Activity of catalase and superoxide dismutase in *Lobaria pulmonaria* from forest communities of middle and northernmost boreal zone (NW Russia)

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Abstract

The present study was carried out to evaluate the activity of antioxidant enzymes and the protein content in *Lobaria pulmonaria* thalli of various ontogenetic stages (studied groups: juvenile and mature) in forest communities of the middle and northernmost boreal subzones (*i.e.* two habitat groups). Obtained results showed that the protein content in thalli of different ontogenetic stages and habitats did not differ significantly between the two studied groups and ranged from 3.4 to 3.8 mg g^{-1}. Peroxidase (POD) activity was low and did not exceed 0.01 μmol TG mg^{-1} of protein. No significant differences between the studied groups (ontogenetic and habitat) in POD was found. On the contrary, the activity of superoxide dismutase (SOD) and catalase (CAT) showed significant differences. SOD activity ranged from 5.1 to 6.3 U mg^{-1} of protein. Significant differences were found between the SOD activity in juvenile and mature *L. pulmonaria* thalli in the communities of the northern boreal subzone ($p = 0.043$) and in juvenile thalli from different habitats ($p = 0.020$). In general, CAT activity ranged from 187 to 605 μmol H_2O_2 mg^{-1} protein. Significant differences in CAT activity were apparent between the thalli from the middle and northernmost boreal subzone communities ($p=0.040$). The differences in CAT activity values in juvenile and mature thalli in the northernmost boreal subzone ($p = 0.006$) were found as well. Juvenile thalli from different habitats ($p = 0.008$) differed. As a result we suggest that juvenile thalli might be more suitable for the purposes of bioindication of environmental conditions, such as temperature, humidity, since they are more sensitive in comparison to mature thalli.

Key words: *Lobaria pulmonaria*, antioxidant, catalase, superoxide dismutase

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Introduction

Lichens symbiosis is regarded as an organism with biochemical uniqueness and broad spectrum of unique and active secondary metabolites are described and investigated. Within the last decade, several reviews have focused on different effects of a variety of lichen substances (Molnára and Farkas 2010, Thadhani et al. 2011, White et al. 2014, Fernarndez-Moriano et al. 2016). The studies indicated that secondary metabolites content are of key importance of ecophysiological plasticity of lichens and their high stress tolerance (Aossar et al. 2018).

Nowadays a great interest is brought to the activity of antioxidant system (AOS) in lichens regarding to their ability to survive at extreme environmental conditions (Sun et al. 2003, De Vera et al. 2004) and reactive oxygen species (ROS) formation. Antioxidative enzymes have an important role in the protection of lichens from the harmful effects of ROS. Lichens, in general, exhibit a high level of defense against the oxidative stress (Beckett et al. 2005, Liers et al. 2011). Within the last few years, it was revealed that activity of superoxide dismutases (SOD), catalases (CAT), peroxidases (POD) as well as multi-copper oxidases activity (laccases, tyrosinases) of lichens fits different taxonomic and substrate group in different environmental conditions (Zavarzina and Zavarzin 2006, Weissman et al. 2006, Kranner et al. 2003, Kranner and Birtić 2005, Beckett and Minibayeva 2007, Beckett and Zavarzina 2013, Balasubramanian and Nirmala 2014).

Lobaria pulmonaria (L.) Hoffm is an epiphytic foliose cephalodial chlorolichen which inhabits old-growth forests in the boreal region. The species is considered extremely sensitive to environmental conditions and its occurrence strongly depends on different habitat characteristics: availability of light and the risk of desiccation under high-light-intensity damage (Gauslaa and Solhaug 1999, 2000; Gauslaa et al. 2006, Gauslaa and Coxson 2011); substrate properties (Gustafsson and Eriksson 1995); cover of mosses (Öckinger et al. 2005, Rubio-Salcedo et al. 2015).

The world population of *L. pulmonaria* has declined considerably during the past 60 years, and at present time it is endangered in many countries of Western Europe (Gauslaa 1995, Zoller et al. 1999, Jüriado et al. 2011, Scheidegger et al. 2012).

