Ecophysiological Responses of Lichens to Environmental Stressors: Assessing the Role of Stress-Tolerant Mechanisms in Sun and Shade Adapted Species of Afromontane Lichens

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Submitted in fulfilment of the academic requirements for the degree of

Doctorate of Philosophy

in the Discipline of Biological Sciences

School of Life Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Pietermaritzburg Campus

November 2024

ABSTRACT

Lichens are a relatively understudied yet significant component of South African ecosystems. They are capable of inhabiting a wide range of habitats and in these habitats are susceptible to abiotic stressors. Amongst these, drought, temperature fluctuations, and intense light exposure pose significant threats, primarily due to accumulation of reactive oxygen species (ROS), which can be lethal to lichens. Remarkably, lichens have evolved various mechanisms to counteract such stresses and mitigate the impact of ROS. These mechanisms involve either avoidance through the accumulation of secondary metabolites and non-photochemical quenching (NPQ), scavenging by antioxidants, or repairing the damage inflicted by ROS through the PSII repair cycle. However, our understanding of how these mechanisms contribute to stress tolerance in lichens remains limited. Hence, this research aims to investigate and understand stress tolerance mechanisms in lichens in response to common abiotic stresses. Furthermore, a secondary aim is to identify mechanisms in lichens that could improve stress tolerance in crops. In particular, phenotypic plasticity was assessed by comparing the roles of NPQ, melanin and glutathione in stress tolerance among lichens from shaded and exposed habitats. The findings reveal that pre-treating lichens such as *Crocodia aurata* under moderate light conditions increases tolerance to photoinhibition by enhancing NPQ. Comparison between sun and shade lichens revealed that rapid NPQ relaxation in shade forms optimizes light use efficiency; the higher NPQ levels in shade lichens more likely enables them to cope with rapid light changes. Interestingly, melanised thalli from slightly exposed habitats display NPQ patterns resembling shade forms. Additionally, comparison of glutathione (GSH) accumulation in melanized and pale thalli of *Lobaria pulmoria* during drying and wetting cycles demonstrates that melanized thalli are less affected by oxidative stress, necessitating lower levels of GSH to scavenge

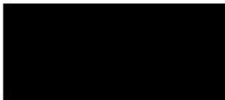
ROS. Overall, this research underlines the adaptability of lichen photobionts to modulate NPQ and electron transport rates (rETR) under varying light conditions, ensuring efficient photosynthesis. Moreover, melanins play a crucial role in lichen tolerance to various stresses beyond protecting from harmful UV-B and PAR radiation. Therefore, this thesis contributes to our understanding of the impacts of climate change induced abiotic stressors and may potentially aid in enhancing stress tolerance in crop species facing the challenges of global warming.

Keywords: Reactive oxygen species (ROS), photoinhibition, non-photochemical quenching (NPQ), electron transport rates (rETR), melanin, glutathione (GSH).

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 January 2019 to November 2024.

This thesis, submitted for the degree of Doctor of Philosophy in Science in the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, School of Life Sciences, Pietermaritzburg campus, represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.



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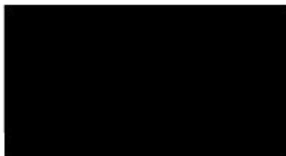

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Professor Richard. P. Beckett

Supervisor

November 2024

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis.

Publication 1

Beckett, R.P., Minibayeva, F.V., Mkhize, K.G.W., 2021. Shade lichens are characterized by rapid relaxation of non-photochemical quenching on transition to darkness. The Lichenologist 53, 409–414.

Author contributions:

RPB conceived paper with FVM. RPB, MFV and KGWM collected and analysed data, and wrote the paper.

Publication 2

Mkhize, K.G.W., Minibayeva, F.V., Beckett, R.P., 2022a. Lichen photobionts can be hardened to photoinhibition by pretreatment with light. Acta Physiologiae Plantarum, 2022, 44:122.

Author contributions:

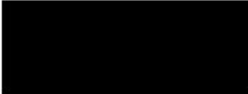
KGWM conceived paper with RPB and FVM. KGWM collected and analysed data, and wrote the paper. RPB and FVM contributed valuable comments to the manuscript.

Publication 3

Mkhize, K.G.W., Minibayeva, F.V., Beckett, R.P., 2022b. Adaptions of photosynthesis in sun and shade in populations of some Afromontane lichens. The Lichenologist 54(5), 319-329.

Author contributions:

KGWM conceived paper with RPB and FVM. KGWM collected and analysed data, and wrote the paper. RPB and FVM contributed valuable comments to the manuscript.



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Kwanele Mkhize

November 2024

ACKNOWLEDGEMENTS

I would like to sincerely express my deep gratitude to my supervisor, Prof. R.P. Beckett. From the beginning of my postgraduate studies, he has shown unwavering support, provided guidance in all my work, and offered valuable feedback that has enabled me to grow in my career. I am especially grateful for the extra mile of financial support he provided through difficult times and for his personal advice and encouragement. You really played a positive role in my life. I also thank Dr Farida Minibayeva for positive inputs towards experiments conducted here.

Special thanks to NRF (South Africa) for funding my PhD and also the University of KwaZulu-Natal for providing me with all the necessary facilities for my research, I'm truly grateful.

I would like to thank Dr Thomas Rouche from University of Innsbruck for being a great host in Innsbruck, the valuable research techniques that you taught me and the support you and your family gave me throughout my stay.

Huge appreciation to Dr Nqobile Ndhlovu for everything that you have done for me. The support, assistance, personal advice and many more of great memories we have shared throughout our academic journey. I wish you all the best in life and strong growth in your career. God bless!

Lastly, but certainly not least, I am thankful for the support, encouragement, and prayers from my family and friends. My friends from UKZN, in particular have played a huge role towards the success of this project. Their motivation, assistance and the love they showed me provided me with the hope and strength to perform at my best.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AOX	alternative oxidase
APx	ascorbate peroxidase
ASC	ascorbate
c.	Approximately
CAT	catalase enzyme
CEF	cyclic electron flow
Cf	final conductivity
CFC	chlorofluorocarbons
Chl	chlorophyll fluorescence
CO	carbon monoxide
CO_2	carbon dioxide
CP	cytochrome respiratory pathway
CPD	cyclobutane pyrimidine dimers
Cv	initial conductivity
Cyt	cytochrome
DHA	Dehydroascorbate
DHAR	dehydroascorbate reductase
DHN	1,8-dihydoxynaphtalene
DM	dry mass
DNA	deoxyribose nucleic acid

DOPA	dihydroxyphenylalanine
DT	desiccation tolerant
ER	endoplasmic reticulum
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
FLV	flavodiiron proteins
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
GP_{MAX}	maximal gross photosynthesis
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide / oxidized glutathione
HCL	hydrochloric acid
HSP	heat shock proteins
H_2O_2	hydrogen peroxide
L-DOPA	L-3,4-dihyroxyphenylalanine

LEA	late-embryogenesis abundant
LHC	light-harvesting complex
LCHSR	light-harvesting complex stress-related proteins
MDA	monodehydroascorbate
MDAR	monodehydroascorbate reductase
NOx	nitrogen oxides
NPQ	non-photochemical quenching
$O_2^{\cdot -}$	superoxide radicals
$^1O_2$	singlet oxygen
$^3O_2$	triplet oxygen
OCP	orange carotenoid protein
OH•	hydroxyl radical
PAR	photosynthetically active radiation
PCEF	pseudocyclic electron flow
POX	peroxidase
PPFD	photosynthetic photon flux densities
PQ	plastoquinone
PSBS	photosystem II 22 kDa protein
PSII	photosystem II
PSI	photosystem I
rETR	relative electron transport rate
$rETR_{MAX}$	maximum relative electron transport rate
RH	relative humidity

ROS	reactive oxygen species
SLC	secondary lichen compounds
SOD	superoxide dismutase
SO_2	sulfur dioxide
SOx	sulphur oxides
SE	standard error
SPSS	Statistical Package for the Social Sciences
TM	turgid mass
UV	ultraviolet
VDE	violaxanthin de-epoxidase
WC	water content

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CHAPTER 1

INTRODUCTION

1.1 Background

Lichenized ascomycetes, known as "Lichens", are a relatively understudied yet vital component of South African ecosystems. Lichens comprise a fungus (usually an ascomycete) termed the mycobiont, which derives its carbon from photosynthesis carried out by an alga or cyanobacterium termed the photobiont (Nguyen et al., 2013). In return, it is assumed that the fungus protects the photobiont from environmental stresses. This mutually beneficial relationship enables lichens to thrive in extreme environments that would be too harsh to be inhabited by either of the individual partners (Williams et al., 2017). Lichens play important roles in facilitating the formation of newly formed soil through the decomposition of organic matter (Beckett et al., 2013) and mechanical fragmentation of rocks (Chen et al., 2000). Additionally, they serve as bioindicators of environmental health (Yang et al., 2023). In extreme habitats, lichens can be the dominant life form, and display resistance to a wide range of environmental stresses, including desiccation, temperature fluctuations, and intense light and UV-radiation (Beckett et al., 2008, Meessen et al., 2013). For this reason, lichens are also referred to as "extremophiles" (Beckett et al., 2008).

While lichens can withstand a range of environmental stresses, prolonged or intense exposure to stressful conditions at the cellular level can lead to the formation of reactive oxygen species (ROS) (Sachdev et al., 2021; Beckett et al., 2008). ROS are produced during normal metabolism and have signalling roles; furthermore, they can regulate diverse functions such as cellular homeostasis, programmed cell death (Francoise et al., 2014), and lignocellulosic decomposition, which is the natural decomposition of the substrate's cellulose, hemicellulose, and

lignin (Beckett et al., 2013). A series of enzymatic and non-enzymatic antioxidants aids in detoxification and ensure that ROS concentrations do not become excessive. However, when ROS accumulation surpasses the detoxification system, it can damage cells by targeting nucleic acids, lipids, and proteins (Beckett et al., 2005). Examples of ROS includes singlet oxygen ($^1O_2$), the superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the highly destructive hydroxyl radical (OH•). However, ROS are not only produced during high-stress conditions but also during stress recovery. For example, Weissman et al. (2005a) showed that during the desiccation / rehydration of *Ramalina lacera*, particularly strong ROS production occurred during rehydration.

Apart from the detoxification system, tolerance to stress often increases through prior exposure to stress, a phenomenon known as acclimation or hardening (Beckett et al., 2021). In this context, gene expression is modified to enhance stress tolerance (Williams et al., 2017). Remarkably, photobionts can coexist within a single thallus and be able to switch when confronted with unfavourable conditions (Chrismas et al., 2023). This enables them to associate with better-adapted photobionts for a specific microhabitat, allowing lichens to acclimate to changing environments (Williams et al., 2017). These mechanisms significantly contribute to lichens' survival and dominance in extreme environments. For instance, research by Chrismas et al. (2023) revealed that a marine cyanolichen *Lichina pygmaea* has a complex photobiont community including the cyanobionts *Rivularia* and *Pleurocapsa* which are dominant in lichens growing at high and low tide, presumably associated with high salinity and desiccation tolerance respectively.

It is important to highlight that various mechanisms that contribute to lichen stress tolerance. The mechanisms are involved in the prevention of ROS formation, scavenging of ROS once formed, and repairing damage caused by ROS accumulation. Overall, the ability of lichens

to tolerate extreme conditions is primarily influenced by their lifestyle and adaptation to their natural habitat (Meessen et al., 2013).

This review aims to highlight the effect of abiotic stresses on lichens and elucidate the mechanisms they employ to withstand such stressful conditions. In particular, the introduction outlines the role of different defence mechanisms in stress tolerance in lichens from shaded and exposed microhabitats, a specific focus of the work described here. However, first we review the significance of symbiosis in lichens and explore how each symbiotic partner contributes to lichen survival.

1.2 Understanding the symbiotic relationship in lichens

The nature of the symbiotic relationship in lichens has sparked intense debate among scientists. The prevailing belief among most researchers is that it represents a mutualistic relationship, where the fungus benefits by receiving "food" in the form of carbohydrates from the photobiont, while the photobiont gains shelter. However, some scientists believe that the photobiont is primarily responsible for the survival of the partnership, and the symbiosis is a parasitic relationship. Joneson (2009) has provided a comprehensive overview of the data supporting both theories, offering a broader understanding of lichen symbiosis.

A lichen thallus is predominantly composed of specialized fungi, and the classification and naming of a lichen is based on the fungal partner (Muggia et al., 2016). Lichen-forming fungi can only survive by obtaining fixed carbon from photobiont cells through photosynthesis. In cyanobacterial lichens, the fungus also obtains nitrogen through nitrogen fixation. In return, the photobiont presumably directly receives water and micronutrients from the fungi through structures like appressoria, haustoria, or intragelatinous protrusions (Honegger, 2009).

Furthermore, the fungal thallus directly contributes to the protection of the photobionts from excessive light radiation due to densely packed fungal hyphae serving as the primary defence (Calcott et al., 2018), and by secretion of secondary metabolites and melanins (Muggia et al., 2016). Rikkinen (2003) also proposed that "a wide range of compatible mycobionts can effectively enhance the photobiont's resistance against grazing, pathogenic fungi, and other harmful factors".

Research by Beckett et al. (2019) showed that in *Cetraria islandica* tolerance of the photobiont depends on a combination of fungal cortical screening pigments and photobiont-based mechanisms. However, it remains uncertain whether this holds as a general feature of the photobionts in other lichens. Conversely, the photobiont of *Cladonia vulcani* exposed to high light intensities, contains higher photoprotective pigments involved in non-photosynthetic quenching of light energy to minimize $^1O_2$ formation (Kranner and Birtic, 2005). Furthermore, while the fungus of *C. vulcani* contains the antioxidant glutathione (GSH), this antioxidant has a low effectiveness without the alga (Kranner and Birtic, 2005). Marini et al. (2011) have suggested that the type of photobiont partner may play a crucial role in the ability of lichens to adapt to climate and land use changes. It is postulated that the photobiont can mitigate the effects of desiccation by increasing the osmotic potential of algal cells through production of sugar alcohols like ribitol, mannitol, and arabitol (Balarinova et al., 2014).

The balance of information available suggests that a lichen can only survives through a mutualistic relationship, regardless of the type of lichen or symbionts (Figure 1.1). The contrasting opinions arise from weighing the symbionts individual abilities to withstand adverse environmental conditions. Nonetheless, it is evident that the photobiont, although it only comprises a small proportion of the thallus, makes a significant contribution to the survival of a lichen. One

useful approach could involve isolating the symbionts and subjecting them to identical stress conditions while monitoring their responses.

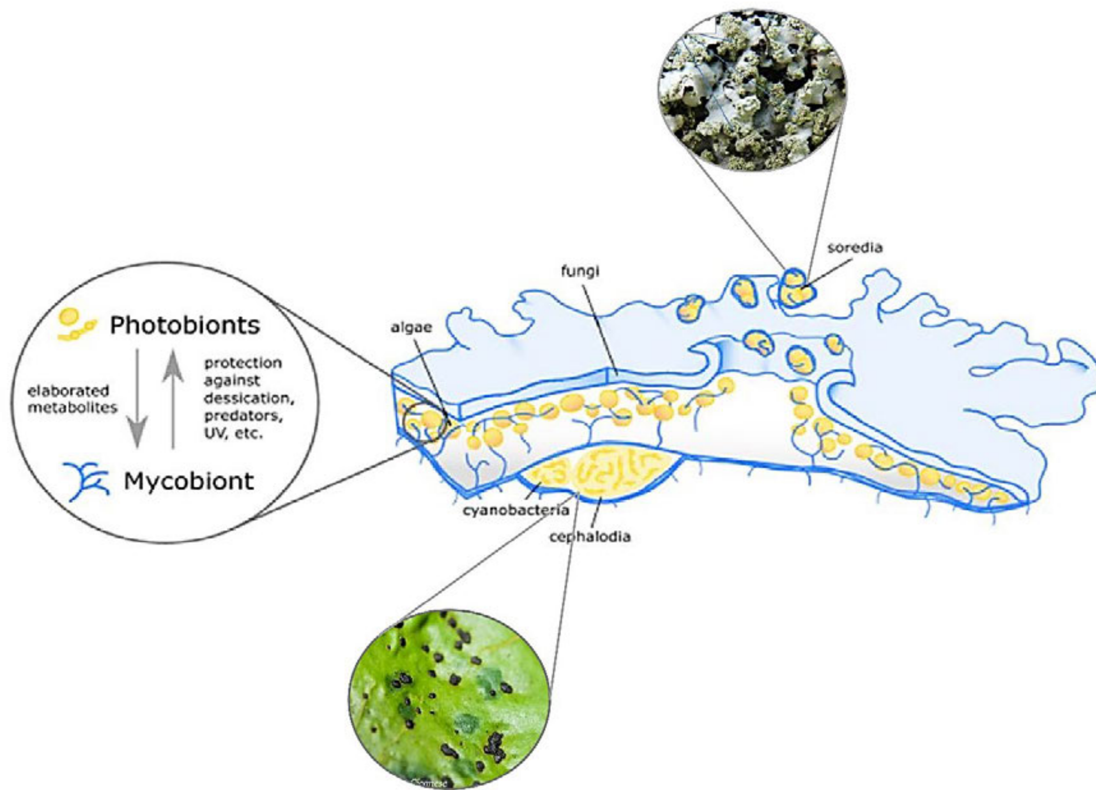


Figure 1.1: A schematic representation of lichen thallus and symbiotic relationship between the photobionts and mycobionts. Pictures display true presentation of how the cephaloidia containing cyanobacterial symbionts and soredia look like on a thallus (Duran-Nebreda and Valverde, 2023).

Additionally, a diverse array of prokaryotic and eukaryotic organisms, both epibionts and endobionts, have been discovered in lichen thalli, with no clearly defined roles documented thus far. These include symptomless fungal endophytes, parasitic fungi, lichenicolous fungi, lichen-eating invertebrates, gall-forming organisms, epibiotic bacterial films, airborne cyanobacteria, diatoms, other algae, and tardigrades (Honegger, 2009). Most commonly found on or in the lichen thallus are the lichenicolous fungi which are believed to be obligate parasites, saprophytes, or

parasymbionts. Lichens appear to provide a rich nutritional base for these organisms (Asplund et al., 2016). These organisms can kill their hosts (Hawksworth, 2003), taking over the photosynthetic partner or even forming colonies independently (Figure 1.2).

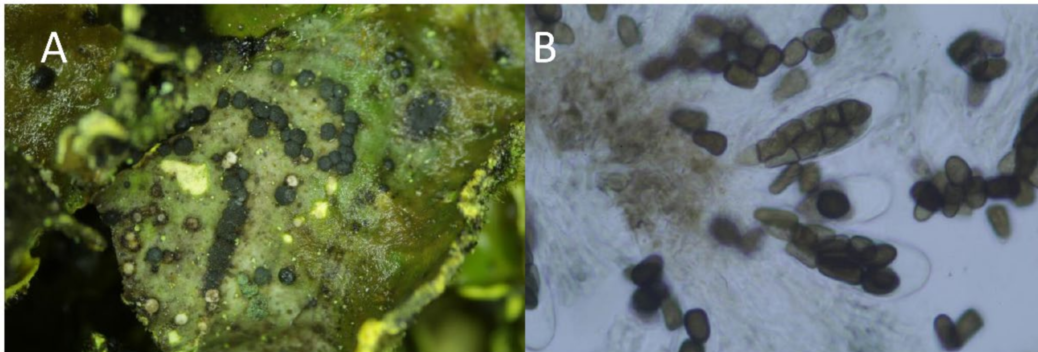


Figure 1.2: Microscopic images of *Crocodia aurata* invaded by *Abrothallus* spp. of lichenicolous fungi on the surface of the thalli (A) and inside forming colonies (B).

1.3 Human impact on lichen communities

Lichens are considered as keystone species. Unfortunately, a variety of anthropogenic factors are currently causing substantial environmental challenges, such as climate change, and these changes directly or indirectly impact the diversity and abundance of lichens and plants in general. For example, any disturbance in a forest ecosystem can cause biodiversity loss and contribute to climate change (Goudie, 2013), particularly for species like lichens that heavily rely on such environments for survival. Forests, however, represent potential sources of bioenergy (Stewart et al., 2011) and agricultural resources (Goudie, 2013). Consequently, they are subjected to repeated disturbances. In tropical regions, many forests are being cleared for agricultural purposes, leading to the destruction of the natural habitats of lichens. Deforestation is the most important cause of biodiversity loss in tropical ecosystems, and epiphytic species such as lichens and bryophytes are particularly vulnerable (Chuquimarca et al., 2019). Lichens are sensitive to exposure following

deforestation, exposing “shade” or “sunflecks” species found beneath the canopy to high light and UV-B (see below for discussion of these terms). One such species, *Lobaria pulmonaria*, has seen a decline across Europe not only due to its sensitivity to sulfur dioxide (SO_2) but also because of factors like global change and forestry practices and is currently listed as a threatened species in several European countries (Paoli et al., 2019; Ravera et al., 2023). Another major consequence may be a reduction in cyanobacterial lichens, which will reduce nitrogen inputs to the system (Trest et al., 2015).

