

Response to cobalt toxicity in lichen *Pseudevernia furfuracea*; uptake, photosynthetic quantum yield, membrane integrity and deoxyribonucleic acid fragmentation

Pseudevernia furfuracea'da kobalt toksisitesine yanıt; alım, fotosentetik kuantum verimi, membran bütünlüğü ve deoksiribonükleik asit fragmentasyonu

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ABSTRACT

Objectives: This study aims to examine the toxic potential of Cobalt (Co) on photosystem II photosynthetic quantum yield, membrane integrity, and deoxyribonucleic acid (DNA) fragmentation formation.

Materials and methods: Oligonucleosomal DNA fragmentation was detected by terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay. Lipid peroxidation was determined with malondialdehyde analyzing.

Results: The Fv/Fm ratio decreased in *Pseudevernia furfuracea* following exposure to various concentrations of Co (NO₃)₂ (5, 15 and 30 mM) for one, three and 24 hours. Co²⁺-treatment caused the accumulation of Co in lichen, induced severe oxidative stress by the generation of hydrogen peroxide, impaired the membrane integrity, and induced lipid peroxidation as measured by malondialdehyde. Samples treated with 15 mM and 30 mM of Co (NO₃)₂ had higher percentage of cell death than 5 mM-treated group.

Conclusion: To our knowledge, this is the first study detecting a high rate of DNA fragmentation *in situ* in phycobiont layer of *Pseudevernia furfuracea*; while it reveals that mycobiont layer has a lower rate of TUNEL-positive cells. It has been concluded that Co exposure results in impaired photosynthesis accompanied by oxidative stress and DNA fragmentation in *Pseudevernia furfuracea*; all these effects were concentration-dependent.

Keywords: Cobalt; deoxyribonucleic acid fragmentation; lichen; oxidative stress; *Pseudevernia furfuracea*; TUNEL.

ÖZ

Amaç: Bu çalışmada fotosistem II fotosentetik kuantum verimi, membran bütünlüğü ve deoksiribonükleik asit (DNA) fragmentasyonu formasyonu üzerinde kobaltın (Co) toksik potansiyeli incelendi.

Gereç ve yöntemler: Oligonükleozomal DNA fragmentasyonu terminal deoksinükleotidil transferaz dUTP çentik uç işaretleme (TUNEL) testi ile tespit edildi. Lipid peroksidasyonu malondialdehit analizi ile belirlendi.

Bulgular: Bir, üç ve 24 saat boyunca farklı Co konsantrasyonlarına (NO₃)₂ (5, 15 ve 30 mM) maruziyetten sonra *Pseudevernia furfuracea*'da Fv/Fm oranı azaldı. Co²⁺-tedavisi likende Co birikimine yol açtı, hidrojen peroksit üretimi ciddi oksidatif stres başlattı, membran bütünlüğüne zarar verdi ve malondialdehit ile ölçüldüğü üzere lipid peroksidasyonu oluşturdu. On beş mM ve 30 mM Co (NO₃)₂ ile tedavi edilen örneklerin hücre ölümü yüzdesi 5 mM ile tedavi edilen gruptan daha yüksek idi.

Sonuç: Bildiğimiz kadarıyla, bu çalışma *Pseudevernia furfuracea*'nın fikobiyont katmanında *in situ* yüksek oranda DNA fragmentasyonu tespit ederken mikobiyont katmanda daha düşük oranda TUNEL pozitif hücre olduğunu gösteren ilk çalışmadır. Kobalt maruziyetinin *Pseudevernia furfuracea*'da oksidatif stres ve DNA fragmentasyonunun eşlik ettiği bozulmuş fotosentez yolu açtığı sonucuna varıldı; tüm bu etkiler konsantrasyona bağımlı idi.

Anahtar sözcükler: Kobalt; deoksiribonükleik asit fragmentasyonu; liken; oksidatif stres; *Pseudevernia furfuracea*; TUNEL.

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Cobalt (Co) is an element that occurs naturally in many different chemical forms in the environment. It is an essential element for the synthesis of vitamin B, which is required for human and animal nutrition.^[1] However, high concentrations of Co are inhibitory for growth, chlorophyll synthesis and induce changes in photosynthetic activity.^[2-4] Toxicity symptoms due to excess Co are chlorosis, necrosis, and root tip damage in higher plants.

