Cryoresistance of Antarctic endemic lichen *Himantormia lugubris*: Analysis of photosystem II functionality using a constant-rate cooling approach

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Abstract

It is well established that lichens from polar regions of the Earth are capable to perform photosynthesis at sub-zero temperatures. Majority of them show a high degree of cryoresistance, however, species-specific differences exist. Therefore, the aim of our study was to evaluate behaviour of primary photochemical processes of photosynthesis in Antarctic endemic species *Himantormia lugubris* at sub-zero temperature. For the purpose, the method of constant rate (2°C min⁻¹) cooling (from +20 to -40°C) with simultaneous measurements of chlorophyll fluorescence parameters related to photosystem II (PSII) was used. During the cooling, potential yield of photosynthetic processes in PSII ($F_{V}/F_{M}$), and effective quantum yield of PSII ($\Phi_{PSII}$) were measured in 30 s interval. From the $F_{V}/F_{M}$ and $\Phi_{PSII}$ data sets, $S$-curves reflecting temperature dependence of the two chlorophyll fluorescence parameters were constructed and analyzed. The $S$-curves were found tri-phasic in response to sample temperature decline: (1) slight or no decline phase, (2) rapid decline phase, followed by (3) slow change reaching critical temperature at which the primary photosynthetic processes were fully inhibited. Critical temperature was found -30 and -20°C for $F_{V}/F_{M}$, and $\Phi_{PSII}$, respectively. The latter critical temperature was accompanied by an increase in background chlorophyll fluorescence ($F_{0}$) indicating inhibition of energy transfer from light-harvesting complexes to core of PSII. A newly-designed chlorophyll fluorescence parameter (a differential, i.e. the difference between the maximum value-normalized $F_{V}/F_{M}$, and $\Phi_{PSII}$) was used in order to evaluate the temperature at which the processes related to photosynthetic electron flow through thylakoid membrane carriers ($\Phi_{PSII}$) and the energy flow through PSII ($F_{V}/F_{M}$) differed to a largest extent. This parameters proved to be temperature-dependent and useful in the evaluation of cryoresistance. Based on our study, *H. lugubris*, its primary photosynthetic processes in particular, might be considered as higly resistant to sub-zero temperature.

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**Key words:** freezing, photosynthetic apparatus, cooling point, King George Island, differential method

**Introduction**

Lichens are important components of vegetation oases on the South Shetlands (Antarctica) (Rodriguez et al. 2018). Among them, Antarctic endemic species *Himantormia lugubris* (Hue) I.M. Lamb forms lichen-dominated communities with different *Usnea* spp. (Kappen and Redon 1987). Within last decades, several studies focused on different aspects of ecophysiological performance of *H. lugubris* in the field, such as e.g. low rate of photosynthesis associated with low photobiont populations in lichen thallus (Sojo et al. 2003), and generally low chlorophyll content (Kappen et al. 1991). Complex field study was performed by Sancho et al. (2020) who used gas exchange measurements and chlorophyll fluorescence approach to evaluate photosynthetic performance of the species at the Livingston Island, Antarctica. The study reported that positive net photosynthesis was only possible at low temperatures, high relative air humidity and low light, despite the fact *H. lugubris* grows at open sunny habitats. The study suggests that the maritime Antarctica, a part of which the South Shetland archipelago is, provides such climatic characteristics, i.e. low temperature, high cloudiness and hydration due to the regular precipitation in austral summer season.

In spite of the fact that the range of *H. lugubris* covers generally wet terrestrial ecosystems in maritime Antarctica (western coast of the Antarctic peninsula), resistance of primary photosynthetic processes to thallus desiccation is well comparable to other Antarctic lichens both with partly and severely dehydrated thallus (Barták et al. 2021).

