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EXPERIMENTAL ARTICLES

Laccase and Tyrosinase Activities in Lichens

A. G. Zavarzina[a, 1] and A. A. Zavarzin[b]

[a] *Department of Soil Chemistry, Faculty of Soil Science, Moscow State University, Moscow, 119992 Russia*
[b] *Biological Research Institute, St. Petersburg State University, St. Petersburg, 199034 Russia*

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Abstract—Phenoloxidase activity was found in lichenized ascomycetes belonging to different taxonomic groups. Most of the epigeic and epilithic lichens of the order *Peltigerales* were found to possess both laccase and tyrosinase activities; the lichens of the order *Lecanorales* possessed only laccase activity, which was an order of magnitude lower than that of *Peltigerales*. Water-soluble phenoloxidases were present only in peltigerous lichens: activity that could be washed out from intact thalli comprised 10% of that released from disrupted thalli. The activity of the peltigerous lichens and the release of soluble phenoloxidases into the medium increased when the thalli were rehydrated quickly. In some of the lichens tested, the phenoloxidase activity was stimulated by desiccation–rehydration cycles. The oxidases discovered may play an important role in the phenolic metabolism of lichens and be involved in the biochemical reaction of humus synthesis during primary soil formation, which may be a previously unknown geochemical function of these symbiotic microorganisms.

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Lichens are symbiotic microorganisms representing an association of a fungus (usually an ascomycete) and a phytobiont, which may be an alga and/or a cyanobacterium. The presence of a photosynthesizing partner allows the lichens to inhabit substrates that are virtually completely devoid of organic matter; in addition, lichens possess a number of morphological and chemical adaptations enabling them to survive stressful conditions and to quickly restore their metabolic activity [1, 2]. These and other characteristics enable lichens to exist in oligotrophic habitats and severe climatic zones and to dominate in the communities on approximately 6–8% of the terrestrial surface.

Lichens are pioneer organisms that colonize rocky substrates; they participate in at least two global-scale processes: weathering of rocks with the formation of fine earth [3] and the accumulation of organic matter on its surface [1], resulting in the formation of primitive soils. The involvement of lichen enzymes in pedogenic processes is currently unknown [3].

In the course of field interdisciplinary investigations of primitive soil formation under lichen vegetation, we discovered that many lichens are able to produce phenoloxidases of the laccase and tyrosinase type and to release them into the environment. Laccase (EC 1.10.3.2., diphenol : O_2 oxidoreductase; *p*-diphenol oxidase) and tyrosinase (EC 1.14.18.1., monophenol monooxygenase; *o*-diphenol oxidase) are copper-containing oxidases catalyzing the oxidation of a wide spectrum of phenolic and related substrates by molecular oxygen. In the course of the reaction, oxygen is reduced to water, and the substrate is oxidized to unstable phenoxy radicals and quinones, which undergo spontaneous polymerization [4, 5]. Laccases and tyrosinases occur in higher plants, free-living fungi, and bacteria, participating in phenolic metabolism and pigment, lignin, and melanin syntheses [5–7]. In addition, being released into the environment, these enzymes are involved in delignification [8, 9] and in the synthesis [10–12] and degradation [13, 14] of humus. The important physiological and biospheric functions of laccases and tyrosinases determine the necessity of unraveling their role in lichen metabolism, as well as their possible involvement in biochemical reactions of pedogenesis, which may be a so far unknown geochemical function of lichens.

This paper presents quantitative data on the laccase and tyrosinase activities in 32 lichens representing various taxonomic groups and the results of investigation of the factors that influence the activity of phenoloxidases of lichen thalli and their release into the environment.

MATERIALS AND METHODS

Microorganisms and the field screening of enzyme activity. The lichens were collected at the Kivach State Nature Reserve (Republic of Karelia, Russia) in summer 2004 under rainy weather condi-

[1] Corresponding author; e-mail: zavarzina@mail.ru

tions and at the same site, as well as on the Karelian Isthmus (Leningrad oblast) and in the Khibiny Mountains (Kola Peninsula, Murmansk oblast) in summer 2005 under dry weather conditions.

Preliminary screening for phenoloxidase activity of lichens was carried out under field conditions by introducing thalli into 5 mM sodium–acetate buffer (pH 4.5) containing 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). ABTS oxidation was judged by the development of green coloration of the solution. Additionally, a test for the presence of tyrosinase activity was performed by placing thalli in 50 mM sodium–acetate buffer (pH 5.5) containing L-tyrosine (Serva, Germany) and monitoring development of pink coloration of the solution. The reaction mixture to which sodium azide (1 mM) was added served as the control. The lichen thalli with a positive reaction in the field experiments were collected, air dried, and stored at room temperature for further study in the laboratory.

The laboratory experiments used intact or disrupted thalli. In the first case, whole thalli (*Solorina crocea*, *Peltigera venoza*, *P. elizabethae*) were used, or 2-cm cuttings, if thethallus was large; in the second case, the thalli were reduced to 1–2-mm pieces. The samples thus prepared were air-dried and then rehydrated by dropwise addition of distilled water until full saturation of the tissues.

Determination of the enzyme activity. The enzyme activity was determined at 22°C. The phenoloxidase activity was determined from the oxidation rate of 0.2 mM ABTS (Sigma, USA) in 50 mM sodium–acetate buffer (pH 4.5), as well as from the oxidation rate of 5 mM L-dihydroxyphenylalanine (L-DOPA, Serva, Germany) in 50 mM sodium–acetate buffer (pH 5.0). The oxidation of ABTS to a stable cation radical was monitored by the increase in absorption at 436 nm (ε_{436} 29 mM^{-1} cm^{-1}). The oxidation of L-DOPA to dopachrome was monitored by the increase in absorption at 475 nm (ε_{475} 3.6 mM^{-1} cm^{-1}). In both cases, the absorption was measured every minute for the lichens of the order Peltigerales and every 10 min for the lichens of the order *Lecanorales*.

