THE ROLE OF SECONDARY METABOLITES IN PROTECTING LICHENS FROM CLIMATE CHANGE-INDUCED STRESS

By

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PREFACE

The research contained in this thesis was completed while based in the School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal Pietermaritzburg, South Africa under the supervision of Prof. R.P Beckett from August 2019 to December 2022

These studies represent original work by the candidate and have not otherwise been submitted in any form of any degree or diploma to another University. Where use has been made of the work by other authors it has been duly acknowledged in the text

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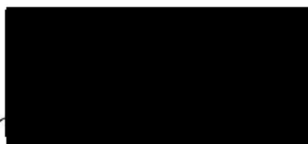

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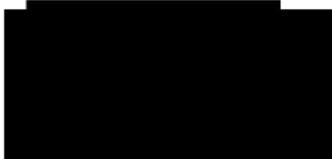
Student: 

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PUBLICATIONS

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ABSTRACT

Lichen secondary metabolites play a great diversity of roles in lichen biology by acting as UV screens, antimicrobials, herbivore deterrents or allelopathic compounds. The main aim of the work described in this thesis was to test the potential roles of secondary metabolites in the tolerance of lichens (twelve species) to a variety of abiotic stresses. The first stress considered was that of tolerance to high levels of photosynthetically active radiation (PAR). Photosynthetic organisms possess a great diversity of mechanisms to protect themselves from the potentially stressful effects of high PAR. In addition to non-photochemical quenching and antioxidant mechanisms, it has been suggested that lichens use secondary metabolites for photoprotection. A few studies have shown that even faintly pigmented or unpigmented lichen substances can reduce photoinhibition. Here, the acetone rinsing technique was used to harmlessly remove secondary metabolites from all lichen thalli and then the chlorophyll fluorescence technique was used to measure their tolerance to photoinhibition in desiccated and hydrated states. Results showed that colorless lichen substances can increase the tolerance of lichen photobionts to photoinhibition when thalli are hydrated, apparently by increasing reflectance. Interestingly, substances can also photoprotect lichens in the dry state, while having no effect on reflectance. The acetone rinsing technique was also used to compare the relative importance of lichen substances in photoprotection in sun and shade collections of four species of Afromontane lichens. Results showed that lichens collected from sunny microhabitats have higher tolerance to photoinhibition than those from shaded locations. Furthermore, removal of lichen substances increases sensitivity to photoinhibition much more in sun than shade collections. Results further emphasized the importance of lichen secondary metabolites in photoprotection. This study also considered whether melanins, a pigmented

secondary metabolite can cause problems with using fluorimetry techniques for e.g., to measure NPQ. A dissecting technique was used to remove the lower cortices and medullas of two lichen species so that NPQ could be measured from the underside of the thallus with an imaging PAM. Results confirmed that NPQ can be satisfactorily assessed with a standard fluorimeter by taking measurement from above using intact thalli. However, interestingly, photobionts from the bottom of the photobiont layer tended to have slightly lower rates of photosynthetic activity and lower NPQ than those at the top, i.e., display mild "shade" characteristics. The results presented in the final chapter looked at the protective role of secondary metabolites against desiccation-induced stress. It was hypothesized that secondary metabolites may act as antioxidants that protect lichens from desiccation-induced stress. Unfortunately, the findings of this part of the work did not present a consistent story, but rather indicated that according to species, the secondary metabolites can act as antioxidants or prooxidants. Nevertheless, taken together, the work presented in this thesis clearly shows that lichen secondary metabolites, whether pigmented or unpigmented, play important roles in photobiont photoprotection.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APx	ascorbate peroxidase
c.	approximately
CAT	catalase enzyme
Chl	chlorophyll fluorescence
Cf	final conductivity
Cv	initial conductivity
DART-MS	direct analysis in real time mass spectrometry
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DHN	1,8-dihydoxynaphtalene
DNA	deoxynucleic acid
DM	dry mass
DT	desiccation tolerant
ETR	electron transport rate
ETC	electron transport chain
Fig.	Figure
F_M	maximum fluorescence
$F_{M'}$	maximum fluorescence when saturating pulse given in the light
FM	fresh mass
F_O	minimum fluorescence
F_t	stable fluorescence signal in the light
F_v	variable fluorescence
F_V/F_M	maximal quantum yield of PSII photochemistry
GC	gas chromatography
GSH	reduced glutathione

GSSG	glutathione disulphide
GRX	glutaredoxin
GR	glutathione reductase
G6PDH	glucose-6-phosphate dehydrogenase
h	hour(s)
HPLC	high performance liquid chromatography
HPTLC	high performance thin-layer chromatography
K^+ loss	ion leakage (membrane damage)
KZN	KwaZulu-Natal
L-dopa	L-3,4-dihyroxyphenylalanine
LEA	late embryogenesis abundant
LC-NMR	liquid chromatography nuclear magnetic resonance
LED	light-emitting diode
LHCII	light harvesting complex II
LHC	light harvesting complex
MDHA	monodehydroascorbate
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
nm	nanometer
NPQ	non-photochemical quenching
OCP	orange carotenoid pigment
PAM	pulse amplitude modulation
PAR	photosynthetically active radiation
PPFD	photosynthetic photon flux density
PSI	photosystem I
PSII	photosystem II
PFD	photon influx density
PKS	polyketides

PSBS	photosystem II 22 kDa protein
PPFR	photosynthetic photon fluence rate
rETRMAX	maximum relative electron transport rate
rETR	relative electron transport rate
ROS	reactive oxygen species
RWC	relative water content
SOD	superoxide dismutase
SE	standard error
SPSS	Statistical Package for the Social Sciences
TM	turgid mass
unp.	unpublished
UV	ultraviolet
w/w	weight in weight
ϕ_{PSII}	quantum yield of PSII
µmol	micro mole
µmol m^{-2} s^{-1}	micromole per second and square meter
µS cm^{-1}	electrical conductivity

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CHAPTER 1: LITERATURE REVIEW

1.1. Introduction to lichens

A lichen is a stable, ecologically obligate, self-sustaining symbiotic association between a fungal partner (mycobiont) and one or more photobiont partners, either algal, cyanobacterial or both (Seaward 1988; Hawksworth and Honegger 1994; Calcott et al. 2018; Hawksworth and Grube 2020). Currently, more than 18 000 lichen species have been described (Feuerer and Hawksworth 2007; Muggia and Grube 2018). Of the two common photosynthesizing partners, most mycobionts are associated with green algae (Levis and McCourt 2004; Lutzoni and Miadlikowska 2009), 10% with cyanobacteria (e.g., *Nostoc*) (Henskens et al. 2012) and only 3% with both photosynthesizing partners (Hawksworth et al. 1995; Okasanen 2006). Lichens occur in three common morphological forms; namely, fructicose (or fruticose, shrubby and tree-like), foliose (leaf-like) and crustose (crust-like biofilm) (Hawksworth et al. 1995; Brodo et al. 2001), with each form adapted to enable the photobiont to optimally carry out photosynthesis (Honegger 2012**;** Sanders and De Los Ríos 2016). As c. 21% of fungi can form associations with photobionts, this makes lichens the largest group of mutualistic fungi (Molnár and Farkas 2010; Muggia et al. 2011). The association of the mycobiont and the photobiont allows for the development of life forms that rarely or never exist in the isolated symbionts (Pino-Bodas and Stenroos 2021). These life forms increase the chances of adaptation or survival under conditions such as harmful ultraviolet radiation, extreme temperatures, and desiccation that may be lethal, conditions that when the symbionts are isolated may not survive (Okasanen 2006; Calcott et al. 2018).

Lichens grow in a wide range of habitats, ranging from polar regions to tropical areas, plateaus, mountainous regions (e.g., Alpine areas) (Müller 2001; Grimm et al. 2021), xeric environments and urban areas (Jayanthi et al. 2012). In these habitats, lichens can be found growing on tree trunks, rocks, soil, leaves, and in urban areas on artificial structures such as concrete, glass, wood, metal, and plastic (Brightman and Seaward 1977; Seward 2008; Adamska and Juśkiewicz 2018). Lichens are known for the ability to survive harsh environments that are mostly not suitable for other life forms such as vascular plants. In these environments organisms experience extreme temperatures, drought, and high irradiance (Kershaw 1985; Vrablikova et al. 2005; Gasulla et al. 2021). They also include nutrient-deficient regions, saline habitats, and environments with high concentrations of some pollutants (Nash 2008). Despite their generally high stress tolerance, certain species are sensitive to some specific air-pollutants and heavy metals and are therefore used as biological monitors of air pollution (see Gries 1996; Fernández-Salegui et al. 2007; Sheppard et al. 2007; Geiser and Neitlich 2007; Gerdol et al. 2014; Van der Wat and Forbes 2015). Lichens have several important ecological roles, for example cyanobacterial lichens can provide nitrogen fixation in nitrogen deficient environments (Nash 1996; Marks et al. 2015). This can prevent desertification and increase water holding capacity and the availability of nutrients in the soil (Davidson et al. 2002; Okasanen 2006).

1.2. Climate change and Lichens

Climate change is defined as long term shifts in climatic and weather patterns such as temperatures and precipitation (Wadanambi et al. 2020; United States Environmental protection 2022). These shifts may be natural but recently human activities such as burning of fossil fuels, deforestation and any activities that produce house trapping gases have been the major drive of climate change

since the start of the industrial revolution in the 1800s (Short and Neckles 1999; Wadanambi et al. 2020). These shifts can be increases in temperatures (i.e., frequent heat waves, increasing UV radiation), rising sea levels (sea warning and melting ice glaciers), droughts, floods, and extreme hydrological cycles (Short and Neckles 1999; Thuiller 2007). Different ecosystems will be affected differently by climate change, for example, marine ecosystems are at a risk of experiencing thermal stratification (different temperatures in water layers), decreased pH etc. Terrestrial ecosystems may likely experience changes in precipitation (floods) and temperature (i.e., occurrence of wildfires) (Thuiller 2007).

Lichens' ecological and physiological requirements to grow make them sensitive to environmental changes (Kuldeep and Prodyut 2015). This makes them the perfect candidates for long term monitoring studies (such as air quality or atmospheric changes). For example, a study in the Netherlands dating back to 1979 has identified major changes in epiphyte distribution (Aptroot and van Herk 2007). An observation where lichen species growing in warm-temperate habitats significantly increased and species adapted to cold environments have either decreased or disappeared was made and climate change was statistically found to be the driving factor in this shift in patterns (van Herk 2002). Watson et al. (2004) state that many lichen species are heavily dependent on climate and it has a profound influence on the distribution of these sensitive organisms. In a study titled "Lichens as an indicator of climate and global change" investigated the vulnerability of lichens growing in mountain tops in the tropics which are the most susceptible to possible extinction of lichens because of global warming and discussed the predicted, observed and uncertain effects related to lichen and climate change together (for review see Aptroot et al. 2021).

1.3. Lichen secondary metabolites

The presence of secondary metabolites is clear in lichens because of the range in colour, from bright orange to shades of white grey, to brown and even black (Stocker-Wörgötter 2008). Secondary metabolites are generally deposited as crystals or amorphous structures on the surfaces of the hyphae (Singh and Arya 2019). Interestingly, c. 1050 secondary metabolites have been isolated from lichenized fungi and their symbionts (Jayanthi et al. 2012), and of these, c. 700 are only produced by lichens. Most species produce just a few compounds each, with a few exceptions. For example, *Pseudocyphellaria glaucescens* and *P. rubella* each produce at least 20 secondary metabolites (Galloway et al. 2001). Secondary metabolites occur in the cortex, and are known as cortical pigments (e.g., atranorin, parietin, usnic acid and melanins) (Molnár and Farkas 2010), while some occur in the medulla such as protocetraric and physodic acid. When lichen mycobionts are grown axenically, it has been shown that the production of secondary metabolites requires specific temperatures, nutrients, sugars, and pH values (Hager et al. 2008). Various analytical techniques are used to identify secondary metabolites. These methods include high performance liquid chromatography (HPLC), high performance thin-layer chromatography (HPTLC), liquid chromatography nuclear magnetic resonance (LC-NMR), direct analysis in real time mass spectrometry (DART-MS), nuclear magnetic resonance (NMR), and gas chromatography (GC) (Thadhani et al. 2021)

Most lichen secondary metabolites are derived from the acetyl-polymalonyl pathway (Stocker-Wörgötter 2008). This includes usnic acids, phenolic compounds such as anthraquinones, xanthones, dibenzofuranes, depsides and depsidones (Solarova et al. 2020) and polyketide aromatic compounds amongst others (Elix and Stocker-Wörgötter 2008). The shikimic and the

mevalonic pathways produce steroids and pulvinic acid, respectively (Stocker-Wörgötter 2008) (Fig. 1.1).

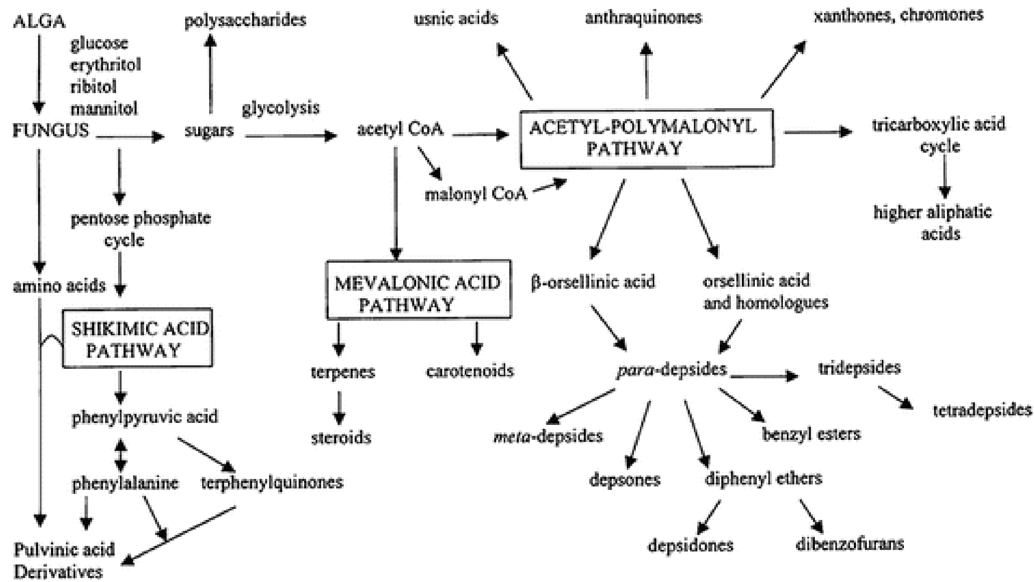

Figure 1.1: Biosynthetic pathways of secondary metabolites found in lichens (Elix and Stocker-Wörgötter, 2008).

1.4. Melanins

Melanins are complex ubiquitous polymers that occur across all biological domains (Fortuna et al. 2017). Although they can occur in a variety of colours, they are normally brown or black (melanin comes from the Greek word melanos meaning dark or black) (Solano 2014). Melanins can be classified into categories namely: eumelanins, pheomelanins, neuromelanin and allomelanins (Ambrico 2016). Melanins have complex chemical structures, and are hydrophobic, not dissolving in many aqueous solvents. They are also resistant to chemical degradation and hence have proven difficult to study and/or define (Cordero and Casadevall 2017). Although only limited information

is available on the structure of melanin, microscopic studies have suggested that melanins have a granular structure (Chen et al. 2014; Kim et al. 2016). These granules occur mostly in the cell wall or extracellularly, depending on the fungal species. Melanisation can be constitutive or an environmentally induced trait. For example, in the family Parmeliaceae many species invariably produce a brown-black lower cortex (Fortuna et al. 2017). Melanins always occur in the cilia of *Heterodermia spp* or throughout the entire thallus in species from genus *Bryoria*. In species such as *Lobaria pulmonaria,* melanin biosynthesis can occur in the upper cortex in response to UV light (Matee et al. 2016).

1.4.1. Melanin Biosynthesis

Many fungal species synthesize melanins via polymerization of DHN (1,8-dihydoxynaphtalene) pathway, but there are actually two main biosynthetic pathways (see Fig 1.2).

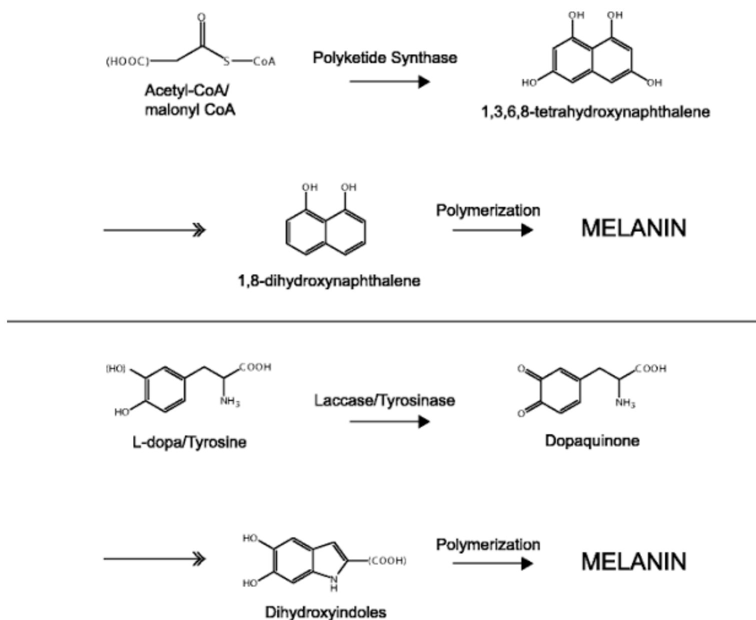

Figure 1.2: Two pathways of melanin synthesis in lichens, DHN (top) and L-dopa (bottom) (Matee et al. 2016; Rassabina et al. 2020).

Polyketide synthase (PKS) genes are responsible for biosynthesis of DHN melanins (Bell and Wheeler 1986; Muggia and Grube 2010). In the DHN pathway, Acetyl-CoA or malonyl CoA are the precursor molecules. The precursor of the other pathway is L-dopa (L-3,4-dihyroxyphenylalanine). In this pathway, tyrosine or L-dopa are precursor molecules which are first converted/oxidized to dopaquinone using the enzyme tyrosinase (Eisenman and Casadevall 2012).

1.4.2. Melanins in the fungal Kingdom

It is well known that black free-living fungi can survive in harsh environments where stresses such as drought and light stress are common (Magan 2007; Selbmann 2019; Coleine and Selbmann 2021; Tesei 2022. Melanins appear to enable them to colonize habitats such as bare rocks which receive very high amounts of light (Cordero and Casadevall 2017). Such habitats are often oligotrophic, and the fungi growing there appear to partner with other organisms such as algae or cyanobacteria. Interestingly, some black fungi are phylogenetically related to lichen-forming fungal clades (Gostinćar et al. 2012). Like black fungi, lichenized fungi with melanins are often resistant to harsh conditions and grow in places where they experience high light, desiccation, and low nutrient availability (Armstrong 2017). It has been suggested that the very first lichens were derived from black free-living fungi (Gueidan et al. 2008).

1.5. Functional roles of secondary metabolites (including melanins) in lichens

Synthesis of secondary metabolites is an energy demanding process, and in organisms like lichens they can make up to 10% w of the dry thallus (Calcott et al. 2018). While this suggests that they are likely to play important roles in lichen biology, many of these roles remain unknown. Similar secondary metabolites tend to occur in the same genus or species and therefore have been used in taxonomic studies (Nordin et al. 2007; Fehrer et al. 2008). However, it cannot be inferred that the same compound will have a similar function in different species (Molnár and Farkas 2010). Furthermore, the presence of the same compounds does not necessarily indicate that species are closely related phylogenetically (Nelsen and Gargas 2008). For example, barbatic and diffractaic acid are structurally related with differences in one functional unit. However, barbatic acid inhibits the growth of the photobiont and delays mitosis in *Treboxia jamesii*, whilst diffractiac acid promotes algal growth and mitosis in lichens (Hager et al. 2008).

As will become clear in this thesis, in many cases the functions of lichen secondary metabolites are poorly understood. However, significant evidence exists that they have important ecological and physiological roles. Some of these roles include protection against UV light (Millot et al. 2012), and high photosynthetically active radiation (PAR) (Mafole et al. 2019), acting as antioxidants (Luo et al. 2010), acting as allelochemicals (Marante et al. 2003), and having antibacterial, antifungal or antiherbivory activities (Halama and Haluwin 2004, Molnár and Farkas 2010). In addition, some have been reported to increase tolerance to general oxidative and heavy metal stress (Lawrey 1986).

1.5.1. Antioxidant activity of secondary metabolites

A universal effect of stress on living organisms is the increased production of reactive oxygen species (ROS) such as hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2) or singlet oxygen ($^1O_2$). While ROS are important for certain processes in the cell such as signalling, when production is excessive, they can have detrimental side effects e.g., attacking cell components such as proteins and nucleic acids (Jayanthi et al. 2012). This damage is known as oxidative stress and is common in lichens (Françoise et al. 2014). In addition to the normal enzymatic and non-enzymatic radical scavengers it has been suggested that lichen secondary metabolites can act as strong antioxidant compounds that can aid in protection (Marante et al. 2003; Yamamoto et al. 2015). Secondary metabolites achieve this function by reacting with free radicals, chelating free catalytic metals and by acting as oxygen scavengers (Kosanić and Ranković 2011; Anjitha et al. 2021).

1.5.2. Photoprotection mechanisms in lichens

Lichens have developed mechanisms to protect themselves against high levels of PAR and damaging effects of ultraviolet (UV) light (Nguyen et al. 2013). Photoprotection mechanisms include an increase in the thickness of the upper cortex, especially for species growing in habitats with high amount of light (Tobler 1925; Gauslaa and Ustvedt 2003; Gauslaa and McEvoy 2005), reduced light transmittance (Ertl 1951; McEvoy et al. 2007), increased surface reflectance of the upper cortex (Gauslaa 1984; Kuusinen et al. 2020), and an increase in nonphotochemical quenching (NPQ) (Gasulla et al. 2019; Beckett et al. 2021a), conformational change in the chlorophyll-protein complex (Heber et al. 2007) and desiccation-induced NPQ (Heber et al. 2001;2006a; Kopecky 2005). However, these photoprotective mechanisms alone are not entirely sufficient to protect lichens against light stress (Solhaug and Gauslaa 1996; Balarinová et al. 2014)

and can be supplemented by the production of secondary metabolites in the upper cortex (Solhaug and Gauslaa 2012; Molnár and Farkas 2010) and in the medulla (Farkas et al. 2020). The precise ways in which secondary metabolites may help lichens to tolerate light stress are discussed in detail in section 1.6.1.

In humans, melanin acts as a sunscreen for skin (Wolbarsht et al. 1981). While other screening compounds only absorb light at certain light wavelengths, melanins absorb the whole UV-visible portion of the light spectrum, and very well in the green to blue region of the visible spectrum (Meredith and Sarna 2006). The ability of melanin to offer protection from harmful UV is a result of a series of processes such as the scattering of photons, and possibly also by acting as an antioxidant (Khajo et al. 2011; Revskaya et al. 2012). Good evidence exists that melanins can help protect lichens from the harmful effects of high irradiance and/or UV-induced photoinhibition (Mafole et al. 2019).