The burning of forests significantly contributes to climate change and global warming, particularly by reducing carbon sequestration (Stewart et al., 2011). Global warming primarily occurs due to an increase in the emission of greenhouse gases (e.g. carbon dioxide, methane and nitrous oxide) as a result of human activities (Riebeek, 2010). Moreover, atmospheric nitrogen deposition resulting from such activities can alter the competitive dynamics within lichen communities (Pereira et al., 2012; Prather, 2017). Another threat to lichens comes from air pollution originating from industrial sources and cars, particularly the primary pollutants such as sulfur oxides (SOx), nitrogen oxides (NOx) and carbon monoxide (CO), poses a severe threat to all living organisms. Combustion of fossil fuels stands out as a major global contributor to air pollution, ultimately generating secondary pollutants like acid rain and contributing to the phenomenon of global warming (Wakefield and Bhattacharjee, 2011). Furthermore, increased deposition of nitrogen and sulfur compounds can have detrimental effects on lichens (Pescott et al., 2015; Wakefield and Bhattacharjee, 2011). These effects include "reductions in respiration and photosynthesis, increased membrane permeability, heightened influx of potassium ions and loss of other ions, and structural changes" that can damage algal cells and consequently affect the entire thallus (Hutchinson et al., 1996). Several other pollutants have been identified as having harmful

effects, including fluoride, secondary pollutants such as ozone and peroxyacetyl nitrate, agricultural pesticides, trace metals, and metalloids (Hutchinson et al., 1996).

Therefore, climate change represents a complex and dynamic series of changes in environmental conditions affecting life on earth (Chaudhry and Sidhu, 2022). Human activities have led to an increase in atmospheric carbon dioxide (CO_2) levels, impacting temperature, rainfall patterns, intensity of sunlight, and various environmental factors such as droughts and waterlogging (Onyekachi et al., 2019). These changes pose significant challenges to food security and ecological balance, prompting extensive research into adaptive strategies for plants. This involves developing plants and crop species that can better withstand the effects of climate change (Onyekachi et al., 2019). Below we review the impacts of abiotic stresses as a result of climate change on lichens and explore the mechanisms they employ for tolerance.

1.4 Effects of common abiotic stresses on lichens

1.4.1 Impact of Ultraviolet Radiation Stress

Ultraviolet (UV) radiation remains one of the major stressors causing health and environmental concerns. UV can be categorized into UV-C (100-280 nm), UV-B (280-320 nm), and UV-A (320-400 nm), referred to as shortwave, medium wave, and long wave, respectively (Sanchez et al., 2014). UV-C is mostly absorbed by the earth's atmosphere. In plants and lichens, UV radiation, especially UV-B, can cause the formation of DNA lesions, specifically cyclobutane pyrimidine dimers (CPDs) and 6,4 photoproducts (6,4-PPs) (You et al., 2001). These molecules not only have mutagenic effects but also hinder DNA replication (Sanchez et al., 2014). UV-A on the other hand cannot be absorbed by DNA, therefore has a less damaging effect on DNA, although in conjunction

with visible light can result in ROS generation, especially $^1O_2$, indirectly causing DNA damage (Gill et al., 2015).

Ozone depletion in the stratosphere has increased levels of UV-B radiation reaching the Earth's troposphere (Nguyen et al., 2013). Ozone concentrations have decreased by 5% in the past 50 years, mostly due to the release of pollutants such as chlorofluorocarbons (CFC's) into the atmosphere (Gill et al., 2015). "Though most of the extraterrestrial UV-B is absorbed by the stratospheric ozone, remaining UV-B can produce adverse effects on diverse habitats" (Gill et al., 2015). In addition to DNA damage, exposure to UV-B can result in protein damage, photosynthesis inhibition, and the generation of highly energetic ROS (Hideg et al., 2013).

1.4.2 Impact of high and low photosynthetically active radiation (PAR) and fluctuating light levels

Photosynthetically active radiation (PAR) comprises wavelengths of 400-700 nm. When lichens are exposed to PAR at levels above those that can be used to fix CO_2, ROS can be formed that can have detrimental effects on lichens (Bianchi et al., 2019). In the field, lichens encounter high light stress, often accompanied by an increase in thallus temperature resulting in desiccation (Bartak et al., 2004). For many lichen species, thallus desiccation offers significant protection from light stress. This is probably mostly due to the greatly reduced metabolic activity in the desiccated state, but also due to reduced light penetration of the upper cortex (Ertl, 1951) (see the "Physiological and Morphological Adaptations to Extreme Environments and Other Mechanisms" section below). Conversely, metabolically active or hydrated thalli are more susceptible to high light stress due to lower reflectance and increased cortex translucency, which exposes the photosynthetic apparatus to light. Therefore, hydration status is a key determinant in how lichens respond to radiation (Morillas et al., 2021). For example, in *Lobaria pulmonaria*, high light exposure exceeding one

day is most detrimental in desiccated compared to hydrated thalli (Gauslaa and Solhaug, 1996). However, excessive light exposure for a relatively short period of time can also be detrimental.

Lichens adapted to shaded habitats often encounter fluctuating light conditions, characterized by short-term variations (ranging from seconds to minutes) rather than consistent low light levels. For instance, lichens growing on tree bark experience rapid alterations in light intensity due to openings in the canopy, influenced by daily changes in sunlight angles, tree architecture, and movements of branches (Beckett et al., 2021; Mkhize et al., 2022). These lichens experience rapid shifts in irradiance levels, with brief intervals of intense light exposure referred to as 'sunflecks' (Beckett et al., 2021; Mkhize et al., 2022). However, sudden increase in light intensity can potentially trigger oxidative stress, necessitating the presence of constitutive photoprotective mechanisms (Mkhize et al., 2022). The effects of a sudden increase in light intensity have been well-documented in epiphytic lichens such as *Seirophora villosa* and *Lobaria pulmonaria*, which have been declining in their natural habitats due to habitat fragmentation (Bianchi et al., 2019; Gauslaa and Solhaug, 1996). Studies have shown that after logging or habitat fragmentation, epiphytic lichens are exposed to sudden increases in PAR and rapid changes in water availability. When these changes surpass the typical ecological range, they can have adverse effects on the photosynthetic efficiency of the lichens (Bianchi et al., 2019).

In addition to canopy cover, clouds can significantly reduce incoming light, causing irregular variations in light intensity (Slattery et al., 2018). Commonly, this leads to a decrease in photosynthesis proportional to the optical thickness of the cloud cover. Besides impairing photosynthesis, the cooling effect of cloud cover can affect respiration and water status (Hughes et al., 2024). The direct and diffuse photosynthetic photon flux densities (PPFD) under cloud cover correspond with reductions in PAR, with occasional bright intervals during cloud openings / gaps,

resulting in fluctuating light levels (Hughes et al., 2024). Cloud gaps can cause rapid temporal changes in light levels, and depending on the type, size, and movement of clouds, these changes can last from minutes to hours. Such rapid variations can significantly impact plant ecophysiology (Hughes et al., 2024). However, clouds generate more diffuse solar radiation, which can be captured more effectively than direct solar radiation (Aartsma et al., 2020).

While lichens from exposed or open habitats generally exhibit high photosynthetic rates and carbon gain, those frequently exposed to fluctuating light levels under canopies and cloud cover can respond quickly to these brief periods of light, achieving positive carbon balances (Lakatos et al., 2006). To understand how lichens survive in variable light environments and utilize sunflecks to enhance photosynthesis, laboratory experiments on cortical lichens simulating natural lightflecks revealed that two species of lichens could reach 50–100% of their maximal gross photosynthesis (GP_{MAX}) within 32–54 seconds during sunfleck events. Furthermore, their CO_2 uptake rates during these events can sometimes exceed those recorded under steady-state light conditions (Lakatos et al., 2006). Despite this, the potential for oxidative stress due to sudden increases in light levels during sunflecks is undisputable (Beckett et al., 2021; Mkhize et al., 2022). As Hampp et al. (2018) postulated, lichens adapted to low light are sensitive to increase in PAR and rely on protective mechanisms like heat dissipation for survival.

Given that lichens from exposed habitats generally have a higher capacity for photoprotection (Lakatos et al., 2006), the mechanisms used by shade-adapted lichens or those adapted to fluctuating light environments are still not fully understood. This makes lichen species such as *Crocodia aurata*, which is adapted to shaded, moist microhabitats with frequent light level fluctuations, ideal model species for studying how they adapt to rapid changes in light conditions. Indeed, the lichen species used in this research effectively address adaptations found in shade

species subjected to occasional sunflecks. Research on the tolerance mechanisms of these lichens offers valuable insights for improving the productivity of higher plants, especially crop species, in environments with rapidly changing or fluctuating light levels.

1.4.3 Impact of Extreme Temperature Stress

The rise in temperature attributed to global warming has resulted in the extinction of numerous plant and animal species (Brusowankin, 2022). Among affected organisms, lichens emerge as particularly sensitive to global warming (Pisani et al., 2007). Many lichen species show specific climate preferences, and changes in climate such as global warming, can significantly influence their geographical distribution (Pisani et al., 2007). Factors like decreased rainfall, higher temperatures, and increased light intensity can collectively reduce the duration of hydration and metabolic activity in lichen thalli (Gasulla et al., 2012).

Mallen-Cooper et al. (2023) suggest that with current changing climate conditions, both lichens and mosses will experience shifts in their niche or distribution range in the near future, with some species potentially expanding their ranges while others may face reduction or even extinction. For instance, species adapted to dry-lands are expected to decline due to carbon imbalances, particularly during summer seasons. While lichens and mosses are well adapted to extreme conditions, recovery from desiccation comes with carbon costs, and the predicted rapid evaporation of rainfall will leave these species with less time for carbon intake, making dry-lands unsuitable for them. Conversely, some species like *Stereocaulon paschale* with primarily boreo-arctic distribution are expected to expand across the boreal and high arctic zones. Currently there is evidence of lichens and mosses dominating these areas, and these species have been shown to regenerate after 400 years of burial under a glacier. Therefore, this study highlights temperature as the most critical climate variable influencing the distributions of lichens and mosses.

Higher temperatures have adverse effects on metabolism, growth, and reproduction (Steinhäuser, 2015), with exceptions noted in Antarctica lichens where temperature increases can benefit lichens and bryophytes. Furthermore, there is a correlation between a decrease in the total number of species and increasing latitude, and higher productivity of lichens has been observed at lower latitudes (Sancho et al., 2019). However, even minor temperature fluctuations can cause significant changes in the net photosynthetic rate of sensitive species (Pisani et al., 2007). Therefore, it seems likely that the response to heat stress depends largely on the degree of exposure. For instance, prolonged exposure of the epiphytic lichen *Evernia prunastri* to 80°C can inhibit chlorophyll synthesis, degrade chlorophyll, and damage cell membranes, while exposure to 40°C only reduces chlorophyll *b* (Pisani et al., 2007). Moreover, species like *Cladonia rangiferina* cannot endure prolonged temperature stress between 35°C and 45°C, resulting in impaired respiration and net photosynthetic rate (Tegler and Kershaw, 1981). However, this species can somewhat adjust its stress tolerance according to the season (Tegler and Kershaw, 1981).

A notable study by Almer et al. (2023) demonstrated that cyanobacteria and green algae coexisting in *Peltigera britannica* react differently to heat stress, with heat stress genes expressed at 15°C and 25°C, respectively. This highlights the distinct responses of each symbiont to environmental cues, their specific ecological preferences, and the tolerance abilities, suggesting that the green algal partner can tolerate heat stress better than the cyanobacterial and fungal partners (Almer et al., 2023). This however can be attributed to the distinct tolerance mechanisms employed by the symbionts.

1.4.4 Impact of Desiccation Stress

Lichens are classified as "poikilohydric" and have an ability to survive prolonged periods of desiccation. They lack any form of cuticle, and rely on precipitation, fog, dew, and airborne

moisture for water (Gasulla et al., 2021). Chlorolichens can resume photosynthesis activity when hydrated by even airborne moisture, while in contrast, cyanolichens require liquid water (Honegger, 2009). Some species of lichens can survive near-complete water loss (<0.1 g H_2O g^{-1} dry mass) (Gasulla et al., 2021), and can even recover after being stored desiccated at -20°C for years (Honegger, 2009). Nonetheless, significant water loss can be detrimental, for example, leading to overproduction of ROS (Kranner et al., 2008). Other effects include an alteration in membrane structures resulting in loss of cellular integrity resulting in K^+ ion loss upon rehydration, and eventually cellular collapse, reduced net photosynthesis, respiration and nitrogen fixation. As mentioned earlier, desiccation is often accompanied by high light stress, which can further increase ROS formation (Kranner et al., 2005). Beckett et al. (2021) proposed at least two reasons why lichens can be susceptible to light stress while desiccated. First, increased electron leakage from thylakoid membrane to O_2 occurs during drying when carbon fixation ceases before photophosphorylation. Second, normal repair processes are hindered in desiccated thalli, including the repair of the D1 protein (Holzinger and Karsten, 2013). Below we review how ROS can be formed from desiccation and high light stress.

1.5 Understanding how ROS are formed in two major stresses, desiccation and high light stress.

While different stresses are known to cause specific impacts on lichens, commonly they cause the formation of ROS (Beckett et al., 2008). ROS play a significant role in various contexts, both advantageous and detrimental. They operate as signals that initiate and stop biological processes and are inherently linked to cellular operations. ROS are typically present at minimal levels in healthy cells and are only harmful at high levels enhanced during abiotic and biotic stresses

(Weissman et al., 2005a; Françoise et al., 2014). As mentioned in the introduction above, ROS comprise of $^1O_2$, $O_2^{\cdot -}$, H_2O_2, and OH•, formed in either membranes, chloroplasts, mitochondria or peroxisomes, and can be lethal, causing extensive damage to protein, DNA and lipids and thereby affects normal cellular functioning (Das and Roychoudhury, 2014).

1.5.1 ROS formation by high light

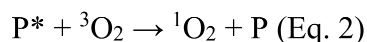
Under optimal conditions, lichen photobionts can photosynthesize efficiently by absorbing light energy through the light-harvesting complex (LHC) and transferring it to the photosystem reaction centers through a process known as resonance energy transfer, which initiates photosynthesis. In lichens, photosynthesis occurs within a limited timeframe when there is an adequate amount of light and, crucially, when the thalli are at least partially hydrated (Gasulla et al., 2012). However, when abiotic stress levels rise, light absorbed exceeds light needed for CO_2 fixation. This may lead to ROS formation, and as a result photoinhibition. Accumulation of ROS will occur as a result of limited CO_2 fixation and impaired ATP synthesis within the chloroplast electron transport chain (ETCs) of PSI and PSII during the photophosphorylation process (Muhammad et al., 2021). It is often a case that when CO_2 fixation is constrained or inhibited, electron transport continues to operate, transferring energy from excited chlorophyll pigments to triplet oxygen ($^3O_2$), ultimately generating $^1O_2$, $O_2^{\cdot -}$ and H_2O_2 (Gasulla et al., 2012).

1.5.2 ROS formation by desiccation

While lichens are known to be poikilohydric, desiccation and drought remain two of the strong contributors to increased ROS production in different cellular compartments, namely in the endoplasmic reticulum (ER), chloroplasts, peroxisomes and the mitochondria (Cao et al., 2022; de Carvalho, 2008). While peroxisomes and chloroplasts exhibit a faster rate of ROS generation

during stress, the same is true for mitochondria, and mitochondria are particularly more susceptible to oxidative damage due to their comparatively lower levels of antioxidant protection (de Carvalho, 2008). Furthermore, like other biotic and abiotic stresses, desiccation can cause loss of control mechanisms that maintain low ROS concentrations such as antioxidants (Kranner, 2002). In vegetative photosynthetic tissues, restricted photosynthesis upon desiccation and in the presence of light can result in the formation of $^1O_2$ from light energy transferred from photo-excited pigments onto $^3O_2$ (equation 1 and 2), thus also increase in excitation or inhibition of photophosphorylation increases $^1O_2$ production (See review by Kranner and Birtic, 2005). It has been estimated that about 1-2% of the oxygen consumed by plant mitochondria is utilized to generate $O_2^{\cdot -}$ in complexes I and III of the ETC (de Carvalho, 2008). In the ER, $O_2^{\cdot -}$ is generated through oxidation and hydroxylation reactions, involving NADPH as an electron donor and cytochrome P450 reductase (Cao et al., 2022). Kranner et al. (2008) outlines the effect of desiccation (Figure 1.3) and tolerance mechanisms involved.

$P + light \rightarrow P^*$ (excited pigment) (Eq. 1)

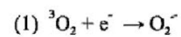


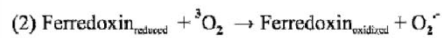
$P^* + {}^3O_2 \rightarrow {}^1O_2 + P$ (Eq. 2)

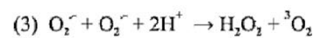
Desiccation of Lichen Thalli

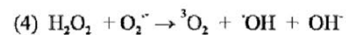
ROS Production Reactions

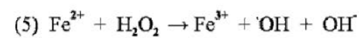
Formation of Reactive Oxygen Species

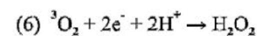


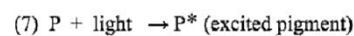


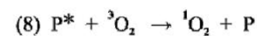


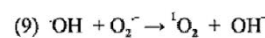






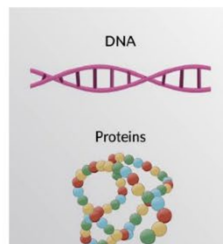




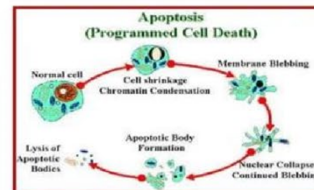


Damage **Initiation**

Results



Lipids



Loss of Viability

Figure 1.3: Desiccation induced ROS production in lichens. Equations (1) to (9) give examples of some reactions that typically produce ROS (Kranner et al., 2008).

1.6 Mechanisms involved in stress tolerance

To survive in challenging microhabitats lichens must be able to endure harsh environmental stresses. Lichens employ a combination of strategies, including avoidance mechanisms, scavenging of ROS, and repair mechanisms of damage caused by ROS, which are particularly effective when the partners are in symbiosis. For example, the cyanobacterium *Nostoc commune*, commonly found in lichens, exhibits at least three responses to dehydration, which involve deactivation of PSII reactions, efficient dissipation of absorbed light energy as heat, and cyclic electron flow around PSI (Kosugi et al., 2009). On the other hand, the mycobiont can employ various mechanisms to shield the photobiont beneath, such as the production of secondary lichen compounds and production of antioxidants (Kosugi et al., 2009). Therefore, the lichen's ability to withstand abiotic stresses is a result of the intricate interactions between these symbiotic partners. Below, some of the mechanisms employed by lichens to combat ROS are reviewed. Here, particular emphasis will be given to adaptations of the photobiont.

1.6.1 Mechanisms that prevent / avoid ROS formation

1.6.1.1 Protection by Secondary Lichen Compounds (SLCs)

Lichens produce secondary lichen compounds (SLCs) that form tiny crystals on the surface of their hyphae, acting as an effective "sunscreen". Generally, these compounds are synthesized in response to long-term exposure to high light by the mycobiont but protect both mycobiont and photobiont from the harmful effects of excess solar radiation (Ndhlovu et al., 2023). While some of these compounds are pigmented (e.g. parietin and vulpinic acid) and can absorb PAR and UV, most of them are colourless (e.g. atranorin). However, even colourless substances can protect the photobiont from high PAR by reflecting excess light energy from the cortex (Solhaug et al., 2010). One way they do this is to increase the hydrophobicity of the hyphal surface within the thallus

(Honegger, 2009) by forming air filled cavities that can prevent water entering the cortex of the thallus and directly reflect light (Ndhlovu et al., 2022; Solhaug et al., 2010). Furthermore, as may be predicted, sun adapted species contain higher concentrations of SLC than shade species, and as a result removal of these substances increases photobiont sensitivity to photoinhibition more than in shade adapted species (Ndhlovu et al., 2023).

Over 700 different lichen secondary compounds / metabolites with known structures have been reported so far (Huneck and Yoshimura, 1996), and the compounds are probably not primarily involved in photoprotection, but protection against biotic factors (e.g., antiherbivore and antibacterial defences) (Calcott et al., 2018; Phinney et al., 2019). The compounds are very diverse, and include anthraquinones, xanthones, shikimic acid derivatives (e.g., calycin, mycosporines, and scytonemin), phenolic compounds (e.g., depsidones, depsides, and diphenyl ethers) and classical pigments (e.g., melanin and carotenoids) (Nguyen et al., 2013). These compounds are synthesized by the mycobiont through various biosynthetic pathways such as polymalonate, shikimic acid and mevalonic acid pathways (Boustie and Grube, 2005).

Many of these compounds play a significant role in photoprotection, and more specifically UV protection. Compounds such as polyaromatic scytonemin, mycosporines and xanthones are produced by cyanolichens for protection against UV-A (Trest et al., 2015), while UV-B is controlled by depsidones, depsides, dibenzofuranes, diphenyl ethers and chromones (see review by Murugan et al., 2021). Phinney at al. (2019) showed that vulpinic acid in *Letharia vulpina* and other yellow pigmented compounds are effective in absorbing and screening of blue-light. It is worth noting that other SLC’s are found in the medulla layer and are believed to have a different function to those found in the upper cortex (Boustie and Grube, 2005). These includes physodic acid, physodalic acid and protocetraric acid (Molnár and Farkas, 2010).

