Tolerance to photoinhibition within lichen species is higher in melanised thalli

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Abstract

Some lichenized ascomycetes synthesize melanic pigments when exposed to ultraviolet light and high solar radiation. Here, we used chlorophyll fluorescence in order to test the effect of melanisation on the ability of the lichens *Cetraria islandica*, *Lobaria pulmonaria*, *Peltigera aphthosa*, *P. membranacea*, *Pseudocyphellaria gilva*, and *Sticta sublimbata* to tolerate high-light stress. For each species, melanised and pale thalli were selected from relatively exposed and shaded individuals of the same population. For all species, melanised thalli displayed significantly less photoinhibition than pale thalli following a controlled exposure of hydrated thalli to high light. Melanised thalli were less photoinhibited than pale thalli in lichens from both high light and more shaded habitats, and those that possess either green-algal or cyanobacterial photobionts. Photoinhibition also occurred when dry lichens were exposed to high light; melanised thalli showed again less photoinhibition. Melanised thalli are less sensitive to the adverse effects of high light.

Additional key words: acclimation, desiccation, photoprotection, UV-B.

Introduction

Lichens represent a symbiosis between an ascomycete and one or more photosynthesizing algae or cyanobacteria, often with small amounts of basidiomycete yeasts (Spribille *et al*. 2016). Arguably, what makes lichens special, and what separates them from most other eukaryotic organisms, is their ability to tolerate extreme stress (Beckett *et al*. 2008). Lichens frequently grow in exposed microhabitats where they experience high light. In photosynthetic tissues, excessive solar radiation can cause a temporary or more permanent reduction of photosynthesis known as "photoinhibition". Photoinhibition is caused, directly or indirectly, by the stimulation of production of reactive oxygen species (ROS) that occurs when photosystems cannot use the light energy they are absorbing, and this energy rather activates oxygen (Gururani *et al*. 2015, Pospíšil 2016). The D1 protein, a key component of PSII, is believed to be the most sensitive to damage during photoinhibition. Some damage appears to occur even in moderate light intensities, therefore photo-synthesizing organisms must continuously repair the damage to this protein. The "PSII-repair cycle", occurring in chloroplasts and in cyanobacteria, involves degrading and re-synthesizing the D1 protein, followed by activation of the reaction center (Nath *et al*. 2013). Recent studies of photoinhibition have suggested that light-induced ROS formation causes most damage by inhibiting D1 protein repair, rather than directly affecting PSII (Nishiyama and Murata 2014). Irrespective of how it occurs, photoinhibition reduces the ability of lichen photobionts to photosynthesize. Strong evidence exists that photoinhibition regularly occurs in lichens in field situations (*e.g.*, Leisner *et al*. 1997, Jairus *et al*. 2009, Míguez *et al*. 2017).

Plants possess a variety of mechanisms that help to overcome high light-induced photoinhibition. Some of these mechanisms involve reduction of ROS formation by converting excess light energy into thermal energy, a process termed nonphotochemical quenching (NPQ). Briefly, an increase in the gradient of protons across the thylakoid membrane triggers rearrangements and aggregation of LHCII and the formation of "NPQ quenchers". It has been

estimated that each unit of NPQ can protect against light of *ca*. 400 µmol(photon) m^{-2} s^{-1} (Ruban 2016). However, NPQ is clearly a plastic character that varies between and within species, and increases with increasing ambient light (Gururani *et al*. 2015). Interestingly, recent evidence suggests that, unlike higher plants, photorespiration is not important for dissipating excess energy under excess light in algae, and therefore presumably is not important in lichens (Hanawa *et al*. 2017).