Lichens are potentially useful in the monitoring of heavy metal pollution. Their reaction to pollution is a consequence of the fact that, comparing to plant species, they do not have an outer covering tissue which facilitates direct penetration of gases, dusts and solutions inside the thalli. Some lichen species can accumulate large quantities of metals.^[5] However, high concentrations of heavy metals may cause damage to the lichen thalli. Cobalt bioaccumulation and biosorption has been recently studied; however, there are no data available on the effect of Co on the cellular processes in lichens. This study aimed to investigate the potential toxic effect of Co on photosynthesis, oxidative stress, and DNA fragmentation.^[6,7]

MATERIALS AND METHODS

Pseudevernia furfuracea (L.) Zopf samples were collected from bark of Pinus in Karagöl, Izmir-Turkey (38°33' N 27°13' E, 840 m). Lichens transferred to the laboratory were rinsed three times (10 s each) to minimize dust contamination and divided into groups. Experiments were conducted within 3-4 days of collection. The test solutions were prepared just before the experiments. Samples were incubated for 1 h in solutions of $\text{Co}(\text{NO}_3)_2$ dissolved in bidistilled water (5 mM, 15 mM and 30 mM); control samples were soaked in distilled water. After incubation, lichen thalli were kept for 24 h under laboratory conditions.

For the determination of Co content, the three-stage microwave-assisted digestion procedure was applied using a microwave oven (Berghof, speedwave) under a pressure of 8 atm. In the first stage, lichen sample (0.5 g) was washed three times with distilled water and decomposed with 5 mL of HNO_3 for 10 min. The sample was cooled, vented to remove NO_2 and CO_2 and 1 mL

of H_2O_2 was added. The vessel was capped and subjected to microwave attack for 10 min (second stage). In the third stage, 1 mL of hydrogen fluoride was added and the sample was processed for 10 min. After cooling, the sample was transferred to a polytetrafluoroethylene (PTFE) evaporating dish and evaporated to dryness. The residue was then processed as described above. Co concentration was measured by inductively coupled plasma optical emission spectrometer (ICP-OES). Each treatment was comprised of three replicates.

For the detection of photosynthetic capacity of lichen, chlorophyll a fluorescence of samples was measured with a plant efficiency analyzer (Handy PEA, Hansatech). Before measurement of chlorophyll fluorescence, the thalli were dark-adapted for 15 min. The Fv/Fm parameter was calculated by the instrument from fluorescence induction curves of 5 s duration recorded at an irradiance of 1800 $\mu\text{mol m}^{-2}\text{s}^{-1}$ from light emitting diodes. The Fv/Fm parameter defines the maximum quantum yield efficiency of photosystem II (PSII) and was used as a stress indicator. Time-dependency of the effect of Co on PSII was also tested in thalli samples exposed to 5 mM, 15 mM and 30 mM $\text{Co}(\text{NO}_3)_2$ for 1, 3 and 24 h.

Electrical conductivity (EC) as an index of cellular membrane integrity was measured. Batches of lichen thalli were divided into subsamples of 1 g and immersed in 100 mL of double-distilled water for 60 min. Electrical conductivity of the water was measured by an EC meter (WTW Cond 340i) and is used as a measure of electrolyte leakage to the incubation solution.

Formation of malondialdehyde (MDA) was evaluated as an indicator of lipid peroxidation. Determination of MDA was performed by the thiobarbituric acid reactive substances method.^[8] The absorbance differences between 532 and 600 nm was used to calculate MDA formation as a by-product of lipid peroxidation. Each treatment was comprised of three replicates.

Hydrogen peroxide (H_2O_2) levels were determined according to Sergiev et al.^[9] Leaf tissues of 0.5 g were homogenized in ice bath with 5 mL 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 g for 15 min and 0.5 mL of supernatant was added to

0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M potassium iodide. The absorbance of supernatant was read at 390 nm. The content of H₂O₂ was calculated based on a standard curve.

For DNA fragmentation assay, the thallus was cut into the small pieces and then fixed with FAA solution (50% ethanol, 5% glacial acetic acid, 3.7% formaldehyde, freshly prepared) and incubated in this solution overnight. Fixed samples were dehydrated through an ethanol series (50%, 70%, 80%, 95%, 100% for 15 min each concentration), immersed in ethanol-xylene (1:1) for 10 min and then in 100% xylene for 10 min. The samples were embedded in paraffin, and cut to 5 µm sections in Leica RM 2145 microtome (Leica Microsystems, RM2145, Glattbrugg, Switzerland).

