South African Journal of Botany 162 (2023) 688–693

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Allelopathic effects of three lichen secondary metabolites on cultures of aposymbiotically grown lichen photobionts and free-living alga *Scenedesmus quadricauda*

Martin Bačkor[a,b,*], Michal Goga[b], Dajana Ručová[b], Dana Urminská[a], Miriam Bačkorová[c], Bořivoj Klejdus[d]

[a] Department of Biochemistry and Biotechnology, Institute of Biotechnology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Tr. A. Hlinku 2, Nitra 949 76, Slovak Republic
[b] Department of Botany, Institute of Biology and Ecology, Faculty of Science, Šafárik University, Mánesova 23, Košice 041 67, Slovak Republic
[c] Department of Pharmacognosy and Botany, University of Veterinary Medicine and Pharmacy, Komenského 73, Košice 041 81, Slovak Republic
[d] Department of Chemistry and Biochemistry, Mendel University in Brno, Zemědělská 1, Brno 613 00, Czech Republic

ARTICLE INFO

Article History:
Received 5 April 2023
Revised 28 August 2023
Accepted 3 October 2023
Available online xxx

Edited by: Dr M. M. El-Sheekh

Keywords:
Allelopathy
Algae
Antioxidants
Glutathione
Lichens
Organic acids
Phenolic compounds
Secondary metabolites

ABSTRACT

In the present work, we studied the influence of lichen secondary metabolites (evernic acid, orcinol, usnic acid) on selected species of algae: *Trebouxia erici*, photobiont of lichen *Cladonia cristatella* producing typical lichen secondary metabolites, *Coccomyxa solorinae-saccatae*, photobiont of lichen *Solorina saccata* which does not produce typical secondary metabolites of lichens, and free-living alga *Scenedesmus quadricauda.*
For the determination of the cytotoxic effects of these compounds, algae were cultivated on the surface of disks composed of glass microfibers, in quantities of 0.1 mg/disk for each metabolite. After 14-days prolonged cultivation, we analysed selected physiological parameters: growth of algae, chlorophyll *a* fluorescence, the content of ascorbic acid, content of reduced and oxidized glutathione and contents of selected organic acids. The presence of secondary compounds typically inhibited the growth of tested algae and chlorophyll *a* fluorescence. In the presence of evernic acid and usnic acid, we observed a decrease of ascorbic acid, glutathione, and significant changes in the composition of organic acids.
Lichen metabolites, except for their other biological and ecological roles, may act as phytotoxic substances, causing oxidative stress in photobiont cells. These metabolites may play an important role in the "controlled parasitism" of fungal partner on algal/cyanobacterial partner of symbiosis. Differences in the sensitivity of algal cells from different habitats to lichen compounds are, however, not understood sufficiently.

1. Introduction

It is generally known that lichens form a vegetative body (thallus), consisting of cells of a fungal partner (mycobiont) and an algal and/or cyanobacterial partner (photobiont). The resulting "organism" does not resemble parental organisms and due to mutualistic symbiosis can survive in extreme environments, e.g. cold, hot, dry, UV-exposed, or metal-polluted habitats, where the existence of single symbionts without a mutualistic partner of symbiosis is not possible (Bačkor and Loppi, 2009). They may be considered more like an ecosystem or community than a typical organism (Fahselt, 1994; Pichler et al., 2023). Lichens play also an important role in boreal forest ecology as they can affect the metabolism of plants which form natural ecosystems with them (Pizňak and Bačkor, 2019).

Lichens are producing more than 1000 unique substances (Goga et al., 2020), typical for this group of organisms, called secondary metabolites. While primary metabolites, which are directly involved in the normal growth, development, and reproduction of lichens (e.g. carbohydrates, proteins, amino acids, vitamins and other) are known also from another living organisms, secondary metabolites of lichens are not typically present in other organisms and play mostly diverse biological and ecological roles. These compounds are typically extracellular phenols, which are secreted by the fungal partner, and mostly deposited on the surface of hyphae, but also photobionts to a lesser extent. These substances in lichens occur in very considerable amounts, typically constituting from 0.1 to 5.0 % (w/w) of thallus dry weight. However, it is not rare when their content in lichen thalli exceeds these values (Fahselt 1994; Pichler et al., 2023). Typical

Abbreviations: AsA, ascorbic acid; dw, dry weight; fw, fresh weight; GSH, reduced glutathione; GSSG, oxidized glutathione

* Corresponding author at: Department of Biochemistry and Biotechnology, Institute of Biotechnology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Tr. A. Hlinku 2, Nitra 949 76, Slovak Republic
E-mail address: martin.backor@uniag.sk (M. Bačkor).

https://doi.org/10.1016/j.sajb.2023.10.005
0254-6299/

secondary metabolites of lichens include dibenzofuran derivatives, depsides, and depsidones.

Lichen metabolites have many biological and ecological functions. These roles include mostly antimicrobial activity, allelopathy, antiherbivory, chelating of heavy metals and UV protection of symbionts, mostly photobionts (Lawrey, 1986; Pöykkö et al., 2005; Latkowska et al., 2006; Solhaug et al., 2009; Pizňak et al., 2019).

