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Research article

Fluorescence quenching in the lichen *Peltigera aphthosa* due to desiccation

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ABSTRACT

Photoprotective mechanisms were studied on the tripartite lichen *Peltigera aphthosa* that exhibits external cephalodia. Using the methods of steady-state and time-resolved fluorescence microscopy, we studied the dynamics of the rehydration process in different parts of the lichen thalli. It was found that apical, medial and basal parts of the thallus are not only morphologically different, but also show completely different chlorophyll induction curves and other spectral characteristics. In dry state, significant contribution to the fluorescence spectrum of lichen gives a green fluorescence of hyphae forming the upper crust, which is rapidly and almost completely quenched during the rehydration process. Probably this is one of the protective mechanisms that reduce the amount of light reaching the PS II reaction centers in the dry state. In the process of rehydration, we observed an increase in the intensity of the chlorophyll fluorescence of the photobiont at 680 nm, with significant changes of the fluorescence lifetimes and the amplitude ratios of fast and slow components of fluorescence decay kinetics. While in dry state, chlorophyll fluorescence is strongly quenched (opposite to the fluorescence of the hyphae), and the fluorescence time constants recover to the typical decay times of active photosynthetic organisms during rehydration. The quantitative behavior of these changes differs largely between the apical, medial and basal parts of the thallus, probably due to the complex interactions of the fungus, algae and cyanobacteria.

1. Introduction

Free-living cyanobacteria and algae form symbioses with heterotrophic organisms. The most diverse group of such symbiosis are lichens. Lichens are composed of two or three genetically isolated organisms, but morphologically, physiologically and biochemically they are closely related (Rikkinen et al., 2002). Lichen symbiosis is generally obligate, because most species of mycobionts are not able to exist apart from the lichen (Hawkswoth et al., 1995).

Green algae *Trebouxia* and *Coccomyxa* play the role of photobionts in most lichen species (Honegger, 1991; Palmqvist, 2000). Algae cells enter the hyphae of mycobiont from the surrounding soil (Fried, 1997). Algae are able to produce products of photosynthesis for the mycobiont, while the mycobiont can assimilate mineral nitrogen from the environment. Also the cyanobionts have the ability to fix atmospheric nitrogen that makes cyanolichens independent from the mineral forms of nitrogen. One example of bipartite cyanolichen is *Peltigera scabrosa*. The filaments of *Nostoc* cells are distributed in its thallus. The ratio of heterocysts and vegetative cells is similar to that in populations of free-living cyanobacteria. It can be explained by the fact that in two-component lichens, cyanobacteria provide not only nitrogen but also carbon. During the evolution of tripartite lichens, cyanobacteria localized in specialized structures, called cephalodia. Cephalodia can be located in the interior layers of the thallus or on the surface. The internal cephalodia are almost completely isolated from the light (Palmqvist, 2000) preventing the cyanobacteria from photosynthesis. Therefore cyanobacteria depend on nutrients delivered through the mycobiont hyphae. In lichens with external cephalodia, the cyanobacteria use their own carbondioxide for photosynthesis (Stocker-Worgotter, 2001). An

Abbreviations: Chl, chlorophyll; *PSII*, photosysyem II; *RC*, reaction center; *PBS*, Phycobilisomes; τ, fluorescence lifetime; ϕ_{fl}, fluorescence quantum yield; TCSPC, time-correlated single photon counting; F_0, Initial fluorescence after the onset of actinic illumination; F_m, Fluorescence maxima under saturating illumination; F_v, Variable fluorescence.

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example of tripartite lichens with external cephalodia is given by *Peltigera aphthosa*.

