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RESEARCH PAPERS

Morphological, Physiological, and Biochemical Traits of Melanized Thallus of the *Cetraria islandica* Lichen

A. G. Daminova[a, *], E. I. Galeeva[a], D. F. Rakhmatullina[a], L. V. Viktorova[a], and F. V. Minibayeva[a, b]

[a] *Kazan Institute of Biochemistry and Biophysics, Kazan Scientific Center, Academy of Sciences, Kazan, 420111 Russia*

[b] *"Biomarker" Research Laboratory, Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, 420008 Russia*

**e-mail: daminova.ag@gmail.com*

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Abstract—Lichens are extremophilic symbiotic associations possessing phenomenal resistance to abiotic stress-factors. In this regard, melanization of thalli in response to UV is one of the mechanisms protecting lichens from excessive insolation. However, microstructure and biochemical properties of the melanized thalli are still poorly investigated. In the present study, morphological, nanomechanical, and physiological, and biochemical traits of naturally melanized thalli of the *Cetraria islandica* (L.) Ach. lichen were examined. In the upper cortex of its thallus, the nature of the pigment layer was verified using typical qualitative reactions for melanins. It was found that melanization leads to changes in microstructure of the upper cortex of the mycobiont, in particular, thickening of the cell walls and extension of the interhyphal space. The melanized and pale (nonmelanized) thalli were found to differ from each other in their nanomechanical properties, including the parameters of adhesion and rigidity. This implies the possible formation of complex associates of melanin with cell wall components in the melanized mycobiont. In addition, higher antioxidant activity and lower respiratory activity were found in the melanized thalli of *C. islandica* in comparison with the pale thalli. Presumably, the found modifications in the microstructure and nanomechanical, physiological, and biochemical properties of thalli occurring in the course of melanization make lichens more resistant to intense insolation.

Keywords: *Cetraria islandica*, antioxidant activity, respiration, lichens, melanization, thallus morphology, topography

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INTRODUCTION

Lichens are photosynthetic symbiotic associations whose thallus is composed of two major partners—mycobiont and photobiont [1]. Lichens are classified as extremophilic organisms because of their phenomenal resistance to unfavorable environmental factors, such as dehydration, sharp changes in temperature, and ultraviolet irradiation [2]. Among causes of the high stress resistance of lichens, an appreciable role may be played by synthesis of secondary metabolites, including melanins. Dark pigment melanin, which is formed in several lichen species under natural intense insolation or sole UV irradiation, is situated in a pigmented layer on a surface of the lichen's thalli. Melanins are products of oxidative polymerization of phenol or indole compounds. These pigments absorb light over a wide spectrum including gamma rays, X-rays, and UV that makes them photoprotectors [3–5]. In the lichen's thallus, melanins exhibit photoprotective and antioxidant properties as well [6]. Publications characterizing morphology of pigmented thalli of lichens are scarce probably because of a complexity of the thallus anatomy, interactions between fungal and photosynthetic symbionts, and diversity of pathways of metabolites' biosynthesis. We earlier analyzed microstructure and elementary composition of the pigmented layer in the upper cortex of the *Lobaria pulmonaria* lichen subjected to UV-B light [7]. It was found that the thallus pigmentation was a consequence of eumelanin synthesis induced by this radiation. In addition, melanization of *L. pulmonaria* changed nanomechanical parameters, namely, decreased the adhesion capacity of the pigmented layer of the cells in the upper cortex of the thalli [8].

The object of the present work was the *Cetraria islandica* (L.) Ach. lichen (Iceland moss). Its mycobiont is the *Cetraria* fungus and phytobiont is the green alga *Trebouxia* sp. This lichen is branched and bushy, with the upright foliose thallus up to 7 cm in height. Its color varies from grayish-white to deep brown. This species represents arctic, subarctic, and alpine flora all over the northern hemisphere. Its thallus is widely used in pharmacology as antimicrobial preparation and biologically active supplement. The high biologi-

cal activity of this lichen is accounted for by unique lichen metabolites to a large extent, in particular, lichenin polysaccharide. In the nonpigmented thalli of the *C. islandica* and *Evernia prunastri* lichens, low-temperature scanning electron microscopy and immunocytochemistry visualized lichenin as a structural element of the fungal cell wall mainly participating in the water metabolism [9]. It is shown that UV irradiation induces melanin biosynthesis in the *C. islandica* thallus [10, 11] but particulars of the thallus microstructure related to melanization seem to be unknown.