Although lichens possess some of the biochemical adaptions to high light, which are found in higher plants (*e.g.*, Adams *et al*. 1993), they often respond to UV-B and high light by synthesizing cortical compounds (Solhaug and Gauslaa 2012). The most widespread cortical pigments are classic lichen substances, such as usnic acid, atranorin, and parietin (Solhaug and Gauslaa 2012). In some lichens, high light or UV induce the synthesis of a dark-coloured melanic pigments. There is little evidence that higher plants produce melanins in their leaves, although they may do so in their roots (Soares *et al*. 2011) or seeds (Al-Mufarrej *et al*. 2006). Rather, in higher plants, flavonoids appear to play the same role as melanins in lichens (Carletti *et al*. 2014). Melanin-producing lichens come from a range of lichen orders, and grow on a variety of substrata (Gauslaa and Solhaug 2001). There are few studies directly demonstrating that melanins protect lichens against photoinhibition. McEvoy *et al*. (2007) transplanted thalli of *Lobaria pulmonaria* to three sites with low, medium, and high light intensities for 100 d. Lichens at the site with the highest light became significantly melanised. Thalli from each of these sites were then transplanted to a highly-exposed area for 12 d. The melanised thalli displayed a much smaller reduction of F_V/F_M than the material at the more shaded sites, strongly suggesting that melanins are involved in photoprotection. Further suggestion that melanins play protectives role came from the study of Färber *et al*. (2014) on pendulous lichens that dominate canopies of boreal forests. Typically, dark *Bryoria* species grow in the upper canopy, while pale *Alectoria* and *Usnea* species occupy the lower canopy. Exposing these lichens under controlled conditions to a light of 400 µmol(photon) m^{-2} s^{-1} for 7 d caused much less photoinhibition in the melanised *Bryoria* species than that of the non-melanised *Alectoria* and *Usnea*. The main aim of the work presented was to systematically carry out within-species comparisons of the tolerance to photoinhibition in melanised and pale individuals of a range of lichens, including species with chlorophycean and cyanobacterial species, which grow in shaded or more light-exposed habitats.

A further aim of our study was to test whether melanisation is more effective in protecting dry or wet thalli. It is well known that lichens suffer from light stress even when desiccated (Kershaw and MacFarlane 1980). In bryophytes, desiccation apparently does not stop the transfer of excitation energy from the light-harvesting pigments to the reaction centers (Heber *et al*. 2006). Furthermore, no water is necessary for the formation of singlet oxygen from a chlorophyll triplet (Pospíšil 2106). It may be also relevant that changes in gene expression and/or protein synthesis have been observed in dry seeds of *Nicotiana* (Bove *et al*. 2005, Leubner-Metzger 2005) and *Arabidopsis* (Cadman *et al*. 2006, Chibani *et al*. 2006). These results have been interpreted as indicating the existence of hydrated pockets. Possibly, similar pockets in dry lichens allow ROS such as H_2O_2 to form when desiccated thalli are illuminated. Even if light only causes the formation of tiny amounts of ROS, in desiccated thalli, normal processes, which repair DNA and other biomolecules, do not take place (Buffoni-Hall *et al*. 2003). In *L. pulmonaria*, cortical transmittance to PAR is typically between one third and one half lower in the air-dry compared to the hydrated state (Gauslaa and Solhaug 2001). Melanisation significantly reduces transmittance, and interestingly, the transmittance of the upper cortex of melanised thalli to PAR is about 40% lower than pale thalli when the thalli are dry, but only about 30% lower when the thalli are wet. We therefore hypothesized that melanisation may give a greater photoprotection when the thalli are air-dry.

Materials and methods

Plant material: Lichen material was collected from the localities listed below. For each species, melanised and pale thalli were selected from exposed and shaded individuals of the same population, never more than 5 m apart. If collected wet, material was slowly dried at room temperature. Material was stored at –20ºC until ready for experimentation for a maximum of two weeks. One day before each experiment, 2-cm discs were cut, or for small thalli, thallus fragments were selected. Lichens were stored overnight on wet filter paper in Petri dishes under dim light [5 µmol(photon) m^{-2} s^{-1}] at a cool temperature (15ºC, thermostatically controlled room). Before high-light treatment, chlorophyll (Chl) fluorescence parameters were measured, and then material was either slowly dried for 4 h, or used immediately.

