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Review

Occurrence and possible roles of melanic pigments in lichenized ascomycetes

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photosynthetically available radiation

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reactive oxygen species

ABSTRACT

Many species of lichenized ascomycetes are capable of synthesizing darkly colored melanin pigments by polymerizing phenolic compounds. Available data suggest that different clades of lichens produce various kinds of melanins, with N₂-fixing Peltigeralean lichens producing N-rich DOPA or “eumelanins” and lichens from other orders producing N-poor “allomelanins.” In general, melanic lichens seem to be more common in environments with high levels of abiotic stress such as polar and montane regions, but they are by no means restricted to these habitats. Here, we review the occurrence of melanins in lichens, their chemical structure, methods of quantification and biological roles. Good evidence exists that melanins in lichens protect the mycobiont from high UV, and the photobiont against high PAR. However, given their many unique properties, it seems likely that melanins help lichens to survive a range of biotic and abiotic stresses.

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1. Introduction

The word “melanin” comes from the ancient Greek, melanos, meaning “dark,” and in a general sense refers to dark pigments that occur throughout all domains of life (Solano, 2014). All

phyla of the fungi contain species that form melanin. Chemically, melanins are difficult to define due to their structural complexity, but in fungi they mostly belong to either the eumelanin or allomelanin groups (Butler and Day, 1998). Shared properties include broadband absorption spectra,

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paramagnetism, charge transport and remarkable structural stability. It is therefore perhaps not surprising that melanins have been suggested to play many roles in fungal biology (Cordero and Casadevall, 2017). The biosynthesis of fungal melanins was recently reviewed by Belozerskaya et al. (2015). Briefly, most fungal melanins are generated from the polymerization of either tyrosine (DOPA or eumelanins) using tyrosinase or in some case laccases, or the synthesis of 1,8-dihydroxynaphthalene (DHN or allomelanins) using polyketide synthases, followed by a polymerization step involving an oxidase e.g. laccase. During the final stages of melanin synthesis, the phenolic precursors undergo multiple oxidation and reduction steps, which can occur enzymatically or passively by spontaneous polymerizations. Melanins form granules that may accumulate at the cell surface or be released into the extracellular space. The main aim of the present review is to summarize the literature data on melanins in lichens, with an emphasis on recent findings on their ecological role.

2. Evolution and occurrence of melanins in lichens

Gostinçar et al. (2012) suggest that the earliest lichens were derived from oligotrophic heavily melanised fungi growing on surfaces with almost no usable organic-carbon e.g. bare rock. Such fungi inevitably attempted to improve their carbon supply by attaching to microscopic algae. Rock-inhabiting fungi tend to develop into “lichenoid structures” within months when co-cultured with lichen algae (Gorbushina et al., 2005). Gorbushina and Broughton (2009) interpreted the rock surface as a kind of “symbiotic playground,” where competitive interactions between species are probably rare. It seems likely that the transition from a rock-inhabiting to a lichenized lifestyle in early ascomycetous evolution was driven by the high stress of their habitat. Desiccation and high levels of UV and PAR increase the formation of ROS in both fungi and algae, but the lichen symbiosis increases the efficiency of the ROS scavenging mechanisms compared to isolated symbiotic partners (Kranter et al., 2005). A relationship between lichens and heavily melanised fungi is also suggested by molecular phylogenetic studies. Rock-inhabitants are basal to the lichenized lineages of Arthoniomycetes and Verrucales (Gueidan et al., 2008; Ruibal et al., 2009).

In present day lichens, melanins may be synthesized constitutively as part of development, e.g. the cilia of *Heterodermia*, in the lower cortex of genera such as *Parmelia* and *Nephroma*, or throughout the whole thallus in some species of *Bryoria*. In other cases, melanins are environmentally induced, for example by UV in the upper cortex of genera such as *Lobaria* (Solhaug and Gauslaa, 2012). Melanins remain common in many species of lichens from a variety of habitats.