1.6.1.2 Protection by Melanins

Melanins are important SLCs, and in addition to lichens, are also found in plants, free-living fungi, bacteria and animals. In lichens they occur as blackish / dark pigment-containing granules on the surface of the hyphae or between the plasma membrane and the cell wall (Daminova et al., 2023). Lichen melanins are only produced by the mycobiont and belong to either eumelanin or allomelanin groups. Eumelanins are formed from the polymerization of tyrosine L-dihydroxyphenylalanine (DOPA) using either tyrosinase or laccases, while allomelanins are produced from 1,8-dihydroxynaphthalene (DHN) using polyketide synthases (Mafole et al., 2019). The precise pathways for melanin biosynthesis in lichens have not been elucidated, and it seems likely that more than one lichen redox enzyme can catalyse melanin formation, including laccases, tyrosinases and peroxidases (Beckett et al., 2013). Both Peltigeralean and non-Peltigeralean lichens can produce melanins. Many Peltigeralean lichens make N-rich eumelanins, probably because they often include a cyanobacterial photobiont, while non-Peltigeralean species appear to produce N-poor DHN melanins (Matee et al., 2016). Belozerskaya et al. (2015), postulated that some free-living fungi can have more than one type of melanin, as in *Aspergillus fumigatus*; possibly the two types have different ecological roles.

Melanins can protect against harmful levels of both PAR and UV by absorbing these radiations (Mafole et al., 2019). However, the trigger for melanin biosynthesis appears to be UV rather than high PAR (see general reviews by Solhaug and Gauslaa (2012) and Mafole et al. (2019)). Apart from their obvious roles in protection against high PAR and UV-B, in other organisms they have been shown to be involved in the detoxification of heavy metals, ability to withstand hyperosmotic conditions, defence against microbes, and ability to tolerate pH shock

(Mafole et al., 2019). In addition, they are believed to increase tolerance to desiccation and ionising radiation.

When acting as light screens, melanins effectively convert light energy into heat and dissipate it at a rate of 99.9 %, which will reduce ROS formation (Meessen et al., 2013). Butler and Day (1998) postulated that melanins form a partially impermeable barrier at the thallus surface. For lichens, this may reduce the rate at which they desiccate. Furthermore, they offer a protective advantage when thalli are dry, lowering PAR transmittance and photoinhibition (Mafole et al., 2019); as discussed above, when desiccated, active repair does not take place (Solhaug et al., 2003). In melanised *L. pulmonaria*, the transmittance of the upper cortex to PAR is reduced by about 40% compared to pale thalli in desiccated thalli, whereas it is 30% lower in wet melanised thalli (Gauslaa and Solhaug, 2001). However, a negative consequence of melanisation is that CO_2 fixation is reduced by more than 40% at light levels lower than 100 μmol photons m^{-2} s^{-1} (Mafole et al., 2019). A further disadvantage of melanisation is the increase in overall thalli temperature (Mafole et al., 2019).

1.6.1.3 Mediated Xanthophyll Response / Regulation of Light Energy in Photobionts

In addition to possessing SLCs, lichen photobionts can also dissipate excess light energy to heat through non-photochemical quenching (NPQ) (Messant et al., 2021). NPQ can be readily measured by fluorimetry. NPQ works in different ways in cyanobionts and chlorobionts (Beckett et al., 2021). Cyanobionts contain pigments known as phycobilins, specifically phycocyanin and allophycocyanin, while chlorobionts possess light-harvesting complex (LHC) antenna proteins composed of carotenoids and chlorophyll pigments (Beckett et al., 2021). Three xanthophyll pigments, namely violaxanthin, antheraxanthin and zeaxanthin participate in NPQ in chlorobionts (Gasulla et al., 2012). These compounds undergo a cycle to efficiently regulate light energy in

photobionts. The xanthophyll cycle involves the conversion of violaxanthin to zeaxanthin during sunrise for carbon fixation and back to violaxanthin at sunset (Ralph et al., 2002), regulated by the enzyme violaxanthin de-epoxidase (VDE). Zeaxanthin dissipates excess energy as heat by quenching singlet excited states of chlorophyll molecules (Vrablikova et al., 2005). Overall, these carotenoids safeguard the chloroplast thylakoids from damage caused by excessive light (Gasulla et al., 2012). However, it is worth noting that the exact mechanism of the xanthophyll cycle remains uncertain.

In addition to the xanthophyll cycle, there are several other components to NPQ, which can to some extent be distinguished by their "relaxation" time in the darkness. Xanthophyll cycle-based NPQ is termed qE, and relaxes quickly, over a time scale of seconds to minutes. NPQ caused by state transitions is termed qT, and relaxes over a time scale of minutes. Photoinhibitory NPQ is termed qI and relaxes over a time scale of tens of minutes (Messant et al., 2021). Typically, NPQ gradually increases during exposure to light, and depending on the species can take 5 min or more to reach stable values.

Normally, energy-dependent quenching (qE) is the major component of NPQ and is the component that allows plants and lichens to rapidly modulate the amount of NPQ in response to changes in light conditions (Müller et al., 2001; Zhao et al., 2017). qE involves the activation of light-harvesting complex stress-related (LHCSR) proteins along with zeaxanthin, facilitating a quick and reversible reorganization of the PSII antenna system in response to a rise in ΔpH. This reorganization converts the antenna from light harvesting to light energy dissipating antenna (Derks et al., 2015). Unlike higher plants, where PsbS is responsible for high light sensing, LHCSR proteins in lichens play a crucial role in sensing lumen pH within the thylakoid and quenching excess light energy (Truong, 2011). Conversely, state transition quenching (qT) represents a more

gradually induced and sustained component of NPQ that involves a reconfiguration of LHC and the thylakoid membrane, leading to the creation of energy sinks capable of dissipating excessive energy as heat (Müller et al., 2001; Zaks et al., 2012). During high light stress, qT facilitates the migration of LHCII from PSII to PSI, enhancing PSI's light absorption while balancing the excitation of both photosystems (Minagawa, 2013). This reduces excessive excitation of PSII and helps optimize the efficiency of photosynthesis and minimizes the risk of photodamage. As light levels decrease, qT reverses within minutes, relocating LHCII back to PSII to once more optimize light capture for photosynthesis (Müller et al., 2001). Finally, photoinhibitory quenching (qI) is a slower and more prolonged component of NPQ, yielding a long-term photoprotective response. This component arises from photoinhibition and is not subject to rapid reversal (Müller et al., 2001; Zhao et al., 2017). It revolves around the creation of inactive reaction centers, shielding PSII from photodamage and relaxes very slowly (Zhao et al., 2017).

In contrast, cyanolichens appear to be generally more vulnerable to high light, with the level of protection varying among species (Shrader, 2011). While cyanolichens can produce zeaxanthin, potentially from β-carotene, they don't appear to possess the full xanthophyll cycle (Adams et al., 1993). Research by Adams et al. (1993) showed that *Peltigera rufescens* displays a broad range of zeaxanthin levels under high light conditions, which increases with increasing growth light intensity. Additionally, β-carotene was found in large quantities in all cyanophycean lichens, reaching up to 200 mmol carotenoid/mol chlorophyll a. Furthermore, rehydration of the cyanolichen *Peltigera polydactyla* was found to increase the concentrations of both zeaxanthin and violaxanthin, but interestingly these concentrations never exceeded those of β-carotene and α-tocopherol (Kranner et al., 2003). Therefore, it appears that rather than using the xanthophyll cycle,

cyanolichens use the "orange carotenoid protein" to dissipate excess light energy collected by phycobilisomes (Beckett et al., 2021).

The orange carotenoid protein (OCP) is a soluble protein in cyanobacteria and plays a crucial role in photoprotection as a light sensor and by quenching $^1O_2$ generated in the thylakoid membranes and dissipating excessive excitation energy harvested by the phycobilisome, contributing to high light tolerance (Sedoud et al., 2014). This process is induced by blue-green light triggering a conformational switch of the OCP from its dark-stable, orange form (OCP^O) to its light-activated, red form (OCP^R) through absorption by OCP's associated carotenoid, 3-hydroxyechinenone (Mach, 2014). OCP^R binds to the light-harvesting phycobilisome complex, where it quenches excess energy, diverting energy away from the photosystems and dispersing it as heat (Mach, 2014). This in turn reduces energy arriving at the photosynthetic reaction centers (Sedoud et al., 2014). Furthermore, OCP can also quench $^1O_2$ induced by exposure of light-harvesting complexes (antennae) and photochemical centers to high light, probably due to a reaction of molecular oxygen with the triplet states of the phycobilins (Sedoud et al., 2014; Kirilovsky and Kerfeld, 2016).

1.6.1.4 Adaptation of PSl to high light / cyclic- and pseudocyclic electron flow around PSI

Another essential element of photosystem protection involves the activation of NPQ by cyclic electron flow (CEF). This process entails the cycling of electrons around photosystem I (PSI) without generating NADPH (Suorsa, 2015; Beckett et al., 2023), and is believed to activate NPQ by acidifying the lumen. Despite limited knowledge about CEF's role in photoprotection, it appears to be a significant feature in lichens. In other organisms it has been shown that under high light, CEF is activated to prevent over-reduction of the electron transfer chain, safeguarding PSI from photodamage (Huang et al., 2015). CEF redirects electrons from PSI to the thylakoid

plastoquinone (PQ) pool, reducing NADPH synthesis and enhancing proton pumping by cytochrome (Cyt.) b6f (Beckett et al., 2023). NPQ is then rapidly induced by the trans-thylakoid proton gradient during photosynthesis, which requires PGR5/PGRL1-dependent CEF (Naranjo et al., 2021).

In algae, CEF exists in two main forms, one based on PGR5/PGRL1 and the other on NDH-2. PGR5/PGRL1 pathway is believed to be responsible for CEF in light conditions whereas NDH-2 is believed to primarily function in chlororespiration during darkness, reducing the PQ pool's NAD(P)H (Beckett et al., 2023). The PGR5-PGRL1-mediated CET plays a major role in electrochemical proton gradient ($\Delta\mu H^+$) formation and ATP synthesis, increasing the ATP/NADPH ratio to meet metabolic needs. On the other hand, NDH-mediated CET assists in balancing the redox state of electron carriers by cooperation of NDH and plastoquinol oxidase (PTOX) to deliver and remove electrons from the PQ pool respectively, thus preventing over-reduction, and providing additional ATP for metabolic reactions (Zolotareva and Polishchuk, 2022). Consequently, an imbalance in chlororespiration activity is considered the main trigger for CEF (Figure 1.4).

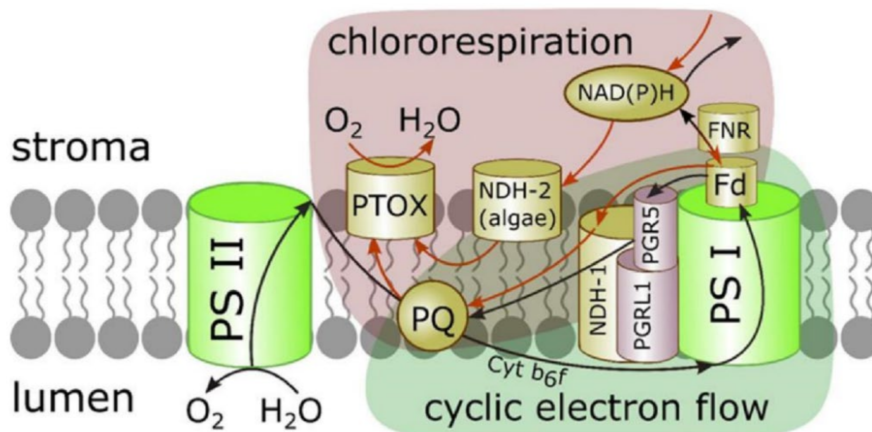


Figure 1.4: A schematic representation of chlororespiration and cyclic electron transport pathways in the chloroplast (Zolotareva and Polishchuk, 2022).

CEF serves at least three crucial biochemical roles in photosynthesis. Firstly, under high light, it prevents the overreduction of PSI, thereby reducing ROS formation and damage to PSI. Secondly, by pumping more protons into the thylakoid, it enhances ATP production, maintaining the ATP:NADPH balance. Thirdly it is involved in expediting NPQ induction to protect PSII (Beckett et al., 2023).

Photosynthetic organisms except for Angiosperms can employ pseudocyclic electron flow (PCEF) as an alternative electron transport pathway. While PCEF shares some similarities with CEF, it is distinct and facilitated by enzymes called flavodiiron proteins (FLV's or FDP's), which accept electrons downstream of PSI to reduce oxygen to water (Beckett et al., 2023). These enzymes are mainly active in the first moments of increased light intensity before increase in NADPH consumption by the Calvin-Benson cycle, resulting in prevention of over-reduction of PSl and protecting both PSl and PSll (Beckett et al., 2023). PCEF is known to regulate various physiological processes, such as photoprotection, high ATP demand, adaptation to environmental stress and regulation of electron transport, maintaining balance between light availability and the capacity of the photosynthetic apparatus (Storti et al., 2020).

Evidence of CEF, chlororespiration and PCEF in lichens and photobionts have been documented in very few studies. In the chlorobiont *Asterochloris erici*, darkness induces PQ reductase activity related to chlororespiration and LHC phosphorylation (Gasulla et al., 2019). This phosphorylation is presumed to detach LHC from PSII, reducing exciton-trapping and increasing light energy dissipation, preventing damage during dawn or under fluctuating light conditions when lichens are hydrated (Gasulla et al., 2019). Moreover, research by Beckett et al. (2023) on various lichens from different microhabitats indicates higher PCEF in sun-exposed

species, elevated FLV activity in melanised compared with pale forms of two shade species, and consistently high CEF across all lichens, with a tendency for higher values in sun-exposed species.

1.6.1.5 Reduction of ROS Formation by Alternative Oxidase

As indicated above, given that most of the work described in this thesis is on light stress, the emphasis of this Introductory chapter is on lichen photobionts. Nevertheless, it is worth noting that undoubtedly lichen mycobionts possess a variety of mechanism to reduce stress-induced ROS formation e.g. the alternative oxidase (AOX). The alternative oxidase works in mitochondria to provide an alternative route for electrons passing through the ETC (Shelyakin et al., 2022). In general, the ETC in mitochondria of plants, algae and fungi is structured in a way that allows electrons within the ubiquinone pool to pass to oxygen through either the typical cytochrome respiratory pathway (CP) associated with ATP synthesis, or through an alternative route involving AOX (Jayawardhane et al., 2020; Shelyakin et al., 2022). AOX bypasses the CP at the level of ubiquinone, reducing oxygen to water and dramatically reduces ATP generation and dissipating energy as heat (Saha et al., 2016). The overreduction of ubiquinone due to elevated stress such as UV is a major source of mitochondrial ROS. The AOX then accepts electrons from ubiquinone and reduce molecular oxygen to water, preventing overreduction of the ubiquinone pool and lowering ROS formation (Shelyakin et al., 2022).

1.6.2 Mechanisms that scavenging ROS during stress

1.6.2.1 Effective ROS Scavenging Antioxidants

1.6.2.1.1 Enzymatic Antioxidants

Should NPQ and other mechanisms fail to adequately prevent ROS formation, ROS can be scavenged by a range of antioxidants. With the exception of some anaerobic bacteria, these antioxidants are found in all living organisms, including humans. These antioxidants can be

broadly categorized into two groups: enzymatic (such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX)) and non-enzymatic antioxidants (including glutathione (GSH), ascorbate (ASC), and tocopherols) (Kranner et al., 2008). An increase in production of ROS e.g. during stress tends to correlate with an upregulation of antioxidants in organisms in general, although in some cases they appear to be constitutive (Kusvuran et al., 2016; Mkhize et al., 2020), particularly in organisms growing in microhabitats where the onset of stress is very rapid. However, different species possess different antioxidants that are involved in various defence strategies. In lichens, many studies have focused on desiccation and rehydration stress. For example, transcriptomic analysis has revealed the upregulation of MnSOD in the lichen photobiont *Trebouxia gelatinosa* during dehydration, suggesting a role in enabling rapid recovery during rehydration (Carniel et al., 2016). Another study revealed that during rehydration following desiccation, the photobiont of *Ramalina lacera* produces four Fe-SOD and four Mn-SOD enzymes, while the mycobiont produces Cu/Zn-SOD and Mn-SOD isoforms (Weissman et al., 2005b). Additionally, there is evidence suggesting a link between POX and SOD and lichen resistance to SO_2 pollution (Pescott et al., 2015), but in lichens this has been poorly studied. In other organisms Class III peroxidases are often upregulated by a variety of stresses, but lichen mycobionts, being mostly ascomycetes, do not possess these enzymes (Morgenstern et al., 2008), and Class III peroxidases are absent from chlorophycean algae (Mathé et al., 2010). However, the chlorophycean photobionts do possess Class I (ascorbate) peroxidases (APX) (Maruta et al., 2016). Liers et al. (2011) described an unusual haem peroxidase from a jelly lichen that does not belong to the classical higher plant or fungal groups of peroxidases. However, it would seem unlikely that fungal peroxidases can reduce oxidative stress in the photobionts.

1.6.2.1.2 Non-Enzymatic Antioxidants

Among the non-enzymatic antioxidants, glutathione (GSH) is regarded as one of the most important antioxidants (Bartak et al., 2004) and is important in modulating oxidative stress by scavenging ROS (Kranner et al., 2005; Kranner et al., 2008). During stress, GSH is oxidized to glutathione disulfide (GSSG), which can inhibit protein synthesis and induce programmed cell death. To prevent this, and regenerate GSH, GSSG is reduced back to GSH by the enzyme glutathione reductase (GR) following the relief of stress (Kranner et al., 2005). It is postulated that glutathione undergoes different phases in response to photo-oxidative stress, such as (1) dynamic (or the initial phase of a stress response) and (2) a steady-state acclimation stage (Vrablikova et al., 2005; Tausz et al., 2004). The importance of GSH in desiccation tolerance was demonstrated in a classical study by Kranner (2002) and Kranner et al. (2003) with the lichens *Pseudevernia furfuracea*, *Peltigera polydactyla* and *Lobaria pulmonaria*. Results showed that differences in desiccation tolerance are correlated with the redox status of GSH, particularly the ability to reduce GSSG during rehydration. Other functions of GSH and GSSG includes storage and transport of reduced sulfur and detoxification of xenobiotics (Balarinova et al., 2014). GSH is also involved in regeneration of dehydroascorbate to ascorbate (vitamin C) (Vrablikova et al., 2005).

Similar to GSH, ascorbate is also a low-molecular-weight, non-enzymatic antioxidant. It serves as a potent substrate for neutralizing H_2O_2. In higher plants, the combined action of ascorbate and GSH enhances osmoregulation, plant water levels, nutrient status, water use efficiency, photosynthetic performance, and overall plant productivity (Hasanuzzaman et al., 2019). The application of exogenous ascorbate and GSH can improve the plant's antioxidant defence and its ability to withstand various environmental stresses (Hasanuzzaman et al., 2019).

The Asada-Halliwell pathway, also referred to as the Ascorbate-Glutathione cycle, is a metabolic process aimed to detoxify H_2O_2. While it actively occurs in higher plants, ferns and bryophytes, it remains unclear whether this pathway works in unicellular green algae (Beckett et al., 2021). It involves ascorbate, glutathione, and four enzymes: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Hasanuzzaman and Bhuyan, 2021). This pathway consists of several steps, as outlined by Hasanuzzaman and Bhuyan (2021) and Hasanuzzaman et al. (2019). H_2O_2 is converted into water by APX, with ASC providing the necessary electrons. The oxidized ASC, known as monodehydroascorbate (MDA), is then regenerated by MDAR. However, MDA is a radical, and if it's not promptly reduced, it decomposes into ASC and dehydroascorbate (DHA). The enzyme dehydroascorbate reductase (DHAR) then subsequently reduces DHA back to ASC, using GSH as the electron source. The oxidized GSH is then reconverted into GSH by GR, with NADPH serving as the electron donor (Figure 1.5).

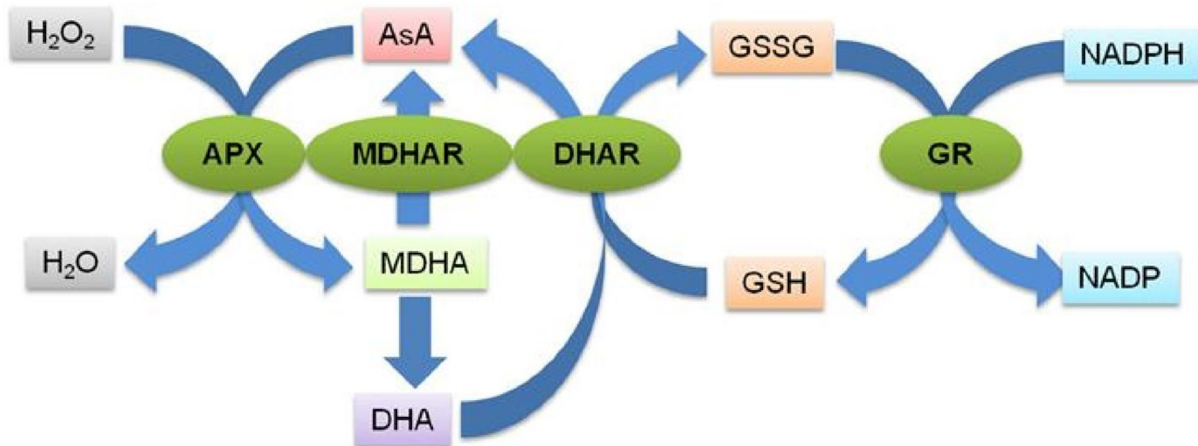


Figure 1.5: A schematic representation of the Asada-Halliwell pathway (Ascorbate-Glutathione pathway) ensuring effective detoxification of ROS and maintenance of redox balance under abiotic stress conditions (Pandey et al., 2015).