Usnic acid (UA) is a yellowish pigment and dibenzofuran derivative. UA is one of the most common cortical secondary metabolites of lichens. It is especially abundant in members of the genera *Alectoria, Usnea*, or *Xanthoparmelia*. So far, antibiotic, antiviral, antimycotic, antiprotozoal, antiherbivoral, antiproliferative, anti-inflammatory, analgesic, antipyretic, allelopathic and UV-protecting effects of UA have been documented previously (for review see Cocchietto et al., 2002; Goga et al., 2020).

Evernic acid (EA) is a *para*-depside, which belongs to the orcinol series derived by the acetyl-malonate pathway. EA is a major secondary metabolite in lichen *Evernia prunastri* and antifungal (Halama and Van Haluwin, 2004), antibacterial (Renzaka and Sigler, 2007), antitubercular (Tasdemir and Franzblau, 2007), antioxidant (Kosanić et al., 2013) activity was confirmed. EA isolated from lichen *Usnea longissima* showed herbicidal activity on plant growth and acts as an inhibitor of photosystem II (Nishitoba et al., 1987; Endo et al., 1998).

Orcinol is an organic compound that occurs in many species of lichens including members of the genera *Roccella* and *Lecanora*. Orcinol isolated from lichen *Umbilicaria esculenta* showed anti-inflammatory potential. Cytotoxic activity of orcinol and other secondary metabolites isolated from lichen *Parmelia subrudecta* was shown (Ivanova et al., 2010; Krishna et al., 2022).

We know that the presence of secondary metabolites, including metabolites used in the present study, has a significant influence on lichen biology and ecology. Lichens are slow-growing and long-lived organisms and due to the toxic effect of their metabolites on a wide range of organisms, are in part, protected against pathogens and herbivores. On the other side, they may be potentially toxic to their own algal partners, which form with mycobiont lichen symbiotic association and are in direct contact with extracellular crystals of lichen metabolites. Previous studies demonstrated that the presence of usnic acid, or atranorin may regulate photobiont populations in lichen thallus (Bačkor et al., 1998; Buďová et al., 2006). Production of secondary compounds in lichens may be a critical step in maintaining the required ratio of mycobiont to photobiont biomass in thalli.

The mechanisms of the allelopathic effects of secondary compounds from lichens on plants, including their own photobionts and algal cells are still not understood sufficiently. It is known that they include inhibition of growth and biomass production, chlorophyll *a* fluorescence (F_V/F_M), a decrease of carotenoid and chlorophyll contents in the plant cells (Cardarelli et al., 1997; Endo et al., 1998; Buďová et al., 2006; Lechowski et al., 2006; Pizňak et al., 2019).

As lichen photobionts are in direct contact with cortical and medullar extracellular metabolites produced by mycobiont, the main aim of this study was to evaluate their potential phytotoxic effects on aposymbiotically grown lichen photobionts *Trebouxia erici* and *Coccomyxa solorinae-saccatae.* These photobionts may be evolutionarily adapted to phytotoxicity of secondary metabolites of lichens through co-evolution with lichen mycobionts which produce them. Free-living alga *Scenedesmus quadricauda* was chosen as another model organism for the assessment of phytotoxicity of lichen secondary compounds.

2. Materials and methods

2.1. Organisms, culture conditions and lichen metabolites

The lichen photobiont *Trebouxia erici* Ahmadjian (UTEX 911), isolated from the lichen *Cladonia cristatella* Tuck. containing typical lichen secondary metabolites (e.g. usnic acid, barbatic acid and didimic acid; Brodo et al., 2001); photobiont *Coccomyxa solorinae-saccatae* Chodat (SAG 216-12), isolated from the lichen *Solorina saccata* (L.) Ach. containing no typical lichen secondary metabolites and free-living alga *Scenedesmus quadricauda* (Turpin) Brébisson (UTEX 76) were used in this study. All algae belong to the phylum Chlorophyta. Algae were cultivated on agar medium (2 %) previously developed for *Trebouxia* by Ahmadjian (1993). This was Bold's Basal Medium (BBM 3N) plus 10 g casein acid hydrolysate and 20 g glucose per liter with the pH adjusted to 6.5 (Bačkor et al., 2003). Cultures were maintained at 22°C under a 16-h photoperiod and 30 μmol.m^{-2}.s^{-1} artificial irradiance ("cool white" tubes).

For assessment of the role of lichen secondary metabolites on the growth and metabolism of algae, three selected compounds were chosen. Usnic acid was provided by Sigma-Aldrich (Sant Louis, MO, USA), while evernic acid and orcinol were provided by Extrasynthese (Genay, France). The purity of compounds was assessed by High-Performance Liquid Chromatography (HPLC).

2.2. Culturing of algae on glass fiber disks

Algae were cultivated on the surface of glass fiber filter disks (Whatman CF/C filters), 25 mm in diameter, as described in previous studies (Bačkor et al., 2003). Two inoculation loops of algal cells of three species used in this study grown on stock solid *Trebouxia* media were transferred into 50 ml of liquid *Trebouxia* media in an Erlenmeyer flasks and suspended by gentle stirring on a magnetic stirrer for 1 h. Cultures were maintained for 5 days in a cultivation room under the conditions described previously, with daily stirring on a magnetic stirrer for about 1 h. The homogeneity of algal suspensions was verified microscopically and the number of cells was calculated using a standard haemocytometer. The cell density of cultures was adjusted to approximately 10^6 cells ml^{-1} of the medium.

