

Comparison of the Phytotoxic Effects of Usnic Acid on Cultures of Free-Living Alga *Scenedesmus quadricauda* and Aposymbiotically Grown Lichen Photobiont *Trebouxia erici*

Martin Bačkor · Katarína Klemová ·
Miriam Bačkorová · Veneta Ivanova

Received: 17 November 2009 / Accepted: 11 March 2010 / Published online: 20 March 2010
© Springer Science+Business Media, LLC 2010

Abstract The phytotoxic effects of the lichen secondary metabolite—usnic acid on cultures of free living alga—*Scenedesmus quadricauda* (UTEX 76) and aposymbiotically grown lichen photobiont *Trebouxia erici* (UTEX 911) were assessed. We found a relatively strong inhibition effect of usnic acid on the growth of alga *Scenedesmus*, accompanied by an increase of cell size, an alteration of assimilation pigment composition, followed by strong degradation of chlorophyll *a*, a decrease of chlorophyll *a* fluorescence, and an increase of reactive oxygen species in the cells. The content of soluble proteins remained a stable parameter. Phytotoxicity of usnic acid on cultures of *Trebouxia* photobiont was significantly lower. Usnic acid in lichens may act as an allochemical that controls the division of photobiont cells, thereby regulating the balance between the photobiont and mycobiont forming

thallus. Higher tolerance to usnic acid in *Trebouxia* cultures may be an adaptation resulting from the long term co-evolution of these algae with fungi that produce secondary metabolites.

Key Words Algae · Lichens · Secondary metabolites · Toxicity · Usnic acid

Introduction

Lichens are a stable and self-supporting association between fungi (mycobionts) and photoautotrophic algae or cyanobacteria (photobionts). Interactions within a lichen thallus are, however, complex, and lichens may be considered more like an ecosystem or community than a typical organism (Fahselt 2008).

Lichens produce many unique compounds that are considered to have important biological and ecological roles. These roles include antimicrobial activity, allelopathy, antiherbivory, chelating of heavy metals, light screening, and other proposed functions not yet sufficiently supported by experimental evidence under laboratory conditions (Lawrey 1986; Pöykkö et al. 2005; Latkowska et al. 2006; Hauck et al. 2009; Solhaug et al. 2009). Secondary metabolites may be responsible for the ecological success of lichenization, allowing lichens frequently to be the dominant organisms in environments characterized by extreme ecological conditions. Lichen compounds are secreted by the fungal partner, and they are deposited on the surface of hyphae, as well as on lichen algae, typically constituting 0.1 to 5.0% (w/w) of thallus dry weight (Fahselt 1994).

M. Bačkor (✉) · K. Klemová
Department of Botany, Institute of Biology and Ecology,
Faculty of Science, Šafárik University,
Mánišova 23,
041 67 Košice, Slovak Republic
e-mail: martin.backor@upjs.sk

M. Bačkorová
Department of Cell and Molecular Biology, Institute of Biology
and Ecology, Faculty of Science, Šafárik University,
Moyzesova 11,
040 01 Košice, Slovak Republic

V. Ivanova
The Stephan Angeloff Institute of Microbiology,
Bulgarian Academy of Sciences,
Acad. G. Bonchev Str. 26,
1113 Sofia, Bulgaria

Usnic acid, a yellowish pigment and dibenzofuran derivative, is a common lichen metabolite, especially abundant in members of the genera *Alectoria*, *Usnea*, or *Xanthoparmelia*. The antibiotic, antiviral, antimycotic, antiprotozoal, antiherbivoral, antiproliferative, anti-inflammatory, analgesic, antipyretic, allelopathic, and UV protecting effects of usnic acid have been stated previously (for review see Cocchiello et al. 2002).

There is little doubt that the presence of secondary metabolites, including usnic acid, has significant influence on lichen survival. Lichens are slow growing and long lived organisms, and due to the toxic effect of usnic acid to a wide range of organisms, their thalli are relatively well protected against pathogens and herbivores. However, usnic acid potentially can be toxic to cells that come in direct contact with it, even the algal partner of the lichen that produces it. Crystals of lichen cortical secondary metabolites may be deposited directly on the surface of photobiont cells (Sarret et al. 1998). Previous studies have suggested that the presence of usnic acid may regulate photobiont populations (Bačkor et al. 1998; Bud'ová et al. 2006). Production of usnic acid may be a critical step of maintaining the required ratio of mycobiont to photobiont biomass in thalli for mutualistic symbiosis.

