Responses of thallus anatomy and chlorophyll fluorescence-based photosynthetic characteristics of two Antarctic species of genus *Usnea* to low temperature

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Abstract

Biometrical parameters of two fruticose lichens from Antarctica (*Usnea aurantiaco-atra*, *U. sphacelata*) were studied using thallus cross-sections at basal, middle, and apical parts of thallus. The thallus diameter (TD), the thickness of the upper cortex (UCT), distribution of symbiotic alga (*Trebouxia* sp.) in the algal layer, the thickness of medulla (MT), central cord diameter, and area (CCD, CCA) were measured. *U. sphacelata* had comparable relative UCT (0.080–0.110, relative to diameter) to *U. aurantiaco-atra* (0.085–0.130). The relative MT was higher in *U. sphacelata* (0.240–0.300) than *U. aurantiaco-atra* (0.080–0.180). In *U. aurantiaco-atra*, the CCA was two times larger than that in *U. sphacelata*. Rapid freezing of thalli in liquid nitrogen led to an increase in TD, UCT, CCD because of intrathalline ice crystals formation. Cultivation of symbiotic alga at different temperatures (1.5, 6.0, 15.0, 22.0, and 28.0°C) with repetitive chlorophyll fluorescence parameters measurements showed growth optimum of 15.0°C for potential and effective quantum yield.

Introduction

The genus *Usnea* has large inter and intraspecific variability in anatomy and morphology (Randlane *et al.* 2009). This genus is most diverse in temperate and tropical forests, particularly in montane rainforests (*see e.g.*, Truong *et al.* 2013). However, it is distributed and abundant in many boreal forests as well as in the Southern Hemisphere including Antarctica (Øvstedal and Lewis Smith 2001). *Usnea sphacelata* and *Usnea aurantiaco-atra* are dominant components of Antarctic lichen flora in the South Shetlands archipelago. The two species have been studied both in the field and in a laboratory experiment in order to evaluate the species responses to environmental factors and determine the key factors affecting their growth and morphology. Within the last decade, attention

Highlights

- Temperature-response curves of F_V/F_M and Φ_{PSII} are triphasic in the two *Usnea* species
- Shock freezing of wet lichen thalli leads to an increase in thallus and cord diameter
- Isolated photobiont (*Trebouxia*) shows optimum of Chl fluorescence parameters at 15°C

was devoted to taxonomical problems (Mark et al. 2016), distribution, and ecological requirement (Ruiz-Fernández et al. 2017), in situ monitoring of growth rate (Sancho et al. 2017), and ecophysiology of Usnea sp. (Laguna-Defior et al. 2016). Particular physiological responses to limiting environmental factors, such as resistance to photoinhibition (Balarinová et al. 2014), temperature (Colesie et al. 2018), freezing tolerance (Hájek et al. 2016), and strategies of water uptake, and dehydration resistance (Jonsson Čabrajić et al. 2010), were studied as well. Last but not least, several studies have addressed photosynthetic performance evaluated by gas exchange (Kappen et al. 1995, Cao et al. 2015), chlorophyll (Chl) fluorescence exploiting (1) fast (OJIP) transients (Bednaříková et al. 2020a,b), (2) slow Kautsky kinetics with quenching analysis (Hájek et al. 2001, 2009) or (3) monitoring of in situ effective quantum yield of PSII (Cho et al. 2020). Some studies combine gas exchange and Chl fluorescence measurements (e.g., ten Veldhuis et al. 2020).

Ecophysiological characteristics of the two representatives of Antarctic Usneaceae reflect the concept of structure and function. In this respect, understanding of inter and intraspecific variability in thallus morphology is of crucial importance for the evaluation of the behaviour of the species in situ and their potential to cope with ongoing environmental changes in Antarctic terrestrial vegetation oases. It is well established that morphology may alter water storage strategies in representatives of genus Usnea (Eriksson et al. 2018). Such function-related morphological approach comprises studies of the branching pattern of thalli, 3-D structure and arrangement of lichen thalli clusters, and variability in morphological characteristics (e.g., soralia, or papillae; for details, see below). These studies are rather rare and there is still a substantial gap in knowledge regarding the morphometric parameters in Antarctic Usenaceae.

Seymour et al. (2007) focused on morphologies of the principal Antarctic species of genus Usnea. They described the most important interspecific differences between five species (U. antarctica, U. aurantiaco-atra, U. sphacelata, U. subantarctica, and U. trachycarpa). The authors compiled data from Walker (1985) and Øvstedal and Lewis Smith (2001) and classified the following morphological characteristics: presence/absence of soralia, papillae, fibrils, the pattern of thallus pigmentation, apothecia, and apothecia rays. They also paid partial attention to the cross-sections of the thalli and examined diameters of both the central axis and the whole branch and evaluated the relative diameter of the axis (expressed as a percentage of the whole thallus diameter). Morphological differences, however, might be found even within a single species. It has been reported for U. auratiaco-atra (Cao et al. 2018) that four morphological subgroups may be distinguished according to the presence/absence and proportion of apothecia to soredia.

In our study, we focused on the detailed biometrical analysis of the components forming cross-sections in two Usnea species: Usnea aurantiaco-atra and U. sphacelata. We also addressed the question of whether the proportion of individual structural components (upper cortex, medulla, and cord) to cross-section diameter have similar numerical value when evaluated for the basal, middle, and upper parts of individual branches. We hypothesized that the size of structural components of thallus might be changed and injured after fast freezing episodes happening to wet thalli in the field. To evaluate freezing-induced changes of the two Usnea species, we used a rapid freezing approach (see e.g., Orekhova et al. 2018) which focuses on anatomical properties and physiological processes in cells surviving a short-term immersion of wet lichen thalli to liquid nitrogen. Moreover, we addressed intraspecific differences in cryoresistance of the two species. We expected species-specific differences in photosynthetic performance (monitored by several Chl fluorescence parameters) in Usnea thalli exposed to a short-term progressive cooling from room temperature to –25°C. We also hypothesized that the photosynthetic performance of symbiotic alga would differ even during long-term cultivation at several low temperatures. Therefore, we cultivated isolated algal cells (Trebouxia sp.) in a gradient cultivator and monitored the effective quantum yield of photochemical processes in PSII in order to identify optimum growth temperature and the species-specific differences in the rate of acclimatory changes to low temperature.

