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RESEARCH PAPERS

Superoxide Dismutase and Catalase Activity As an Indicator of the Ontogenetic State of the Threatened Lichen *Lobaria pulmonaria* (L.) Hoffm. in the Middle Boreal Subzone

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Abstract—It is well-known that the switch of an organism ontogenetic state is accompanied with the change in metabolic status, especially, the activity of antioxidant system enzymes. However, there is still lack of knowledge about the internal changes which occur during the shift between ontogenetic stages in lichens. In this study, superoxide dismutase (SOD) and catalase (CAT) activities were measured for *Lobaria pulmonaria* thalli from middle boreal subzone on the Karelian Republic territory. We have shown that the transition between ontogenetic stages in *L. pulmonaria* could be observed not only by the set of morphological features, but also by changes in the activity of SOD and CAT for the first time. Therefore, we have made a conclusion that the enzyme activity could be the basis for more justified separation of different ontogenetic stages and identifying processes associated with aging.

Keywords: *Lobaria pulmonaria*, antioxidant enzymes, ontogenetic stages

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INTRODUCTION

It is extremely important to take into account the ontogenetic state of thalli for a full assessment of the lichen population structure [1]. The change in the activity of antioxidant system (AOS) enzymes as a biochemical marker during the transition between ontogenetic stages is of great interest. According to the literature the changes in the antioxidant enzyme activity seem to be growth and development indicators during plant ontogeny [2, 3]. Currently, there is only limited information available on the role of antioxidant enzymes in eliminating reactive oxygen species (ROS) in lichens [4–6].

Considerable role in the oxidative stress elimination belongs to the antioxidant system components, which maintain a high level of protection from the ROS destructive effects in lichens [7–9]. It is interesting to note that increased ROS levels are highly reactive and affecting various cellular, biochemical and physiological functions such as disruption of the cell membrane through lipid peroxidation; damage to DNA, pigments and enzymes; carbohydrate deoxidation; and protein denaturation [10–12]. During last few decades, studying ROS functional significance and the accompanying antioxidant response in the growth, development, and differentiation of organisms have become important [10, 12, 13].

Lobaria pulmonaria (L.) Hoffm. is an epiphytic cyanolichen which is widely distributed in boreal, temperate, mountainous and oceanic regions of the world [14]. *L. pulmonaria* is three-component lichen formed by a mycobiont (ascomycete fungi), a primary photobiont, the eukaryotic green alga *Dictyochloropsis reticulate* Tschermak-Woess [15], and a secondary photobiont, the nitrogen-fixing cyanobacteria *Nostoc* sp. [16].

In the last century in Europe, there has been a rapid decline in the species populations. A particular sharp decrease in the abundance of *L. pulmonaria* has been recorded in densely populated and industrial areas due to increased forest management practices and air pollution [17, 18]. In this regard, in most northern and central Europe countries, the species is included in the IUCN Red Lists. In Russia, the lichen is listed in the Red Data Book of the Russian Federation (2008) with the status of a vulnerable species with a declining population (2b).

It should be noted that the ontogeny of the *L. pulmonaria* species has been studied in detail [19, 20], however, research aimed at analysing the state of the

Abbreviations: CAT—catalase; SOD—superoxide dismutase.