The mechanisms of the phytotoxic effects of usnic acid on plants, including on its own photobionts are not understood sufficiently; however, inhibition of chlorophyll *a* fluorescence (F_v/F_M), decrease of carotenoid and chlorophyll levels in the cells, and decrease of viability have been demonstrated as responses of plants to the presence of usnic acid (Cardarelli et al. 1997; Endo et al. 1998; Bud'ová et al. 2006). Decrease of biomass production also has been demonstrated (Bačkor et al. 1998; Bud'ová et al. 2006; Lechowski et al. 2006).

Concentrations of secondary metabolites around photobiont cells are high, and lichen photobionts are in direct contact with cortical metabolites produced by the mycobiont (Takahagi et al. 2008). Although lichen compounds may act as allelochemicals and are responsible for maintaining balance between bionts inside lichen thalli, photobionts must be protected from the toxicity of these compounds. We hypothesized that lichen photobionts have evolved protective mechanisms against phytotoxicity of secondary metabolites through co-evolution with lichen mycobionts. To test this hypothesis, we evaluated the potential phytotoxic effect of usnic acid on the aposymbiotically grown lichen photobiont *Trebouxia erici*. For assessment of the potential differences in phytotoxicity of usnic acid to free-living algae without evolutionary experience with the presence of lichen secondary metabolites, *Scenedesmus quadricauda* also was chosen.

Methods and Materials

Organisms and Culture Conditions Free-living alga *Scenedesmus quadricauda* (Turp.) Bréb. (UTEX 76) and aposymbiotically grown lichen photobiont *Trebouxia erici* Ahmadjian (UTEX 911) were used. Both species belong to Chlorophyta, a division of green algae. Algae were cultivated on agar medium (1.5%) previously developed for cultivation of lichen algae by Ahmadjian (1993). This was Bold's Basal Medium (BBM 3N) plus 10 g casein acid hydrolyzate and 20 g glucose per liter with the pH adjusted to 6.5 (Bačkor et al. 2004). Cultures were maintained at 22°C under a 16:8 hr, L:D photoperiod and 30 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ artificial irradiance ("cool white" tubes).

Toxicity Assay Algae were cultivated on the surface of Whatman CF/C filters (glass fiber filter disks, 25 mm diam). Glass fiber filter disks were used instead of the cellulose-acetate disks, previously used for lichen photobiont cultivation (Bačkor et al. 2003, 2004). Glass fiber filter disks may be used directly for disruption in the mortar of algal cells grown on their surfaces. Crystals of usnic acid on the surface of fibers of the disk simulate the situation in natural lichens, where extracellular secondary metabolites located on the surface of hyphae are in the contact with algal cells in the photobiont layer. Briefly, cells from both algal species (approximately two inoculation loops) grown on stock solid *Trebouxia* media were transferred into 50 ml of liquid *Trebouxia* media in an Erlenmeyer flask. Cells were suspended by gentle stirring on a magnetic stirrer for 1 hr. Cultures were maintained for 5 d in a cultivation room under conditions described previously, with daily stirring on a magnetic stirrer for about 1 hr. Homogeneity of the algal suspension was verified microscopically, and the number of cells was calculated by using a standard haemocytometer. Cell density of cultures was adjusted to approximately 10^6 cells ml^{-1} of medium before quantitative cultivation of algae.

For cultivation of both algal species, sterilized Whatman CF/C filters (25 mm diam) were subjected to three different pretreatments. Usnic acid (0.01 and 0.1 mg / disk) dissolved in acetone (volume 30 μl) was applied by automatic pipette on the surface of disks, while the same volume of acetone was applied to control disks. We used (+)-usnic acid enantiomer (Sigma-Aldrich). After evaporation of acetone for 4 hr, one disk was transferred to the surface of solid *Trebouxia* medium in a separate Petri dish, (6 cm diam), and 20 μl of algal suspension were inoculated into the center of each disk. Disk pores allowed supplemental nutrient media to pass through the disk and permitted growth to be determined easily from changes in biomass (Bačkor et al. 2003, 2004). The total mass of cultures was calculated by subtracting the mean fresh weight (fw) of a

Whatman CF/C disk saturated by identical medium, from the fw of a disk supporting algal cultures after 14 d. Each treatment was replicated eighteen times for both algal species assessed.