There are numerous reports in the literature dealing with ecology and physiology of *L. pulmonaria*. Some of them reported antioxidant properties of phenolic compoundds (Odabasoglu et al. 2004, Karakus et al. 2009, Atalay et al. 2015) while only limited data is available for activity of antioxidant enzymes of *L. pulmonaria* (Matee et al. 2016, Golovko et al. 2018). In fact, it was assumed by several authors that ontogenesis stage might be responsible for the species ability to adapt to a changing environment and the more vulnerable the juvenile stages of thalli (Gauslaa et al. 2006, Larsson and Gauslaa 2011, Eaton and Ellis 2012). For example, it was noticed that juvenile thalli showed high variability in growth rates, pointing to environmental sensitivity (Scheidegger et al. 1998, Giordani and Brunialti 2002). Others pointed molecular evidence for environmental sensitivity during the early stages of thallus development (Werth et al. 2006).

It is well established that AOS enzymes activity and ROS formation can be involved in plant growth, development and differentiation during organism ontogeny (Zhukova et al. 1996, Gupta and Datta 2003, Apel and Hirt 2004, Molassiotis et al. 2004, Foyer and Noctor 2005, Imin et al. 2005, Obert et al. 2005, Pasternak et al. 2005, Kwak et al. 2006, Blázquez et al. 2009, Barba-Espin et al. 2010). In lichens, however, such relation has not yet been noticed. The likely reason is that the antioxidant system functioning is very little studied in lichens. Information is still lack-

ing on ecophysiology of different ontogenetic stages of *L. pulmonaria*.

In the present study, we aimed to study activity of the CAT and SOD in thalli of *Lobaria pulmonaria* from the forest communities of middle and northernmost boreal zone with emphasis on ontogenetic stages. We expect that juvenile thalli may be more suitable for the purpose of bioindication of environmental conditions by the using of antioxidative enzymes activity as a diagnostic characteristic.

Material and Methods

Study sites and data collection

Lichen sampling was carried out in spruce forest communities in the middle and northernmost boreal subzone of Northwest Russia (Fig. 1).

Sample plots within middle boreal subzone were established in the Kivach Strict Nature Reserve (62°17'N, 34°00'E, 10,880 ha) and the Zaozersky Sanctuary (61°50'N, 34°20'E, 13,500 ha) on the territory of the Republic of Karelia. The climate is predominantly temperate and intermediate ranging from oceanic to continental and characterized by having relatively mild, long winters and cool, short summers. The mean annual temperature is +3°C and the mean annual precipitation is 450–750 mm (Nazarova 2003).

Fig. 1. The location of the study areas (NW Russia): 1 – Zaozersky Sanctuary, 2 – Kivach Strict Nature Reserve, 3 – Vodlozersky National Park.

The studied communities in northernmost boreal subzone were located in the Arkhangelsk region on the territory of the Vodlozersky National Park (63°30'N, 37° 28'E, 337,600 ha). The climate is characterized by frequent weather changes. The cyclones bring precipitation, cloudy weather, and warming in winter, the cold Arctic air flows cause a strong temperature decline and frosts. The annual precipitation ranges from 400 to 540 mm with up to 200 days with precipitation – snowfalls of short duration in winter and long drizzles in autumn. The mean annual temperature is +2°C (Humala and Polevoi 2008).

The sampling was carried out in the phytocoenosis of two formations – spruce and aspen forests. Thalli of *Lobaria pulmonaria* were collected from trunk of aspen at heights of 1.3-1.5 m from the ground. The ontogenetic stage was determined for each thalli (Ignatenko and Tarasova 2018): *virginal 1* (distinctive mature appearance – well formed lobes and tuberosity, but sterile, *i.e.* without reproductive structures), *virginal 2a* (with marginal soralia), *virginal 2b* (with abundance of marginal and single laminal soralia), *virginal 2c* (soralia were abundantly distributed on the margins and ribs of thalli), *generative* (with apothecia), *subsenile* (with partly destroyed thallus in centre, regenerative structures were formed on dying lobes and not able to spread) and *senile* (destroyed thallus over most part of the area, the remaining lobes had a large necrotic areas). Sampled thalli of *L. pulmonaria* were divided in 2 group: juvenile (*virginal 1*, *virginal 2a, 2b*, *2c*) and mature (*senile*, *subsenile*).

