

# Copper induced physiological changes and oxidative damage in lichen *Ramalina farinacea*

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Copper is an essential element for all organisms but can reach toxic concentrations as a result of human activities. The aim of the present study was to investigate physiological effects of copper on the lichen *Ramalina farinacea* (L.) Ach. The effect of copper on the chlorophyll content, fluorescence and oxidative status was assessed. The chlorophyll *a* content was not significantly different from the control. The chlorophyll *b* content was significantly higher at any tested  $\text{Cu}^{2+}$  concentration in comparison with the control.  $F_v/F_m$  values decreased significantly in *R. farinacea* following 50 mg Cu and higher concentrations. MDA increased significantly after exposure to 100 and 500 mg Cu concentrations.

**Key words:** fluorescence, cell membrane damage, copper, *Ramalina farinacea*, oxidative stress

## INTRODUCTION

Copper is an essential micronutrient for all living organisms. It is also a component of several enzymatic systems, participating in electron flow and catalysis of redox reactions (Moura et al., 2012). A sufficient intracellular copper ion level must be maintained in order to establish these important mechanisms. Although essential as a nutrient, copper can become toxic at concentrations that exceed the level which is required by organisms. Copper released to the environment by anthropogenic activities (industrial and domestic waste water, combustion processes, wood and phosphate fertilizer production) results in elevated concentrations in the environment (Georgopoulos et al., 2001).

Due to redox activity, copper ions can cause toxicity when present in excess (Bačkor, Loppi, 2009). Concerning the toxicity of different heavy

metals, the most harmful cation was copper, which alters the structure of the cell wall and plasma membrane of the algae causing them to lose their selective permeability (Garty et al., 1992). This allows copper cations to enter the cytoplasm, initiating degenerative processes that probably cause alteration in different metabolic pathways. The chlorophyll degradation of photobiont was observed as a sign of damage (Garty et al., 1992; Chettri et al., 1998; Bačkor, Dzubaj, 2004; Carreras, Pignata, 2007; Vantová et al., 2013). Excessive Cu causes a significant concentration-dependent increase in chlorophyll *b* and a decrease in chlorophyll *a*, consistent with accelerated conversion of one to the other (Bačkor et al., 2009). The reduced  $F_v/F_m$  values measured in copper-treated lichen thalli indicate damage to PSII (Branquinho et al., 1997; Bačkor et al., 2007; Vantová et al., 2013). The total carotenoid content was decreased under short-term exposure to increased copper concentrations (Bačkor et al., 2009; Vantová et al., 2013). At high concentrations, copper is involved in the

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formation of  $\text{OH}^-$  from  $\text{H}_2\text{O}_2$  via Haber-Weiss and Fenton reactions (Moura et al., 2012). The concentration of malondialdehyde (MDA) increased with the increase in copper concentration in the nutrient solution (Monnet et al., 2005). The presence of copper negatively affected the oxidative status of the lichen photobiont (Piovár et al., 2011).

The aim of this study was to determine physiological responses of copper excess exposure in the lichen *Ramalina farinacea* (L.) Ach.

## MATERIALS AND METHODS

Samples of the epiphytic lichen *Ramalina farinacea* (L.) Ach. were picked up in November 2013, on *Quercus* sp. trees in the Dubrava forest, an area 12 km south-west of Kaunas, Lithuania. Lichen samples were transferred to a laboratory in plastic bags, cleaned from extraneous materials.

The experiments were undertaken 2–3 days after collection. The solutions were prepared using double-deionized water. All the reagents used were of analytical grade (Merck). In order to study the impact of different concentrations of  $\text{Cu}^{2+}$  on some physiological parameters, 2 g of fresh thalli were soaked for 30 min in 50 mL solutions containing such Cu concentrations: 0, 50, 100 and 500 mg Cu  $\text{L}^{-1}$ . Copper was supplied as  $\text{CuCl}_2$ . Control samples of lichen thalli were soaked for 30 min in deionized water. The pH of the solutions was adjusted at 6.5 immediately before the treatment, avoiding acid conditions known to be detrimental to chlorophyll (Gauslaa et al., 1996; Chettri et al., 1998). After treatments, the treated lichen thalli were then thoroughly washed twice with double-deionized water, gently shaken to remove any excess water and allowed to dry at room temperature.