Materials and methods

Lichen samples: Two fruticose lichens of Usnea genus were used for the study of anatomical changes after rapid freezing in liquid nitrogen. Usnea sphacelata samples were collected in February and March 2018. The collection site was located close to the Panorama Pass (63°48'51"S, 57°49'53"W, 242 m a.s.l.) at James Ross Island, Antarctica. Thalli of U. aurantiaco-atra were collected at the La Cruz Plateau in the Fildes Peninsula, King George Island (62°12'S, 58°57'W, 41 m a.s.l.). For more details of the collection site, see Casanova-Katny et al. (2016). After collection, the thalli were cleansed of substrate and dirt and subsequently dried out naturally (shade and windy place, at the temperature of 2 to 5°C), stored in a dry state in a refrigerator (at the temperature of 5°C for two weeks), and then transported to the Czech Republic.

Cross-sections and microscopy: In the laboratory at Masaryk University, Brno, the thalli were stored in a dry state for two months in a refrigerator at 5°C. Before experiments, the thalli were put in between two wet papers and rehydrated for 24 h at 5°C and under dim light [10 μmol(photon) m^{-2} s^{-1}]. Cross-sections were then prepared by razor blade on rubber pad in saturated state of lichen thallus. The cross-sections of thalli were made in different parts of thallus: nearly to the base, in the middle of the thallus (before first branching), bellow the top of the thallus (before the last branching). For preparing the cross-sections, 5% of the whole thallus length was used for basal cross-sections, and the middle part was defined by nearly 50% of the whole length. The part before the last branching of thallus was used for the top cross-sections. The cross-sections from every part were placed on a microscope slide into a drop of distilled water. About 20–30 cross-sections

suitable for photographing (undamaged, undistorted, without cracks, with the same width in the whole cross-section) were prepared from each part of the thallus (different thalli for each species). Before microscopy and photographing on a digital light microscope, the excess water was soaked by absorbent paper. The cross-sections were observed by digital light microscope *KEYENCE VHX-900F* (*Keyence Corp.*, Belgium).

Evaluation of anatomical parameters: Evaluated parameters related to the individual layers distinguishable at cross-sections were: (*1*) the width of the upper cortex, (*2*) the width of medulla with cells of the photobiont, (*3*) the width of central cord, and (*4*) the width of the whole thallus. In the case of distinguishable papillae on a cross-section, the width and length of papillae were measured. The width of the central cord and whole thallus was measured in five replicates, the width of the upper cortex and medulla in 20 replicates in different positions at the cross-section. All pictures were evaluated in graphic *VHX-500_900F* software *ver. 1.7.0.3* (*Keyence Corp.*, Belgium).

Rapid freezing effects on lichen anatomy: Samples of both lichen species were hydrated by distilled water in the Petri dish on/under a wet piece of filter paper until they reached the fully hydrated state. Hydration of thalli lasted 24 h at a room temperature (23°C). Whole hydrated thalli were then frozen by immersion in liquid nitrogen (–196°C) for 5 min. After the freezing procedure, the thalli were naturally thawed at laboratory temperature (23°C) and moistened with distilled water again. The control treatment was represented by the thalli fully hydrated by distilled water by the same method but not exposed to the freezing in liquid nitrogen. In both groups, *i.e.*, (*1*) untreated control and (*2*) rapid frozen and thawed thalli, anatomical characteristics were evaluated by optical microscopy in 20 replicates (cross-sections from five different thalli in each species).

Cultivation of symbiotic alga: For the photobiont growth experiments, we used the stock culture of *Trebouxia* sp. isolated from thalli of *Usnea sphacelata* (EEL – Collection of the Extreme Environments Life Laboratory Department of Experimental Biology, Masaryk University). The alga was inoculated on solid Bold basal medium (BBM) solidified with 1.5% agar and cultivated for six weeks before the experiments. Then it was cultivated in liquid BBM medium [temperature of 15°C, the light of 20 µmol(photon) m^{-2} s^{-1}] and used for experiments after reaching sufficient density (tested by digital light microscope).

Evaluation of optimum growth temperature: The algal culture (10 ml) was transferred from the liquid medium onto glass filters (porosity of 0.42 µm) by vacuum filtration to form a homogeneous layer of algal inoculum. Then, the filters with algal cultures were placed in Petri dishes on a solid BBM medium and the algae were allowed to grow for 14 d in aerophytic conditions [17°C, 35 µmol(photon)

m^{-2} s^{-1}]. During the growth, the cultures were checked regularly by Chl fluorescence measurements (*FluorCam HFC-010, Photon Systems Instrument*). After reaching optimal values of potential primary photosynthesis (F$_V$/F$_M$ > 0.6, for method, *see e.g.*, van Kooten and Snell 1990), the cultures on filters were used for evaluation of the optimum growth temperature. Petri dishes were placed on a temperature gradient cultivator (*LABIO*, Prague, Czech Republic) and cultivated at the temperature of 1.5, 6.0, 15.0, 22.0, and 28.0°C for 4 d. The cultures were exposed to PAR of 35 µmol(photon) m^{-2} s^{-1} and 16/8 h (L/D) photoperiod. Chl fluorescence parameters were measured every 24 h, exploiting the approach of slow Kautsky kinetics of Chl fluorescence supplemented with saturation pulses (for method *see e.g.*, Roháček *et al.* 2008). In order to evaluate the effect of cultivation temperature on primary photosynthetic processes, the following parameters were measured and analysed as dependent on the cultivation time: maximum yield of PSII photochemistry (F$_V$/F$_M$), effective quantum yield of PSII photochemistry (Φ$_{PSII}$), background (minimum) Chl fluorescence (F$_0$), peak Chl fluorescence on light (F$_P$), and maximum Chl fluorescence induced by saturation light pulse (F$_M$) – for parameters definition and application in lichen studies focused on temperature effects, *see e.g.*, Marečková and Barták (2016).