1.5.3. Allelopathy

Allelopathy is a mechanism used by plants to inhibit or eliminate competition in their adjacent microhabitat (Latif et al. 2017). This is achieved by secreting allelochemicals that reduce the growth of potential competitors or by making the environment unfavourable for other organisms (Cheng and Cheng 2015). Lichens often grow in the same microhabitats as other organisms such as mosses which they may compete with for sunlight, nutrient availability, and space (Armstrong and Welch 2007; Favero-Longo and Piervittori 2010). Secondary metabolites in lichens can function as allelochemicals to reduce such competition. The release of allelochemicals can affect protein synthesis, respiration, and cell permeability (Chou 2006). For example, the 4-O-methylated depsides evernic and squamatic acids can inhibit the germination and growth of the common

mosses *Cetratodon purpurens*, *Funaria hygometrica* and *Mnium cuspidatum* that co-occur with lichens (Lawrey 1977). Lichens that co-occur with vascular plants e.g., *Cladonia* species growing in boreal forests such as *C. stellaris* and *C. rangiferina* can apparently reduce the nitrogen and phosphorus concentrations of jack pine seedlings (*Pinus banksiana*) and white spruce seedlings (*Picea glauca*) (Pyatt 1967). Phenolic compounds produced by *Lethariella canariansis* can inhibit the germination of common garden plants such as tomato, lettuce, and pepper (Marante et al. 2003).

1.5.4. Antiherbivore activity

Lichens serve as a food source for organisms such as spiders, insects, mites (Mukherjee et al. 2010) and reindeer (*Rangifer tarandus*) (Storeheier et al. 2002). However, grazing is generally not considered to be a problem for lichens, because they have a combination of morphological structures such as a thick cortex and the production of secondary metabolites (Lawrey 1986). The idea of secondary metabolites acting as antiherbivory compounds was first proposed by Zukal (1895) and has been supported by more recent studies (see Gauslaa 2005; Nimis and Skert 2006; Asplund and Gauslaa 2007).

Surprisingly, in free-living fungi, melanisation reduces the quality of them being a food source and deters any organisms that may be interested in feeding on them (Scheu and Simmerling 2004). It is not known if this is true for lichens; if true, then pale species or pale individuals of species in which melanisation is environmentally induced would suffer more from grazing than the dark ones. Melanins strengthen or toughen cell walls and hence reduce the chances of physical attacks

from pathogens and / or herbivores (Daminova et al. 2022). It is clear that much needs to be learned about the role of melanins in protecting lichens against biotic and abiotic stresses.

1.5.5. Thermoregulation (protection against temperature stress)

The concept of thermoregulation by melanins is by a combination of two processes: absorption of solar radiation and dissipation in the form of heat. Melanins re-emit very little of the energy they absorb – this is known as having a low radioactive yield (Meredith and Riesz 2004). Although absorbed energy could trigger the formation of ROS (Khajo et al. 2011) most of it is dissipated as harmless heat (Meredith and Riesz 2004). Melanin-induced heat gain is important for "ectotherm" organisms which rely on conduction and convention mechanisms to maintain temperature homeostasis. In contrast, for "endotherms", body temperature is regulated using their metabolism (Norris and Kunz 2012). A consequence of melanisation is that it enables organisms to heat up and reach equilibrium faster than non-melanised organisms (Cordero and Casadevall 2017). Therefore, melanisation could be beneficial for "ectotherms" found in cold environments but detrimental in hot conditions because of overheating (Cordero and Casadevall 2017). Although the role of fungal melanins in thermoregulation has rarely been explored, studies have shown that melanins may help maintain thallus temperature in lichens. For example, the presence of melanins in *L. pulmonaria* increases thallus temperature by 3°C compared to pale thalli (McEvoy et al. 2007). In the free-living fungus *Cryptococcus neoformans* melanins regulate heat and cold temperature to decrease susceptibility to temperature stress (Rosas and Casadevall 1997). Melanins have also been reported to reduce the formation of ROS as a response to stressful conditions such as high temperature (Cordero and Casadevall 2017).

1.5.6. Protection from desiccation stress

Desiccation is a protection mechanism against photodamage (Veerman et al. 2007), lichens being poikilohydric organisms cannot really protect themselves against desiccation but can slow down the process and suffer less damage when dry. Melanins have been reported to aid in protection of other stresses other than just high light and lethal temperatures. Lichens are poikilohydric organisms and can tolerate long periods of desiccation and be reactivated in just minutes of rehydration (Beckett et al. 2008). But surprisingly, no attempt has been made to investigate the role of melanic compounds in the latter phenomenon. In free-living fungal species e.g., *Cenococcum geophilum* the absence of melanins resulted in susceptibility to osmotic stress and desiccation (Fernandez and Koide 2013). It remains unclear as to exactly how melanins can increase stress tolerance, although possibly melanins help reduce the rate of desiccation, scavenge desiccation-induced ROS or toughen cell walls (Mafole et al. 2019). Studies with synthetic and natural melanin revealed that melanins associate with water that is weakly bound that can be easily removed by drying or heating above 60°C (Albanese et al. 1984) and changing porosity of the cell wall (Eisenman et al. 2005; Kogej et al. 2007), therefore affecting osmolyte exchange, making cells more hypertonic and reducing water loss.

1.6. Acetone rinsing

One approach to studying the role of secondary metabolites in lichen metabolism is to artificially remove these substances using the "acetone rinsing technique", first proposed by Solhaug and Gauslaa (2001). In this technique, secondary metabolites can be removed by washing dry thalli

(with relative water content (RCW) <3%) for periods of a few minutes to a few hours with pure acetone. Relative water content is the balance between the water supply to the leaf tissue and transpiration rate (Lugojan and Ciulca 2011). If done carefully, this has been shown to not affect the vitality of the lichens (Solhaug and Gauslaa 2001). This technique enables the physiological roles of secondary metabolites in lichens to be studied in such phenomena as tolerance to high light (Solhaug and Gauslaa 2010) by comparing thalli with and without these substances (Carniel et al., 2017). As mentioned in section 1.3 above, secondary metabolites in lichens are synthesised in the mycobiont but can be found extracellularly in the medullary hyphae (Fahselt and Alstrup 1997) and photobiont (Honegger and Peter 1994) which means that when rinsing, the acetone does not have to enter the cells to remove these compounds.

It is worth noting that lichens differ in their tolerance to acetone rinsing. Solhaug and Gauslaa (2001) tested acetone tolerance in 12 different species by submerging them in acetone. Results from this study showed significant tolerance differences. Toxic effects were noted in *Peltigera aphthosa* after 30 min of exposure, while the vitality of the species *Lasallia pustulata* only started to decline after 250 h. In the present study, it was found that different lichen species were tolerant to different times of exposure to acetone rinsing, measured as damage to PSII. According to Solhaug and Gauslaa (2001), it is important to test for the sensitivity of lichens to acetone rinsing before using the technique to study the role of secondary compounds in lichens. Solhaug and Gauslaa (1996) studied the role of the blue-light absorbing pigment parietin in photoprotection of the photosystem in three species: *Xanthoria parietin*, *Xanthoparmelia conspersa* which contain usnic acid and atranorim, respectively and *Parmelina tiliacea* which contains a colorless extract. In this study, results showed that lichen substance removal increased susceptibility to high light (measured as reductions in F_V/F_M (a parameter used as an indicator for plant photosynthetic

performance – when F_V/F_M are low this could be an indication that the plant is stressed [Jägerband and Kudo) and rETR which is relative electron transport rate, a product of effective photochemical yield of PSII and photosynthetic photon flux density (PPFD) [Masojídek et al 2001]). As will be discussed in more detail later, an important aim of the current study was to test the role of secondary metabolites in stress tolerance, particularly tolerance to light, using the acetone rinsing method.

Solhaug and Gauslaa (2001) also reported that in general, based on physiological measurements (e.g., F_V/F_M) lichens with *Trebouxia* as a photobiont were more tolerant to acetone rinsing compared to cephalo- and cyanolichens. Certainly, care needs to be exercised when using the acetone rinsing method. For example, Carniel et al. (2017) showed that acetone rinsing affected membrane permeability (K^+ leakage) and photosynthetic activity of *Flavoparmelia caperata*.

1.7. Light stress

Not all light absorbed is used for photosynthesis, too much irradiation can reduce photosynthetic activity of the PSII complex (Kalaji et al. 2014; Lu et al. 2017). Photoinhibition is defined as sustained depression of maximal photochemical quantum yield of PSII, although in a wider sense, as "dynamic photoinhibition", it is used to describe all reactions that decrease the efficiency of photosynthesis when plants are exposed to light (Barták et al. 2006). Luckily, plants have developed mechanisms that defend the photosynthetic apparatus, in particular the structure of thylakoids and PSII (Kalaji et al. 2017). Physiological traits such as F_V/F_M or the quantum yield of PSII (ϕ_{PSII}) can be used as measure of stress in plants. For example, F_V/F_M values around 0.8 are typical for plants that grow in stress free environments, although even healthy lichens often

have initial values of F_V/F_M that are below 0.8 (Jensen 2002). Lower values of F_V/F_M (0.2-0.3) would indicate that the plant is stressed and that the PSII reaction centers are possibly damaged (Basu et al. 1998; Seppanen 2000). In *Pinus leucodermis*, F_V/F_M decreased with an increase in light intensity (see Figure 1.3)

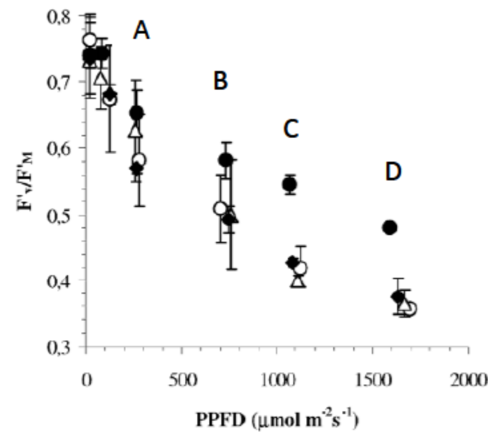

Figure 1.3: F_V/F_M measured for four populations A, B, C and D of *Pinus leucodermis* (adapted from Colom et al. 2003)

1.7.1. Light stress in lichens

A. Why are lichens as poikilohydric organisms susceptible to high light stress?

As for vascular plants, in lichens light is essential to enable the photobiont to carry out photosynthesis (Beckett et al. 2021). The amount of light received by lichens daily during thallus hydration may determine the success of some species of forest lichens (Dahlman and Palmqvist 2003). At the same time, light represents a form of stress in lichens, not only because it results in desiccation, but because too much solar radiation may result in long-term photoinhibition (Gauslaa and Solhaug 2000; Veres et al. 2020). Lichens are known for their ability to survive harsh conditions, including high levels of PAR. In some cases, photoinhibition occurs seasonally; for

example, Míguez et al. (2017) showed that the lichen species *Lecanora muralis* shows annual photoinhibition in winter. However, even under normal mild temperate conditions, continuous field measurements of photosynthesis in *Lecanora muralis* over several days indicated that photoinhibition is a regular occurrence (Leisner et al. 1997).

Thallus drying can be viewed as a means of protection against high light. The increase in thallus temperature that accompanies high light can cause them to desiccate rapidly, and when their water content is below 15%, they become photosynthetically inactive (Hájek et al. 2001). Even though in their desiccated state lichens are less affected by high irradiances because metabolic activity is reduced or completely shut down photodamage damage can still occur (Gauslaa and Solhaug 1999; Solhaug et al. 2003). Furthermore, less solar radiation is transmitted through the upper cortex (Ertl 1951; Gauslaa and Solhaug 2001; Gasulla et al. 2021). However, desiccation does not exempt lichens from suffering from light stress (MacFarlane and Kershaw 1980; Kosuga et al. 2009; Leprince et al. 2015). In bryophytes, desiccation does not stop the transfer of excitation energy from the light-harvesting pigments to the reaction centres (Heber et al. 2006b). Nevertheless, the highly quenched state of chlorophyll upon desiccation shows extremely efficient dissipation of photons, lowering $^1O_2$ formation. However, even if light only causes the formation of tiny amounts of ROS in desiccated thalli, normal repair processes do not take place (Buffoni Hall et al. 2003). It remains unclear whether in the field most photoinhibition occurs in the desiccated or hydrated states.

Interestingly, when fully hydrated, lichens are highly susceptible to photoinhibition because there is a reduction in CO_2 availability for photosynthesis caused by the hydration-induced resistance to CO_2 diffusion into the thallus (Lange et al. 2001). It has been suggested that lichens are most susceptible to photoinhibition in early spring when there is enough moisture from mist/fog, rainfall

or melting snow in combination with low temperatures and high light. In vascular plants it is well established that cold exacerbates the effects of photoinhibition (see Öquist and Hunner 1993; Zhang and Scheller 2004; Szalai et al. 2018; Mattila et al., 2020),

Lichens with cyanobacterial photobionts appear more sensitive to photoinhibition than those with green photobionts (Demmig-Adams et al. 1990). However, while lacking zeaxanthin, cyanobacterial lichens possess other mechanisms to resist photoinhibition such as the orange carotenoid pigment (OCP), which the species use to dissipate excess energy collected, and by this process it can reduce excess ROS formation (Beckett et al. 2021b).

B. Overview of light protection mechanisms in lichens

i. Screening

The production of secondary metabolites by the mycobiont is likely an important mechanism of protection against light stress in lichens. Some pigments such as parietin and vulpinic acid in *Xanthoria* spp. and *Letharia vulpina* absorb light in the visible spectrum and directly protect the photosynthetic apparatus of the photobiont (Rundel 1978; Galloway 1993; Gauslaa and Ustvedt 2003; Solhaug et al. 2010; Beckett at al. 2021a). However, lichens also contain many unpigmented or faintly pigmented lichen substances such as atranorin. The extinction coefficients of these compounds (see Huneck and Yoshimura 1996) show that most absorb UV-B radiation very efficiently. Interestingly, although not absorbing visible light, removal of atranorin from *Physcia aipolia* by acetone rinsing considerably reduced the reflectance of moist thalli, presumably because crystals either directly reflect light or raise reflection by preventing water from entering air spaces in the cortex (Solhaug et al. 2010). Nguyen et al. (2013) showed that the effect of usnic acid is due

to absorbing (not reflecting) light. The ability of other unpigmented lichen substances to work in photoprotection however, remains to be tested.

Species like *Lobaria pulmonaria* produce melanins in their upper cortex (Gauslaa and Solhaug, 2001), which reduces transmission of PAR and thereby helps protect against photoinhibition (Mafole et al. [2017], see section 1.5.2 above). In addition, cyanobacterial photobionts produce a yellow-brown pigment called scytonemin which has a high UV screening efficiency (Büdel et al. 1997).

ii. NPQ

Non-photochemical quenching (NPQ) is a mechanism that dissipates excess light safely as heat. NPQ comprises several components that can, to some extent, be resolved using chlorophyll fluorescence (for review see Liu et al. [2019]). qT is a component of NPQ where the antenna chlorophyll molecules are switched between the PSI and the PSII system to equilibrate electron transport. In qE levels of zeaxanthin (Z) in the pigment antennae increase at the expense of violaxanthin (V) which is an irreversible mechanism resulting in dissipation of some of the excess energy as heat (Kalaji and Guo 2008) (Fig 1.4). When PAR levels are low, this process is reversed (Schindler and Lichtenthaler 1996). In addition to Z, qE is dependent on factors such as lumen pH and PsbS (a Four-helix proteins belonging to the Lhc family, essential for NPQ (Kirilovsky and Büchel 2019). qZ is another type of NPQ which differs from qE as it forms between 10-30 min and is dependent on zeaxanthin (Brooks et al. 2013). As for qE, the process is dependent on zeaxanthin, but is independent of PsbS proteins (Dall'Osto 2005). Lastly, qI is a type of quenching that is not rapidly reversible and is therefore referred to as sustained thermal dissipation (Liu et al.

2019). It was originally thought to be caused by photoinhibition, but it is now known that various other mechanisms contribute to qI (Kopecky et al. 2005).

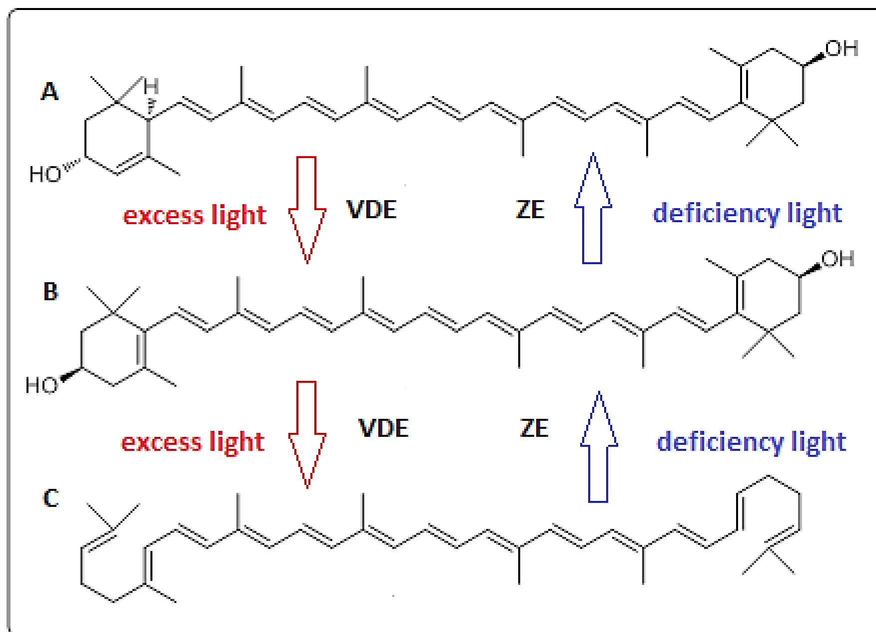

Figure 1.4: The xanthophyll cycle of carotenoid biosynthesis - the conversion of violaxanthin and zeaxanthin. A - violaxanthin, B - antheraxanthin, C - zeaxanthin, VDE - violaxanthin deepoxidase , ZE - zeaxanthin (Kmiecik and Dziembowska 2018)

Although there are relatively few studies involving lichens, there is no doubt that lichens with chlorophycean photobionts display classical NPQ (for review see Beckett et al. [2021a]). In general, high levels of thermal dissipation have been found in lichens that need photoprotection. For example, Calatayud et al. (1997) showed that NPQ tends to be induced in drying thalli of *Parmelina quercina*. Vráblíková et al. (2006) studied seasonal variation of NPQ in *Xanthoria parietina* sampled in one location in Norway for one year. NPQ rapidly increased from early spring until summer solstice, suggesting a higher need for photoprotection in the season with the highest solar irradiance. The effect of seasonal variation and microhabitat exposure on photoprotection

and photoacclimation were investigated in a study by Veres et al. (2022) on six lichen species. The data collected from the four-year study showed that species growing on the north facing slope are characterized by high humidity and low light intensities, while species growing on the southwest facing side are experiencing drier and high light intensities – influencing the metabolism of both sun and shade populations of different species. The study also showed that during bright and dry seasons on sunny microhabitats, rapid NPQ mechanisms protect the photobiont especially when in a hydrated state, when desiccated the main protective mechanism is provided for by secondary metabolites and mechanical actions (i.e., lobe curling) (Veres et al. 2022)

iii. Antioxidants

Should the amount of light absorbed by plants exceed the amount that the plant can quench, the resulting consequence is the formation of ROS which need to be scavenged before they cause damage. Although there have been few studies specifically on light stress in lichens, general "abiotic stresses" have been shown to increase the production of enzymatic and non-enzymatic ROS scavenging systems (Deltoro et al. 1998; Kranner and Birtic 2005; Weissman et al. 2005). Non-enzymatic antioxidant substances include reduced glutathione (GSH), tocopherols (vitamin E), ascorbic acid (vitamin C) and light-induced xanthophyll cycle pigments, while antioxidant enzymes include superoxide dismutase (SOD), peroxidases (POX), and catalase (CAT). In vascular plants, these are often organised into specific pathways such as the Halliwell-Asada pathway which helps to remove H_2O_2 (Figure 1.5) (Hasanuzzaman et al. 2019). It remains unclear whether the classical Haliwell-Asada pathway operates in lichens (Beckett et al. 2021a). Interestingly, instead of making use of ascorbate or glutathione, some photobionts (e.g.,

Trentepohlia) contain high amounts of β-carotene. While this compound is known for its light screening function, it shows some antioxidant behaviour; however, the ability of β-carotene to scavenge ROS has not been directly tested (Beckett et al. 2021a).

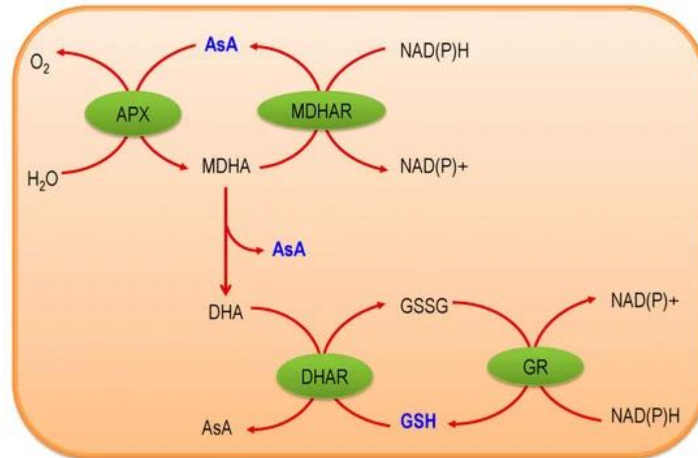

Figure 1.5: Ascorbate-Glutathione (AsA-GSH) (Halliwell - Asada) pathway. From Hasanuzzaman et al. (2019).

iv. PSII repair

Should the antioxidant system of a lichen photobiont be unable to scavenge stress-induced ROS, an early target is the PSII complex. The D1 and D2 proteins are the major components of the PSII complex, and are sensitive to stress, and when damaged need to be repaired (Beckett et al. 2021a). Although not directly tested, studies on free living algae and cyanobacteria suggest that the "PSII repair cycle" is likely to occur in both chlorolichens and cyanolichens (Beckett et al. 2021a). In this cycle damaged proteins are repaired, new protein subunits are produced, incorporated, and activated into the PSII complex (Vass et al. 2014, Kim et al. 1993).

v. Mechanical protection

Lichen communities are more visible when hydrated because during desiccation they shrink or curl. Such movements are distinct in most foliose species that roll up into a ball and only expose their lower surface during desiccation (Rogers 1971; Lumbsch and Kothe 1988; Barták et al. 2006). Such movements have been assumed to protect the light sensitive algal layer (Rogers 1971). Sometimes the lobe tips and margins curl or fold to a vertical position during desiccation, protecting the photobiont layer from high solar radiation (Barták et al. 2006). Rolling up, bending, or curling during desiccation have also been proposed to reduce the effects of light-induced damage in poikilohydric vascular plants (Lebkuecher and Eickmeier 1993; Farrant et al. 2003).