Species	Collection locality	Photobiont	Shade/sun species
Cetraria islandica (L.) Ach.	Soil, Langangen, Norway	Chlorophycean	Sun
Lobaria pulmonaria (L.) Hoffm.	Oak trees, Langangen, Norway	Chlorophycean	Shade
Peltigera aphthosa (L.) Willd.	Soil, outskirts of Ås, Norway	Chlorophycean	Sun
Peltigera membranacea (Ach.) Nyl.	Soil, outskirts of Ås, Norway	Cyanobacterial	Sun
Pseudocyphellaria gilva (Ach.) Malme	*Leucosidea sericea* trees, Fort Nottingham, RSA	Cyanobacterial	Shade
Sticta sublimbata (J.Steiner) Swinscow & Krog	*Leucosidea sericea* trees, Fort Nottingham, RSA	Cyanobacterial	Shade

Reflectance measurements to estimate melanin content: Reflectance spectra of the upper cortex were recorded on dry thalli as described by Solhaug *et al.* (2003). Briefly, an integrating sphere (*model ISP-50-REFL OceanOptics*, Eerbeek, The Netherlands) was pressed against the thalli, and thalli were then illuminated with a halogen lamp (*model DH2000, OceanOptics*) through a 600-μm optical fibre (thick) connected to the input port of the integrating sphere. Reflectance (400–1,050 nm) was measured with a spectrometer (*model SD2000, OceanOptics*) connected to the output port of the sphere with a 400-μm fibre. Reflection was calculated relative to a reference spectrum derived from a white reference tile (*WS-2, OceanOptics*). The browning reflectance index (BRI), calculated as $(1/R_{550} - 1/R_{700})/R_{750}$ (Solovchenko *et al.* 2001), was used as a quantitative estimate of melanic compounds.

Chl *a* fluorescence was measured using the red version of the *PAM 2000* fluorimeter (*Walz*, Effeltrich, Germany). The maximal efficiency of PSII (F_V/F_M) and the operating efficiency of PSII (Φ_{PSII}) were measured. Thalli were initially dark-adapted at room temperature for 10 min. At the start of a run, a saturating flash was applied to determine F_V/F_M. Thalli were then exposed to PAR at 30 μmol(photon) m^{-2} s^{-1} for 10 min, after which fluorescence had reached a stable value. Another saturating flash was then applied, and F_V/F_M and Φ_{PSII} was calculated as:

$$F_V/F_M = (F_M - F_0)/F_M$$

where F_M is the maximum fluorescence (reaction centers closed) and F_0 is the minimum fluorescence (reaction centers open), and:

$$\Phi_{PSII} = (F_{M'} - F_t)/F_{M'}$$

where F_t is the stable fluorescence signal in the light, and $F_{M'}$ is the maximum fluorescence at saturating light intensity pulse.

High-light stress was applied by a white LED light-source panel (*Model SL-3500, Photon System Instruments*, Brno, Czech Republic). Light was set at 800 or 1,500 μmol(photon) m^{-2} s^{-1} until the maximum quantum yield of PSII photochemistry (F_V/F_M) was reduced from values of around 0.7 to 0.3–0.4, with each species needing different times as indicated in Table 1.

Chl fluorescence measurements were made in moist material before high-light treatment ("time zero"), at the end of the high-light treatment, and then at intervals for up to 40 h. Photoinhibition in thalli exposed to high light in the dry state was measured at intervals following the hydration of replicate samples. When sufficient inhibition had occurred, they were immediately hydrated following exposure by being placed on moist filter paper and then sprayed with additional distilled water. During recovery, lichens were maintained at room temperature (20ºC) and dim light [5 μmol(photon) m^{-2} s^{-1}] as recommended by Solhaug (2018).

Results were analyzed using a two-way analysis of variance (*ANOVA*, *Microsoft Excel*), with melanisation and time as the two factors.

Results

Lichens were more tolerant to high light when dry; compared to wet thalli, much longer exposures were needed to create similar photoinhibition (Table 1). In general, lichens collected from open habitats required longer times or high light levels to create similar levels of photoinhibition compared to species from more shaded localities.

Measurement of thallus reflection indicated that the extent of melanisation varied greatly between different lichen species (Table 2). For example, in *Pseudocyphellaria gilva*, the BRI of melanised thalli was only 20% higher than that of pale thalli, while in *Cetraria islandica*, the BRI was more than one hundred times greater (Table 2).