3. Quantification and structure of lichen melanins

The melanin content of lichens can be quantified either by extracting melanins and determining their absorption, or alternatively by measuring reflectance. In the first method, melanins are extracted from thalli, for example as described

by Matee et al. (2016), and quantified by measuring the absorption e.g. at 400 nm. Alternatively, melanins can be quantified based on thallus reflection (Solhaug et al., 2003). A reflectance spectrum is first obtained, and then a Browning Reflectance Index or BRI calculated as $(1/R550 - 1/R700)/R750$, with the numbers referring to reflectance at those specific wavelengths of light (Solovchenko et al., 2001). While the first method works well with all the Peltigeralean lichens we have tested so far, the black pigments from some non-Peltigeralean lichens do not behave as classic melanins, and it can be difficult to separate them from other pigments such as chlorophylls. Furthermore, while the first method determines total melanins in a sample, thallus reflection will estimate the amount of light screening melanins in the upper cortex, which for some studies may be of more interest.

The chemical nature of lichen melanins remains unclear. The melanins of *Lobaria pulmonaria* behave as classic DOPA melanins; they are extracted with NaOH, and precipitated with HCl (Matee et al., 2016). The resulting black precipitate is insoluble in acetone, but readily dissolves in DMSO, and has an absorption spectrum resembling that of commercially available melanins. The low C:N ratio of these melanins (10:1, Matee et al., 2016) indicates that, as in humans, they were most likely synthesized from L-DOPA rather than hydroxylated naphthalene-derived molecules that produce “allomelanins”, which have a C:N ratio close to 100:1 (Loganathan and Kalyanasundaram, 1999; Solano, 2014). It is well known that in general the overall N content of a lichen is directly proportional to its ability to fix N₂ (Green et al., 1980). Matee et al. (2016) suggested that it is the N₂-fixing cephalodia in *L. pulmonaria* that provide enough N to allow the synthesis of N-rich melanins. The biosynthetic pathways of lichen melanins are unknown. Interestingly, the field study of Matee et al. (2016) with *L. pulmonaria* suggested that melanisation was accompanied by an increase in laccase rather than tyrosinase activity. Our preliminary results, based on C:N ratio of extracted melanins, suggest that inducible DOPA melanins occur throughout the N₂-fixing Peltigeralean lichens, including both species with cyanobacteria in cephalodia and those in which the main photobiont is cyanobacterial (Mafole, 2018).

By contrast, the nature of melanins in non-Peltigeralean lichens remains uncertain, particularly in species that appear to synthesize constitutively high concentrations of melanins. While the pigments from some species e.g., the very dark members of the genus *Bryoria*, can readily be extracted by NaOH, they are not precipitated by acidification, making their chemistry difficult to study. Addition of FeCl₃ to acidic solutions, however, gives precipitates with a C:N ratio of around 100:1, typical for allomelanins (Mafole, 2018). These species contain only green algae rather than N₂ fixing cyanobacteria as the main photobiont or in cephalodia, and therefore presumably have less available nitrogen.

4. What roles do melanins play in lichen biology?

Melanisation is particularly common in lichens growing in environments with high levels of abiotic stress such as polar and montane regions (Gostinçar et al., 2012), and intuitively it

seems likely that melanins will play a general role in stress tolerance. [Cockell and Knowland \(1999\)](#) list four criteria that should be fulfilled to confirm a specific light screening role to pigments such as melanins, and these criteria can be generalized to include other roles of pigments in stress tolerance. The criteria are:

1. The compound must be shown in the laboratory to have properties that protect against the stress;
2. The compound should be induced or boosted by the stress;
3. A role in stress tolerance should be demonstrated *in vivo* e.g. by comparing the stress tolerance of melanised and pale individuals;
4. The compound should protect against ecologically relevant stress levels.

As will become clear in this review, the only reasonably safe deductions are first that melanins protect first the mycobiont from high UV, and second the photobiont against high PAR. While it seems likely that melanins play other roles in the tolerance of lichens to a diversity of biotic and abiotic stresses, these roles must be confirmed by carrying out more work.

Protective effects of melanins against UV and high PAR

In species where melanins are environmentally rather than developmentally induced, the trigger for melanisation is UV, particularly UV-B (wavelengths 280–315 nm, e.g. [Solhaug et al., 2003](#)). A recent short-term experiment using growth chambers with and without UV-B radiation showed that while moderate UV-B levels do not affect pure photobiont responses, they can substantially reduce growth driven by both symbionts together ([Chowdhury et al., 2017](#)). A likely implication is that the mycobiont is more sensitive to UV-B than the photobiont, and melanins in lichens protect the fungus from the harmful effects of UV radiation. Photobionts, however, may not need melanins for UV protection. In *Lobaria pulmonaria*, the presence of even an intact unmelanised upper cortex is enough to protect the underlying algal layer from UV-induced photoinhibition ([Gauslaa et al., 2017](#)). While removing the upper cortex of this lichen (exposing the algal-layer) renders the photobionts sensitive to UV, if the upper cortex is left intact even high doses of UV have no effect on photosynthesis. Therefore, the upper cortex does not need to be melanised to protect the photobiont against UV.