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 lichen samples were ground and homogenized in the medium of the following composition: 67 mM K, Na – phosphate buffer (pH = 7.8), 0.5 mM EDTA; ratio tissue: buffer – 1: 10. After 20 min. extraction at 4°C, the homogenate was centrifuged twice at 10000 g for 20 min. (Centrifuge MPW-351R, Poland) according to Nikerova et al. (2016).

The CAT was determined by the enzymatic decomposition of hydrogen peroxide. The incubation medium contained 67 mM K, Na-phosphate buffer (pH 7.8) and 10.3 mm hydrogen peroxide, the amount of supernatant 50-200 µl depending on enzyme activity. Linear dependence of CAT activity on the reaction time was observed for 30 min. Incubation time of 20 min. for the reaction was chosen, because the activity in samples differed a lot between each other, and some samples had very low activity, that was not detectable in the first 5-10 min. To determine the activity of CAT, the decrease of optical density at 240 nm was measured (SF-2000 spectrometer, OKB-Spektr, Russia). The hydrogen peroxide content was calculated according to a pre-constructed calibration in the range of 1.5-20.6 mM hydrogen peroxide. CAT activity was expressed in µmol of reduced hydrogen peroxide per 1 mg of protein in 20 min. (µmol H_2O_2 mg^{-1} of protein) according to Nikerova et al. (2016).

The activity of superoxide dismutase (SOD) was determined as the inhibition of photo recovery of nitro blue tetrazolium (NBT). The incubation medium for determination of SOD activity consisted of 67 mM K, Na-phosphate buffer (pH 7.8), 172 µM HBT, 210 µM methionine, 24 µM riboflavin, 0.1% Triton X-100. The amount of supernatant was 100 µl. Besides, we made additional series of experiments in which the incubation medium did not contain methionine and riboflavin, respective-

ly. This was to eliminate the interfering effects that could be caused by the presence in enzyme preparations such substances as methionine and riboflavin and others. To determine the activity of SOD, a decrease in optical density was measured at 560 nm after 30 min. of incubation under fluorescent lamps. SOD activity was expressed in Units per 1 mg of protein in 30 min. (U mg^{-1} of protein) according to Nikerova et al. (2019).

Peroxidase (POD) activity determination based on guaiacol that was used as a hydrogen donor. Hydrogen peroxide was used as a substrate. The incubation medium for the POD activity measurements contained 67 mM K, Na-phosphate buffer (pH 7.8), 2.6 mM hydrogen peroxide and 21.5 mM guaiacol. The amount of supernatant was 200 µl. The incubation time was 30 min. POD activity was determined as the rate of formation of the reaction product of tetraguaiacol (TG). To determine the content of the formed TG, an increase in the optical density at 470 nm was measured, and the amount of TG was calculated taking into account the extinction coefficient ε (470 nm) = 0.0266 μM^{-1} cm^{-1}. The activity of POD was expressed in µmol of the formed TG per 1 mg of protein for 30 min. (µmol TG mg^{-1} of protein according of Galibina et al. (2016), and Nikerova et al. (2019).

The protein content was determined by Bradford method and expressed in mg of protein per g of tissue (mg g^{-1}).

Data processing and statistical analysis

Data were statistically analyzed by analysis of variance (one-way ANOVA, Microsoft Excel 2007, StatGraphics for Windows) and the *p*-value was used to evaluate the statistically significant differences (Ivanter and Korosov 2010). The data in the diagrams are shown as arithmetic means from a biological replicates in triple analytical replicates. The bars represent standard errors. Differences were considered significant under $p < 0.05$ (* – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$). The total set of collected sample had 66 individual thalli: 23 – juvenile (middle boreal subzone – 13 and northernmost – 10) and 43-mature (middle – 27, northernmost boreal subzone – 16).

Results

The protein content of the studied *L. pulmonaria* thalli of different ontogenetic stages (juvenile and mature) and habitats is shown in Table 1.

Protein content did not differed significantly between ontogenetic and habitat groups. No differences were also found in protein content between the thalli from different habitats of the Zaozersky Sanctuary, the Kivach Strict Nature Reserve, the Vodlozersky National Park (from southern to northern locality) that might be caused by a high variation of protein content in the thalli of this lichen species (Riga-Karandinas and Karandinos 1998, Cansaran-Duman et al. 2015).