Importantly, morphology and topography of the thallus may determine its physicochemical properties and physiological functions. In this respect, the goal of the present study was the analysis of normally melanized thallus of the *C. islandica* lichen. We examined the structural parameters, including morphology and nanomechanical properties together with physiological and biochemical features, in particular, antioxidant properties and respiratory activity.

MATERIALS AND METHODS

Material collection. Pale and melanized thalli of *Cetraria islandica* (L.) Ach. lichen were collected in bedimed or well-lighted areas in the suburb of Syktyvkar city. The material was cleaned, slowly dried out at room temperature and 60–70% RH to be stored at –20°C. Before experiments, the thalli were hydrated on wet filter paper at 10°C for 24 h.

Qualitative reactions for melanin. We prepared 50-μm-thick cross sections from pale and melanized thalli using a Leica VT 1000S vibratome (Leica, Germany). The sections were stained with different techniques as follows:

1. Incubation in 0.2% L-3,4-dihydroxyphenylalanine (L-DOPA) for 1 h in the darkness [12].

2. Incubation in 1% Azure II for 30 min followed by addition of 0.5% eosin for several minutes according to Rejniak [13].

3. Incubation in 2.5% $FeSO_4$ for 1 h followed by 30-min incubation in 1% $K_3[Fe(CN)_6]$ and then in ethanol-xylol mixture (50 : 50, v/v) according to Lillie [14].

The stained sections of the lichen's thalli were observed under a Leica DM1000 epifluorescence microscope (Leica, Germany) furnished with a digital camera (Sony, China). To measure the thickness a pigmented layer, 15 sections were examined in three biological replications by means of the microscope's software.

Scanning electron microscopy (SEM). The cross sections of pale and melanized lichen's thalli were fixed for 24 h in 2.5% solution of glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The sections were dehydrated in a series of ethanol solutions and were sequentially incubated in mixtures of hexamethyldisilazane with ethanol in the ratios 1 : 3, 1 : 1, and 3 : 1 (v/v) for 30 min in each. Finally, the sections were placed in 100% hexamethyldisilazane for 60 min. To study morphology of the pale and melanized thalli, they were sputter-coated with gold at a Q150T ES high-vacuum coater system (Quorum Technologies, Great Britain). A high-resolution scanning electron microscope (Merlin, Germany) was used at an accelerating voltage of 5 kV. To estimate relative thicknesses of the cell walls in the pale and melanized thalli, the numbers of hyphae per a unit of cross-sectional area of SEM images were counted with the help of the ImageJ graphic program.

Atomic force microscopy (AFM). Pale and melanized thalli were fixed in 2.5% glutaraldehyde followed by postfixation in osmium tetroxide (Sigma, United States) and dehydration in sequentially increasing concentrations (from 30 to 96%) of ethanol in acetone. The samples were polymerized in LR White resin (Sigma, United States) at 60°C for 24 h. The upper surfaces of the sample blocks were leveled with a diamond knife. The upper cortex of thalli was visualized in cross sections using a Bruker Dimension FastScan microscope (Bruker, United States) with ScanAsyst-Air cantilevers (Bruker, United States) in PeakForceQNM mode. Images were acquired at a resolution of 512 lines per scan.

Enzymatic activities of lichen's thalli. Crude enzymatic extract was prepared from a rehydrated lichen by its disruption in liquid nitrogen followed by 1-h extraction in 50 mM sodium phosphate buffer, pH 7.0. The homogenate was centrifuged at 10000 *g* for 30 min. The extract was grained with ammonium sulfate in a 30–80% saturation gradient. After centrifugation, the sediment was resuspended in a minimal volume of 25 mM Tris-HCl buffer, pH 7.5. The activities of enzymes were assayed spectrophotometrically with a UV-1600 device (Shimadzu, Japan). The laccase activity was measured by the rate of oxidation of 1 mM *o*-dianisidine (the extinction coefficient at 460 nm is 30.0/(mM cm)) in 80 mM acetate buffer, pH 4.5. The tyrosinase activity was estimated by the oxidation rate of L-DOPA (the extinction coefficient at 475 nm is 3.6/(mM cm)) in 50 mM sodium phosphate buffer, pH 6.0 [15].

Detoxification of hydrogen peroxide. Fragments of the pale and melanized thalli were incubated in the dark in 0.05 mM sodium phosphate buffer, pH 7.0, containing 500 μM H_2O_2. After 20, 40, and 60 min, the H_2O_2 concentration in the incubation medium was measured through oxidation of xylenol orange (by A_{560}) [16] at a UV-1600 spectrophotometer. A calibration curve was built with standard H_2O_2 solutions. There were no changes in the determined H_2O_2 concentration in control samples without thallus fragments.