In contrast to UV, high PAR (with wavelengths from 400 to 700 nm) probably has little effect on the mycobiont, except indirectly by causing heat stress, and possibly a faster rate of drying after hydration, which will reduce the time a lichen remains metabolically active. In the photobiont however, high PAR can cause oxidative stress and temporary or permanent photoinhibition. Photoinhibition is caused, directly or indirectly, by stimulation of the production of harmful 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](#)). Convincing evidence exists that photoinhibition is a common phenomenon for lichens in field situations (e.g. [Leisner et al., 1997](#); [Jairus et al., 2009](#)). However,

photosynthetic organisms have several mechanisms to reduce photoinhibition. For example, ROS formation can be reduced by converting the excess light energy into thermal energy, a process termed non-photochemical quenching ([Duffy and Ruban, 2015](#)). Lichens, in addition to these mechanisms, reduce photoinhibition by synthesizing cortical pigments ([Solhaug and Gauslaa, 2012](#)). [Dietz et al. \(2000\)](#) showed that cortical transmittance in general tends to be lower in lichens growing in habitats with high light levels. In a field study specifically on melanins, [McEvoy et al. \(2007\)](#) transplanted *L. pulmonaria* to three sites with low, medium and high light levels for 100 d. Lichens at the site with highest light level became significantly melanised. Thalli from each of these sites were then transplanted to an area with high light levels for 12 d. The melanised thalli displayed a much smaller reduction in F_v/F_M than the material that had been in the more shaded sites. While other mechanisms may have been involved, results strongly suggest that fungal melanins are involved in the photoprotection of photobionts. Recently, [Sadowsky and Ott \(2016\)](#) showed that while intact melanised thalli of *Umbilicaria decussata* from Antarctica were highly resistant to photoinhibition, photobionts isolated from the thalli were highly sensitive. Further support for the role of melanins in photoprotection came from the study of [Färber et al. \(2014\)](#) on pendulous lichens that dominate canopies of boreal forests. Typically, dark *Bryoria* species grows in the upper canopy, and pale *Alectoria* and *Usnea* species in lower canopy. Exposing these lichens under controlled conditions to a light level of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 7 d caused much less photoinhibition in the melanised *Bryoria* species than the non-melanised *Alectoria* and *Usnea*. In a recent laboratory study [Mafole et al. \(2019\)](#) collected pale and melanised thalli from the same populations of six species of lichens and subjected them to light stress in the wet and dry states. High light caused significantly less photoinhibition in melanised thalli, particularly when lichens were dry. Interestingly, [Gauslaa and Solhaug, 2001](#) showed that in *L. pulmonaria* melanisation reduces the transmittance of the upper cortex by about 40 % when thalli dry, but only by about 30 % when wet, possibly explaining why melanisation is particularly effective at protecting dry lichens from photoinhibition.

Cost of producing melanins to photobiont photosynthesis

Although effective at reducing photoinhibition, melanins may shade the photobiont, potentially reducing photosynthesis should light levels fall. Recently, [Mafole et al. \(2017\)](#) studied the cost of photo-protection by melanic pigments in *L. pulmonaria*. While this lichen normally grows on trees in relatively shaded habitats ([McCune and Geiser, 1997](#)), it may periodically receive more light, for example immediately after leaf-fall in autumn, or when forest openings are created for various reasons. [Mafole et al. \(2017\)](#) collected unmelanised *L. pulmonaria* from a shaded site and transplanted half the thalli to an open site for four weeks to induce melanic pigments. Melanised thalli had normal chlorophyll contents and at moderate light levels normal maximum rates of photosynthesis. It was

clear that melanisation had effectively protected photobionts from the harmful effects of high light. However, at light levels lower than $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, typical for the natural habitat of this lichen, melanisation of the upper cortex of the lichen reduced rates of CO_2 fixation by more than 40 %. In practice, falling light levels may occur by reductions in light availability following canopy development, development of new branches above lichens, or by the growth of plants surrounding the host tree. The latter is realistic, as the juvenile stage of *Lobaria* species can extend over 20 y (Gustafsson et al., 2013; Larsson and Gauslaa, 2011; Rhoades, 1983). Furthermore, it would seem unlikely melanins can be broken down in living tissue, although this needs to be tested explicitly. However, in soil, the melanin from dead fungi can only be degraded by strong Class II peroxidases (Butler and Day 1998). Thus, melanised lichens returned to more shaded conditions may display lower carbon fixation rates than pale thalli, and therefore have significantly lower growth rates.