The laccase activity was determined from the oxidation rate of 0.5 mM syringaldazine (Sigma, USA) in 30% ethanol solution (pH 5.2). The oxidation of syringaldazine to the corresponding quinone was monitored by the increase in absorption at 525 nm (ε_{525} 65 mM^{-1} cm^{-1}). The lichens or thallus water extracts were preincubated with the substrate until stable coloration appeared (usually 10 min), and then measurements at 1-min intervals were made.

The tyrosinase activity was determined from the oxidation rate of 5 mM L-tyrosine (Serva, Germany) in 50 mM sodium–acetate buffer (pH 5.5). The absorption was measured at 470 nm (ε_{470} 3.7 mM^{-1} cm^{-1}) every 30 min. Since tyrosine appeared to be too slowly metabolized by lichen water extracts, the tyrosinase activity in them was also determined as the difference between the oxidation rates of ABTS or L-DOPA in the absence and presence of the tyrosinase inhibitors CO [15, 16] and 4-hexylresorcinol [17], respectively. In the former case, 200 μl of the water extract in 600 μl of acetate buffer (pH 4.5) was saturated with CO and kept for 10 min in a closed Eppendorf tube. After that, 400 μl of 0.8 mM ABTS was added (the final concentration in the reaction mixture was 0.2 mM), and the activity was measured according to the standard procedure. In the latter case, 200 μl of the water extract from the lichen thallus was incubated with 100 μl of hexylresorcinol for 10 min; 700 μl of L-DOPA in acetate buffer (pH 5.0) was then added and the activity was measured as described above.

The reaction mixtures, to which sodium azide was added to attain a concentration of 1 mM, were used as the controls of abiotic substrate oxidation. The enzyme activity was expressed in arbitrary units as a change in the absorption induced in 1 min by 1 l of lichen extract (ΔA_λ min^{-1} l^{-1}) or by 1 kg of the dry mass of lichen (ΔA_λ min^{-1} kg^{-1}). All the spectrophotometric measurements were made using a Specol 11 spectrophotometer (Germany). The tables show the mean activity values and their standard deviations.

The activity of water extracts from the lichen thalli. The phenoloxidases were extracted from the lichens 20 min after thallus rehydration. Distilled water at a thallus/water ratio of 1 : 50 was added to intact thalli. In order to assess the extraction kinetics of the enzymes from the thalli, the mixtures were gently agitated on a shaker for 2 h, with taking solution aliquots for activity measurements every 15 min. The sample volume for determining the enzyme activity was 50 to 100 μl (200 μl for measurements in the presence of the inhibitors); the total volume of the reaction mixture was 1 ml. Data in the tables on the enzyme activities in the lichen water extracts correspond to the measurements made 1 h after the beginning of extraction. The activities were determined in three biological replicates.

The lichen thallus activity. After extracting the soluble enzymes, the thalli were rinsed with distilled water and placed in an open exsiccator over water vapors for 24 h. The thallus enzyme activity was determined by adding 2 ml of substrate to 50 mg (the order *Peltigerales*) or 100 mg (the order *Lecanorales*) of thallus and measuring an increase in the absorption of the liquid phase as described above. The activities were determined in five replicates.

Effect of rehydration conditions on the phenoloxidase activity of the lichens. The activity of the lichens rehydrated by the dropwise addition of water to the air-dry thallus (fast rehydration) was compared to the activity of the thalli rehydrated in closed exsiccators over water vapors for 24 h (slow rehydration). In the first case, the initial thallus activity was measured 1 h after rehydration, immediately after the extraction of the soluble phenoloxidases. In the second case, the initial thallus activity was measured 24 h after placing the

lichens in the exsiccator. The lichens were then rinsed thoroughly with water and placed in open exsiccators over the water vapors for 24 h with subsequent repeated measurements of the thallus activities. In addition, the enzyme activities in water extracts from the lichens were determined. The water extracts were also used for determining the loss of K^+ ions, i.e., for assessing the level of stress caused by rehydration. The potassium concentration was measured by the atom absorption method using the AAS-3 spectrophotometer (Germany).

The influence of desiccation–rehydration on the phenoloxidase activity of the lichens. The thalli of five species of lichens were subjected to three sequential rehydration–desiccation cycles. Air-dry thalli were rehydrated by dropwise addition of distilled water, soluble enzymes were removed by extraction as described above, and the initial thallus activity was measured with ABTS. The thalli were then air dried at room temperature, after which the rehydration procedure and measurement of the oxidase activity were repeated. The data reported were obtained as a result of parallel experiments in which the activity of each thallus was measured only once, i.e., after the first, second, or third rehydration/desiccation cycle, in order to rule out the possible influence of ABTS on the lichen phenoloxidase activity.

Comparison of phenoloxidase release from intact and disrupted thalli. Five species of the *Peltigerales* lichens were used for analysis: *Peltigera aphthosa, P. praetextata, Lobaria pulmonaria, Nephroma arcticum*, and *Solorina crocea*, as well two species of the order *Lecanorales*: *Cladonia arbuscula* and *C. rangiferina.* Intact thalli (2-cm cuttings or whole thalli) or thalli reduced to 1–2-mm pieces were rehydrated by dropwise addition of distilled water with subsequent enzyme extraction with distilled water. The thallus–water ratio was 1 : 50; the extraction time was 1 h. The enzyme activity in the extracts was determined as described above.