The habitat of lichens spreads from the shores of the Arctic Ocean to the Antarctica, which is mostly explained by their high adaptability to extreme environments. The fast restoration of the metabolism after the dehydration of the thallus is one of these adaptive capabilities. It is achieved through conservation of photosynthetic reactions of the photobionts, and by the synthesis of fluorescence quenchers (Kranner et al., 2003). The reaction center of PSII is sensitive to desiccation-induced damage because water plays role of the electron donor for the oxidized $P680^+$ (Allakhverdiev et al., 2005; Ohnishi et al., 2005). To avoid formation of reactive oxygen, lichens evolved two groups of protection mechanisms. The first group developed techniques to minimize the exposure of the photobiont to sunlight and minimize light absorption by structural changes of the thallus that lead to cell shading and enhanced light scattering (Scheidegger, 1994). In addition, some lichen acids (group of organic compounds synthesized and accumulated in the lichen thalli) can exhibit properties of the light filter (Hamada, 1991). Seasonal changes of the concentration of usnic acid have been registered in the lichen *Usnea aurantiaco-atra* in the Antarctica (Quilhot et al., 1991). Monthly measurements made over three years showed that the highest concentrations of usnic acid in the thalli can be observed during the periods of the largest solar activity. The second group of protection mechanisms mostly protects lichens from excessive illumination by alteration of the energy transfer between the antenna and reaction centers (Stadnichuk et al., 2012; Mullineaux and Holzwarth, 1991; Horton et al., 1996; Petrášek et al., 2005; Horton et al., 1991). It is well known that under normal conditions the PSII activity is assessed by variable chlorophyll *Chl* a fluorescence, which characterizes the reduction of quinone acceptors (Govindjee, 1995). A decrease in the variable *Chl a* emission observed in desiccated lichens is likely associated with their phototolerance and may arise from multiple mechanisms. Veerman et al. (2007) have investigated the origins of fluorescence quenching in the lichen *Parmelia sulcata* by steady-state and time-resolved fluorescence spectroscopy. They found the main target of quenching is PSII, which produces negligible levels of fluorescence in desiccated lichens. They also reported that the fluorescence decay in desiccated lichens was dominated by a component with fast decay time which is spectrally red-shifted and energetically coupled to the PSII (Veerman et al., 2007). Energy is transferred in this process and quenched. However, the nature of this long-wavelength quenching species remains unclear.

In this paper we present a study of protective mechanisms of *Peltigera aphthosa* based upon the results obtained by steady-state and time-resolved spectroscopy and optical microscopy. This approach allowed us to show the complexity and inhomogeneity of the lichen thallus and led to conclusions about its structural and functional organization.

2. Materials

Lichen thalli were collected on Kindo peninsula, Karelia, near the White Sea Biological Station of the Lomonosov Moscow State University in August 2013. Thalli were dried in the dark in open air until the weight of the thalli was constant. Rehydration of desiccated samples was achieved by placing the sample on a section of filter paper and adding distilled water to filter paper.

3. Methods

Fluorescence measurements were performed by time- and wavelength-correlated single photon counting with the equipment described in Becker and Hickl GmbH (2006); Schmitt (2011). The setup consists of a photomultiplier system with a Hamamatsu R5900 16-channel multi-anode photomultiplier tube with 16 separate output (anode) elements and a common cathode and dynode system (PML-16, Becker & Hickl, Berlin, Germany). The polychromator was equipped with 600 or 1200 grooves/mm grating resulting in a spectral bandwidth of the PML-16 of about 200 or 100 nm (resolution of 12.5 to 6.25 nm/channel, respectively). Excitation was performed with a pulsed 405 nm laser diode (LDH-405, Picoquant, Berlin, Germany) delivering 60 ps FWHM pulses, driven at repetition rate of 20 MHz and 405 nm laser (IOS, Russia) delivering 26 ps pulses, at repetition rate of 50 MHz. To study the dynamics of the fluorescence quenching the signal was recorded as 200 cycles (f(t,T) mode of B&H SPC (Becker and Hickl GmbH, 2006)) with a duration of the signal accumulation of 1 s for each cycle. This technique allowed us to record the dynamics of the transition of the samples from dry to the moisturized state, by measuring the intensity of fluorescence and simultaneously the fluorescence lifetimes with a global resolution of 1 s. Thus, each experiment provided 16 fluorescence decay curves in the spectral range for 200 cycles (seconds) resulting in 3200 fluorescence decay curves.

For time-integrated measurements we used Fluoromax 4 (Horiba Jobin Yvon, France) and the USB-connected fluorometer system with CCD array USB4000 (Ocean Optics, USA). When investigating the processes of thallus hydration, the fluorescence signal was accumulated for 500 ms, the procedure was repeated 500 times (i.e., 500 fluorescence spectra were recorded in steps of 500 ms). Then changes in fluorescence intensity in range of 525–535, 680–690 and 730–735 nm were analyzed.