Underlying mechanisms affecting physiological characteristics of the species have been measured under controlled conditions only scarcely. Specific studies analyzed antioxidant contents and selected enzymes in *H. lugubris* (Areche et al. 2022). In photosynthesiis research, however, several studies addressed short-term (laboratory experiments) and long-term warming approach (Open Top Chambers) to evaluate a potential of the species to cope with microclimate in Antarctica witnessing global warming effects. Among them, Marin et al. (2022) focused on photosystem II activity under desiccation, and high temperature treatments and stated that the species can tolerate warming and high temperature better than desiccation. Resistance of *H. lugubris* to low temperature was studied by Folgar-Cameán and Barták (2019) by a constant-rate cooling approach. The study focused on the evaluation of critical temperature at which primary photosynthetic processes associated with PSII are fully inhibited. The study reports a high resistance of both potential quantum yield of photochemical processes of photosynthesis in PSII (Fv/Fm) for *H. lugubris*. Critical temperature was found at -20°C, i.e. lower than in majority of species investigated by this method so far.

The aim of our study was to bring a new insight into the knowledge of *H. lugubris* cryoresistance by exploiting the constant-rate cooling method in order to analyze physiological mechanisms attributed to PSII functioning at subzero temperature. For this purpose, we measured and analyzed several chlorophyll fluorescence parameters during a constant-rate cooling of *H. lugubris* thalli. Attention was devoted to a newly suggested parameter (Puhovkin et al. 2023), temperature-response curve of the differential between normalized Fv/Fm and ΦPSII values.
Material and Methods

Species description

*Himantormia lugubris* is a lichen species belonging to the Parmeliaceae family, which forms extensive lichen communities associated mainly with species of the genus *Usnea*. It grows usually in areas of high ambient humidity, between Graham Land, in the South Shetland Islands and up to the Anvers Islands, in the Paradise Bay area, Antarctica. The genus *Himantormia* is monospecific, and is well distinguished from other lichenicolous species in the area by its dark fuscous to cinerous color (Lamb 1964). *H. lugubris* species has a prothallus at the base, which does not contain algae lager and is rough and aeruginous. The metathallus branches are erect, elongate, terete and flattened dorsiventrally on some old branches. The thallus has an internal chondroid axis that gives consistency to the thallus. In young terete branches, the thallus is black at the base, but upward is covered with a smooth, matt, subnitid cream mantle. As the thallus increase the size, the branches become flattened and verrucose, which disrupts along the branches in several lumps or lobulate patches, on the surface of the black chondroid ground tissue. In this flattened old branches, the ground surface corresponds to the central chondroid axes, with carbonized appearance. The algae layer is found underneath the cortex, in areas of the patches. *Himantormia lugubris* is growing on rocks or on moss carpets of *Sanionia uncinata*.

Collection and handling of the samples

Thalli of *H. lugubris* were collected from the sites neighbouring the Artigas station, King George Island, Antarctica (-62.187762° S, -58.923369° W), where *Usnea sp.* was abundant as reported in previous studies (Piñeiro et al. 2012, Smykla et al. 2005, Mróz et al. 2018). The thalli were collected from inclined rock surfaces (see Fig. 1) and transferred in dry state into the EEL laboratory for experiments. In the laboratory, *H. lugubris* thalli were stored at -5°C. The samples were re-wetted by demineralized water at +5°C before the experiments. For this purpose dry thalli were put in between two sheets of paper placed in a Petri dish under dim light (5 μmol m⁻² s⁻¹ of photosynthetically active radiation – PAR) and sprayed by water for 24 h. When full hydration was reached and restoration of photosynthetic processes was apparent (checked by maximum and constant values of $F_\text{V}/F_\text{M}$) thalli were used for the cooling experiments.