The less studied molecule nitric oxide (NO), widely known as a signalling molecule may act as an antioxidant by regulating lipid peroxidation, acting as a chain-breaking antioxidant to scavenge peroxyl radicals and photobiont photo-oxidative stress. This has been shown in *Trebouxia sp*. and *Asterochloris erici* isolated from *Ramalina farinacea* (Gasulla et al., 2012). Just like ROS, NO is an important major signalling molecule in plants that is induced by stress, but which can also be toxic, causing cell death and ROS production (Gasulla et al., 2012). Interestingly, it has been suggested that NO may help stabilize PSll and reduce photobiont photo-oxidative stress during rehydration (Gasulla et al., 2012). In *Ramalina lacera,* a burst of intracellular ROS was detected from both symbionts upon rehydration, and yet was accompanied by NO production in the fungus but not in the algae (Weissman et al., 2005a). Other findings suggest that NO is also involved in phototaxis and the general "stress response" in algae, whereas NO control of the formation and growth of fruiting bodies have been reported in fungi (Song et al., 2000). Thus, available data suggest that NO is likely to play a role in lichen stress tolerance, but more work is required to confirm this.

1.6.2.2 Accumulation of Heat Shock Proteins (HSP's)

Heat shock proteins (HSP's) are classified by their molecular sizes into various classes like small HSP's, HSP40, 60, 70, 90, and 110 families. These proteins are important in the response of almost all organisms to stresses such as temperature, UV light, and biotic factors (Gong et al., 2017; Garcia-Garcia et al., 2012). HSP's are considered as an almost universal response to high temperature, hence they are called 'heat shock' proteins. They play a crucial role in protein folding and stability during stress (Park and Seo, 2015; Muggia et al., 2016). Many appear to be chaperones as they can aggregate when denatured by heat stress (Park and Seo, 2015). HSP's may also be involved in desiccation tolerance, and it has been speculated that they aid in maintaining general

cell structure and the stability of macromolecules during desiccation (Gasulla et al., 2021). Various lichen species express different HSP genes in response to dehydration and rehydration. An isolated lichen phycobiont *Asterochlori erici* was found to express Hsp90 genes during dehydration, whereas Hsp70 were constitutively expressed in *Trebouxia gelatinosa* (Gasulla et al., 2021). In the lichen *Cladonia rangiferina,* Hsp98 were upregulated in response to rehydration (Junttilla et al., 2013).

1.6.2.3 LEA Proteins and Desiccation Tolerance

LEA proteins, a family of hydrophilic proteins, are crucial for environmental stress tolerance, particularly drought and desiccation. They provide structural support for macromolecules and cellular structures in the cytoplasm, and in conjunction with sugars, they increase glass transition temperature and hydrogen bonding of the glass formed under stress (Beckett and Minibayeva, 2007). D-11 proteins, also known as dehydrins are an important group of LEAs. These proteins are important in improving desiccation tolerance, particularly in seeds, and are involved in maintenance of cytosolic desiccation tolerance and cell wall mechanical failure (Layton et al. 2010). While they appear not to be present in the mycobiont, they certainly occur in the photobiont (Carniel et al. 2016). Various LEAs have been discovered in *Trebouxia gelatinosa*; desiccation increased the expression of dehydrin (group 2 LEAs). Two group 3 LEAs appeared to be constitutively expressed (Carniel et al. 2016). Furthermore, expression of LEA4 and LEA5 was upregulated in the free-living green algae *Zygnema circumcarinatum* upon desiccation stress (Rippin et al., 2017).

1.6.3 Mechanisms that repair cellular damage caused by ROS

Plants and lichens have developed various mechanisms to repair DNA damage resulting from ROS. Once they reach a critical level, ROS can inhibit the de novo synthesis of the D1 protein, suppress ROS-scavenging enzymes, and disrupt the thylakoid membrane (Gururani et al., 2015). Only limited information is available on the specific mechanisms that repair ROS-induced cellular damage in lichens. So far, much of our knowledge has been directed towards the repair cycle of PSII induced by high light stress.

1.6.3.1 PSll Repair Cycle

The PSII reaction center complex includes two crucial proteins, namely the D1 and D2 proteins, which form the core of the reaction center and are highly sensitive to UV-B exposure and, in some cases, even moderate light intensities (Beckett et al., 2021). In the PSII repair cycle, only the damaged reaction center D1 protein and occasionally also the D2, CP43, and PsbH subunits are replaced (Järvi et al., 2015). This repair cycle begins in the grana stacks where the damage typically occurs but then continues in non-appressed thylakoid regions, where many steps are common to both the repair and de novo assembly of PSII (Järvi, et al., 2015). These steps were extensively detailed by Järvi et al. (2015) (Figure 1.6) and Theis and Schroda (2016) and are as follows: (1) Photodamaged PSII undergoes reversible phosphorylation of PSII core subunits, triggering the disassembly of PSII and the degradation of the damaged D1 protein. (2) The phosphorylated PSII complex becomes monomeric and migrates laterally from grana to stroma thylakoids. (3) The monomeric PSII complex then partially disassembles. (4) The damaged D1 protein is proteolytically degraded. (5) The damaged D1 protein is then replaced with a new copy. (6) The PSII monomers are reassembled and migrate back to grana thylakoids for dimerization and supercomplex assembly, effectively restoring PSII function.

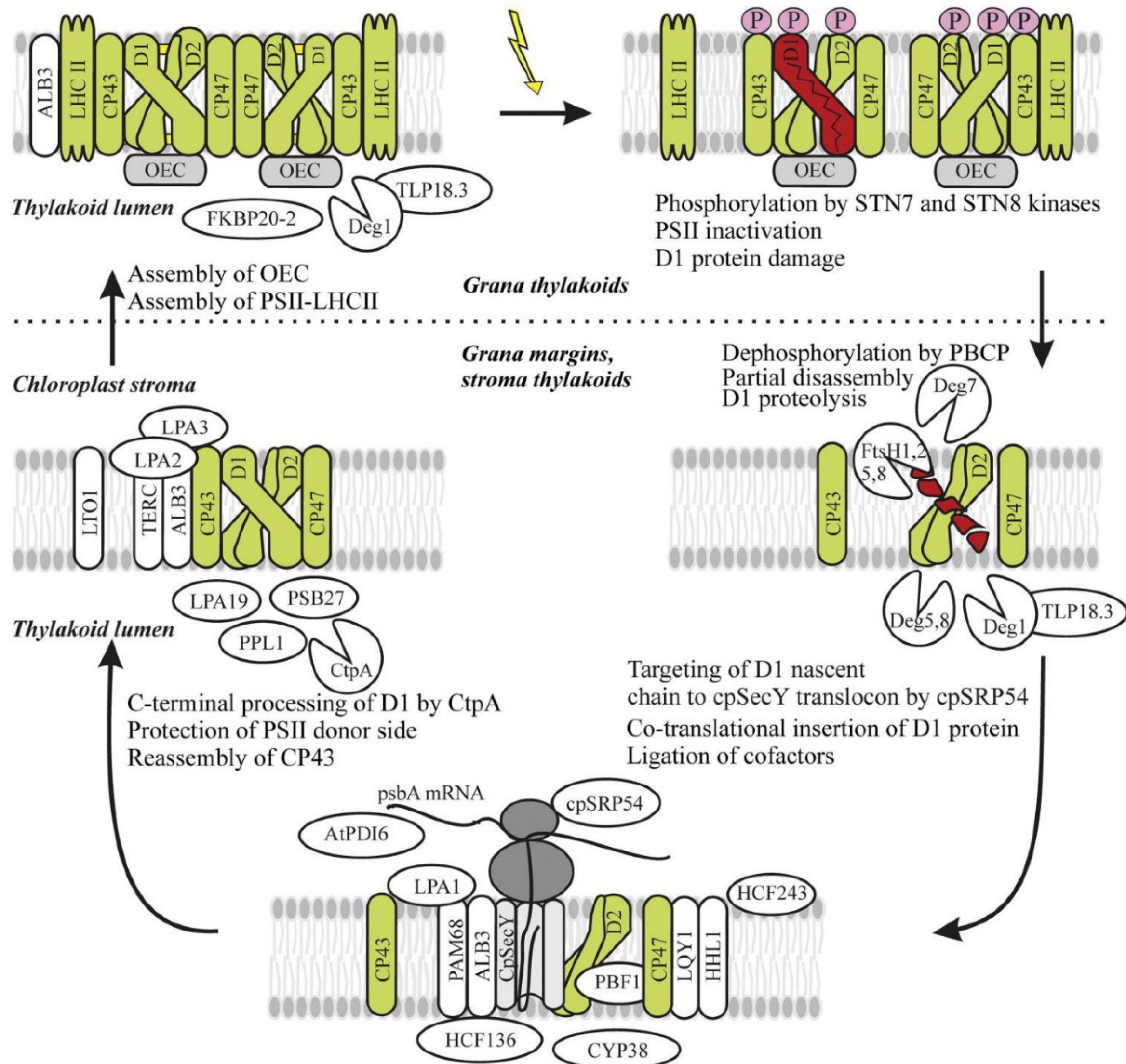


Figure 1.6: A schematic representation of the PSII repair cycle responsible for repair of light induced damage of D1 protein in plant chloroplasts. The damaged D1 protein is highlighted by the red color (Järvi et al., 2015).

1.7 Physiological and Morphological Adaptations

In addition to stress tolerance mechanisms mentioned above, stress can induce some physiological or morphological changes in the lichen thallus (Sadowsky and Ott, 2015). These adaptations include changes in colour, thallus structure, and physiological alteration to mitigate the effects of environmental stresses. Lichens, including other poikilohydric organisms, can endure extended periods of desiccation by mechanisms such as a temporary suspension of photosynthesis, and the accumulation of protective compounds. In an event of thallus folding or curling, both partners shrink and the fungal cells cavitate (Honegger, 2009). In species like *Lobaria pulmonaria*, curling has been associated with photoprotection with less visible bleaching in curled lobe tips compared to normal lobes on sun transplanted material (Bartak et al., 2006). Overall, these mechanisms allow them to quickly recover their metabolism and photosynthesis upon rehydration. Nevertheless, the ability to recover relies on water availability and most importantly the type of photobiont. However, in some species, too much of water saturation can hinder the diffusion of CO_2 to the algae cells. It has been suggested that this may lead to the photobiont translocating all its products to the fungus, which therefore will lead to the death of the photobiont (Gasulla et al., 2012). Furthermore, desiccation could be conceived as an advantage as a photoprotective mechanism, because, as discussed above, dry thalli are much more tolerant to photooxidative damage.

1.8 Concluding Remarks and Introduction to the Present Study

Many studies have shown that lichens are able to thrive in extreme environments. It is intriguing how both components, the mycobiont and photobiont, possess adaptations facilitating survival and growth in extreme microenvironments. However, more research is still required in this field. Undoubtedly, lichens can show great plasticity in their response to environmental stresses such as

high light and/or UV, temperature and desiccation. Sun adapted species are probably in general more tolerant to stress than shade species. With increase in global warming and deforestation, shade species in particular experience increased exposure to unfavourable conditions.

Therefore, the work presented here aims to compare and understand the tolerance mechanisms employed by both chlorolichens and cyanolichens in response to stresses that are exacerbated by climate change, such as desiccation and light stress. In particular, the study aims to assess phenotypic plasticity by comparing the role of melanin, NPQ, and glutathione in stress tolerance in lichens from shaded and exposed microhabitats. Additionally, the study aims to identify the underlying mechanisms of stress tolerance, with the intention of exploring potential applications of the findings in biotechnological or agricultural contexts, such as transferring the responsible genes into crop species. Therefore, results from this study will enhance our understanding of the mechanisms of stress tolerance in lichens, which may help to improve tolerance in crop species.

Throughout at least the last 11,000 years agriculture has sustained humanity (Walter and Kromdijk, 2022). However, with the world's population projected to reach around 11 billion by 2100, there's a pressing need to significantly increase food production. This poses a significant challenge as available agricultural land is becoming more and more limited (Walter and Kromdijk, 2022). While agricultural areas in the northern hemisphere have remained stagnant or even decreased over the past three decades, tropical regions have witnessed a surge of up to 20% in agricultural land use, often at the expense of forests being cleared for food and animal feed. This raging deforestation has already resulted in over 50% loss of rainforests (Walter and Kromdijk, 2022).

Recent studies exploring potential increases in atmospheric CO_2 levels have demonstrated that genetic modifications can enhance photosynthesis, leading to improved crop yields (Leister, 2022). As suggested by the title of a keynote address at a recent conference, photosynthesis is "Ancient, essential, complex, diverse… and in need of improvement in a changing world" (Niinemets et al., 2017). Modelling studies suggest that improving quantum yield and electron transport capacity hold greater potential for increasing crop productivity compared to other photosynthetic mechanisms such as improving rubisco activity (Walter and Kromdijk, 2022). This is partly due to the inherent variability of sunlight. Even on a sunny day, the upper leaves in a canopy can experience fluctuations in light exposure and spectral features when clouds cover the sun (Walter and Kromdijk, 2022). However, more subtly, it is now realized that plants, particularly those in lower canopy levels, experience rapid changes in light levels, known as "sunflecks" (as detailed above) due to factors such as shifting sun angles and wind-induced leaf movements. These fluctuations mean that plants must protect themselves from photoinhibition, where excessive light absorption exceeds the capacity for carbon fixation. Therefore, manipulating aspects of photosynthesis like quantum yield and electron transport cycle to enable a rapid response to fluctuation in light availability could enhance crop yield by facilitating faster relaxation of NPQ and recovery of CO_2 fixation rate and potentially maintaining higher photoprotection levels under intense light conditions (Simkin et al., 2019). However, it is worth noting that rubisco activation during the transition from darkness to light is comparatively slower, potentially limiting photosynthesis. Therefore, increasing rates of rubisco activation represent another potential way to improve crop yields (Simkin et al., 2019; Walter and Kromdijk, 2022).

The specific objectives of the research presented here were to:

1. Compare the role of NPQ in the adaption to light in sun and shade species of lichens.

2. Test whether within a range of lichens species, whether within one species sun and shade collections display different adaptations to light.

3. Testing the phenotypic plasticity of lichens responses to light stress by examining how laboratory manipulations can alter the tolerance of photosynthesis and general characteristic of photosynthesis.

4. Testing the role of melanisation in desiccation stress, by measuring the GSH redox potential during a drying / wetting cycle in pale and melanised thalli of the lichen *Lobaria pulmonaria*.

1.9 Structure of the thesis

The main body of this thesis is organised as manuscripts prepared for publication in peer-reviewed journal articles. The first chapter (this chapter, Chapter 1) is the Introduction which comprises a literature review of the concepts covered in this study. The next four chapters (Chapters 2, 3, 4 and 5) are experimental chapters with each one covering a specific objective. Each chapter is formatted according to the journal in which it has been published, or for Chapter 5, the journal which it is intended to be submitted to. Because of this thesis format, a certain degree of repetition, especially in the methods section, was unavoidable. However, this is deemed to be of little concern as this format allows the reader to read each chapter separately without losing the overall context of the thesis. Chapter 2 investigated the ability of the lichen photobiont to harden to photoinhibition by pretreatment with light. Chapter 3 investigated the induction and relaxation of NPQ components on transition to darkness as well as electron transport rates (rETR), in lichens from exposed and shaded locations. In Chapter 4 the induction and relaxation of NPQ and the induction of rETR in a range of Afromontane lichens that generally grow in sunny sites with shade collections of the

same species are compared. In addition, the induction and relaxation of NPQ in melanized and pale thalli of three shade lichens are compared. Chapter 5 investigated the role of melanins in stress tolerance and GSH metabolism in the lichen *Lobaria pulmonaria.* Chapter 6 presents a summary of the findings and recommendations.

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CHAPTER 2

Lichen photobionts can be hardened to photoinhibition by pretreatment with light

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Formatted for: Acta Physiologiae Plantarum

Abstract

Lichens often grow in microhabitats where they receive more light than they are capable of using to fix carbon. Unless regulated, this excess energy can end up activating oxygen, thereby forming reactive oxygen species (ROS). These ROS can damage the photosynthetic apparatus and other cellular components, causing photoinhibition and photo-oxidative stress. Tolerance to high light theoretically can be achieved in a variety of ways, but for lichen photobionts, our knowledge of the precise mechanisms involved is rather fragmentary. Here, we show that tolerance to photoinhibition in the cephalolichen *Crocodia aurata* could be increased by pretreating thalli at a moderate light intensity for 48 h. Increased tolerance was correlated with increased ability to avoid oxidative stress by dissipating the excess energy as heat, here assessed by measuring non-photochemical quenching (NPQ). Increased tolerance only occurred when thalli were pretreated hydrated. The same pretreatment did not increase tolerance in the cyanolichen *Sticta fuliginosa*, which grows in similar habitats to *Crocodia*, suggesting that cephalo- and cyanobacterial lichens may require different pretreatment conditions. Similarly, tolerance was not increased in collections of the chlorolichen *Ramalina celastri* from exposed habitats, although additional experiments showed that fluorescence parameters in *Ramalina* can display considerable plasticity. It seems likely that in "sun" populations tolerance is fully expressed and cannot be further increased. However, the ability to harden *Crocodia* to photoinhibitory stress could provide a foundation for more detailed investigations into the mechanism of photoprotection in lichen photobionts such as the type of NPQ or the role of antioxidant enzymes.

Keywords: Photoinhibition · High light stress · Acclimation · Non-photochemical quenching (NPQ) · Electron transfer reaction (ETR)

2.1 Introduction

Light is essential for photosynthesis but when a photosynthetic organism absorbs more light than they can use in carbon fixation, the photosynthetic apparatus can be damaged by a process often termed "photoinhibition" that will eventually reduce growth. While there is currently no consensus on how exactly photoinhibition occurs (Zavafer and Mancilla 2021), most workers believe that photoinhibition occurs when an excess of energy causes the production of reactive oxygen species (ROS) (Pospíšil 2016). ROS can cause lipid peroxidation or damage to the PSll complexes, mainly the D1 and D2 proteins in the reaction centre (Foyer 2018). For a variety of reasons, lichen photobionts are often exposed to high light stress. For example, lichens growing on trees can sometimes be exposed to sudden increases in light intensity (for example if surrounding trees are felled), and it has been shown that this causes long-term depression of photosynthesis (Gauslaa and Solhaug 2000; Jairus et al. 2009). Leisner et al. (1997) showed that even in *Lecanora muralis* growing under mild temperate conditions photoinhibition occurs regularly. Photoinhibition can also occur when lichens are desiccated (Kershaw and MacFarlane 1980; Mafole et al. 2019). Although it is not entirely clear how this occurs, in bryophytes, energy can move from the light-harvesting pigments to the reaction centres in desiccated thalli (Heber et al. 2006). It remains unknown whether most photoinhibition occurs while lichens are hydrated or desiccated, but it is clear that in the field lichens, are regularly photoinhibited (e.g. Gauslaa et al. 2019).

As for all photosynthetic organisms, lichen photobionts have developed extensive mechanisms to either protect themselves or cope with the effects of high light stress. Theoretically, tolerance mechanisms can be broadly divided into those most likely to be important for changes in light availability in the longer or shorter terms, although there is overlap between the two categories. Tolerance mechanisms to long term (over a range from weeks to months) and possibly

more pronounced light stress include the fungal symbiont synthesizing cortical light screening pigments (Solhaug and Gauslaa 2012), and the photobiont regulating the amount of LHC proteins or changing the ratio of PSII to PSI (Kim et al. 2020). Tolerance to short-term changes in light availability (over a range from minutes to a few days) can be improved in photobionts first by increasing the dissipation of excess energy absorbed without radiation as heat using non-photochemical quenching (NPQ), second by increasing the ability to scavenge ROS formed during photoinhibition, and third by increasing the ability to repair ROS-induced damage. However, the relative importance of these mechanisms for the photoprotection of lichens in field situations is largely unknown. For example, while it has been reported that populations of *Peltigera aphthosa* from open habitats are much more tolerant to light stress than material from shaded locations (Kershaw and MacFarlane 1980), the tolerance mechanisms responsible are unknown. More generally, the relative importance of various protecting strategies in different types of lichens from various habitats has been little studied. It could be expected that the relative importance of the mechanisms of tolerance to high light in lichens such as *Lobaria pulmonaria*, which normally grows in habitats with a maximum light level rarely exceeds 100 µmoles m^{-2} s^{-1} (Gauslaa and Solhaug 2000), will differ from those in lichens that form exposed soil crust communities.

One potential approach to studying the tolerance of photobionts to high light would be to investigate tolerance mechanisms in material collected from locations in the field differing in light availability such as those described by Kershaw and MacFarlane (1980). However, a limitation to this approach is that the habitats of lichens collected from "sun" and "shade" populations may differ in more than just available light; for example, N supply may vary (Piccotto and Tretiach 2010). As an alternative, a laboratory-based approach could be used, particularly to test for short-term adaptations to light stress. In this approach, the ability of lichens to acclimate to a

photoinhibitory light stress would be tested by giving a prior exposure to moderate light over, for example, a few days. Should hardening occur, mechanisms of tolerance to high light could be compared in hardened and non-hardened material. Perhaps surprisingly, the potential for using this approach has not yet been tested with lichens. In addition to elucidating tolerance mechanisms, it could be used to compare phenotypic plasticity in tolerance to light stress in lichens from different habitats. Some lichens grow in habitats with continuously changing light availability. For example, temperate corticolous species experience seasonal changes in solar radiation and canopy cover. In addition to seasonal changes, such species often experience rapidly changing light during the day, and many can probably be classified as "sunfleck" species. These species could be predicted to display significant plasticity. By comparison, lichens growing in exposed sites in sub-tropical or tropical areas where there are less diurnal and seasonal variations in light availability could be predicted to display less ability to harden.