The reflection spectra were recorded using a USB2000 spectrometer with DT-MINI-2-GS deuterium tungsten halogen light source (Ocean Optics, USA), reflection was recorded at 90°, mirror was used as a standard.

Fluorescence induction curves were recorded by Handy-PEA (Plant Efficiency Analyzer) Hansatech (Great Britain) with 655 nm excitation wavelength (Maksimov et al., 2011).

The fluorescence decay kinetics were approximated by a sum of exponential functions. To compare different kinetic patterns, we calculated the average decay time according to the expression: $\tau_{av} = \sum_i^n \tau_i a_i$, where τ_i is the lifetime of the i-th component and a_i is the fraction of the amplitude of i-th component of the fluorescence decay normalized to $\sum_i^n a_i = 1$. To obtain the time integrated fluorescence spectra, the number of photons in each spectral channel was summed up.

All calculations were performed using the Origin 8.0 (OriginLab Corporation, United States) and SPCImage (Becker and Hickl, Germany) software packages (Becker et al., 2001).

The lichen cross sections were obtained using a freezing microtome HM-560 (Thermo Scientific Microm, Walldorf, Germany). For this purpose lichen preliminary has been rehydrated in water, the excess fluid was removed with filter paper, after that piece of lichen was frozen in frozen section medium NEG-50™ (Richard-Allan Scientific®, Thermo Scientific). The cross sections thickness was 50 microns. After mounting sections on a microscope slide, they were dried.

The multi parameter fluorescence microscope is based on a *Nikon TI Eclipse* microscope that can combine different excitation wavelengths, detectors and detection pathways. The setup allows doing fluorescence lifetime imaging microscopy (FLIM) and time resolved fluorescence microscopy with a resolution of 200 ms exciting the sample with different wavelengths and imaging the emission with several filter combinations. To distinguish mycobiont, cyanobacteria and alga images taken with 530/25, 660/25 and 682/12 nm emission filters (AHF Analysentechnik, Tüingen, Germany) are used for the separation of the fluorescence of hyphae,

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phycobilisomes and *Chl a*, respectively. For further details regarding the microscopic setup see (Schmitt et al., 2013). For some experiments we also used laser scanning microscope LSM-710 (Carl Zeiss, Jena, Germany).

4. Results and discussion

Traditionally, the lichen thallus can be divided into three morphologically distinct zones. The basal (B) area is in the immediate vicinity of the point of attachment to the substrate and characterized by large cephalodia. It is followed by the medial (M) area with small cephalodia. The apical (A) zone is allocated closer to the edge of the thallus. Despite the fact that there are no sharp edges of these areas the thallus structure can vary significantly (Fig. 1). It can be seen from the fluorescent micrographs of lichen sections that the apical zone is characterized by a dense plexus of hyphae in the lower crust and lack of tubular hyphae, which act as aquifer channels in medial and basal areas. Using a set of lasers with different wavelengths allowed us to visualize the structural and functional organization of the lichen thalli. Fig. 2 shows images of dry lichen thallus 50 μm cross-sections from the basal area, obtained with Carl Zeiss LMS710 fluorescence microscope. Excitation with 458 nm laser light induces intense green fluorescence of the fungus hyphae, and red fluorescence of algae (see Fig. 3) and cyanobacteria. We assume that the chitin-glucan complex of the fungal cell walls is the source of the green fluorescence. The spectral characteristics of red and near IR emission shows that the *Chl a* of algae is causing this emission. Excitation with 561 nm laser light leads to intense fluorescence of cyanobacterial phycobilisomes (PBS), with a characteristic shoulder at 570 nm indicating phycoerythrin (Maksimov et al., 2013). Obviously, the pigments of the fungi cell walls in the upper crust may screen the pigments of the photobionts. The extinction coefficients of the PBS are low in the blue part of the spectrum, so the cephalodium may seem “empty” at 458 nm fluorescence excitation. The difference between structures of fungal hyphae morphology in the upper cortex and in the lower layer should be noted. Those morphological differences are probably correlated with the different functional roles of these structures.