Chl fluorescence parameters in response to cooling: The culture of *T. erici* was filtered through a filter paper which was placed into the cooling chamber of the *Kryo-Planer* unit (United Kingdom). The chamber was linked to a 20-L Dewar flask with liquid nitrogen and cooled from 20 to −25°C at a constant rate of 2°C min^{-1}. Before cooling, an individual sample was equilibrated to 20°C for 15 min. The process of cooling was PC-controlled, and chamber (T$_{ch}$) and sample temperature (T$_s$) were regularly measured by in-built thermocouples. Simultaneously with cooling, Chl fluorescence parameters (F$_V$/F$_M$, Φ$_{PSII}$) were measured on algal culture by a *PAM 2000* fluorometer (*Walz*, Germany). The distance between the probe end and the sample was typically 3 mm to ensure a satisfactorily high Chl fluorescence signal. Repeated saturation pulses of 5,000 µmol(photon) m^{-2} s^{-1} (0.8 s) of PAR were applied each 30 s to induce maximum Chl fluorescence signals (F$_M$ and F$_M$' for dark- and light-adapted state, respectively). For F$_V$/F$_M$ determination, the following values were used: background (F$_0$) and maximum (F$_M$) measured on dark-adapted samples. For Φ$_{PSII}$ evaluation, actinic light of 30 µmol(photon) m^{-2} s^{-1} provided by a fluorometer was used. It was switched on 5 min before the cooling protocol started and lasted until the end of the cooling period. Three repeated measurements were conducted for F$_V$/F$_M$ and Φ$_{PSII}$. Means of F$_V$/F$_M$ and Φ$_{PSII}$ were calculated for a class of 1°C and plotted against temperature.

Statistical analysis: For the statistical evaluation, we used factorial analysis of variance (*ANOVA*) with the software *Dell Statistica ver. 13* (*Dell Inc.*, Tulsa, USA). The significant differences between tested variants of biometrical thalli parameters and indices were evaluated

by *Fisher*'s LSD test at *p*=0.05 after the normality of the data was verified and homogeneity of data variance was confirmed.

Results

Analysis of biometric parameters derived from cross-sections revealed anatomical structures typical for *Usneaceae* in both species, *i.e.*, thick cortex layer and underlying medulla with irregularly arranged clusters of *Trebouxia* sp. forming interrupted ring (Figs. 1, 2). In the central part of cross-sections, mechanical pseudo tissue (cord) was found.

The diameter of cross-sections of wet branches showed a relatively large variation among individual thalli in both species (Fig. 3). However, it generally decreased from the base to apex of individual branches, more apparently in *U. aurantiaco-atra*. *U. aurantiaco-atra* had about two times higher thallus diameter in the basal part of individual branches. The thickness of cortex, medulla, and cord all increased with the branch diameter.

Cells of algal photobiont were found beneath the upper cortex in the outermost part of the medulla. They were irregularly distributed forming clusters of different sizes (typically ranging from 10 to 120 μm). No regular ring of an algal layer was observed in both species, contrastingly

to other species of the genus (*e.g.*, *U. flammea*, *U. sub-floridana*, *U. cornuta* – Eriksson 2016).

The relative thickness of the upper cortex and medulla along the radius (*see* index values in Fig. 4) varied between the two species. *U. antarctica* had a comparable relative thickness of cortex (0.080–0.110) to *U. aurantiaco-atra* (0.085–0.130). The relative thickness of medulla, however, showed high interspecific differences: 0.240–0.300 (*U. sphacelata*) and 0.080–0.180 (*U. aurantiaco-atra*). Relative index for the cord (cord diameter to thallus thickness) was found much lower for *U. sphacelata* (0.260–0.300) than that of *U. aurantiaco-atra* (0.400–0.650). Species-specific differences in central cord diameter were also reflected in the relative cord area (*see* Fig. 3). In *U. sphacelata,* much lower value (0.230–0.300) was found than that for *U. aurantiaco-atra* (0.440–0.620).

A central cord was formed by thick-walled, agglutinated hyphae. In both species, a single threadlike elastic cord was found. The cord formed a central axis which provides the requisite tensile and skeletal strength to an individual part of a single branch. The filaments forming the central cord were packed and observed as integrated tissue in which no individual cells/cell walls were distinguishable by digital microscopy (Fig. 1).

The cord formed much larger part of overall cross-section in *U. aurantiaco-atra* than in *U. sphacelata* (Fig. 1), and reached higher values of cord diameter in basal, middle, and apex parts (Fig. 3). The relative thickness of the upper cortex, medulla, and cord are given as index values (relative to thallus diameter) in Fig. 4. In the majority of cases, they differed between the two species, and before and after shock freezing.
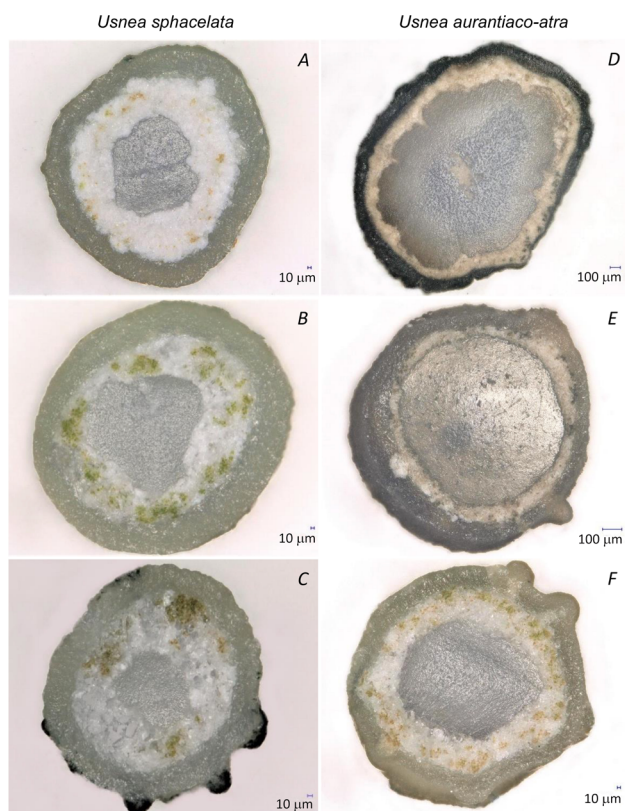
Chl fluorescence parameters in response to cooling: In *T. erici*, F_V/F_M values decreased with decreasing sample temperature forming a triphasic curve (Fig. 5). The three phases were distinguished as follows: an initial linear decrease found at the sample temperature decreasing from 20 to 8°C (Phase I), an intermediate phase typical of more or less constant F_V/F_M value (0.32, decrease from 8 to –11°C, Phase II), and the end part of the curve (a decrease to close-to-zero values of F_V/F_M found at –25°C, Phase III). Similar phases were distinguished for Φ_{PSII}, which decreased rapidly with temperature from 20 to 8°C (Phase I). Then, Phase II was typical by a slight decrease of Φ_{PSII} from 8 to –16°C. Phase III was in the temperature range of –19 to –25°C. At –25°C, zero of Φ_{PSII} was reached. Within the whole range of decreasing temperature, the decline of Φ_{PSII} was more rapid and pronounced than F_V/F_M (*see* inset in Fig. 5).