For quantitative algal cultivation, sterilized 25 mm (in diameter) glass fiber disks were subjected to four different pretreatments. Usnic acid, evernic acid and orcinol (0.1 mg/disk) dissolved in acetone, volume 30 μl, were applied by automatic pipette on the surface of disks while the same volume of acetone was used for control disks. After evaporation of acetone for 4 h, one disk was transferred to the surface of solid *Trebouxia* medium in a separate Petri dish, 6 cm in diameter, and 20 μl of algal suspensions were inoculated into the center of each disk. Disk pores allow supplemental nutrient media to pass through the disk and permit growth to be easily determined from changes in biomass (Bačkor et al., 2003). The total mass of cultures was calculated by subtracting the mean fresh weight (fw) of a glass fiber disk saturated by identical medium, from the fw of a disk supporting algal cultures after 14 days of cultivation. Each treatment was replicated eight times.

2.3. Activity of photosystem II

Chlorophyll *a* fluorescence was measured in algae grown on glass fiber disks on the surface of *Trebouxia* agar media. While still on the surface of media in Petri dishes to minimize desiccation, algae were dark-adapted for 30 min prior to measurement. The potential quantum yield of photosystem II (PSII) was measured using FluorCam MF-800 fluorescence-imaging camera (Photon Systems Instruments Ltd., Czech Republic), by applying a saturating flash of light 2000 μmol s^{-1} m^{-2} for 1s. The maximum efficiency of the PSII was assessed by the F_V/F_M ratio. $F_V/F_M = (F_M - F_0)/F_M$, where F_0 represents ground fluorescence in the dark-adapted state and F_M represents maximum fluorescence at a saturating radiation pulse in the dark-adapted state. Chlorophyll fluorescence parameters were taken from three separate positions on each disk and the mean value was used as the overall observation. Each treatment was replicated three times.

2.4. Determination of ascorbic acid (AsA), glutathione (GSH, GSSG) and organic acids

Reduced (GSH), oxidized glutathione (GSSG) and AsA were extracted from algal samples with 0.1 M HCl (0.1 g fw/1 ml) and quantified using LC-MS/MS (Agilent 1200 Series Rapid Resolution LC system coupled on-line to a MS detector Agilent 6460 Triple quadrupole with Agilent Jet Stream Technologies) at m/z values 308/76, 613/231 and 177/95 in positive MRM mode, respectively. Separation was done using column Zorbax EC-C18 100×4.6 mm, 2.7 μm particle size and mobile phase consisting of 0.2% acetic acid and methanol (95:5). The flow-rate was 0.6 ml/min and the column temperature was set at 25°C. Freshly prepared standards were used for calibration and quantification (Bačová et al., 2019).

Liquid chromatography with tandem mass spectrometry using a triple-quadrupole MS detector was used to analyse organic acids in the algae. A volume of 1 ml of 80 % methanol and glass beads 0.5 mm in diameter were added to samples of algal cells. Samples were homogenized at 6800 rcf, centrifuged at 16,000 rcf and then filtered through Whatman Mini-UniPrep syringeless filters 0.45 μm before analysis by liquid chromatography with mass detection. The samples were analysed using an Agilent 1200 Rapid Resolution LC system. The system was connected to an Agilent Technologies 6460 triple-quadrupole MS detector with an Agilent Jet Stream all from Agilent Technologies, Waldbronn, Germany. This system is described in more detail in ~~a~~ previous publications (Bačová et al., 2019; Koláčková et al., 2020).

2.5. Statistical analysis

One-way analysis of variance and Tukey's pairwise comparisons (MINITAB Release 11, 1996) were used to determine the significance ($P < 0.05$) of differences in all measured parameters.

3. Results

3.1. Algal growth

Biomass production (mg/g fw) of free-living alga *Scenedesmus* was significantly higher than biomass production of lichen photobionts, both, *Trebouxia* as well as *Coccomyxa* (Table 1). The presence of medular compound evernic acid did not affect the growth of *Scenedesmus*, while biomass production was significantly affected in the case of *Coccomyxa* and *Trebouxia* photobionts. Orcinol significantly affected only the growth of both lichen photobionts tested. Usnic acid, a cortical lichen compound, had a significant inhibition effect on both algae tested. This effect was most pronounced in the case of *Scenedesmus* cultures, where we observed approximately 90% inhibition effect when compared to corresponding controls (Table 1).

3.2. Activity of photosystem II

Chlorophyll *a* fluorescence (expressed as F_V/F_M ratio) was comparable in controls of all three tested algae (Table 1). The presence of evernic acid caused a significant decrease of chlorophyll *a* fluorescence in all tested algal species, while the presence of orcinol did not affect this parameter (Table 1). The presence of usnic acid decreased chlorophyll *a* fluorescence in both tested photobionts, in *Coccomyxa* was this decrease strong enough to be significant. In *Scenedesmus* was chlorophyll *a* fluorescence signal due to the presence of usnic acid on the surfaces of the disks not even detectable (Table 1).