Initial values of F_V/F_M and Φ_{PSII} were very similar in melanised and pale thalli (Figs. 1–3). In most cases, 24 h after the photoinhibitory stress, almost all lichens fully recovered, although the recovery was not complete in some cases, *e.g.*, non-melanised *C. islandica* (Fig. 1*B,D*), wet *Peltigera membranacea* (Fig. 2*E,F*) and *Pseudo-cyphellaria gilva* (Fig. 3*B*). In all cases, melanised thalli showed significantly lesser photoinhibition than that of pale thalli in both wet and dry thalli. The differences between melanised and pale thalli were the greatest in those species that showed the greatest relative increases in BRI between pale and melanised thalli. For example, the greatest difference between melanised and pale thalli was in *C. islandica* (Fig. 1*A–D*), the species that showed the greatest increase in BRI (Table 2). By contrast, melanised thalli of *Pseudocyphellaria* and *Sticta* had BRI values only slightly greater than those of pale thalli, and in these two species, melanised thalli displayed only small (but

Table 1. Light intensity and time of exposure used to induce photoinhibition.

Species	Dry Time [h]	Light intensity [μmol m^{-2} s^{-1}]	Wet Time [h]	Light intensity [μmol m^{-2} s^{-1}]
Cetraria islandica	36	800	3	1,500
Lobaria pulmonaria	12	800	8	800
Peltigera aphthosa	12	800	5	1,500
Peltigera membranacea	12	800	12	800
Pseudocyphellaria gilva	6	800	5	800
Sticta sublimbata	4	800	2	800

Table 2. Browning reflectance index (BRI) of the lichens used in this study. Figures are given ± 1 SD, n = 12. BRI was calculated as described by Solovchenko *et al.* (2001).

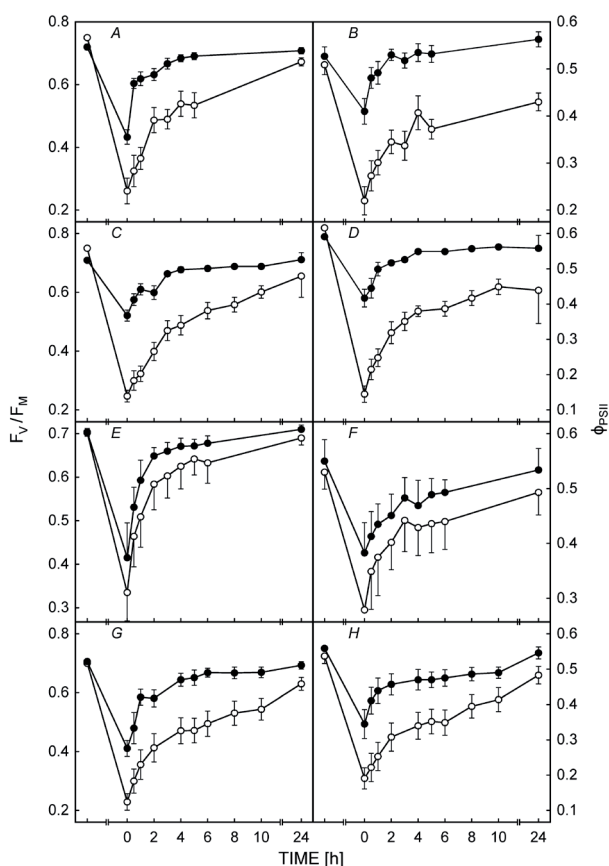| Species	BRI Non-melanised thalli	BRI Melanised thalli	Increase in BRI in melanised thalli
Pseudocyphellaria gilva	7.5 ± 1.9	9.3 ± 1.9	1.2
Sticta sublimbata	8.2 ± 2.6	24.8 ± 4.0	3.0
Lobaria pulmonaria	4.4 ± 1.1	48.4 ± 42.4	11.0
Peltigera membranacea	2.8 ± 0.5	31.6 ± 4.1	11.3
Peltigera aphthosa	2.1 ± 2.1	24.8 ± 15.6	11.8
Cetraria islandica	0.6 ± 0.8	87.9 ± 27.1	146.5