Measurements of chlorophyll fluorescence during a constant-rate cooling

The samples of *H. lugubris* were placed into the cooling chamber of the KryoPlaner unit (Great Britain) linked to a 20 l Dewar flask with liquid nitrogen and cooled from +20°C (denoted as $T_0$) to -50°C at a constant PC-controlled rate of 2°C min⁻¹. Chamber ($T_\text{ch}$) and sample temperatures ($T_\text{s}$) were monitored by in-built thermocouples during the cooling procedure and recorded. Simultaneously with cooling, two chlorophyll fluorescence parameters ($F_\text{V}/F_\text{M}$ and $\Phi_{\text{PSII}}$) were measured by a PAM 2000 fluorometer (H. Walz, Germany). Since *H. lugubris* is brown-blackish or black, there is generally low chlorophyll fluorescence signal emitted from the thalli exposed to light.
The distance between the probes’ end and the sample was optimized (2 mm) so that a satisfactorily-high chlorophyll fluorescence signal was reached. Repeated saturation pulses of 5000 μmol m$^{-2}$ s$^{-1}$, lasting 0.8 s each, were applied every 30 s during the cooling in dark to induce maximum chlorophyll fluorescence signals ($F_{M}$). Then, the values of background ($F_{0}$) and maximum ($F_{M}$) values were recorded and used for $F_{V}/F_{M}$ (maximum quantum yield of photochemical processes in PSII) calculation. Similarly, in light adapted samples (continuous actinic light of 30 μmol m$^{-2}$ s$^{-1}$ provided by a fluorometer during cooling), saturation pulses were applied in the same interval in order to evaluate effective quantum yield of photosystem II ($\Phi_{PSII}$), a proxy of photochemical part of photosynthesis.

**Data processing and analysis**

$F_{V}/F_{M}$ and $\Phi_{PSII}$ data recorded during the constant rate cooling were plotted against the sample temperatures and fitted by a 5 parameter log-logistic model. It was used to construct S-curves (see Hájek et al. 2016 for details).

From the best fit, the following parameters were evaluated: (1) sample temperature at which temperature-dependent inhibition of $F_{V}/F_{M}$ and $\Phi_{PSII}$ starts (T1), (2) critical temperature at which $F_{V}/F_{M}$ and $\Phi_{PSII}$ reach zero (T2), and (3) LT$_{50}$ defined...
as the temperature at which $F_v/F_M$ and $\Phi_{PSII}$ reach 50% of their maxima. Apart of the $S$-curves of $F_v/F_M$ and $\Phi_{PSII}$, a new chlorophyll fluorescence parameter was analyzed.

To evaluate the temperature at which the largest difference appears between the temperature response curves of $F_v/F_M$ and $\Phi_{PSII}$, differential (D) between $F_v/F_M$ and $\Phi_{PSII}$ was calculated. The values of $F_v/F_M$ and $\Phi_{PSII}$ presented in Fig. 2 were normalized (division of actual $F_v/F_M$ and $\Phi_{PSII}$ value by maximum $F_v/F_M$ and $\Phi_{PSII}$ value). The normalized values were used for differential (D) evaluation using the below equation (Hajek et al. 2022).

$$D = (F_v/F_M)_{norm} - (\Phi_{PSII})_{norm}$$  \hspace{1cm} Eqn. 1

where, D is the differential, $F_v$ is variable chlorophyll fluorescence, $F_M$ is maximal chlorophyll fluorescence induced by a saturation pulse applied in dark-adapted state, and $\Phi_{PSII}$ is effective quantum yield of photosynthetic processes in photosystem II.

**Results and Discussion**

In order to estimate the effect of cold stress on photosynthesis during the constant-rate cooling, several chlorophyll fluorescence parameters were measured and analyzed. Temperature-response curves of $F_v/F_M$, $\Phi_{PSII}$ formed typical $S$-curves with well-distiguishable three phases (Fig. 2).