3.3. Algal antioxidants

The content of AsA (μg/g dw) in photobiont cells was significantly higher than in *Scenedesmus* when compared to the unit of dw (Table 1). The presence of evernic acid caused strong inhibition of AsA in photobiont cells, when compared to controls, however in the *Scenedesmus* was this decrease not strong enough to be significant. Cultivation of algae on the disks supported with orcinol caused a significant decrease of AsA content only in the case of *Scenedesmus*. In the *Coccomyxa* cells, we observed a decrease in AsA content due to the presence of usnic acid, however, it was not strong enough to be significant (Table 1). On the other hand, AsA content was significantly decreased in *Trebouxia* photobiont due to the presence of usnic acid, in the case of *Scenedesmus* cells, we did not even observe a detectable amount of AsA (Table 1).

Scenedesmus contained a significantly lower amount of GSH, when compared to photobionts (Table 1). The presence of evernic acid caused a strong decrease of GSH content only in photobiont cultures. On the other hand, the presence of orcinol did not cause significant changes in the GSH content of all tested algal species. Usnic acid caused the decrease of GSH content in photobiont cells, and in *Scenedesmus* was GSH content due to the presence of usnic acid not even detectable (Table 1).

We observed strong differences between the content of GSSG in selected algal species, the highest was measured in the cells of *Trebouxia* control photobionts (Table 1). The ratio between GSH and GSSG (GSH/GSSG) was lowest in *Trebouxia* photobiont cells from all three tested algal species (Table 1). The presence of evernic acid strongly decreased this parameter only in photobiont cells. Orcinol decreased the GSH/GSSG ratio only in *Coccomyxa* cells (Table 1). Usnic acid did not change the GSH/GSSG ratio in *Trebouxia* photobiont cells, while in *Coccomyxa* was this ratio strongly decreased

Table 1.
Effects of selected lichen secondary metabolites (0.1 mg/disc), namely evernic acid, orcinol and usnic acid on selected parameters of 14-days old cultures of *Scenedesmus, Coccomyxa* and *Trebouxia* algae cells cultivated on discs with presence of metabolite, supported with nutritional medium. Data are means ± SDs ($n = 3$), nd = not detectable. Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

		Growth (mg/g fw)	F_V/F_M	AsA (μg/g dw)	GSH (μg/g dw)	GSSG (μg/g dw)	GSH/GSSG
Scenedesmus	**Control**	195 ± 14.0**a**	0.66 ± 0.04**a**	32.1 ± 4.0**a**	19.7 ± 1.63**b**	1.79 ± 0.34**b**	11.2 ± 1.64**a**
	Evernic acid	221 ± 17.7**a**	0.45 ±0.08**b**	22.5 ± 6.02**ab**	44.9 ± 8.33**a**	5.30 ± 1.50**a**	8.66 ± 1.39**a**
	Orcinol	216 ± 28.0**a**	0.60 ± 0.03**a**	19.1a ± 2.52**b**	26.5 ± 6.54**b**	2.99 ± 0.19**b**	8.99 ± 2.67**a**
	Usnic acid	18.3 ± 7.64**b**	nd	nd	nd	nd	nd
Coccomyxa	**Control**	100 ± 2.65**a**	0.65 ± 0.07**a**	88.7 ± 3.29**a**	367 ± 28.2**a**	37.2 ± 4.26**b**	9.88 ± 0.47**a**
	Evernic acid	46.6 ± 8.02**c**	0.38 ± 0.03**c**	0.29 ± 0.06**b**	12.1 ± 5.35**c**	3.61 ± 0.44**c**	3.29 ± 1.09**b**
	Orcinol	71.3 ± 15.4**b**	0.56 ± 0.04**ab**	107 ± 33.0**a**	345 ± 39.6**a**	70.1 ± 3.15**a**	4.93 ± 0.34**b**
	Usnic acid	35.0 ± 6.24**c**	0.47 ± 0.09**bc**	64.2 ± 20.6**a**	217 ± 77.2**b**	58.8 ± 19.4**ab**	3.68 ± 0.32**b**
Trebouxia	**Control**	86.3 ± 8.14**a**	0.62 ± 0.04**a**	84.7 ± 14.7**a**	605 ± 98.0**a**	274 ± 32.4**a**	2.19 ± 0.17**a**
	Evernic acid	35.0 ± 10.6**b**	0.37 ± 0.03**b**	0.24 ± 0.05**b**	10.9 ± 2.11**b**	31.3 ± 8.19**b**	0.35 ± 0.03**b**
	Orcinol	46.6 ± 3.79**b**	0.59 ± 0.07**a**	71.5 ± 8.92**a**	622 ± 90.9**a**	308 ± 40.1**a**	2.01 ± 0.11**a**
	Usnic acid	56.3 ± 8.74**b**	0.51 ± 0.05**a**	5.11 ± 2.42**b**	96.2 ± 34.6**b**	37.1 ± 17.8**b**	2.38 ± 0.29**a**

when compared with control, and in *Scenedesmus* was this parameter even not detectable (Table 1).

3.4. Algal organic acids

The organic acid content of all algae tested was species-specific in control variants (Tables 2-3). The content of citric acid decreased as a result of the presence of evernic acid and orcinol on cultivation disks. This effect was negatively correlated with the content of lactic acid. We observed a strong increase of lactic acid content as a result of the presence of orcinol and evernic acid on the surface of disks. The content of tartaric acid was very low, so we observed significant variability in its content in *Scenedesmus* cells. Contents of fumaric acid, glutamic acid, ketoglutaric acid, malic acid, pyruvic acid, quinic acid and succinic acid were not affected by the presence of evernic acid and orcinol on the surfaces of cultivation disks. Contents of algal acids in *Scenedesmus* cells due to treatment by usnic acid were not detectable (Tables 2-3).

