen spore germination by lichen acids. In. Bryologist 87:42–43