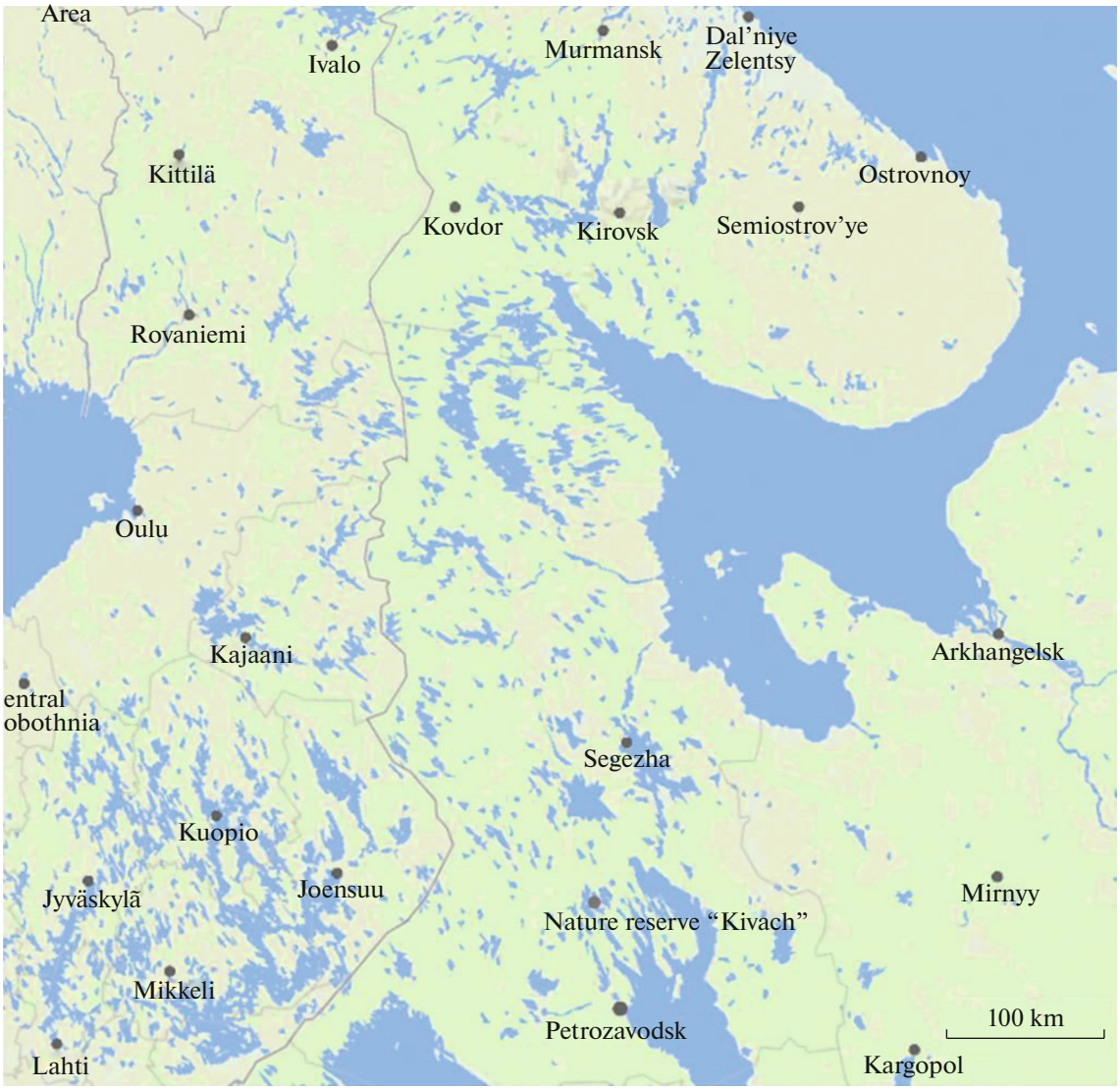

Fig. 1. Map of the study areas location (highlighted with black dots).

antioxidant system in thalli of different ontogenetic states are rare, while researchers usually divide thalli into young and old [5, 21–23].

The aim of our study is to determine the activity of AOS enzymes of endangered lichen *L. pulmonaria* at different ontogenetic stages, as well as to evaluate their role as biochemical markers of ontogeny changes.

MATERIALS AND METHODS

Study sites. The study was carried out in 2016 in the middle boreal subzone blueberry feathermoss spruce forests on the Karelian Republic territory, including the Kivach Strict Nature Reserve, the Zaozersky sanctuary (Fig. 1).

Petrozavodsk is the capital of the Karelian Republic, the industrial, transport and tourist centre, one of the largest cities in the Russian Northwestern Federal District, located on the southwestern shore of Lake Onega (61°50′ N, 34°20′ E) [24]. The city area is 113.0 km^2; population—266.2 thousand people. The natural forests of the city are mainly represented by spruce, pine and aspen forests, which area is ~6933.3 ha. There are two protected natural areas on the territory of the Petrozavodsk District: PetrSU Botanic Garden (367 ha) and the part of the Zaozersky Sanctuary (400 ha).

Landscape sanctuary "Zaozersky" (area 2700 ha, founded in 1991) is located on the western shore of Lake Onega, Baraniy Bereg peninsula. Spruce and pine forests predominate in the structure of forest cover, the share of which is approximately the same. Birch and alder forests occupy about 9% of the forest area.