Respiration rate of the pale and melanized thalli was measured manometrically in a Warburg device (Warburg-Apparat VEB Glaswerke Stutzerbach, Germany) [17] and were expressed as μL O_2/(h g dry wt).

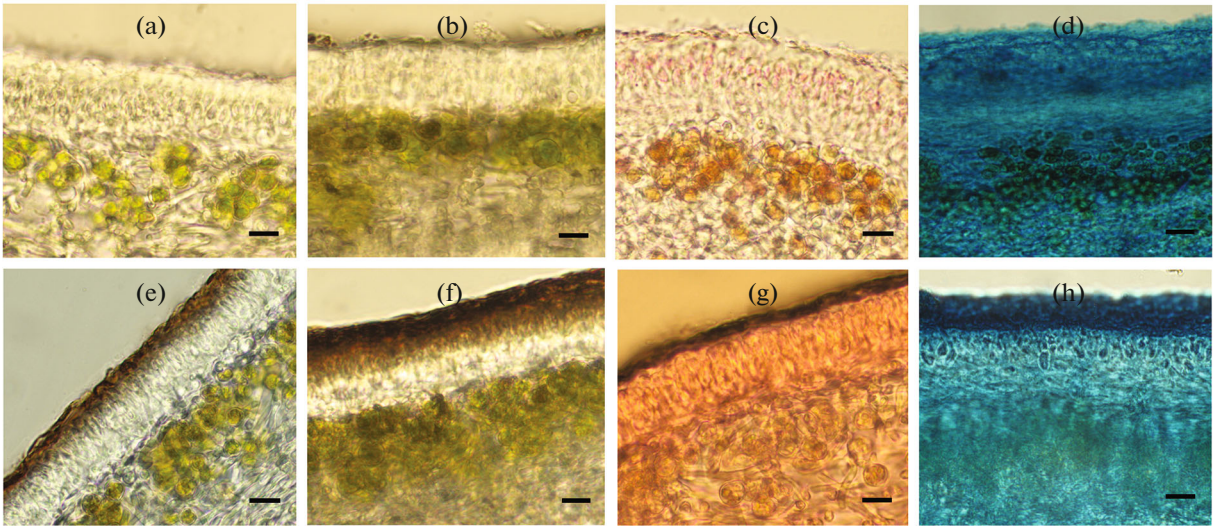

Fig. 1. Cross section of (a–d) pale and (e–h) melanized thalli of *C. islandica* lichen. Staining technique: (a, e) without staining; (b, f) staining with L-DOPA; (c, g) staining by Rejniak; (d, h) staining by Lillie. Scale bars correspond to 10 μm.

Statistics. The experiments were carried out with three to six biological and three to 14 analytical replications. Means and their SEs are reported. All experimental data represent normal distribution. To compare mean values, one-way ANOVA was performed with estimation of pair differences using Tukey and Bonferroni criteria.

RESULTS

Morphological Analysis of C. islandica Thalli Using Qualitative Reactions

Light microscopy of cross sections of pigmented thalli of *C. islandica* enabled visualization of a deep brown layer on a surface of the upper cortex under which nonpigmented fungal hyphae were located together with algae (Fig. 1e). The thickness of the pigmented layer varied from 5 to 8 μm on average. Qualitative reactions for melanin were carried out to test the nature the pigment. Thus, the cross sections were stained with L-DOPA, which is a substrate of phenoloxidases, including tyrosinases and laccases, involved in melanin synthesis [18]. After such treatment, the upper bark layer of the pigmented but not pale thalli intensively turned to brown (Figs. 1b, 1f). The method of Rejniak involving eosin visualized deep brown cells on cross sections of the pigmented thalli (Fig. 1g). The staining by Lillie, based on iron oxidation, visualized the dark pigment only in the pigmented thalli as well (Fig. 1h).

Microstructure of C. islandica Thalli Visualized by Scanning Electron Microscopy

This technique revealed that the upper cortex of the melanized thallus was thicker than that of the pale counterpart. Thus, in the pale thallus, the upper bark layer consisted of fungal hyphae with multiple pores (Fig. 2a), while the pore number per area unit of a cross section was 3.8 times fewer in the melanized thallus (Fig. 2b). This witnesses to thickening the hyphal cell walls associated with melanization.