Fig. 1. The effect of melanisation on the recovery of F_V/F_M and Φ_{PSII} from a photoinhibitory exposure to light in *Cetraria islandica* (*A–D*) and *Lobaria pulmonaria* (*E–H*). For this experiment, and those that follow, the exposure time and light levels are given in Table 1. The initial data points are the values in freshly collected, hydrated material. *Open symbols* indicate non-melanised thalli, while solid symbols represent melanised. *A* and *E* are results for F_V/F_M for wet material, *B* and *F* indicate Φ_{PSII} for wet material, *C* and *G* are data for F_V/F_M for dry material, and *D* and *H* indicate Φ_{PSII} for wet material. Data is given ± SE of the mean, n = 15. Error bars smaller than the size of the symbols are omitted.

significant) improvements in recovery after high-light stress (Fig. 3). In general, Φ_{PSII} appeared to be a more sensitive indicator of recovery from photoinhibition than F_V/F_M. For example, in *C. islandica*, F_V/F_M indicated that after 24 h pale thalli completely recovered from photoinhibition (Fig. 1*A,C*), while Φ_{PSII} measurements showed that recovery was incomplete (Fig. 1*B,D*).
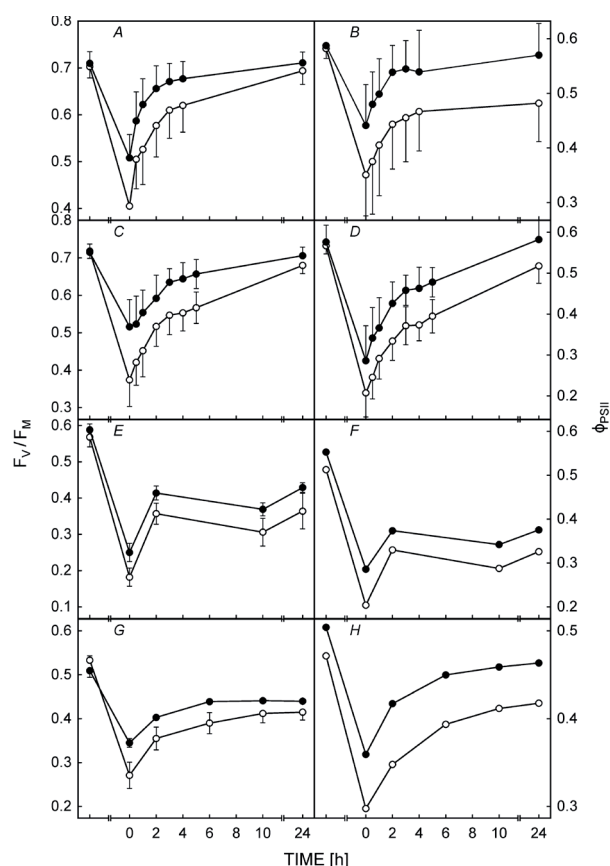

Fig. 2. The effect of melanisation on the recovery of F_V/F_M and Φ_{PSII} from a photoinhibitory exposure to light in *Peltigera aphthosa* (*A–D*) and *P. membranacea* (*E–H*).