The complex arrangement of components of the lichen may explain the differences in the fluorescence spectra of the whole thalli in the dry state and during hydration. In the dry state (with excitation at 405 nm) an intense fluorescence signal is registered from the pigments of fungi cell walls. These compounds have a wide range of fluorescence, which overlaps with the spectra of chlorophyll and phycobiliproteins. Moreover, the visualization of the internal components of whole lichen thalli in dry condition is difficult because algae and cyanobacteria are covered with hyphae, which are impervious to 405 nm light. But almost immediately after rehydration the fluorescence of hyphae is quenched and microscopy can reveal elements of the internal structure of the lichen. It should be noted that moisturizing quenches the fluorescence of the fungi cell walls inhomogeneously with stronger quenching of the green fluorescence in hyphae of the lower layer in medial and basal zones (see Figs. 1 and 2). This is probably also indicating different functional purposes of the upper layer of the hyphae (crust), which protects the lichen from external influences, and the lower layers, which accumulate and retain water in the thallus.

Fig. 3 shows the reflection spectra of the whole lichen thalli (medial part). The reflection spectra of dry and rehydrated thalli have characteristic differences. The dry thallus is highly reflective, but after rehydration it starts to absorb light efficiently, reflecting only a small part of the spectrum in the green and infrared regions. Thus, the components of the cell walls of hyphae by absorbing water can significantly change their optical properties.

Fluorescence spectra of dry and rehydrated samples are presented in Fig. 4A. In dry conditions, the emission of algae cells has two characteristic spectral bands. One with a maximum at 685 nm is typical for the *Chl a* fluorescence. The other broad band is peaking at approximately 730 nm. Based on the observation of an enhanced 730 nm fluorescence in desiccated lichens, it was suggested previously that a red-shifted form of *Chl* acts as a long-wavelength quencher (Veerman et al., 2007; Heber et al., 2006). However, long-wavelength emission can originate from a number of sources (Melkozernov et al., 2004; Mullineaux et al., 1993; Melkozernov et al., 1998; Karapetyan et al., 1992). Probably such long-wavelength bands are the result of the interaction of multiple chlorophylls (coupling) due to formation of supercomplex of antenna proteins. Such red antenna states of the distal antenna complexes associated with the PS I in higher plants, known as LHCI, have been studied and analyzed by measurement of their pre-equilibrium fluorescence upon direct excitation at room temperature by Jennings et al. (2003), (2004). It has been suggested that the main biological function of these red states is light harvesting by leaves exposed to a light environment enriched in wavelengths above 690 nm, due to shading by other leaves (Rivadossi et al., 1999). However it is now generally accepted that they do not increase the rate of energy flow from the antenna to the primary donor chlorophylls (Croce et al., 2002; Gobets et al., 2001; Fischer and Hoff, 1992; Trissl, 1993; Byrdin et al., 2000; Croce et al., 1998). Another reason might be that at high packing densities of pigments the phenomenon of reabsorption can be observed, which can lead to significant distortions of the fluorescence spectrum, namely the reduction in intensity of the band at 685 nm, compared with the one at 730 nm.

Fig. 4B shows the characteristic time-courses of different emission bands during the rehydration of lichen thalli obtained by steady-state spectroscopy. The quenching of green fluorescence and rise of Chl emission occur in all parts of lichen thalli with minor