Nanomechanical Properties of C. islandica Thalli Visualized by Atomic Force Microscopy

To examine the topography of the thalli's upper cortex, we visualized the relief, adhesion, and rigidity of a surface of cross sections of the pale and melanized thalli by atomic-force microscopy (Fig. 3).

The images by height, obtained by scanning along the main axis of a probe sensor, markedly differed between the pale and melanized thalli. The color saturation of the relief was 1.2 times higher in the pale than in the melanized thallus (Figs. 3a, 3d). The adhesion of the surface of the melanized thallus was approximately four times stronger than that of the pale thallus (Figs. 3b, 3e). Meanwhile, the rigidity of the melanized thallus was more than tenfold lower than that of the pale one (Figs. 3c, 3f).

Redox and Respiratory Activities of C. islandica Thalli

Among phenoloxidases, laccases manifested higher activity than tyrosinases. The laccase activity was 167 ± 12 and 131 ± 7 ncat/g dry wt in the melanized and pale thalli, respectively. Thus, the enzyme was 1.3 times more active in the pigmented thallus. The tyrosinase activity was very low irrespective of thallus melanization (data are not shown).

The important index of antioxidant capacity is the rate of hydrogen peroxide decomposition by a sample. The melanized thalli were found to degrade exogenous 500 μM H_2O_2 much more efficiently (by 50%) than the pale thalli over 1 h (Fig. 4a).

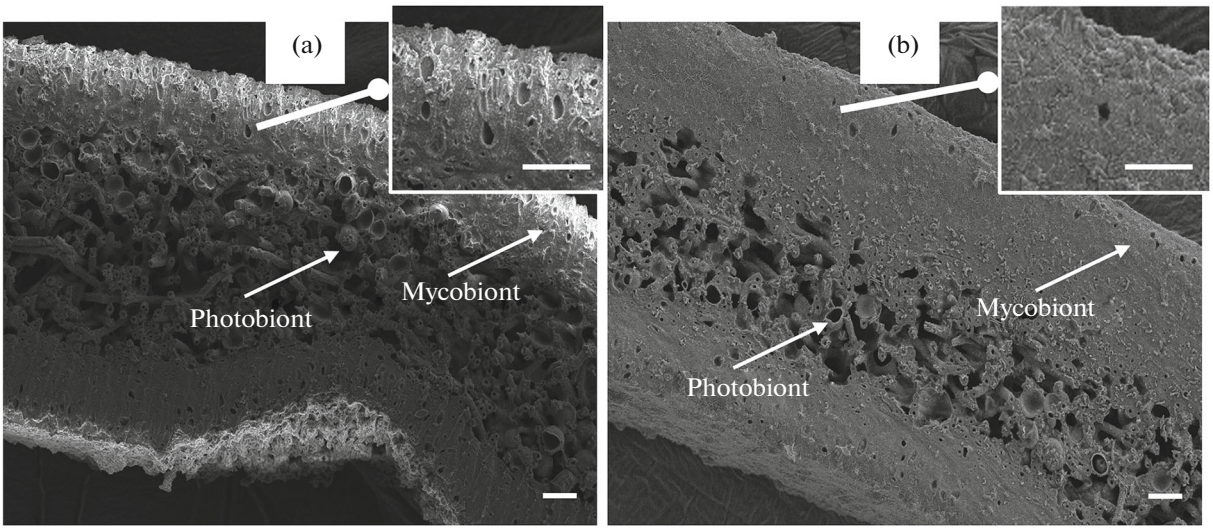

Fig. 2. Scanning electron micrographs of cross sections of (a) pale and (b) melanized thalli of *C. islandica* lichen. Scale bars correspond to 10 μm.