Electrical conductivity (EC) is a relative measure of membrane integrity. 100 mg of each sample were soaked for 1 h in 50 ml of distilled water. The electric conductivity of the water (expressed in  $\mu\text{S cm}^{-1}$ ) was measured before and after lichen immersion using a conductivity meter. Final values, obtained as differences in electric conductivity after and before soaking, and normalized on the water volume and dry

weight of samples ( $\mu\text{S cm}^{-1} \text{ ml mg}^{-1}$ ), were taken as an indicator of cell membrane integrity.

Chlorophyll *a* fluorescence of samples was measured with a plant efficiency analyzer (Handy PEA, Hansatech). Before measurement of chlorophyll fluorescence, thalli were dark-adapted for 15 min (Maxwell, Johnson, 2000). Fluorescence was measured on well-wet samples, applying a saturating flash of light of 2 400  $\mu\text{mol s}^{-1} \text{ m}^{-2}$  for 1 s (Pisani et al., 2009). The  $F_v/F_m$  parameter (maximum quantum yield efficiency of PSII) was used as a stress indicator.

Extracts of photosynthetic pigments were obtained using dimethylsulfoxide (DMSO) (Boonpragob, 2002). The optical density of the solutions was measured at 665 and 648 nm with a spectrophotometer. Concentrations of chlorophyll *a* and *b* were calculated using the equations of Wellburn (Wellburn, 1994).

Malondialdehyde (MDA) was measured by a colorimetric method (Heath, Packer, 1968). The absorbance of the supernatant was measured at 532 nm and corrected for non-specific absorption at 600 nm. Concentration of MDA was calculated using the extinction coefficient 155  $\text{mM}^{-1} \text{ cm}^{-1}$  and expressed as  $\mu\text{mol g}^{-1}$  fresh weight (Fw).

Significance of differences ( $p < 0.05$ ) was calculated using the Mann-Whitney nonparametric test.

## RESULTS

Exposure of *Ramalina farinacea* to enhanced  $\text{Cu}^{2+}$  concentrations resulted in no any signs of chlorosis. An increase in chlorophyll *a* content was observed but it was not significantly different from the control ( $p > 0.05$ ) (Fig. 1). Chlorophyll *b* content was significantly higher at any tested  $\text{Cu}^{2+}$  concentration in comparison with the control ( $p > 0.05$ ). At 50 and 100 mg Cu the chlorophyll *b* content was more than 2 times higher than without Cu in the solution (Fig. 1).

The increase in pigment content was accompanied by a decrease in chlorophyll fluorescence in the treated lichens:  $F_v/F_m$  values decreased significantly in *Ramalina farinacea* following 50 mg Cu treatment and higher concentrations

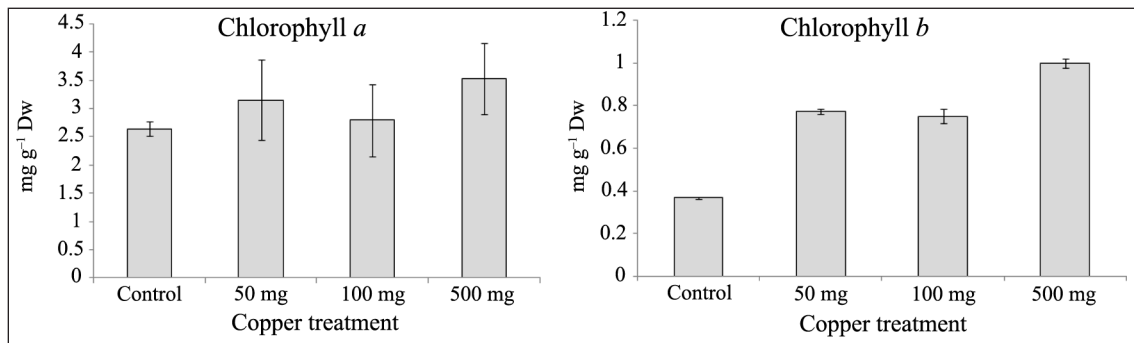


Fig. 1. Impact of Cu<sup>2+</sup> on the photosynthetic pigment content of the treated *Ramalina farinacea*

( $p < 0.05$ ) (Fig. 2). At 500 mg Cu the chlorophyll fluorescence was lower by 15% than in the control while at lower Cu concentrations (50–100 mg Cu) the  $F_v/F_m$  ratio was about 6% lower.

