SYMBIOSIS (2006) 42, 169–174 ISSN 0334-5114

Usnic acid and copper toxicity in aposymbiotically grown lichen photobiont *Trebouxia erici*

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(Received September 13, 2006; Accepted November 16, 2006)

Abstract
Effects of copper and usnic acid on the lichen photobiont *Trebouxia erici* were assessed in order to determine whether usnic acid detoxifies copper. Copper and usnic acid were phytotoxic: each inhibited growth, decreased viability and chlorophyll *a* fluorescence, and altered assimilation pigments of lichen algae. Although usnic acid and increased copper were not synergistic, usnic acid did not significantly protect photobionts from negative physiological effects of copper.

Keywords: Assimilation pigments, chlorophyll *a* fluorescence, growth inhibition, heavy metals, secondary metabolites, viability

1. Introduction

Secondary metabolites of lichens include extracellular phenols secreted by the fungal partner, or mycobiont, which are deposited mainly on the surface of hyphae. Usually these substances, which include dibenzofuran derivatives, depsides, and depsidones, occur in considerable amounts, often constituting 0.1 to 5.0%, or even more, of thallus dry weight (Fahselt, 1994).

Lichen substances have many functions, including antimicrobial activity, which may be responsible for long-term survival of thalli (Lawrey, 1986) and antiherbivory (Pöykkö et al., 2005); allelopathy, which may involve inhibition of seed and spore germination (Follmann and Peters, 1966; Lawrey, 1986). Lichen products have also been reported to inhibit root elongation and mitosis (Rathore and Mishra, 1971; Reddy and Rao, 1978) and they are implicated in rock mineralization and pedogenesis, as well as nutrient acquisition through chelation (Lawrey, 1986). These metabolites may also regulate photobiont populations (Bačkor et al., 1998), maintain the symbiosis and protect the light-sensitive algal symbiont.

Usnic acid, one of the commonest lichen metabolites, exerts antibiotic and antimicrobial activity against G^+ bacteria and other organisms, both aerobic and anaerobic (Vicente and Cifuentes, 1981; Yamamoto et al., 1998), and has allelopathic effects as it reduces starch utilization during germination, inhibits amylase synthesis and ATP-hydrolysing phosphatase during germination (Dalvi et al., 1972). Usnic acid also causes heavy mortality and growth retardation of *Spodoptera littoralis* (Lepidoptera) and extends its larval period (Emerich et al., 1993). Usnic acid can be potentially toxic to photosynthesizing cells with which it may be in direct contact; for example, some lichen metabolites, including usnic acid, inhibit chlorophyll *a* fluorescence (F_v/F_m) in spinach leaves (Endo et al., 1998). Usnic acid also reduces protoplast viability of *Nicotiana tabacum* and inhibits plant cell proliferation (Cardarelli et al., 1997).

Enantiomers of usnic acid, (–) and (+) forms, have different phytotoxic effects (Romagni et al., 2000); for example, (–)-usnic acid causes a strong decrease of carotenoid and chlorophyll levels in cucumber cotyledons and is associated with visible bleaching, while none of these effects are produced by (+)-usnic acid. Lechowski et al. (2006) found that (+)-usnic acid has a significant negative effect on leaf blade area of hydroponically cultivated tomato plants, although leaf thickness is increased about 40% in plants cultivated with 40 μM (+)-usnic acid added to the medium for 21 days.

Excess heavy metals, including copper, are toxic for the algal partner in the lichen symbiosis. Copper has been

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reported as a redox-reactive metal (Sanità di Toppi and Gabrielli, 1999) and at the tested concentrations in our previous studies (Bačkor et al., 2003, 2004, 2006) was capable of increasing the levels of membrane lipid peroxidation (TBARS), decreasing chlorophyll *a* fluorescence and the rate of oxygen evolution, as well as altering assimilation pigments. Metal binding properties of usnic acid have been studied by synthetic and spectroscopic methods, and the structure of copper (II) complexes established by X-ray diffraction (Takani et al., 2002), but to date, the possibility of a protective role for secondary metabolites against heavy metals has not been tested experimentally. Therefore, the main aim of our work was to develop a system for assessing whether usnic acid provides protection against toxic levels of copper within lichen thalli. A secondary objective was to compare the toxicity of usnic acid and copper at selected concentrations and thereby experimentally determine the ability of usnic acid to chelate copper.