1.8. Temperature stress

Generally, lichens growing in open habitats usually have high heat tolerance (Beckett et al. 2008). In early studies photosynthesis, respiration and nitrogenase activity were used as parameters to indicate thermal stress (Tegler and Kershaw 1980). When dry, lichens shut down metabolic activity. Lange's classic (1953) study on several lichen species clearly showed that dry lichens are resistant to high temperatures, with resistance to heat stress ranging from 70-101°C for *Alectoria sormentosa* and *Cladonia pyxidata* respectively. However, in hydrated thalli, the resistance to temperature stress ranged between 35-46°C (Tegler and Kershaw 1981), which is clearly lower than that of desiccated thalli. In two *Peltigera* species resistance was reported to be different: *P. canina* var. *praetexta* was sensitive to moderate temperatures (35°C) whilst *P. canina* var. *rufescens* was resistant to fairly high temperatures (45°C) (MacFarlane and Kershaw 1980). These different responses to heat stress were attributed to the ecology of these two populations and

suggested that thermotolerance may be responsible for shaping parameters in the ecology of many other lichen species (MacFarlane and Kershaw 1980). In some mosses (*Ctenidium, Fissidens* and *Syntrichia spp*) that occupy a similar ecological niche to lichens, seasonal changes in heat tolerance can occur, with thalli being tolerant by at least 10°C more during summer months (Tegler and Kershaw 1980), but interestingly comparable studies appear not to have been carried out on lichens.

In the field, high temperatures increase evaporation, and it can be difficult to decide if lichens are responding to extremely high temperatures or desiccation (Kappen et al. 1973). It seems likely that different stages of the lichen life cycle differ in tolerance. In a study by Thomas (1939) spores of *Xanthoria parietina* did not germinate under moderate temperatures (higher than 24°C).

1.9. Desiccation tolerance

Desiccation tolerance (DT) is described by Kranner (2002) as the ability of cells to survive under water contents below 10% (w/w) but resume physiological abilities upon rehydration. DT is common in fungi, bryophytes, and algae, and probably all lichenized ascomycetes are desiccation tolerant (Kranner and Lutzoni 1999; Scott 2000; Cao et al. 2020; Gasulla et al. 2021). Although desiccation tolerant, constant hydration is harmful for lichens (Farrar 1976; Dietz and Hartung 1999, Honegger 2006). Understanding desiccation tolerance of cryptogams can help devise strategies that may increase drought tolerance in vascular plants (Kranner et al. 2008). For organisms to be regarded as desiccation tolerant, they must meet certain criteria: a) maintain physiological integrity even in the desiccated state; b) recover rapidly upon rehydration; c) repair damage caused by desiccation (Oliver et al. 2000). Various mechanisms have been proposed for

protection against desiccation-induced injury. First, late embryogenesis abundant (LEA) proteins (Dirk et al. 2020) e.g., dehydrins and rehydrins can protect biomolecules by acting as “chaperones”. Second, non-reducing sugars can protect membranes (Elbein et al. 2003). Third, desiccation-induced free radicals can be actively scavenged (Kranner et al. 2008; Dinakar and Bartels 2013; Georgieva et al. 2017; Hell et al. 2019).

Adopting a symbiotic lifestyle probably increases the DT of both partners. A major effect of desiccation stress in lichens is an increase in ROS formation (Catalá et al. 2010). Isolated symbionts are very sensitive to oxidative stress when desiccated, but in lichenized fungi each partner appears to up-regulate protective systems in the other. In particular, without the algal partner, the glutathione-based antioxidant system of the mycobiont is slow and ineffective (Kranner and Birtic 2005). However, it should be noted that the relationship between DT and antioxidant activity is not always straightforward. For example, Kranner (2002) reported no correlation between differences in DT and glutathione reductase (GR) of *Lobaria pulmonaria*, *Peltigera polydactyla* and *Pseudevernia furfuracea*. Weissman et al. (2005) reported a 50-70% reduction in CAT and SOD activity in *Ramalina lacera* following rehydration. Mayaba and Beckett (2001) observed decreases or small changes in CAT, SOD and ascorbate peroxidase (APx) activities of *Peltigera polydactyla*, *Ramalina celastri* and *Teloschistes capensis* following desiccation – rehydration cycles. These three species occur mostly in very different habitats: moist, xeric and extremely xeric habitats, respectively. Clearly more work is needed on the role of antioxidative systems in DT of lichens.

1.10. Introduction to the study

Lichens often grow in habitats where they experience extreme conditions such as desiccation, high light intensities and temperature extremes (Beckett and Minibayeva 2007). What makes lichens interesting and unique is their ability to tolerate these stresses. Possible tolerance mechanisms to these abiotic stresses have been reviewed in detail above. However, recently the production of secondary metabolites has been suggested as one of the ways in which lichens can tolerate stress.

Apart from the study by Solhaug et al. (2010) only limited information is available on the role of unpigmented substances in protection against PAR. As a result, the aim of the work presented in the first chapter was to use the acetone rinsing to test for the ability of usnic acid and atranorin to protect lichen photobionts from high light. The aim of the work presented in the second chapter was to compare NPQ in melanised and pale thalli of the same species. An additional aim was to understand if presence of a melanised cortex will or will not prevent the use of conventional chlorophyll fluorimetry to compare NPQ in pale and melanised lichens. In the work described in the third chapter, intraspecific variation was tested by comparing the relative importance of secondary metabolites in protection against photoinhibition in shade and sun forms of the same species of lichens. Finally, the ability of secondary metabolites to protect lichens against damage caused by desiccation stress was tested.

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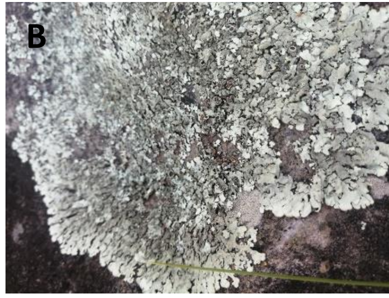
CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1. Lichen material

Lichen material shown in Figure 2.1 was collected from various locations in South Africa, and Europe (see Table 2.1). Collections were made between April 2019 – July 2022, in this period, approximately 43 trips were made to the various locations for collections. The trips were made depending on what type of lichen material was required (i.e., sun vs shady), for what type of experiments (i.e., chlorophyll fluorescence or ion leakage) as a result collections were made throughout all seasons. In general, the species used in this study were chose because they were readily available and could be collected sustainably. Essentially, they represented the common macrolichens in the Afromontane region of KwaZulu Natal. The taxonomic identity of the species used in this study is currently confused and changes all the time. The work we did for my MSc, proved to us that even when using molecular techniques (DNA barcoding) to accurately identify lichen species, it is not an easy task because species that have different morphological characteristics may belong to the same species and vice versa, so it was hard for the taxonomic identification to be 100% correct and accurate but it was very close to what we needed to use in this study.

Table 2.1: Localities where lichen material was collected from

Species	Collection locality	Location description
Parmotrema perlatum	Fort Nottingham Nature Reserve, South Africa	Small pocket of an Afromontane Forest in the mist belt of KZN
Ramalina celastri	Fort Nottingham Nature Reserve, South Africa	Small pocket of an Afromontane Forest in the mist belt of KZN
Xanthoparmelia conspersa	Queen Elizabeth Park, South Africa (shade populations)	Shaded area with lichens growing in rocks and branches
	Cascades (sun populations)	Rocky outcrops near the Cascades Lifestyle Center
Usnea angulata	Fort Nottingham Nature Reserve, South Africa	Small pocket of an Afromontane Forest in the mist belt of KZN
Leucodermia leucomelos	Fort Nottingham Nature Reserve, South Africa	Small pocket of an Afromontane Forest in the mist belt of KZN
Cladonia foliacea	Cumberland Nature Reserve, South Africa	Collected in soil surfaces of a highly sunny area
Cetraria islandica	Syktyvkar, Russia	Exposed site in boreal vegetation
Crocodia aurata	Fort Nottingham Nature Reserve, South Africa	Small pocket of an Afromontane Forest in the mist belt of KZN
Peltigera aphthosa	Ås, Norway	Nemoral boreal vegetation on the outskirts
Lobaria pulmonaria	Langangen, Norway	Collected from the bark of oak trees from nemoral boreal vegetation
Usnea cornuta	Fort Nottingham Nature Reserve, South Africa	Small pocket of an Afromontane Forest in the mist belt of KZN
Peltigera malacea	Syktyvkar, Russia	Exposed site in boreal vegetation



9 cm | 20 cm | 6.1 cm

7.2 cm | 11 cm | 16 cm

8.7 cm | 15.1 cm | 9 cm

17 cm | 6 cm | 7 cm

Figure 2.1: Species used in this study. **A** – *Parmotrema perlatum*; B – *Xanthoparmelia conspersa*; C – *Ramalina celastri*; D – *Usnea angulata*; E – *Leucodermia leucomelos*; F – *Cladonia foliacea*; **G** – *Peltigera malacea*; **H** – *Crocodia aurata*; **I** – *Usnea dasea*; **J** – *Lobaria pulmonaria*; **K** – *Peltigera aphthosa*; **L** – *Cetraria islandica*. Scale represents the actual size of the thalli presented. Pictures taken by Nqobile Ndhlovu

Table 2.2: Lichen species and the secondary metabolite(s) that occur in the cortex and/or medulla

Species	Secondary metabolite(s)	Reference(s)
Parmotrema perlatum	Usnic and stictic acid	Manojlović et al. 2020; Hussain et al 2022
Ramalina celastri	Parietin and usnic acid	Fazio et al. 2007
Crocodia aurata	Calycin and pulvinic acid	Fernándes et al. 2018
Usnea angulata	Usnic acid	Reddy et al. 2019; Popovici et al. 2021
Cetraria islandica	Protolichesterinic acid and fumarprotoctraric acid	Xu et al. 2016
	Melanin	Beckett et al., 2019
Leucodermia leucomelos	Atranorin; Salazinic acid	Muhoro and Farks 2021
Xanthoparmelia conspersa	Stictic acid and usnic acid	Molnár and Farkas, 2010
Cladonia foleacea	Cortical substances: Dibenzofuran; usnic acid	Farkas et al. 2020
	Medullary substances: Despsidone; fumarprotocetraric acid	Farkas et al. 2020
Lobaria pulmonaria	Melanin, stictic acid, constictic acid, norstictic acid	Gauslaa and Goward 2020 Pejin et al. 2017 Asplund 2011
Peltigera aphthosa	Melanin	Mafole et al. 2019
Peltigera malacea	Melanin	Matee et al. 2015
Usnea cornuta	Usnic acid	Guo et al. 2008

2.2. Storage, selection, and preparation of lichen material

After collection, lichen material was cleaned of debris (bark, leaves, soil) using forceps and fingers and was allowed to air dry in room temperature in the laboratory overnight in the darkness. Drying material below 10% water content allows the material to be stored for longer periods of time, and metabolic activity can be restored within minutes upon rehydration (Kappen 1988; Honegger 2003). After cleaning and air drying, lichen material that was not used immediately was stored by placing lichen material in a plastic bag, putting it in a freezer (Defy freezer) that sits at -24°C (for two weeks to a month at maximum) until needed for experiments. Freezing is a recommended storage method for lichen thalli that will be used for physiological experiments (Larson 1978; Jensen and Feige 1987) such as chlorophyll fluorescence. Frozen thalli can be thawed and refrozen without losing viability (Honegger 2003).

Thalli were selected based on homogeneity of appearance; this selection happened before the start of any experiment. Lichens were collected dry and upon removal from the freezer (Defy freezer) hydrated by placing it over wet filter paper (Whatman filter paper grade 1) (sprayed with distilled water) and placed under a photosynthetic photon fluence rate (PPFR) with a light intensity of *c*. 30 μmol m^{-2} s^{-1} for 24 h and used immediately after this period**.** Thalli that served as control (thalli whose secondary metabolites were not extracted/removed from the cortex) were immediately placed on wet filter paper and placed on a growth cabinet that has a temperature of ±23°C and light intensity of *c*. 30 μmol m^{-2} s^{-1} for 24 – 48 h to acclimatize before the experiments. In some cases, thalli were further desiccated over silica gel to ensure complete desiccation state.

2.3. Acetone rinsing

To compare the effect of lichen substances on sensitivity to photoinhibition in the hydrated and desiccated states, lichen substances were removed using the "acetone rinsing" technique of Solhaug et al. (2010). Before acetone rinsing, lichens were removed from freezer (Defy freezer) to completely thaw and be in their dry state, to ensure that lichens material were completely dry, they were left overnight over silica gel (Analar grade, Merck, Darmstadt, Germany). They were then gently shaken (made by the UKZN workshop) in 100% acetone for 10 min. Acetone (Analar grade, Merck, Darmstadt, Germany) was then discarded, and the process repeated two to four times. Different lichen species have different acetone tolerance (Solhaug and Gauslaa 2001). As per the latter statement the different species used in this study required different acetone rinsing treatments (see Table 2.3). After acetone rinsing, the thalli were left at room temperature overnight to allow residual acetone to evaporate. Specific details of the technique for the different experiments are explained in chapters 3 and 5.

Table 2.3: The acetone rinsing times for the different species.

	Acetone rinsing	
	Wet state	Dry state
Parmotrema perlatum	4 x 5ml for 20 min	4 x 5ml for 20 min
Cetraria islandica	1 x 5ml for 5 min	1 x 5ml for 5 min
Ramalina celastri	2 x 5ml for 10 min	2 x 5ml for 10 min
Usnea cornuta	2 x 5 ml for 10 min	2 x 5 ml for 10 min
Leucoodermia leucomelos	4 x 5 ml for 10 min	4 x 5 ml for 10 min
Xanthoparmelia conspersa	4 x 5 ml for 10 min	4 x 5 ml for 10 min
Crodocodia aurata	2 x 5 ml 10 min	2 x 5 ml 10 min
Cladonia foleacea	1 x 5 ml 10 min	1 x 5 ml 10 min

2.4. Chlorophyll fluorescence measurements

To assess the effects of light stress on photosynthesis, chlorophyll fluorescence was used to measure the maximal efficiency of photosystem II (PSII; F_V/F_M) and the relative electron transfer rate (rETR, a proxy of steady state photosynthesis). In general, both parameters responded similarly to light stress, although occasionally one parameter was more sensitive than another for no obvious reason. Chlorophyll fluorescence was measured using a Hanstech FMS 2 (Hanstech instruments, Kings' Lynn, England) and a PAM 2500 fluorometer (Walz, Effeltrich, Germany) using a red LED throughout.

After a dark adaptation period of at least 10 min, material was given a flash of light (typically 8000 µmol m^{-2} s^{-1} for 0.8 s), and F_V/F_M measured, where

F_M = maximum fluorescence

F_V = variable fluorescence, calculated as F_M - F_o), with F_o = minimal fluorescence yield of the dark-adapted state. Thalli with anomalous values of F_V/F_M were discarded. The actinic light (25 µmol m^{-2} s^{-1}) was then switched on, and when the fluorescence signal was stable, rETR was calculated as:

$$rETR = 0.5 \times \Phi PSII \times PAR$$

where PAR = photosynthetically active radiation and ΦPSII is the effective quantum yield of PSII photochemistry calculated as $(F_{M'} – F_t)/Fm$ (where $F_{M'}$ = maximal fluorescence yield of the light-adapted state and F_t = stable fluorescence signal in the light). Equal distribution of excitation between PSII and PSI was assumed (hence multiplication by 0.5).

NPQ was calculated using a formula from Bilger et al. (1995) as:

$NPQ = (F_M - F_{M'})/F_{M'}$

2.5. Photoinhibition treatments

For each species 1.5 cm (diameter) disks x 40 samples were used (per experiment), 10 for each treatment combination of hydrated and desiccated with and without secondary metabolites. For species where disks could not be cut (i.e., *Usnea*, *Leucoodermia* and *Ramalina*). For *Ramalina*, 2-3 strands were used to represent a sample. All thalli were initially in a desiccated state. To expose hydrated thalli to high light, thalli were acetone rinsed if required, the acetone allowed to evaporate overnight, all (rinsed and unrinsed) thalli placed on wet filter paper (Whatman filter paper grade 1) at 30 μmol $m^{-2}s^{-1}$ overnight and an initial F_V/F_M measurement taken. Thalli were then exposed to high light. To expose desiccated thalli to high light, thalli were acetone rinsed if required, the acetone allowed to evaporate for 24 h, all thalli (rinsed and unrinsed) hydrated overnight as above, an initial F_V/F_M measurement taken, and then allowed to air dry overnight. They were then exposed to the photoinhibitory light, and immediately rehydrated by placing them on wet filter paper (Whatman filter paper grade 1). Lichens were photoinhibited using a LED panel (Model SL – 3500, Photon System 212 Instruments, Brno, Czech Republic) that provides cool white light. The time exposure to light needed to reduce F_V/F_M down to c. 0.2 to 0.3 for untreated lichens was determined in preliminary experiments. Species differed in their sensitivity, and much longer exposures were needed for dry compared to wet material (see Chapter 3). Lichens with and without lichen substances received the same exposure times and intensities. Initial chlorophyll fluorescence measurements were taken at the start of the experiment as indicated above, immediately after the exposure to high light and again at intervals for 14 -50 h. During recovery, lichens were exposed to normal laboratory light (c. 5 ümol m^{-2} s^{-1}) as recommended by Solhaug (2018).

2.6. Statistical analysis

Statistical analysis was done on Excel 2021 (v. 2212) for two-way ANOVAS. The statistics package ‘‘Statistica’’ (Basic Academic Bundle V14, TIBCO Software Inc., Palo Alto, CA, U.S.A.) was used to carry out generalized mixed linear models (repeated measures) analyses following checks for normality and homogeneity of variance.

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CHAPTER 3: UNPIGMENTED LICHEN SUBSTANCES PROTECT LICHENS AGAINST PHOTOINHIBITION OF PHOTOSYTEM II IN BOTH THE HYDRATED AND DESICCATED STATES

3.1. Introduction

To some extent, the fungal upper cortex protects the photobiont against the effects of high light. Visible light transmitted by a moist cortex typically ranges from approximately 90 % for rainforest lichens to only 45 % for lichens from bright exposed sites (Dietz et al. 2000), but can be as low as 10% (Büdel and Lange 1994). Transmittance is much lower in the upper cortex of desiccated than hydrated thalli (Ertl 1951). The optical properties of the fungal hyphae in the upper cortex themselves are partly responsible for screening the photobionts from excessive light. However, the presence of secondary compounds, which occur partlya as hydrophobic crystals on the hyphal cell walls, can greatly reduce transmittance. Melanins (Mafole et al. 2019) and parietin (in *Xanthoria parietina* Solhaug and Gauslaa 1996) are some lichen substances that are heavily pigmented and filter both UV and PAR. In addition, Phinney et al. (2019) reported that susceptibility to photoinhibition in the lichen *Letharia vulpina* (that contains mainly the bright yellow vulpinic acid) is much higher following extraction of lichen acids using the acetone rinsing technique of Solhaug and Gauslaa (2001). However, most other lichen substances are colorless. Perhaps surprisingly, evidence exists that even colorless secondary metabolites can provide photoprotection from high levels of PAR. For example, Legaz et al. (1986) found higher concentrations of usnic acid and atranorin in the thalli of *Evernia prunastri* during the brighter summer months than in winter. Furthermore, extraction of the colorless secondary metabolite atranorin from *Physcia aipolia* significantly increased photoinhibition caused by high light (Solhaug et al. 2010). This increased sensitivity to light appeared to be because the reflectance of

the thallus was greatly reduced, and the visible appearance of the lichen colour changed from pale grey to green in the hydrated state. Differences in the reflectance of dry thalli with and without lichen substances were much smaller. Irrespective of the mechanism, preliminary data suggest that even unpigmented lichen substances can provide significant photoprotection for lichen photobionts.

Apart from the study on *Physcia aipolia* described above, a review by Nguyen et al. (2013) investigates UV-protectant metabolites from their lichens and symbiotic partners and Nybakken et al. 2004 where the role of secondary metabolites in the species Cetraria islandica and Xanthoria elegans is investigated. Expanding from the mentioned studies, the first aim of the work presented in this chapter was to use the acetone rinsing method to test the ability of unpigmented lichen substances to reduce photoinhibition in four common South African Afromontane macro lichens. Three of these lichens contain the faintly yellow lichen substance usnic acid, while the fourth contains the unpigmented atranorin. Secondly, we also tested the effectiveness of lichen substances at preventing photoinhibition in desiccated lichens, which has not previously been tested in any species. Photoinhibition was assessed by measuring the maximal (or optimal) quantum yield ($F_{V/}F_M$) using chlorophyll fluorescence (see Chapter 2 for more details).

3.2. Materials and Methods

3.2.1. Lichen material

The lichens used were *Parmotrema perlatum* (Huds.) M Choisy., *Ramalina celastri* (Sprengel) Krog and Swinscow, and *Usnea cornuta.* Körb., all reported to contain mainly usnic acid (Payal and Sharma 2016; Bannister et al. 2020) and *Leucodermia leucomelos* (L.) Kalb, reported to contain almost exclusively atranorin (Culberson 1966). The identity of the main lichen substances

present in the lichens was confirmed by high performance liquid chromatography as described by Pawlik-Skowrońska and Bačkor (2011), and comparison with standards of usnic acid and atranorin from Sigma-Aldrich (Burlington Massachusetts, United States of America). Lichens were collected dry from an Afromontane forest at Fort Nottingham, KwaZulu Natal, South Africa and most were growing on a small tree, *Leucosidea sericea* Eckl. and Zeyh. Lichens were collected growing close to each under similar environmental conditions. The photobionts of these lichens have been reported to belong to the Chlorophycean genus *Trebouxia* (Rambold et al. 1998). Lichens were stored dry for up to 1 month at in a freezer (− 24 °C) (Defy freezer) until the start of the experiments.

3.2.2. Acetone rinsing

Rinsing four times had no effect on F_V/F_M in *Parmotrema* or *Leucoodermia spp.,* but slightly reduced values in *Usnea* and *Ramalina spp*. The extraction was repeated for the latter two species, and results showed that reducing rinsing from four to two times prevented damage.

3.2.3. Reflectance spectra measurements

Out of the four species tested, only the thalli of the *Parmotrema spp.* were sufficiently large to accurately measure reflectance spectra with the equipment available. Reflectance spectra (300–800 nm) were measured at random positions of the thalli with a spectrometer (SD2000, Ocean Optics, Netherlands) connected to an output port sphere with a 400 µm thick fiber. Measurements were done by directly placing the upper cortex of each lichen thalli (factorial combination of desiccated, hydrated, with and without secondary metabolites). The thalli were then illuminated

by a halogen lamp (model DH2000, Ocean Optics, Netherlands) through a 600 µm optical fiber connected to the input port of the integrating sphere (ISP-50- REFL, OceanOptics, Netherlands). The species were illuminated for different lengths of times (see Table 3.1) and the times needed for photoinhibition were identified in preliminary experiments.

3.2.4. Photoinhibition treatments

Table 3.1: Times of exposure and light intensities used to photoinhibit lichens to F_V/F_M values of c. 0.2 – 0.3.

	Times of exposure to a photoinhibitory light	
	Wet state	Dry state
Parmotrema perlatum	900 µmol m^{-2} s^{-1} for 4 h	900 µmol m^{-2} s^{-1} for 10.5 h
Ramalina celastri	600 µmol m^{-2} s^{-1} for 4.5 h	1200 µmol m^{-2} s^{-1} for 24 h
Usnea cornuta	400 µmol m^{-2} s^{-1} for 5 h	900 µmol m^{-2} s^{-1} for 27 h
Leucoodermia leucomelos	600 µmol m^{-2} s^{-1} for 5 h	1350 µmol m^{-2} s^{-1} for 24 h

3.2.5. Statistics

All data from the photoinhibition experiments were subjected to a two-way analysis of variance (ANOVA) in Microsoft Excel where all data was tested for checks of normality and homogeneity of variance requirements.