Immunohistochemical expression was analyzed in the lichen thallus tissue using peroxidase as described in details below. Paraffin sections were immersed in xylene overnight and immunohistochemical terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed as follows: sections were incubated for 20 min in methanol containing 1% H₂O₂ to reduce endogenous peroxidase activity and then exposed to microwave in sodium citrate solution for five minute 90 watt - 15 minute 360 watt. After washing in 0.2 M Tris-HCl including 0.5% Triton X, the sections were exposed to the TUNEL (Promega TUNEL Systems Cat. # G7130-G7360). Finally, sections were reacted with 0.05% diaminobenzidine (Zymed Histostain Plus Ref/Cat No: 859643 San Francisco California, USA) and H₂O₂ (0.01%). Immunoreaction was assessed by light microscopy (Olympus BX-51 light microscope, Olympus C-5050 digital camera; Olympus Co., Tokyo, Japan) at a magnification of X100. TUNEL (+) cells were counted in the lichen thallus using image software (Image-Pro Express 4.5, Media-Cybernetics Inc., Rockville, MD, USA). Cell density (cells µm²) was calculated for lichen and the average TUNEL (+) cell density was obtained in each group.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was performed for the statistical

analysis of Fv/Fm ratios and nonparametric Kruskal Wallis test for independent samples was performed for the rest of the analysis. P<0.05 was accepted significant; when a P value below 0.05 was determined, Bonferroni test or Mann Whitney U test was carried out in post hoc analysis for the comparison of groups where appropriate SPSS for Windows version 11.0 version software (SPSS Inc., Chicago, IL, USA).

RESULTS

ICP-OES data showed that the concentration of Co in the untreated thallus of *P. furfuracea* soaked in bidistilled water was 3.49±0.04 µg/g dry weight. On the other hand, treatment of thalli with 5 mM, 15 mM, and 30 mM Co(NO₃)₂ for 1 h resulted in a significant dose-dependent increase in the Co content of thallus (64.12±11.37, 108.63±0.58 and 216.17±3.62 µg/g dry weight, respectively; p<0.05 when compared to control group and when compared to exposure groups with each other).

For the estimation of the effect of Co on photosynthesis capacity of the thallus, photoinhibition was measured with permanent reduction in maximal PSII efficiency (Fv/Fm). There was a gradual, time- and dose-dependent decrease in the Fv/Fm values in Co²⁺-exposure

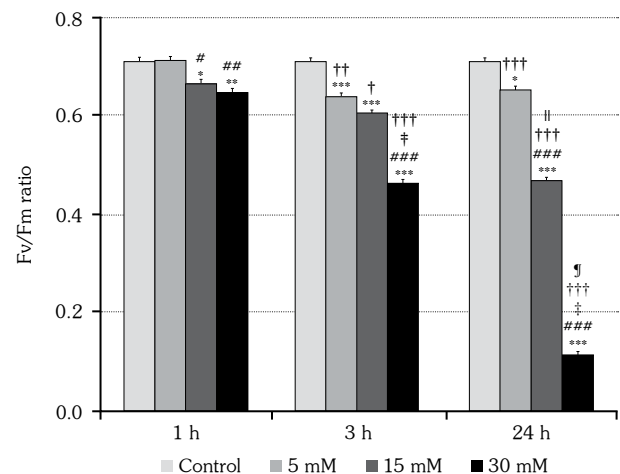


Figure 1. Fv/Fm ratio of *P. furfuracea* thalli immersed in bidistilled water or in different concentrations of Co (NO₃)₂. Data are presented as mean ± SEM of eight individual experiments. * P<0.05, ** P<0.01 and *** P<0.001, when compared to control; # P<0.05, ## P<0.01 and ### P<0.001, when compared to 5 mM group; † P<0.001, when compared to 15 mM group; ‡ P<0.05, †† P<0.01 and ††† P<0.001, when compared to corresponding 1 hour exposure data; § P<0.05, §§ P<0.01 and §§§ P<0.001, when compared to corresponding 3 hours exposure data.