In the *Coccomyxa* cells, evernic acid on the disks caused a strong decrease of citric acid, glutamic acid, ketoglutaric acid, malic acid quinic acid and succinic acid (Tables 2-3). On the other hand, we observed a strong increase in the content of pyruvic acid when compared to the control. The content of fumaric acid, lactic acid and tartaric acid was relatively stable. The presence of orcinol decreased only the content of lactic acid and succinic acid in the *Coccomyxa* tested cells. The presence of usnic acid on the disks caused an increase of fumaric acid and pyruvic acid, while contents of ketoglutaric acid and succinic acid decreased (Table 2-3).

The content of citric acid, glutamic acid, ketoglutaric acid and malic acid decreased as a result of the presence of evernic acid on the disks in *Trebouxia* cells (Tables 2-3). On the other hand, the content of fumaric acid, lactic acid and pyruvic acid increased. The presence of orcinol decreased the content of citric acid, malic acid and succinic acid, while the content of pyruvic acid significantly increased. The presence of usnic acid decreased the contents of citric acid, glutamic acid, ketoglutaric acid and malic acid. The content of pyruvic acid in *Trebouxia* increased as a result of the presence of usnic acid on the disks (Table 3).

4. Discussion

4.1. Algal growth

Algae cultivated on the surface of glass filaments in the disk form resemble mycobionts in stratified lichens (Bačkor et al. 2003). The distribution of metabolites crystals on the surface of filaments is reminiscent of the situation in natural lichens, where extracellular secondary metabolites located on the surface of hyphae may also be in contact with algal cells in the photobiont layer.

It has been previously demonstrated that usnic acid appears to have allelopathic effects on vascular plants (Cardarelli et al., 1997; Lechowski et al., 2006). Similarly, it seems that usnic acid has phytotoxic acid on cultures of lower plants, e.g. mosses (Goga et al., 2017) or free-living algae and photobionts of lichens (Bačkor et al., 1998; Budová et al., 2006; Bačkor et al., 2010; Lokajová et al., 2014). In the present study, we demonstrated that control cultures of free-living alga *Scenedesmus* grew better, when compared to aposymbiotically grown control cultures of lichen photobionts, e.g. *Trebouxia* and *Coccomyxa*. Usnic acid possesses antimitotic effects, previously demonstrated in taxonomically diverse organisms (Cardarelli et al., 1997; Pizňak et al., 2019). It can be the result of the direct effect of usnic acid on the spindle apparatus during the processes of mitosis (Al-Bekairi et al., 1991; Pizňak et al., 2019).

The influence of other extracellular lichen metabolites on the growth of plants is, however, under-researched. Bačkor et al. (2013) tested growth inhibition of lichen photobiont *T. erici* due to the presence of selected secondary metabolites of lichens (atranorin, gyrophoric acid and parietin). They found that atranorin, a cortical compound, was more effective in the phytotoxicity of photobiont when compared to other tested substances. Lokajová et al. (2014) tested mixtures of naturally occurring secondary metabolites of lichens and discovered that extracts containing evernic acid had negative effects on aposymbiotically grown cultures of lichen photobionts *T. erici*. However, we know virtually almost nothing about the cytotoxicity of most secondary metabolites synthesized in lichen thalli.

4.2. Activity of photosystem II

Usnic acid and evernic acid demonstrated the strongest inhibition effects on chlorophyll *a* fluorescence in all tested algal species in this study, while the effect of orcinol was not strong enough to be statistically significant. The influence of lichen secondary metabolites on Photosystem II, including usnic acid and evernic acid was demonstrated previously (Endo et al., 1998; Budová et al., 2006; Takahagi et al., 2008). It seems that other chlorophyll fluorescence methods may help explain the sensitivity or tolerance of complex photosynthetic processes in lichen algal partners (Váczi et al., 2018; Barták et al., 2023).

4.3. Algal antioxidants and organic acids influenced by allelochemicals

It has been shown by Caviglia et al. (2001) that usnic acid extracted from lichen *Parmelia soredians* may act as an antioxidant.

Table 2.
Effects of selected lichen secondary metabolites (0.1 mg/disc), namely evernic acid, orcinol and usnic acid on selected parameters of 14-days old cultures of *Scenedesmus, Coccomyxa* and *Trebouxia* algae cells cultivated on discs with presence of metabolite, supported with nutritional medium. Data are means ± SDs (n = 3), nd = not detectable. Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