3.3. Results

3.3.1. Reflectance spectra of Parmotrema perlatum

Extracting lichen substances from *Parmotrema* greatly reduced reflectance compared to control thalli when the lichen was in the rehydrated state (Figure 3.1). In desiccated thalli reflectance was generally higher but removing secondary lichen substances had little effect on reflectance.

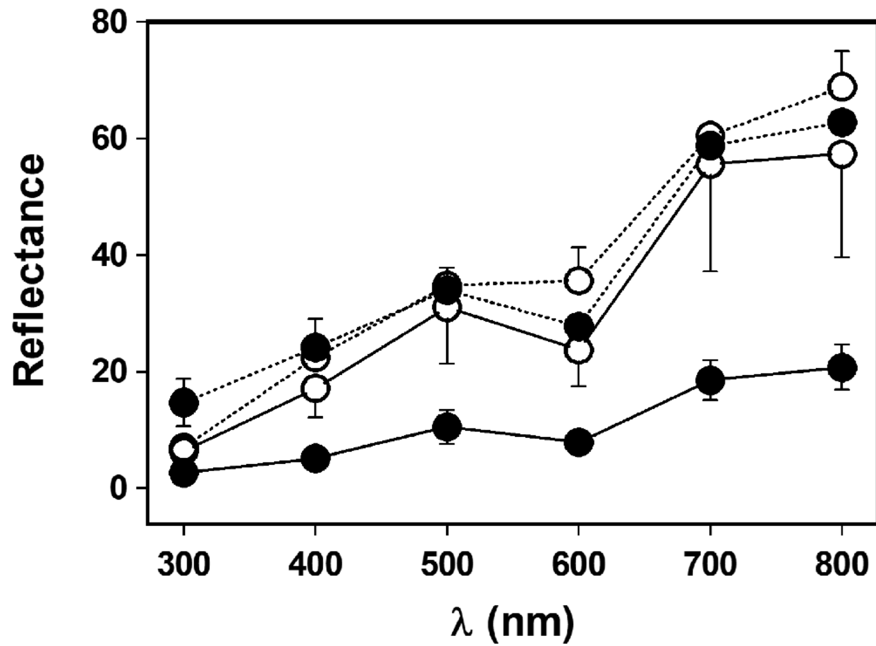

Figure 3.1: The effect of acetone rinsing on reflectance in hydrated and desiccated thalli of *Parmotrema perlatum*. Dashed lines indicate desiccated thalli, while solid lines indicate hydrated thalli. Open symbols are thalli containing lichen substances, while closed symbols indicate thalli that had been acetone rinsed to remove lichen substances. Error bars indicate the standard error, n = 6; overlapping error bars have been removed

Results presented here show that secondary metabolites can protect lichen photobionts from high PAR, even if they are only faintly pigmented or unpigmented, and furthermore photoprotection occurs whether thalli are wet or dry.

3.3.2. Photoinhibition of wet and dry thalli with or without lichen substances

As discussed in Chapter 2, despite all lichen species material being collected from the same place and occupying superficially similar habitats (small twigs at the periphery of the canopy), species differed in their sensitivity to photoinhibition (Table 3.1). Furthermore, much longer exposures were needed to reduce F_V/F_M down to c. 0.2–0.3 for dry compared with wet material (Table 3.1). After exposure to the photoinhibitory light, thalli with lichen substances removed (treatment) usually displayed lower values of F_V/F_M compared with thalli with lichen substances still present, and often displayed reduced rates of recovery back to initial values (Figure 3.2). For *Ramalina* and *Parmotrema spp.,* removal of substances had similar effects on the susceptibility of hydrated and desiccated thalli to photoinhibition; photoinhibition reduced F_V/F_M to lower levels than control thalli (thalli that still has lichen substances intact), and F_V/F_M took longer to recover (Figure 2A–D). In *Usnea* removal of lichen substances had more effect in dry material. In hydrated material removal of lichen substances slightly reduced F_V/F_M after photoinhibition and reduced the rate of recovery (Figure 3.2E). In desiccated material, removal had more effect on F_V/F_M after photoinhibition, and slowed down recovery, particularly during the early stages. By contrast, in *Leucodermia* samples the removal of lichen substances slightly increased the susceptibility of hydrated thalli to photoinhibition but had almost no effect on the susceptibility of desiccated thalli (Figure 3.2G, H). Two-way ANOVA indicated that removing lichen substance significantly ($P < 0.05$) affected the recovery of both hydrated and desiccated lichens from photoinhibition, the only exception being desiccated material of *L. leucomelos*, where there was no significant effect of lichen substance removal ($P > 0.05$).

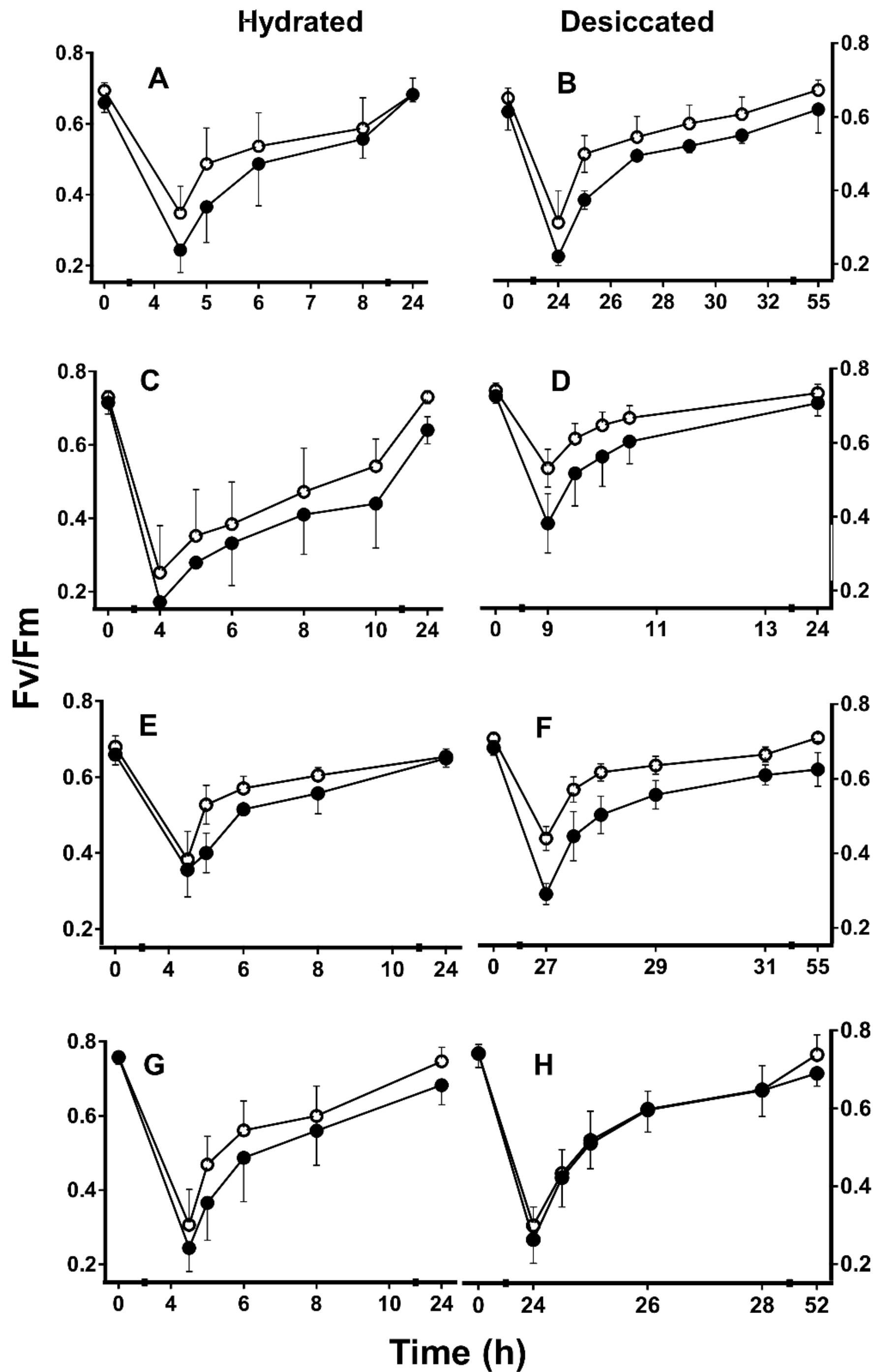

Figure 3.2: The recovery from a photoinhibition in untreated (open symbols) and thalli with lichen substances removed using acetone rinsing (closed symbols) in hydrated (A, C, E, G) and desiccated (B, D, F, H) thalli of *Ramalina celastri* (A, B), *Parmotrema perlatum* (C, D), *Usnea cornuta* (E, F) and *Leucodermia leucomelos* (G, H). Error bars indicate the standard error, n = 10; overlapping error bars have been removed

3.4. Discussion

The results presented here clearly show that even in lichens possessing only faintly pigmented or unpigmented lichen substances, photoinhibition is higher when the lichen substances are removed (Figure 3.2). Unfortunately, our equipment for measuring reflectance required a disk of c. 1 cm in diameter, and we were therefore only able to test *Parmotrema* thalli. In this species, lichen substance removal greatly reduced the reflectance of wet thalli (Figure 3.1). Assuming lichen substance removal affected the reflectance of the other species in the same way, it seems likely that in hydrated thalli reduced reflectance is responsible for the greater photoinhibition in thalli with lichen substances removed. However, except for the atranorin-containing *Leucoodermia*, lichen substances can also protect desiccated lichens, even though in *P. perlatum*, the one species we were able to test, removal of substances has no effect on reflectance when lichens are dry (Figure 3.1). This suggests that there are other reasons why lichen substances can photoprotect photobionts in dry lichens.

3.4.1. Effect of the removal of lichen substances on thallus reflectance in Parmotrema perlatum

Removal of lichen secondary metabolites greatly decreases the reflectance of visible light in hydrated thalli of *Parmotrema*, particularly at longer wavelengths (Figure 3.1). Desiccated thalli have much higher reflectances than wet thalli, and by contrast, lichen substance removal has little effect on reflectance. Results obtained here are very similar to those reported following atranorin removal in *Physcia* thalli by Solhaug et al. (2010). The precise reasons for the decrease in reflectance that occurs following lichen substance removal remain unclear. Probably, at least part of the reason for the effect is that crystals of lichen substances directly reflect light. However, lichen substance removal does not affect the reflectance of desiccated lichens. Possibly, as they

are hydrophobic, the lichen substances stop water from entering the intercellular hyphal spaces in the cortex, and in hydrated thalli it is actually the air-filled cavities that reflect light (Solhaug et al. 2010).

3.4.2. Effect of removal of lichen substances on susceptibility of lichens to high light induced reductions in F_V/F_M

To varying degrees, usnic acid removal significantly increases the sensitivity of F_V/F_M to inhibition following a photoinhibitory light stress in photobionts of *Parmotrema*, *Ramalina* and *Usnea spp.* in both the hydrated and desiccated states (Figure 3.2A–F). By contrast, in *Leucodermia*, atranorin appears to increase tolerance to reductions in F_V/F_M only when the lichen was hydrated and has no effect when the lichen is desiccated (Figure 3.2G, H). The main reason that lichen substances increase tolerance to photoinhibition in the hydrated state is probably because they considerably increase reflectance (Figure 3.1). In an analogous way, the leaves of many vascular plants from bright environments protect themselves from photoinhibition by a high reflectance because of a coat of hairs or wax or even salt crystals (Robinson et al. 1993). However, it is more difficult to explain why lichen substances can improve tolerance to photoinhibition in dry lichens, while apparently not increasing thallus reflectance (Figure 3.1). Various explanations are possible. First, while lichen substances may not increase reflectance, they may reduce transmission, although this does not appear likely given that they are only faintly pigmented or unpigmented. Neither atranorin or usnic acid absorb in the PAR region of the electromagnetic spectrum (Fazio et al. 2009; Medina and Avalos-Chacon 2015). Possibly, the lichen substances can scatter light to some extent, although this does not appear to result in an increase in reflectance. Second, as discussed in the Introduction, lichen substances can have very high antioxidant activity (Kosanić et al. 2011;

Fernandez-Moriano et al. 2016). Although not proven for lichen photobionts, it seems likely that as for bryophytes (Heber et al. 2006), even when desiccated, light shining on chloroplasts may generate harmful radicals. However, it is currently unknown whether lichen substances can scavenge ROS produced by photobiont chloroplasts. Regardless of the mechanism, it seems clear that usnic acid can protect lichen photobionts from photoinhibition even when thalli are desiccated. It is not clear why unlike usnic acid, atranorin does not protect desiccated *Leucoodermia* (Figure 3.2G, H). In all species studied the photobiont belongs to the same genus, *Trebouxia*, so photobiont differences seem unlikely. Possibly atranorin is less effective in decreasing transmittance or is less effective as an antioxidant. However, both atranorin and usnic acid have been reported to possess antioxidant properties (Sepahvand et al. 2021; White et al. 2014).

3.5. Conclusions

Lichen substances play many roles in lichen biology. It now seems clear that in addition to their better elucidated roles in UV screening and acting as antimicrobials or grazing deterrents, they can also protect the photobionts from high PAR. While Solhaug et al. (2010) reported that unpigmented substances can photoprotect hydrated thalli of *Physcia spp.*, it appears that such protection may be a rather general phenomenon. Furthermore, we show here for the first time that lichen substances can even protect photobionts from high light when a lichen is desiccated. Many lichens spend a great proportion of their lives desiccated, even in temperate climates (Leisner et al. 1997). Although it requires longer to photoinhibit photobionts when lichens are desiccated, arguably photoprotection is equally or even more important when they are desiccated as when they are hydrated. However, more work is needed to understand how lichen substances

such as usnic acid can improve photoprotection in desiccated lichens, and why some lichen substances are more effective than others.

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CHAPTER 4: MELANISATION IN BOREAL LICHENS IS ACCOMPANIES BY VARIABLE CHANGES IN NON-PHOTOCHEMICAL QUENCHING

4.1. Introduction

Many lichens grow on tree trunks or on rocks under a tree canopy, and in such microhabitats, they experience rapidly changing light levels. This is a result of gaps in the canopy creating brief periods of high light, known as "sunflecks" (Mkhize et al. 2022). Tolerance to these short-term changes in light availability (over a range from minutes to hours) can be improved by increasing the dissipation of excess energy harmlessly as heat using non-photochemical quenching (NPQ). For lichens, species that grow in microhabitats where much of the light is derived from sunflecks usually display considerably higher NPQ than collections from more open microhabitats (Beckett et al. 2021; Mkhize et al. 2022). Although little studied in lichens, additional short-term mechanisms may include an increased ability to scavenge ROS formed during photoinhibition, an increased capacity to repair ROS-induced damage, and an increase in cyclic electron flow (Shi et al. 2022).

The relative importance of the various tolerance mechanisms for photoprotection in different species of lichens in field situations is unknown. In particular, it is difficult to separate mechanisms based on the synthesis of light screening pigments from more biochemical mechanisms of photoprotection. For lichens that become melanised when they grow in sunny locations it appears intuitive that melanin synthesis would be the most important defense mechanism. As noted, melanised thalli are more tolerant to photoinhibition than pale thalli (Mafole et al. 2019a; Mafole et al. 2019b). Unfortunately, the problem with making such simple comparisons between pale and

brown thalli is that melanised thalli have a history of exposure to higher light levels than pale thalli. As a result, the photobionts of melanised thalli may have developed other mechanisms that increased tolerance to photoinhibition. Recently, the importance of melanisation in tolerance to high light was compared with the importance of other tolerance mechanisms by dissecting away the lower cortices and medullas in a range of species (Beckett et al. 2019). This enabled photobionts to be photoinhibited with light from below i.e., without the presence of a melanised upper cortex. Results showed that in all species, compared with pale thalli, photobionts in melanised thalli were more tolerant to photoinhibition when exposed from above. However, when photoinhibited from below, the tolerances of photobionts from melanised thalli of *Crocadia aurata* and *Lobaria pulmonaria* were rather similar to those of photobionts from pale thalli. In these species, melanin synthesis does indeed appear to be the main tolerance mechanism. However, in *Cetraria islandica* the tolerance of photobionts in melanised thalli photoinhibited when exposed from below was still significantly higher than that of pale thalli. This suggests that for *C. islandica* protection from high light appears to derive from a mixture of both cortical pigments and biochemical mechanisms.

Apart from the dissection study by Beckett et al. (2019) discussed above, there have been few attempts to investigate the importance and nature of other mechanisms of tolerance to high light in pale and melanised thalli of the same species of lichens. When growth is measured in differently melanised thalli of *L. pulmonaria,* results suggested that the mycobiont adjusted to the light received by the photobiont beneath the screening upper cortex to rather uniform levels, for example across a gradient in tree canopy openness (Gauslaa and Goward 2020). The implication would be that photosynthetic and photoprotective parameters, for example NPQ, should not differ between pale and melanic thalli. Recently, Mkhize et al. (2022) compared the induction and relaxation of

NPQ in melanised and pale thalli of members of three species of shade lichens, including *L. pulmonaria*. Consistent with the study that involved growth measurements (Gauslaa and Goward 2020), in *L. pulmonaria*, NPQ was rather similar in pale and melanised thalli. However, in *Lobaria virens* and *C. aurata* melanised forms have more, and faster relaxing NPQ than pale forms. Interestingly, in a similar study, It was also showed that in five species of non-melanising lichens, shade forms generally display higher NPQ than sun forms (Mkhize et al. 2022). For these species, it seems likely that the higher NPQ in the shade forms may protect photobionts from occasional rapid increases in light that occur during sunflecks. As both pale and melanised material of *L. virens* and *C. aurata* grow in relatively shaded habitats, the higher NPQ in the melanised thalli may be induced simply to provide additional protection over and above melanins. The implication is that, unlike *L. pulmonaria,* melanins in *L. virens* and *C. aurata* may not adequately reduce light levels at the photobiont layer. At the moment, the relative importance of NPQ in the photoprotection of melanising species that grow in more open habitats is unknown.

An inherent problem with comparing chlorophyll fluorescence parameters in pale and melanised thalli is that in melanised thalli the photobiont layer is being analysed through a pigmented upper cortex. As discussed above, our preliminary data suggest that the characteristics of the induction and relaxation of NPQ can differ in melanised and pale thalli from the same species (Mkhize et al. 2022). The widely accepted method of estimating NPQ is based on measuring ratios of fluorescence signals, not absolute values (Bilger et al. 1995). Therefore, the partial attenuation of the measuring beam by a melanised cortex should in theory not have a great effect on the validity of measurements at the intact thallus level. However, attenuation of the actinic light used for NPQ determination may increase $F_{M'}$ (maximal fluorescence yield of the light-adapted state) and therefore decrease NPQ. In other words, at the level of the photobionts, values of NPQ may be

measured at a slightly lower light level, and NPQ is well known to depend on light intensity. Therefore, our first hypothesis was that the presence of a melanised cortex should not prevent the use of conventional chlorophyll fluorimetry to compare NPQ in pale and melanised thalli. Our aim was to compare the induction of NPQ in melanised and pale thalli of *L. pulmonaria* and *C. aurata* using the dissection technique (Beckett et al. 2019). Essentially, the lower cortices and medullas were dissected away in pale and melanised thalli, and the induction and relaxation of NPQ were measured as a function of time following illumination and subsequent darkness.

Our second aim was to assess the role of NPQ in pale and melanised thalli of the lichens *Peltigera aphthosa* and *Cetraria islandica*. Unlike the species in the earlier study by Mkhize et al. (2022), these two lichens grow in rather open microhabitats, i.e., habitats characterized by slow rather than rapid fluctuations in light.

4.2. Materials and Methods

4.2.1 Lichen material

Three cephalolichens were used in this study. *Crocodia aurata* (Ach.) Link. was collected from *Leucosidea sericea* trees growing in Afromontane vegetation at Fort Nottingham, South Africa. *Lobaria pulmonaria* (L.) Hoffm. was collected from the bark of oak trees from nemoral boreal vegetation at Langangen, Norway. *Peltigera aphthosa* (L.) Willd. was collected from an exposed locality in nemoral boreal vegetation on the out-skirts of Ås, Norway. The chlorolichen *Cetraria islandica* (L.) Ach. and the cyanobacterial lichen *Peltigera malacea* were collected from an exposed site in boreal vegetation on the outskirts of Syktyvkar, Russia. For each site, collections of pale (shaded) and melanic (more exposed) thalli from the same population (a few cm to up to 5

m apart) were made at the same time. The lichen material was dried at room temperature between sheets of filter paper in the laboratory overnight and then stored refrigerated for a maximum of two weeks. One day before each experiment, discs 1 cm in diameter were cut. For *C. aurata* and *L. pulmonaria* under a dissecting microscope a small section (c. 2 – 4 mm) of the lower cortex and the adjacent medulla was scraped away using the tip of a Pasteur pipette until the lower part of the continuous green photobiont layer was fully exposed. All discs were then allowed to hydrate overnight on moist filter paper (Whatman filter paper grade 1) in Petri dishes under dim lighting (5 µmol m^{-2} s^{-1}) at 15 °C in a thermostatically controlled chamber (Labcon, model LTGC 40). For all the other species, disks were cut from pale and melanised thalli, and hydrated in the same way. Chlorophyll fluorescence parameters were measured the following day.

4.2.2. Chlorophyll fluorescence

For *Crocodia aurata* and *Lobaria pulmonaria* chlorophyll fluorescence was measured using the "maxi" red version of the Imaging PAM fluorimeter (Walz, Effeltrich, Germany). At each sampling event, an image of each disc was captured. In the intact discs, fluorescence parameters were integrated over a large area. For the scraped discs, parameters were integrated over the area from which the lower cortex had been removed, typically several mm^2 in size. Typical images are shown in Figure 4.1. For all the other species chlorophyll fluorescence parameters were measured using a PAM 2500 fluorometer (Walz, Effeltrich, Germany) using the red LED throughout. As quenching in the cyanobacterial *Peltigera malacea* cannot be measured with a conventional PAM, only rapid light curves were constructed.

Rapid light response curves of relative electron transport rates (rETR) were measured by increasing the actinic light in small steps for 10 to 20 s at each light level from 0 to 250 µmol

photons m^{-2} s^{-1} for *Lobaria pulmonaria* and *Cetraria islandica* and from 0 to 578 µmol photons m^{-2} s^{-1} for *Peltigera aphthosa*, *Cetraria islandica* and *Peltigera malacea*, with saturating flashes at the end of exposure to each light level. For the induction of rETR and NPQ as a function of time, parameters were calculated as described in Chapter 2. In initial experiments we tested the induction of these parameters using a variety of light intensities, but in a laboratory setting values much above 100 µmol photons m^{-2} s^{-1} tended to cause photoinhibition in some species.

4.2.3. Statistical Analysis

The statistics package "Statistica" (TIBCO Software Inc. Palo Alto, California, USA) was used to carry out generalized mixed linear models (repeated measure) for NPQ and rETR in discs of *L. pulmonaria* and *C. aurata* exposed to light at 100 µmol m^{-2} s^{-1} for 11 min followed by darkness for 10 min.

4.3. Results

Results presented here show that chlorophyll fluorescence can be validly used to compare NPQ in pale and melanised thalli without the need for dissection experiments. However, the dissection technique revealed that there may be subtle differences in the characteristics of the photobionts at the top and bottom of the photobiont layer. When results from the four species tested here are considered together, it appears that no simple relationship exists between melanisation and NPQ.