The Kivach Nature Reserve (62°20′ N, 34°00′ E) is located in the northwestern part of the Zaonezhsky Peninsula. The strict reserve (area 10450 ha, founded

in 1931) is a 12 km long from the north to the south 14 km from the west to the east forest territory, bounded by lakes. Forests occupy about 90% of its territory. Coniferous communities predominate, including pine forests accounting 42%, spruce forests 32%, and deciduous forests 7% of the reserve area [25].

Lichen material and sampling. The study of *Lobaria pulmonaria* thalli parameters was carried out by the method of continuous counting on 32 trunks of *Populus tremula* at the height of 0–2 m from the ground. For each thallus, the total area and necrosis area (cm^2) were recorded using 25 × 25 cm frame. A total of 175 thalli were studied.The ontogenetic stages were determined for each thalli following Ignatenko et al. [20] classification. All analyzed thalli were divided into 4 ontogenetic groups: virginile (v2a, v2b, v2c)—thalli with pronounced soralia, cephalodia and emerging isidia; generative (g)—large thalli with apothecia and abundant soralia; subsenile (ss)—a collapsing thalli with non-spreading isidia and lobules; senile (s)—thalli with abundant necrotic formations and regenerative structures.

To determine the enzymatic activity of the *L. pulmonaria* thalli, 51 samples were collected from trees at the height of 1.3–1.5 m from the ground.

Estimation of enzymatic activity. The enzymatic activity measurements were carried out using the equipment of the Core Facility of the Karelian Research Centre of the Russian Academy of Sciences.

The crude extract was prepared by grounding and homogenization of lichen samples in buffer medium containing: 67 mM K, Na—phosphate buffer (pH 7.8), 0.5 mM EDTA; ratio tissue : buffer—1 : 10. After 20-minute extraction at 4°C, the homogenate was centrifuged twice at 10000 *g* for 20 min (Centrifuge MPW-351R, Poland), and the supernatant was collected [3, 5].

Superoxide dismutase (SOD) activity was determined by measuring inhibition of nitroblue tetrazolium (NBT) photoreduction. In the light, riboflavin generates superoxide radicals, which, in the presence of methionine, oxidize NBT to formazan, which has a blue colour. SOD catalyzes the hydrogen peroxide formation using riboflavin as a superoxide radicals' source and methionine as a hydrogen donor. NBT oxidation does not occur in this case, and, accordingly, a blue colour does not develop, which correlates with the SOD activity [3, 5]. The assay mixture contained 67 mM K,Na-phosphate buffer (pH 7.8), 172 μM NBT, 210 μM methionine, 24 μM riboflavin, 0.1% Triton X-100. The amount of supernatant was 100 μL. Moreover, we conducted additional series of experiments in which the incubation medium did not contain methionine and riboflavin, respectively. This was done to eliminate the interfering effect that can be caused by the presence of substances such as methionine, riboflavin, and others in enzyme preparations. To define the activity of SOD, an optical density reduction was measured at 560 nm after 30 min of incubation under fluorescent lamps. To calculate SOD activity (A), the colour inhibition degree (B) was first determined:

$$B = 1 - \frac{(D2 - D4 - D6)}{(D1 - D3 - D5)}, \quad (1)$$

$$A\left(\frac{U}{\text{mg protein}}\right) = \frac{B \times V1 \times V2 \times m2}{m1 \times V3 \times l}, \quad (2)$$

where D1 and D2—the optical density of control and test variants with methionine and riboflavin in the incubation medium; D3 and D4—the optical density of control and test variants without methionine in the incubation medium; D5 and D6—the optical density of control and test variants without riboflavin in the incubation medium; *V*1—the total volume after the preparation, mL; *V*2—the volume of the incubation mixture, μL; *V*3—the supernatant volume, μL; *m*1—the protein content in the sample, mg; *m*2—the mass of the sample, g; *l*—the optical pathlength, mm. SOD activity was indicated in U mg^{-1} of protein.