		Citric acid	Fumaric acid	Glutamic acid (μg/g dw)	Ketoglutaric acid	Lactic acid	Malic acid
Scenedesmus	**Control**	44.1 ± 2.09**a**	3.95 ± 0.31**a**	41.8 ± 7.10**a**	1.22 ± 0.23**a**	19.68 ± 0.48**b**	13.8 ± 2.21**a**
	Evernic acid	23.1 ± 12.6**b**	3.66 ± 0.65**a**	40.5 ± 12.6**a**	3.62 ± 1.41**a**	157 ± 15.7**a**	15.8 ± 5.59**a**
	Orcinol	16.8 ± 3.61**b**	5.53 ± 2.75**a**	29.7 ± 15.4**a**	11.1 ± 7.63**a**	136 ± 40.3**a**	15.6 ± 4.99**a**
	Usnic acid	nd	nd	nd	nd	nd	nd
Coccomyxa	**Control**	212 ± 14.5**a**	8.03 ± 0.52**b**	72.7 ± 8.65**a**	11.6 ± 7.31**a**	256 ± 21.7**a**	85.1 ± 7.92**ab**
	Evernic acid	6.81 ± 0.81**b**	13.4 ± 3.59**ab**	8.84 ± 1.35**b**	1.45 ± 0.18**b**	174 ± 34.5**ab**	5.47 ± 0.83**c**
	Orcinol	241 ± 18.8**a**	7.5 ± 1.56**b**	64.3 ± 20.8**a**	2.75 ± 0.27**ab**	130 ± 20.3**b**	108 ± 5.12**a**
	Usnic acid	194 ± 67.9**a**	18.2 ± 3.54**a**	57.3 ± 20.4**a**	2.04 ± 0.62**b**	203 ± 48.6**ab**	66.0 ± 20.7**b**
Trebouxia	**Control**	66.6 ± 14.7**a**	6.52 ± 1.32**b**	88.1 ± 13.8**a**	4.04 ± 0.67**a**	63.1 ± 7.20**b**	28.5 ± 5.76**a**
	Evernic acid	18.6 ± 4.89**bc**	16.2 ± 4.31**a**	14.4 ± 3.94**b**	2.59 ± 0.65**b**	138 ± 41.1**a**	10.2 ± 3.78**bc**
	Orcinol	31.0 ± 4.18**b**	11.2 ± 1.70**ab**	105 ± 10.2**a**	2.82 ± 0.082**ab**	62.3 ± 16.2**b**	15.5 ± 2.27**b**
	Usnic acid	4.51 ± 1.35**c**	7.32 ± 0.81**b**	21.8 ± 5.62**b**	0.72 ± 0.13**c**	44.6 ± 10.1**b**	3.98 ± 1.18**c**

Table 3.
Effects of selected lichen secondary metabolites (0.1 mg/disc), namely evernic acid, orcinol and usnic acid on selected parameters of 14-days old cultures of *Scenedesmus*, *Coccomyxa* and *Trebouxia* algae cells cultivated on discs with presence of metabolite, supported with nutritional medium. Data are means ± SDs (n = 3), nd = not detectable. Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

		Pyruvic acid	Quinic acid (μg/g dw)	Succinic acid	Tartaric acid
Scenedesmus	**Control**	134 ± 10.3**a**	0.25 ± 0.55**a**	10.6 ± 2.91**a**	0.06 ± 0.01**b**
	Evernic acid	148 ± 12.5**a**	0.30 ± 0.07**a**	14.5 ± 2.22**a**	0.15 ± 0.06**a**
	Orcinol	180 ± 115**a**	0.27 ± 0.01**a**	15.8 ± 3.98**a**	0.12 ± 0.03**ab**
	Usnic acid	nd	nd	nd	nd
Coccomyxa	**Control**	119 ± 2.05**b**	4.12 ± 0.74**ab**	27.6 ± 2.40**a**	0.23 ± 0.03**a**
	Evernic acid	307 ± 55.5**a**	0.95 ± 0.14**c**	5.60 ± 1.09**c**	0.39 ± 0.20**a**
	Orcinol	150 ± 73.1**b**	4.85 ± 0.63**a**	11.3 ± 1.60**b**	0.23 ± 0.05**a**
	Usnic acid	369 ± 71.4**a**	2.29 ± 1.01**bc**	10.1 ± 1.77**bc**	0.47 ± 0.17**a**
Trebouxia	**Control**	127 ± 8.86**b**	0.50 ± 0.10**a**	10.7 ± 4.47**ab**	0.26 ± 0.09**a**
	Evernic acid	330 ± 90.1**a**	0.56 ± 0.24**a**	15.1 ± 5.13**a**	0.32 ± 0.11**a**
	Orcinol	267 ± 22.9**a**	0.84 ± 0.21**a**	5.57 ± 1.35**b**	0.34 ± 0.14**a**
	Usnic acid	241 ± 28.9**ab**	0.47 ± 0.23**a**	3.31 ± 0.45**b**	0.25 ± 0.11**a**

They found that this compound may detoxify reactive oxygen species produced after the application of the herbicide Paraquat. On the other hand, Han et al. (2004) demonstrated that usnic acid may induce oxidative stress in the living cells.

Aliphatic organic compounds produced by various plants were reported as potential chelators of metals under stress conditions (Dresler et al., 2014). The response of plants (e.g. algae) to the presence of allelochemicals, which are produced by their symbiotic partners or free-living forms, is still enigmatic. Algae used in these experiments reacted differently to the presence of secondary metabolites of lichens.

Pyruvic acid ensures the energy to the cells by the citric acid cycle and it is a key product between catabolism and anabolism of carbohydrates, fats and proteins. The highest content of pyruvic acid was observed with orcinol in *Scenedesmus*, with usnic acid in *Coccomyxa* and evernic acid in *Trebouxia*. The increase in pyruvate in response to allelochemicals responds similarly to that in response to heavy metals and is likely directed toward the production of ATP and NADH for other metabolic processes (Kováčik et al., 2015).