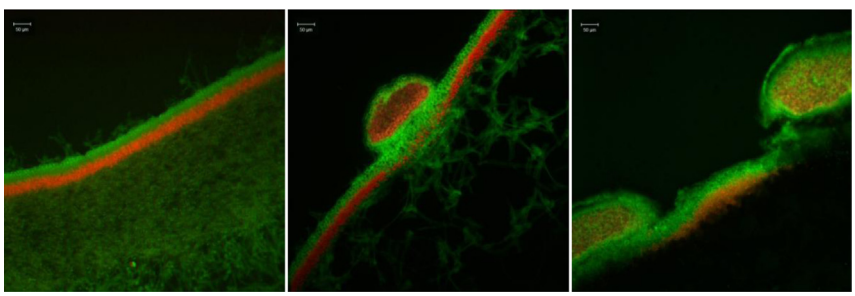

Fig. 1. From left to right autofluorescence (recolored) of 50 μm cross-sections of rehydrated apical, medial and basal zones of *Peltigera aphthosa*. The figure overlay pictures taken with 530/25 nm (green) 660/25 and 682/12 nm emission (both red) to separate cyanobacteria, algae and hyphae. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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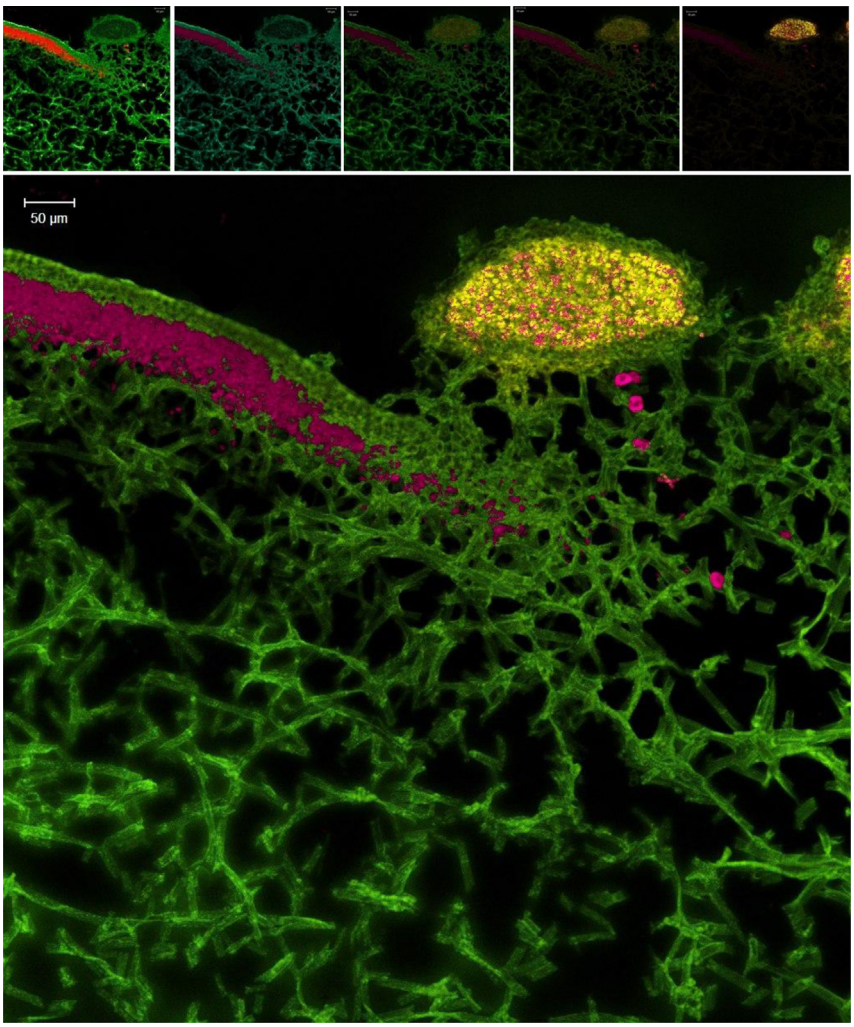

Fig. 2. Confocal re-colored fluorescent image of dry lichen thallus (50 μm cross-section). Bottom – image overlay. LSM-710 (Carl Zeiss, Jena, Germany). Top row, left to right excitation 445, 458, 488, 514 and 561 nm. Image is imposition of fluorescence in three spectral ranges: green – 521–544 nm – cell walls of fungi, magenta – 675–702 nm – Chlorophyll of algae and cyanobacteria, yellow 568–658 nm. Pinhole 35 mkm. Objective Ec plan-Neofluar 10×/0.30 M27. X:Y – 849.98 μm × 849.98 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