Pigment Analysis and Measurement of Chlorophyll *a* Integrity The influence of usnic acid on the algae was determined by using cultures grown on Whatman CF/C disks. Weighed disks were extracted in the dark for 1 hr at 65°C in 5 ml of dimethyl sulfoxide (DMSO). To maximize chlorophyll extraction, cell aggregates were homogenized with a mortar, and glass fibers of disks facilitated disruption of cell walls of algae. After cooling to ambient temperature, the absorbance of the extract, as a reflection of turbidity, was determined at 750 nm with a spectrophotometer to be certain that it was always less than 0.01. Absorbance of extracts was read at 665, 649, 435, and 415 nm to assess chlorophyll content and the possibility of chlorophyll *a* degradation (Barnes et al. 1992; Wellburn 1994). To utilize the linear portion of the response curve, extracts from disks with very high cell densities (absorbance at 665 nm higher than 0.8) were diluted with fresh DMSO to fall into the absorbance range 0.2–0.8. To determine the content of “total” carotenoids, absorbance was read at 480 nm. Chlorophyll *a*, chlorophyll *b*, chlorophyll *a+b*, and total carotenoids were calculated by using equations derived from specific absorption coefficients for pure chlorophyll *a* and chlorophyll *b* in DMSO. Chlorophyll *a/b* was used to assess the physiological competence of algal cells.

The ratios of absorbances at 435 and 415 nm (A_{435}/A_{415}), termed the phaeophytinization quotient, were calculated as a reflection of the ratio of chlorophyll *a* to phaeophytin *a* and to provide an indication of integrity of photobiont chlorophyll. Each treatment was replicated three times.

Activity of Photosystem II Chlorophyll *a* fluorescence was measured in algae grown on Whatman CF/C disks on the surface of *Trebouxia* agar medium. While still on the surface of the medium 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 with a Plant Stress Meter (PSM Mark II, Biomonitor, SCI AB), with a sensor 5 mm diam, and results were expressed as F_V/F_M calculated as the maximal fluorescence (F_M) less the minimal fluorescence (F_0), divided by F_M of dark adapted plants: $(F_M - F_0) / F_M = F_V/F_M$. Chlorophyll fluorescence parameters were taken from three separate positions on each disk, and the mean value used as one observation. Each treatment was replicated three times.

Content of Soluble Proteins Algae grown on Whatman CF/C disks (controls, as well as with addition of usnic acid on the

surface of disks) were homogenized with disks in an ice-cold mortar in phosphate buffer (50 mM). After centrifugation at 15,000 g at 4°C for 20 min, the water-soluble protein content of supernatants was measured using the Bradford (1976) method. Supernatants (100 μ l) were pipetted into 900 μ l of Bradford assay kit (Biorad) in a spectrophotometric cuvette and mixed. After 10 min, absorbance of samples was spectrophotometrically measured at 595 nm. Bovine serum albumin was used as calibration standard. Each treatment was replicated three times.

Detection of Oxidative Status Hydrogen peroxide and superoxide were measured in homogenates with potassium phosphate buffer prepared for assay for determination of soluble proteins. Hydrogen peroxide was quantified by the $TiCl_4$ method (Kováčik and Bačkor 2007). Superoxide was estimated according to Elstner and Heupel (1976) by monitoring formation of nitrite from hydroxylamine at 530 nm. Reaction mixture contained 0.27 ml of potassium phosphate buffer, 0.03 ml of 10 mM hydroxylamine, 0.3 ml of supernatant, 0.3 ml of 17 mM sulphanilamide, 0.3 ml of 7 mM α -naphthylamine, and 0.3 ml of diethyl ether. Each treatment was replicated three times.

Cell Size Algae grown on Whatman CF/C disks from each treatment were separated gently from the surface of the disks by a scalpel. Cells were suspended immediately in 5 mM HEPES buffer, pH 6.5 and viewed under a light microscope at 800 \times . Cell size parameters (width and length in the case of *S. quadricauda* cells, or length in the case of *T. erici* spherical cells) were measured with an eyepiece micrometer. Twenty individual cells from each treatment and both algal species were evaluated.

Statistical Analysis The one-way analysis of variance and Tukey’s pairwise comparisons (MINITAB Release 11, 1996) were applied to determine significance ($P < 0.05$) of differences in all measured parameters. In addition, two-way ANOVA with interactions (SPSS version 16, 2008) was applied to evaluate species effect (species), usnic acid effect (UA), and interactions between species effect and UA effect (species \times UA) where applicable.