Activity of POD was low and did not exceed 0.01 µmol TG mg^{-1} of protein. No significant differences were found between the studied ontogenetic groups and habitats. Low POD activity in lichens was reported by other authors (Morgenstern et al. 2008, Golovko et al. 2018).

Protein content mg g^{-1}	Zaozersky S M±m	Kivach SNR M±m	Vodlozersky NP M±m
Juvenile thalli	3.4±0.26	3.6±0.14	3.6±0.28
Mature thalli	3.4±0.10	3.4±0.13	3.8±0.34

Table 1. Protein content in juvenile and mature thalli of *L. pulmonaria* from different habitats.
Notes: S - Sanctuary, SNR - Strict Nature Reserve, NP - National Park, M - average value, m - standard error.

SOD activity in *L. pulmonaria* thalli ranged from 5.1 to 6.3 U mg^{-1} of protein. Comparison of SOD activity between the thalli from the studied habitats showed no significant differences.

Due to the fact that no significant differences were found in the enzyme activity in the separation of thalli by latitude of the selection sites, the analyzed thalli were grouped into two groups according to their sampling sites, different boreal subzones in particular: the middle (The Kivach Reserve, the Zaozersky Sanctuary) and the northernmost (Vodlozersky National Park).

There were no significant differences in SOD activity between *L. pulmonaria* thalli from the forest communities of middle and northernmost boreal subzones (5.5 and 5.7 U mg^{-1} of protein respectively).

The SOD activity in juvenile and mature thalli from the forest communities of the middle boreal subzone averaged 5.4 and 5.6 U mg^{-1} of protein, respectively. The SOD activity differed from the communities of the northern boreal subzone – 6.2 and 4.9 U mg^{-1} protein, respectively. Significant differences were recorded between the SOD activity in juvenile and mature *L. pulmonaria* thalli in the communities of the northern boreal subzone (p = 0.043), as well as in juvenile thalli from different habitats (p = 0.020) *see* Fig. 2.

However, it is difficult to compare obtained quantitative data with the results of other researchers, since the SOD enzyme activity is expressed in conventional units. Therefore, there may be significant differences and modifications in the biochemical conditions and the equations used.

CAT activity in analyzed *L. pulmonaria* thalli showed significant differences in the values between the *L. pulmonaria* thalli from the communities of the Kivach Reserve (614 μmol H_2O_2/mg protein) and the Vodlozersky National Park (378 μmol H_2O_2 mg^{-1} protein, p = 0.048). Thus, lower CAT activity was found in thalli from more northern habitats.

Moreover, according to obtained data, significant differences in CAT activity were found between the *L. pulmonaria* thalli from the middle and northernmost boreal subzone communities where it averaged 605 and 421 μmol H_2O_2 mg^{-1} protein, respectively (p=0.040).

CAT activity in the juvenile and mature thalli in communities of the middle taiga subzone averaged 441 and 578 μmol H_2O_2 mg^{-1} protein, respectively, and in communities of the northern taiga subzone – 187 and 522 μmol H_2O_2 mg^{-1} protein, respectively. Significant differences were found between the CAT activity values in juvenile and mature thalli in the northernmost boreal subzone (p = 0.006), as well as in juvenile thalli from different habitats (p = 0.008) – *see* Fig. 3.

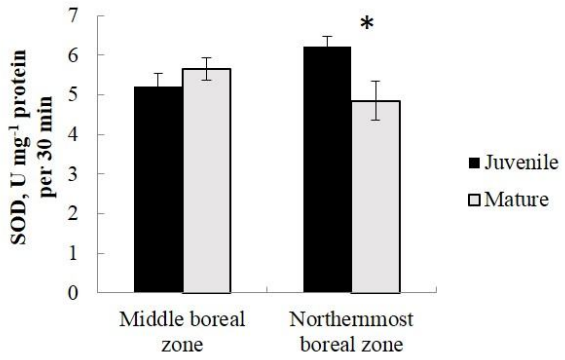

Fig. 2. SOD activity of juvenile and mature *L. pulmonaria* thalli in forest communities of middle and northernmost boreal zone.