RESULTS AND DISCUSSION

General assessment of the phenoloxidase activity in lichens. In the field experiment, the presence of phenoloxidase activity (as determined from the capacity to oxidize ABTS) was studied in 72 species of lichens. All the lichens studied represented a symbiotic association of an ascomycete with an alga (the genera *Dyctyochloropsis*, C*occomyxa, Trebouxia*) and/or a cyanobacterium (of the genera *Nostoc* and *Stigonemia*) [18]. In the 2004 field test, a positive reaction was established in 22 of the 25 lichens studied (Table 1), except for C*ladonia stellaris*, *Parmelia sulcata*, and *Vulpicida pinasti.* In summer 2005, an additional 47 species of lichens were investigated; moreover, repeated tests with the lichens studied during the previous field season were carried out. ABTS and L-tyrosine were used as test substrates. A positive reaction with ABTS was revealed in 19 newly tested species (Table 1) and was absent in the following 28 lichens: *Alectoria nigricans, A. ochroleuca, Aspicillia* cf. *caesiocinerea, Baeomyces placophyllus, B. rufus, Cetraria muricata, Cetrariella delisei, Cladonia cenotea, C. furcacta, C. squamosa, C. stellaris, Flavocetraria cuculata, Lasallia pustulata, Lecanora argentata, Parmelia saxatilis, P. sulcata, Physcia aipolia, Pseudephebe pubescens, Pseudevernia furfuracea, Sphaerophorus fragilis, Stereocaulon evolutum, Thamnolia vermicularis, Umbilicaria deusta, U. hyperborea, U. polyphylla, U. torrefacta*, and *U. vellea.* Moreover, the reaction with ABTS was absent for *Cladonia uncialis, C. arbuscula, C. borealis*, and *C. rangiferina*, which oxidized ABTS in the first year of our studies. ABTS oxidation was completely inhibited upon the addition of sodium azide (1 mM) to the reaction mixture, which rules out the abiogenic nature of the reaction and implies the presence of phenoloxidases in the lichens.

Thus, in the field experiments, phenoloxidase activity was revealed in all the lichens representing the order *Peltigerales*; many of them also oxidized L-tyrosine (data not shown). Of the Lecanorales representatives, phenoloxidase activity was detected only in 21 of the 52 lichens studied. These lichens oxidized ABTS but did not oxidize L-tyrosine. Based on the data obtained, 32 of 43 species of lichens showing a positive reaction with ABTS in different field seasons were chosen for further laboratory experiments. The choice was determined by the presence of a sufficient amount of material for studies.

Before carrying out the experiments on quantitative determination of the activity of lichens, the temporal dynamics of the phenoloxidase activity was studied for 72 h, beginning from the moment of thallus rehydration, i.e., form the moment when the lichens were caused to be metabolically active (data not shown). The phenoloxidase activity in most peltigerous lichens attained the maximum value 24 h after rehydration and then reached a plateau or slightly decreased. Thus, 72 h after rehydration, the activity of the peltigerous lichens constituted 80–90% of that recorded after 24 h, except for *Solorina crocea*, for which the activity was maximum immediately after rehydration and then decreased over 72 h to 30% of the initial activity. In representatives of the genus *Cladonia*, the capacity for ABTS oxidation was virtually absent immediately after rehydration but then increased and peaked in the subsequent 24–48 h. *Arctoparmelia centrifuga* and *Stereocaulon vezuvianum* exhibited the highest phenoloxidase activity 72 h after rehydration. A slow increase in the activity in the Lecanorales lichens after their rehydration may explain the differences between the results of the 2004 and 2005 field experiments (Table 1). In 2004, the lichens were collected wet because of rainy weather, and in 2005, these species were collected in the dry state and placed immediately in the buffer with ABTS; thus, the time of their rehydration might have been insufficient. Since the highest phenoloxidase activity in

Table 1. Screening for phenoloxidase activity in lichens

Lichens	Field test		Laboratory experiments	Lichens	Field test		Laboratory experiments
	2004	2005			2004	2005	
The order *Peltigerales*				*Bryoria oroarctica* (Krog) Goward	+	–	
Lobaria pulmonaria (L.) Hoffm.	+	+	+	*Cetraria islandica* (L.) Ach.	+	–	
Nephroma arcticum (L.) Torss.		+	+	*Cladonia amaurocracea* (Florke)			
N. bellum (Spreng.) Tuck.	+		+	Schaer.	+	–	
N. parile (Ach.) Ach.	+		+	*C. arbuscula* (Wallr.) Flot.	+	–	+
N. resupinatum (L.) Ach.		+	+	*C. borealis* S.Stenroos	+	–	
Peltigera aphthosa (L.) Willd.		+	+	*C. gracilis* (L.) Willd.	+	–	+
P. canina (L.) Willd.	+	+	+	*C. pleurota* (Florke) Schaer.		+	+
P. didactyla (With.) J.R.Laundon		+	+	*C. rangiferina* (L.) F.G.Wigg.	+	–	+
P. elizabethae Gyeln.		+	+	*C. stygia* (Fr.) Ruoss		+	+
P. extenuata (Vain.) Lojka		+	+	*C. sulphurina* (Michx.)Fr.	+	–	
P. horizontalis (Huds.) Baumg.		+		*C. uncialis* (L.)Weber & F.H.Wigg.	+	–	+
P. leucophlebia (Nyl.) Gyeln.		+	+	*Flavocetraria nivalis* (L.)			
P. malacea (Ach.) Funck		+	+	Karnefelt &Thell		+	+
P. neopolydactyla (Gyeln.) Gyeln.	+	+	+	*Hypogymnia physodes* (L.) Nyl.	+	–	
P. polydactylon (Neck.) Hoffm.		+		*Melanelia olivacea* (L.) Essl.	+	–	
P. ponojensis Gyeln.		–	+	*Platismatia glauca* (L.)			
P. praetextata				W.L.Culb. &C.F.Culb.		+	+
(Florke ex. Sommerf.) Zopf	+	+	+	*Stereocaulon vesuvianum* Pers.	+	–	+
P. rufescens (Weiss.) Humb.	+	+	+	*Stereocaulon.* sp.		+	+
P. scabrosa Th.Fr.		+	+	*Usnea filipendula* Stirt.	+		+
P. venosa (L.) Hoffm.		+	+	*Vulpicida pinastri* (Scop.)			
Solorina crocea (L.) Ach.		+	+	J.-E.Mattsson & M.J.Lai	–	–	
The order *Lecanorales*				Xanthoparmelia somloensis			
Arctoparmelia centrifuga (L.) Hale	+	–	+	Gyeln.) Hale		+	+
Brodoa intestiniformis (Vill.) Goward	+	–					