With initial decrease in thallus temperature from +20 to -18°C, $F_v/F_M$ values remained more or less constant showing only a slightly decreasing trend phase I. Then, with thallus temperature decrease below -18°C, an intermediate phase (phase II) was found. It was typical by a rapid decrease ending at the temperature of about -30°C. The end of the curve showed a slow decrease to constant or close-to-zero values of $F_v/F_M$ found at the temperature below -30°C (phase III). The temperature response curve of $F_v/F_M$ had similar shape as the one reported by Folgar-Cameán et Barták (2019). Critical temperature ($T_2$, see Fig. 2), however, was found lower indicating a higher of cryoresistance of *H. lugubris* thalli collected close to Artigas station then that from the Yardly peninsula (Folgar-Cameán et Barták 2019, see supplementary data). Critical temperature, however, was comparable with other lichen species (e.g. Marečková et al. 2019 for *Dermatocarpon polyphyllizum*). Contrastingly to $F_v/F_M$ temperature response curve, $\Phi_{PSII}$ exhibited more pronounced decrease within phase I, i.e. when the samples were exposed to the temperature decreasing from +20 to +5°C. With further cooling from +5 to -20°C (phase II), a rapid decrease in $\Phi_{PSII}$ values was apparent with the cooling. Phase II was hardly distinguishable for $\Phi_{PSII}$.

Temperature-response curve of $F_0$ was found triphasic. With the temperature decline from +20 to -20°C, $F_0$ decreased (phase I) finding the minimum value at about -20°C. With further decrease of temperature to -30°C, $F_0$ increased (phase II), while $F_0$ values was found more or less constant at the temperature below -30°C (phase III). The reason for the $F_0$ increase with the cooling within the phase II might be attributed to full inhibition of PSII due to temperature-induced block of linear electron trasport chain demonstrated as $\Phi_{PSII} = 0$. The increase in $F_0$ is attributed to ice nucleation and subsequent freezing of extracellular water as reported for *Sphagnum capillifolium* (Buchner and Neuner 2010). The above-decribed triphasic behaviour of $F_0$ in lichens is well comparable to the previous data reported for the same species by Folgar-Cameán and Barták (2019).
Fig. 2. Temperature-response curves for maximum quantum yield of photochemical photosynthetic processes in PSII (F_v/F_M), effective quantum yield of PSII photosynthetic processes (Φ Hoàng), and background chlorophyll fluorescence (F_0) recorded for H. lugubris during a constant-rate cooling form +20°C to -40°C.

The turning points (see Fig. 2, the arrows) were, however, found at higher temperatures (-10 and -20°C) in previous study dealing with H. lugubris (Folgar-Cameán and Barták 2019) than in the recent study.

Underlying mechanism could be (1) site effect, i.e. that fact that the samples were collected from different sites and in different times of austral summer season or other reasons related to physiological properties of the samples. Among them, either (2) different number and arrangement of light harvesting complexes (LHCs) and their attachment to core of PSII, or (3) the differences in PSII functioning might be considered.

Lichens as well as lichen symbiotic algae have a high freezing tolerance and survive rapid freezing as shown in controlled experiments (Kvidervá et al. 2013). Similarly, Antarctic free-living terrestrial algae and cyanobacteria exhibit a high degree of freezing tolerance as reported for shock-frozen samples in liquid nitrogen (Sabacká and Elster 2006, Orekhova et al. 2019, Hejduková and Nedbalová 2021). Such capability of these polar microorganisms is achieved thanks to adaptation and acclimation to low temperature. For several algal species from polar regions, different cryoresistance is reported for different living forms as shown for pre- akinetes of Zygnema sp. (Trumhová et al 2019). Underlying physiological mechanisms for cryoresistance were reviewed by several authors (e.g. Lyon and Mock 2014, Pichrtová et al. 2020) and comprise membrane fluidity control (i.e. maintaining fluidity even at sub zero temperature), temperature adjustment of key enzymes, and synthesis of cryoprotective compounds.