Results

Biomass production of the control *Scenedesmus* cultures was almost five times higher after 2 weeks of cultivation when compared to control *Trebouxia* cultures (Table 1). Presence of usnic acid on the disks significantly affected biomass production of both algal cultures in a dose dependent manner. However, a 0.01 mg/disk of usnic acid decreased biomass production of *Scenedesmus* cultures by a factor of 4,

Table 1 Biomass production (mg fw/disk), chlorophyll integrity (435/415), chlorophyll *a/b* ratio (A/B), total carotenoid/total chlorophyll ratio (CAR/CHL) and chlorophyll *a* fluorescence (F_V/F_M) of 2 week old *Scenedesmus* and *Trebouxia* cultures cultivated on disks with addition of usnic acid (0, 0.01 and 0.1 mg)

	Usnic acid conc. (mg/disk)	Biomass (mg fw/disk)	435/415	A/B	CAR/CHL	F_V/F_M
<i>Scenedesmus</i>	0	246±39.9a	1.45±0.02a	3.57±0.22a	0.25±0.02a	0.65±0.02a
	0.01	66±25.5b	1.40±0.03a	3.22±0.29a	0.33±0.06a	0.51±0.04b
	0.1	22±8.5b	0.94±0.09b	1.17±0.38b	0.22±0.05a	0.14±0.07c
	<i>F</i> value	<i>F</i> =54.93	<i>F</i> =75.17	<i>F</i> =54.35	<i>F</i> =4.11	<i>F</i> =84.69
	<i>P</i> value	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> =0.075	<i>P</i> <0.001
<i>Trebouxia</i>	0	55±4.0a	1.40±0.04a	2.82±0.21a	0.27±0.01a	0.60±0.04a
	0.01	45±9.7ab	1.36±0.08a	2.66±0.06a	0.27±0.02a	0.57±0.01ab
	0.1	25±6.1b	1.32±0.07a	3.24±0.78a	0.29±0.02a	0.52±0.01b
	<i>F</i> value	<i>F</i> =12.29	<i>F</i> =1.22	<i>F</i> =1.25	<i>F</i> =0.87	<i>F</i> =8.50
	<i>P</i> value	<i>P</i> =0.008	<i>P</i> =0.36	<i>P</i> =0.351	<i>P</i> =0.468	<i>P</i> =0.018

Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test ($P<0.05$)

and a 0.1 mg/disk decreased biomass production by a factor of 11, while in *Trebouxia* culture biomass production was decreased only at the highest tested usnic acid dose of a 0.1 mg/disk by a factor of approximately 2.2 (Table 1). Two-way ANOVA for biomass production: species ($F=63.25$, $P<0.001$), UA ($F=52.98$, $P<0.001$), species \times UA ($F=40.98$, $P<0.001$).

The highest tested usnic acid doses significantly decreased chlorophyll *a* integrity in *Scenedesmus* cultures, while in *Trebouxia* cultures none of the tested usnic acid concentrations significantly altered chlorophyll integrity (Table 1). Two-way ANOVA for chlorophyll *a* integrity: species ($F=40.75$, $P<0.001$), UA ($F=10.84$, $P=0.006$), species \times UA ($F=23.75$, $P<0.001$).

Similarly, the highest tested usnic acid doses significantly decreased chlorophyll *a/b* of *Scenedesmus* cultures. In the *Trebouxia* cultures, we did not observe significant changes of chlorophyll *a/b* under any of the tested concentrations (Table 1). Two-way ANOVA for chlorophyll

a/b: species ($F=10.26$, $P=0.002$), UA ($F=1.88$, $P=0.194$), species \times UA ($F=24.11$, $P<0.001$).

Total carotenoid/total chlorophyll ratio was stable in both algal cultures regardless of usnic acid application (Table 1). Two-way ANOVA for total carotenoid/total chlorophyll ratio: species ($F=2.32$, $P=0.139$), UA ($F=0.13$, $P=0.716$), species \times UA ($F=4.81$, $P=0.03$).

When calculated per unit of fresh weight (mg/g fw) of algal biomass, we observed a significant decrease of chlorophyll *a*, chlorophyll *b*, chlorophyll *a+b*, and total carotenoids in *Scenedesmus* cultures with the presence of a 0.1 mg/disk of usnic acid (Table 2). In the *Trebouxia* cultures, we did not observe any decrease in these parameters when calculated per unit fresh weight (Table 2). Two-way ANOVA for chlorophyll *a*: species ($F=11.04$, $P=0.002$), UA ($F=20.69$, $P<0.001$), species \times UA ($F=6.73$, $P=0.01$). Two-way ANOVA for chlorophyll *b*: species ($F=3.91$, $P=0.049$), UA ($F=14.81$, $P=0.002$), species \times UA ($F=0.44$, $P=0.651$). Two-way ANOVA for chlorophyll *a+b*: species ($F=8.74$,