2. Materials and Methods

Organisms and culture conditions

The lichen photobiont *Trebouxia erici* Ahmadjian (UTEX 911) was cultivated on agar medium (2%) previously developed for *Trebouxia* by Ahmadjian (1993); namely 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., 2004). Copper enrichment was achieved using 0.4 mM and 4.0 mM copper chloride, and cultures were maintained at 22°C under a 16-h photoperiod and 30 $\mu mol.m^{-2}.s^{-1}$ artificial irradiance ("cool white" tubes).

The computer program GEOCHEM-PC was used to estimate copper speciation of the low-copper culture media (control medium, 6.3 µM) as follows: free Cu^{2+} activity $3.94E^{-14}$ M, free Cu^{2+} concentration $6.47E^{-14}$ M. Speciation of the culture medium enriched by 4 mM copper was: free Cu^{2+} activity $5.00E^{-6}$ M, free Cu^{2+} concentration $8.33E^{-6}$ M; about 63.5% of Cu^{2+} in this medium was in solid form with PO_4, 32.1% in solid form with OH^-, 4.16% complexed with EDTA, 0.03% complexed with OH^-, 0.01% complexed with SO_4, and about 0.21% as a free metal.

Culturing on cellulose-acetate disks

Photobionts were cultivated on the surface of cellulose-acetate disks as described in previous studies (Bačkor et al., 2003, 2004). Briefly, two inoculation loops of *T. erici* cells grown on stock solid *Trebouxia* medium were transferred into 50 ml of liquid *Trebouxia* medium in an Erlenmeyer flask and suspended by gentle stirring on a magnetic stirrer for 1 h. Cultures were maintained for five days in a cultivation room under conditions described previously, with daily stirring on a magnetic stirrer for about 1 h. The homogeneity of algal suspension was verified microscopically and the number of cells calculated using a standard haemocytometer. Cell density of cultures was adjusted to c. 10^6 cells ml^{-1} of medium.

For photobiont cultivation, sterilized 25 mm (in diameter) cellulose-acetate disks (AcetatePlus) with pore size 0.25 µm 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 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 15 µl of algal suspension were inoculated into the center of each disk. Disk pores allowed supplemental nutrient media to pass through the disk and permit growth to be easily determined from changes in biomass (Bačkor et al., 2003, 2004). The total mass of cultures was calculated by subtracting the mean fresh weight of a cellulose-acetate disk saturated by identical medium, from the fresh weight of a disk supporting the photobiont culture after 14 days of cultivation. Each treatment was replicated eight times.

Viability assessment

Photobiont cells from each treatment were gently separated from the surface of the cellulose-acetate filters by scalpel and, to disrupt cell aggregates, the cell mass was passed through a phytoplankton net with pore size 50 µm. Cells were immediately suspended in 5 mM HEPES buffer, pH 6.48. The prepared cell suspension was diluted with 0.4% trypan blue solution (1:1) and loaded into the counting chambers of a haemocytometer. Trypan blue dye was excluded from cells with an intact membrane, while cells with damaged membranes took up the coloring agent. After 1 minute, the number of stained cells and total number of cells were recorded. The calculated percentage of unstained cells was assumed to represent the percentage of viable cells. Each treatment was replicated four times.