Table 1. Electrical conductivity and malondialdehyde and hydrogen peroxide content of *P. furfuracea* thalli incubated in bidistilled water (control) or in different concentrations of $\text{Co}(\text{NO}_3)_2$ for 24 hours

	Electrical conductivity ($\mu\text{S cm}^{-1}$)	Malondialdehyde (μM)	H_2O_2 (μM)
	Mean \pm SEM	Mean \pm SEM	Mean \pm SEM
Control	2.53 \pm 0.26	1.72 \pm 0.54	1.66 \pm 0.12
5 mM $\text{Co}(\text{NO}_3)_2$	3.33 \pm 0.60	2.43 \pm 0.01	6.23 \pm 0.10*
15 mM $\text{Co}(\text{NO}_3)_2$	9.40 \pm 0.56*, \ddagger	7.51 \pm 0.67*, \ddagger	11.79 \pm 0.20*, \ddagger
30 mM $\text{Co}(\text{NO}_3)_2$	18.77 \pm 0.59*, \ddagger , J	14.38 \pm 0.26*, \ddagger , J	51.53 \pm 2.07*, \ddagger , J

SEM: Standard error of the mean; H_2O_2 : Hydrogen peroxide; * $P < 0.05$, when compared to control; \ddagger $P < 0.05$, when compared to 5 mM group; J $P < 0.05$, when compared to 15 mM group.

groups, indicating a significant photodestructive effect on PSII (Figure 1).

Cellular membrane integrity was assessed by the measurement of EC in the incubation solution. It was noticed that Co^{2+} exposure of thalli for 24 h increased the EC at 15 and 30 mM concentrations significantly when compared to the control group ($P < 0.05$, Table 1) and this effect was concentration-dependent; indicating a marked impairment in the membranal integrity of the thalli.

Table 1 shows the MDA and H_2O_2 content of thalli after exposure to Co^{2+} for 24 h. Co^{2+} exposure significantly increased the MDA content of thalli at 15 mM and 30 mM, but not at 5 mM, concentrations ($p < 0.05$), while it increased H_2O_2 content at all concentrations. The increase in MDA level, as an index of lipid peroxidation, and H_2O_2 level was in a concentration-dependent manner.

Tissue sections of thallus from *P. furfuracea* were examined for TUNEL-positive nuclei, which