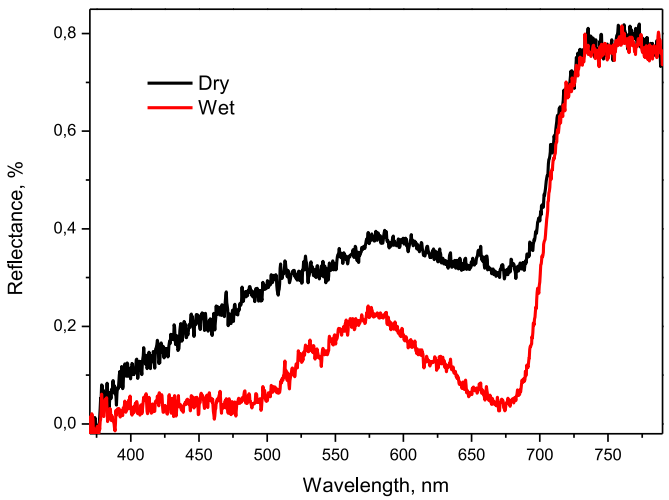

Fig. 3. Reflection spectra of dry (black curve) and rehydrated (red) lichen thalli. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

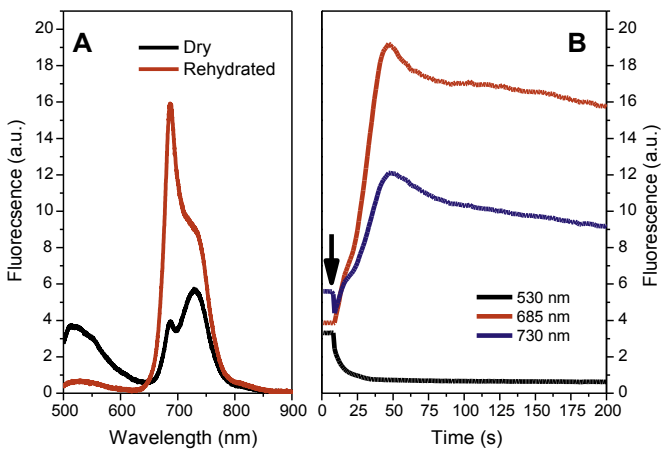

Fig. 4. Room temperature fluorescence emission spectra of dry and rehydrated apical zone of the thallus (A) and time-courses of the fluorescence intensity at 530, 685, and 730 nm during rehydration (B). The moment of addition of water is indicated by the black arrow. Excitation is performed at 405 nm.

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differences (data not shown). The whole curve of fluorescence intensity changes can be divided into five temporal sections (Fig. 4B). The initial section, until the moisture, shows a high fluorescence intensity of fungi cell walls. When water is added (Section 2), a sharp decrease in the intensity of green fluorescence is observed (with characteristic time of 11 and 1.5 s). Simultaneously, there is a slight reduction of the fluorescence intensity in the IR region of the spectrum, which is probably due to fast uncoupling of the antenna supercomplexes and vanishing of long-wavelength states (also the overlap of chlorophyll fluorescence and green fluorescence of the fungus hyphae should be noted). Then, there is a gradual increase in the intensity of chlorophyll fluorescence, reaching a maximum of about 40 s after the start of rehydration (Section 3). Chlorophyll fluorescence intensity increases up to five-fold (which is in good agreement with (Honegger, 1991)). As discussed previously, this change may arise from changes of the thallus morphology, including changes in its light-scattering properties (Fig. 3) as well as changes in excited state quenching within the photosynthetic apparatus of the photobiont. It should be noted that after humidification, the 685 and 730 nm bands ratio may vary considerably in different zones of the thallus (Fig. 5). In addition, it should be noted that 730 to 685 nm bands ratio may significantly vary for different lichen thalli, probably due to environmental differences. After about 60 s, the fluorescence of *Chl a* drops slightly with a fast component (Section 4) and a somewhat slower component (section 5).

The fast drop in Section 4 fits to the activation of the Q_A^- reoxidation and adaption to the equilibrated photosynthetic activity and therefore leads to higher photochemical quenching than in the maximum at 40 s. The slower processes of fluorescence decay (section 5) might be related to photobleaching.