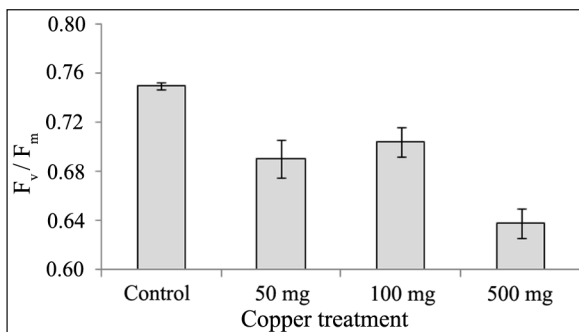


Fig. 2.  $F_v/F_m$  values in the lichen *Ramalina farinacea* treated with selected Cu concentrations

The concentration of MDA in the control samples of *Ramalina farinacea* was not significantly higher than at 50 mg Cu exposure treated lichens, with a mean of 2.1  $\mu\text{mol g}^{-1}$  Fw (Fig. 3). MDA increased significantly after exposure

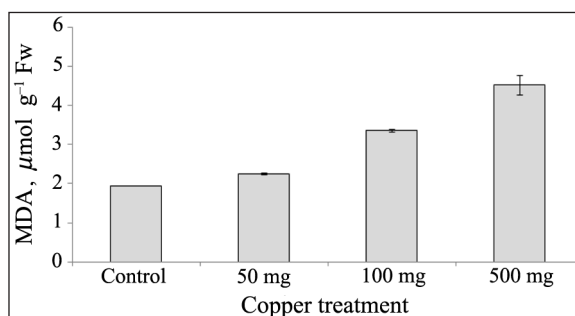


Fig. 3. MDA content in the lichen *Ramalina farinacea* treated with different Cu concentrations

to 100 and 500 mg Cu concentrations and the content was significantly higher in comparison with the control ( $p < 0.05$ ). The content of MDA ranged between approximately 3.3 and 4.5  $\mu\text{mol g}^{-1}$  Fw after exposure of these concentrations.

## DISCUSSION

Copper is an essential microelement for many organisms and plays an important role in plants. As other metals at elevated concentrations it can be toxic. Many physiological and morphological parameters were used as sensitive markers assessing the degree of environmental stress including heavy metals (Bačkor, Loppi, 2009). The experiment results showed that chlorophyll *a* was sensitive to the presence of copper but chlorophyll *b* increased in response to copper exposure. This is in agreement with the results obtained by the Bačkor and Dzubaj (2004) experiment with lichen photobionts: chlorophyll *a* was more sensitive to metals than chlorophyll *b*, and its decrease was attributable to conversion of chlorophyll *a* to chlorophyll *b* by copper. Chlorophyll *a* as a sensitive marker of Cu excess was also proved by an experiment on the influence of long-term exposure to copper on the lichen photobiont *Trebouxia erici* and free-living algae *Scenedesmus quadricauda* (Piovár et al., 2011).

Treating lichens with different Cu concentrations it could have various effects on the chlorophyll content. Chettri with colleagues (1998) observed that higher Cu level in *Cladonia*

*rangiformis* had no effect on the total chlorophyll content, while in *Cladonia convoluta* high Cu concentrations induced a decrease in the total chlorophyll content. Treatment with copper decreased the chlorophyll *a* concentration of lichens *Cetraria islandica* and *Flavocetraria cucullata* (Bačkor, Zetikova, 2003). Our results are in agreement with results of other experiments (Piovár et al., 2011; Vantová et al., 2013): a significant decrease in chlorophyll *a* and a significant increase in chlorophyll *b* were observed at the highest external Cu doses tested.

Chlorophyll *a* degradation is also an often used parameter for assessing effects of air pollution and heavy metals. A significant decrease in the phaeophytinization quotient (the ratio of optical densities at 435 and 415 nm) with copper excess indicated reduced integrity of photobiont chlorophyll (Bačkor et al., 2009). After prolonged copper exposure (24 h) a degree of chlorophyll *a* degradation – a significant decrease in chlorophyll *a* integrity was observed at 500  $\mu\text{M}$  Cu doses (Vantová et al., 2013). The phaeophytization quotient did not change during Cu exposure (Pawlik-Skowronska et al., 2006).