In the present study, we selected three model species from contrasting habitats, and tested their ability to display short-term hardening to light stress. As acclimation to some stress factors e.g., high temperature can occur even when lichens are dry (Kershaw 1985), we compared the effect of hardening pretreatments in desiccated and hydrated lichens. The first two species were the cephalolichen *Crocodia aurata* with the chlorophycean photobiont *Symbiochloris*, and the cyanolichen *Sticta fuliginosa* with the photobiont *Nostoc*. Both species normally grow on the trunks of deciduous trees inside forests, and are rarely found in sun-exposed microhabitats. These species can be regarded as "sunfleck" lichens, as they experience rapid changes in light levels. In support of this, Gauslaa et al. (2007) showed that transplanting *Lobaria pulmonaria* to exposed localities (simulating "clearcutting") significantly reduced chlorophyll fluorescence parameters and chlorophyll concentrations. As *L. pulmonaria* has the same photobiont as *Crocodia aurata*

and often occurs in similar habitats, the results of Gauslaa et al. (2007) suggest that both species are best adapted to shade.

The third model species was the chlorolichen *Ramalina celastri*, which normally grows in exposed locations on the peripheral branches of trees, and is exposed to relatively high and constant levels of light. This appears to be typical for the habitat of *R. celastri* from other parts of the world (e.g. Nash et al. 2004). However, *R. celastri* can be collected from more shaded microhabitats, similar to those occupied by *C. aurata* and *S. fuliginosa*, although it is much less common in the shade. The photobiont of *R. celastri* is the chlorophycean alga *Trebouxia*. It may be relevant that Nelsen et al. (2021) recently suggested that early *Trebouxia* lineages largely occurred as symbionts in temperate forest lichens, but later diversified so that this genus now also forms the photobiont of lichens growing in more stressful non-forested habitats. We hypothesized that *Crocodia* and *Sticta*, which grow under conditions of greater fluctuations of light, will display greater ability to harden to light stress than *Ramalina* collected from the periphery of the canopy, which grows under more constant light conditions. As *Crocodia* and *Ramalina* have chlorophycean photobionts we also tested whether any hardening is correlated to changes in NPQ, a readily measurable short-term tolerance mechanism. Furthermore, as *Ramalina* can also be collected from more shaded habitats, we investigated the plasticity of photosynthesis in the species. We compared the characteristics of photosynthesis in "sun" populations with collections from uncharacteristically shaded habitats, and also sun populations treated in the laboratory to fluctuating low light intensities for several days.

2.2 Materials and methods

2.2.1 Lichen material

All lichens were collected dry from a small patch of Afromontane forest in Fort Nottingham, KwaZulu Natal, South Africa. The area occurs at an altitude of between 1500 and 1600 m; the climate is characterized by warm, wet summers, and dry cold (down to freezing temperatures) winters. Most lichens were collected from the small tree species, *Leucosidea sericea* Eckl. and Zeyh. Most of the *Ramalina celastri* (Sprengel) Krog & Swinscow was collected from minor twigs at the periphery of the canopy, while *Crocodia aurata* (Ach.) Link and *Sticta fuliginosa* (Hofm.) Ach. were collected from shaded microhabitats on the main branches or tree trunks. Lichens are henceforth referred to by the respective genus names. *Ramalina* was also collected from more shaded microhabitats, similar to those occupied by the other two species. Lichens were thoroughly cleaned, allowed to air dry between sheets of paper toweling, and stored at – 24 °C for up to four weeks. Thallus segments (1 cm or longer) of mature *Ramalina* and 1 cm discs from between the margins and the centre of *Crocodia* and *Sticta* thalli were used. The disk size used was large in relation to the overall thallus size, and probably comprised both younger and older material. When hydrated material was required, before experimentation lichens were placed on moist filter paper and stored for 24 h at 20 µmol photons m^{-2} s^{-1} at 12 °C in a growth cabinet. We have found that using these conditions, hydrated lichens remain in a healthy state for at least one week, longer than the time needed to complete all experiments described here. All treatments comprised ten replicates.

2.2.2 Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured using a the Hansatech Instruments (King's Lynn, UK) FMS 2 fluorimeter. After a dark adaptation period of at least 10 min, a saturating pulse of light was given, and the maximal efficiency of PSII (F_V/F_M) measured, where F_M = maximum fluorescence and F_V = variable fluorescence or ($F_M – F_0$), where F_0 = minimal fluorescence yield of the dark-adapted state. Thalli with anomalous values of F_V/F_M were discarded. An actinic light at 50 µmol m^{-2} s^{-1} was switched on, and when the fluorescent signal was stable (typically c. 1 min) another saturating pulse of light given. The relative ETR was calculated as:

rETR = 0.5 x φPSII x PAR

where PSII is the effective quantum yield of PSII photochemistry calculated as ($F_{M'}$ – F_t)/$F_{M'}$, where $F_{M'}$ = maximal fluorescence yield of the light-adapted state and F_t = stable fluorescence signal in the light. NPQ was calculated using the formula of Bilger et al. (1995):

$NPQ = (F_M – F_M')/F_M'$

2.2.3 Hardening to light stress

In preliminary experiments, hydrated material of *Crocodia* and *Ramalina* was pretreated with light at 200 µmol m^{-2} s^{-1} at 20 °C for 6 h using a LED panel (cool white light, Model SL – 3500, Photon System Instruments, Brno, Czech Republic), while control samples were kept hydrated in a growth cabinet at 20 µmol m^{-2} s^{-1}. F_V/F_M was measured at time zero, and after 2, 4 and 6 h. All material was then transferred to 20 µmol m^{-2} s^{-1}, 12 °C until 24 h after the start of exposure to light at 200 µmol m m^{-2} s^{-1}. F_V/F_M was again measured, and then lichens were photoinhibited using the same LED panel by exposing them to 400 µmol m^{-2} s^{-1} for 6 h. Lichens were kept on Petri dishes lined with moist filter paper and were kept fully hydrated by the addition of distilled water when required. Chlorophyll fluorescence parameters were measured every 2 h. After photoinhibition,

lichens were allowed to recover at 20 µmol m^{-2} s^{-1}, 12 °C, and measurements taken 48 h after the start of exposure to light at 200 µmol m^{-2} s^{-1}.

As preliminary experiments to harden lichens were unsuccessful, two alternative hardening treatments were used. Lichens were pretreated with 100 µmol m^{-2} s^{-1} light intensity at 20 °C for 48 h in either a dry state or in a hydrated state, while control samples were kept hydrated or desiccated in the growth cabinet at 20 µmol m^{-2} s^{-1}. After pretreatment, dry lichens (both controls and lichens receiving the light pretreatment) were hydrated by placing them on moist filter paper in the growth cabinet at 20 µmol m^{-2} s^{-1}, 12 °C for 24 h, while the moist material was kept hydrated under the same conditions. F_V/F_M, ETR and NPQ were then measured, and then lichens were photoinhibited by exposing them to 800 µmol m^{-2} s^{-1} for 8 h. Chlorophyll fluorescence parameters were measured every 2 h. After photoinhibition, lichens were allowed to recover at 20 µmol m^{-2} s^{-1}, 12 °C, and measurements taken after 24 and 48 h.

2.2.4 Plasticity of photosynthesis in Ramalina celastri

To further investigate the flexibility of photosynthesis in *Ramalina*, a collection was also made from the limited material growing in shaded microhabitats similar to those occupied by *Crocodia* and *Sticta*. Furthermore, additional material from the sun population was collected dry. Both categories of thalli were hydrated at 20 µmol m^{-2} s^{-1}, 12 °C for 24 h and then exposed to fluctuating light for 8 h a day for 2 d using a cool white led light at 150 µmol m^{-2} s^{-1}. The LED light alternated with dim laboratory lighting (c. 5 µmol m^{-2} s^{-1}) on a 3 min of/on cycle. After exposure, material was then kept overnight at 20 µmol m^{-2} s^{-1} before assessing acclimation traits. To determine the induction of rETR, and the induction and relaxation of NPQ, thalli were dark adapted for 10 min, and F_V/F_M measured. An actinic light of 100 µmol photons m^{-2} $s^{-1}$was then turned on, and saturating flashes applied at increasing intervals for 11 min. The actinic light was

then turned off, and relaxation measured for 8 min, with saturating flashes given at increasing intervals.

2.2.5 Statistical analysis of data

were checked for homogeneity of variance and normality, and ANOVA analysis carried out using IBM SPSS statistics 27.

2.3 Results

Preliminary experiments showed that exposing *Crocodia* and *Ramalina* to 200 µmol m^{-2} s^{-1} for 6 h did not increase their tolerance to a subsequent exposure to 400 µmol m^{-2} s^{-1} (Fig. 2.1). For both species, exposure to light at 200 µmol m^{-2} s^{-1} mildly inhibited F_V/F_M, although values recovered after 24 h. During the subsequent photoinhibition at 400 µmol m^{-2} s^{-1}, lichens that had received the 200 µmol m^{-2} s^{-1} pretreatment were more severely inhibited than the untreated controls.

Exposing hydrated *Crocodia* to a lower light intensity (100 µmol m^{-2} s^{-1}) for 48 h significantly improved tolerance to photoinhibition), whether photosynthesis was assessed as maximal efficiency of PSII (F_V/F_M) or rETR (Figs. 2.2A, 2.3A; $P < 0.001$, Table 2.1). Although NPQ values were initially similar in hardened and untreated (“control”) lichens, NPQ significantly increased throughout light stress in hardened thalli ($P < 0.001$, Table 2.1), while NPQ in the control changed very little (Fig. 2.4A). By contrast, exposing desiccated *Crocodia* to the hardening light intensity had a much smaller, but still significant effect on tolerance to photoinhibition assessed using F_V/F_M ($P < 0.05$, Table 2.1, Figure 2.2B). However, the effect of pretreatment was not significant for rETR (Table 2.1, Figures 2.3B) and NPQ (Table 2.1, Figure 2.4B). For *Ramalina*, pretreatment of hydrated material caused a small, non-significant, increase in tolerance to

photoinhibition (Table 2.1, Figures 2.2C, 2.3C). Pretreating desiccated material caused a small but significant increase in tolerance to photoinhibition assessed with the fluorescence parameter F_V/F_M, but not rETR (Table 2.1, Figures 2.2D, 2.3D). NPQ was significantly increased by light pretreatments in material pretreated both hydrated and desiccated (Table 2.1, Figures 2.4C,D). Pretreating *Sticta* in the wet or dry states had little effect on the tolerance of the lichen to photoinhibition; the only significant effect was a slight increase in the tolerance of material pretreated dry when photosynthesis was assessed by measuring rETR (Table 2.1, Figures 2.2E,F, 2.3E,F).

Table 2.1: Two-way ANOVA of the effects on F_V/F_M, rETR and NPQ during photoinhibition of hardening by pre-treatment with light at 100 µmol m^{-2} s^{-1} for 48 h in *Crocodia aurata*, *Ramalina celastri* and *Sticta fuliginosa.* Error degrees of freedom = 61.

	F_V/F_M			rETR			NPQ		
Species	*Crocadia*	*Ramalina*	*Sticta*	*Crocadia*	*Ramalina*	*Sticta*	*Crocadia*	*Ramalina*	df
Hydrated									
Hardening	P < 0.001	n.s.	n.s.	P < 0.001	n.s.	n.s.	P < 0.001	P < 0.001	1
Time	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	6
Acclimation x Time	P < 0.01	n.s.	n.s.	P < 0.01	n.s.	n.s.	P < 0.01	P < 0.001	6
Desiccated									
Hardening	P < 0.05	P < 0.001	n.s.	n.s.	n.s.	P < 0.01	n.s.	P < 0.001	1
Time	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.01	6
Acclimation x Time	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	6

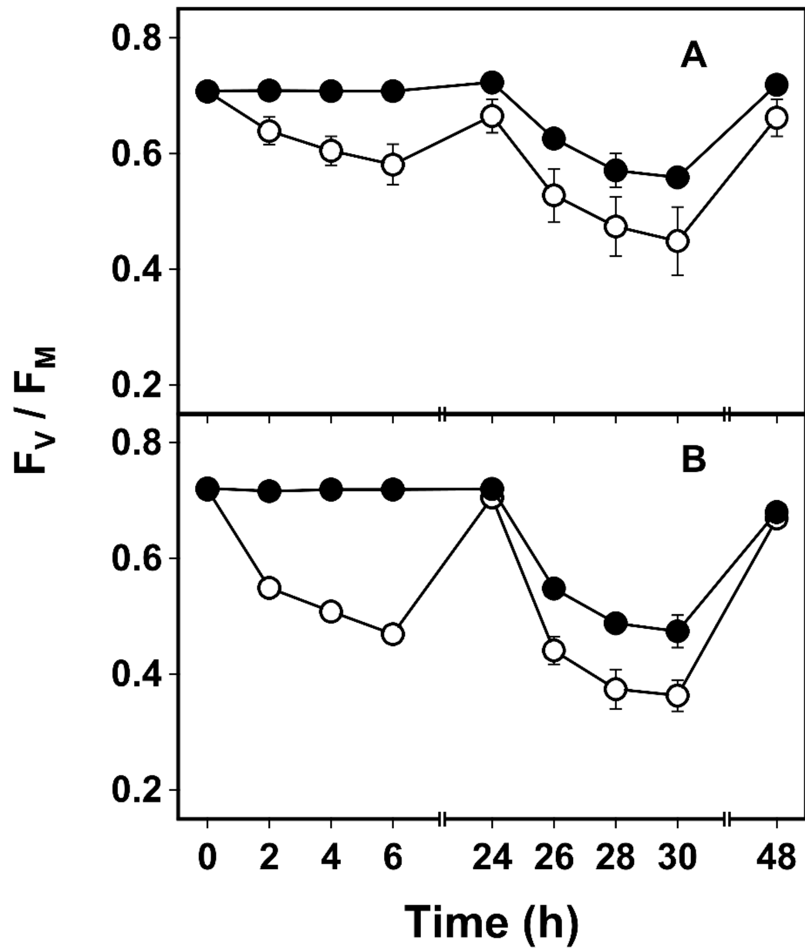

Figure 2.1: The effect of pretreating hydrated material of *Crocodia aurata* (A) and *Ramalina celastri* (B) (open symbols) with light at 200 µmol m^{-2} s^{-1} for 6 h followed by storage overnight at 20 µmol m^{-2} s^{-1} on tolerance to photoinhibition 400 µmol m^{-2} s^{-1} for 6 h. Control samples were kept hydrated at 20 µmol m^{-2} s^{-1} during pretreatment (closed symbols). After photoinhibition, all lichens were allowed to recover at 20 µmol m^{-2} s^{-1}. Error bars denote the SE, n = 10.

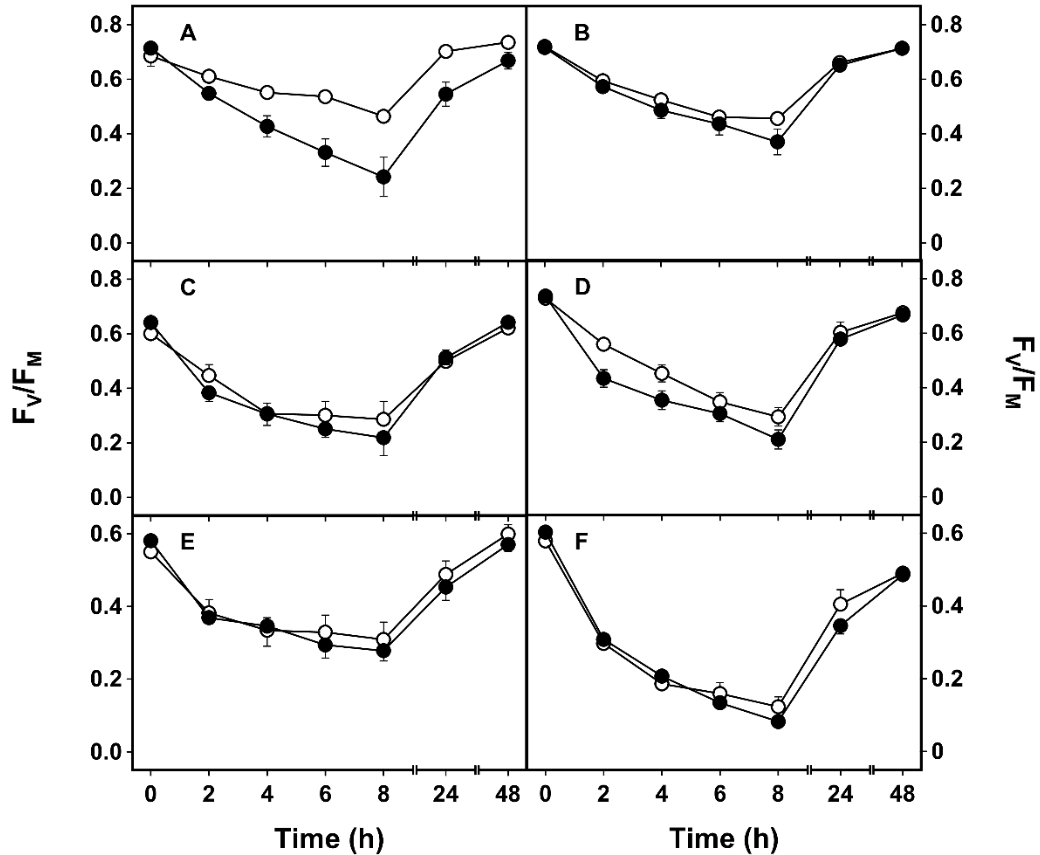

Figure 2.2: The effect of pretreating hydrated (A,C,E) and desiccated (B,D,F) material of *Crocodia aurata*, *Ramalina celastri* and *Sticta fuliginosa* respectively with light at 100 µmol m^{-2} s^{-1} for 48 h followed by storage overnight at 20 µmol m^{-2} s^{-1} (open symbols) on the tolerance of the maximal efficiency of PSII (F_V/F_M) to photoinhibition at 800 µmol m^{-2} s^{-1} for 8 h. Control samples were kept hydrated at 20 µmol m^{-2} s^{-1} in a Conviron during pretreatment (closed symbols). After photoinhibition, all lichens were allowed to recover at 20 µmol m^{-2} s^{-1}. Error bars denote the SE, n = 10.

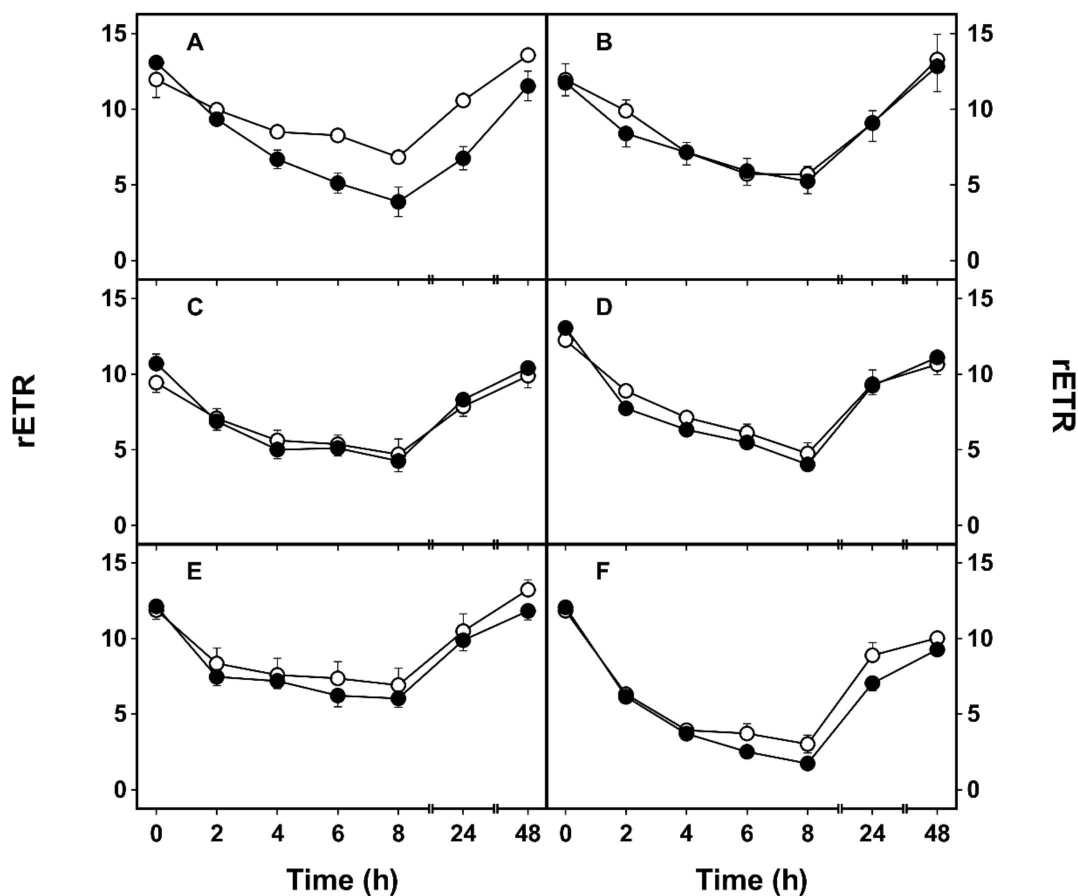

Figure 2.3: The effect of pretreating hydrated (A,C,E) and desiccated (B,D,F) material of *Crocodia aurata*, *Ramalina celastri* and *Sticta fuliginosa* respectively with light at 100 µmol m^{-2} s^{-1} for 48 h followed by storage overnight at 20 µmol m^{-2} s^{-1} on the tolerance of the relative electron transfer rate (rETR) to photoinhibition at 800 µmol m^{-2} s^{-1} for 8 h. rETR was measured after induction for 1 min at 50 µmol m^{-2} s^{-1}. Control samples were kept hydrated in the Conviron during pretreatment. After photoinhibition, all lichens were allowed to recover at 20 µmol m^{-2} s^{-1}. Symbols and error bars as for Figure 2.2.