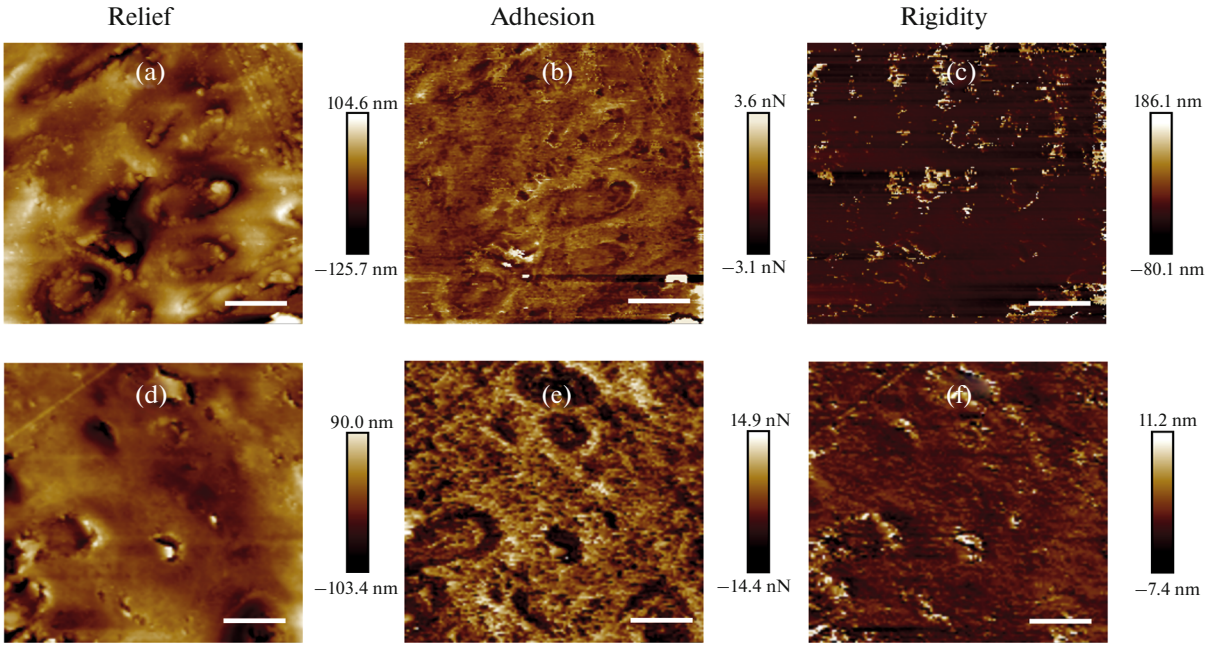

Fig. 3. (a, d) Relief, (b, e) adhesion, and (c, f) rigidity of the surface of cross sections of (a–c) pale and (d–f) melanized thalli of *C. islandica* lichen. Atomic force microscopy was used. Scale bars correspond to 2 μm.

The respiration rate of the melanized thallus was almost twofold lower than that of the pale counterpart (Fig. 4b).

DISCUSSION

Lichens are extremophilic organisms that successfully survive under the harshest environmental conditions [19]. In response to stress impact caused by intense solar insolation or sole UV irradiation, thalli of the *C. islandica* lichen acquire dark pigmentation resulting from a synthesis of melanin pigment in the upper cortex of the thallus. Melanins are universal pigments that are widespread in many organisms. At present, most studies of morphology and other features of melanins have been carried out on pathogenic fungi [20–22], bacteria [23, 24], and human melanosomes [25, 26]. We earlier analyzed physicochemical properties of melanins isolated from *C. islandica*, *Lobaria pulmonaria*, and *Leptogium furfuraceum*