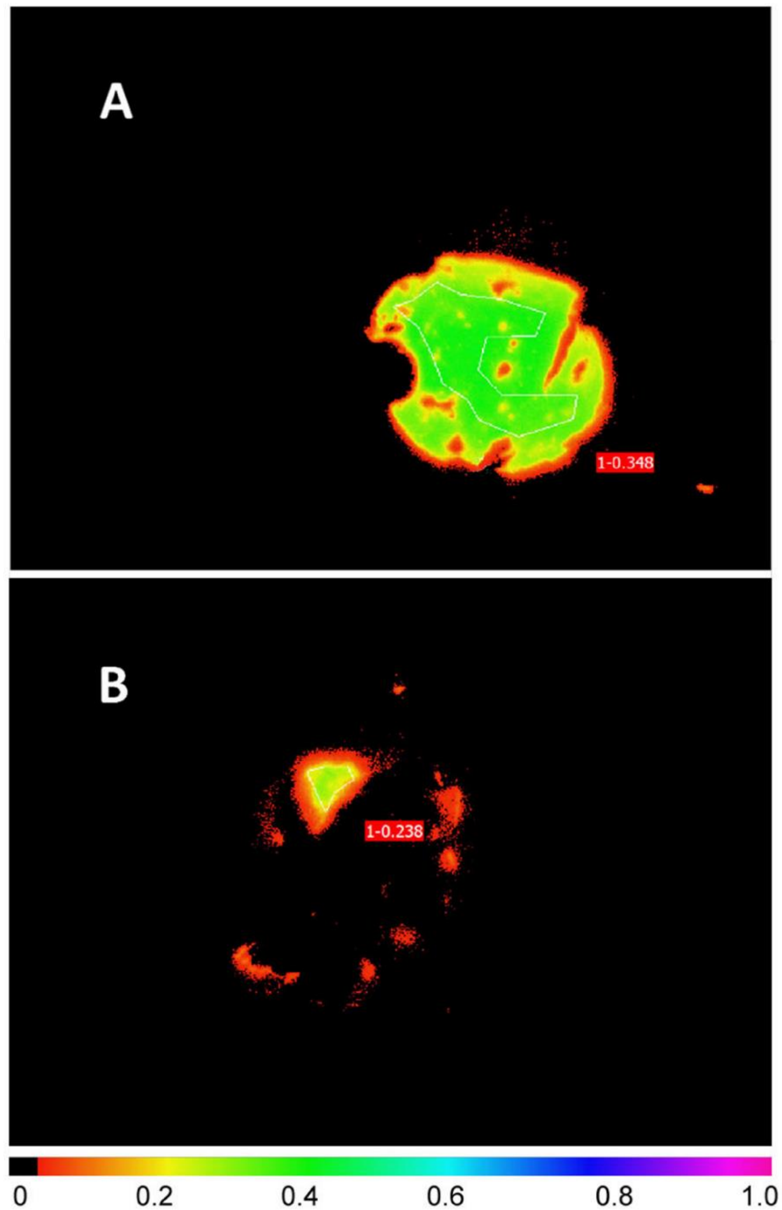

Figure 4.1: Typical screenshot from the Walz Imaging PAM fluorimeter. Here a melanised disk of *Crocodia aurata* was measured from above (A) and melanised disk of *C. aurata* with the lower cortex and medulla partially removed was measured from below (B). The disks were 1 cm in diameter. The parameter illustrated here with false colour is $F_{M'}$ (= maximal fluorescence yield of the light-adapted state). The white lines show the areas of interest used for calculation of the $F_{M'}$ values shown.

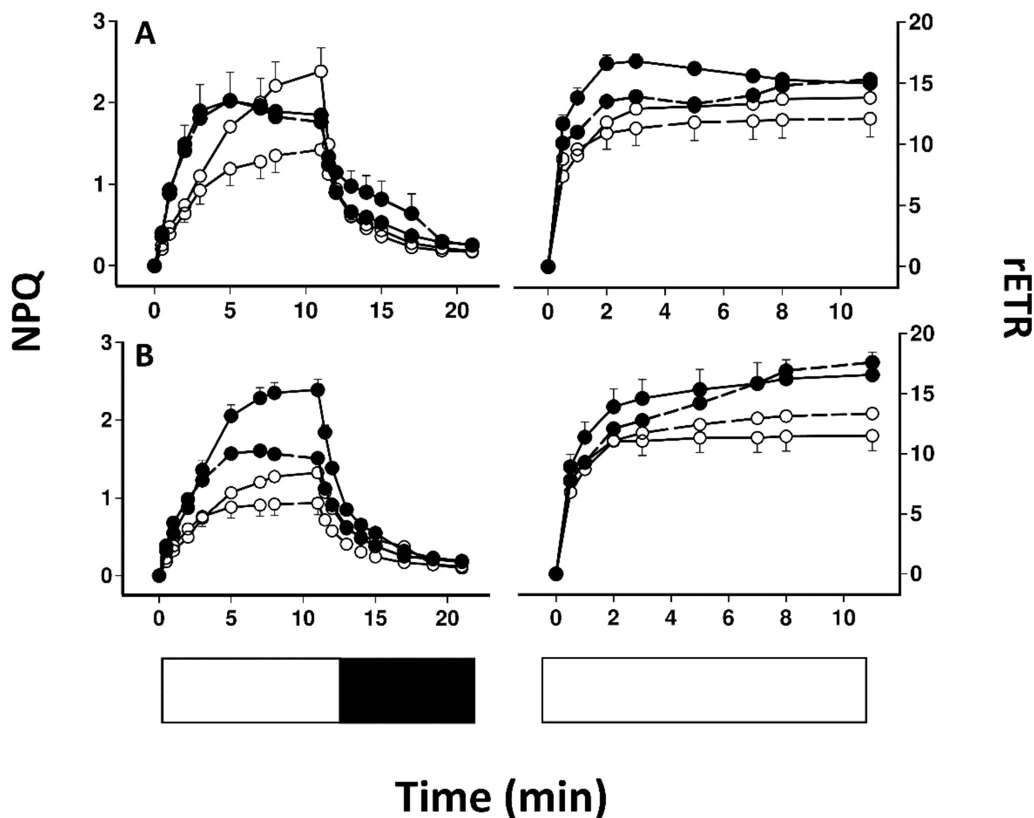

Figure 4.2: The effect of melanisation on the induction and relaxation of NPQ and the induction of rETR following exposure to light at 100 µmol m^{-2} s^{-1} in *Lobaria pulmonaria* (A) and *Crocodia aurata* (B). Open symbols indicate pale thalli and solid symbols melanised thalli. Solid lines represent intact material exposed to high light from above, while dashed lines represent material with part of the lower cortex and medulla removed and illuminated from below. Error bars show the mean 1 ± SE (n = 10) when smaller than symbol size were removed. The white section in the row at the base of each graph indicates the time periods when samples were exposed to light, and the dark when samples were exposed to dark. Statistical analyses of these graphs are present in Table 4.1.

Figure 4.2 compares the induction and relaxation of NPQ and the induction of rETR in pale and melanised collections of *L. pulmonaria* and *Crocodia aurata* illuminated with light at 100 µmol m^{-2} s^{-1} from above and from below with the lower cortex and medulla removed. Results of a repeated measure ANOVA for these data are presented in Table 4.1. NPQ was induced more rapidly in melanised than pale collections of both species.

Table 4.1: Generalized mixed linear models (repeated measured) for NPQ and rETR in discs of *Lobaria pulmonaria* and *Crocodia aurata* exposed to light at 100 µmol m^{-2} s^{-1} for 11 min followed by darkness for 10 min. Fixed factors: melanisation (M, pale versus melanic); position (P), exposed from above through an intact upper cortex or from below with the lower cortex and medulla removed); time (T) during exposure to light and then darkness. Interactions between the factors are indicated with as e.g. M x P, meaning the interaction between melanisation and position. There were ten replicates for each treatment combination. *** P < 0.001; ** P < 0.01; * P < 0.05.

Effect	Degrees of freedom for NPQ	*Lobaria* NPQ	*Crocodia* NPQ	Degrees of freedom for ETR	*Lobaria* rETR	*Crocodia* rETR
Melanisation (M)	1	*	***	1	***	0.202
Position (P)	1	0.206	*	1	0.057	*
M x P	1	0.081	0.337	1	0.163	0.312
Time (T)	15	***	***	7	***	***
T x M	15	***	***	7	***	**
T x P	15	***	***	7	***	***
T x M x P	15	***	0.059	7	0.411	***

In *L. pulmonaria* illuminated from above, pale thalli displayed slightly higher NPQ than melanised thalli after 11 min, while in material illuminated from below, NPQ values were slightly lower in pale thalli. The effect of melanisation on NPQ was significant (Table 4.1). While melanised thalli displayed a similar induction and relaxation of NPQ whether illuminated from above or below, in pale thalli NPQ was lower when illumination was from below, although overall the effect of position (above or below) was not significant (Table 4.1). In *C. aurata* NPQ was always higher in melanised than pale thalli, and always higher when thalli were illuminated from above. The effect of melanisation on NPQ was highly significant (P<0.001), and the effect of position significant at P < 0.05 (Table 4.1). For all thalli of both species, the induction of rETR was rapid, and rETR was slightly higher in melanised thalli, although only the effect of melanisation was significant for *L.*

pulmonaria, (P<0.001) and only the effect of position significant for *C. aurata* (P<0.05) (Table 4.1).

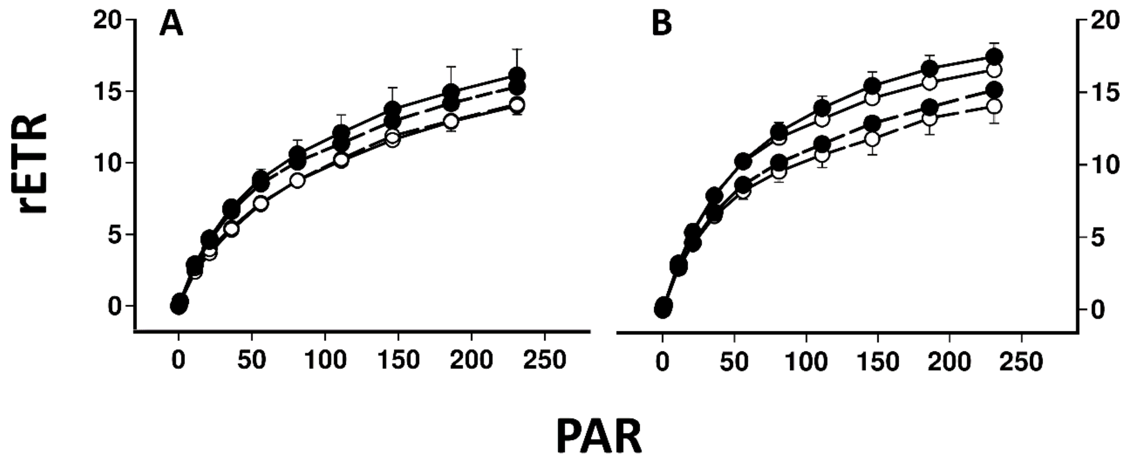

Figure 4.3: Rapid light curves (rETR as a function of light level) in *Lobaria pulmonaria* (A) and *Crocodia aurata* (B). Open symbols indicate pale thalli and solid symbols melanised thalli. Open symbols indicate pale thalli and solid symbols melanised thalli. Solid lines represent intact material exposed to high light from above, while dashed lines represent material with part of the lower cortex and medulla removed and illuminated from below. Error bars show the mean 1 ± SE (n = 10) when larger than symbol size.

Rapid light curves for both species suggested that rETR was slightly higher in melanised forms, and slightly higher when thalli were illuminated from above (Figure 4.3). Values of rETR obtained when light intensities above 250 μmol m^{-2} s^{-1} were used were anomalous (appearing to rise rapidly) and are not presented here.

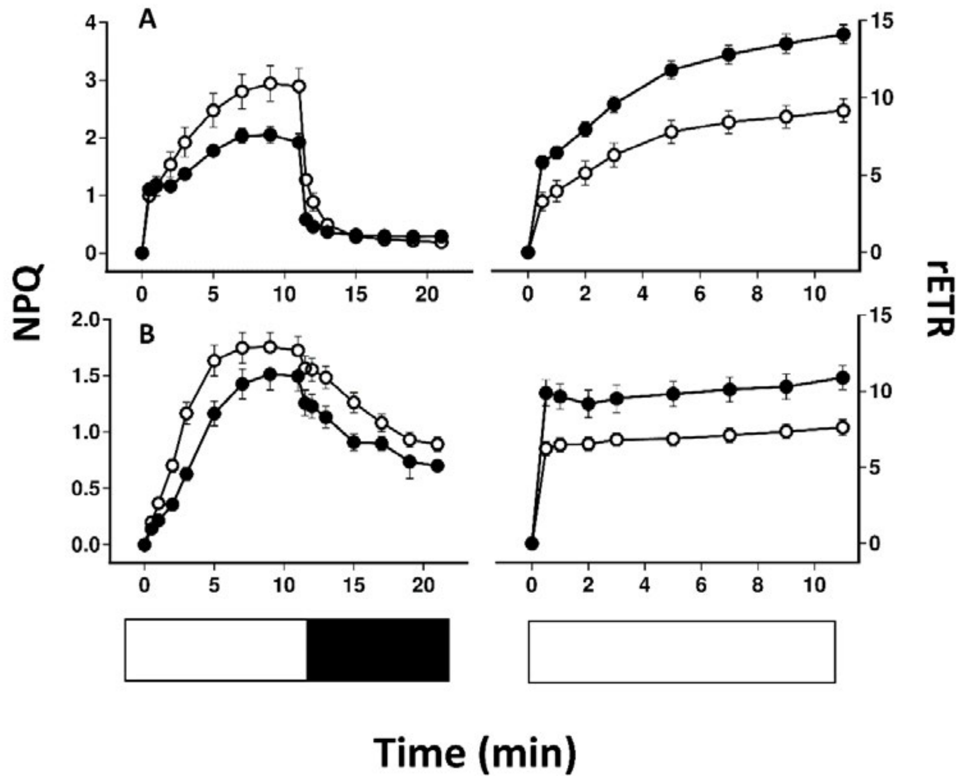

Figure 4.4: The effect of melanisation on the induction and relaxation of NPQ and the induction of rETR following exposure to light at 100 µmol m^{-2} s^{-1} in *Peltigera aphthosa* (A) and *Cetraria islandica* (B). Open symbols indicate pale thalli and solid symbols melanised thalli. Error bars show the mean 1 ± SE (n = 10) when larger than symbol size. The white section in the row at the base of each graph indicates the time periods when samples were exposed to light, and the dark when samples were exposed to dark.

Figure 4.4 compares the induction and relaxation of NPQ and the induction of rETR in pale and melanised collections of *P. aphthosa* and *Cetraria islandica* illuminated with light at 100 µmol m^{-2} s^{-1}. For both species, NPQ was higher in pale than melanised thalli. Relaxation of NPQ was fast in *P. aphthosa* but relatively slow in *Cetraria islandica*. Induction of rETR was relatively slow in *P. aphthosa* but rapid in *Cetraria islandica*. In both species, rETR after 11 min was higher in melanised than pale forms.

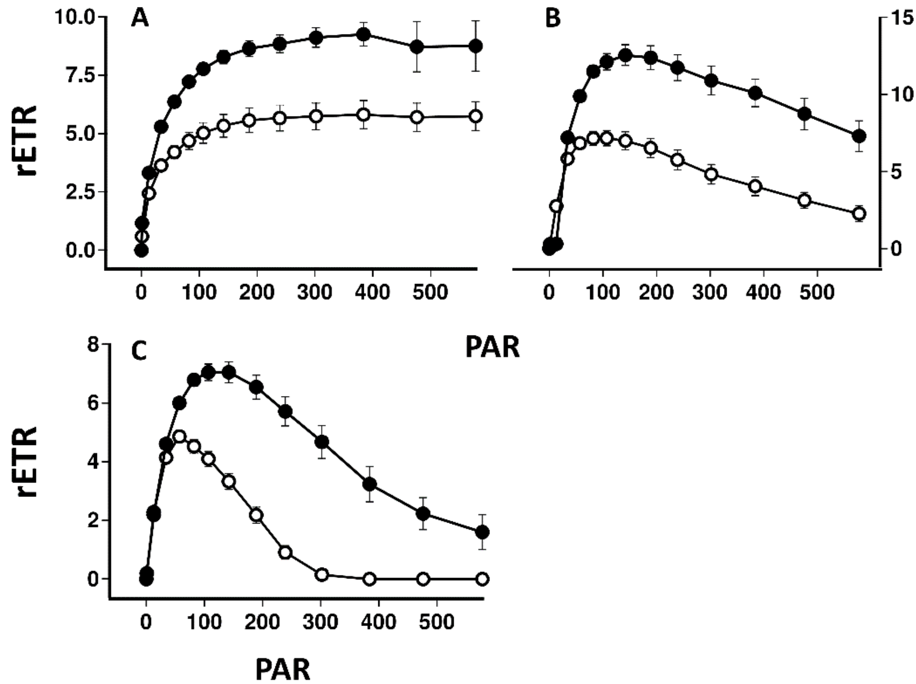

Figure 4.5: Rapid light curves (rETR as a function of light level) in *Peltigera aphthosa* (A), *Cetraria islandica* (B) and *Peltigera malacea* (C). Open symbols indicate pale thalli and solid symbols melanised thalli. Error bars show the mean 1 ± SE (n = 10) when larger than symbol size

The tendency for melanised thalli to have higher rETR was confirmed by rapid light curves (Figure 4.5). Melanised thalli of the cyanobacterial lichen *Peltigera malacea*, collected from adjacent to the *C. islandica* tested here, also displayed higher values of rETR. In both *C. islandica* and *P. malacea*, compared with pale forms, melanised forms were apparently more resistant to photoinhibition induced during the construction of the rapid light curves.

4.4. Discussion

Melanisation and NPQ are two mechanisms of photoprotection used by lichens to tolerate long term light stress, although the balance between these two mechanisms in field situations remains unclear. Here we used a dissection technique to show that despite potential problems with measuring lichen photobionts through a variously melanised upper cortex, NPQ can be

satisfactorily assessed with a standard fluorimeter. Interestingly, however, the dissection method revealed that photobionts from the top and bottom of the photobiont layer can display characteristic "sun" and "shade" features, respectively. Taking together the data from all four species studied here, it is clear that no simple relationship exists between melanisation and NPQ.

4.4.1 NPQ can be measured through a melanised cortex

It seems likely that lichens protect themselves against the harmful effects of high radiation using a combination of cortical light screening pigments and other, biochemical mechanisms (Beckett et al. 2021). Recently, we tested the relative importance of melanisation by dissecting away the lower cortex and medulla, and then photo-inhibiting photobionts by light exposure from both above and below (Beckett et al. 2019). Results showed that for some species, tolerance to photoinhibition was still higher when photobionts were exposed to light in the absence of a melanised upper cortex. This indicates that melanisation is not the only tolerance mechanism present in these species. However, further study of these additional mechanisms of tolerance e.g., measurement of NPQ could be hindered by the presence of a melanised upper cortex, even though in theory the equation used to calculate NPQ is based on ratios rather than absolute estimates of fluorescence parameters (Bilger et al. 1995). Unfortunately, our dissection technique is not applicable to all species (Beckett et al. 2019), and furthermore requires the use of an imaging PAM. The first aim of the present study was to test whether valid comparisons of NPQ can be made in collections with and without a melanised upper cortex without resorting to the dissection technique. For example, recently Mkhize et al. (2022) measured the induction of NPQ as a function of time in pale and melanised collections of two of the species tested here. In *L. pulmonaria* NPQ induced more rapidly in melanised than pale forms but was similar after 11 min in the light. In contrast, in *Crocodia aurata*

NPQ induced more rapidly and reached much higher values in melanised than pale forms. The implication is that NPQ may be an important additional tolerance mechanism in melanised *Crocodia aurata* but less so in *L. pulmonaria*. In the collections of *L. pulmonaria* used here, when the photobionts were exposed in the same way as a previous study (Mkhize et al. 2022) (i.e., from above, through the upper cortex) the pattern of NPQ induction was similar, although in the present study NPQ after 11 min was slightly higher in pale material (Figure 4.2). Melanised *L. pulmonaria* exposed from below behaved similarly to material exposed from above, while pale material exposed from below displayed generally lower values of NPQ than material exposed from above. For *Crocodia aurata*, NPQ in lichens exposed from below is generally lower than when exposed from above, but melanised material always displays much higher values of NPQ than material exposed from above. There are likely explanations for the differences in the values of NPQ observed when thalli are exposed from above or below (see next section). Nevertheless, the conclusions on the relative importance of NPQ are similar, whether lichens are exposed from above, or from below, without the presence of a melanised cortex. In other words, results suggest that valuable information about the potential role of NPQ in the tolerance of photobionts to high light can be obtained simply by taking measurements from above using intact thalli with a standard PAM.

4.4.2. Photobionts from top of the photobiont layer display relative sun properties

Results suggest that differences may exist between the characteristics of photobionts at the tops and bottoms of the photobiont layers in *L. pulmonaria* and *Crocodia aurata* (Figure 4.2). It can be difficult to estimate how deeply a fluorimeter samples within a leaf or thallus (see Terashima et al. [2016] for discussion of this point). However, it is probable that the photobionts sampled when the

thallus is measured from above differ from those sampled when measurements are made from below, with the lower cortex and medulla removed. In *Cladonia arbuscula* little light penetrates more than c. 30 µm into a thallus, and if this is generally true for other lichens the implication is that the lower parts of the photobiont layer receive less light than those on the top (Buffoni et al. 2002). It could be predicted therefore that the upper parts of the photobiont layer may show more "sun" characteristics while the lower part more "shade" characteristics. Specifically, photobionts at the top of the photobiont layer may display more efficient photosynthesis, but also higher quenching to protect themselves from high light. In the present study, light response curves and rates of rETR after 11 min at 100 µmol m^{-2} s^{-1} were quite similar for pale and melanised thalli, whether measured from above or below (Figure 4.2). Nevertheless, as predicted, in both species maximum rates of rETR were slightly lower when measurements were made for the lower surface of both pale and melanised thalli (Figure 4.3). In contrast to rETR, larger differences in NPQ were usually found between the top and bottom of the photobiont layer. Only in melanised *L. pulmonaria* did the induction of NPQ display similar kinetics whether measured from the top or bottom of the thalli. In all other cases, lower values of NPQ were recorded for the lower side. The simplest explanation for this is that shading means that less photoprotection is needed for photobionts in the lower parts of the photobiont layer. There have been very few studies on gradients in photosynthetic acclimation within one lichen thallus. In one study, a microscope imaging PAM was used to study gradients of chlorophyll fluorescence parameters in the desert crust squamulose lichens *Placidium* and *Peltula* and a crustose *Collema* sp. (Wu et al. 2014). Gradients in the effective quantum yield of PSII (ΦPSII) were more or less as would have been predicted from the higher plant literature, with the top of the photobiont layers displaying higher quantum yield of PSII; however, changes in quenching were not always as predicted. In *Placidium*,

qN (the proportion of energy absorbed dissipated as heat) decreased with depth in the thallus, but in *Petula* qN increased, while in *Collema* there was no change. Possibly in squamulose soil crust lichens, reflectance from the substratum may mean that some species receive higher light from below than would be expected. More information is available for higher plant leaves where gradients of light can also be quite steep, and are modulated by the presence of pigments, surface waxes and trichomes (Terashima et al. 2016; Karabourniotis et al. 2021). For example, the chloroplasts isolated from paradermal sections prepared from the leaves of *Camellia japonica* and *Spinacia oleracea* were compared (Terashima and Inoue 1984; Terashima and Inoue 1985a; Terashima and Inoue 1985b). A clear gradient in the chloroplast properties from sun to shade exists within these leaves. Components such as rubisco and cytochrome f expressed on a chlorophyll basis were much greater in the sun-type chloroplasts than in shade type. Similar trends in efficiency have been observed in *Quercus* leaves, and further, NPQ is lower towards the lower surfaces of the leaves (Peguero-Pina et al. 2009). Therefore, the trends of photosynthetic efficiency and NPQ reported in vascular plants resemble those found here. It is unclear why values for NPQ are rather similar for the tops and bottoms of melanised *L. pulmonaria* thalli, although possibly in melanised samples of this species the algal layer is thinner. However, results presented here suggest that photobionts from the bottom of the photobiont layer tend to have slightly lower rates of photosynthesis and less quenching than those at the top, i.e., display mild shade characteristics.