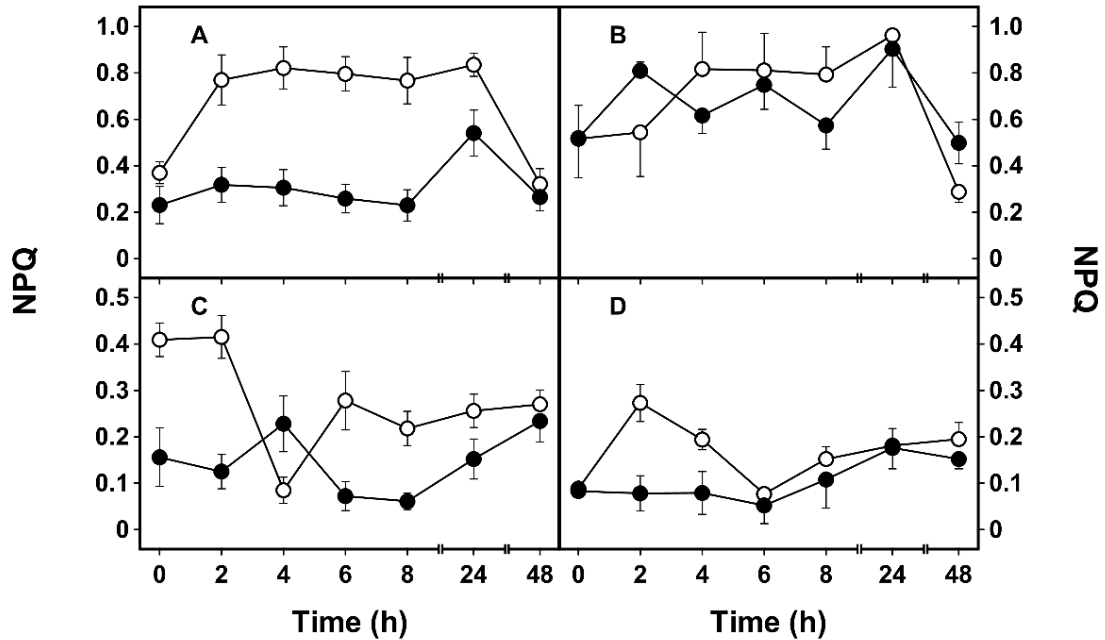

Figure 2.4: The effect of pretreating hydrated (A,C) and desiccated (B,D) material of *Crocodia aurata* and *Ramalina celastri* respectively with light at 100 µmol m^{-2} s^{-1} for 48 h followed by storage overnight at 20 µmol m^{-2} s^{-1} on NPQ measured after induction for 1 min at 50 µmol m^{-2} s^{-1} during photoinhibition at 800 µmol m^{-2} s^{-1} for 8 h. Control samples were kept hydrated in the Conviron during pretreatment. After photoinhibition, all lichens were allowed to recover at 20 µmol m^{-2} s^{-1}. Symbols and error bars as for Figure 2.2.

Photosynthesis in *Ramalina* can display considerable plasticity (Figure 2.5). Compared to material from the normal, exposed, microhabitat of the species, after illumination for 4 min material collected from shaded field locations displayed considerably higher NPQ, which relaxed much more quickly on transition to the dark. Rates of ETR at 100 µmol m^{-2} s^{-1} were much lower in *Ramalina* from shade than exposed microhabitats. Exposing *Ramalina* from exposed habitats to flashing light at relatively low light intensities (150 µmol m^{-2} s^{-1}) changed the photosynthetic

parameters such that they resembled those of lichens collected from shaded field microhabitats, although NPQ was induced more rapidly.

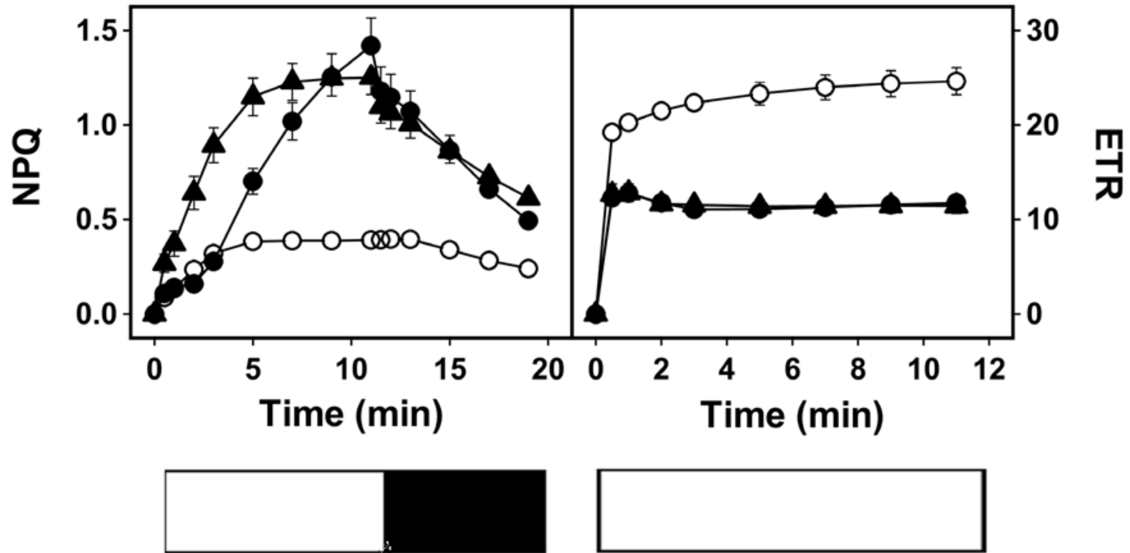

Figure 2.5: Induction and relaxation of NPQ, and induction of rETR in *Ramalina celastri* in response to light at 100 µmol m^{-2} s^{-1}. Open circles denote material freshly collected from exposed locations, closed circles denote material freshly collected from shaded locations, and closed triangles denote material from exposed locations exposed to fluctuating light for 8 h a day for 2 d using a cool white led light at 150 µmol m^{-2} s^{-1} alternating with dim laboratory lighting (c. 5 µmol m^{-2} s^{-1}) on a 3 min off / on cycle. Error bars denote the standard error, n = 10. The white and black sections in the box at the base of the graph indicates the times when samples were exposed to light or darkness respectively.

2.4 Discussion

Here we show that tolerance to photoinhibition in the cephalolichen *Crocodia* can be increased by pretreating hydrated thalli with light at relatively low intensities for 48 h. The increased tolerance to photoinhibition seems at least in part to be a result of an increase in the ability of the lichen to

dissipate excess energy as heat, here assessed by measuring NPQ. Pretreatment was much less effective in increasing tolerance in the cyanolichen *Sticta*, which grows in similar habitats to *Crocodia*, or collections of the chlorolichen *Ramalina* from exposed habitats. Possibly, cyanolichens may need different pretreatment conditions, and in "sun" populations of *Ramalina* tolerance to photoinhibition is fully expressed and cannot be further increased. However, the ability to harden *Crocodia* to tolerance to photoinhibition under controlled conditions could provide a foundation for more detailed investigations into the mechanism of photoprotection in lichen photobionts.

2.4.1 The effect of pretreatment with low light on tolerance to photoinhibition

Our early attempts to increase tolerance to photoinhibition in *Crocodia* and *Ramalina* were not successful. We initially reasoned that a mildly photoinhibitory light stress followed by a recovery period would harden photobionts to a more severe subsequent light stress. Figure 2.1 illustrates the results of a typical experiment, in which hydrated material of *C. aurata* and *R. celastri* were pretreated with light at 200 μmol m^{-2} s^{-1} for 6 h, lichens allowed to recover overnight and then photoinhibited by exposing them to 400 μmol m^{-2} s^{-1} for 6 h. While apparently completely recovering from the mildly inhibitory pretreatment, pretreated lichens were actually more, not less, sensitive to a subsequent photoinhibitory stress. Testing a variety of pretreatment conditions indicated that no pretreatment that caused even slight photoinhibition improved tolerance to a subsequent harsher stress (data not shown). Therefore, in our subsequent experiments, we tried using a lower light intensity for longer.

Tolerance to photoinhibition in hydrated *Crocodia* can be significantly increased by pretreating hydrated lichens to light at 100 μmol m^{-2} s^{-1} for 48 h (Figs. 2.2A, 2.3A). The mechanism of increased tolerance to photoinhibition was not studied in detail in the present study,

but probably at least in part results from an increase in NPQ (Fig. 2.4A). In Angiosperms it is well known that increases in NPQ can improve tolerance to photoinhibition (e.g. Ruban 2017). However, pretreating desiccated *Crocodia* with light at the same intensity causes a much smaller increase in tolerance to photoinhibition (Figs. 2.2B, 2.3B) and has little effect on NPQ (Fig. 2.4B). Adaptation to some stresses such as temperature can take place in desiccated lichens (for review see Kershaw (1985)), and transcription has been demonstrated in desiccated tissues such as seeds, possibly in residual micro-pockets of H_2O (Leubner-Metzger 2005). More work is needed to determine whether tolerance to photoinhibition can be increased in desiccated thalli of *Crocodia*, possibly using alternative pretreatment conditions. In contrast to hydrated *Crocodia*, pretreating *Sticta* with light at 100 µmol m^{-2} s^{-1} for 48 h only causes a small increase in tolerance to photoinhibition, whether thalli were exposed hydrated or desiccated (Figs. 2.2E, F and 2.3E, F). The cyanolichen *Sticta* was collected from a very similar habitat to that of the cephalolichen *Crocodia* (trunk of a small tree in moderate shade). In cyanobacteria, a significant proportion of photoprotection is mediated by “Orange Carotenoid Proteins” (OCPs). These proteins are highly conserved, and are present in *Nostoc*, the photobiont of *Sticta*, and indeed most sequenced cyanobacterial genomes (Kerfeld et al. 2017). OCPs can rapidly dissipate any excess energy absorbed by the phycobilisomes (Wilson et al. 2008), which will reduce ROS formation by the photosystem reaction centres. Interestingly, the synthesis of OCPs is strongly upregulated by desiccation in *Nostoc flagelliforme* (Yang et al. 2019) suggesting that dissipation is probably active at low water contents, and is important in the desiccation tolerance of cyanobacteria. It seems likely that different pretreatment conditions may be needed to modulate the activity of OCPs in cyanobacteria.

Similar to *Sticta*, pretreating *Ramalina* with light at 100 µmol m^{-2} s^{-1} for 48 h only causes small increases in tolerance to photoinhibition (Figs. 2.2C, D and 2.3C, D). *Ramalina* was collected from a much more exposed habitat than *Crocodia* and *Sticta*. In hydrated *Ramalina* pretreatment caused an initial increase in NPQ, but NPQ was rapidly reduced during photoinhibition to similar values to those of the controls (Fig. 2.4C), although the fluorescent data on their own do not suggest an obvious explanation for this. There is a general view that the survival of lichens growing in more extreme microhabitats may depend largely on constitutive tolerance mechanisms (Gasulla et al. 2021). This is because in harsh environments, conditions typically change rapidly, and there is insufficient time to put inducible tolerance mechanisms in place. Possibly, this may explain why tolerance could be increased in *Crocodia* but not *Ramalina*. However, there are other possible explanations for the apparently lower plasticity in *Ramalina*. First, although *Ramalina* experiences higher light intensities than *Crocodia*, in exposed microhabitats the light intensities are more stable, and therefore less plasticity could be needed. Second, in the exposed localities in which *Ramalina* grows, drying occurs rapidly after re-wetting. More photoinhibition may occur when the lichens are desiccated, and different mechanisms may be needed to enhance tolerance to "dry photoinhibition". Third, as photosynthesis in *Ramalina* is clearly quite flexible (Fig. 2.5), our lack of success in increasing tolerance to photoinhibition may have been because in the sun populations that we sampled, tolerance was already fully expressed and could not easily be increased.

2.4.2 Plasticity of photosynthesis in Ramalina

Although hardening treatments did not increase tolerance to photoinhibition in *Ramalina* (Figs. 2.2, 2.3), photosynthesis can display considerable plasticity in this species (Fig. 2.5). In freshly collected material the induction of ETR, and the induction and relaxation of NPQ during

exposure to 100 µmol m^{-2} s^{-1} were typical of those of other sun lichens (Beckett et al. 2021). However, both freshly collected (atypical) material from shaded localities, and material given light fluctuating between moderately low levels of 5 and 150 µmol m^{-2} s^{-1} on a 3 min cycle for 3 d in the laboratory display first lower rETR and second higher, faster relaxing NPQ (Fig. 2.5). The reduction of rETR in lichens subjected to natural (shade collections) or artificial (laboratory manipulated) fluctuating light levels is probably an adaptation to lower light availability. Compared to shade plants, sun plants can display high rates of photosynthesis by having higher concentrations of cytochromes involved in electron transport (e.g. Cyt b559, Cyt b563 and Cyt f), and having more Calvin cycle enzymes (Greer 2022). While enabling high rates of photosynthesis, such adaptations come at a metabolic cost, and are likely to be downregulated if higher capacities can only rarely be realized. The higher, faster relaxing NPQ that occurs in shade and laboratory treated *Ramalina* (Fig. 2.5) tends to suggest that *Trebouxia* has limited ability to use the light energy present in "sunflecks" and rather increases capacity for protective quenching. However, the additional NPQ relaxes rapidly, presumably enabling photobionts to use more efficiently the lower light available after a sunfleck. Excessive NPQ can reduce rates of photosynthesis, particularly at lower light levels (Murchie and Ruban 2020). We were surprised at the speed of acclimatization, as significant changes in the chlorophyll fluorescence parameters of sun collections of *Ramalina* occurred after only 3 d. As discussed in the Introduction, it seems likely that *Trebouxia* was originally a photobiont in forest lichens, but later diversified into species that grow in more extreme habitats (Nelsen et al. 2021). Results presented here suggest that while photosynthetic characteristics such as maximum rates of ETR and NPQ changed as they emerged from shaded habitats, they retained the ability to revert to shade forms. This is consistent with our field observation that lichens that normally grow in exposed microhabitats can sometimes be

collected from more shaded locations. In shaded microhabitats, photosynthesis will need to adapt to lower and most likely fluctuating light intensities. While we were unable to increase tolerance to photoinhibition in sun collections of *Ramalina*, even in this species photosynthesis can still display considerable plasticity.

2.5 Conclusions and prospects

Results presented here show that for hydrated material of *Crocodia*, pretreatment with light at 100 μmol m^{-2} s^{-1} for 48 h increases tolerance to photoinhibition. Increased tolerance in pretreated material is accompanied by an increase in NPQ during photoinhibition compared with the controls. Conversely, in *Ramalina* pretreatment did not increase tolerance, and while pretreatment caused an initial increase in NPQ, during photoinhibition NPQ quickly reduced to similar values to those of the controls. Thus, results suggest that there is a general correlation between increased tolerance to photoinhibition and increased NPQ, although more work is needed to explain how the increase in NPQ occurs. Other possible mechanisms of increased tolerance also need to be explored. For example, it is possible that hardening increases the capacity of the photobionts to scavenge ROS, or the efficiency of the PSII repair cycle. Furthermore, it would be useful to study the form of NPQ (e.g., qE or qI) induced by hardening treatments, and other aspects of quenching such as the xanthophyll pool size and the expression of enzymes such as violaxanthin epoxidase. In future, we are planning to study how the transcriptomes of our photobionts change during pretreatment and photoinhibition. This will provide a more mechanistic basis for the changes in NPQ observed here, and suggest which other tolerance mechanisms may be involved. There is currently great interest in working out how higher plants dissipate excess light energy (Kaiser et al. 2019), and the signaling pathways that mediate tolerance to high light stress (Gollan and Aro 2020; Biswas and

Mano 2021). While the main aim of much of this research is to find ways of increasing the yield of crop species, it seems likely that studying tolerance to light stress in lichen photobionts may provide fresh insights into how photosynthetic performance can be improved.

2.6 Acknowledgements

Richard Beckett thanks the University of KwaZulu-Natal Research Fund, and also acknowledges that the work presented here was supported by the Kazan Federal University Strategic Academic Leadership Program (PRIORITY-2030). Kwanele Mkhize thanks NRF-DAAD for a PhD bursary and Farida Minibayeva thanks the Russian Science Foundation (Grant No. 18-14-00198) and the Russian government assignment of FRC Kazan Scientific Center of RAS for financial support.

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CHAPTER 3

Shade lichens are characterized by rapid relaxation of non-photochemical quenching on transition to darkness

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Abstract

Non-photochemical quenching (NPQ) plays an important role in protecting photosynthetic organisms from photoinhibition by dissipating excess light energy as heat. However, excess NPQ can greatly reduce the quantum yield of photosynthesis at lower light levels. Recently, there has been considerable interest in understanding how plants balance NPQ to ensure optimal productivity in environments in which light levels are rapidly changing. In the present study, chlorophyll fluorescence was used to study the induction and relaxation of non-photochemical quenching (NPQ) in the dark and the induction of photosynthesis in ten species of lichens, five sampled from exposed and five sampled from shaded habitats. Here we show that the main difference between sun and shade lichens is the rate at which NPQ relaxes in the dark, rather than the speed that photosynthesis starts upon illumination. During the first two minutes in the dark, NPQ values in the five sun species declined only by an average of 2%, while by contrast, in shade species the average decline was 40%. For lichens growing in microhabitats where light levels are rapidly changing, rapid relaxation of NPQ may enable their photobionts to use the available light most efficiently.

Key words: chlorophyll fluorescence, photoprotection, photosynthesis, sunfleck, xanthophyll cycle

3.1 Introduction

Many lichens possess a variety of tolerance mechanisms that enable them to grow in habitats where they are exposed to levels of light that are far greater than lichen photobionts can use in carbon fixation (Beckett *et al.* 2021). However, some species grow in more shaded microhabitats. Long-term adaptations of lichens growing in shade include having lower light saturation and compensation points than those from sun-exposed habitats (Green *et al.* 1997), and also less cortical pigments (Dietz *et al.* 2000). Many lichens from shaded habitats experience short-term (s-min) changes in light levels. For example, for lichens growing on the trunks of trees, gaps in the canopy expose the lichens to rapidly changing light levels in ways that depend on the diurnal variations in the angle of sunlight, tree architecture and movements of the tree branches. Lichens in such habitats experience rapidly changing levels of irradiance; the relatively brief periods that lichens are exposed to high light levels are known as 'sunflecks' (Greer 2021). In higher plants, it is known that the ability of photosynthesis to adapt to these fluctuations is under genetic control (Cruz *et al.* 2016) and that the speed of these responses can be improved by exploiting natural genetic variation (Morales & Kaiser 2020).

Lichen photobionts need to optimally use the light that becomes available to them in a sunfleck. A few minutes of illumination at least are needed for Calvin cycle intermediates to reach critical levels, and this 'induction requirement' of photosynthesis determines how fast a lichen photobiont can respond to an increase in photon irradiance. Previous workers emphasized the need for a rapid increase in photosynthesis following illumination in both lichens (Lakatos *et al.* 2006) and bryophytes (Kubasek *et al.* 2014) growing in shaded environments. However, it appears that no comprehensive survey of the speed of induction of photosynthesis in 'sun' and 'shade' lichens has been carried out. In general, induction of photosynthesis occurs more quickly in lichens and

bryophytes than in higher plants, probably at least in part because the former do not possess stomata that need to be opened (Lakatos *et al.* 2006).