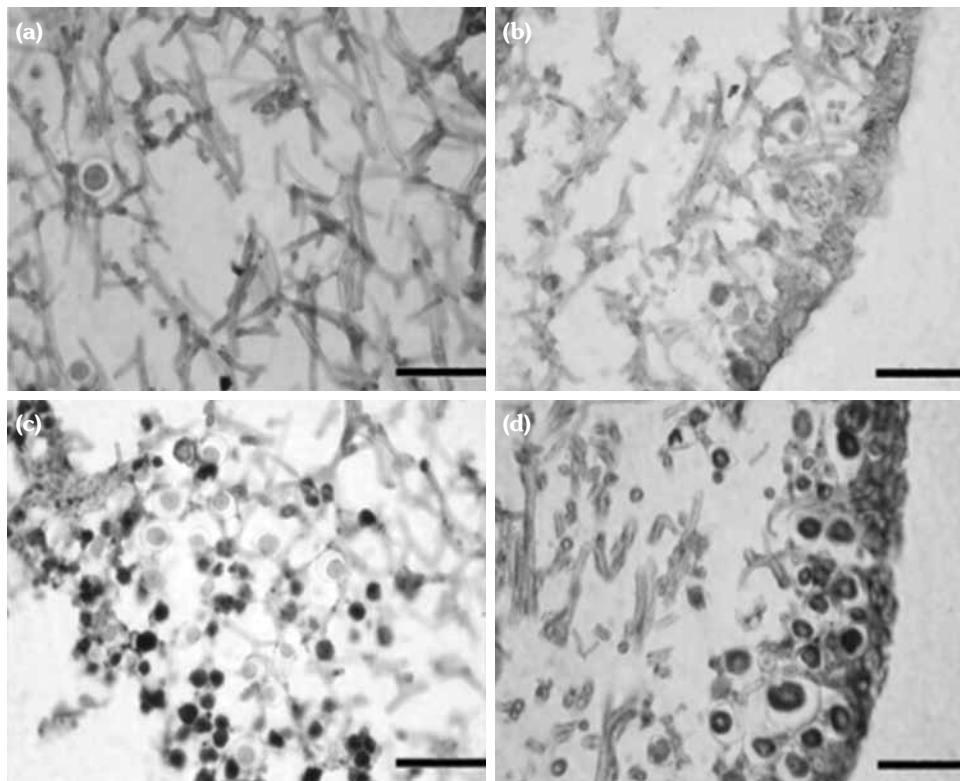


Figure 2. TUNEL staining of the sections of lichen incubated in (a) bidistilled water (control group), (b) 5 mM $\text{Co}(\text{NO}_3)_2$, (c) 15 mM $\text{Co}(\text{NO}_3)_2$ or (d) 30 mM $\text{Co}(\text{NO}_3)_2$. Original magnification $\times 40$. Scale bar= 125 μm . Dark nuclei are TUNEL (+) cells.

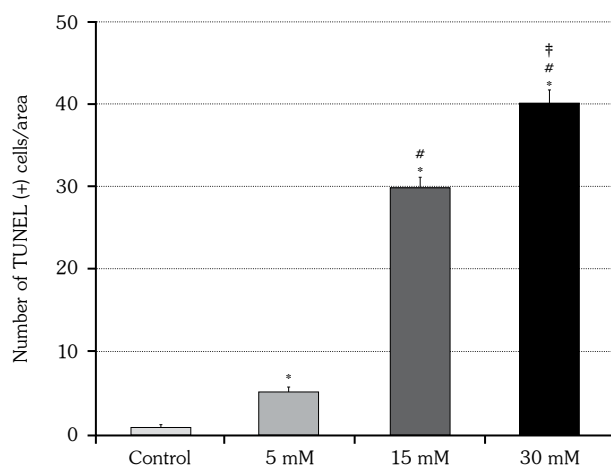


Figure 3. Number of TUNEL (+) cells in lichen thallus sections. Data are presented as mean \pm SEM of three individual experiments. * $P < 0.001$, when compared to control; # $P < 0.001$, when compared to 5 mM group; † $P < 0.001$, when compared to 15 mM group.

were noticed as dark brown spots (Figure 2). In mycobiont layer, TUNEL-positive nuclei were less frequently scattered in Co^{2+} exposure groups comparing to the intensity of TUNEL-positive nuclei in the phycobiont layer of thallus (Figure 2). In total, exposure of thalli to Co^{2+} resulted in a significant increase in the number of TUNEL-positive cells in a concentration-dependent manner ($p < 0.001$, Figure 3). When the distribution of TUNEL-positive cells among the two layers of lichen was statistically examined; it was noticed that the percentage of TUNEL-positive cells in the photobiont was higher than the mycobiont both in the control group ($p < 0.05$) and in the Co^{2+} exposure groups ($p < 0.01$ in 5 and 30 mM groups and $p < 0.001$ 15 mM group) (Table 2).

Table 2. The percentage of TUNEL positive nuclei of mycobiont and photobiont layers of *P. furfuracea* thalli immersed in bidistilled water (control) or in different concentrations of $\text{Co}(\text{NO}_3)_2$ for 24 hours

	TUNEL positive nuclei (%)	
	Mycobiont	Photobiont
	Mean \pm SEM	Mean \pm SEM
Control	0.70 \pm 0.08	1.30 \pm 0.20 †
5 mM $\text{Co}(\text{NO}_3)_2$	4.02 \pm 0.17 *	23.33 \pm 1.67*, §
15 mM $\text{Co}(\text{NO}_3)_2$	10.52 \pm 0.77*, †	65.65 \pm 0.59*, †, ††, †††
30 mM $\text{Co}(\text{NO}_3)_2$	37.24 \pm 3.08*, †, ††	82.02 \pm 1.52*, †, ††, †††

SEM: Standard error of the mean; $P < 0.05$, when compared to control; † $P < 0.05$, when compared to 5 mM group; †† $P < 0.05$, when compared to 15 mM group; ††† $P < 0.05$, § $P < 0.01$, ††† $P < 0.001$, when compared to mycobiont layer.