4.4.3. Melanised thalli display different NPQ responses compared to pale thalli

Melanised thalli of the four species tested here, compared with pale thalli, can display values of NPQ that are higher (*Crocodia aurata*), similar (*L. pulmonaria*) or lower (*P. aphthosa* and *Cetraria islandica*) (Figures 4.2, 4.4). As discussed in the Introduction, growth measurements of

L. pulmonaria suggest that the mycobiont appears to adjust the melanins present in the upper cortex so that the photobiont receives uniform light levels, for example across a gradient in tree canopy openness (Gauslaa and Goward 2020). Consistent with this suggestion, in the present study NPQ and parameters such as rETR were rather similar in pale and melanised thalli of *L. pulmonaria*. However, in the other species tested, differences were found in NPQ when comparing pale and melanised thalli. For melanised thalli of *Crocodia aurata* melanins seem to provide insufficient photoprotection for the extra light they experience in their microhabitat, and as result, they possess additional NPQ (Figure 4.4). *P. aphthosa* and *Cetraria islandica* were collected from more open habitats than *Crocodia aurata* and *L. pulmonaria*. Interestingly, although our earlier results (Beckett et al. 2021b; Mkhize et al. 2022) would suggest that in general NPQ relaxes faster in sun than shade lichens, *P. aphthosa* was an exception in that, for no obvious reason, NPQ relaxes quickly on transition to darkness. Melanised thalli from *P. aphthosa* and *Cetraria islandica* display lower maximum values of NPQ than pale, suggesting that their photobionts may experience less light stress than those from pale thalli. The most straightforward explanation is that melanins may have been synthesised following exposure to particularly bright light at one time of the year, and at the time of collection the resulting melanisation was excessive. Once synthesised, melanins cannot be broken down. Higher estimates of rETR in melanised thalli of these species, and in the cyanobacterial lichen *P. malacea* may have been obtained because the PAR used to estimate rETR was higher than the actual PAR reaching the photobiont (Figure 4.5). However, it is also possible that the higher rETR in melanised forms indicates that they are showing some sun characteristics, and actually use alternative mechanisms of photoprotection in addition to NPQ. Similarly varied results have been reported from other photosynthetic organisms that may produce pale or pigmented leaves. For example, much higher NPQ occurs in dark red collections of the liverworts

Jamesoniella colorata and *Isotachis lyallii* collected from exposed sites compared with pale green individuals from more shaded sites (Hooijmaijers and Gould 2007). Conversely, pigmented leaves in *Ophiopogon*, *Vitus* and *Prunus* all display lower values of NPQ than pale forms (Hatier et al. 2022; Liakopouos et al. 2006; Lo Piccolo et al. 2016). For these species, the implication is that strong pigments allow leaves to maintain a smaller content of xanthophyll cycle components and depend less on xanthophyll cycle energy dissipation. However, if excessive, pigment-based photoprotection could be predicted to reduce photosynthesis under conditions of non-inhibitory light levels (e.g., Gould et al. 2002). Interestingly, a more wide-ranging study found rather similar values of NPQ in both young red leaves and paler mature forms (Gong et al. 2020). In conclusion, as for results from other pigmented or pale photosynthetic organisms, it seems impossible to generalize as to whether in lichens the photobionts of melanised thalli show higher or lower NPQ than pale forms. Presumably, depending on the light history of the microhabitat and presence of other tolerance mechanisms, all combinations are possible.

4.5. Conclusions

From the literature on free-living algae and vascular plants, it is becoming increasingly clear that photosynthetic organisms use a great diversity of mechanisms to protect themselves against the potentially harmful effects of excess light (Shi et al. 2022). A uniquely lichenological mechanism is the production of light-screening melanins by the fungi that form the upper cortex (Mafole et al. 2019a). In addition, what appears to be classical NPQ has been widely reported in lichens (Beckett et al., 2021). The present study suggests that the relationship between melanisation and NPQ is species specific. In all species tested here, rETR was higher in melanised than pale forms. This is probably at least in part because the photobionts in melanised thalli may receive less light due to

screening. Therefore, rETR will be overestimated because it is based on a higher PAR than the photobionts actually receive. In some species, NPQ is similar in pale and melanised forms, suggesting that mycobionts can adjust the melanins present in the upper cortex so that the photobiont receives uniform illumination. In other species, melanisation appears to offer insufficient photoprotection, and the photobionts display additional NPQ. In still other species, NPQ is actually less in melanised than pale forms, suggesting that either melanisation may have been excessive earlier in the growing season, or alternatively that these species use other mechanisms to protect themselves from the effects of high light. In these species there is a clear need to investigate other potential mechanisms of tolerance to high light stress.

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CHAPTER 5: LICHEN SUBSTANCES ARE MORE IMPORTANT FOR PHOTOPROTECTION IN SUN THAN SHADE POPULATIONS WITHIN LICHEN SPECIES

5.1 Introduction

While a significant number of lichen species "melanise" on exposure to high light, it appears more common for the mycobiont to produce colourless or lightly pigmented lichen substances such as usnic acid and atranorin in their upper cortexes (Goga et al 2020). While there is good evidence that the presence of these compounds can assist in photoprotection, their relative importance compared with other tolerance mechanisms remains untested. For many foliose lichens such as *Usnea* and *Ramalina*, it is not possible to test the significance of substances present in the upper cortex by surgically removing the lower cortex and medulla and exposing lichens from below. However, it is possible to harmlessly remove lichen substances using the "acetone rinsing" technique of Solhaug et al. (2010). Tolerance to photoinhibition can then be compared in thalli with and without lichen substances, enabling the relative importance of these compounds to be assessed. One approach to assess the relative importance of tolerance mechanisms is to compare tolerance mechanisms present in "sun" forms with "shade" forms of members of the same species. While it has been shown that lichens can, as for vascular plants, display sun and shade forms (Piccotto and Tretiach 2010), there have been surprisingly few attempts to test whether shade collections of the same species of lichens are more sensitive to photoinhibition than those of sun forms. Kershaw and MacFarlane (1980) reported that populations of *Peltigera aphthosa* collected from the dense shade of spruce are extremely sensitive to high light, while populations collected from open habitats are much more tolerant. However, the mechanisms responsible for the increased tolerance of the sun collections were not studied. Our preliminary results suggested that

sun forms of a range of Afromontane lichens possess greater tolerance to photoinhibition than shade forms. Therefore, the main aim of the present study was to test the relative importance of secondary metabolites in the additional tolerance to photoinhibition of sun forms in both hydrated and desiccated states in lichens. We reasoned that first, if the major role of lichen substances is to protect photobionts from photoinhibition, then removal of these substances will increase the sensitivity of the sun forms more than that of the shade forms. Second, if other tolerance mechanisms are also important (e.g., enhanced NPQ, higher levels of antioxidant enzymes or PSII repair cycle enzymes), then even after removal of lichen substances, sun forms should still display greater tolerance to photoinhibition than shade forms.

5.2. Materials and Methods

5.2.1. Lichen material

All species used in this study were collected in Afromontane vegetation in KwaZulu Natal, South Africa. Both sun and shade collections of *Parmotrema perlatum* (usnic and stictic acid) and *Usnea angulata* (usnic acid) were collected from a forest at Fort Nottingham Nature reserve. Sun collections were made from minor twigs at the periphery of the canopy (the more normal microhabitat of these species), while shade collections were made c. 1 m away, from deep inside the canopy, usually on main branches or tree trunks. Shade populations of *Xanthoparmelia conspersa* (stictic and usnic acid) and *Ramalina celastri* (parietina and usnic acid) were collected from shaded rocks and trees, respectively, in Queen Elizabeth Park, Pietermaritzburg. Sun populations of *X. conspersa* were collected from rocky outcrops near the Cascades Lifestyle Center, Pietermaritzburg, c. 3 km from the shade population. Sun populations of *R. celastri* were collected from unshaded tree bark in Clarendon, Pietermaritzburg, c. 5 km from the shade

population. The local climates at the collection sites were very similar. The photobionts of these lichens have been reported to belong to the chlorophycean genus *Trebouxia* (Rambold et al. 1998). After collection, lichen material was allowed to airdry at room temperature between filter paper (Whatman filter paper grade 1) overnight and then stored at -24°C until needed. For chlorophyll fluorescence measurements, see details under subheading 2.4 (Chapter 2).

5.2.2. Acetone rinsing

While we did not quantify the concentrations of lichen substances present in the thalli used in this chapter, usnic acid was qualitatively determined to be the main lichen substance present in all species. In preliminary experiments, simple acetone extracts of dry thalli were analysed using high performance liquid chromatography as described by Pawlik-Skowrońska and Bačkor (2011), and comparisons made with standards from Sigma-Aldrich, USA. In the main experiment, lichen substances were removed using the "acetone rinsing" technique of Solhaug et al. (2010). Acetone rinsing is described in detail on the General Materials and Methods chapter 2.

5.2.3. Photoinhibition treatments

Table 5.1: Light intensities and times of exposure used to induce photoinhibition. Lichens with and without lichen substances received the same exposure times and intensities. These are light intensities and time exposures that have already triggered photoinhibition (according to the results of a previous investigation)

Species	Light intensity and duration	
	Hydrated	Desiccated
R. celastri	600 µmol m^2 s^{-1} for 6 h	1500 µmol m^2 s^{-1} for 50 h

X. conspersa	850 µmol m^2 s^{-1} for 6 h	2000 µmol m^2 s^{-1} for 70 h
P. perlatum	700 µmol m^2 s^{-1} for 5 h	1800 µmol m^2 s^{-1} for 18 h
U. filipemdula	750 µmol m^2 s^{-1} for 5 h	1000 µmol m^2 s^{-1} for 45 h

5.2.4. Statistical analysis

Statistical analyses (two- and three-way ANOVA) were carried out using "IBM SPSS Statistics" (v28.0.0.0, 2021) following checks for normality (what tests did we use) and homogeneity of variance. For thalli stressed in both the hydrated and desiccated states, two-way ANOVA was used to make four sets of comparisons using subsets of our data. First, we tested whether collection site (sunny or shaded) affected the sensitivity of F_V/F_M and rETR in photobionts exposed to a photoinhibitory light stress. Second and third, we separately tested whether the presence or absence of lichen substances affected the sensitivity of both sun and shade collections of thalli to light stress. Finally, we tested whether collection site (sunny or shaded) affected the sensitivity of thalli to light stress when lichen substances have been removed. In addition, a three-way ANOVA was carried out to test if lichen substance removal affected tolerance to photoinhibition differently in collections of lichens from sun and shade habitats. All 235 data from hydrated material and all data from desiccated material were combined into two data sets and a three-way ANOVA used to test for the interaction of collection site (sun or shade) and the presence or absence of lichen substances.

5.3. Results

Results presented here show that for most species, the additional tolerance present in sun collections is derived from a combination of both lichen substances and other tolerance mechanisms.

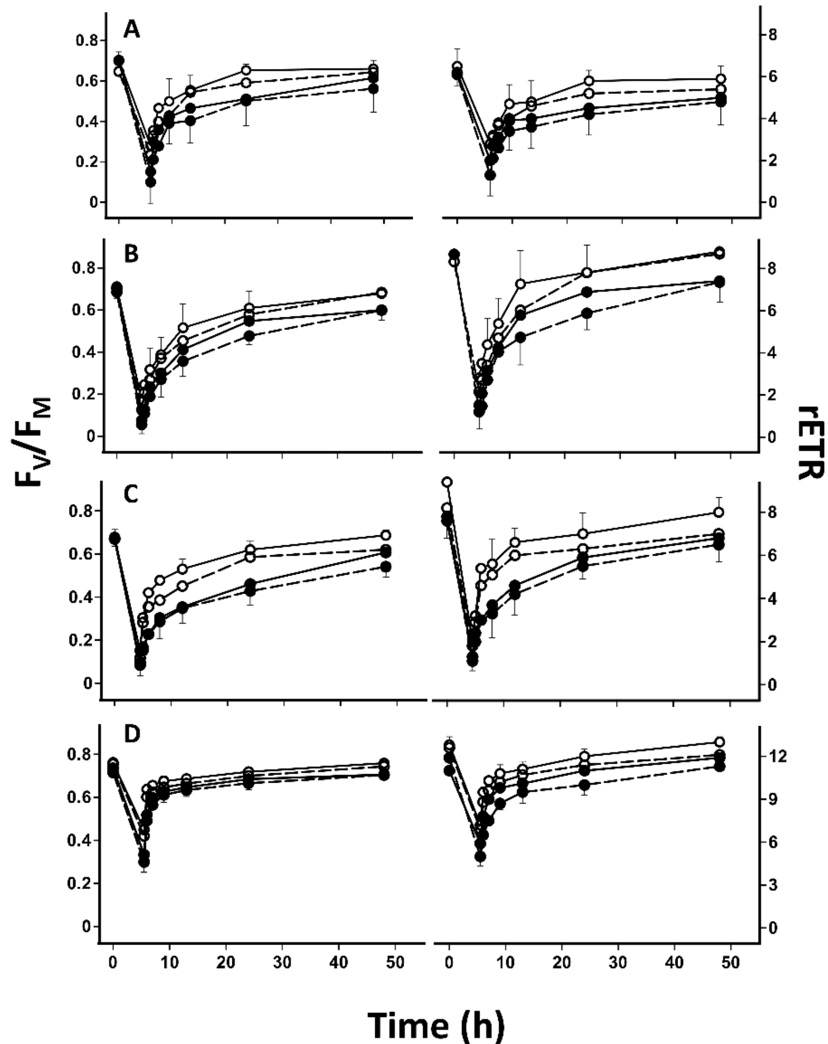

Figure 5.1: The effect of acetone rinsing on the maximal efficiency of PSII (F_V/F_M – left) and relative electron transport rate (rETR – right) in hydrated material of sun (open circles) and shaded populations (closed) of *Ramalina celastri* (A), *Parmotrema perlatum* (B), *Usnea cornuta* (C) and *Xanthoparmelia conspersa* (D). Solid lines indicate thalli with lichen substances present, while dashed lines indicate thalli with lichen substances removed. Vertical error bars indicate the standard error of the mean, n = 10.

5.3.1 Tolerance of PSII activity to photoinhibition in sun compared with shade collections

Much longer exposure times and higher light intensities were needed to photoinhibit the desiccated compared with the hydrated lichens (Table 5.1). When lichen substances were present (i.e., there was no acetone rinsing), for both hydrated and desiccated lichens, the tolerance of PSII activity to high light in sun collections was always significantly greater than that of shade collections (Figures 5.1; 5.2 and Tables 5.2; 5.4). When lichen substances were extracted using acetone, again for both hydrated and desiccated lichens, the tolerance of PSII activity to high light in sun collections was always significantly greater than that of shade collections (Figures 5.1; 52 and Tables 5.2; 5.4). The only exception here was for desiccated material of *U. cornuta* when PSII activity was assessed as F_V/F_M when *P* was 0.11.

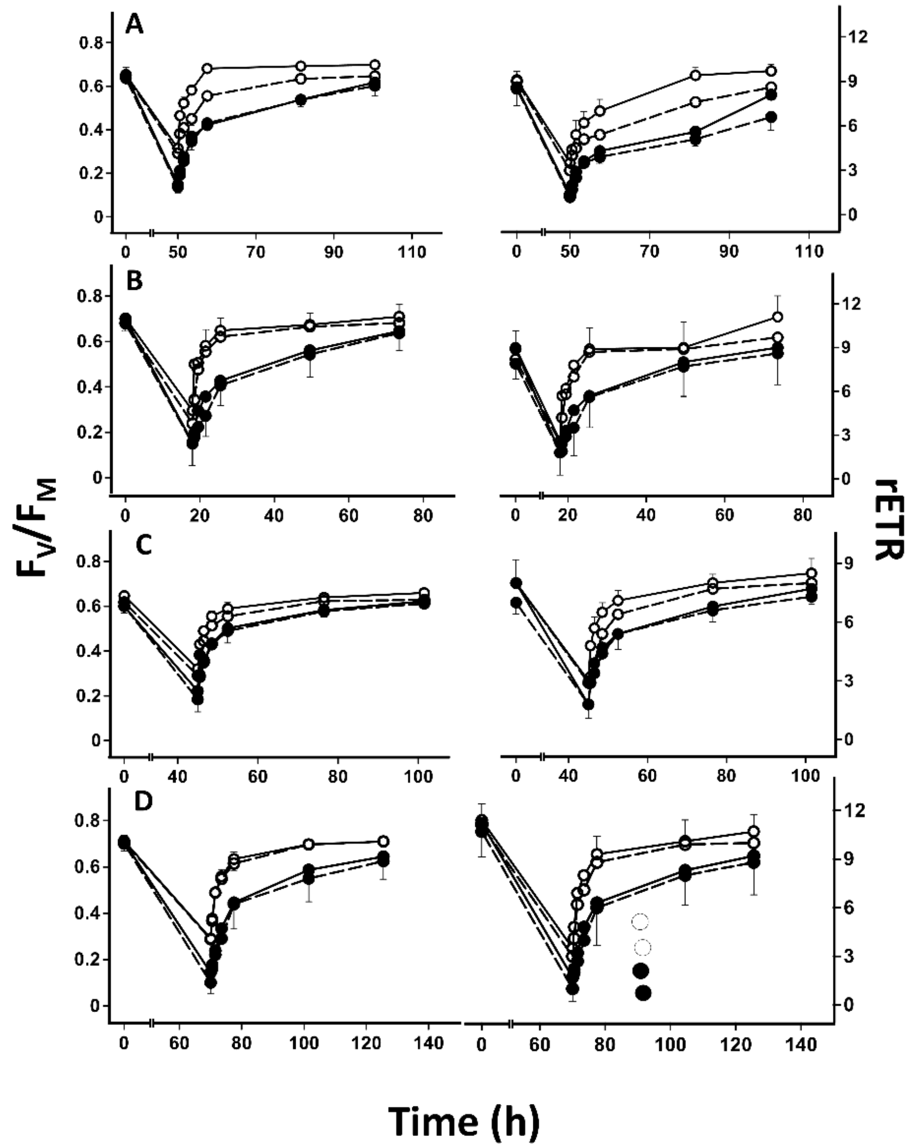

Figure 5.2: The effect of acetone rinsing on the maximal efficiency of PSII (F_V/F_M – left) and relative electron transport rate (rETR – right) in desiccated material of sun (open circles) and shaded populations (closed) of *Ramalina celastri* (A), *Parmotrema perlatum* (B), *Usnea cornuta* (C) and *Xanthoparmelia conspersa* (D). Solid lines indicate thalli with lichen substances present, while dashed lines indicate thalli with lichen substances removed. Vertical error bars indicate the standard error of the mean, n = 10.

Table 5.2: Statistical analysis (2- way ANOVA) of the effects of time and presence of lichen substances on the sensitivity to photoinhibition in sun and shade collections of hydrated thalli of *Ramalina celastri*, *Parmotrema perlatum*, *Usnea cornuta* and *Xanthoparmelia conspersa*. For all comparisons, the error had 159 degrees of freedom. Significance: * = P < 0.05, ** = P < 0.01, *** =P < 0.001

		Ramalina celastri		*Parmotrema perlatum*		*Usnea cornuta*		*Xanthoparmelia conspersa*	
Comparison	**Factor**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**
Sun/Shade with substances present	**Sun or shade**	***	***	***	***	***	***	***	***
	Time	***	***	***	***	***	***	***	***
	Interaction	0.084	0.764	0.183	0.317	***	**	***	0.650
With/without substances in sun collections	**Substances**	0.224	0.686	0.262	**	***	**	**	***
	Time	***	***	***	***	***	***	***	***
	Interaction	0.627	0.121	0.322	0.288	0.146	0.163	0.302	0.207
With/without substances in shade collections	**Substances**	0.129	0.100	**	0.177	*	0.515	0.399	0.147
	Time	***	***	***	***	***	***	***	***
	Interaction	0.421	0.479	0.614	**	0.074	0.633	0.437	0.357
Sun/Shade with substances removed	**Sun or shade**	*	*	***	*	***	***	***	***
	Time	***	***	***	***	***	***	***	***
	Interaction	0.793	0.834	0.680	0.197	*	***	***	0.188

Table 5.3: Statistical analysis (3-way ANOVA) of the effect of time and the presence of lichen substances on the sensitivity to photoinhibition in sun and shade collections of hydrated lichen species of *Ramalina celastri*, *Parmotrema perlatum*, *Usnea cornuta* and *xanthoparmelia conspersa*. For all comparisons, the error had 287 degrees of freedom. Significance: * = P < 0.05, ** = P < 0.01, *** =P < 0.001

	Ramalina celastri		***Parmotrema perlatum***		***Usnea cornuta***		***Xanthoparmelia conspersa***	
	F_V/F_M	**rETR**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**
Time	***	***	***	***	***	***	***	***
Sun/Shade	***	***	***	***	***	***	***	***
With/Without Substances	0.501	0.113	0.304	0.062	***	0.113	*	***
Time*Sun/Shade	0.327	0.627	0.118	0.116	*	0.627	***	0.160
Time*With/Without Substances	0.527	**	0.316	*	0.117	*	0.159	*
Sun/Shade*With/Without Substances	*	0.259	*	**	*	0.259	0.255	0.161
Time*Sun/Shade*With/Without Substances	0.417	0.948	0.664	0.707	0.691	0.312	0.733	0.887

5.3.2 Effect of lichen substance removal on tolerance of PSII activity to photoinhibition in sun compared with shade collections

In hydrated material of sun collections, removal of lichen substances increased sensitivity to photoinhibition in all species except *R. celastri* and F_V/F_M for *P. perlatum* (Figure 5.1and Table 5.2). In contrast, in shade collections, the only significant effect of removal were small reductions in sensitivity in F_V/F_M for *P. perlatum* and *U. cornuta*. Similarly, in desiccated material, removal of lichen substances significantly increased sensitivity to photoinhibition in all species except for rETR in *P. perlatum* and F_V/F_M for *X. conspersa* (Figure 5.2 and Table 5.4), while in shade collections, removal only had a significant effect in *U. cornuta* (both parameters) and rETR for *R. celastri.*

Table 5.4: Statistical analysis (2- way ANOVA) of the effects of time and presence of lichen substances on the sensitivity to photoinhibition in sun and shade collections of desiccated thalli of *Ramalina celastri*, *Parmotrema perlatum*, *Usnea cornuta* and *Xanthoparmelia conspersa*. For all comparisons, the error had 159 degrees of freedom. Significance: * = P < 0.05, ** = P < 0.01, *** =P < 0.001

		Ramalina celastri		***Parmotrema perlatum***		***Usnea cornuta***		***Xanthoparmelia conspersa***	
Comparison	**Factor**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**
Sun/Shade with substances present	**Sun or shade**	***	***	***	***	***	**	***	***
	Time	***	***	***	***	***	***	***	***
	Interaction	***	***	***	**	*	0.559	***	***
With/without substances in sun collections	**Substances**	***	***	*	0.625	***	***	0.639	**
	Time	***	***	***	***	***	***	***	***
	Interaction	***	**	***	0.082	***	**	0.903	0.987
With/without substances in shade collections	**Substances**	0.442	***	0.261	0.284	***	***	0.342	0.423
	Time	***	***	***	***	***	***	***	***
	Interaction	0.804	***	0.909	0.777	0.189	0.939	0.804	0.912
Sun/Shade with substances removed	**Sun or shade**	***	***	***	***	0.107	***	***	***
	Time	***	***	***	***	***	***	***	***
	Interaction	***	**	***	***	0.773	**	***	**

Table 5.5: Statistical analysis (3-way ANOVA) of the effect of time and the presence of lichen substances on the sensitivity to photoinhibition in sun and shade collections of desiccated lichen species of *Ramalina celastri*, *Parmotrema perlatum*, *Usnea cornuta* and *Xanthoparmelia conspersa*. For all comparisons, the error had 287 degrees of freedom. Significant difference: * = P < 0.05, ** = P < 0.01, *** =P < 0.001

	Ramalina celastri		***Parmotrema perlatum***		***Usnea cornuta***		***Xathoparmelia conspersa***	
	F_V/F_M	**rETR**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**	**F_V/F_M**	**rETR**
Time	***	***	***	***	***	***	***	***
Sun/Shade	***	***	***	***	***	***	***	***
With/Without Substances	***	***	*	0.693	***	***	0.291	**
Time*Sun/Shade	***	***	***	***	0.929	0.136	***	***
Time*With/Without Substances	0.145	***	0.199	0.225	***	*	0.929	0.976
Sun/Shade*With/Without Substances	***	**	0.796	***	0.235	*	0.484	0.787
Time*Sun/Shade*With/Without Substances	0.060	0.070	0.112	***	*	*	0.685	0.863

5.3.3 *Interaction of lichen substance removal and characteristics of the microhabitat of the collected lichen thalli*

Whether the effect of lichen substance removal on tolerance to photoinhibition differs between sun and shade lichens was tested by combining all data from hydrated material and all data from desiccated material into two data sets and carrying out three-way ANOVA (Tables 5.3 and 5.5). For both hydrated material and desiccated material, results showed that a significant interaction between lichen substance removal and whether lichens were collected from sun or shade locations occurred for at least one parameter in *R. celastri*, *P. perlatum* and *U. cornuta* but not *X. conspersa*.