In addition to the need for rapid induction of photosynthesis, lichens must protect themselves from damage that could result from a sudden increase in light. Excess light energy can result in elevated levels of reactive oxygen species (ROS) produced by chlorophyll ($^1O_2$) and electron transport chains ($O_2^{\cdot-}$ and H_2O_2), which can cause photo-oxidative damage (Roach & Krieger-Liszkay 2019). Photobionts use several processes to regulate the efficiency with which light energy is used, collectively referred to as non-photochemical quenching (NPQ). Lichens possessing green (chlorophycean) photobionts have light harvesting complex (LHC) antenna proteins and, as a result, dissipate excess energy using strategies similar to those found in bryophytes and higher plants (Beckett *et al.* 2021). In the enzyme-catalyzed xanthophyll cycle, the carotenoid violaxanthin is converted to zeaxanthin in a pH-regulated process that occurs during increases in light intensity. However, NPQ plays both positive and negative roles in ensuring optimal plant productivity in environments in which light levels are rapidly changing (Murchie & Ruban 2020). Positively, NPQ delays the onset of photoinhibition by reducing ROS production. However, negatively, while not affecting photosynthesis in high light, NPQ can greatly reduce the quantum yield of photosynthesis at lower light levels. In other words, under low light a lichen 'expressing' high NPQ will require a higher irradiance to achieve the same photosynthetic rate as one without it. Recently, NPQ induction and relaxation in higher plants was accelerated by over-expressing violaxanthin de-epoxidase and zeaxanthin epoxidase. When grown in the field, these plants possessed higher biomass and yield, and in particular CO_2 assimilation rates were enhanced during the transition to low light (Kromdijk *et al.* 2016). The implication for lichens could be that shade species growing in habitats subjected to rapidly changing light levels will benefit from more

rapid relaxation of NPQ on transition to low light, enabling lichens to efficiently utilize the lower light levels available after a sunfleck has passed.

Perhaps surprisingly, the relaxation of NPQ on transition from light to dark has not been systematically studied in lichens. We therefore used chlorophyll fluorescence to measure both the induction of photosynthesis on exposure to light and the rates of NPQ relaxation in the dark in lichens. We compared species growing in exposed habitats with those growing in generally shaded habitats, but ones in which lichens experience rapidly changing light levels. Results showed that the main differences between lichens that grow in full sun and those in more shaded habitats are not in the speed of activation of photosynthesis, but rather that sunfleck species show much faster relaxation of NPQ, particularly during the few minutes of transition to the dark.

3.2 Materials and Methods

3.2.1 Lichen material

The list of species used and their microhabitats are described in Table 3.1. Lichens were collected from a small patch of Afromontane forest in Fort Nottingham, KwaZulu Natal, South Africa and some surrounding drier savannah. Lichens were cleaned and stored refrigerated for up to 2 weeks. For uniformity, before the start of each experiment all material was initially hydrated by spraying with distilled water followed by moist storage for c. 24 h in dim light (20 μmol photons m^{-2} s^{-1}) at 12 °C.

Table 3.1. Collection sites, habitats and photobiont types of the lichen species used in this study.

Species	Collection site	Probable Photobiont	Microhabitat
Shade Species			
Cetrelia cetrarioides	Afromontain forest, Fort Nottingham, KwaZulu Natal	*Trebouxia*	Tree trunk, deep shade
Crocodia aurata	Afromontain forest, Fort Nottingham, KwaZulu Natal	*Symbiochloris*	Tree trunk, deep shade
Lepraria incana	Afromontain forest, Fort Nottingham, KwaZulu Natal	*Asterochloris*	Tree trunk, deep shade
Lobaria quercizans	Afromontain forest, Fort Nottingham, KwaZulu Natal	*Symbiochloris*	Tree trunk, deep shade
Roccella montagnei	Afromontain forest, Umgeni Nature Reserve, Howick, KwaZulu Natal	*Trentepohlia*	Base of tree-shaded cliffs
Sun Species			
Cladonia coniocraea	Savanna, Cumberland Nature Reserve, KwaZulu Natal	*Asterochloris*	Exposed rocky outcrops
Parmelia conspersa	Savanna, Cumberland Nature Reserve, KwaZulu Natal	*Trebouxia*	Exposed rocky outcrops
Parmelia saxatilis	Savanna, Cumberland Nature Reserve, KwaZulu Natal	*Trebouxia*	Exposed rocky outcrops
Ramalina celastri	Afromontain forest, Fort Nottingham, KwaZulu Natal	*Trebouxia*	Periphery of canopy
Usnea undulata	Afromontain forest, Fort Nottingham, KwaZulu Natal	*Trebouxia*	Periphery of canopy

3.2.2 Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured using a PAM 2500 fluorimeter (Walz, Effeltrich, Germany) using the red LED. After a dark adaptation period of at least 10 min, the maximal efficiency of PSII (F_V/F_M) was measured, where F_M = maximum fluorescence and F_V = variable fluorescence or ($F_M - F_0$), with F_0 = minimal fluorescence yield of the dark-adapted state. Thalli with anomalous values of F_V/F_M were discarded. Rapid light response curves of ETR were measured by increasing the actinic light in 11 small steps of 10 to 20 s each from 0 to 475 µmol photons m^{-2} s^{-1} (at 12, 33, 56, 81, 106, 141, 185, 238, 301, 383 and 475 µmol photons m^{-2} s^{-1}) with saturating flashes at the end of exposure to each light level. The relative ETR was calculated as:

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

where ΦPSII is the effective quantum yield of PSII photochemistry calculated as $(F_{M'} - F_t)/F_{M'}$, where $F_{M'}$ = maximal fluorescence yield of the light-adapted state and F_t = stable fluorescence signal in the light.

The equation derived by Eilers & Peeters (1988) was used to calculate the following parameters:

$rETR_{MAX}$: The maximal relative electron transport rate reached during light curve recording, reflecting the light saturated capacity of the sample (units: µmol electrons m^{-2} s^{-1}).

Ik: The light intensity at which PAR saturation sets in. This is estimated by constructing a linear regression of the initial part of the light response curve, and extrapolating it until it hits a ETR value corresponding to the estimate of $rETR_{MAX}$. The light intensity where the two lines intersect is lk (units: µmol photons m^{-2} s^{-1}).

To determine the induction of rETR, and the induction and relaxation of NPQ, thalli were dark adapted for 10 min, and F_V/F_M measured; thalli with anomalous values were discarded. An actinic light of 100 µmol photons m^{-2} s^{-1} was then turned on, and saturating flashes applied at increasing intervals for 11 min. The actinic light was then turned off, and relaxation measured for 8 min, with saturating flashes given at increasing intervals. NPQ was calculated using the formula of Bilger *et al.* (1995):

$NPQ = (F_M - F'_M)/F'_M$

In initial experiments we tested the induction of NPQ using a variety of light intensities, but in a laboratory setting, values much above 100 µmol photons m^{-2} s^{-1} tended to cause some photoinhibition. To avoid progressive development of any slow relaxing photoinhibitory quenching (qI) we therefore elected to standardize on 100 µmol photons m^{-2} s^{-1}.

3.3 Results

Table 3.2 presents a summary of the data derived from the rapid light curves. Comparing the sun and shade lichens, both $rETR_{MAX}$ and the PAR where saturation sets in (lk) were more than double in the sun compared with the shade species. Figures 3.1 and 3.2 compare the induction and relaxation of NPQ and the induction of rETR in shade and sun species respectively. Induction of rETR by 100 μmol m^{-2} s^{-1} was rapid, and similar in sun and shade species. The proportion of $rETR_{MAX}$ induced after 30 s was almost the same in sun and shade species (Table 3.2). NPQ tended to be induced more slowly in shade than sun species, and was not complete even after 11 min in *Lepraria* and *Roccella*. However, the final values of NPQ (after 11 min) induced in shade species were on average almost double that of sun species (Table 3.2). Shade and sun lichens differed mainly in their rate of relaxation of NPQ, particularly in the first 2 min of darkness. While in the five sun species NPQ declined only by an average of 2%, in shade species the average decline was 40%, with two species (*Lobaria* and *Roccella*) declining by more than 50%. Correlation analysis showed that the PAR where saturation sets in was very strongly correlated with $rETR_{MAX}$ (Fig. 3.3A) and was significantly negatively correlated with the proportion of NPQ relaxed after 2 min (Fig. 3.3B).

Table 3.2. Summary of photosynthetic parameters of sun and shade lichen species. The start of light saturation (lk) and maximal relative electron transport rate ($rETR_{MAX}$) were derived from rapid light curves, while other values were derived by illuminating dark-adapted lichens to light at 100 μmol m^{-2} s^{-1} and measuring the time course of the induction of ETR and non-photochemical quenching (NPQ) for 11 min, and the relaxation of NPQ for 8 min after switching off the light. Figures are given as ± SE, n = 10–15.

Species	$rETR_{MAX}$	lk (start of light saturation) (μmol m^{-2} s^{-1})	% $rETR_{MAX}$ after 0.5 min	NPQ after 11 min	% NPQ relaxed after 2 min*
Shade Species					
Cetrelia cetrarioides	15.0 ± 2.0	43 ± 7	71	0.67 ± 0.08	15 ± 1
Crocodia aurata	13.1 ± 0.8	50 ± 3	70	0.95 ± 0.08	46 ± 2
Lepraria incana	8.6 ± 0.6	31 ± 3	65	0.49 ± 0.10	31 ± 3
Lobaria quercizans	12.9 ± 1.1	39 ± 4	66	1.44 ± 0.07	54 ± 1
Roccella montagnei	6.9 ± 2.1	29 ± 11	71	0.66 ± 0.11	54 ± 4
Mean for shade species	**11.3**	**38**	**69**	**0.84**	**40**
Sun Species					
Cladonia coniocreae	21.1 ± 1.1	73 ± 4	84	0.58 ± 0.09	-1 ± 4
Parmelia conspersa	28.9 ± 2.2	97 ± ±7	67	0.54 ± 0.06	-2 ± 3
Parmelia saxatilis	25.2 ± 1.7	93 ± 7	66	0.51 ± 0.04	7 ± 2
Ramalina celastri	30.8 ± 2.4	105 ± 10	57	0.39 ± 0.05	-5 ± 4
Usnea undulata	32.6 ± 2.9	128 ± 14	67	0.43 ± 0.03	4 ± 3
Mean for sun species	**27.7**	**99**	**67**	**0.49**	**2**

* Negative values indicate stimulation of NPQ

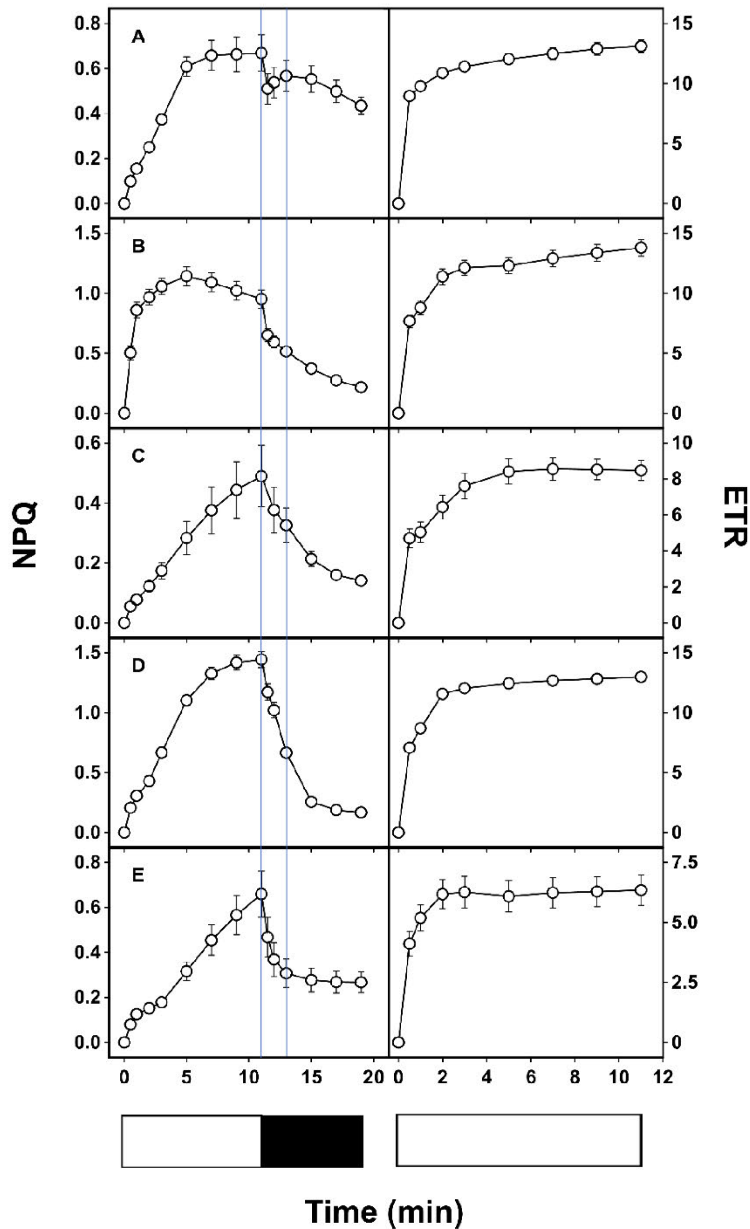

Figure 3.1: Induction and relaxation of NPQ, and induction of rETR in shade species of lichens in response to light at 100 µmol m^{-2} s^{-1}. A, *Cetrelia cetrarioides*; B, *Crocodia aurata*; C, *Lepraria incana*; D, *Lobaria quercizans*; and E, *Roccella montagnei*. Error bars denote the standard error, n = 10 to 15. The vertical lines on the graph delimit NPQ during the first two minutes of darkness. The white and black sections in the box at the base of the graph indicates the time periods when samples were exposed to light or darkness respectively.

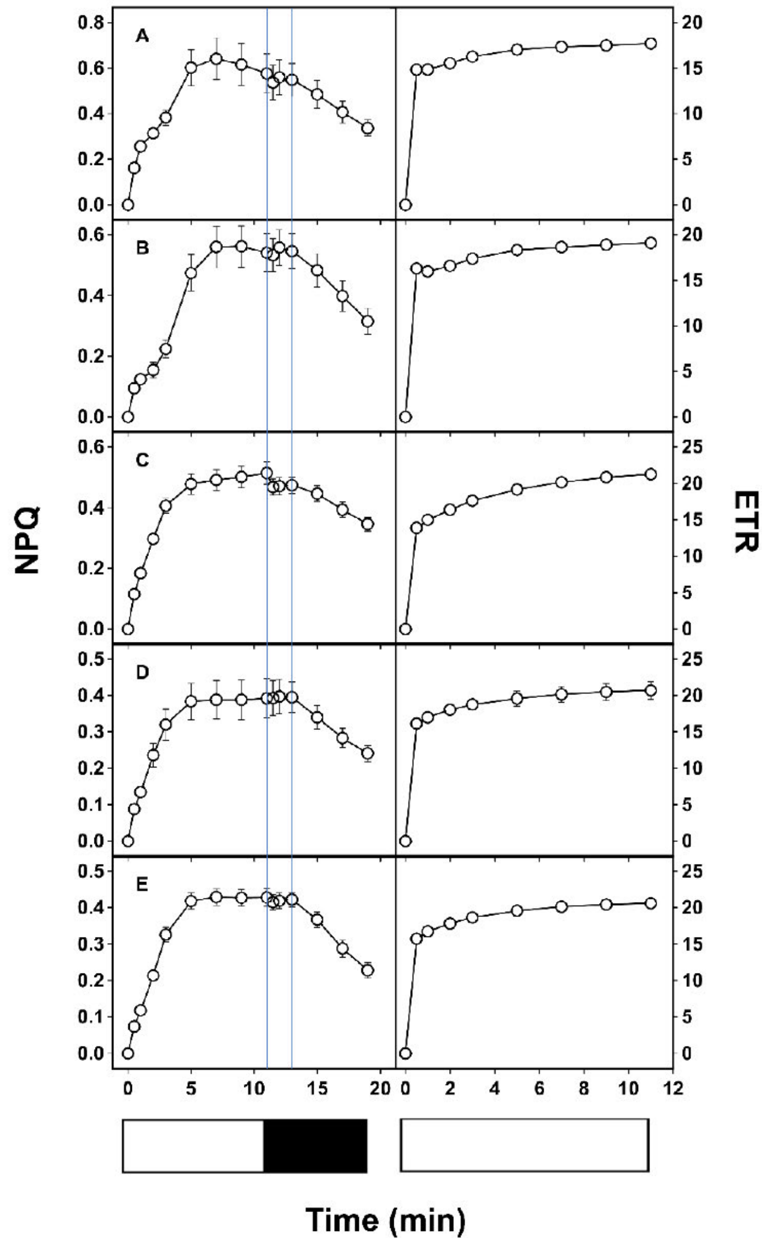

Figure 3.2: Induction and relaxation of NPQ, and induction of rETR in sun species of lichens in response to light at 100 µmol m^{-2} s^{-1}. A, *Cladonia coniocreae*; B, *Parmelia conspersa*; C, *Parmelia saxatilis*; D, *Ramalina celastri* and E, *Usnea undulata*. Error bars denote the standard error, n = 10 to 15. The vertical lines on the graph delimit NPQ during the first two minutes of darkness. The white and black sections in the box at the base of the graph indicates the time periods when samples were exposed to light or darkness respectively.

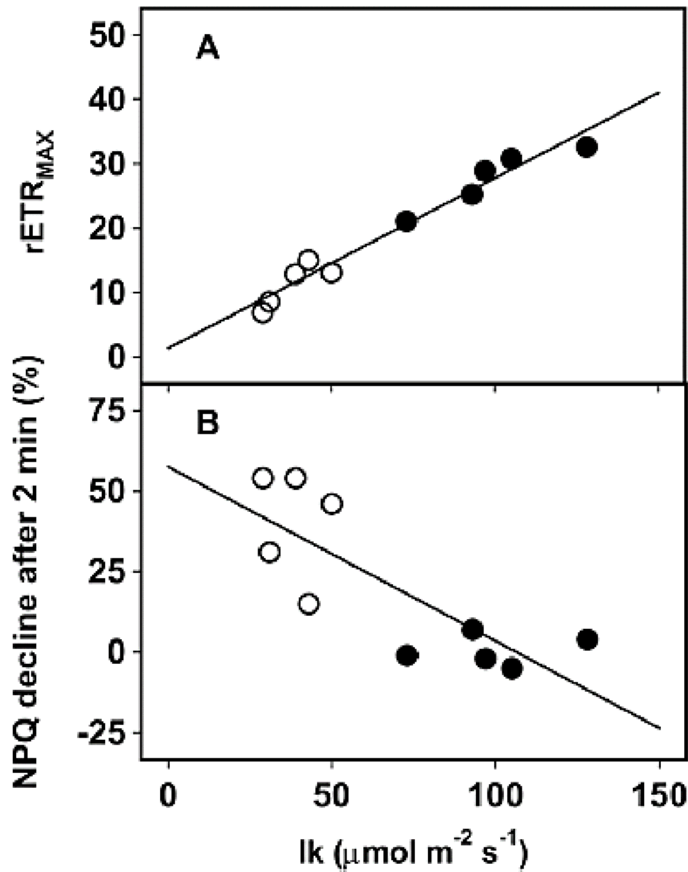

Figure 3.3: Correlation between lk (the PAR where light saturation sets in) and A, $rETR_{MAX}$ ($P < 0.001$); and B, the percentage drop in maximum values of NPQ (after illumination for 11 min) during the first two minutes of darkness ($P < 0.01$). Each point is the average of at least ten values. Solid symbols represent sun species, open symbols represent shade species.

3.4 Discussion

Lichens growing in shaded habitats often experience short-term (s-min) changes in light levels. The duration of periods of relatively bright and dim light varies greatly between habitats, but the average duration of sunflecks in subtropical Afromontane forests is probably c. 2 min (Pallardy 2008). While lichens need to optimize the use of brief periods of high light, at the same time they must also protect themselves from damage that could result from ROS formation. One of the most powerful ways photobionts can reduce ROS formation is by inducing NPQ. However, while strong

quenching will delay the onset of photoinhibition during a sunfleck, it will simultaneously greatly reduce the quantum yield of photosynthesis when light returns to lower levels. Results presented here show that sun and shade lichens differ mainly in the rate of relaxation of NPQ during the first few minutes that light levels fall. Rapid relaxation of NPQ probably has little selective advantage for lichens growing in exposed sites, where during the day the only major changes in light levels result from changes in cloud cover and occur over periods of hours rather than minutes. By contrast, the rapid relaxation of NPQ observed in lichens that grow in microhabitats where light levels are rapidly changing will enable their photobionts to efficiently utilize the lower light levels that occur once a sunfleck has passed.

3.4.1 Rapid light curves

Parameters derived from the rapid light curves indicate that the light intensity where saturation of photosynthesis sets in (lk) is much lower in the shade species than in the sun species (38 compared with 99 μmol m^{-2} s^{-1}; Table 3.2). Furthermore, the average $rETR_{MAX}$, the maximal relative electron transport rate reached during light curve recording (reflecting the light saturated rate of photosynthesis), is much lower in shade than sun species (11.3 compared with 27.7). It is well known that higher plants growing in bright habitats have a greater capacity for photosynthetic electron transport (greater abundance of transport components such as Cyt b559, Cyt b563, Cyt f and plastoquinone) and a greater capacity for ATP synthesis per unit of chlorophyll compared with shade plants (Greer 2021). This results in higher rates of photosynthesis in sun plants, and photosynthesis that saturates at higher light levels, as found in the present study for lichen photobionts. Although there are few comparable studies with lichens, Piccotto & Tretiach (2010) surveyed a range of lichens from contrasting habitats and found that the 'potential solar irradiation' of each site was significantly correlated to lk and maximum rates of photosynthesis. In the present

study, $rETR_{MAX}$ and lk were highly significantly correlated (Fig. 3.3A). While no actual measurements of field light intensities were taken in the present study, visual inspection suggests that lk, or the PAR where saturation starts, appears to be a good quantitative measure of the light regimes of the habitats that the lichens were collected from.