Due to the fact that the fluorescence intensity of the photobiont may vary due to morphological transformations of the thallus, which leads to changes in light absorption, it is necessary to measure the fluorescence lifetime – a parameter directly related to the value of the fluorescence quantum yield ϕ_{fl} (Lakowicz2nd ed, 1999), independent from the geometry of the object. A 405 nm picosecond laser at 50 MHz repetition rate allowed us to obtain good signal to noise ratio to analyze fluorescence decay kinetics and calculate average lifetimes for different spectral regions. It was shown that in dry state the overall fluorescence of the photobiont is red-shifted and exhibits short average lifetime equal to 250 ps, but after rehydration the average lifetime values increases up to 600 ps. Such changes are characteristic for all zones of the lichen thalli. The comparison of changes in the total numbers of photons and the average fluorescence lifetime is presented in Fig. 6. It must be noted that in the 50 MHz pulse regime the overall intensity of picosecond laser is low, so intensity of *Chl* fluorescence correspond to F_0 level (however, in the CW mode used for steady-state spectroscopy (Fig. 4B), light intensity is large enough to cause reduction of the quinone pool and activate non-photochemical quenching of *Chl* fluorescence (Demmig-Adams and Adams, 1990)). The rise of the average lifetime was approximated by an exponential function and showed a typical kinetics with 58 s rise time. Simultaneously, an increase in overall fluorescence intensity has a more complicated dependency and is characterized by a rise time of approximately 25 s. The comparison of the fluorescence intensity and average lifetime shows that up to 75% of changes of the fluorescence intensity are caused by the changes of the *Chl* quantum yield ϕ_{fl}. In other words, the change in fluorescence intensity is related to changes of time constants for energy transfer between antenna and reaction centers or possible non-photochemical quenchers which are deactivated after rehydration. The remaining 25% of change are probably related to alteration of cell shading and light scattering, or the deactivation of strong quenchers localized at some reaction centers, which are visible as amplitude change only due to the high rate constant for quenching. The most interesting fact is the discrepancy between the sharp change in the ratio of the fluorescence bands at 685 nm and 730 nm, and the smooth change in the fluorescence lifetime. The band at 730 nm, which is associated with a long-wavelength quencher, disappears almost immediately after the beginning of rehydration (Fig. 4), but the system remains in a quenched state for a considerable time (Fig. 6). This phenomenon requires further experimental investigation.

The analysis of the *Chl* fluorescence induction curves confirmed that dried lichen thalli do not possess variable fluorescence (data not shown). Shortly after rehydration, the initial level of fluorescence (corresponding to F_0) increases strongly, as it was previously shown by steady-state spectroscopy (Fig. 4). But most surprising was the difference between the induction curves from different zones of the lichen (Fig. 7). While all parts of lichen demonstrate high (0.7) values of F_v/F_m, we observed significantly faster

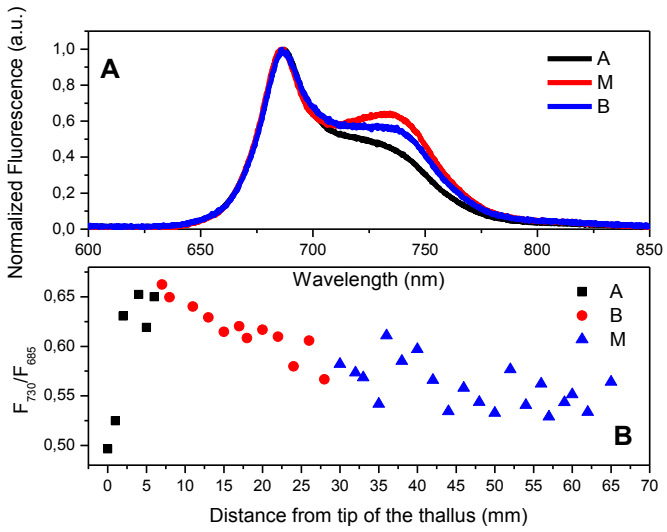

Fig. 5. Fluorescence spectra (A) and ratio of bands 730 and 685 nm (B) of rehydrated lichen in apical (A), medial (M) and basal (B) zones. Excitation wavelength is 405 nm.