DISCUSSION

Lichens do not shed plant parts as readily as vascular plants.^[5,10] The lack of a waxy cuticle and stomata allows them to adsorb many contaminants through the whole lichen surface.^[5] Lichens are capable of accumulating various elements to concentrations that vastly exceed their physiological requirements and therefore, deposition patterns are distinguishable from normal element loadings.^[5] Data obtained in lichen samples incubated in $\text{Co}(\text{NO}_3)_2$ in our study show that Co content rapidly increases in *P. furfuracea* with the increasing Co concentration in the incubation solution. Exposure to $\text{Co}(\text{NO}_3)_2$ produced an accumulation of Co in *P. furfuracea* thallus at a considerably high level (i.e. 216.73 $\mu\text{g/g}$ dry weight at exposure to 30 mM Co^{2+}) that had a capacity to inhibit photosynthesis significantly.

Maximal PSII efficiency is frequently used to monitor stress in photosynthetic organisms. Under non-stressed conditions, lichens typically possess a Fv/Fm ratio in the range of 0.45-0.65.^[11] The decrease of Fv/Fm ratio in the lichen *P. furfuracea* is in accordance with studies on the impact of Co^{2+} stress on algae.^[12] Plekhanov and Chemeris^[12] showed that the Fv/Fm ratio in *Chlorella pyrenoidosa* treated with 0.1, 1, and 10 mM Co^{2+} , rapidly decreased to 0.45, 0.25, and 0.15, respectively. In the present study, 24-hour exposure to Co^{2+} resulted in Fv/Fm values of 0.47 and 0.1 at 15 mM and 30 mM concentrations; suggesting that particularly at 30 mM, Co^{2+} decreases the photosynthesis capacity of lichen below the non-stressed levels and induces significant photo-destructive effects on PSII, respectively.

Oxidative stress caused lipid peroxidation and thereby destruction of cell membranes.^[13] Although the toxicity of Co is quite low compared to many other metals in soil, the toxic action of Co was found to be altering membrane permeability.^[14,15] Turton et al.^[16] suggested that the presence of MDA in biological systems can be related to the peroxidation of unsaturated fatty acids constituting cellular membranes. The consequences of the changes in lipid and protein structure are the loss of membrane integrity and selective permeability. Thus, the increase in MDA levels suggests that higher concentration

of Co^{2+} has a damaging effect on the cellular membranes of *P. furfuracea*. In present study, the concentration-dependent increase in MDA levels shows a positive correlation with the EC values in such a manner that the higher MDA levels in lichen, the higher EC in the incubation solution. Electrical conductivity is considered to indicate injury to cell membranes and has been previously used for the assessment of electrolyte leakage in lichen.^[17,18] Although 5 mM Co^{2+} -exposure did not significantly change EC in thalli, marked higher EC values were observed in thalli treated with 5 mM and 30 mM concentration of Co^{2+} ; indicating the leakage of electrolytes through the damaged membranes of the lichen.

H_2O_2 is toxic for most animal cells at levels of about $10\text{-}10^2 \mu\text{M}$.^[19] Experiments with plant material have demonstrated that plant tissues can tolerate high concentrations of H_2O_2 in the range of $10^2\text{-}2 \times 10^5 \mu\text{M}$.^[20] H_2O_2 increase has been reported in *Arabidopsis thaliana* and tomato plants after treatment with copper, cadmium and mercury.^[21-23] Photosynthetic efficiency data in our study has shown that thallus of *P. furfuracea* can tolerate concentrations of H_2O_2 at an approximate range of $6\text{-}52 \mu\text{M}$ (see Table 1). de Pinto et al.^[24] showed that only simultaneous increase of H_2O_2 in tobacco cells induced cell death that had typical cytological and biochemical features of DNA fragmentation. Similarly, our results have shown that increasing production of H_2O_2 induces a marked increase in the density of TUNEL-positive cells in *P. furfuracea* thalli. TUNEL-positive nuclei were examined less frequently in mycobiont layer compared to the photobiont layer, suggesting the photobiont layer is more sensitive to Co-induced toxicity than mycobiont layer.

In conclusion, our data have clearly indicated that exposure to Co^{2+} increases the Co content of the lichen and consequently results in decreased photosynthetic quantum yield. This functional Co toxicity is accompanied by lipid peroxidation and consecutive damage to cellular membrane integrity and oxidative stress-induced formation of DNA fragmentation especially in the photobiont layer. Results of the present study, however, do not clearly identify that the feature of DNA fragmentation is directly related to apoptotic-like formation or necrosis. Further focused research

is required to investigate the characteristics of the formation of DNA fragmentation related to necrosis or apoptosis in lichen under heavy metal stress conditions.

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