Optimum growth temperature: Chl fluorescence parameters related to primary photosynthetic processes (F_V/F_M, Φ_{PSII}) of *T. erici* showed an adjustment to cultivation temperature, more pronounced with cultivation time (Fig. 6). After 96 h, the optimum temperature of 15°C was found in both species. Higher or lower temperatures led to the limitation of F_V/F_M and Φ_{PSII}. Apart from the two parameters, Chl fluorescence signals showed temperature dependence. For background Chl fluorescence (F_0), an

Fig. 1. Typical cross-sections of *Usnea sphacelata* and *Usnea aurantiaco-atra* taken in basal (*A,D*), middle (*B,E*), and apical parts (*C,F*) of individual branches of thallus. The photos taken by a *Keyence 500* digital microscope and its graphic software.
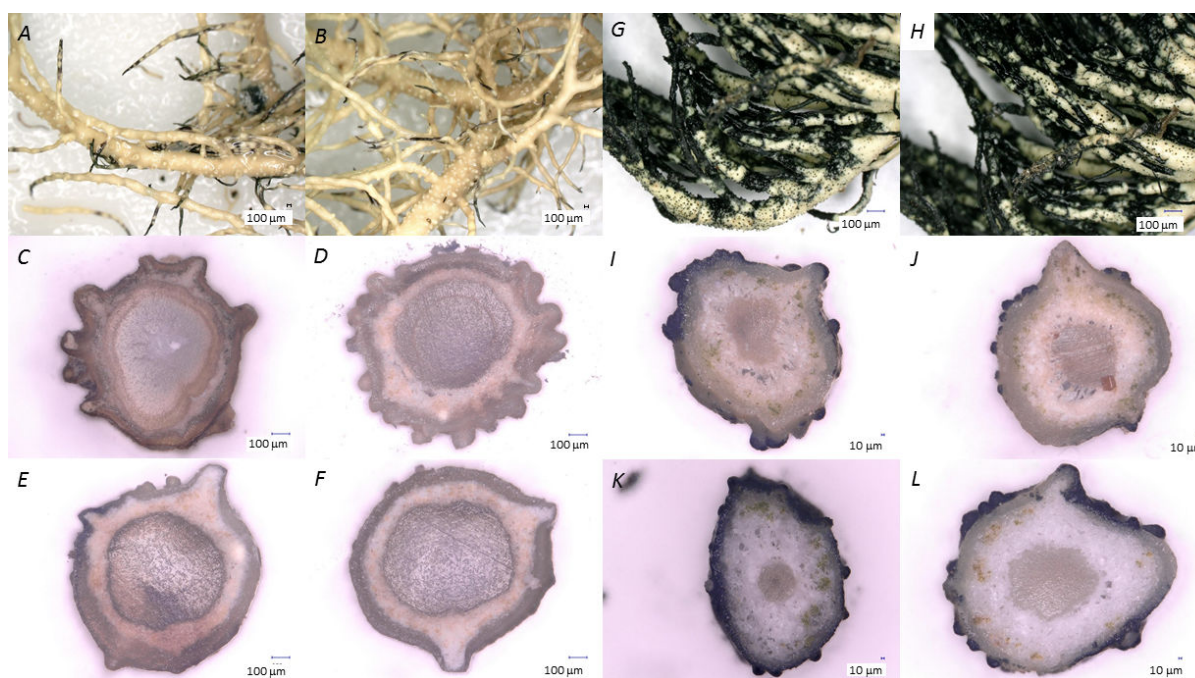
Fig. 2. Papillae distribution over a branching structure of *Usnea aurantiaco-atra* (*A,B*), and *Usnea sphacelata* (*G,H*). Selected cross-sections showing papillae number and size in *U. aurantiaco-atra* (*C–F*) and *U. sphacelata* (*I–L*). The photos taken by a *Keyence 500* digital microscope and its graphic software.

increase was found in the above-optimum temperature within the first 24 and 48 h, followed by a decrease after 96 h. At 1.5°C, no change from control (*i.e.*, before cultivation experiment) was found. Maximum Chl fluorescence signal (F_M) showed a time-dependent decrease in high (28°C) and low (1.5°C) temperatures. Chl fluorescence signal at P point (F_P) showed a time-dependent increase in optimum temperature (15°C), and a decrease at high (28°C) and low (1.5°C) temperature.

Discussion

Thallus anatomy: Cross-sections of *Usnea sphacelata* and *U. aurantiaco-atra* revealed that the thickness of particular layers differed in separate thalli parts, *i.e.*, basal, middle, and apex. Interspecific differences were apparent as well. Overall thallus morphology was classified according to Cao *et al.* (2018) for each species. Our data showed that *U. aurantiaco-atra* belonged to the morphological groups 1 and 2: 1 – thallus with apothecia without soredia, 2 – thallus with apothecia with soredia, while *U. sphacelata* fitted to the group 3 – thallus without apothecia and with soredia. For *U. sphacelata*, however, rare apothecia are reported by Walker (1985). *U. aurantiaco-atra*, on the other hand, has numerous apothecia, and soralia are either observed or missing (Seymour *et al.* 2007). The species, however, is found without soralia more frequently, similarly to the thalli examined in our study. The presence of papillae on the upper side of the cortex is typical for *U. sphacelata*. Only a limited number of papillae was observed in *U. aurantiaco-atra*. The species was typical

by numerous secondary branches which were differentiated from papillae according to cross-sections as well (Fig. 2*B–F*).