Citric acid, glutamic acid and lactic acid and were most present in studied algae, whereas fumaric acid, ketoglutaric acid, quinic acid, succinic acid, tartaric acid were found in very low content or as trace elements. However, we know almost virtually nothing about the presence and the role of organic acids in lichen photobionts.

5. Conclusions

Lichen photobionts, algae, and/or cyanobacteria are in permanent contact with their mycobionts forming lichen thalli. Because this symbiosis is a long-term association, we assume that the production of secondary metabolites in lichens by mycobionts is a key element for maintaining homeostasis in lichen thallus, preventing overgrowth of biomass of photobiont resulting in an imbalance between bionts. We studied allelopathic effects of lichen secondary metabolites (evernic acid, orcinol, usnic acid) on selected species of algae: *Trebouxia erici*, photobiont of lichen *Cladonia cristatella* producing typical lichen secondary metabolites, *Coccomyxa solorinae-saccatae*, photobiont of lichen *Solorina saccata* which do not produce typical secondary metabolites of lichens, and free-living alga *Scenedesmus quadricauda*.

Allelopathic effects of lichen compounds, so far studied in recent studies, involving growth (biomass production) inhibition of photobionts, a decrease of their chlorophyll *a* fluorescence, the content of ascorbic acid in the cells, and affecting the content of reduced and oxidized glutathione and contents of selected organic acids. In general, lichen metabolites may act as phytotoxic substances, causing oxidative stress in photobiont cells. Due to this reason, lichen symbiosis may be considered, in part, as controlled parasitism of the fungal partner of thalli on the algal/cyanobacterial partner of symbiosis. However, we did not observe a clear response of algae with different habitat preferences to lichen metabolites.

Declaration of Competing Interest

Authors declare no conflict of interests.

Acknowledgments

This paper is dedicated to the memory of our dear co-worker Prof. Bořivoj Klejdus, who passed away while this paper was being prepared. Bořivoj took a significant part in the biochemical analyses. This work was financially supported by the Slovak Research and Development Agency under contract No. APVV-21-0289 and Slovak Grant Agency KEGA under contracts No. 008SPU-4/2023 and 009UPJŠ-4/2023.

References

Ahmadjian, V., 1993. The Lichen Symbiosis. John Wiley & Sons, Inc., New York.
Al-Bekairi, A.M., Qureshi, S., Chaudhry, M.A., Krishna, D.R., Shah, A.H., 1991. Mitodepressive, clastogenic and biochemical effects of (+)-usnic acid in mice. J. Ethnopharmacol. 33, 217–220.
Bačkor, M., Loppi, S., 2009. Interactions of lichens with heavy metals. Biol. Plantarum 53, 214–222.
Bačkor, M., Hudák, J., Repčák, M., Ziegler, W., Bačkorová, M., 1998. The influence of pH and lichen metabolites (vulpinic acid and (+) usnic acid) on the growth of lichen photobiont *Trebouxia irregularis*. Lichenologist 30, 577–582.
Bačkor, M., Fahselt, D., Davidson, R., Wu, C.T., 2003. Effects of copper on wild and tolerant strains of the lichen photobiont *Trebouxia erici* (Chlorophyta) and possible tolerance mechanisms. Arch. Environ. Con. Tox. 45, 159–167.
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, 405–411.
Bačkor, M., Ivanova, V., Laatsch, H., Lokajová, V., Bačkorová, M., 2013. Allelopathic effects of lichen secondary metabolites on photobiont *Trebouxia erici*. Allelopathy J 31, 189–198.
Bačová, R., Klejdus, B., Ryant, P., Cernei, N., Adam, V., Húska, D, 2019. The effects of 5-azacytidine and cadmium on global 5-methylcytosine content and secondary metabolites in the freshwater microalgae *Chlamydomonas reinhardtii* and *Scenedesmus quadricauda*. J. Phycol. 55, 329–342.
Barták, M., Hájek, J., Halici, M.G., Bednaříková, M., Casanova-Katny, A., Váczi, P., Puhovkin, A., Mishra, K.B., Giordano, D., 2023. Resistance of primary photosynthesis to photoinhibition in Antarctic lichen *Xanthoria elegans*: Photoprotective mechanisms activated during a short period of high light stress. Plants 12, 2259.
Brodo, I.M., Sharnoff, S.D., Sharnoff, S., 2001. Lichens of North America. Yale University Press, New Haven, Connecticut, USA.
Buďová, J., Bačkor, M., Bačkorová, M., Židzik, J., 2006. Usnic acid and copper toxicity in aposymbiotically grown lichen photobiont *Trebouxia erici*. Symbiosis 42, 169–174.