Apart from melanins, the most widespread cortical pigments in lichens are “lichen substances” such as usnic acid, atranorin and parietin (Solhaug and Gauslaa, 2012). Synthesis of some of these compounds is also induced by UV light, and they reduce the transmittance of the upper cortex to UV. Although most of these compounds show little absorption in the visible region when extracted (Rao and LeBlanc, 1965), their crystalline structure reflects PAR, significantly reducing light levels in the algal layer (Solhaug et al., 2010). Anecdotal observations suggest that lichens use classic lichen substances such as usnic acid, atranorin and parietin more frequently than melanins to protect themselves against the effects of high light (Solhaug and Gauslaa, 2012). Interestingly, lichens with lichen substances or melanic compounds often coexist in open habitats, and some lichens with atranorin or usnic acid such as *Flavocetraria* and *Cladonia* additionally produce melanic compounds under very sun-exposed conditions (Solhaug and Gauslaa, 2012). There are various possible explanations as to why typical lichen compounds may be more important than melanins as light screening pigments in lichens. First, lichen substances can play other roles in lichen biology e.g. deter herbivores (Gauslaa, 2005). Secondly, even in lightly melanised species such as *Lobaria pulmonaria* melanisation can increase thalli temperature by up to 3°C (McEvoy et al., 2007). In black lichens belonging to the Parmeliaceae that absorb close to 100 % of near infrared radiation, the increases in temperature caused by melanisation are likely to be even greater (Gauslaa, 1984). While melanins may protect fungi from potentially lethal high temperatures (see below), it is hard to predict the long-term effects of smaller, sustained increases in thallus temperature on overall thallus health. As discussed above, typical lichen compounds reflect rather than absorb PAR, and therefore have less effect on the heat balance of lichens. Thirdly, a recent study by Siletti et al. (2017) that involved surveying of a wide range of free-living fungi showed a negative correlation between melanisation and hyphal growth. While the reasons for this remain unclear, the implication is that melanised lichens may be unable to grow as fast as their pale counterparts. Clearly, more work is needed to understand the relative advantages and disadvantages of melanins and typical lichen compounds as sun screens in lichens.

Melanins and temperature stress

There is some evidence that in free-living fungi melanins protect against temperature stress. Examples include melanin-deficient mutants of *Monilinia fructicola* that produce conidia that are more susceptible to high temperatures (Rehnstrom and Free 1996). In *Cryptococcus neoformans*, melanisation increases tolerance to heat and cold stresses (Rosas and Casadevall, 1997). Exactly why melanin reduces temperature stress is unknown, although it has been suggested that melanins can reduce stress-induced ROS formation (Cordero and Casadevall, 2017). It is currently unknown whether melanins increase the heat or cold tolerance of lichen fungi. In theory, melanins may reduce heat-induced ROS formation in the mycobiont, a potential disadvantage of melanisation is that it increases thalli temperature. As discussed above, even the relatively lightly melanised thalli of *L. pulmonaria* are typically up to 3°C warmer than pale thalli (McEvoy et al. 2007), possibly increasing rather than decreasing heat stress. However, considering that melanised lichens are particularly common in high stress habitats where light intensities are often high (Gostinçar et al., 2012), it seems most likely that benefits of photoprotection more than offset any negative effects of increased thallus temperature.