Activity of photosystem II

Chlorophyll *a* fluorescence was measured in photobionts grown on cellulose-acetate disks on the surface of the *Trebouxia* agar medium. Photobionts were dark-adapted for 30 min prior to measurement while still on the medium in Petri dishes to minimize desiccation. The potential quantum yield of photosystem II (PSII) was measured using a Plant Stress Meter (PSM Mark II, Biomonitor, SCI AB), with sensor 5 mm in diameter, and results were expressed as F_v/F_m calculated as the maximal fluorescence (F_m) less the minimal fluorescence (F_o),

divided by F_m of dark adapted photobionts: $(F_m - F_o) / F_m = F_v/F_m$. Chlorophyll fluorescence parameters were taken from three separate positions on each disk, and the mean value used as one observation. Each treatment was replicated four times.

Pigment analysis and measurement of chlorophyll a integrity

The influence of usnic acid on the photobiont response to excess copper was determined using cultures grown on cellulose-acetate disks. Weighed disks were directly extracted in the dark for 1 h at 65°C in 3 ml of dimethyl sulfoxide (DMSO). To maximize chlorophyll extraction, cell aggregates were then homogenized in a Vortex mixer in plastic conical 15 ml centrifuge tubes for c. 10 sec with the cellulose acetate disk. After cooling to ambient temperature, the absorbance of the extract was determined at 750 nm with a spectrophotometer to be certain that it was always less than 0.01. The absorbance of extracts was then read at 665.1, 649.1, 435 and 415 nm to assess chlorophyll content and the possibility of chlorophyll *a* degradation (Barnes et al., 1992; Ronen and Galun, 1984; Wellburn, 1994). To utilize the linear part of the response curve, extracts from disks with very high cell densities (absorbance at 665.1 nm higher than 0.8) were diluted with fresh DMSO to fall into the absorbance range 0.2–0.8. However, this was not possible for cultures grown on 4 mM Cu as the absorbance of chlorophyll was less than 0.2. 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 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* in order to provide an indication of photobiont chlorophyll integrity. Each treatment was replicated four times.

Usnic acid yield

HPLC was used to analyze the concentration of (+)-usnic acid remaining on the surface of the cellulose-acetate disk at the end of experiments. Filters with usnic acid were placed in Eppendorf tubes and extracted in 1 ml of cool acetone for 60 min (Feige et al., 1993). Extraction of each disk was repeated at least three times, and the extracts from each disk combined. Extracts were evaporated and the residues re-dissolved in 1.5 ml fresh acetone. Filtered acetone extracts were analyzed by gradient HPLC (Feige et al., 1993; Lumbsch, 2002) using a Tessek SGX C18 5µm (4 × 250 mm) column with flow rate 0.7 ml. min^{-1}. The mobile phase was A = H_2O : acetonitrile : H_3PO_4 (80 : 19 : 1) and B = 95% acetonitrile. The solvent gradient programme was 0 min 25% B, 5 min 50% B, 20 min 100% B, 25 min 25% B. Detection was performed at 245 nm (detector Ecom LCD 2084) and commercially available (+)-usnic acid (Aldrich) was used as standard. Each treatment was replicated four times.

Statistical analysis

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

3. Results

Fig. 1a shows biomass production (mg fresh weight) of the *Trebouxia erici* photobiont on the cellulose-acetate disks after two weeks of cultivation. Growth of the photobiont was significantly reduced due to presence of usnic acid on the surface of cellulose-acetate disks and increased copper concentrations in the media. Although biomass on disks with a combination of usnic acid and 4 mM Cu was slightly higher than with cultures treated with 4 mM Cu, the differences were not large enough to be statistically significant.

The viability of photobiont cultures in axenic cultures was reduced by usnic acid and copper treatments (Fig. 1b). Two usnic acid concentrations tested caused only a small (c. 10%) decrease of viability of photoiont cells. The higher, 4 mM, copper concentration tested caused a more pronounced decrease in photobiont cell viability, i.e., up to c. 60%. A combination of usnic acid (0.1 mg/disk) with the highest copper concentration treatment was equally phytotoxic.