The upper cortex averaged 9.1% of the cross-section diameter in *U. sphacelata* and 10.8% in *U. aurantiaco-atra* (Fig. 3). The upper cortex consisted of dense, parallel-arranged rows of thick-walled fungus cells. The upper cortex is considered a protective layer for lichens (He *et al.* 2012), in which a wide variety of phenolic secondary metabolites is localized. Among them, usnic acid is reported to be localized on the surface of the upper cortex of *Usnea* sp. (Gerlach *et al.* 2017). The amount of usnic acid, however, may differ within a season in Antarctica (Quilhot *et al.* 1991).

Relative area of cord: In the central part of thalli, an axis of the thallus (cord), is found, consisting of fibers longitudinally oriented and closely connected. The hyphae are formed by tightly ordered chondroid cells (*see e.g.*, Tõrra and Randlane 2007). In our study, a wide range of relative area of the cord was found (51.5, 41.7, and 19.9% for basal, middle, and apical parts of *U. aurantiaco-atra*, respectively, while 10.7, 9.8, and 6.9% for basal, middle, and apical parts of *U. sphacelata*, respectively). For the basal part of *U. aurantiaco-atra*, Seymour *et al.* (2007) reported 56 and 61% area of cord, respectively, which is well comparable to our data. Data for the middle and top of the thallus are missing. A more detailed analysis of the ratio of the central section (cord) of the thallus to the overall width of the thallus *U. sphacelata* allows comparison with the data published in the experimental study by Zvěřina
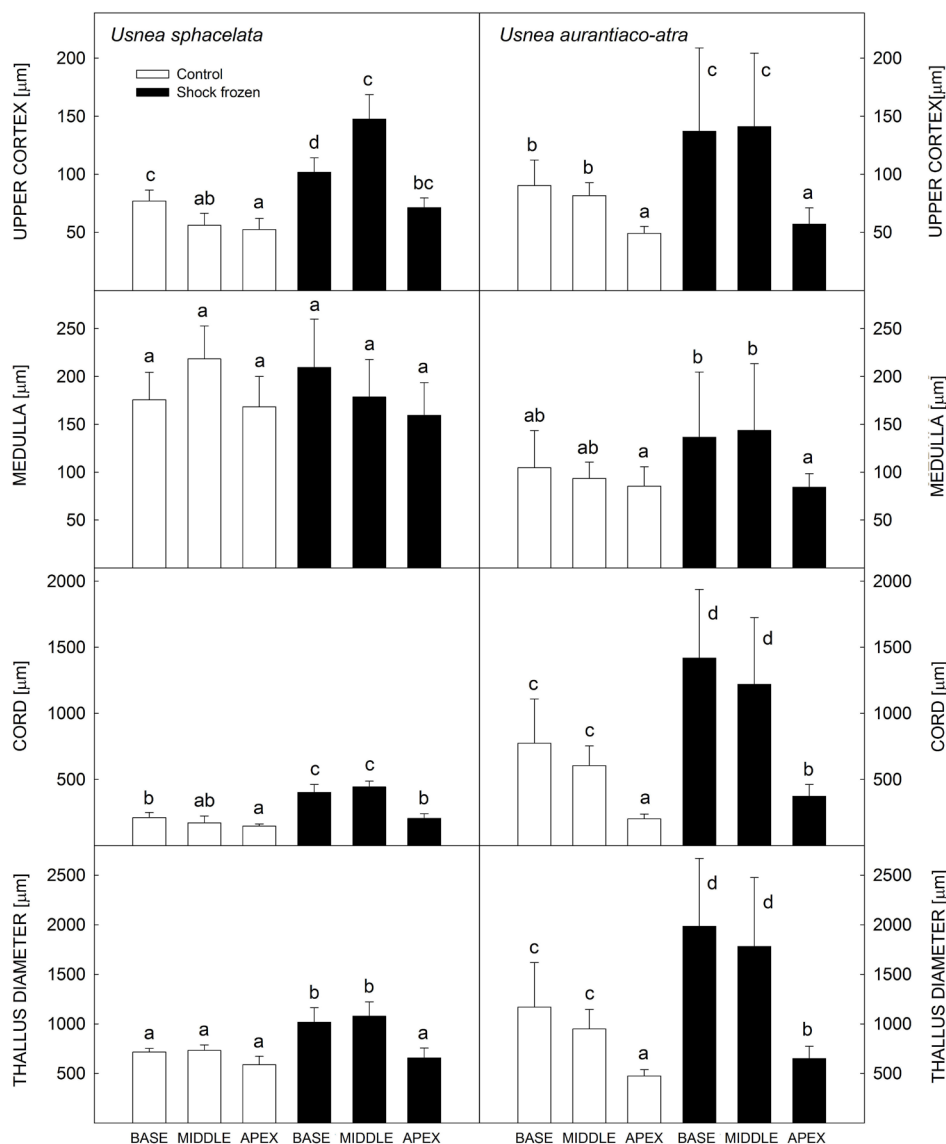
Fig. 3. Biometrical thalli parameters evaluated from cross-sections of *Usnea sphacelata* and *Usnea aurantiaco-atra*: upper cortex thickness, medulla thickness, cord diameter, and thallus diameter. The data represent mean of 60 replicates ± SD for particular parameters measured in untreated control and the thalli treated by shock freezing. *Different letters* show significant differences (*ANOVA*, *Fisher*'s LDS test, $p<0.05$).

et al. (2018), which recalculated the proportions of the basal – middle – apical part of *U. sphacelata* thallus as 48 – 28 – 11%.

In other species of *Usnea* genus, the ratio evaluated for basal part tends to reach somewhat lower values: 41% (*U. florida*; data from the Department of Botany, University Hamburg), 38 and 44% (*U. intermedia* and *U. fulvoreagens*; http://www.lichenes.de/), 44% (*U. longissima*; Sanders and de los Ríos 2012). For *Usnea* lichens from tropical areas, which are characterized by long, pendulous thalli, the ratio of the central section (cord) area to the overall area of the cross-section of the thallus is often less than for lichens from polar regions. This is evidenced by the data published in Ohmura (2012), which, after calculating the ratio, gives a value of 45% for *U. ceratina*, 21% for *U. bicolarata*, and 32% for *U. rubrotineta*.