Catalase activity (CAT) was measured spectrophotometrically (SF-2000 spectrometer, OKB-Spektr, Russia) by monitoring the decrease in optical density at 240 nm outcoming from the decomposition of hydrogen peroxide after 20 min of incubation. The incubation medium contained 67 mM K,Na-phosphate buffer (pH 7.8) and 10.3 mM hydrogen peroxide. The hydrogen peroxide content was calculated according to a pre-constructed calibration in the range of 1.5–20.6 mM hydrogen peroxide. The activity was expressed as micromoles H_2O_2 decomposed per minute per milligram protein in 20 min (μmol H_2O_2 mg^{-1} protein) [3, 5].

The protein content was assayed according to the method of Bradford [26] and expressed in mg of protein per g of thallus mass (mg g^{-1}).

Data processing and statistical analysis. Statistical data processing was performed using Microsoft Excel 2007 and PAST (version 4.0). Bars in the diagrams are average means of experimental runs with standard errors. Sample sizes are denoted as n. Before starting the statistical analysis, the raw data were initially checked for normality using the Shapiro–Wilk test. The significance of differences between variants was estimated by Mann–Whitney U-test. The difference (indicated by letters) was considered significant at $P < 0.05$.

RESULTS

Based on the results, it was found that in the overall ontogenetic spectrum, thalli belonging to virginile 1 (26%), virginile 2a (31%), and senile (22%) stages predominate. Apothecia were recorded in 5% of the examined thalli. According to the data obtained the thalli area varied greatly from 27.1 to 496.5 cm^2 (Fig. 2) in studied samples. It was noted that as the ontoge-

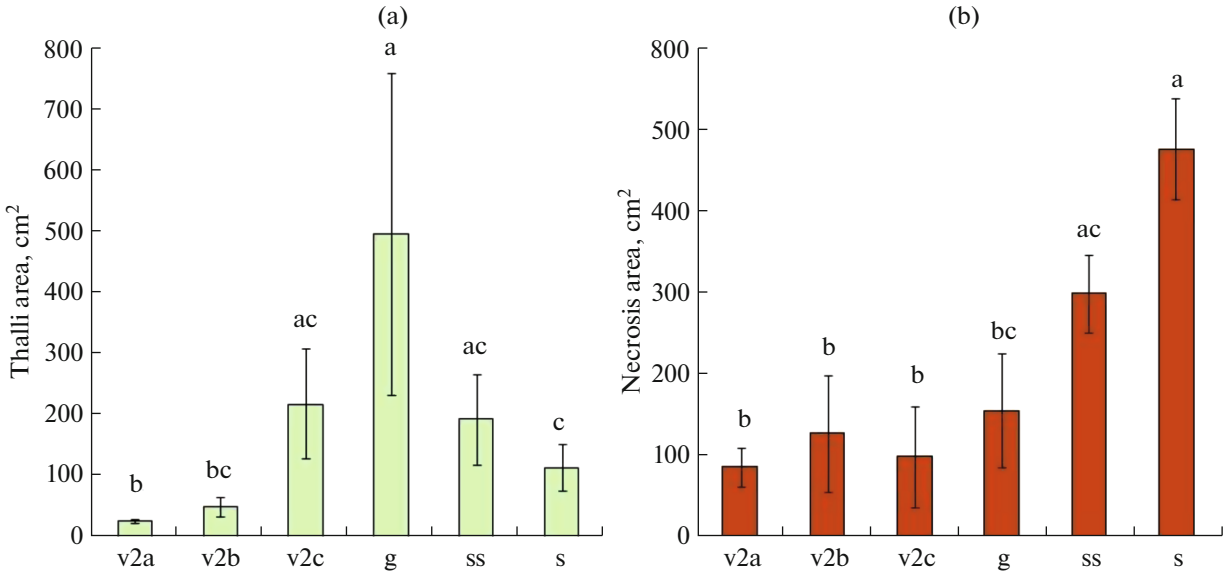

Fig. 2. Thalli area (a) and the percentage of necrosis (b) per area of *Lobaria pulmonaria* thalli of different ontogenetic stages. Values represent mean ± SE (n = 55 for v2a; n = 8 for v2b; n = 7 for v2c; n = 9 for g; n = 13 for ss; n = 38 for s).

netic stage changed, the average thalli area increased from virginile to generative individuals and further decreased for subsenile and senile individuals. The highest value was found for mature generative thalli and varied from 18.7 to 2093.7 cm^2 within the studied group. At the same time, the percentage of necrosis between ontogenetic groups ranged from 4.4 to 23.9% (for virginile 2a to senile, respectively).