The systematic position of the species is according to [35]; the nomenclature, according to [36].
Designations: "+," positive reaction with ABTS; "–," absence of reaction with ABTS; blank space means that the test was not performed.

most lichens was recorded 24 h after rehydration, this time period was chosen for further comparative experiments.

The highest phenoloxidase activity was revealed in the lichens of the order *Peltigerales* (Table 2). All the peltigerous lichens oxidized the laccase substrates: ABTS and syringaldazine. The soil-stabilizing lichen *Solorina crocea* exhibited the highest activity. In addition, the peltigerous lichens oxidized L-DOPA, which is the substrate commonly used for determining the tyrosinase activity, although it may also be oxidized by laccases [17, 19]. The presence of the tyrosinase activity in the peltigerous lichens is confirmed by the capacity of most representatives of this group for oxidation of the specific tyrosinase substrate L-tyrosine (the highest activity was shown by *Peltigera leucophlebia, P. scabrosa*, and *P. elizabethae*). As for the representatives of the order Lecanorales, the capacity for oxidizing ABTS was an order of magnitude lower in them (Table 3), and no oxidation of syringaldazine, L-tyrosine, and DOPA was revealed at all.

The experimental technique allowed the possible contribution of peroxidases to the oxidation of the substrates used to be completely excluded. First, measurements of the activity were carried out in the absence of added hydrogen peroxide. Second, it was shown earlier [20] that lichens produce hydrogen peroxide in response to stress (rehydration immediately after desiccation); however, it disappears quickly: its concentration in the medium decreases fourfold over 1 h. In our work, determinations of the lichen activity were not performed until 1 h after rehydration (stress). And,

Table 2. Phenoloxidase activity of *Peltigerales* lichens

Lichen	Activity, ΔA_λ min^{-1} kg^{-1}			
	ABTS	Syringaldazine	L-DOPA	L-tyrosine
Peltigera aphthosa	1470 ± 30	1815 ± 92	4150 ± 50	78 ± 14
P. canina	1420 ± 180	2442 ± 586	1800 ± 200	51 ± 13
P. didactyla	1580 ± 140	1345 ± 148	2142 ± 415	28 ± 9
P. elizabethae	402 ± 10	560 ± 32	530 ± 50	133 ± 40
P. extenuata	1360 ± 250	–	–	75 ± 4
P. leucophlebia	1420 ± 80	1170 ± 184	2000 ± 100	103 ± 11
P. malacea	1150 ± 150	1345 ± 111	1330 ± 150	27 ± 3
P. neopolydactyla	1100 ± 150	509 ± 45	3400 ± 990	51 ± 5
P. ponojensis	1390 ± 130	–	–	–
P. praetextata	1140 ± 80	2538 ± 196	2400 ± 300	54 ± 8
P. rufescens	630 ± 30	1321 ± 120	1637 ± 405	320 ± 94
P. scabrosa	1240 ± 80	1010 ± 129	1900 ± 100	120 ± 10
P. venosa	140 ± 10	–	–	0 ± 0
Lobaria pulmonaria	200 ± 10	1200 ± 33	470 ± 160	31 ± 6
Nephroma arcticum	880 ± 20	1179 ± 98	1750 ± 250	42 ± 9
N. bellum	740 ± 130	–	–	0 ± 0
N. parile	120 ± 10	–	–	–
N. resupinatum	210 ± 20	–	–	–
Solorina crocea	2130 ± 420	2465 ± 205	1300 ± 100	11 ± 1

finally, preliminary experiments showed that the addition of hydrogen peroxide (0.2–5 mM) to the reaction mixture containing ABTS did not increase the activity as compared to the hydrogen-peroxide-free experiments (data not shown). Despite the fact that the presence of peroxidases in lichens has been shown earlier [21, 22], our data provide evidence of either the absence of peroxidases in the species studied or the absence of the peroxidase activity under the conditions of our experiments.

Table 3. Phenoloxidase activity of the *Lecanorales* lichens

Lichen	Activity with ABTS, ΔA_{436} min^{-1} kg^{-1}
Arctoparmelia centrifuga	64 ± 10
Cladonia arbuscula	32 ± 12
C. pleurota	15 ± 5
C. rangiferina	24 ± 5
C. uncialis	11 ± 3
C. stygia	15 ± 2
Flavocetraria nivalis	11 ± 3
Hypogymnia physodes	21 ± 3
Platismatia glauca	27 ± 11
Stereocaulon vezuvianum	34 ± 5
Stereocaulon sp.	75 ± 5
Usnea fillipendula	32 ± 9
Xanthoparmelia somloensis	21 ± 3