Cardarelli, M., Serino, G., Campanella, L., Ercole, P., De Cicco Nardone, F., Alesiani, O., Rosiello, F., 1997. Antimitotic effects of usnic acid on different biological systems. Cell. Mol. Life Sci. 53, 667–672.
Caviglia, A.M., Nicora, P., Giordani, P., Brunialti, G., Modenesi, P., 2001. Oxidative stress and usnic acid content in *Parmelia caperata* and *Parmelia soredians* (Lichenes). Farmaco 56, 379–382.
Dresler, S., Bednarek, W., Wójcik, M., 2014. Effect of cadmium on selected physiological and morphological parameters in metallicolous and non-metallicolous populations of *Echium vulgare L*. Ecotoxicol. Environ. Saf. 104, 332–338.
Endo, T., Takahagi, T., Kinoshita, Y., Yamamoto, Y., Sato, F., 1998. Inhibition of photosystem II of spinach by lichen-derived depsides. Biosci. Biotechnol. Biochem. 62, 2023–2027.
Fahselt, D., 1994. Secondary biochemistry of lichens. Symbiosis 16, 117–165.
Goga, M., Antreich, S., Bačkor, M., Weckwerth, W., Lang, I., 2017. Lichen secondary metabolites affect growth of *Physcomitrella patens* by allelopathy. Protoplasma 254, 1307–1315.
Goga, M., Elečko, J., Marcinčinová, M., Ručová, D., Bačkorová, M., Bačkor, M., 2020. Lichen metabolites: An overview of some secondary metabolites and their biological potential. Co-Evol. Second. Metab. 1–36.
Halama, P., van Haluwin, C., 2004. Antifungal activity of lichen extracts and lichenic acids. BioControl 49, 95–107.
Han, D., Matsumaru, K., Rettori, D., Kaplowitz, N., 2004. Usnic acid-induced necrosis of cultured mouse hepatocytes: inhibition of mitochondrial function and oxidative stress. Biochem. Pharmacol. 67, 439–451.
Ivanova, V., Bačkor, M., Dahse, H.M., Graefe, U., 2010. Molecular structural studies of lichen substances with antimicrobial, antiproliferative and cytotoxic effects from *Parmelia subrudecta*. Prep. Biochem. Biotechnol. 40, 377–388.
Kosanic, M., Manojlovic, N., Jankovic, S., Stanojkovic, T., Rankovic, B., 2013. *Evernia prunastri* and *Pseudoevernia furfuraceae* lichens and their major metabolites as antioxidant, antimicrobial and anticancer agents. Food Chem. Toxicol. 53, 112–118.
Kováčik, J., Klejdus, B., Babula, P., Hedbavný, J., 2015. Nitric oxide donor modulates cadmium-induced physiological and metabolic changes in the green alga *Coccomyxa subellipsoidea*. Algal Res. 8, 45–52.
Krishna, B.R., Gundoju, N.R., Somasekhar, T., Elkhateeb, W., Daba, G., 2022. Biologically active orcinol-based secondary metabolites originated from lichens. Nova Biotechnol. Chim. 21, e1075 art. no..
Latkowska, E., Lechowski, Z., Bialczyk, J., Pilarski, J., 2006. Photosynthesis and water relations in tomato plants cultivated long-term in media containing (+)-usnic acid. J. Chem. Ecol. 32, 2053–2066.
Lawrey, J.D., 1986. Biological role of lichen substances. Bryologist 89, 111–122.
Lechowski, Z., Mej, E., Bialczyk, J., 2006. Accumulation of biomass and some macroelements in tomato plants grown in media with (+) usnic acid. Environ. Exp. Bot. 56, 239–244.
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). S. Afr. J. Bot. 93, 86–91.
Nishitoba, Y., Nishimura, H., Nishiyama, T., Mizutani, J., 1987. Lichen acids, plant growth inhibitors from *Usnea longissima*. Phytochemistry 26, 3181–3185.
Pichler, G., Muggia, L., Candotto-Carnier, F., Grube, M., Kranner, I., 2023. How to build a lichen: from metabolite release to symbiotic interplay (Tansley review). New Phytol. 238, 1362–1378.
Pizňak, M., Bačkor, M., 2019. Lichens affect boreal forest ecology and plant metabolism. S. Afr. J. Bot. 124, 530–539.
Pizňak, M., Kolarčik, V., Goga, M., Bačkor, M., 2019. Allelopathic effects of lichen metabolite usnic acid on growth and physiological responses of Norway spruce and Scots pine seedlings. S. Afr. J. Bot. 124, 14–19.
Pöykkö, H., Hyvärinen, M., Bačkor, M., 2005. Removal of lichen secondary metabolites affects food choice and survival of lichenivorous moth larvae. Ecology 86, 2623–2632.
Renzaka, T., Sigler, K., 2007. Hirtusneanoside, an unsymmetrical dimeric tetrahydroxanthone from the lichen *Usnea hirta*. J. Nat. Prod. 70, 1487–1491.
Solhaug, K.A., Lind, M., Nybakken, L., Gauslaa, Y., 2009. Possible functional roles of cortical depsides and medullary depsidones in the foliose lichen *Hypogymnia physodes*. Flora 204, 40–48.
Takahagi, T., Endo, T., Yamamoto, Y., Sato, F., 2008. Lichen photobionts show tolerance against lichen acids produced by lichen mycobionts. Biosci. Biotech. Bioch. 72, 3122–3127.
Tasdemir, D., Franzblau, S.G., 2007. *In vitro* antituberculotic activity of several lichen metabolites. Planta Med. 73, 174.
Váczi, P., Gauslaa, Y., Solhaug, K.A., 2018. Efficient fungal UV-screening provides a remarkably high UV-B tolerance of photosystem II in lichen photobionts. Plant Physiol. Biochem. 132, 89–94.