Discussion

The main conclusion of the work presented here is that melanised lichens are more tolerant to photoinhibition than the pale thalli, for both desiccated and hydrated thalli. Apart from the field experiment of McEvoy *et al.* (2007) with just one species (*Lobaria pulmonaria*), the present study is the first to directly correlate within-species melanisation to increases in tolerance to photoinhibition in lichen photobionts. In general, the greatest differences in the effects and rate of recovery from high light occurred in species with the largest difference in BRI between melanised and pale thalli. For example, *C. islandica* had the largest difference in BRI between pale and melanised
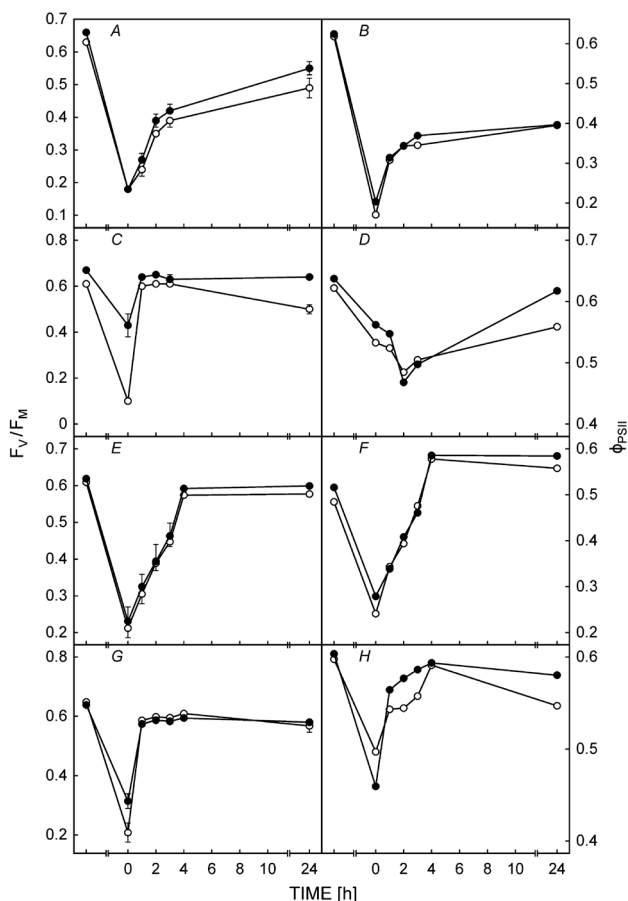
Fig. 3. The effect of melanisation on the recovery of F_V/F_M and Φ_{PSII} from a photoinhibitory exposure to light in *Pseudocyphellaria gilva* (*A–D*) and *Sticta sublimata* (*E–H*).

thalli (Table 2), and also displayed the largest difference in the effects of high-light stress (Fig. 1*A–D*). By contrast, for species in which differences in BRI were lesser, *e.g.*, *Pseudocyphellaria gilva* and *Sticta sublimbata* (Table 2), the differences in the effects of high-light stress were much lesser (Fig. 3). An inherent problem with the approach used here is that although melanised and pale thalli were collected only a few meters apart, the melanised thalli probably had a history of exposure to higher light levels in contrast to pale thalli. As a result, melanised thalli might therefore possess some of the biochemical mechanisms that confer tolerance to photoinhibition, *e.g.*, increased NPQ. In theory, we could have produced melanic and non-melanic thalli by using UV-transmitting and UV-absorbing screens as in our earlier work (*e.g.*, Mafole *et al*. 2017). However, the problem would remain as melanin synthesis would cause differential screening of the algae, and therefore possibly induction of alternative mechanism that protect the photosynthetic apparatus. In future, we plan to attempt to separate the effects of melanisation from biochemical mechanisms, for example by removing a melanised or non-melanised cortex and directly exposing the underlying algal layer (*see* Gauslaa *et al*. 2017 for how this might be done). However, results presented here clearly show that, compared to pale thalli, melanised thalli

in species, which grow in both high and more shaded habitats and which possess either green or cyanobacterial photobionts, have significantly greater tolerance to photoinhibition, strongly suggesting that melanins play a key role in photoprotection.

Melanised lichens are more resistant to photoinhibition than the pale ones: Melanins are probably important for mycobiont and photobiont symbionts for different reasons. The fungal symbiont is responsible for melanin synthesis, and the trigger for synthesis is UV light (Matee *et al*. 2016). Fungi apparently need more protection from UV than the photobionts, as elevated UV affects overall lichen growth more than purely photobiont responses, such as Chl content and the quantum yield of photosynthesis (Chowdhury *et al*. 2017). Photobionts *in vivo* avoid UV levels currently being experienced by lichens in the field, as even in pale thalli the cortex effectively blocks transmittance of light with wavelengths lesser than 325 nm (Gauslaa *et al*. 2017). However, while not needing UV protection, results presented here show that in melanised thalli, the photobiont are more tolerant to photoinhibition by high PAR. Therefore, although the mycobiont benefits directly from melanin synthesis by having increased UV protection, protecting the photobionts from high PAR indirectly benefits the mycobiont by increasing the supply of photosynthates to the whole thallus.