The capacity for ABTS oxidation in the absence of hydrogen peroxide by the representatives of different taxonomic groups of lichens suggests wide distribution of phenoloxidases among lichens. The oxidation of syringaldazine, considered to be a specific laccase substrate [23], as well as of L-tyrosine, a specific tyrosinase substrate [16], by the *Peltigerales* lichens leads us to assume the presence of both the laccase and tyrosinase types of enzymes in their thalli. The production of laccases by the peltigerous lichens is confirmed by the recent data on the extracellular laccase from *Peltigera malacea* [24] and by our data on purification and characterization of laccases from *Solorina crocea* (Lisov et al., unpublished). No data on tyrosinase activity in lichens have been published so far; however, the recent investigations of the R. Beckett and F. Minibaeva in this field (personal communication) support our suggestion that tyrosinases are present in representatives of the peltigerous lichens. The question as to whether lichens of other taxonomic groups also produce tyrosinases awaits further studies: the attempts to obtain positive results with DOPA and L-tyrosine, substrates specific to this groups of enzymes, have so far been unsuccessful.

Table 4. Phenoloxidase activity of the water extracts from intact thalli of the *Peltigerales* lichens

Lichen	Activity, ΔA_λ min^{-1} l^{-1}					
	ABTS	Syringaldazine	*L*-DOPA	*L*-tyrosine	ABTS + CO	*L*-DOPA + HR[a]
Peltigera aphthosa	190 ± 43	+	20 ± 5	0 ± 0	149 ± 21 (78)[b]	20 ± 5 (100)
P. praetextata	173 ± 32	+	11 ± 2	0 ± 0	118 ± 20 (30)	6 ± 1 (55)
P. canina	131 ± 31	+	20 ± 2	0 ± 0	46 ± 5 (35)	20 ± 2 (100)
P. didactyla	158 ± 65	+		0 ± 0	85 ± 11 (54)	
P. elizabethae	200 ± 21	+	9 ± 2	0 ± 0	85 ± 13 (53)	6 ± 1 (67)
P. extenuate	120 ± 41			0 ± 0	36 ± 9 (30)	
P. leucophlebia	354 ± 42	+	120 ± 40	0 ± 0	246 ± 33 (67)	36 ± 18 (40)
P. malacea	363 ± 40	+	60 ± 10	0 ± 0	100 ± 15 (28)	39 ± 6 (65)
P. neopolydactyla	270 ± 31	+	4 ± 1	0 ± 0	270 ± 40 (100)	2 ± 1 (50)
P. ponojensis	370 ± 13			0 ± 0		
P. rufescens	130 ± 21	+				
P. scabrosa	120 ± 20	+	34 ± 9	0 ± 0	50 ± 7 (42)	26 ± 4 (76)
P. venosa	70 ± 20					
Lobaria pulmonaria	100 ± 10	–	18 ± 6	0 ± 0	33 ± 6 (23)	9 ± 2 (50)
Nephroma arcticum	80 ± 10	+	16 ± 4	0 ± 0	22 ± 3 (28)	8 ± 2 (50)
Solorina crocea	1700 ± 50	300 ± 21	276 ± 36	0 ± 0	600 ± 72 (34)	218 ± 46 (80)

[a] Hexylresorcinol.

[b] In parentheses, the percentage of activity without the inhibitors is indicated.

In the course of measurement of the enzyme activities in lichens of the order Peltigerales, we noted a change in the color of the lower (fungal) thallus surface. *Peltigera neopolydactyla* showed coloring of the whole lower surface. In *Peltigera aphthosa* and *P. malacea*, the thallus edge was colored (as a wide band); in the former species, coloration subsequently involved the whole of the lower surface. The veins acquired coloration in *Peltigera canina, P. praetextata*, and *P. rufescens*; in *Lobaria pulmonaria* and *Nephroma arcticum*, the coloration appeared as a reticular pattern on the lower surface. All the lichens indicated stained violet in the presence of ABTS, orange in the presence of L-DOPA, and wine-red in the presence of syringaldazine. These observations may be indirect evidence of the lichen mycobiont being responsible for the phenoloxidase synthesis.

Water extraction of phenoloxidases from the lichen thalli. Sixteen species of lichens from the order *Peltigerales* and several representatives of the order *Lecanorales* (*Cladonia* spp.) were studied for the presence of water-soluble phenoloxidases that can be extracted from thalli with distilled water (simulation of rainfall). The phenoloxidase activity was revealed only in the water extracts from representatives of the peltigerous lichens of the genera *Peltigera, Solorina*, and *Lobaria* and one species of the genus *Nephroma* (*N. arcticum*) (Table 4). *Solorina crocea* appeared to be the most active producer of the water-soluble enzyme (Table 4). The phenoloxidases were extracted from the thalli relatively quickly: complete extraction was normally achieved within 30–60 min (Fig. 1). The activity was virtually absent in repeated water extracts from thalli (data not shown). The water extracts of most of the peltigerous lichens oxidized syringaldazine, which gives evidence of the presence of laccases; however, the reaction rate was too low for quantitative assessment to be made, except for the *Solorina crocea* extract. The water extracts from peltigerous lichens did not oxidize L-tyrosine; however, the inhibition of the enzyme activity by carbon monoxide and hexylresorcinol when ABTS and DOPA, respectively, were the substrates allows us to suggest the presence of soluble tyrosinases as well. In the lecanorous lichens studied, phenoloxidase activity was strongly associated with thalli; no water-soluble enzymes were found.

The influence of stressful conditions on the phenoloxidase activity of the lichen thalli and/or on the extraction of the enzymes. Under natural conditions, lichens are subject to different stressful conditions, primarily to periodical desiccation, rapid rehydration, and mechanical injury. A possible influence of stressful conditions on the activity of phenoloxidases and their release from thalli was studied by in the *Peltigerales* lichens.