The total soluble protein content of the examined thalli decreased gradually from 3.53 to 3.20 mg^{-1} of thallus mass from earlier ontogenetic stages to generative one and differed significantly between virginile and senile one (P = 0.044) (Fig. 3). No differences in protein content were recorded for generative and subsenile thalli.

Results showed significant differences in SOD activity in senile and generative thalli (6.48 and 4.81 U mg^{-1} protein, respectively; P = 0.037) (Fig. 4). Thus, the old senile thalli showed a higher SOD activity. No differences in the values were recorded for virginile and subsenile thalli averaged 5.27 and 5.36 U mg^{-1}protein, respectively.

According to the data obtained, CAT activity increased with the change in ontogenetic stage from virginile to senile thalli (223 and 787 μmol H_2O_2 mg^{-1} of protein, respectively; $P < 0.05$) (Fig. 5). The group of generative and subsenile specimen did not vary significantly between each other but differed from the

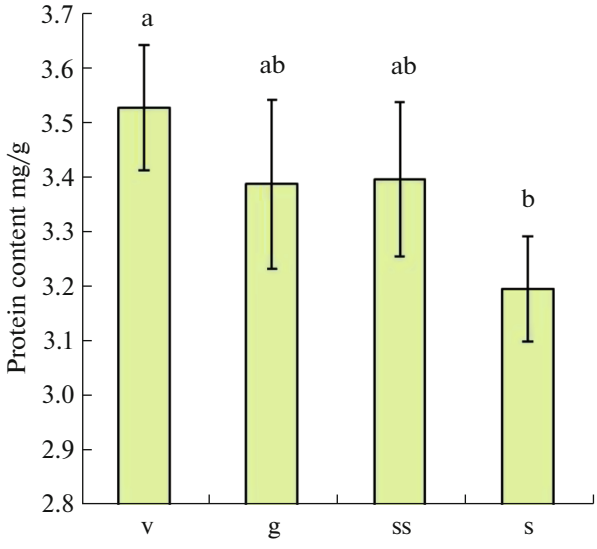

Fig. 3. Protein content in *Lobaria pulmonaria* thalli of different ontogenetic stages. Values represent mean ± SE (n = 17 for v; n = 7 for g; n = 14 for ss; n = 13 for s).

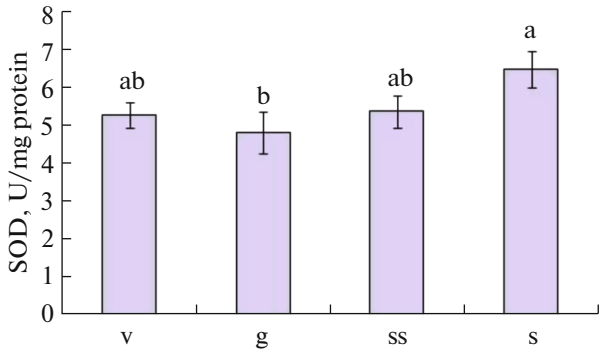

Fig. 4. SOD activity of *Lobaria pulmonaria* thalli at different ontogenetic stages. Values represent mean ± SE (n = 17 for v; n = 8 for g; n = 13 for ss; n = 14 for s).

rest. Thus, the senile thalli with the lowest protein content had the highest CAT activity, while an inverse correlation was observed for the virginile group.

DISCUSSION

Over the past few decades the identification of various cytological, biochemical and molecular markers has been very acute to determine the transition between ontogenetic stages in various organisms [27].

It is believed that the protein content can be used as an indicator of reversible and irreversible metabolism changes, as well as a biomarker of adverse environmental conditions and pollution [28]. However, according to the literature, no significant changes in this indicator were found depending on the pollution level [28, 29] or habitat [5]. This was also confirmed by other researchers. For example, von Arb and Brunold [30] on the example of another foliose epiphytic lichen *Parmelia sulcata*, often found in communities together with *Lobaria*, observed no significant differences in protein content between thalli growing in habitats with different pollution levels. Thus, it can be assumed that the use of this parameter as a biomarker was not so sensitive to environmental pollution [29]. However, these studies did not take into account the ontogenetic state of the examined thalli. In the present paper, we have shown that there are significant differences in protein content between thalli of different ontogenetic states. Possibly, the greater variability of this parameter [28] may be associated with the use of thalli of different ontogenetic stages, which levels out the existing differences in protein content.