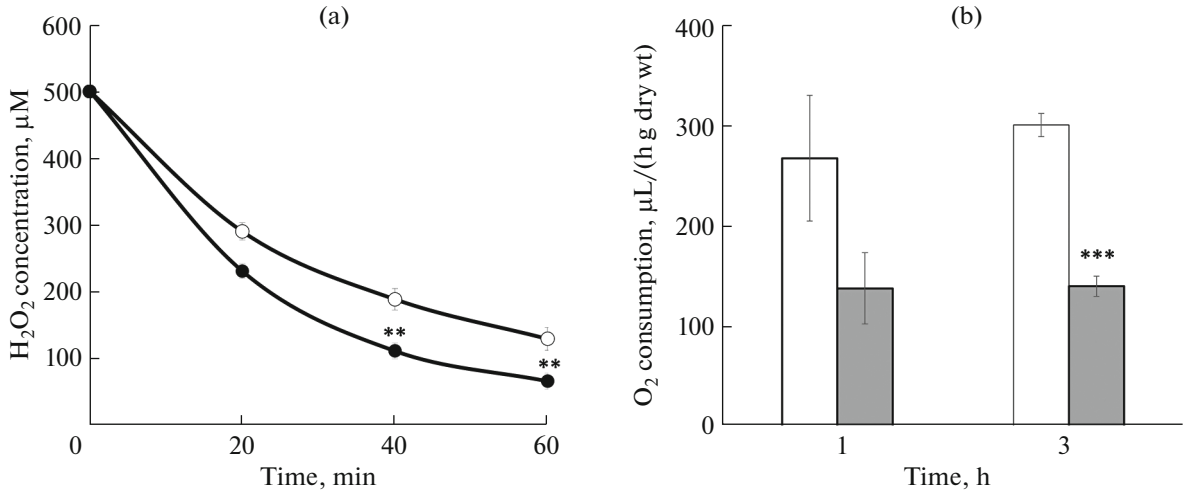

Fig. 4. Redox and respiration activities of thalli of *C. islandica* lichen. (a) Decomposition of exogenous 500 μM H_2O_2 by (open circles) pale and (closed circles) melanized thalli. (b) Rate of respiration of (white bars) pale and (grey bars) melanized thalli. Significance of differences was estimated with one-way ANOVA at ** $P < 0.01$ or *** $P < 0.001$.

lichens [27–29]. Although the role of melanization in lichens' protection from solar insolation is well-investigated [30, 31], only sporadic publications deal with the architecture of melanin layer in lichen's thalli in situ [7, 8].

The present work revealed particulars of surface morphology and topography of the bark layer in the melanized thallus of the *C. islandica* lichen. It was found that the melanized thalli were characterized by surface darkening of the upper cortex and positive staining reactions typical of melanins (Fig. 1). Simultaneously, thickening of the cell walls and expanding of the interhyphal space of the upper bark layer were visualized on cross sections of the thalli (Fig. 2). We earlier demonstrated that melanization of the *L. pulmonaria* lichen was accompanied by enlargement of the interhyphal space and accumulation of melanin-like granules [7]. The expanded interhyphal space in the upper bark layer of the thallus appears to be a typical morphological trait of lichens undergoing UV-induced melanization. Besides, we found that the thallus melanization of *L. pulmonaria* affected not only morphological but also physical properties of the thalli due to formation of melanin complexes [8]. In the present work, atomic-force microscopy made possible characterization of the topography and nanomechanical properties of the upper bark layer on cross sections of *C. islandica*. Here, the melanized thalli displayed an increase in adhesion of the core layer, while the indexes of rigidity were considerably lower than those of the pale thalli (Fig. 3). This evidences to the high chelating capacity of melanin and possible formation of complex associates of it with polysaccharides and proteins of the cell walls. Preliminary estimation supposes that melanin associates of *C. islandica* contain higher percentage of polysaccharides (including lichenin and isolichenin) than those of *L. pulmonaria*. This difference may increase the sorption capacity and alter topographical characteristics of the melanized upper bark layer of thalli (data are not shown). Therefore, the changes in nanomechanical parameters confirm the changes in topography, measured at a nano-level, in the *C. islandica* thalli upon their melanization.

It is likely that such considerable changes in the thallus morphology and topography affect the physiological and biochemical state of a lichen. This suggestion is supported by the data on antioxidant and respiratory activities. In fact, the melanization was accompanied by the enhanced antioxidant activity, namely, efficient decomposition of exogenous hydrogen peroxide by the melanized thallus of *C. islandica*. These results confirm the high antioxidant activity of melanin preparation extracted from this species [27]. Melanization of lichen thalli may diminish efficiency of the main cytochrome-dependent pathway of respiration. Actually, measurements of respiration rate revealed the substantially lower total respiratory activity in the pigmented thalli in comparison with the pale ones (Fig. 4). This might be accounted for by melanin-associated changes in physicochemical properties of the cellular surface of thalli and semiconducting nature of melanin [32]. Finally, this may rearrange the electron stream so that the effectiveness of energy-producing metabolic pathways is diminished. The decreased thallus respiration may indicate decreased metabolic activity of the mycobiont partner in the melanized thallus. It is known that the melanin content negatively correlates with the biomass accumulation of hyphae in free-living fungi [33]. These data agree with the hypothesis of "physiological compromise" between melanin biosynthesis and cellular metabolism [33].

Therefore, our results support the concept of a barrier function of the melanin layer in the upper cortex

of lichen thallus. Upon lichen melanization, the microstructural and topographical changes in the upper cortex, together with the subsequent changes in physiological and biochemical state of the thallus, indicate the diversity of melanin-mediated protective mechanisms against intense penetration and toxic action of strong solar radiation in *C. islandica*.

ABBREVIATIONS AND NOTATION

AFM	atomic force microscopy
L-DOPA	L-3,4-dihydroxyphenylalanine
SEM	scanning electron microscopy

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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