Apart from the differences found in the relative cord area for basal, middle, and apex part, one must consider also age-dependent differences related to the development of particular parts of the anatomical structure. It was shown by Ott (2004) that intrathalline anatomical differentiation starts in the early developmental stages of *U. antarctica* (form soredia). Partial components, *i.e.*, the upper cortex, algal layer, medulla, and central axis (cord) can be easily distinguished. For 6-year-old *U. antarctica* thalli, the author reported central cord occupied most of the space inside such young thallus.

Rapid freezing resulted in statistically significant changes in some anatomical parameters. It might be attributed to the mechanisms of tissue injury caused by a fast cooling rate. In such a case, intracellular ice and extracellular ice crystals are formed (Ba *et al.* 2013), that cause mechanical damage to the cell membranes. Rapid freezing (immersion into liquid N), however, does not lead to freezing-induced cellular dehydration, because extracellular ice formation is not, due to too short time, supported by water transport from the cells to extracellular spaces. Upper cortex thickness, thallus thickness, and cord diameter responded most sensitively in both species and increased after rapid freezing and melting. Such response
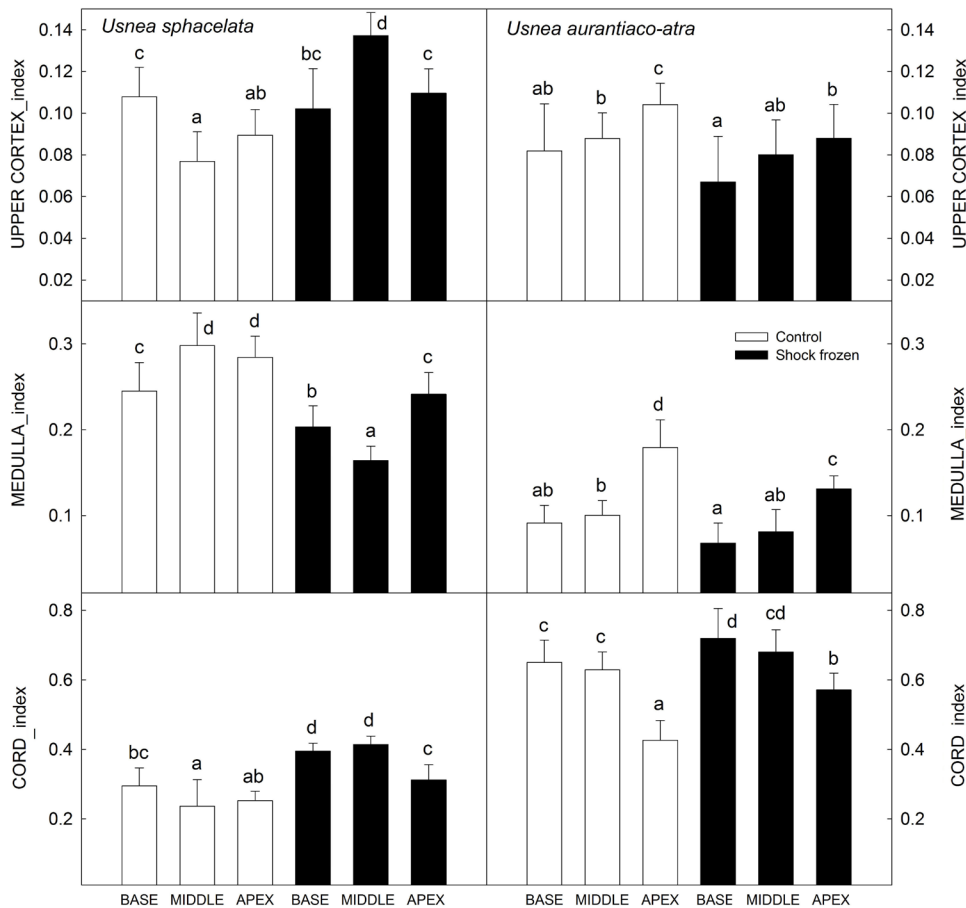
Fig. 4. Biometrical indices (relative to thallus diameter) evaluated for cross-sections of *Usnea sphacelata* and *Usnea aurantiaco-atra*: upper cortex thickness, medulla thickness, cord diameter, and thallus diameter. The data represent mean of three replicates ± SD for particular parameters measured in untreated control and the thalli treated by shock freezing. *Different letters* show significant differences (*ANOVA, Fisher*'s LSD test, *p*<0.05).
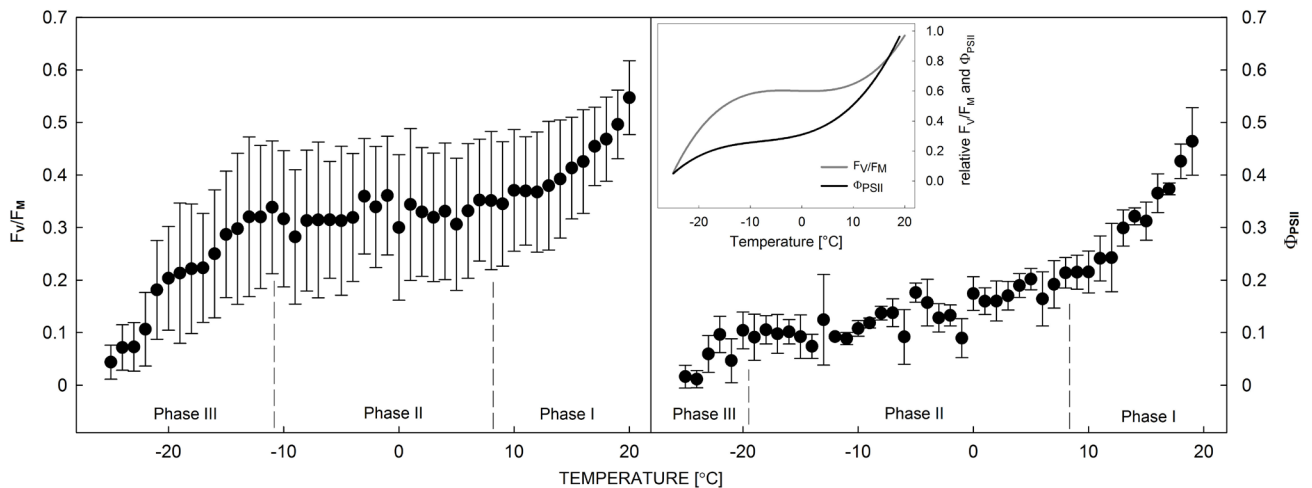