5.4. Discussion

Photosynthetic organisms protect themselves from the stress of high PAR using mechanisms that can be divided into those that work mainly to guard against short term fluctuations and those that are found following longer-term exposure to high light (Beckett et al. 2021; Shi et al. 2022). In the present study, by collecting sun and shade populations of the same species of lichens our focus was on longer-term adaptations. In lichens, long-term exposure to high light can induce the mycobiont to synthesize secondary metabolites in the upper cortex (Solhaug and Gauslaa 2012), and these metabolites can protect photobionts against high PAR (Solhaug et al. 2010, Ndhlovu et al. 2022). Other adaptations to sun and shade have been less studied in lichens. For vascular plants, adaptations can include changes in the ratio of chlorophyll a to chlorophyll b, changes in chloroplast architecture, general adjustments in the maximum photosynthetic rate, and changes in the activities of ROS scavenging enzymes, NPQ and the PSII repair cycle (for reviews see Greer (2022) and Shi et al. (2022)). Here we tested the relative importance of cortical screening pigments

compared to other more biochemical adaptations. Results from the present study show that in four Afromontane lichens, thalli growing in sun locations have higher tolerance to photoinhibition than those from shaded locations. Furthermore, in three of the four species tested, substances play a greater role in photoprotection in sun than shade collections. Interestingly, after removing lichen substances, sun collections still possessed higher tolerance to photoinhibition than shade collections. Therefore, the additional tolerance to photoinhibition found in sun collections appears to be derived from a combination of both lichen substances and other tolerance mechanisms.

5.4.1 Collections of lichens from sunny microhabitats are more tolerant to photoinhibition than those from shaded microhabitats

Differences in the tolerance to photoinhibition of sun and shade collections of lichens has been surprisingly little studied since the early work of Kershaw and MacFarlane (1980). Since that study, our understanding of the tolerance mechanisms displayed by photosynthetic organisms has greatly expanded (Shi et al. 2022). Here we show that for all four species tested, sun populations are more tolerant to photoinhibition than shade, whether photoinhibited in the hydrated (Figure 5.1) or desiccated states (Figure 5.2). While the lichens used here are more tolerant to high light stress when desiccated than hydrated (Table 5.1), as has been reported earlier for other species (Mafole et al. 2019), given sufficient PAR they nevertheless can become inhibited. The precise mechanism is unclear, but in bryophytes, desiccation does not stop the transfer of excitation energy from the light-harvesting pigments to the reaction centres (Heber et al. 2006). Even if light only causes the formation of tiny amounts of ROS in desiccated thalli, normal repair processes do not take place (Buffoni-Hall et al. 2003). Enzyme reactions are severely restricted by the 'rubbery' cytoplasmic states that occur at the onset of desiccation and are totally restricted in the glassy

cytoplasmic states that are found in air-desiccated lichens during the day (Fernandez-Marin et al. 2013). Interestingly, in *U. cornuta* the differences in sensitivity between sun and shade collections are 3-4 smaller in desiccated material than hydrated material, while for *X. conspersa* the differences are smaller in hydrated rather than desiccated thalli. It seems likely that different mechanisms of photoprotection occur in different species. Irrespective of the mechanisms involved, collections of Afromontane lichens from sunny habitats are more tolerant to photoinhibition than thalli from the same species collected in the shade.

5.4.2 Removal of lichen substances in sun collections increases sensitivity to photoinhibition

In sun collections of lichen thalli, removal of lichen substances generally increases the sensitivity of the photobionts to photoinhibition for at least one parameter (F_V/F_M or rETR), whether thalli are stressed hydrated (Figure 5.1 and Table 5.2) or desiccated (Figure 5.2 and Table 5.4). As discussed in the Introduction, for hydrated thalli, the presence of lichen substances can increase tolerance to photoinhibition by increasing thallus reflectance (Solhaug et al. 2010; Ndhlovu et al. 2022). It is more difficult to explain how lichen substances improve tolerance to photoinhibition in desiccated lichens, as substance removal has little effect on reflectance when thalli are dry (Solhaug et al. 2010; Ndhlovu et al. 2022). Possibly, while not increasing reflectance, they may help to screen photobionts by reducing transmission. Alternatively, lichen substances can have very high antioxidant activity (Kosanić et al. 2011; Fernández-Moriano et al. 2016) and may scavenge ROS produced by photobiont chloroplasts. The only instance where lichen substance removal had no significant effect on tolerance was for hydrated thalli of both sun and shade collections of *R. celastri*. This is not consistent with our earlier findings (Ndhlovu et al. 2022), which indicated that the presence of lichen substances reduces photoinhibition in hydrated sun

collections of this species. While sun collections of *R. celastri* were clearly more tolerant to photoinhibition than shade collections (Figures 5.1A and Table 5.2), for hydrated material of this species, lichen substances seemed to be not involved. It may be relevant that Ndhlovu et al. (2022) used *Ramalina* collected c. 80 km from the thalli used in the present study. High PAR is not the only driver of the synthesis of cortical lichen substances. For example, Gauslaa et al. (2013) showed that in *Lobaria pulmonaria* herbivory, rather than light exposure, was the main determinant of cortical usnic acid levels. However, even for *R. celastri*, the presence of lichen substances improved the tolerance of desiccated thalli to photoinhibition (Figure 5.2A and Table 5.4). Thus, while lichen substances play a variety of roles in lichen biology, one of the most important seems to be that of protecting photobionts against high light stress.

5.4.3 *Removal of lichen substances has more impact on the sensitivity to photoinhibiotion in sun than in shade lichen thalli*

Compared with sun collections, lichen substance removal from thalli collected from shaded microhabitats has much less effect on photobiont sensitivity to photoinhibition (Figure 5.2 and Table 5.4). Removal only has a significant effect in hydrated thalli of *P. perlatum* (F_V/F_M) and *U. angulata* (F_V/F_M) and in desiccated thalli of *R. celastri* (rETR) and again *U. cornuta* (F_V/F_M and rETR). As discussed in the Introduction, there are several reports that the levels of lichen substances can approximately track changes in light availability (Solhaug and Gauslaa 2012). Assuming higher PAR induces the synthesis of cortical substances in the lichens used here, sun collections probably contain higher concentrations than shade collections. Therefore, it is not surprising that substance removal has more effect on sensitivity to photoinhibition in sun than shade collections. To test this statistically for the data presented here, three-way ANOVAs were

carried out with the combined data sets from experiments with hydrated and desiccated thalli (Tables 5.3 and 5.5). Results confirm that, for both hydrated and desiccated thalli, there is a significant interaction between collection site (sun or shade) and lichen substances (presence or absence) for at least one parameter for all species except *X. conspersa*. For this species, the statistical analyses suggest that lichen substance removal has a similar effect in both sun and shade collections. Possibly for the shade collection of *X. conspersa* factors other than high PAR may have caused cortical lichen substance biosynthesis. For example, as noted above, herbivory can be more important than light in some populations of lichens in controlling the levels of cortical substances (Gauslaa et al. 2013). However, in general, lichens growing in shaded microhabitats seem to depend less on lichen substances for photoprotection. This is probably a reflection of the type of light stress they typically receive. Lichens growing on the trunks of trees or on rocks under a tree canopy are exposed to rapidly changing light levels because gaps in the canopy vary depending on diurnal variations in the angle of sunlight, tree architecture and movement of the tree branches. The relatively brief periods that lichens are exposed to high light levels are known as 'sunflecks'. Sunflecks probably present a real hazard to lichen photobionts, and in theory cortical light screening substances could protect photobionts against them. However, cortical pigments would also reduce photosynthesis during the lower light levels available after a sunfleck has passed. Mkhize et al. (2022) recently showed that shade collections of lichens have NPQ that is higher and more rapidly inducing and relaxing compared with sun collections of the same species. It seems likely that NPQ, probably together with other biochemical mechanisms, is a more energetically efficient way to protect lichens against sunflecks. It is therefore not surprising that cortical substances are less important in the photoprotection of shade than sun collections of lichens.

5.4.4 *After removal of lichen substances sun collections of lichens are still more tolerant to photoinhibition than shade collections*

Even following the removal of lichen substances, the photobionts of sun collections of lichens still have greater resistance to photoinhibition than those from the shade (Figures 5.1; 5.2 and Tables 5.2; 5.4), the only exception being when PSII activity is assessed using 373 ng F_V/F_M for desiccated *Usnea* (Figure 5.2, and Table 5.4). The implication is that, in addition to synthesising lichen substances, sun collections use other mechanisms to tolerate photoinhibition. Results are analogous to the study of Beckett et al. (2019) that compared the resistance to photoinhibition in melanised and pale thalli of *Cetraria islandica*. In that study, lichens were photoinhibited without any influence of a melanised upper cortex by removing the lower cortices and medullas and exposing the photobionts to light from below. Results showed that the photobionts of melanised thalli possess significantly higher tolerance to photoinhibition than those from pale thalli. Further work is needed to determine the nature of these mechanisms. However, sun collections of a range of species have higher rETRMAX than shade collections (Piccotto and Tretiach 2010; Mkhize et al. 2022); using more light energy in photophosphorylation will reduce the excess available for ROS formation. As discussed above, for vascular plants, adaptations include modifications in chloroplast architecture, and changes in the activities of ROS scavenging enzymes, NPQ and the PSII repair cycle (Greer (2022). Future studies therefore need to focus on which additional ultrastructural and biochemical tolerance mechanisms are present in sun lichens.

5.5. Conclusions

The present study investigated tolerance to photoinhibition in four Afromontane lichens. All species have trebouxioid (have green algae) as their photobionts and normally grow in more exposed microhabitats but can also be readily collected from more shaded locations. Results showed that for all species, collections from the sunny microhabitats are more tolerant to photoinhibition in the hydrated or desiccated states than collections from shaded microhabitats. While removal of lichen substances generally increases the sensitivity of the photobionts to photoinhibition, for three of the four species, removal increases sensitivity significantly more in sun than shade collections. However, even after the removal of lichen substances, sun collections remain more tolerant to photoinhibition than shade collections. In future, more detailed studies need to be carried out to elucidate the biochemical basis of the additional tolerance. However, the main conclusion of the work presented here is that the additional tolerance to high light in the photobionts of lichens from exposed sites is usually derived from a combination of both light screening cortical substances and other tolerance mechanisms.

5.6. References

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CHAPTER 6: THE ROLE OF SECONDARY METABOLITES ON DESSICATION-INDUCED STRESS

6.1. Introduction

Lichens are pioneer organisms that can successfully grow in a wide range of habitats, including those that could be classified as "extreme" i.e., desiccation (Goga et al. 2020). It has been suggested that the ability of lichens to colonize such environments is at least in part due to the presence of secondary metabolites that increase their protection against abiotic stress (Mitrović et al. 2011). From the discussion in Chapter 1 on the roles of secondary metabolites in lichens, it is clear that only very limited information is available on the roles of secondary metabolites in abiotic stress tolerance. The aim of the work presented in this chapter was to test whether lichen secondary metabolites can reduce the effects of desiccation stress on the photobiont or mycobiont of various species of lichens. The approach used here was the removal of the secondary metabolites from lichens using the acetone rinsing technique of Solhaug and Gauslaa (2001) and Solhaug et al. (2010). The effects of stress can then be compared in thalli with and without substances.

In the first set of experiments, the effects on photosynthetic parameters of a slow desiccation followed by storage in the desiccated state and then rapid rehydration was tested in thalli of *Cetraria islandica*, *Parmotrema perlatum*, *Ramalina celastri*, *Usnea angulata*, *Cladonia foliacea* and *Crocodia aurata*. The effects of lichen substances on mycobiont responses to desiccation were tested by desiccating lichens for three weeks over silica gel and then measuring ion leakage (as a proxy for most mycobiont membrane damage) during sudden rehydration in thalli with and without lichen substances.

6.2. Materials and Methods

6.2.1. Plant material collection

All lichen plant material used in this study was collected dry in a small pocket of an Afromontane Forest in the mist belt of the Fort Nottingham Nature Reserve, KwaZulu Natal, South Africa. Main lichen substances were identified and confirmed (see table 1) by high performance liquid chromatography as described by Pawlik-Skowrońska and Bačkor (2011). Storage, preparation and selection of lichen material was discussed in detail in Chapter 2.

Table 1: Lichen species and the secondary metabolite(s) that occur in the cortex and or medulla

Species	Secondary metabolite(s)
Cetraria islandica	Protolichesterinic acid and fumaprotetraric
Parmotrema perlatum	Usnic and stictic acid
Ramalina celastri	Parietin and usnic acid
Usnea angulata	Usnic acid
Cladonia foliacea	Cortical substances: Dibenzofuran usnic acid Medullary substances: Despsidone fumaprotetraric
Crocodia aurata	Calycin and pulvinic acid

6.2.2. Desiccation treatments

Lichen material was desiccated by placing 20 x 2 cm discs (10 for the control and 10 thalli with substances removed) in a desiccator over silica gel for 2 weeks for fluorescence measurements, and 200 mg lichen thalli for 3 weeks for ion leakage measurements. In cases where material could not be cut into disks 3-5 thallus fragments from the shrub-like species were used. This took place

in the laboratory with a PPFR of 5 µmol m^{-2} s^{-1}. Water content was determined by weighing blotted material that had been hydrated (placed in wet filter paper) for at least 24 h (turgid mass) then subtract dry mass obtained by drying (placing it in an oven at 80°C for 48 h). Relative water content (RWC) was calculated as: RWC = (FM-DM)/(TM-DM)

6.2.3. Desiccation induced ion leakage (K^+ loss)

Following desiccation over silica gel for 3 weeks, lichen samples of five replicates of c. 200 mg dry mass were immersed in a beaker containing 100 ml distilled water stirred with a magnetic stirrer. The electrical conductivity (µS cm^{-1}) of the water was measured at 2 min intervals for 30 min using a water conductivity meter (Mettler-Toledo AG, Analytical Schwerzenbach, Switzerland) (Cv). For final conductivity (Cf) the lichen material was boiled in 5 ml of water for 30 min, 5 ml added final conductivity (Cf) was measured. The average of the 2 blanks (just water) was subtracted from the solution measurements to correct the electrical conductivity values. Damage to the membrane was assessed as initial conductivity expressed as the percentage of the total conductivity lost, calculated as: Cv*100/Cf

6.3. Results

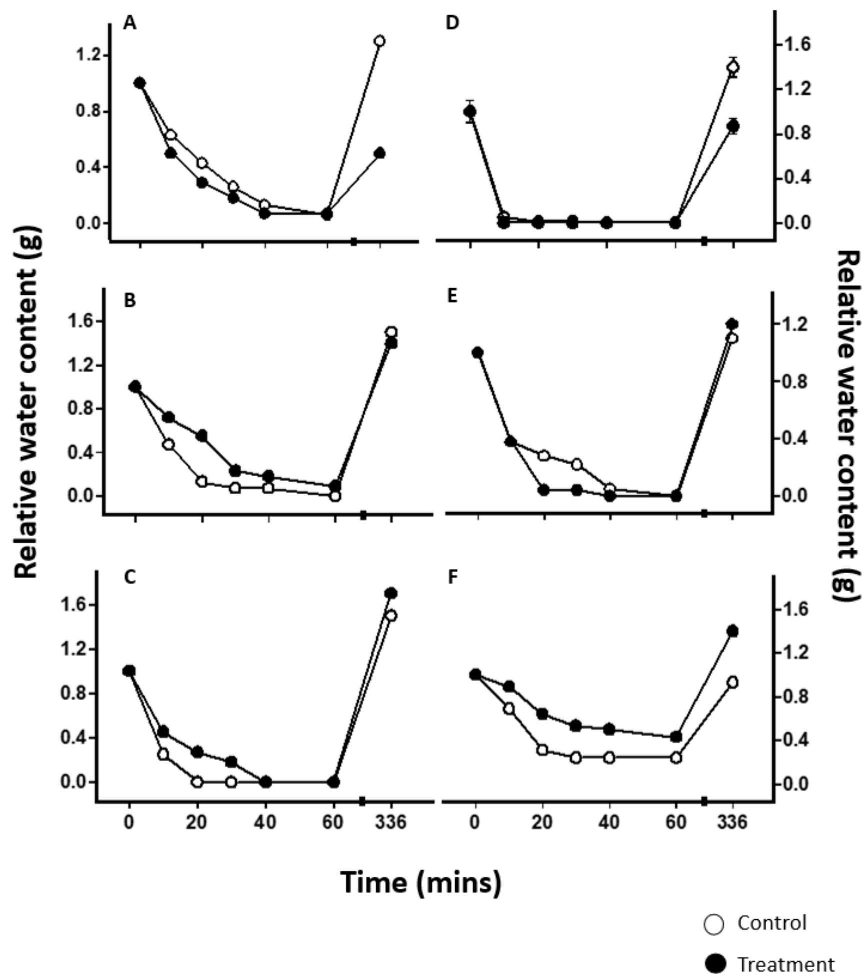

Figure 6.1: The relative water content (RWC) of control (open circles) and acetone-rinsed (closed circles) of *Cetraria islandica* (A), *Parmotrema perlatum* (B), *Ramalina celastri* (C), *Usnea angulata* (D), *Cladonia foliacea (E), Crocodia aurata* (F) and desiccated for 1hr – 2 weeks over silica gel, followed by rapid rehydration. n=20 per species and error bars show ± SE

Figure 6.1 shows the changes in water content during slow desiccation in *Cetraria islandica*, *Parmotrema perlatum*, *Ramalina celastri*, *Usnea angulata*, *Cladonia foliacea* and *Crocodia aurata* with and without lichen substances. Rates of desiccation were lower in thalli with lichen

substances present in *P. perlatum, R. celastri* and *Crocodia aurata*, but similar in other species (Table 6.2).

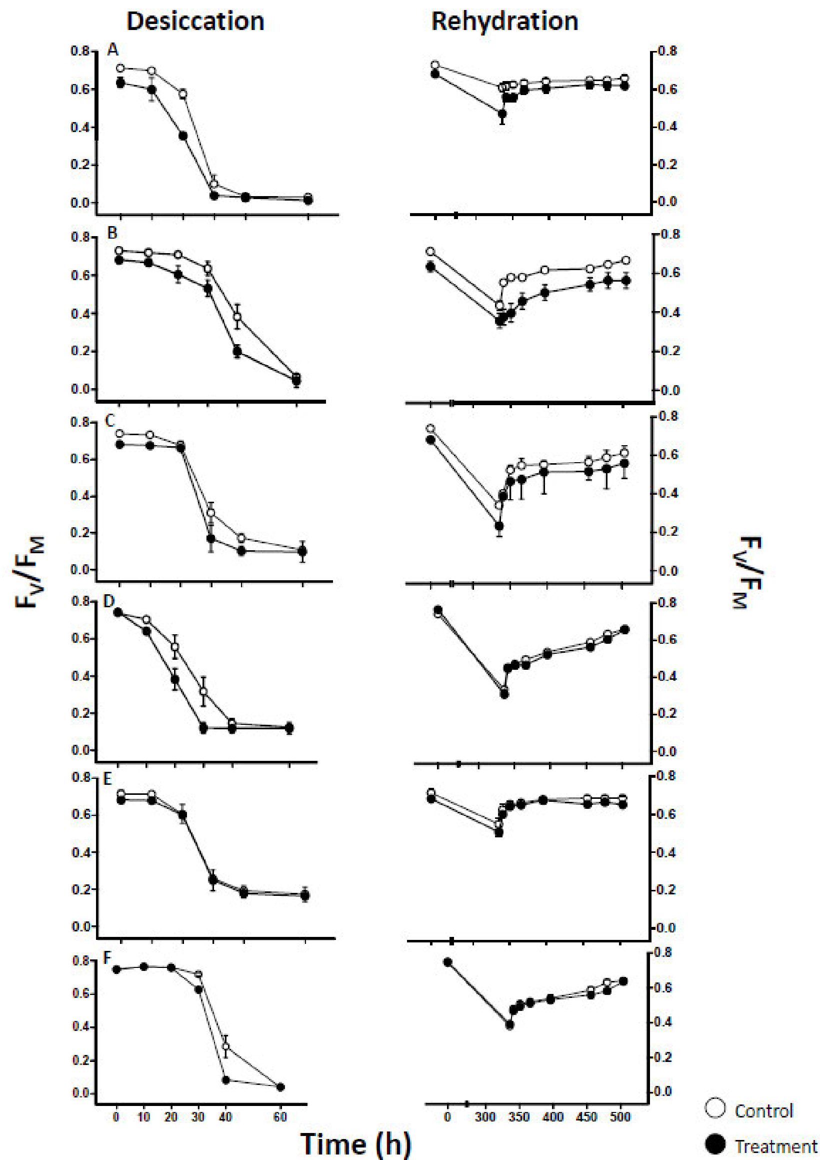

Figure 6.2: The effect of desiccation on F_V/F_M for control (open symbols) and acetone-rinsed (treatment) lichen discs of *Cetraria islandica* (A), *Parmotrema perlatum* (B), *Ramalina celastri* (C), *Usnea angulata* (D), *Cladodia foliacea* (E) and *Crocodia aurata* (F) following 2 weeks of desiccation followed by rapid rehydration. n=20 per species and error bars show ± SE

The response of photobionts to desiccation was measured by measuring F_V/F_M and NPQ during a desiccation / rehydration cycle (Figures 6.2; 6.3) respectively. During desiccation, for most species

F_V/F_M was more sensitive to desiccation following removal of lichen substances. The only exception was *Cladonia foliacea*, where F_V/F_M declined at a similar rate in thalli with and without lichen substances. Following desiccation over silica gel for 3 weeks, initial values of F_V/F_M during rehydration, and general recovery from desiccation were lower following lichen substance removal in *C. islandica*, *P. perlatum* and *R. celastri*, but similar in other species (Figure 6.2; Table 6.1).