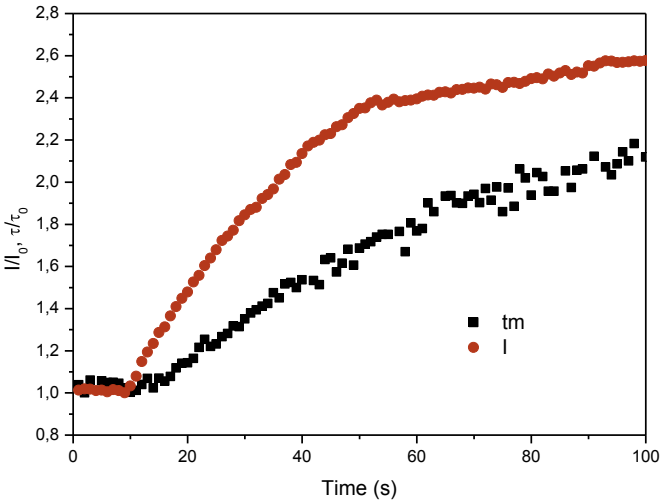

Fig. 6. Comparison of changes in fluorescence intensity (I – total numbers of photons) at 685 ± 6.25 nm and average lifetime (tm) during rehydration of basal zone of lichen. Initial values of fluorescence intensity and lifetime are set to 1.

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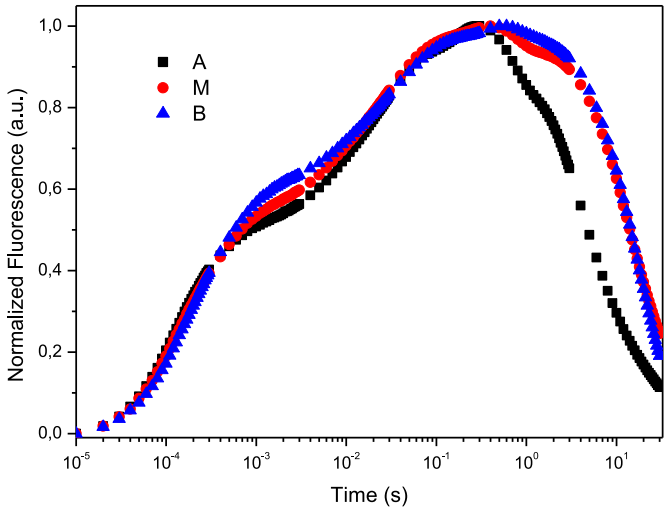

Fig. 7. Fluorescence induction curves of *Peltigera aphthosa* at apical (A), medial (M) and basal (B) zones after rehydration. The fluorescence was excited by a LED with an emission maximum at 655 nm. The minimum value of the fluorescence (F_0) is set to 0, maximum (corresponding F_m) is set to 1.

fluorescence quenching after the F_m level in the apical zone. This result once again shows differences in structural and functional organization of the lichen thallus. However, those differences may be as due to different rates of non-photochemical quenching (NPQ) activation and different kinetics of plastoquinone pool oxidation (StrasserGovindjeeArgyroudi-Akoyunoglou, 1992; Strasser and StrasserMathis, 1995; Vasil'ev and Bruce, 1998), as due to singularity of apical zone morphology and following differences in light scattering and absorption properties.

To summarize, the tripartite lichen *Peltigera aphthosa* is a complex multi-component system with a complex morphology and functional organization. The interaction of three very different organisms leads to the emergence of new properties, such as resistance to changing environmental conditions, independency from certain resources and adaptivity. Even after prolonged period of dehydration and intense sunlight exposure *Peltigera* is capable of rapid recovery of the photosynthetic activity. This is possible due to the combination of protective mechanisms acting both on the molecular and the organism level. One way to prevent the occurrence of reactive oxygen species is the reduction of the energy through the reaction centers of photosystem II due to the formation of long-wavelength quenchers, effectively competing with reaction centers for the capture of excitation energy. Despite of its simplicity, the lichen thallus is not a homogeneous structure, and cells of the same species can have very different spectral and temporal characteristics depending on the external environment and the interaction of all lichen components. Understanding the nature of these differences and mechanisms of regulations is necessary not only for the development of fundamental science, but also for the development of new methods of environmental monitoring.

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