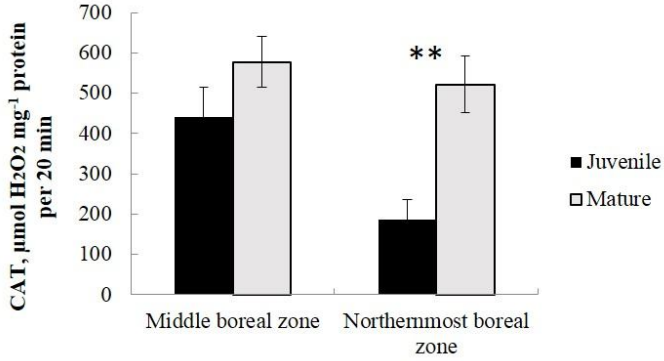

Fig. 3. CAT activity of juvenile and mature *L. pulmonaria* thalli in forest communities of middle and northernmost boreal zone.

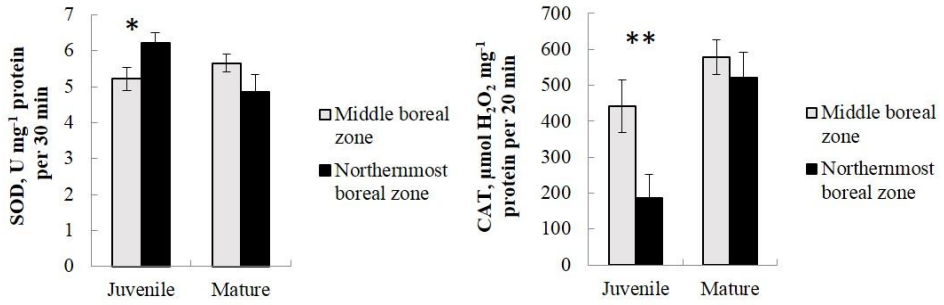

Fig. 4. SOD and CAT activity of *L. pulmonaria* thalli: 1) juvenile and mature thalli of northernmost boreal zone, 2) juvenile thalli from different study area.

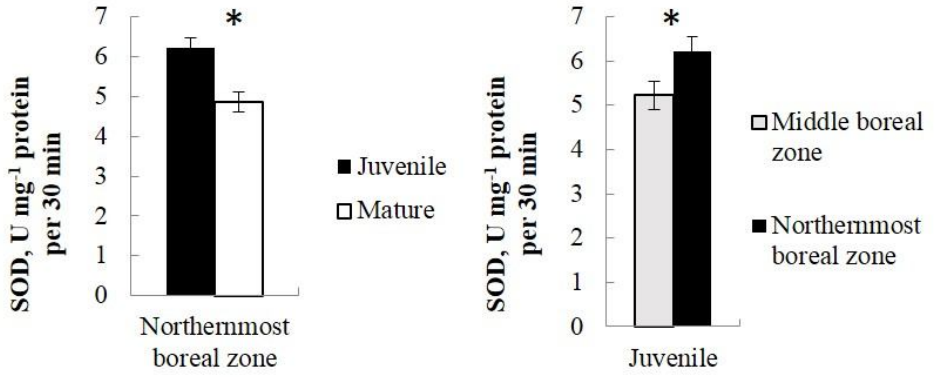

Fig. 5. SOD activity of *L. pulmonaria* thalli: 1) juvenile and mature thalli from northernmost boreal zone, 2) juvenile thalli from different study area.

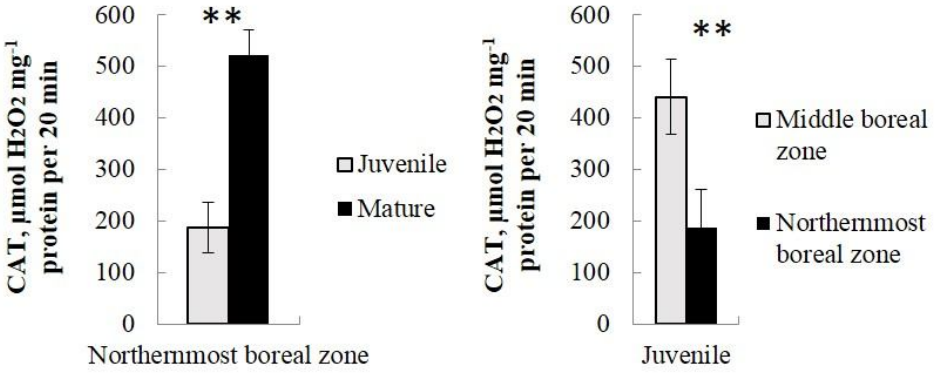

Fig. 6. CAT activity of *L. pulmonaria* thalli: 1) juvenile and mature thalli of northernmost boreal zone, 2) juvenile thalli from different study area.