Dry melanised lichens are also more tolerant to high light than pale thalli: For both wet and dry thalli, melanised thalli are more tolerant to photoinhibition than the pale ones (Figs. 1–3). All species were highly desiccation tolerant, and when not photoinhibited completely, they recovered from desiccation very quickly during rehydration (data not shown). However, some of the recovery that occurs during the first 30 min, when photoinhibited dry lichens are rehydrated, undoubtedly represents recovery from desiccation stress. While lichens are more tolerant of light stress when dry, they eventually become photoinhibited when exposed to even moderate light intensities (Table 1). This is perhaps surprising, as at low thallus water contents metabolism is almost inactive, presumably reducing light-induced ROS formation and the resulting photoinhibition. Furthermore, the cortical transmittance to PAR is much lower (between one third and one half lower) in air-dry compared with hydrated thalli (Gauslaa and Solhaug 2001), probably in part because desiccation enhances light scattering, lengthening the path of the light in the cortex, and thereby increasing the absorption by melanins. While more research is needed to understand the mechanism of photoinhibition in dry lichens, recovery may take many hours (Figs. 1–3), reducing carbon fixation for the symbiosis. Interestingly, comparing wet and dry lichens, melanisation is sometimes more effective at preventing photoinhibition when thalli are dry (*e.g.*, compare Fig. 1*F* with 1*G*, Fig. 3*A* with 3*C* and Fig. 3*E* with 3*G*). One explanation for this result is that melanisation has different effects on cortical transmittance in wet and dry lichens. While in wet thalli the transmittance of the upper cortex of melanised thalli of *L. pulmonaria* to PAR

is about 30% lower than that of pale thalli, in dry thalli, the reduction is about 40% (Gauslaa and Solhaug 2001). Assuming a similar difference occurs in other lichens, a greater reduction in cortical transmittance when lichens are dry thalli may explain why melanisation can be more effective at reducing photoinhibition in these thalli.

Φ_{PSII} can be a more sensitive indicator of photoinhibition than F_V/F_M: Results presented here suggest that for some lichens, measuring the operating efficiency of PSII (Φ_{PSII}) can give a more sensitive measure of the effects of high-light stress than simply measuring maximum quantum yield of PSII photochemistry (F_V/F_M). F_V/F_M can be quickly and easily measured, and is therefore commonly used in photoinhibition studies (*e.g.*, Míguez *et al.* 2017, Solhaug 2018). However, Φ_{PSII} can be also readily derived from Chl fluorescence measurements. Measurement of Φ_{PSII} takes longer than that of F_V/F_M, as an actinic light must be switched on and fluorescence must reach a stable value (F_t) before a saturating pulse is given and $F_{m'}$ measured. However, in the present study, in both wet and dry non-melanised *C. islandica* (Fig. 1) and wet *Pseudocyphellaria gilva* (Fig. 3) simply measuring F_V/F_M would indicate that lichens almost completely recovered from photoinhibition (Fig. 1*A*,*C*; Fig. 3*A*), while Φ_{PSII} shows that some residual effects remained (Fig. 1*B*,*C*; Fig. 3*B*). In general, for all species tested, Φ_{PSII} measurements show more clearly that melanised thalli recover faster and more fully from photoinhibition than F_V/F_M. Therefore, although more time consuming to measure, Φ_{PSII} can be a more sensitive indicator of high-light stress.

Conclusion: Results presented here clearly show that melanised thalli are more tolerant to high light than pale ones. As discussed in the Introduction, melanisation is not the only mechanism of photoprotection, and more work is needed to compare the importance and efficiency of melanisation with, for example, other lichen secondary metabolites and biochemical adaptations such as quenching. Melanisation may not invariably be beneficial for lichens. Mafole *et al.* (2017) recently showed that melanised thalli of *Lobaria pulmonaria* are disadvantaged if light levels fall below about 100 µmol(photon) m^{-2} s^{-1}, due to reduced light interception by the photobionts. Falling light levels may occur for several reasons, *e.g.*, reductions in light availability during autumn, development of leaves in branches above lichens or by the growth of plants surrounding the host tree. A further potential disadvantage of melanisation is that it increases thallus temperature by up to 3°C (McEvoy *et al.* 2007), possibly inducing heat stress. However, if light levels remain high, in the field, melanised thalli are likely to spend significantly less time photoinhibited, which could promote productivity of the whole thallus.

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