The eleven species studied revealed, on the whole, a similar effect of the rehydration conditions on the phenoloxidase activity (Table 5). Rapid rehydration

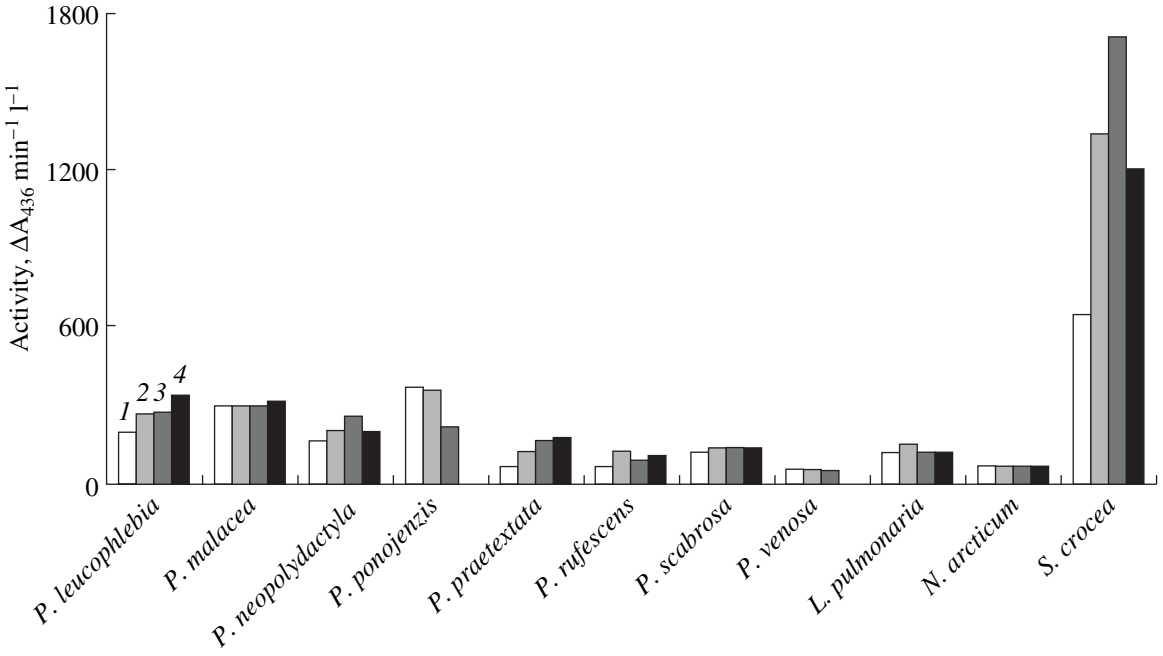

Fig. 1. Kinetics of the release of soluble phenoloxidases from intact lichen thalli: (*1*) 5 min; (*2*) 30 min; (*3*) 60 min; (*4*) 120 min.

increased the release of phenoloxidases from the thalli of most of the lichens studied, this tendency being most markedly pronounced in *Peltigera leucophlebia* and *Solorina crocea.* More significant release of phenoloxidases during rapid rehydration was accompanied by a substantial loss of potassium by the lichen thalli. The potassium loss can be used as indirect estimate of the rehydration stress, because virtually all the potassium in the lichens is present in a soluble form in the cytoplasm [25], and fast addition of water with a low mineral content to a dry lichen causes the cells to lose potassium.

Table 5. Phenoloxidase activity of the lichens and the release of soluble phenoloxidases upon rapid and slow thallus rehydration

Lichen	Rapid rehydration			Slow rehydration			ΔK^{+a}, μM
	Activity in the water extract, ΔA_{436} min^{-1} l^{-1}	Thallus activity, ΔA_{436} min^{-1} kg^{-1}		Activity in the water extract, ΔA_{436} min^{-1} l^{-1}	Thallus activity, ΔA_{436} min^{-1} kg^{-1}		
		initial	after 24 h		initial	after 24 h	
P. aphthosa	190 ± 40	921 ± 22	1470 ± 30	200 ± 30	1380 ± 162	1290 ± 240	0.23
P. canina	130 ± 20	1221 ± 180	1221 ± 180	70 ± 10	1082 ± 203	982 ± 201	0.12
P. leucophlebia	350 ± 40	1430 ± 141	1422 ± 81	90 ± 10	1340 ± 140	1110 ± 182	0.54
P. malacea	360 ± 40	952 ± 33	1152 ± 150	270 ± 20	1071 ± 110	1100 ± 100	–0.3
P. neopolydactyla	270 ± 30	1000 ± 42	1100 ± 151	200 ± 20	622 ± 52	992 ± 150	1.08
P. praetextata	170 ± 30	981 ± 101	1140 ± 40	150 ± 20	661 ± 101	701 ± 150	0.83
P. scabrosa	120 ± 20	960 ± 60	1242 ± 81	120 ± 20	572 ± 50	630 ± 112	0.50
P. venosa	70 ± 10	203 ± 10	140 ± 11	70 ± 10	200 ± 60	200 ± 52	0.08
L. pulmonaria	100 ± 10	200 ± 22	240 ± 10	50 ± 2	180 ± 50	242 ± 60	–0.2
N. arcticum	80 ± 10	682 ± 140	881 ± 20	60 ± 2	620 ± 120	831 ± 200	0.27
S. crocea	1700 ± 50	3310 ± 91	2130 ± 420	900 ± 10	1901 ± 173	1430 ± 90	0.65

[a] The difference between potassium concentrations in the water extracts from the lichens upon rapid and slow thallus rehydration.