Studied enzyme	**Middle boreal zone M±m**	**Northernmost boreal zone M±m**
SOD, U mg^{-1} protein per 30 min	5.4±0.22	5.6±0.39
CAT, µmol H_2O_2 mg^{-1} protein per 20 min	605±64	421±83

Table 2. SOD and CAT activity in *L. pulmonaria* thalli from different habitats.
Notes: M - average value, m - standard error.

Discussion

Total soluble protein content is an important indicator of reversible and ireversible alterations in metabolism, and can also be used as a biomarker of environmental conditions, for example, pollution by metals (Singh and Tewari 2003). It was noted Arb and Brunold (1990) that the protein content within thalli of one species can vary significantly. The obtained data on protein content values are in accordance with previous research of *L. pulmonaria* (Riga-Karandinas and Karandinos 1998, Cansaran-Duman et al. 2015) for the total quantitative content as well as for a significant variation in the its values within the species. However, significant differences in protein content were not revealed within a single species study under the influence of various biotic and abiotic factors, for example, pollution (Arb and Brunold 1990). At the present study, we also showed that significant differences in the protein content had not been found either with a change in ontogenetic stages or with change in the habitat. Of course, it can be just natural intraspecific variability tending to show more or less constant value throughout the habitats and environmental factors. It seems to indicate the maintenance of metabolic activity in the thalli of different ontogenetic stages, since there is no degradation of the protein.

It was reported by some authors that lichens can quickly scavenge hydrogen peroxide formed by superoxide radical dismutation (Beckett and Minibayeva 2007). Possible reason is high rate of their metabolic activity. Other reason is the ability of hydrogen peroxide to activate the antioxidant mechanism quickly by involving different enzymes and complex of antioxidants. In non-stressed thalli, peroxide is practically undetectable (Beckett et al. 2005). Probably, such ability to scavenge ROS makes these organisms quite stress-resistant within a wide area.

The obtained data on the AOS enzymes activity showed that within studied samples, SOD did not differ significantly in the *L. pulmonaria* thalli, and the CAT activity the thalli was lower in the communities of northernmost boreal subzone (*see* Table 2). We assume that this may indicate a decrease in the rate of splitting of hydrogen peroxide formed in the superoxide dismutase reaction in thalli from the forest communities of the northernmost boreal subzone.

It is important to note that hydrogen peroxide, that is not neutralized by catalase, has a high permeability across membranes (Allan and Fluhr 1997) and finally migrate through the cell wall. Thus, the role of peroxidase in the neutralization of hydrogen peroxide in the *L. pulmonaria* thalli may increase in forest communi-ties of the northernmost boreal subzone.

We showed that the values of activity of guaiacol-peroxidase were at the detection limit. That makes it impossible to evaluate any differences. Such low values of guaiacol-peroxidase as detected in our study are confirmed by other authors (Plat et al. 1987, Kranner et al. 2003, Morgenstern et al. 2008). They attributed the low values to the ability of several lichens to metabolize guaiacol in the presence of H_2O_2 at low rates.

However, some studies demonstrated that some lichens possess readily detectable peroxidase activities (Liers et al. 2011, Beckett et al. 2013). Probably, it may be necessary to investigate peroxidase activity during the oxidation of other substrates, for example ABTS (Laufer et al. 2006) or pyrogallol (Silberstein et al. 1996). The role of peroxidase has been studied, as well as laccases and tyrosinases in *L. pulmonaria* (*e.g.* Matee et al. 2016), in the case of oxidation of a wide range of secondary metabolites. Laccase production was studied in the other lichens (Lisov et

al. 2007). It can be assumed that laccases and tyrosinases, which actively respond to the changes in environmental conditions, could have played an important role in the processes we studied. They oxidize aromatic phenols to quinones in the presence of oxygen before its superoxide dismutase dismutation and transformation into other ROS. These enzymes, along with peroxidase, are also involved in the synthesis of melanins and various polymers (Ghosh et al. 1998, Thurston 1994, Halaouli et al. 2006, Marusek et al. 2006, Selinheimo et al. 2007), which actively synthesized in *L. pulmonaria* (Gauslaa and Solhaug 2001).