Protection against other abiotic stresses

Almost all stresses induce ROS formation in organisms, and melanins are powerful antioxidants (McGraw, 2005). While no specific data are available on lichens, melanins probably act as general ROS scavengers in the mycobiont. However, it is perhaps unlikely that mycobiont melanins can directly scavenge stress-induced intracellular ROS in the photobiont. Melanins are almost certainly involved in the tolerance of lichens to a variety of abiotic stress in addition to light and heat. For example, although no data are available for lichens, in free-living fungi Pacelli et al. (2018) have shown that melanised fungi are more tolerant to X-rays than non-melanised ones. Furthermore, what makes lichens special, and what separates them from most other eukaryotic organisms, is their ability to tolerate desiccation, and then rapidly recover activity when rewetted (Beckett et al., 2008). Surprisingly, there has been no attempt to determine whether melanins contribute to desiccation tolerance in lichens. In the free-living fungus *Cenococcum geophilum*, inhibiting melanin synthesis increases susceptibility to osmotic stress and desiccation (Fernandez and Koide, 2013). Presumably, melanins increase desiccation tolerance by scavenging desiccation-induced ROS, toughening cell walls, or by reducing the rate of drying by decreasing cell wall porosity. In lichens, melanins probably have little effect on the desiccation tolerance of the photobiont. However, as discussed above, fungi and lichens growing in extreme habitats are often heavily melanised (Gostinçar et al., 2012), suggesting that future studies should test the roles of melanisation in the desiccation tolerance of the mycobiont.

Heavy metal stress may also be reduced by melanin synthesis. Melanins exhibit high binding affinity and high binding capacity for many different metal ions (Enochs et al., 1997), and in free-living fungi have been suggested to be involved

in heavy metal tolerance (Cordero and Casadevall, 2017). Carboxyl, amine, and hydroxyl functional groups of the pigment can potentially bind metals. Mg, Ca, and Zn, are preferentially coordinated by carboxyl groups, Cu by hydroxyl groups and iron by hydroxyl, amine, imine and acetate groups (Cordero and Casadevall, 2017). It is likely that melanins can readily bind potentially toxic metals in lichens. For example, Williamson *et al.* (2004) used X-ray element mapping across a lichen-rock interface to show that in the lichen *Trapelia involuta* melanins are the likely to be molecules responsible for binding U, Fe, and Cu. Melanised Parmeliaceae species have higher Fe and Zn content than non-melanised. Fe seems to be especially associated with the melanised lower cortex, whereas Zn is more evenly distributed throughout the thallus (Fortuna *et al.*, 2017). However, there are no studies showing that melanised lichens are more common on metal rich sites, or that metals can induce melanin biosynthesis. While Spagnuolo *et al.* (2011) found that lichens suspended in a metal polluted area became melanised, this was probably caused by exposure to high light. Therefore, the significance for melanins in heavy metal tolerance requires further investigation.

Protection against biotic stress

Lichens are subjected to biotic stress by grazing and attack by pathogenic organisms. Many lichens that grow directly in contact with the soil often have a heavily melanised lower cortex, suggesting that melanins may be synthesised as a protection against biotic stress. Perhaps surprisingly, this hypothesis appears not to have been tested for lichens, although some data are available for free-living fungi. For grazing, many invertebrates have been observed to feed on lichens (Gerson and Seaward, 1977). Interestingly, Scheu and Simmerling (2004) found that melanin strongly reduces the food quality of free-living fungi for invertebrates feeding on them. If the same is true of lichens, then melanised thalli would be of lower food quality than pale. However, it remains unknown whether melanisation affects the grazing preference of invertebrates that feed on lichens, or whether grazing stimulates melanin synthesis in lichens. While, as noted above, good evidence exists that lichen compounds can deter herbivores, this does not preclude the possibility that melanins may have a complementary role. Similarly, it is unknown whether melanisation increases the resistance of lichens to attack by microbial pathogens. In free-living fungi, melanisation increases the resistance of cell walls to hydrolytic enzymes (Bloomfield and Alexander, 1967; Kuo and Alexander, 1967; Potgieter and Alexander, 1966). Melanisation is also known to increase the mechanical strength of fungal cells walls (Nosanchuk *et al.*, 2015) and will reduce swelling when hyphae are moist, improving the resistance of hyphae to physical attack by penetration pegs from pathogenic fungi. Clearly, more work is needed to assess the importance of melanisation in biotic stress resistance in lichens.

5. Conclusions

While strong evidence exists that melanins protect lichens against the harmful effects of UV and high PAR, it seems likely

that these pigments are involved in the tolerance of lichens to a variety of biotic and abiotic stresses. Interest in the commercial applications of melanins is growing due to their wide spectrum of biological activity. Their antioxidative, anti-inflammatory, immunomodulating, radioprotective, and gastroprotective properties are currently being actively studied (ElObeid *et al.*, 2017). At present, nothing is known about therapeutic uses of melanins from lichenized fungi, but, given their strong association with stress, they could have applications in the treatment of human diseases. It is hoped that this review will stimulate exciting investigations into the roles and potential applications of lichen melanins in the future.

Declarations of interest

None.

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