Both usnic acid concentrations tested caused a small but significant decrease of chlorophyll *a* fluorescence (Fig. 1c). The phytotoxic effect of 4 mM Cu was more pronounced, and the combination of 4 mM Cu and 0.1 mg usnic acid per disk further decreased chlorophyll *a* fluorescence as measured by F_v/F_m. Chlorophylls as well as total carotenoids were sensitive to presence of usnic acid on the surface of the disks and increased copper concentrations in the media (Fig. 1d–i). Chlorophyll *a* (Fig. 1d), chlorophyll *b* (Fig. 1e), chlorophyll *a+b* (Fig. 1f), and total carotenoid content per disk (Fig. 1g) decreased significantly in response to increased usnic acid, and the decreases were even greater with the highest, 4 mM, copper concentration tested. The chlorophyll *a/b* ratio was significantly decreased with 4 mM Cu in the medium, but usnic acid did not change this parameter significantly (Fig. 1h). Chlorophyll *a* / phyeophytin *a* ratio was significantly decreased by

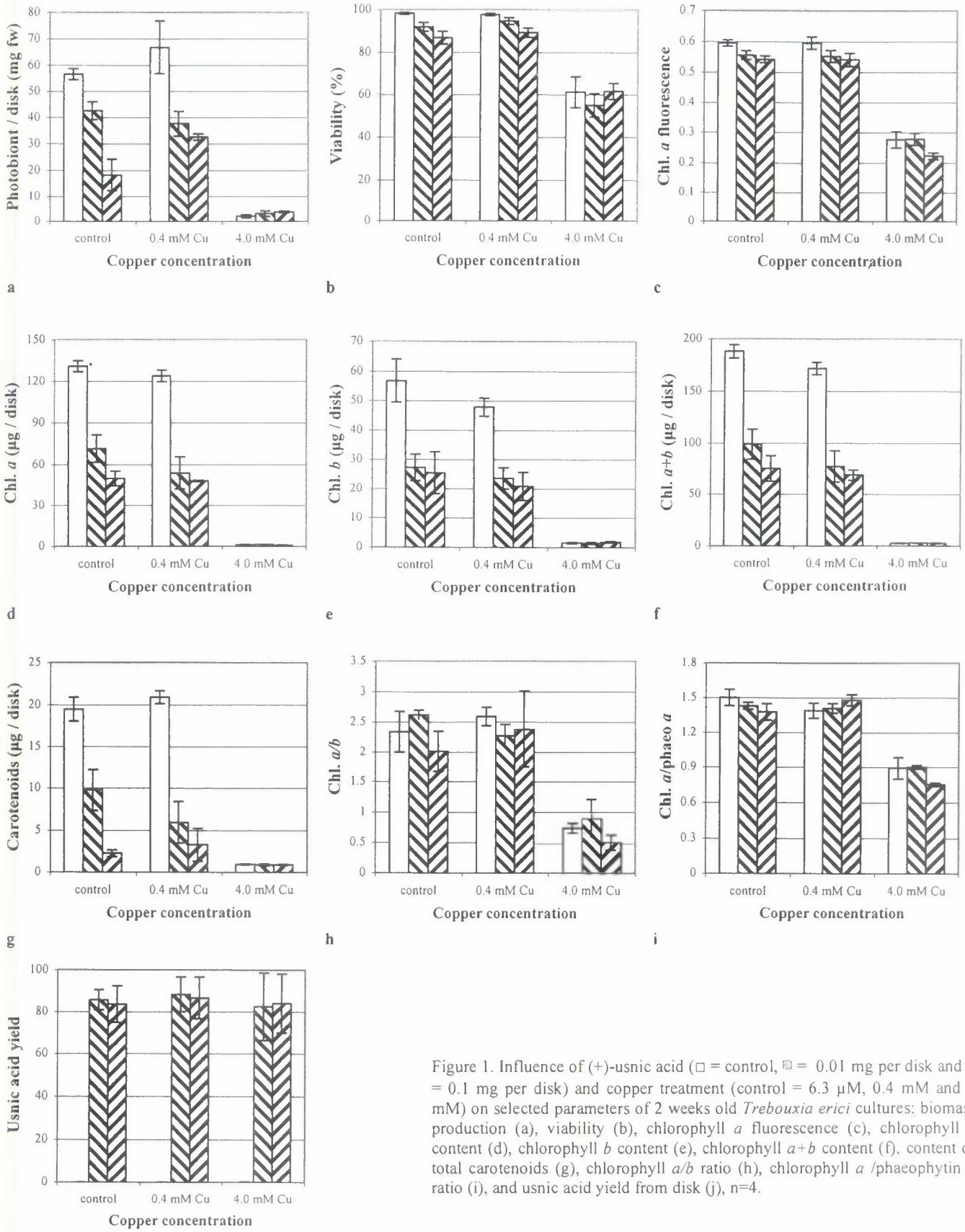

Figure 1. Influence of (+)-usnic acid (□ = control, ▨ = 0.01 mg per disk and ▨ = 0.1 mg per disk) and copper treatment (control = 6.3 µM, 0.4 mM and 4 mM) on selected parameters of 2 weeks old *Trebouxia erici* cultures: biomass production (a), viability (b), chlorophyll *a* fluorescence (c), chlorophyll *a* content (d), chlorophyll *b* content (e), chlorophyll *a+b* content (f), content of total carotenoids (g), chlorophyll *a/b* ratio (h), chlorophyll *a* /phaeophytin *a* ratio (i), and usnic acid yield from disk (j), n=4.

treatment with 4 mM Cu (Fig. 1i), but not with usnic acid.

The yield of the usnic acid from disks after 2 weeks was in the range of 70–100% (Fig. 1j). The colour of usnic acid crystals on the surface of the disks changed at 4 mM copper treatments from yellow-green at the start of experiment to green at the end of the experiment.

4. Discussion