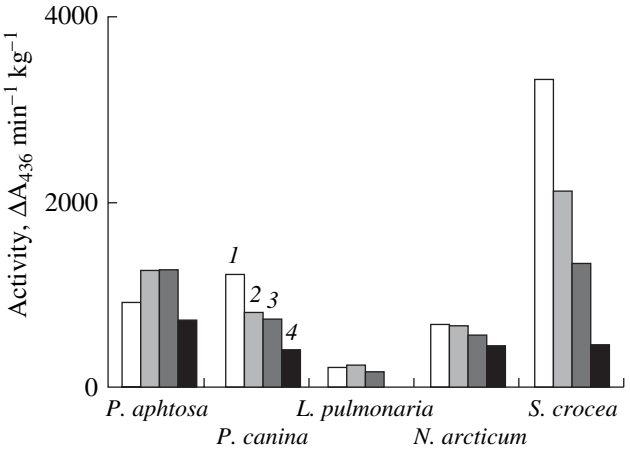

Fig. 2. Effect of desiccation/rehydration cycles on the phenoloxidase activity of lichens: (*1*) the initial thallus activity; (*2*) the activity after the first desiccation/rehydration cycle; (*3*) the activity after the second desiccation/rehydration cycle; (*4*) the activity after the third desiccation/rehydration cycle.

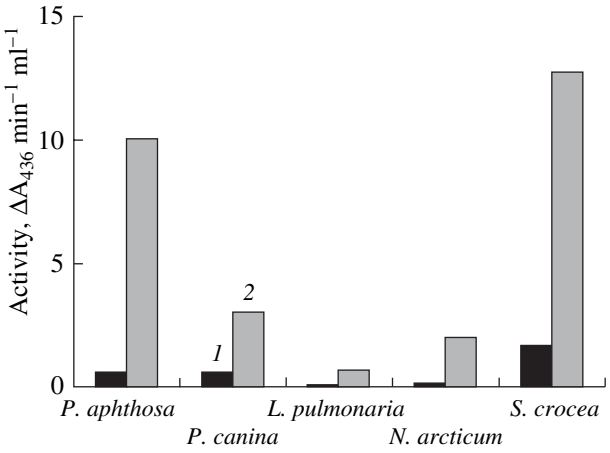

Fig. 3. Release of water-soluble phenoloxidases from (*1*) intact thalli and (*2*) homogenized thalli.

Different modes of rehydration also influenced the phenoloxidase activity of the thalli themselves. In *Peltigera aphthosa*, *P. neopolydactyla, P. scabrosa*, and *Solorina crocea*, the thallus phenoloxidase activity was higher following rapid rehydration. In *Peltigera canina, P. leucophlebia, P. venosa, Nephroma arcticum*, and *Lobaria pulmonaria*, the differences in the rehydration conditions did not markedly influence the phenoloxidase activity of the thalli (Table 5). 24 h after the measurement of the initial phenoloxidase activity, the activity became similar in the same species of lichens subjected to either fast or slow rehydration. The stimulating effect of fast desiccation on the phenoloxidase activity of the lichens agrees with the data of the other studies [26].

The influence of several desiccation/rehydration cycles on the thallus phenoloxidase activity was studied in five representatives of the peltigerous lichens. Except for *Solorina crocea*, whose phenoloxidase activity decreased substantially even after the first desiccation following the first rehydration, two sequential rehydration/desiccation cycles either did not influence significantly (*Nephroma arcticum* and *Peltigera canina*) or even stimulated (*Peltigera aphthosa* and *Lobaria pulmonaria*) the phenoloxidase activity of the lichens (Fig. 2). It may be suggested on the basis of these data that the phenoloxidases associated with a lichen thallus are either comparatively resistant to desiccation, or the transition of the thallus from anabiosis to a metabolically active state stimulates their production. The first suggestion is supported by the fact that the phenoloxidase activity of air-dry lichens remained virtually unchanged throughout several months of storage (data not shown). The second suggestion agrees with the data [24] that showed stimulation of the laccase activity upon rehydration in *Pseudocyphellaria aurata.*

The loss of integrity of the *Peltigera aphthosa*, *P. praetextata, Lobaria pulmonaria*, and *Nephroma arcticum* thalli by their reduction to 1–2-mm particles resulted in a practically tenfold increase in the phenoloxidase activity with ABTS in the extracts (Fig. 3): the activity of the water extracts from intact thalli constituted only 5–10% of the activity of the water extracts from the disintegrated lichens. Even in this experiment, *Solorina crocea* was characterized by maximum enzyme release. In addition to the capacity for oxidizing ABTS, the water extracts also oxidized L-DOPA (data not shown) but did not oxidize L-tyrosine. The extracts from the disintegrated thalli of *Cladonia arbuscula* and *C. rangiferina* were not capable of oxidizing ABTS. A sharp increase in the phenoloxidase activity in extracts from disintegrated thalli (as compared to intact thalli) was probably linked to extraction of intrathallus (intracellular) phenoloxidases but was not caused by stimulation of the phenoloxidase activity by injury, as in the studies [24], since the lichens were destroyed in the dry (anabiotic) state.

The physiological role of phenoloxidases in lichens. The synthesis of phenoloxidases of the laccase and tyrosinase types in the thalli of lichens from different taxonomic groups implies, above all, the involvement of these enzymes in the metabolism of these symbiotic organisms.

Phenoloxidases are important enzymes of secondary metabolism in plants and fungi; they are involved in the synthesis of melanin and other pigments, which act as a screen protecting them from an excess of light and decrease the permeability of cell walls to toxic substances and pathogens. The capacity of phenoloxidases for catalyzing the polymerization reactions of the phenolic compounds implies their involvement in the synthesis of lignin, the main structure-forming cell wall component in plants, as well as in the removal of some secondary metabolites and low-molecular-weight phenols by their conversion to high-molecular-weight non-

toxic compounds. Thus, phenoloxidases in plants and fungi are considered to perform protective functions [5, 16].