Fig. 5. Effect of linear cooling on chlorophyll fluorescence parameters. *Left panel*: F_V/F_M – potential yield of photochemical processes of photosynthesis in photosystem II; *right panel*: Φ_{PSII} – effective quantum yield of photochemical processes of photosynthesis in photosystem II of *Trebouxia* sp. The data points represent mean values of at least three replicates ± SD for a class of 1°C. *The inset* shows fitted curves for F_V/F_M and Φ_{PSII} as dependent on thallus temperature.

can be attributed to the volume growth of frozen water (compared to liquid) that irreversibly increased the size of the thallus structures typical by densely arranged hyphae. Medulla, typical by large intercellular spaces, did not show any freezing-induced increase in the size (thickness) in

both species. Ice crystals formation in Antarctic lichens depends also on the size of intrathalline ice-nucleating particles. For *U. aurantiaco-atra*, different sizes of the particles may shift ice nucleation temperature from –8.3 to –5.1°C (Worland *et al.* 1996).
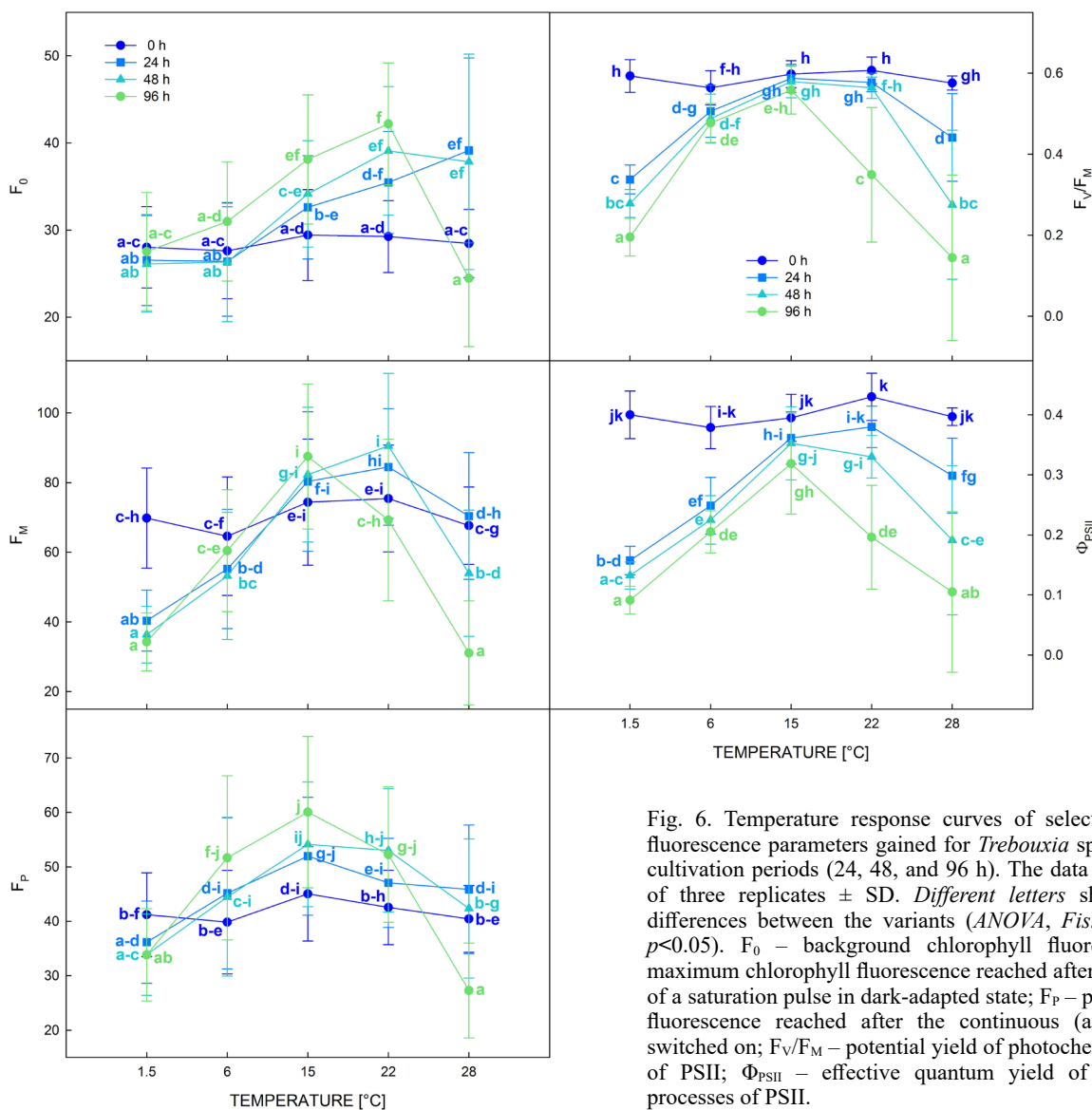
Fig. 6. Temperature response curves of selected chlorophyll fluorescence parameters gained for *Trebouxia* sp. after different cultivation periods (24, 48, and 96 h). The data represent mean of three replicates ± SD. *Different letters* show significant differences between the variants (*ANOVA*, *Fisher*'s LSD test, $p < 0.05$). F_0 – background chlorophyll fluorescence; F_M – maximum chlorophyll fluorescence reached after the application of a saturation pulse in dark-adapted state; F_P – peak chlorophyll fluorescence reached after the continuous (actinic) light is switched on; F_V/F_M – potential yield of photochemical processes of PSII; Φ_{PSII} – effective quantum yield of photochemical processes of PSII.