Table 2 Chlorophyll *a* content (A, mg/g fw), chlorophyll *b* content (B, mg/g fw), chlorophyll *a+b* content (A+B, mg/g fw) and content of total carotenoids (TOT CAR, mg/g fw) of 2 week old *Scenedesmus* and *Trebouxia* cultures cultivated on disks with addition of usnic acid (0, 0.01 and 0.1 mg)

	Usnic acid conc. (mg/disk)	A (mg/g fw)	B (mg/g fw)	A+B (mg/g fw)	TOT CAR (mg/g fw)
<i>Scenedesmus</i>	0	0.43±0.06a	0.12±0.02a	0.54±0.08a	0.14±0.02a
	0.01	0.42±0.08a	0.13±0.03a	0.55±0.11a	0.19±0.06a
	0.1	0.06±0.01b	0.06±0.01b	0.12±0.01b	0.03±0.01b
	<i>F</i> value	<i>F</i> =40.57	<i>F</i> =7.98	<i>F</i> =29.03	<i>F</i> =13.58
	<i>P</i> value	<i>P</i> <0.001	<i>P</i> =0.02	<i>P</i> =0.001	<i>P</i> =0.006
<i>Trebouxia</i>	0	0.52±0.06a	0.18±0.01a	0.71±0.07a	0.19±0.02a
	0.01	0.49±0.16a	0.19±0.06a	0.68±0.23a	0.18±0.04a
	0.1	0.46±0.07a	0.15±0.05a	0.61±0.11a	0.18±0.02a
	<i>F</i> value	<i>F</i> =0.24	<i>F</i> =0.57	<i>F</i> =0.31	<i>F</i> =0.35
	<i>P</i> value	<i>P</i> =0.796	<i>P</i> =0.594	<i>P</i> =0.746	<i>P</i> =0.720

Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test ($P<0.05$)

Table 3 Content of soluble proteins (PROT, mg/g fw), content of hydrogen peroxide (PEROX, $\mu\text{mol/g fw}$), content of superoxide (SUPEROX, $\mu\text{g/g fw}$) and cell size (WIDTH and LENGTH, μm) of 2 week old *Scenedesmus* and *Trebouxia* cultures cultivated on disks with addition of usnic acid (0, 0.01 and 0.1 mg)

	Usnic acid conc. (mg/disk)	PROT (mg/g fw)	PEROX ($\mu\text{mol/g fw}$)	SUPEROX ($\mu\text{g/g fw}$)	WIDTH μm	LENGTH μm
<i>Scenedesmus</i>	0	8.5±0.7a	1.5±0.37b	0.65±0.12b	5.1±1.4b	14±3.7b
	0.01	10±3.6a	2.1±0.31ab	0.69±0.11b	10.5±2.9a	22±3.1a
	0.1	4.8±2.3a	2.9±0.32a	1.12±0.21a	9.6±2.7a	20±5.3a
	F value	F=3.45	F=13.06	F=8.51	F=14.72	F=10.96
	P value	P=0.101	P=0.007	P=0.018	P<0.001	P<0.001
<i>Trebouxia</i>	0	12.2±1.8a	1.35±0.25a	0.66±0.15b	10±3.7a	–
	0.01	13.5±2.3a	1.50±0.21a	0.72±0.18ab	9.7±5.3a	–
	0.1	16.6±3.1a	2.0±0.40a	1.25±0.27a	13±2.8a	–
	F value	F=2.63	F=4.07	F=4.07	F=1.95	–
	P value	P=0.151	P=0.076	P=0.076	P=0.162	–

Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test ($P<0.05$)

$P=0.004$), UA ($F=19.48$, $P<0.001$), species \times UA ($F=4.15$, $P=0.042$). Two-way ANOVA for total carotenoids: species ($F=9.08$, $P=0.004$), UA ($F=14.93$, $P=0.002$), species \times UA ($F=8.14$, $P=0.005$).

Usnic acid decreased the F_V/F_M ratio of *Scenedesmus* cultures. Presence of a 0.1 mg/disk decreased the F_V/F_M ratio more than four times, while in *Trebouxia* cultures the F_V/F_M ratio decreased only 1.15 times at the highest tested usnic acid dose of 0.1 mg/disk (Table 1). Two-way ANOVA for F_V/F_M : species ($F=91.20$, $P<0.001$), UA ($F=55.38$, $P<0.001$), species \times UA ($F=49.48$, $P<0.001$).

Increase of usnic acid on the disk did not cause a significant decrease in the soluble protein content of algal cultures calculated per unit fresh weight according to one-way ANOVA (Table 3). Two-way ANOVA for soluble protein content: species ($F=0.492$, $P=0.623$), UA ($F=29.12$, $P<0.001$), species \times UA ($F=5.60$, $P=0.019$). According to two-way ANOVA, significant UA, as well as species \times UA effects were observed.