In the present study, we focused on different ontogenetic stages of *L. pulmonaria*. However, studies of the antioxidant system of different ontogenetic stages of *L. pulmonaria* thalli have been missing in the literature. The the importance of evaluation of their ecological and physiological features was, however, emphasized by Gauslaa (2011). In fact, the study of AOS enzymes in the ontogenesis of organisms has received considerable interest in plants (Zhukova et al. 1996, Gupta and Datta 2003, Molassiotis et al. 2004, Foyer and Noctor 2005, Imin et al. 2005, Obert et al. 2005, Pasternak et al. 2005, Kwak et al. 2006, Barba-Espin et al. 2010), but there are still gaps for lichens.

Within the integrated samples, significant differences in SOD activity between study areas were not determined. However, the study of enzyme activity in ontogenetic groups showed that juvenile thalli had more pronounced responses in quantitative values to climate gradients, both in superoxide dismutase and catalase activities. Thus, SOD activity in juvenile thalli was significantly higher in northernmost boreal communities. Additionally, the comparison of SOD in the thalli of different ontogenetic stages from communities of the northernmost boreal subzone showed decreasing in superoxide dismutase activity from juvenile to mature thalli (Fig. 5).

It is established that the rate of respiratory processes is connected with the growth place and indirectly age (Sundberg et al. 1999, Palmqvist and Sundberg 2000). In the Northern latitudes, respiratory costs are higher as well as in young organisms. Moreover, a positive correlation of ergosterol – marker of intense respiration, and the rate of formation of superoxide radical were detected, and the greatest superoxide radical formation occurs in young lichen individuals with pronounced metabolism, including respiratory processes (Beckett et al. 2003, Sundberg et al. 1999, Palmqvist and Sundberg 2000). Therefore, it is likely, that SOD activity is higher in juvenile thalli from the northernmost boreal zone.

By contrast, CAT activity decreases with increasing SOD activity, in juvenile thalli, compared with mature ones in the north and within the juvenile thalli towards the more northern habitats (Fig. 6). Probably, based on our assumptions the mature thalli already accumulate more ROS, including hydrogen peroxide, for their vital activity, which related to increasing of catalase activity. Moreover, decrease in CAT activity in juvenile thalli from forest communities of the northernmost boreal subzone may indicate their response to physical stressors, since they are probably more sensitive to the changes in environmental conditions. In this case, there is an active involvement of antistress responses, that lead to an increased energy expenditures. From this point of view, overall metabolic (synthetic) activity can decrease.

It was reported (Kranner 2002, Kranner et al. 2003) that high activities of antioxidant enzymes do not necessarily indicate adaptation to oxidative stress of individual species. Instead of this, the ability to rapidly re-establish redox state is a characteristic of well-adapted species.

Their reaction could be reflected in a decreasing of the rate of metabolic activity and inhibition of CAT against the back-

ground of energy expenditure on increasing SOD activity. Probably, juvenile thalli could manage to adapt to the habitat conditions. A confirmation of this assumption is a substantial decrease in CAT in juvenile thalli in northernmost boreal communities compared with thalli in the middle boreal subzone.

The results of the present work showed that juvenile individuals may be suitable for the purpose of bioindication of environmental conditions, especially temperature and humidity, because of their sensitivity to these factors. The juvenile samples from northern collection sites had higher SOD activity and showed lower CAT activity comparing with mature ones.

Certainly, to clarify the role of peroxidase, it is necessary to study a wide range of its isoforms with regard to the oxidation of various substrates. This can be achieved probably by using phenolic compounds of the lichen, which could be oxidized by peroxidase to neutralize excess hydrogen peroxide amounts. The concentration of hydrogen peroxide should also be determined, since its high content may become a biomarker of a stress state.

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