Despite the substantial interest in the study of AOS enzymes during plant ontogeny [10, 31, 32], there is still a lack of such information for lichens. Single attempts were made to show the difference in diverse parameters with a change of ontogenetic stages [5, 21–23]. However, studies on the antioxidant system of *L. pulmonaria* thalli at different ontogenetic stages have been missing in the literature yet.

It is known that ROS are formed during normal metabolic processes, however, their excessive accumulation can lead to deorganization of organelles, growth restriction and cell death [33, 34], which provokes an increase in AOS enzymes' activity.

It is believed that an increase in the activity of AOS enzymes arise when oxidative stress occurs in response to various environmental conditions. However, it has been reported [35, 36] that higher activity of antioxidant enzymes is not necessarily indicative of adaptation to oxidative stress in individual species. Instead of this, the ability to rapidly reestablish redox state can bea characteristic of thespecies. And the AOS enzyme activity increase can be connected with the changes in metabolic state during ontogeny [37, 38].

We have previously shown that the CAT activity in juvenile thalli was more sensitive to the changes in

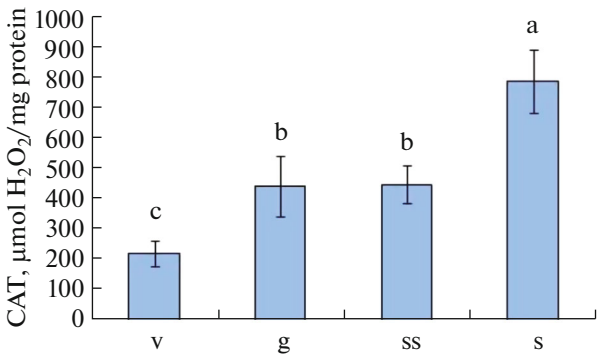

Fig. 5. CAT activity of *Lobaria pulmonaria* thalli at different ontogenetic stages. Values represent mean ± SE ($n = 13$ for v; $n = 7$ for g; $n = 11$ for ss; $n = 9$ for s).

environmental conditions [5]. These results allowed us to consider CAT activity as an indicator of the thalli ontogenetic state. According to the data obtained, the thalli area stopped to increase from generative stage and further. Moreover, it was associated with a dramatic increase in the percentage of necrosis. Thus, during the virginile and generative stages, the studied enzymes were likely to be more involved in maintaining the processes of growth and differentiation, namely there were no significant differences in the protein content, the thalli area increased, and the percentage of necrosis was maintained at a stable low level. At the same time, SOD activity remained constant and catalase activity increased significantly. Probably, under such circumstances CAT was responsible for the active growth processes.

During the transition from the generative to the subsenile stage of development, there was a tendency to a decrease in the thalli area and the increase in the necrosis percentage. However, the work of AOS enzymes did not change significantly, maintaining a stable metabolic status. Thus, we can consider the predominance of morphological changes in relation to the internal state of the thalli, namely no significant differences were found in the protein content as well as in CAT and SOD activities. It seems subsenile stage of development represent the transition stage from the mature generative thalli to the old senile lichen.

With a shift to the senile stage the thalli area decreased, the percentage of necrosis enlarged. The total soluble protein content tended to reduce. The significant increase in SOD activity from generative to senile species and decrease in protein content from virginile ones led to the subsequent rise of CAT activity in senile individuals. Probably, this may indicate that the mature thalli have already accumulated more ROS during their lifespan, which led to the consistent CAT activity increase.

Thus, the transition between ontogenetic stages can be observed not only by the set of morphological features, but also by changes in the internal metabolic

status, namely the change in the AOS enzymes activity. This work is just a step to the search for biochemical markers of the ontogenetic state. And that's of real importance for such rare species like *L. pulmonaria*. Therefore, it is likely that enzyme activity can become the basis for more justified separation of different ontogenetic stages and identifying processes associated with aging, and, consequently, with the onset of oxidative stress.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants as objects of research.

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