The temperature response curve of the difference (F_v/F_M)norm - (Φ Hoàng)norm evaluated for H. lugubris was found similar to the other lichen species investigated in a previous study (Hájek et al. 2022). However, peak 2 and related temperature at which the peak is typically reached (T_p2) was not distinguished for H. lugubris. On the other hand, peak 1 and the corresponding temperature (T_p1) was almost the same as for the lichens involved into the previous study. T_p1 temperature of about -18°C suggests that H. lugubris is cryoresistant to the same extend as the other lichen species (see Fig. 3) from polar
regions investigated so far. The $T_{p2}$ temperature might be considered an effective descriptor of the ice nucleation events in chloroplasts induced by the action of sub zero temperature in cellular compartments, because it is the temperature at which potential photosynthetic processes in PSII.

Therefore, the $(F_V/F_M)_{\text{norm}} - (\Phi_{\text{PSII}})_{\text{norm}}$ might be used in follow-up studies focused on cryoresistance of the lichens from polar and alpine ecosystems as useful parameter evaluating cryoresistance/cryosensitivity. This approach might be useful especially in comparative studies using different lichen ecotypes, samples from different locations and ecological niches with contrasting micrometeorological characteristics. Additionally, the approach might be used in interspecific studies as well.

![Graph](image)

**Fig. 3.** The temperature response curves of $(F_V/F_M)_{\text{norm}} - (\Phi_{\text{PSII}})_{\text{norm}}$ (D – the difference between normalized values of $F_V/F_M$ and $\Phi_{\text{PSII}}$, for normalization see Material and Methods). **Left:** Data for *H. lugubris* investigated in our study. **Right:** Data for other polar lichen species (adopted from Hájek et al. 2022). $T_{p1}$ is a temperature at which the peak of the differential is reached. $T_{\text{min1}}$, $T_{\text{min2}}$ are the temperature at which the differential is equal to 0.

**Concluding remarks**

*Himantormia lugubris* is a species with low net photosynthetic rate but relatively high respiratory rate (Sancho et al. 2020). Such adjustment is likely associated with irregular pattern of a photobiont distribution within lichen thalli. Photobiont (*Treboxia* sp.) cells are typically allocated rather to grey than black parts of a thallus. Therefore, both photochemical and biochemical processes of photosynthesis might be affected by the photobiont allocation within a complex thallus. Ecophysiological studies on the species photosynthesis in relation to thallus size and 3D arrangement, are very scarce. To our best knowledge, appart of gas exchange measurements (Sancho et al. 2020), only limited number of photosynthetic studies focusing on primary photosynthetic processes has been conducted so far in *H. lugubris* (e.g. Barták et al. 2021, Marin et al. 2022) for desiccation-induced limitation of photosystem II using a chlorophyll fluorescence approach). Therefore, our chlorophyll fluorescence study addressing cryoresistance of the species, its primary photosynthetic processes at low temperature in particular, represents only a small part of the species ecophysiological properties. In follow up studies, attention should be paid to different ecotypes of *H. lugubris* thriving in the locations with contrasting availability.
of water. Different morphotypes, and age-related differences in the proportion of black to grey areas over a thallus surface should be taken in consideration as well. Moreover, the response curves of primary photosynthetic parameters (at least $F_{V}/F_{M}$ and $\Phi_{PSII}$) should be evaluated for main environmental factors: (1) photosynthetically active radiation, (2) low but above zero temperature in combination with (3) different intrathalline water contents. Such studies would provide an inside into limiting factors of primary photosynthesis of the species as well as directions of future research of the species, its ecophysiological performance in the field.

Since the decrease in chlorophyll fluorescence parameters reported for sub-zero temperature in our study is associated with the process of intrathalline water freezing, a combination of various biophysical methods is needed to address ice nucleation in lichen species. Future studies should combine chlorophyll fluorescence with $e.g.$ proton NMR spectra that allow to monitor cooperative freezing of bulk water in lichen thallus as shown earlier by Harańczyk et al. (2003) for $H.\ lugubris$ exposed to the temperature range of -5 to -20°C. Exploitation of differential scanning calorimetry (DSC) is also promising (Moffett et al. 2015) for future studies.

References