The highest tested usnic acid dose of 0.1 mg/disk caused a significant increase of hydrogen peroxide content in *Scenedesmus* cultures (Table 3). In the cultures of *Trebouxia*, we also observed an increase of hydrogen peroxide content at a dose of 0.1 mg/disk, however, the increase was not strong enough to be significant (Table 3). Two-way ANOVA for hydrogen peroxide content: species ($F=16.16$, $P<0.001$), UA ($F=11.80$, $P=0.004$), species \times UA ($F=1.96$, $P=0.183$).

The highest tested usnic acid dose of 0.1 mg/disk caused a significant increase of superoxide in cultures of both algal species tested (Table 3). Two-way ANOVA for superoxide content: species ($F=15.49$, $P<0.001$), UA ($F=0.42$, $P=0.526$), species \times UA ($F=0.21$, $P=0.815$).

Both tested usnic acid doses caused significant increases of width and length of *Scenedesmus* cells. In *Trebouxia* cultures, we did not observe any significant increase of cell size at any of the tested usnic acid concentrations (Table 3). Two-way ANOVA for width:

species ($F=6.70$, $P=0.002$), UA ($F=8.53$, $P=0.005$), species \times UA ($F=3.86$, $P=0.026$).

Discussion

Influence of Usnic Acid on Algal Growth Usnic acid appears to have phytotoxic effects on vascular plants (Cardarelli et al. 1997; Lechowski et al. 2006) as well as on the algal partner of lichens (Bačkor et al. 1998; Bud'ová et al. 2006). In the present study, we found that control cultures of free-living alga *Scenedesmus* grew better when compared to aposymbiotically grown control cultures of *Trebouxia*. Presence of usnic acid on the surface of fibers caused a dose-dependent decrease of the growth of both algal cultures, however, the decrease was more pronounced in cultures of *Scenedesmus* compared to cultures of *Trebouxia*. Lower toxicity of usnic acid for photobiont cells may be a result of co-evolution of *Trebouxia* photobionts with filamentous fungi over a long period of time, perhaps hundreds of millions of years (Yuan et al. 2005). Usnic acid possesses antimetabolic effects that have been demonstrated in taxonomically diverse organisms (Cardarelli et al. 1997). Surprisingly, presence of usnic acid in this study caused a significant increase of cell size of *Scenedesmus* cultures. This may be a result of the effect of usnic acid on the spindle apparatus during mitosis (Al-Bekairi et al. 1991). Although a similar trend was observed in the *Trebouxia* cells, the effect was not strong enough to be significant.

Influence of Usnic Acid on Algal Metabolic Processes The mechanisms of phytotoxic effects of usnic acid still are mostly unknown. It has been demonstrated that viability of the protoplasts of *Nicotiana tabacum* decreases due to the presence of usnic acid (Cardarelli et al. 1997). Bud'ová et al. (2006) demonstrated that viability of the *Trebouxia erici* photobiont cells decreased due to increased doses of usnic acid.

Changes in composition of assimilation pigments, as well as in chlorophyll *a* fluorescence of aposymbiotically grown lichen photobionts previously have been found to be excellent markers for assessment of the influence of xenobiotics, including metals (Bačkor et al. 2003). One of the most frequently used parameters in lichen stress physiology is chlorophyll degradation, expressed as the phaeophytinization quotient, which reflects the ratio of chlorophyll *a* to phaeophytin *a* (Bačkor and Loppi 2009). This is defined as the ratio of optical densities at 435 nm and 415 nm (OD_{435}/OD_{415}). In healthy lichens and photobionts, the OD_{435}/OD_{415} ratio is about 1.4, and the presence of xenobiotics, including heavy metals, may cause a marked decrease of this value (Ronen and Galun 1984; Bačkor and Loppi 2009). In the present study, none of the tested usnic acid concentrations caused a significant decrease of phaeophytinization quotient in *Trebouxia* cultures, while the highest tested dose of 0.1 mg/disk caused a significant decrease of the phaeophytinization quotient in the cultures of *Scenedesmus*. An identical trend was observed for the ratio of chlorophyll *a/b*, where only the highest tested dose of usnic acid in the cultures of *Scenedesmus* caused a significant decrease; chlorophyll *a* of *Scenedesmus* cells was more sensitive than chlorophyll *b*. However, when we compared changes of total chlorophyll content to total carotenoid content due to presence of usnic acid, we did not find significant differences in either algal species.