Photobionts on the surface of cellulose filaments form a layer reminiscent of that in stratified lichens (Bačkor et al., 2003). The distribution of usnic acid crystals on the surface of filaments is reminiscent of the situation in naturally-occurring lichens, where extracellular secondary metabolites located on the surface of hyphae may also be in contact with algal cells in the photobiont layer.

As mentioned above, the effects of usnic acid are very well documented (Ingólfsdóttir, 2002), including antimicrobial, antiprotozoal, antiviral, antiproliferative and phytotoxic activities. Although the allelopathic effect of the usnic acid on vascular plants (Lechowski et al., 2006) and algal partner of lichens (Bačkor et al., 1998) has been previously documented, its mode of action is still far from understood. Growth inhibition of photobiont cells by usnic acid, supported by the present study, may be partly explained by the antimitotic effects of usnic acid, a possibility tested throughout taxonomically diverse groups of organisms (Cardarelli et al., 1997). The decreased viability of the photobiont cells due to presence of usnic acid that we observed here is in accordance with results obtained by Cardarelli et al. (1997) for protoplasts of *Nicotiana tabacum*.

Of the optical forms of usnic acid, recent studies have reported that (–)-usnic acid is usually more toxic for organisms tested than the (+) form (Ingólfsdóttir, 2002). Previous work has shown that (–)-usnic acid causes a dose-dependent bleaching of lettuce cotyledonary tissues leading to seedling death, while (+)-usnic acid does not cause any significant change in chlorophyll and carotenoids (Romagni et al., 2000). While chlorophyll *a* degradation and the chlorophyll *a/b* ratio were essentially unchanged in the presence of usnic acid, decreased concentrations of pigments were found, which may have been due to inhibition of photobiont cell division by usnic acid. Chlorophyll *a* fluorescence was decreased as a result of (+)-usnic acid. This is consistent with previous observations that chlorophyll *a* fluorescence, which reflects status of photosystem II, is decreased in spinach leaves in the presence of lichen-derived secondary metabolites (Endo et al., 1998).