It may be suggested, by analogy with free-living fungi and higher plants, that the tyrosinases synthesized by the mycobiont in lichens are involved in the synthesis of melanins and other pigments, which perform the function of photobiont defense against excessive ultraviolet rays, as well as protect the lichens from the effect of radioactive substances [27, 28]. However, the absence of the tyrosinase activity in some of the melanin-synthesizing species studied (e.g., *Cetraria islandica*) implies the involvement of tyrosinases in other physiological processes as well.

By analogy with free-living ascomycetes, possible functions of laccases in the lichens may consist in pigment synthesis and rhizomorph formation [5]. The presence of a lignin-destroying system whose constituent part in free-living fungi is extracellular laccases [6] is not obvious for lichens, and this question requires further study. However, the involvement of laccases in the synthesis of phenol-containing polymers and polyphenols, exemplified by higher plants [19], may also be a function of lichen laccases. It is possible that lichen laccases are involved in the polymerization of mono- and oligophenols in the process of synthesis of specific secondary metabolites (lichen substances).

Beckett et al. [20] suggested that the peltigerous lichen laccases perform a defense function by forming reactive oxygen species. Two alternative defense strategies are considered in the lichens. The first strategy consists in production of oxygen species toxic for pathogens with the involvement of laccases. This strategy is inherent in the peltigerous lichens, characterized by a low-level synthesis of lichen substances and high metabolic activity. The second strategy is realized by lichens of other groups (including macrolichens of the order *Lecanorales*) that have little or no laccases; this strategy consists in accumulation of the lichen substances, many of which have antimicrobial properties. If the hypothesis about the two strategies is confirmed, this may help explain the substantial differences in the levels of phenoloxidase activity revealed in representatives of the order *Peltigerales* and the *Lecanorales* species studied.

The role of lichen phenoloxidases in primary processes of humus formation. The involvement of lichens in weathering of rocks and fine earth formation is considered to be one of their most important biospheric functions. Lichens destroy the mineral substrate both physically, by penetration of the mycobiont hyphae, and chemically, as a result of release of organic acids and specific secondary metabolites, e.g., usnic and psoromic acids and depsides and depsidones [29], which dissolve rock-forming minerals by an acidic attack and chelation [3, 30]. It is evident that lichens also serve as a source of organic carbon and nitrogen in communities, both at the expense of the compounds washed out from alive thalli and at the expense of the accumulation of the thallus destruction products [1]. This results in the formation of primitive soils in which the mineral and organic parts are either separated spatially (organic litter and the underlying mineral substrate) or appear to be a mechanical mixture of detritus and fine soil. For mature soil formation, a chemical interaction should occur between the organic and mineral components with the formation on the surface of the mineral mass of the humus horizon, a soil-specific fertile layer composed predominantly of high-molecular-weight adsorptive organo–mineral complexes. Water-soluble lichen phenoloxidases may be the previously unknown component of these symbiotic organisms that allows the mineral and organic components of primitive soils to be bound together and initiates the synthesis and immobilization of humic substances at the earliest stages of soil formation in the absence of other organisms.

We have recently showed that heterophasic biocatalysis—the synthesis of polymeric (with a mass exceeding 70 kDa) humus-like substances from low-molecular precursors adsorbed on the mineral surface, catalyzed by immobilized fungal laccase—may be an important mechanism of the formation of humus and its organo–mineral compounds [12]. Taking into account the small amounts of the monomers and enzymes that can be washed out from intact lichen thalli, it may be suggested that heterophasic biocatalysis is an important mechanism of primary humus formation under the lichens. The following data are available in favor of this hypothesis. First, the lichens of the order Peltigerales are epigeic and epilithic species; some of them (*Solorina crocea*) belong to the soil-stabilizing species, firmly connected with the weathered mineral substrate (fine earth). Second, the parent rock surface under the lichen thallus is often covered with the products of weathering—iron and aluminum hydroxides, clay minerals [3] known to be adsorbents of proteins [31, 32], and phenolic compounds [33, 34]. Third, the microclimate characterized by both increased humidity and temperature, which may be 10 to 20°C higher than the air temperature, is created under the lichen thallus [30]. Assuming that the average air temperature during the vegetation period in the boreal and tundra zone of the temperate climate is 15°C, the temperature under the lichen thallus is 25–35°C, which is close to the laccase and tyrosinase optima.

Thus, the phenolic and nitrogenous compounds of the lichens released from thalli during rainfall may be adsorbed on the weathered mineral surface and undergo oxidative polymerization, catalyzed by immobilized lichen phenoloxidases, thus forming stable adsorptive organic–mineral complexes. Under natural conditions, these compounds can be observed as dark-colored films (cutanes) covering the rock surface under lichen thalli. Laboratory experiments aimed at confirming the formation of these films from lichen compounds under the

catalytic effect of lichen oxidases are currently in progress.

The present work is one of the first to reveal laccase activity in a broad spectrum of lichens belonging to different taxonomic and substrate groups (epigeic, epilithic, and epiphytic species). The presence of laccase activity in peltigerous lichens has recently been established by Beckett's and Minibaeva's teams in independent investigations [20, 24]. No published data on tyrosinase activity in lichens are available as yet. Research into lichen phenoloxidases is of great importance for understanding functioning of these symbiotic microorganisms both at the cellular and the ecosystem level. Lichen phenoloxidases may be involved both in the phenolic metabolism of cells and in the transformation of the phenolic compounds and humus formation outside the thalli. The present paper is the first to discuss the possible enzymatic role of lichens in pedogenesis.

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