Irrespective of the previously noted parameters, we observed a decrease in the content of chlorophyll *a*, chlorophyll *b*, chlorophyll *a+b*, and total carotenoids of *Scenedesmus* calculated per unit of fresh weight. Although chlorophyll *a* and chlorophyll *b* differ only in the composition of side chain (chlorophyll *a* contains a methyl group and chlorophyll *b* a formyl group), decrease of chlorophyll *a* was notable. Previous studies suggested that plants exposed to pollutants, including acid rain, heavy metals, and herbicides respond by decrease of chlorophyll *a* rather than chlorophyll *b* (Hendry et al. 1987). Degradation of chlorophyll *a* here was accompanied by increased phaeophytinization. Han et al. (2004) demonstrated that usnic acid is toxic to cultured hepatocytes and disrupts electron transport in mitochondria and induces oxidative stress in the cells. A previous study suggested that production of the reactive oxygen species in the cells of the lichen photobiont *Trebouxia erici* caused increase of membrane lipid peroxidation (Bačkor et al. 2007).

The reduced F_V/F_M values measured in our usnic acid treated cultures indicate damage to PSII. Endo et al. (1998) demonstrated a decrease of chlorophyll *a* fluorescence in spinach leaves due the presence of lichen-derived secondary metabolites. However, we found a differential sensitivity of algae to usnic acid as cultures of *Scenedesmus* were

nearly killed (F_V/F_M values lower than 0.2) after higher tested doses of usnic acid, while cultures of *Trebouxia* were decreased only slightly. It has been found previously that PSII in the cells of lichen photobionts is relatively protected against the phytotoxicity of lichen secondary compounds (Bud'ová et al. 2006; Takahagi et al. 2008).

Xenobiotics, including metals, may alter the composition of soluble proteins in cultures of lichen photobionts (Bačkor and Fahselt 2008). Although we observed a significant decrease of protein content due to usnic acid when analyzed for individual disks (data not shown), we did not observe significant changes in soluble protein content calculated per unit of algal biomass either in *Scenedesmus* or in *Trebouxia* cultures. Caviglia et al. (2001) demonstrated that usnic acid in lichen *Parmelia soredians* may act as an antioxidant that detoxifies reactive oxygen species produced by the application of the herbicide Paraquat. However, Han et al. (2004) demonstrated that usnic acid itself may induce oxidative stress in the cells. In the present study, we found that the highest tested dose of usnic acid significantly increased hydrogen peroxide content in cultures of *Scenedesmus*, as well as superoxide radicals in cultures of both tested algal species.

In summary, we confirmed that usnic acid has a phytotoxic effect on algae, but that algae are differentially sensitive, and that the lichen photobiont *Trebouxia* is significantly less affected than *Scenedesmus*. These conclusions are based on analyses of physiological processes including biomass production, composition of assimilation pigments (mostly by decrease of chlorophyll *a* content and increase of its phaeophytinization), chlorophyll *a* fluorescence, soluble proteins, and reactive oxygen species. Higher tolerance of *Trebouxia* cultures to usnic acid may be an adaptation resulting from the long term co-evolution of these algae with fungi that produce secondary metabolites. These also may act as allelochemicals that control cell division of photobiont cells inside lichen thalli, thus regulating balance between symbionts forming lichens.

Acknowledgments This work was financially supported by Slovak Grant Agency (VEGA 1/4337/07), Slovak Research and Development Agency (APVV SK-BG-0013-08) and from National Found Research; Ministry of Education, Youth and Science—Bulgaria (D002-38/09). The authors thank Kenneth Dvorsky (Ontario, Canada) for comments on the manuscript.

References

- AHMADJIAN, V. 1993. The Lichen Symbiosis. Wiley, New York.
- AL-BEKAIRI, A. M., QURESHI, S., CHAUDHRY, M. A., KRISHNA, D. R., and SHAH, A. H. 1991. Mitodepressive, clastogenic and biochemical effects of (+)-usnic acid in mice. *J. Ethnopharmacol.* 33:217–220.