The cytotoxic effects of excess copper on the lichen photobiont *Trebouxia erici* have been better documented than those of usnic acid (Bačkor and Váczi, 2002; Bačkor et al., 2003, 2004) and include retardation of the growth of the *T. erici* cells, decrease of cell viability and chlorophyll *a* content (Bačkor and Váczi, 2002). Chlorophyll *b* content, on the other hand, increased in short term experiments, possibly due to the fact that some chlorophyll *a* was converted to chlorophyll *b* by oxidation of the methyl group on ring II (Chettri et al., 1998). The ratio of chlorophyll *a* to phaeophytin *a*, which indicates the integrity of photobiont chlorophyll, decreases due to the presence of copper (Bačkor and Váczi, 2002). Chlorophyll *a* fluorescence of *T. erici* cells has also been found to be sensitive to the presence of copper excess in culture media (Bačkor et al., 2003, 2004).

Production of water-insoluble complexes of lichen secondary metabolites (including usnic acid) with heavy metals (including copper) has long been considered a detoxification mechanism protecting lichens against heavy metal damage (Purvis et al., 1990). In the present study we observed a change in the coloration of usnic acid crystals caused by the presence of copper in the culture media from yellow-green at start of experiment to green at the end of the experiment, similarly to that observed by Takani et al. (2002); however, this did not affect the yield of usnic acid from the disk at the end of the experiment.

The present study has demonstrated that the presence of usnic acid, as well as excess of copper in the media, reduces growth of photobionts in culture. It is very difficult to compare the cytotoxic effect of usnic acid and copper. Copper availability in photobiont cultivation media is limited by the production of water-insoluble complexes. Usnic acid is only slightly soluble in water (Hrutfiord and Ugolini, 1984), which affects its availability. Bačkor and Fahselt (2004) found by means of wavelength-dispersive X-ray spectrometry analysis that Cu, Ni, Fe and Al may occur within crystals of usnic acid, suggesting that complexing with heavy metals could constitute detoxification. Usnic acid complexes with copper, thereby protecting the photobiont cells in lichens to some extent against copper damage in natural situations.

In the present study, usnic acid did not provide significant protection against copper. Cell wall immobilization (the exclusion mechanism of Cu tolerance) or increased free proline synthesis, as previously observed in the copper tolerant strain of *Trebouxia* (Bačkor et al., 2004), are more probable mechanisms for Cu detoxification by lichen photobionts than chelation by usnic acid.

Acknowledgements

This work was supported financially by the Science and Technology Assistance Agency (Slovak Republic) under contract no. APVT-20-003004. The authors thank Prof. Dianne Fahselt (UWO, Canada) and Prof. Mark Seaward (University of Bradford, England) for comments on the manuscript.

REFERENCES

Ahmadjian, V. 1993. *The Lichen Symbiosis*. John Wiley, New York.

Bačkor, M. and Váczi, P. 2002. Copper tolerance in the lichen photobiont *Trebouxia erici* (Chlorophyta). *Environmental and Experimental Botany* **48**: 11–20.

Bačkor, M. and Fahselt, D. 2004. Physiological attributes of the lichen *Cladonia pleurota* in heavy metal-rich and control sites near Sudbury (Ont., Canada). *Environmental and Experimental Botany* **52**: 149–159.

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. *Archives of Environmental Contamination and Toxicology* **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 Science* **167**: 151–157.

Bačkor, M., Váczi, P., Barták, M., Budová, 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* (in press).

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. *Environmental and Experimental Botany* **32**: 85–100.

Cardarelli, M., Serino, G., Campanella, L., Ercole, P., de Cicco Nardone, F., Alesiani, O., and Rossiello, F. 1997. Antimitotic effects of usnic acid on different biological systems. *Cellular and Molecular Life Sciences* **53**: 667–672.

Chettri, M.K., Cook, C.M., Vardaka, E., Sawidis, T., and Lanaras, T. 1998. The effect of Cu, Zn and Pb on the chlorophyll content of the lichen *Cladonia convoluta* and *Cladonia rangiformis*. *Environmental and Experimental Botany* **39**: 1–10.