Chlorophyll *a* fluorescence is a valuable parameter analyzing changes associated with photosystem II (PSII) in lichens (Garty, 2001). Despite the increase in the pigment content upon exposure to high  $\text{Cu}^{2+}$  concentrations, the quantum yield of photosystem II photochemistry ( $F_v/F_m$ ) in lichen thallus was affected hardly. Reduction in chlorophyll fluorescence parameters in the lichen *Ramalina fastigiata* following an uptake of Cu was also demonstrated by Branquinho et al. (1997). A total inhibition of PSII photochemical reactions was detected when intracellular Cu concentrations exceeded approximately 2.0  $\mu\text{mol g}^{-1}$  level (Branquinho et al., 1999). The reduced  $F_v/F_m$  values in copper treated lichens indicated damage to PSII. Copper excess reduced the  $F_v/F_m$  ratio in *Peltigera rufescens* and *Cladina arbuscula* (Bačkor et al., 2009).

A decreased photosynthetic efficiency of lichens exposed to multiple airborne pollutants

was also reported in lichens growing in the surroundings of metal smelters (Garty, 2001). Closer to the smelters near the Kola Peninsula, Russia, lichens had lower values of  $F_v/F_m$  than those in the clean reference site and the study sites with the greatest distance from the source of the airborne pollutants (Odasz-Albrigtsen et al., 2000).

Copper is known as a redox reactive metal. Its oxidation results in formation of  $\text{O}^{2-}$  and subsequently  $\text{H}_2\text{O}_2$  and a hydroxyl radical via Fenton reactions. Reactive oxygen species may cause unspecific oxidation of proteins, membrane lipids or even induce DNA injury. The increase of MDA content due to excess copper has been observed in the present study. Higher levels of MDA were also found as the concentrations of  $\text{Cu}^{2+}$  increased (Garty et al., 1992). Monnet et al. (2005) demonstrated that the increased content of MDA along with damage of cell membranes in *Dermatocarpon luridum* was associated with excess of copper. The production of thiobarbituric acid reactive substances in the lichen photobiont was increased in response to copper excess (Bačkor et al., 2007). An increase of the superoxide content in lichen *E. prunastri* exposed to higher tested Cu doses was detectable after the first 4 h, while this effect was diminished after longer exposure (24 h) of Cu exposure and a significant increase of hydrogen peroxide was detected (Vantová et al., 2013).

## CONCLUSIONS

The effect of copper on the chlorophyll content, fluorescence and oxidative status was assessed in lichen *Ramalina farinacea*. Exposure of *Ramalina farinacea* to enhanced copper concentrations resulted in no any signs of chlorosis. Increasing the copper content in the solution had no effect on the chlorophyll *a* content of *R. farinacea*, but the chlorophyll *b* content was significantly higher at any tested concentrations in comparison with the control.  $F_v/F_m$  values decreased significantly in *R. farinacea* following 50 mg Cu and higher concentrations. The content of malondialdehyde

increased significantly in the lichen thalli after exposure to the highest tested (100 and 500 mg Cu) copper concentrations.

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## Gintarė Sujetovienė

### VARIO SUKELIAMI FIZIOLOGINIAI POKYČIAI IR OKSIDACINIS STRESAS KERPĖJE *RAMALINA FARINACEA*

#### Santrauka

Varis yra būtinas elementas daugelio organizmų gyvybinėms funkcijoms, bet dėl žmogaus veiklos aplinkoje gali pasiekti gyviems organizmams toksinės koncentracijas. Tyrimo tikslas – ištirti vario poveikį kerpės *Ramalina farinacea* (L.) Ach. fiziologiniams rodikliams bei jo sukeltą oksidacinį stresą. Eksperimento metu vertintas vario poveikis fluorescencijos rodikliams, chlorofilo kiekiui ir oksidacinė būklė. Veiktose kerpėse varis reikšmingo poveikio chlorofilo *a* kiekiui neturėjo; chlorofilo *b* kiekis buvo patikimai didesnis variu veiktose kerpėse, palyginti su kontrole.  $F_v/F_m$  vertės patikimai sumažėjo kerpėje esant 50 mg ir didesnėms vario koncentracijoms. MDA kiekis buvo patikimai didesnis po poveikio 100 ir 500 mg Cu koncentracijomis.

**Raktažodžiai:** fluorescencija, ląstelių membraną pažeidimas, *Ramalina farinacea*, oksidacinis stresas, varis