- BAČKOR, M. and FAHSELT, D. 2008. Lichen photobionts and metal toxicity. *Symbiosis* 46:1–10.
- BAČKOR, M. and LOPPI, S. 2009. Interactions of lichens with heavy metals. *Biol. Plantarum* 53:214–222.
- BAČKOR, M., HUDÁK, J., REPČÁK, M., ZIEGLER, W., and 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., and 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., FAHSELT, D., and WU, C. T. 2004. Free proline content is positively correlated with copper tolerance of the lichen photobiont *Trebouxia erici* (Chlorophyta). *Plant Sci.* 167:151–157.
- BAČKOR, M., VÁČZI, P., BARTÁK, M., BUĐOVÁ, J., and DZUBAJ, A. 2007. Uptake, photosynthetic characteristics and membrane lipid peroxidation levels in the lichen photobiont *Trebouxia erici* exposed to copper and cadmium. *Bryologist* 110: 100–107.
- BARNES, J. D., BALAGUER, L., MANRIQUE, E., ELVIRA, S., and DAVISON, A. W. 1992. A reappraisal of the use of DMSO for the extraction and determination of chlorophylls *a* and *b* in lichens and higher plants. *Environ. Exp. Bot.* 32:85–100.
- BRADFORD, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- BUĐOVÁ, J., BAČKOR, M., BAČKOROVÁ, M., and 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 CICCIO NARDONE, F., ALESIANI, O., and ROSSIELLO, F. 1997. Antimitotic effects of usnic acid on different biological systems. *CMLS* 53:667–672.
- CAVIGLIA, A. M., NICORA, P., GIORDANI, P., BRUNIALTI, G., and MODENESI, P. 2001. Oxidative stress and usnic acid content in *Parmelia caperata* and *Parmelia soredians* (Lichenes). *Farmaco* 56:379–382.
- COCCHIETTO, M., SKERT, N., NIMIS, P.L. and SAVA, G. 2002. A review on usnic acid, an interesting natural compound. *Naturwissenschaften* 89:137–146.
- ELSTNER, E. F. and HEUPEL, A. 1976. Inhibition of nitrite formation from hydroxylammonium chloride: A simple assay for superoxide dismutase. *Anal. Biochem.* 70:616–620.
- ENDO, T., TAKAHAGI, T., KINOSHITA, Y., YAMAMOTO, Y., and 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.
- FAHSELT, D. 2008. Individuals and populations of lichens, pp. 252–273, in T. H. Nash (ed.). *Lichen Biology*, 2nd edition, Cambridge University Press, Cambridge.
- HAN, D., MATSUMARU, K., RETTORI, D., and KAPLOWITZ, N. 2004. Usnic acid-induced necrosis of cultured mouse hepatocytes: inhibition of mitochondrial function and oxidative stress. *Biochem. Pharmacol.* 67: 439–451.
- HAUCK, M., WILLENBRUCH, K., and LEUSCHNER, C. 2009. Lichen substances prevent lichens from nutrient deficiency. *J. Chem. Ecol.* 35:71–73.
- HENDRY, G.A.F., HOUGHTON, J.D., and BROWN, S.B. 1987. The degradation of chlorophyll—a biological enigma. Tansley Review No. 11. *New Phytol.* 107: 255–302.
- KOVÁČIK, J. and BAČKOR, M. 2007. Changes of phenolic metabolism and oxidative status in nitrogen-deficient *Matricaria chamomilla* plants. *Plant Soil* 297:255–265.
- LATKOWSKA, E., LECHOWSKI, Z., BIALCZYK, J., and 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., and BIALCZYK, J. 2006. Accumulation of biomass and some macroelements in tomato plants grown in media with (+)-usnic acid. *Environ. Exp. Bot.* 56:239–244.
- PÖYKKÖ, H., HYVÄRINEN, M., and BAČKOR, M. 2005. Removal of lichen secondary metabolites affects food choice and survival of lichenivorous moth larvae. *Ecology* 86:2623–2632.
- RONEN, R. and GALUN, M. 1984. Pigment extraction from lichens with dimethyl sulfoxide (DMSO) and estimation of chlorophyll degradation. *Environ. Exp. Bot.* 24:239–245.
- SARRET, G., MANCEAU, A., CUNY, D., VAN HALUWYN, C., DÉRUELLE, S., HAZEMANN, J. L., SOLDI, Y., EYBERT-BÉRARD, L., and MENTHONNEX, J. J. 1998. Mechanisms of lichen resistance to metallic pollution. *Environ. Sci. Technol.* 32: 3325–3330.
- SOLHAUG, K. A., LIND, M., NYBAKKEN, L., and 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., and SATO, F. 2008. Lichen photobionts show tolerance against lichen acids produced by lichen mycobionts. *Biosci. Biotech. Bioch.* 72:3122–3127.
- WELLBURN, A. R. 1994. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolutions. *J. Plant Physiol.* 144:307–313.
- YUAN, X., XIAO, S. and TAYLOR, T. N. 2005. Lichen-like symbiosis 600 million years ago. *Science* 308:1017–1020.