Dalvi, R.R., Singh, B., and Salunkhe, D.K. 1972. Physiological and biochemical investigations on the phytotoxicity of usnic acid. *Fyton* **29**: 63–72.

Emmerich, R., Giez, I., Lange, O.L., and Proksch, P. 1993. Toxicity and antifeedant activity of lichen compounds against the polyphagous herbivorous insect *Spodoptera littoralis*. *Phytochemistry* **33**: 1389–1394.

Endo, T., Takahagi, T., Kinoshita, Y., Yamamoto, Y., and Sato, F. 1998. Inhibition of photosystem II of spinach by lichen-derived depsides. *Bioscience, Biotechnology and Biochemistry* **62**: 2023–2027.

Fahselt, D. 1994. Secondary biochemistry of lichens. *Symbiosis* **16**: 117–165.

Feige, G.B., Lumbsch, H.T., Huneck, S., and Elix, J.A. 1993. Identification of lichen substances by a standardized high-performance liquid chromatographic method. *Journal of Chromatography* **646**: 417–427.

Follmann, G. and Peters, R. 1966. Flechtenstoffe und Bodenbildung. *Naturforschung* **21**: 386–387.

Hrutfiord, B.F. and Ugolini, F.C. 1984. Mobility of lichen compounds from *Cladonia mitis* in arctic soils. *Soil Science* **1984**: 40–45.

Ingolfsdottir, K. 2002. Usnic acid. *Phytochemistry* **61**: 729–736.

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. *Environmental and Experimental Botany* **56**: 239–244.

Lumbsch, H.T. 2002. Analysis of phenolic products in lichens for identification and taxonomy. In: *Protocols in Lichenology: Culturing, Biochemistry, Ecophysiology and Use in Biomonitoring*. I. Kranner, R. Beckett, and A. Varma, eds., Springer-Verlag, Berlin, pp. 281–295.

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.

Purvis, O.W., Elix, J.A., and Gaul, K.L. 1990. The occurrence of copper-psoromic acid in lichens from cupriferous substrata. *Lichenologist* **22**: 345–354.

Rathore, J.S. and Mishra, K. 1971. Inhibition of root elongation by some plant extracts. *Indian Journal of Experimental Biology* **9**: 523–524.

Reddy, P.V. and Rao, P.S. 1978. Influence of some lichen substances on mitosis in *Allium cepa* root tips. *Indian Journal of Experimental Biology* **16**: 1019–1021.

Romagni, J.G., Mezza, G., Nanayakkara, N.P.D., and Dayan, F.E. 2000. The phytotoxic lichen metabolite, usnic acid, is a potent inhibitor of plant p-hydroxyphenylpyruvate dioxygenase. *FEBS Letters* **480**: 301–305.

Ronen, R. and Galun, M. 1984. Pigment extraction from lichens with dimethyl sulfoxide (DMSO) and estimation of chlorophyll degradation. *Environmental and Experimental Botany* **24**: 239–245.

Sanità di Toppi, L. and Gabrielli, R. 1999. Response to cadmium in higher plants. *Environmental and Experimental Botany* **41**: 105–130.

Takani, M., Yajima, T., Masuda, H., and Yamauchi, O. 2002. Spectroscopic and structural characterization of copper (II) and palladium (II) complexes of a lichen substance usnic acid and its derivatives. Possible forms of environmental metals retained in lichens. *Journal of Inorganic Biochemistry* **91**: 139–150.

Vicente, C. and Cifuentes, B. 1981. L-usnate and permeability. *Cryptogamie, Bryologie-Lichénologie* **2**: 213–222.

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. *Journal of Plant Physiology* **144**: 307–313.

Yamamoto, Y., Kinoshita, Y., Matsubara, H., Kinoshita, K. Koyama, K., Takahashi, K., Kurokawa, T., and Yoshimura, I. 1998. Screening of biological activities and isolation of biological-active compounds from lichens. *Recent Research Developments in Phytochemistry* **2**: 23–34.