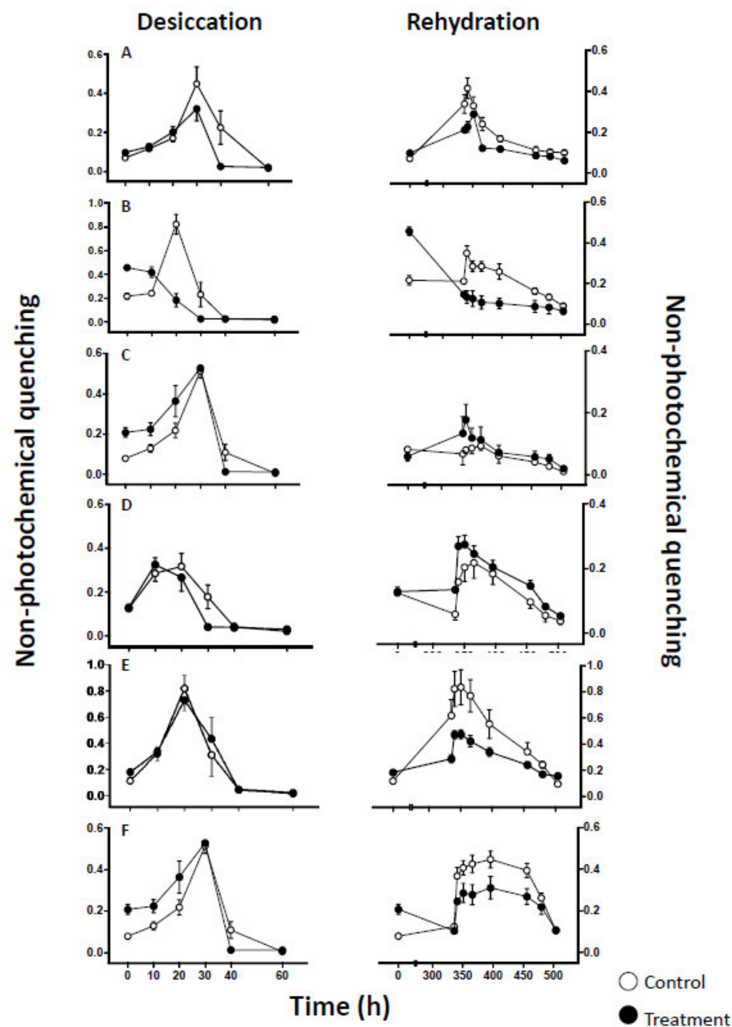

Figure 6.3: The effect of desiccation on NPQ for control (open symbols) and acetone-rinsed (treatment) lichen discs of *Cetraria islandica* (A), *Parmotrema perlatum* (B), *Ramalina celastri* (C), *Usnea angulata* (D), *Cladonia foliacea* (E) and *Crocodia aurata* (F) following 2 weeks of desiccation followed by rapid rehydration. n=20 per species and error bars show ± SE

Slow drying increased NPQ in all species in thalli with or without lichen substances, the only exception being *P. perlatum* with substances removed where no increase occurred (Figure 6.3). Fully desiccated material of all species had low values of NPQ. During rehydration following desiccation, NPQ initially increased, and then declined. Except for *R. celastri* and *U. angulata*, removal of lichen substances may result in reduction of NPQ during rehydration (Figure 6.3; Table 6.1).

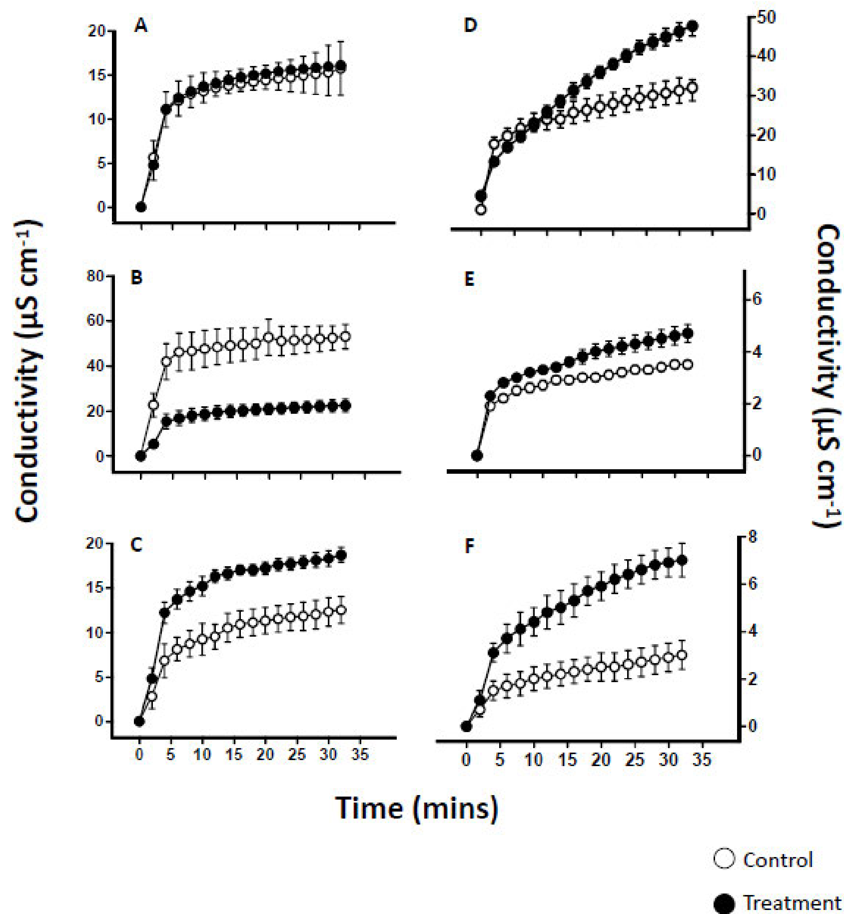

Figure 6.4: Ion leakage from rehydrated control (open circles) and acetone-rinsed (closed circles) of *Cetraria islandica* (A) *Parmotrema perlatum* (B), *Ramalina celastri* (C), *Usnea angulata* (D), *Cladonia foliacea* (E) and *Crocodia aurata* (F) measured as electrical conductivity (µS/cm^{-1}) following 3 weeks of desiccation above silica gel. n=5 per species and error bars show ± SE

Largely mycobiont responses to desiccation were tested by measuring ion leakage as a proxy for membrane damage from thalli desiccated for three weeks over silica gel (Figure 6.4; Table 6.2). In all species, during rehydration following desiccation, ion leakage was initially rapid, and then gradually slowed. Removal of lichen substances had a variable effect on membrane damage. In *P. perlatum* ion leakage was greater in thalli with lichen substances present, while in *Cetraria islandica* ion leakage was similar in thalli with or without lichen substances (Figure 6.4B, A). In the other four species, ion leakage was greater in thalli with lichen substances removed.

Table 6.1: Statistical analysis (2- way ANOVA) of the effect of desiccation on F_V/F_M and NPQ for control and acetone-rinsed lichen discs of *Cetraria islandica*, *Cladodia foliacea, Crocodia aurata Parmotrema perlatum*, *Ramalina celastri*, *Usnea angulata*, and following 2 weeks of desiccation followed by rapid rehydration. For all comparisons, the error had 179 degrees of freedom. Significance: * = P < 0.05, ** = P < 0.01, *** =P < 0.001

		C. islandica		***C. foliacea***		***C. aurata***		***P. perlatum***		***R. celastri***		***U. angulata***	
Comparison	**Factor**	**F_V/F_M**	**NPQ**	**F_V/F_M**	**NPQ**	**F_V/F_M**	**NPQ**	**F_V/F_M**	**NPQ**	**F_V/F_M**	**NPQ**	**F_V/F_M**	**NPQ**
Desiccating	**Control or Treatment**	**	**	0.533	0.981	***	*	***	*	0.552	0.686	***	0.388
	Time	***	***	***	***	***	***	***	***	***	***	***	***
	Interaction	0.371	*	0.987	0.951	***	*	*	***	*	0.875	*	0.361
Rehydrating	**Control or Treatment**	***	***	*	***	0.136	***	***	***	***	0.133	0.853	***
	Time	***	***	***	***	***	***	***	***	***	**	***	***
	Interaction	0.297	**	0.296	**	0.757	***	0.399	***	0.484	0.836	0.309	0.361

Table 6.2: Statistical analysis (2- way ANOVA) of the relative water content (RWC) of control and acetone-rinsed lichen material *Cetraria islandica*, *Cladodia foliacea, Crocodia aurata Parmotrema perlatum*, *Ramalina celastri*, *Usnea angulata* desiccated for 1hr – 2 weeks over silica gel, followed by rapid rehydration. For all comparisons, the error had 139 degrees of freedom. Significance: * = P < 0.05, ** = P < 0.01, *** =P < 0.001

Comparison	Factor	***C. islandica*** **RWC**	***C. foliacea*** **RWC**	***C. aurata*** **RWC**	***P. perlatum*** **RWC**	***R. celastri*** **RWC**	***U. angulata*** **RWC**
Desiccating & Rehydration	**Control or Treatment**	***	0.112	0.204	***	0.190	*
	Time	***	***	***	***	***	***
	Interaction	***	0.237	0.102	0.144	0.103	***

Table 6.3: Statistical analysis (2- way ANOVA) of the Ion leakage from rehydrated control and acetone-rinsed lichen material of *Cetraria islandica*, *Cladodia foliacea, Crocodia aurata Parmotrema perlatum*, *Ramalina celastri*, *Usnea angulata*, measured as electrical conductivity (µS cm^{-1}) following 3 weeks of desiccation above silica gel. For all comparisons, the error had 159 degrees of freedom. Significance: * = P < 0.05, ** = P < 0.01, *** =P < 0.001

Comparison	Factor	*C. islandica* µS cm^{-1}	*C. foliacea* µS cm^{-1}	*C. aurata* µS cm^{-1}	*P. perlatum* µS cm^{-1}	*R. celastri* µS cm^{-1}	*U. angulata* µS cm^{-1}
Desiccating & Rehydration	**Control or Treatment**	0.237	***	***	***	***	***
	Time	***	***	***	***	***	***
	Interaction	0.998	0.064	***	0.359	0.128	***

6.4. Discussion

6.4.1. Effect of lichen substances on rates of desiccation

Rather surprisingly, in some species the presence of lichen substances appeared to increase the rate of desiccation (Figure 6.1, Table 6.2). It was originally thought that substance removal would either have little effect, or rather increase rates of desiccation; possibly substances present in the upper cortex may have formed a structure analogous to the cuticle in vascular plants. However, Solhaug et al. (2010) proposed that when a thallus is wetted, lichen substances may restrict water entry into thalli. This may prevent excessive water uptake or "oversaturation" which will have the effect of restricting CO_2 diffusion to the photobionts for photosynthesis. Removal of substances may therefore have increased total water uptake (on a g H_2O g^{-1} dry mass basis). Therefore, expressing water loss using units of RWC may give the appearance that thalli with substances removed have a slower rate of water loss.

6.4.2. Effect of lichen substances on the sensitivity of the photobionts to desiccation

For most species, the presence of lichen substances increased tolerance to photoinhibition, assessed here as F_V/F_M (the maximum efficiency of PSII) (Figure 6.2, Table 6.1). The most likely explanation for the effects on desiccation tolerance of lichen substances is that these substances have very high antioxidant activity (Kosanić et al. 2011; Fernández-Moriano et al. 2016). Therefore, they may increase desiccation tolerance by scavenging desiccation-induced ROS. Lichen substances occur as crystals on the surface of fungal hyphae (see Introduction) and are therefore spatially well positioned to intercept ROS such as H_2O_2 that may diffuse from the mycobiont to the photobiont during stress. In theory, the ability of lichen substances to increase thallus reflectance observed in Chapter 3 could have contributed to greater tolerance of thalli with

substances present. However, while light can certainly increase the harmful effects of desiccation on poikilohydric organisms (Beckett et al. 2021), here desiccation and rehydration were deliberately carried out under conditions of low laboratory light. Therefore, increased reflection is unlikely to be the explanation for the increased tolerance of thalli with substances present. It is unclear why for *Cladonia foliacea* substance removal had no effect on the sensitivity of the photobionts to desiccation, as the major lichen substance present in this species is usnic acid, which is common in the other species tested here. Interestingly, in general, substance removal had much more effect during desiccation rather than rehydration (Figure 6.2, Table 6.1), possibly suggesting that most ROS are produced as lichens dry.

6.4.3. Desiccation is associated with an increase in NPQ

In all species, slow desiccation is associated with an increase in NPQ, followed by a decrease as thalli finally dry out (Figure 6.3, Table 6.1). As discussed in the Introduction, during drying, carbon fixation often stops before photophosphorylation, increasing the 'leakage of electrons' to ground state oxygen and therefore stimulating ROS production (Challabathula et al. 2018). Under such conditions, lichen photobionts can reduce ROS formation by dissipating the light energy that they cannot use as heat using NPQ. A similar induction of NPQ was found in drying thalli of *Parmelina quercina* by Calatayud et al. (1997). Lichen substance removal reduced NPQ during desiccation in *Cetraria islandica* and *P. perlatum* but had little effect on other species.

During rehydration, NPQ recovered rapidly and then gradually fell to initial values (Figure 6.3). Except for *R. celastri* and *U. angulata*, lichen substance removal tended to reduce NPQ during rehydration. It would seem unlikely that lichen substances have direct effects on NPQ. Rather, the

removal of substances probably increases general oxidative stress during recovery from desiccation. The resulting reduction in photobiont health may reduce the ability of photobionts to increase NPQ. Although in the experiments reported here lichen substance removal had little effect on the recovery of F_V/F_M during rehydration (Figure 6.2, Table 6.1), in the present work lichens were rehydrated under low light. The ability of lichen substances to enable the photobionts to increase NPQ may promote recovery of F_V/F_M for lichens that become hydrated under brighter light.

6.4.4. Desiccation induced membrane damage

In four out of the six species, lichen substance removal increased the susceptibility of the lichens to membrane leakage (Figure 6.4). In *Cetraria islandica* (Figure 6.4A) substance removal had little effect on membrane leakage. For no obvious reason, in *Parmotrema perlatum* substance removal actually improved tolerance to leakage; by contrast, photobiont damage in *P. perlatum* was greater when lichen substances were removed (Figure 6.2B, Table 6.3). For some species, lichen substance removal may affect the photobiont and the mycobiont in different ways. Plant membranes are known to be a major site for oxidative modification during abiotic stress (Anjum et al. 2015). Therefore, the generally protective effects of lichen substances on desiccation-induced membrane damage are likely to be due to their ability to act as antioxidants as discussed above.

6.5. Conclusions

While there is some variation between species, in general the removal of lichen substances increases the susceptibility of both the photobiont and the mycobiont to desiccation-induced

damage. The most likely explanation for these results is that the lichen substance can scavenge desiccation-induced ROS. Future work needs to study why in some cases e.g., for membrane leakage in *Parmotrema perlatum* lichen substances promote rather than reduce damage. Possibly in some species lichen substances act as pro- rather than antioxidants.

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CHAPTER 7: GENERAL CONCLUSIONS AND RECOMMENDATIONS

Having to grow in harsh environments and experience stress daily, the survival of lichens depends on their ability to survive in these conditions. This thesis is entitled: *The role of secondary metabolites in protecting lichens from climate change-induced stress*. The main aim has been to investigate and explore ways in which lichens protect themselves from stress, in particular the role of secondary metabolites in stress tolerance.

7.1. Role of unpigmented lichen substances in protection against high PAR in hydrated and desiccated thalli

The roles of colored and/or pigmented lichen substances (e.g., parietin and melanins) have been well studied. The limited information available on the roles of colorless lichen substances in stress tolerance inspired the work described in Chapter 3. Apart from the study by Solhaug et al. (2010) who tested the role of atranorin in tolerance against photoinhibition, few studies have examined the roles of secondary metabolites in the tolerance of lichens to high light (PAR) stress. The main conclusion from the data presented is that the faintly pigmented (usnic acid) and unpigmented (atranorin) lichen substances can protect lichen photobionts from high PAR and this protection occurs in both hydrated and desiccated states. As a result, it can be concluded that colorless lichen substances need to be added to the list of mechanisms used by lichens to protect themselves against high PAR. Additionally we wanted to find out the effect of lichen substances on the reflectance status of *Parmotrema perlatum*. Results showed that in hydrated thalli reflectance is highly reduced when lichen substances are removed, but removal has little effect when thalli are

desiccated. The mechanism whereby lichen substances can protect lichens against photoinhibition when dry remains unclear.

The one interesting anomaly was that the removal of atranorin in *Leucoodermia leucomelos* had no effect on the susceptibility of the thalli in the desiccated state. It would be interesting to find out why usnic acid is more effective in the desiccated state than atranorin. Is it the presence of the faint color in usnic acid that is not present in atranorin?

7.2. Do melanins alter NPQ measurements in melanized thalli? And how do melanins affect NPQ?

Some lichen substances such as melanins can cause problems when trying to measure physiological parameters (e.g., NPQ) using fluorometry techniques. Can a standard fluorimeter be used to accurately assess the importance of parameters such as NPQ? This was one of the questions we aimed to answer in Chapter 4. A technique was adapted from Beckett et al. (2019) involving the removal of the lower cortex and medulla of lichen thalli. This enabled measurements to be taken from below. Results showed that the presence of melanins does not substantially alter conclusions drawn concerning the importance of NPQ in photoprotection using fluorometry techniques. Careful analysis of the results showed that often the top and bottom of the photobiont layer have slightly different characteristics, with those from the bottom of the layer possessing slightly more "shade" characteristics than those at the top. Comparisons of melanised and pale thalli of a range of different species suggested that there is no simple relationship between melanization and NPQ. NPQ in melanised thalli can be higher, lower or the same as in pale thalli.

Future studies can investigate the precise relationship that exists between the presence of melanins and NPQ in melanised lichen species. Possibly, the poor correlation between melanisation and

NPQ suggest the presence of other biochemical mechanisms that protect lichens from high light stress.

7.3. Is the presence of lichen substances more beneficial to collections of lichens growing in the sun than ones from shaded microhabitats?

To answer this question, lichens from the same species were collected from microhabitats with contrasting light conditions (sun and shade forms) and exposed to similar light stress to see which lichens between these two groups has a higher tolerance to high light. Kershaw and MacFarlane (1980) reported that populations of *Peltigera aphthosa* collected from the dense shade of spruce are extremely sensitive to high light, while populations collected from open habitats are much more tolerant. Similar to Kershaw and MacFarlane (1980), the results from Chapter 5 showed that sun collected lichen species have a higher tolerance to photoinhibition than shade collections. To further study the role that lichen substances play on this high tolerance, lichen substances were removed from both shade and sun forms. This enabled testing whether the sun forms will still have high tolerance to high light in the absence of lichen substances than shade forms. Results showed that while removal of lichen substances increases the sensitivity of most of the sun forms to light stress, there are still more tolerant than those from shaded microhabitats. It appears that in addition to lichen substances, other mechanisms (e.g., antioxidant enzymes, increased NPQ) enable sun forms to maintain high tolerance to light stress. Clearly, these mechanisms will need to be studied in future. Furthermore, it will need to be tested whether tolerance to high light stress is mostly environmentally induced, or whether there are “sun” and “shade” ecotypes of the photobionts of some lichen species.

7.4. Are lichen substances involved in desiccation tolerance in lichens?

Results presented there suggested that the answer to this question can be both yes and no. These answers were obtained after a series of experiments which included, testing the effect of slow desiccation and rapid rehydration on photosynthetic parameters (NPQ, F_V/F_M) of six lichen species. We also tested the effect of desiccation-induced damage to the mycobiont by measuring ion leakage. The first set of results showed that the presence of lichen substances can reduce the rate of desiccation in some species, while having no effect in others. Secondly, the damage done to the photobiont, assessed using photosynthetic parameters, showed that these parameters were more affected by desiccation in thalli with lichen substances removed. With regards to membrane damage, indicative of damage to the mycobiont, the results were highly variable. For some species, ion leakage was higher in thalli with lichen substances present. However, in others leakage was similar in thalli with and without substances present. However, most commonly, leakage was higher following substance removal. Taken together, results suggested that the presence of lichen substances can often increase desiccation tolerance, particularly in the photobiont, but it was not always the case.

So going forward it will be important to understand why some lichen substances (e.g., found in *Parmotrema perlatum*) promote rather than reduce damage caused to membranes. Presumably lichen substances can show both anti- and pro-oxidant characteristics, although there may be other explanations for the variations in the effects of substance removal on desiccation tolerance.

7.5. Will climate change induced changes alter with protective mechanisms (i.e., secondary metabolites) in lichens?

As discussed in the thesis, the author believes there is a correlation between lichen species distribution patterns, ecology and growth, and climate change (see Chapter 1, section 1.2). In the thesis, with support from other authors, we have shown that lichens growing in different macrohabitats (i.e., exposed and shaded) use protective mechanisms differently. With climate change, for example, a lot of UV radiation is expected to reach the Earth's surface and may trigger the upregulation of certain protective mechanisms such as increase in the production of secondary metabolites. Will the presence of lichen secondary metabolites amongst other mechanisms be enough to protect lichen species from stress that will be because of climate change? That is a question we cannot answer at this time but something we can further investigate.

7.6. Overall conclusions

In conclusion, there are still many questions that need to be answered with regards to the role of lichen secondary metabolites in abiotic stress tolerance. For example, why do some lichen substances appear to be more effective than others? Usnic acid appears to be better at reducing light stress than atranorin in dry thalli, but there is no obvious reason for this. Presumably, melanins offer protection, but why do some species melanise in bright light while others produce lichen substances? Are lichen substances the first or last line of defense in stress tolerance? What other additional biochemical processes are working with lichen substances to increase tolerance to abiotic stress? As mentioned above, growing in harsh environments, lichens need/require a diverse array of mechanisms to tolerate these conditions. Although it seems unlikely that genetically

engineering crop plants to produce lichen substance will be a viable strategy for improving yields in the near future, studying alternative or additional stress mechanisms can help us understand how lichens thrive in conditions where other life forms would struggle to survive.

7.7. References

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