Effect of freezing on Chl fluorescence parameters: The polyphasic decrease of the maximum quantum yield (F_V/F_M) of *T. erici* during linear cooling is comparable to the response of poikilohydric organisms, lichens (Hájek *et al.* 2016) and mosses (Folgar-Cameán and Barták 2019). The decrease in F_V/F_M and Φ_{PSII} with temperature decrease is associated with increased limitation of PSII functioning, photosynthetic linear electron flow, and utilization of ATP, NADPH in primary photosynthesis products formation (Genty *et al.* 1989). At sub-zero temperature, a significant decrease in F_V/F_M at the accelerated rate occurred at –12°C. Such a response could be associated with the phenomenon of ice crystals formation (ice nucleation) as reported by Schroeter and Scheidegger (1995) and Harańczyk *et al.* (2003) for fully hydrated lichens exposed to freezing temperature. F_V/F_M decrease is caused by a sub-zero temperature-induced decrease of absolute F_M values simultaneously co-occurring increase in background fluorescence (F_0 relative to F_M) as shown earlier for lichens

(Mishra *et al.* 2015) and vascular plants as well (Mishra *et al.* 2011). The critical temperature for F_V/F_M and Φ_{PSII} is similar, *i.e.* –25°C, for most lichens (Hájek *et al.* 2016). In general, the significant decrease in F_V/F_M and Φ_{PSII} values recorded in the cooling temperature ranged from –12°C to –25°C can be explained by the formation of crystallization cores and the formation of ice crystals, which takes place at nucleation temperature.

The results obtained in this experiment using the linear cooling method for *T. erici* algae are comparable to those obtained by Šabacká and Elster (2006), who investigated 15 species of filamentous cyanobacteria, predominantly of the genus *Phormidium*, and 9 species of unspecified green terrestrial algae. The authors evaluated the viability of algal cells exposed to various methods of cooling, including linear cooling. This characteristic is significantly higher for algal samples taken in continental Antarctica than in coastal Antarctica. These conclusions can also be supported by the results of an earlier study carried out on

various algal species of the genus *Trebouxia* (Hájek *et al.* 2012), which reported a decrease in viability to 60–74% using a similar method of linear cooling. Besides, the reported decreases in F_V/F_M and Φ_{PSII} were species-specific in this work and differed between the *T. asymmetrica*, *T. glomerata*, and *T. erici* species studied.

The ability of *Trebouxia* sp. cells to maintain primary photosynthetic processes is related to the ability to form extracellular ice at sub-zero temperature, which is considered a protective mechanism (Moffett *et al.* 2015). Several phases of ice formation might be distinguished thanks to free and bound water molecules. The process is supported by the substances with nucleation activity, the presence of which has been proven for *U. antarctica* thalli (Harańczyk *et al.* 2006). The temperature of the ice nucleation is, however, species-specific. Moffett *et al.* (2015) determined the nucleation temperature for numerous lichen species from different regions of the Earth and reported –6.5°C for *Usnea*. The value is slightly higher than that found for *T. erici* in our study (–12°C, *see* Fig. 5). This difference can be explained by the fact that the nucleation temperature for the genus *Usnea* was measured on samples from England, while the alga *T. erici* used for our experiments was isolated from the Antarctic lichen *U. antarctica*. This is also consistent with data of Kvíderová *et al.* (2013) who reported the range of –12°C to –16°C for *Trebouxia* sp.

Cultivation optimum for *Trebouxia* sp. assessed by Chl fluorescence: The temperature optima for F_V/F_M and Φ_{PSII} found at 15°C are well comparable to an earlier study of Bayer and Alba (2017) who reported the range of 10–20°C for several Chl fluorescence parameters related to *T. erici* PSII activity. The optima, however, cannot be related to the whole photosynthetic pathway because no biochemical processes were studied in this experiment. Domaschke *et al.* (2013) measured photosynthetic and growth optima for *Trebouxia* sp. Their oximetry data suggest that the temperature optimum of photosynthesis could be as low as 11°C if measured on *Trebouxia* sp. isolated from lichens from polar regions. Another study in *T. erici* (Sehnal *et al.* 2014) reports optimum temperature higher than 12°C based on F_V/F_M, pigments, and biomass production. The ability of Antarctic algae to grow at a higher than optimum temperature is in agreement with the findings of Seaburg *et al.* (1981).

For growth rate and biomass production of Antarctic terrestrial microalgae evaluated by optical density (OD), the range 6–14°C is reported as the optimum growth temperature (*Chlorella* sp., *Stichococcus* sp.; Teoh *et al.* 2004). For *Trebouxia* sp., similar OD-based growth rates are reported for 10 and 15°C during short-term cultivation (16 d) by Balarinová *et al.* (2014). With prolonged cultivation (16–30 d), however, the authors report a faster growth rate at 10°C.

Conclusions: Comparative anatomic study of two species collected from Antarctica (*Usnea aurantiaco-atra*, King George Island; *U. sphacelata*, James Ross Island) revealed that the two species differed in biometrical parameters derived from cross-sections of their thalli. *U. sphacelata* had similar values of the relative thickness of cortex (0.080–0.110, relative to diameter) as *U. aurantiaco-atra* (0.085–0.130). Other parameters showed species-specificity. The relative thickness of medulla was found higher in *U. sphacelata* than *U. aurantiaco-atra*. The cord formed about two times larger part of the overall cross-section area in *U. aurantiaco-atra* than in *U. sphacelata* in basal, middle, and apex parts of the thallus. Since rapid freezing of wet lichen thalli accompanied by the formation of intra- and extracellular ice crystals occur in maritime Antarctica quite frequently, we studied the sensitivity of anatomical parameters to rapid freezing. In the laboratory-based experiment, rapid freezing in liquid nitrogen, followed by gradual thawing led to an increase in thallus diameter, upper cortex thickness, and cord diameter, more apparently in *U. aurantiaco-atra* than *U. sphacelata*. Photosynthetic responses to sub-zero temperature were evaluated for symbiotic alga *Trebouxia* sp. exposed to linear cooling from physiological (20°C) to sub-zero temperature (–30°C). The cooling-induced decrease in potential (F_V/F_M) and effective quantum yield of PSII (Φ_{PSII}) were triphasic showing a fast–slow–fast decline of the two Chl fluorescence parameters with temperature fall. Strong limitation of PSII primary photosynthetic processes occurred at the temperature below –10°C, which might be attributed to ice formation. The critical temperature, at which full inhibition of F_V/F_M and Φ_{PSII} was found, reached –25°C. Despite such cryo-resistance of primary photochemical processes of photosynthesis in *Trebouxia* sp. had their temperature optimum at 15°C in a short-term (96 h) experiment. It revealed that optimum growth temperature was 15°C for both species, and potential (F_V/F_M) and actual (Φ_{PSII}) primary photosynthetic processes declined in a curvilinear manner with temperature fall from 20 to –30°C (critical temperature).

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