3.4.2 Dark relaxation of NPQ

The main differences between the sun and shade species of lichens studied here was in the rate of dark relaxation of NPQ. During the first two minutes of darkness NPQ declined only by on average 2% in the sun species (and in some species NPQ marginally increased), whereas in the shade species NPQ declined by an average of 40% (Table 3.2). The decline in NPQ during the first two minutes of darkness was significantly negatively correlated with lk (Fig. 3.3B). Work with higher plants suggests that there are several possible mechanisms that could promote fast relaxation during the transition from high to low light. First, shade species may possess higher activities of xanthophyll epoxidases (Kaiser *et al*. 2019). Second, the speed of NPQ relaxation is strongly modulated by the K^+ antiporter KEA3 (Armbruster *et al*. 2014, 2016; Correa Galvis *et al*. 2020). KEA3 transfers K^+ into the lumen and H^+ out to the chloroplast stroma, decreasing pH and accelerating NPQ relaxation, leading to a fast recovery of CO_2 assimilation (Armbruster *et al*. 2014). Further work is needed to investigate these possibilities in lichen photobionts, and also to study any metabolic costs associated with rapid relaxation. Interestingly, while the induction (rather than the relaxation) of NPQ in some shade species (e.g. *Lepraria* and *Roccella*; Fig. 3.1C & E) was slower than in sun species and was not complete even after 11 min, average values of NPQ after 11 min were higher in shade than sun species (Table 3.2). High values of NPQ in shade species might appear surprising, but in higher plants fluctuating light has been reported to increase the protective capacity of NPQ (Alter *et al*. 2012; Caliandro *et al*. 2013). Presumably, in shaded

habitats light levels can increase very suddenly, potentially causing oxidative stress, and therefore effective defence mechanisms must be constitutively in place. Theoretically, faster relaxation in shade species could be simply because they contain lower pool sizes of xanthophyll cycle pigments. However, this appears unlikely because in general NPQ is positively correlated with absolute levels of xanthophyll cycle pigments (Demmig-Adams *et al*. 2020), and the higher values of NPQ in shade species suggests that they contain larger, not smaller xanthophyll pool sizes.

It is perhaps surprising that there have been no previous attempts to compare the rates of NPQ relaxation in sun and shade lichens. In the comparable survey of bryophytes by Proctor & Smirnoff (2015), results showed that NPQ on transition to darkness tends to display relatively simple exponential decay curves, unlike the rather complex kinetics of induction and relaxation reported here for lichens. Although relaxation rates in bryophytes appear to be faster than those in lichens, Proctor & Smirnoff (2015) also found that NPQ generally relaxes faster in shade than in sun bryophytes. Limited comparable data is available for microalgae. Environments characterized by particularly large light fluctuations include shallow waters. Here, microalgae employ rapidly reversible NPQ, presumably to cope with more variable light fields, whereas motile benthic algae display sustained NPQ (Fisher *et al*. 2020). For example, Derks & Bruce (2018) compared the induction and relaxation of NPQ in two diatoms from contrasting habitats. *Navicula* grows in a stable high irradiance light environment, while *Nitzschia* grows in churning water with a high particulate content and is exposed to rapid (s-min) changes in light levels. Both species were exposed to 500 μmol m^{-2} s^{-1} for 10 min, followed by 15 min of darkness. NPQ was induced rapidly in both species and was higher in *Nitzschia* than in *Navicula*. Interestingly, however, similar to the results presented here, the main difference between the species was in the rate of relaxation of NPQ, which was much faster in *Nitzschia* than in *Navicula*.

Finally, it is worth noting that differences in rates of relaxation of NPQ are not simply correlated to photobiont type (Table 3.1). The photobionts of the shade species sampled here are more diverse than those of the sun species, and include *Trentepohlia* and *Symbiochloris*. Nevertheless, two shade species, *Cetrelia* and *Lepraria*, contain *Trebouxia* or the closely related *Asterochloris*, possessed by all the sun species sampled here. Interestingly, Nelsen *et al*. (2021) suggested that early *Trebouxia* lineages were largely forest specialists or habitat generalists, and were found in moderate climates. *Trebouxia* then diversified in non-forested and more stressful habitats (Nelsen *et al*. 2021). It seems likely that as *Trebouxia*-containing lichens emerged from shaded habitats, the pattern of NPQ relaxation changed from rapid to more gradual relaxation. Today, both patterns of relaxation are found in trebouxioid lichens.

3.5 Conclusions

Some authors have emphasized the need for lichens growing in habitats with rapidly changing light levels to rapidly induce photosynthesis on illumination (Lakatos *et al*. 2006). However, results from the present study show that rETR induces at very similar rates in shade and sun lichens (Table 3.2). A more fundamental difference between sun and shade lichens appears to be the rate at which NPQ relaxes. Future work needs to investigate at a biochemical level the mechanisms that enable shade lichens to relax NPQ faster than sun species, for example by studying the expression of the xanthophyll epoxidases and the KEA ion transporter. Recently, there has been great interest in understanding how relaxation of NPQ is controlled, with a view to increase yield in crop plants (Kromdijk *et al*. 2016). Comparative studies of sun and shade lichens may facilitate the bioengineering of other organisms to display accelerated responses to natural shading events.

3.6 Acknowledgements

RB thanks the Russian Government Program of Competitive Growth of Kazan Federal University for partial financial support. FM thanks the Russian Science Foundation (grant no. 18-14-00198, rapid light curves) and the Russian government assignment of FRC Kazan Scientific Center of RAS for financial support. KM thanks the DAAD In-Country award (South Africa) for a bursary.

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CHAPTER 4

Adaptions of photosynthesis in sun and shade in populations of some Afromontane lichens

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Formatted for: The Lichenologist

Abstract

Photosynthetic organisms have evolved a variety of mechanisms to optimize their use of sunlight. Some of the clearest examples of adaptations can be seen by comparing photosynthesis in different species and in different individuals of the same species that grow under high and low light levels. While the adaptations of sun and shade higher plants have been relatively well studied, much less information is available on the photobionts of lichenized *Ascomycetes*. An important adaptation that can protect photosynthetic organisms from the potentially harmful effects of excess light is non-photochemical quenching (NPQ); NPQ can dissipate unused light energy as heat. Here we used chlorophyll fluorescence to compare the induction and relaxation of NPQ and the induction of electron transport (rETR) in collections of the same lichen species from exposed and from more shaded locations. All species have trebouxioid photobionts and normally grow in more exposed microhabitats but can also be readily collected from more shaded locations. Shade forms display generally higher NPQ, presumably to protect lichens from occasional rapid increases in light that occur during sunflecks. Furthermore, the NPQ of shade forms relaxes quickly when light levels are reduced, presumably to ensure efficient photosynthesis after a sunfleck has passed. The maximal relative electron transport rate is lower in shade than sun collections, probably reflecting a downregulation of photosynthetic capacity to reduce energy costs. We also compared collections of pale and melanized thalli from three species of shade lichens with *Symbiochloris* as their photobiont. Interestingly, NPQ in melanized thalli from slightly more exposed microhabitats induced and relaxed in a way that resembled shade rather than sun forms of the trebouxioid lichens. This might suggest that in some locations melanization induced during a temporary period of high light may be excessive and could potentially reduce photosynthesis later in the growing season.

Taken together, the results suggest that lichen photobionts can flexibly adjust the amount and type of NPQ, and their levels of rETR in response to light availability.

Key words: chlorophyll fluorescence, photoprotection, photosynthesis, sunfleck, xanthophyll cycle

4.1 Introduction

Plants are adapted to grow in an extraordinarily wide range of light environments, from the deep shade of rainforest understories to high light habitats such as deserts and mountain tops (Greer 2022). This is only possible because plants have evolved various mechanisms to optimize their use of sunlight; furthermore, individual species can display great plasticity in their response to changes in light availability. In higher plants, 'sun species' tend to have more components of the photophosphorylation electron transfer chains, increasing their ability to synthesize ATP and NADPH to fix CO_2. This allows them to possess higher activities of Calvin cycle enzymes. The net result is that sun plants typically display higher light-saturation points and maximum rates of photosynthesis. The maintaining high activities of photosynthetic cytochromes and enzymes will inevitably involve considerable metabolic expenditure.

In addition to balancing light availability with investment in systems involved in photophosphorylation and carbon fixation, plants must protect themselves when the amount of light absorbed exceeds that which can be used. Excess light energy can result in elevated levels of reactive oxygen species (ROS) which can cause photo-oxidative damage (Roach & Krieger-Liszkay 2019). In the short term, a common tolerance mechanism involves increasing the dissipation of excess energy absorbed as heat using non-photochemical quenching. Quenching can be expressed as qN, or the proportion of energy absorbed dissipated as heat, or in absolute terms as NPQ (Liu *et al*. 2019). qN has two main components, qE and qI (Kalaji *et al*. 2017). The component qE, or fast relaxing energy dependent quenching, represents quenching that is relaxed during the first 200 s of darkness with a relaxation halftime of c. 30 s. This parameter is influenced by low lumen pH and the xanthophyll cycle (Gilmore 2004). In absolute terms, this quenching corresponds to NPQ_{fast}. The second component, qI, or slow relaxing quenching is generally thought

to be caused by photoinhibition, and represents the fraction of quenching that takes from c. 8–10 min or longer to relax. In absolute terms, this quenching corresponds to NPQ_{slow}. However, it is now realized that photoinhibition is only one of the many processes responsible for slow relaxing quenching (Liu *et al*. 2019). In higher plants, as might be intuitively predicted, sun plants typically have three to four-fold larger pools of xanthophyll cycle pigments than shade plants, and as a result higher NPQ (Demmig-Adams 1998; Mathur *et al*. 2018). Furthermore, growing individual species of higher plants at increasing light levels increases pigment pool size and NPQ (Demmig-Adams *et al*. 2020). A possible exception to this trend may be plants that grow in fluctuating light levels. For example, exposing *Arabidopsis* to an identical total dosage of light, supplied under either constant or fluctuating conditions, induces higher NPQ in plants receiving fluctuating light (Alter *et al*. 2012). Results suggest that *Arabidopsis* has only a limited ability to utilize short sunflecks, and constitutively high NPQ may be needed in such plants. However, in environments where light levels change rapidly, excessive NPQ reduces the efficiency of photosynthesis when light returns to lower levels (Murchie & Ruban 2020). In such environments, the rapid relaxation of NPQ may confer a selective advantage (Kromdijk *et al*. 2016).

For lichens, different species, and even different populations within one species, can display sun and shade forms (Piccotto & Tretiach 2010). Typically, as for higher plants discussed above, lichens from exposed sites tend to have higher light saturation points and higher maximum rates of photosynthesis than those growing in more shaded microhabitats. Furthermore, lichen photobionts have developed extensive mechanisms to protect themselves from the effects of high light stress (Beckett *et al*. 2021b). Tolerance mechanisms to long-term (over a range of weeks to months) light stress include the synthesis of cortical light screening pigments by the fungal symbiont (Solhaug & Gauslaa 2012). In the shorter term, as for higher plants, photobionts can

reduce high light stress by thermally dissipating excess light using NPQ (Demmig Adams *et al*. 1990). However, unlike the typical pattern found in higher plants, sun lichens do not always display higher NPQ than those growing in the shade. For example, Vráblíková *et al*. (2006) showed that NPQ in *Xanthoria parietina* (L.) Th.Fr from an exposed site increases from early spring until the summer solstice. Intuitively this is consistent with a greater need for photoprotection in the season with the highest solar irradiance and is consistent with most of the higher plant literature. However, MacKenzie *et al*. (2002) found greater levels of photoprotection in *Lobaria pulmonaria* (L.) Hoffm. in spring than in late summer. Similarly, Veres *et al*. (2020) found generally higher NPQ in shaded rather than exposed soil crust lichens. There are various explanations for these differences. First, probably only relatively few lichens growing in shade microhabitats experience uniformly low light. For example, lichens growing on the trunks of trees 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. Lichens in such habitats experience rapidly changing levels of irradiance; the relatively brief periods that lichens are exposed to high light levels are known as 'sunflecks' (Greer 2022). As for higher plants growing under these conditions, photobionts may need at least some constitutive NPQ to protect themselves against photoinhibition. Second, sun lichens may use a variety of mechanisms to protect themselves against high light in addition to NPQ, for example upregulation of ROS scavenging enzymes, or enzymes involved in the PSII repair cycle (Beckett *et al*. 2021b). Third, recent evidence suggests that NPQ (and levels of xanthophyll cycle pigments) in free-living algae may be involved in tolerance to stresses other than light (Fernández-Marín *et al*. 2021). Irrespective of the reasons, for lichens no simple correlation seems to exist between NPQ and light availability.

Recently, Beckett *et al*. (2021a) compared various photosynthetic parameters in a range of sun and shade lichens using chlorophyll fluorescence, and Fig. 4.1 illustrates a summary of this survey. Unlike results from higher plants, shade lichens displayed higher NPQ than sun species (Fig. 4.1A). Consistent with the proposed benefits of rapid relaxation outlined above, a large proportion of the NPQ in shade species relaxed quickly on transition from light to dark (Fig. 4.1A). As discussed above, these species grow in habitats where they experience rapidly changing light levels such as sunflecks, and rapid relaxation of NPQ will enable them to efficiently utilize the lower light levels available after a sunfleck has passed. However, more consistent with results from higher plants, rETR saturated at lower light intensities in shade than sun species (Fig. 4.1B & C), and shade species displayed lower maximum rates of rETR. These observations suggest that a general downregulation of photosynthetic capacity is likely to occur at lower light levels. While the survey of Beckett *et al*. (2021a) compared different lichen species, our preliminary work suggested that similar differences can occur within the same species. Mkhize *et al*. (2022) compared the induction of rETR, and the induction and relaxation of NPQ in sun and shade populations of *Ramalina*. Similar to the results summarized in Fig. 4.1, compared with the sun population, the shade population displayed higher but faster relaxing NPQ and lower maximum rates of rETR. Anecdotal evidence suggests that while shade species are only rarely found in sunny microhabitats, sun species can often be much more readily collected from shaded habitats. Apparently, no comprehensive survey has been carried out on the induction and relaxation of NPQ in different collections of the same species of lichen growing in exposed and shaded habitats. The main aim of the work presented here was to compare the induction and relaxation of NPQ and the induction of rETR in a range of Afromontane lichens that generally grow in sunny sites with shade collections of the same species.

In an additional study, we compared the induction and relaxation of NPQ in melanized and pale thalli of three shade lichens. The melanized and pale forms were collected growing close to each other but typically melanized forms were found in more exposed microhabitats. Gauslaa & Goward (2020) suggested that in *Lobaria pulmonaria* melanic pigments may adjust the light received by the photobiont beneath the screening upper cortex to rather uniform levels, for example across a gradient in tree canopy openness. The implication would be that photosynthetic parameters such as NPQ should not differ between pale and melanic thalli. However, Gauslaa & Goward (2020) also point out that melanin formation is rapid under inducing conditions (Solhaug *et al*. 2003), but it is unknown how fast fungal melanins are removed when pigmented thalli experience lower light levels. Our second aim was to test whether long-lasting excess melanins can cause photobionts to adopt the characteristics of those from shade lichens.

4.2 Materials and Methods

4.2.1 Sun and shade collections of lichen material

Heterodermia leucomelos (L.) Poelt, *Parmotrema perlatum* (Huds.) M. Choisy, *Ramalina celastri* (Spreng.) A. Massal. and *Usnea undulata* Stirt. were collected from Fort Nottingham, KwaZulu-Natal, South Africa. The area is a small patch of Afromontane forest and occurs at altitudes between 1500 and 1600 m; the climate is characterized by warm, wet summers and dry, cold (down to freezing temperatures) winters. Lichens were collected from the small tree *Leucosidea sericea* Eckl. & Zeyh. 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 a few metres away, from deep inside the canopy on main branches or tree trunks. *Xanthoparmelia conspersa* (Ehrh. ex Ach.) Hale was collected at Queen Elizabeth Park Nature Reserve, KwaZulu Natal, South

Africa (altitude c. 850 m) on exposed and shaded rocks. *Xanthoria parietina* was collected on the outskirts of Kazan, Russian Federation on exposed and shaded sides of the same silver birch trees (*Betula pendula* Roth). Lichens were cleaned; generally they were collected dry, but if moist they were allowed to dry overnight between sheets of filter paper at laboratory temperature. Lichens were stored dry at 4 °C in a refrigerator for up to 2 weeks. The photobionts of these lichens have been reported to belong to the chlorophycean genus *Trebouxia* (Rambold *et al*. 1998). For uniformity, and to recover from any field stress, before the start of each experiment all material was initially hydrated by spraying with distilled water followed by moist storage for c. 24 h in dim light (20 µmol photons m^{-2} s^{-1}) at 12 °C.

4.2.2 Melanized and pale collections of lichen material

Crocodia aurata (Ach.) Link was collected from Fort Nottingham, KwaZulu Natal, South Africa growing on *Leucosidea sericea*. *Lobaria pulmonaria* and *L. virens* (With.) J. R. Laundon were collected from the trunks of oak trees in an old forest in Langangen, Norway. All three species tend to grow in shaded habitats and all possess the photobiont *Symbiochloris*. Melanized material was collected from slightly more exposed microhabitats, close to the pale thalli. Material was prepared for experimentation in the same way as the sun and shade collections of the *Trebouxoid* lichens.

4.2.3 Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured using a PAM 2500 fluorimeter (Walz, Effeltrich, Germany) using the red LED. After a dark adaptation period of at least 10 min, the maximal efficiency of photosystem II (PSII; F_V/F_M) was measured, where F_M = maximum fluorescence and F_V = variable fluorescence or ($F_M - F_O$), with F_O = minimal fluorescence yield of the dark-adapted

state. Thalli with anomalous values of F_V/F_M were discarded. Rapid light response curves of electron transport rates (ETR) were measured by increasing the actinic light in 11 small steps of 10 to 20 s each from 0 to 475 µmol photons m^{-2} s^{-1} (at 12, 33, 56, 81, 106, 141, 185, 238, 301, 383 and 475 µmol photons m^{-2} s^{-1}) with saturating flashes at the end of exposure to each light level. The relative ETR was calculated as:

rETR = 0.5 × ΦPSII × PAR

where PAR = photosynthetically active radiation and ΦPSII is the effective quantum yield of PSII photochemistry calculated as $(F_M' - F_t)/F_M$ (where F_M' = maximal fluorescence yield of the light-adapted state and F_t = stable fluorescence signal in the light).

The equation derived by Eilers & Peeters (1988) was used to calculate the following parameters:

α: The initial slope of the rapid light curve, related to the maximal quantum yield of PSII electron transport under light limited conditions (units: electron photon^{-1}).

$rETR_{MAX}$: the maximal relative ETR reached during light curve recording, reflecting the light saturated capacity of the sample (units: µmol electrons m^{-2} s^{-1}).

lk: the light intensity at which PAR saturation sets in. This is estimated by constructing a linear regression of the initial part of the light response curve and extrapolating it until it hits an ETR value corresponding to the estimate of $rETR_{MAX}$. The light intensity where the two lines intersect is lk (units: µmol photons m^{-2} s^{-1}).

To determine the induction of rETR, and the induction and relaxation of NPQ, thalli were dark-adapted for 10 min and Fv/F_M measured; thalli with anomalous values were discarded. An actinic light of 100 µmol photons m^{-2} s^{-1} was then turned on, and saturating flashes applied at increasing intervals for 11 min. The actinic light was then turned off and relaxation measured for

8 min, with saturating flashes given at increasing intervals. NPQ was calculated using the formula of Bilger *et al*. (1995):

$NPQ = (F_M - F_M')/F_M'$

In addition, NPQ was divided into fast and slow relaxing quenching, corresponding approximately to qE and qI respectively using equations in Kalaji *et al*. (2017):

$NPQ_{fast} = (F_M - F_M')/F_M' - (F_M - F_M'')/ F_M''$

$NPQ_{slow} = (F_M - F_M'')/ F_M''$

where F_M'' = maximum fluorescence after 8 min of darkness.

In initial experiments we tested the induction of NPQ 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. However, to test the differences in NPQ and rETR between the sun and shade forms at higher light levels, for *Parmelia perlata* and *Xanthoria parietina* NPQ and rETR were measured as a function of light intensity. Material (eight replicates of sun and eight of shade collections) was allowed to equilibrate for 10 min at each light level before readings were taken.

4.3 Results

Table 4.1 summarizes the parameters derived from the rapid light curves, and from the induction and relaxation of NPQ and rETR experiments (Fig. 4.2). The estimates of the maximum dark-adapted quantum yield of PSII (i.e. F_V/F_M and α) were rather similar in shade and sun collections, although both were usually slightly lower in the sun lichens. Both $rETR_{MAX}$ and the PAR where saturation sets in (lk) were on average much higher in sun compared with shade collections. The

only exception was *Heterodermia leucomelos*, where values were slightly higher in shade than sun collections.

Table 4.1. Summary of photosynthetic parameters of sun and shade collections of the lichen species. Rapid light curves were used to derive alpha (α), the maximal quantum yield of PSII electron transport under light limited conditions quantum efficiency, the start of light saturation (lk) and maximal relative electron transport rate ($rETR_{MAX}$). F_V/F_M values (the maximal efficiency of photosystem II) were measured at the start of the rapid light curves. NPQ values (non-photochemical quenching) were obtained by illuminating dark-adapted lichens with light at 100 µmol m^{-2} s^{-1} and measuring the time course of the induction of NPQ for 11 min, and the subsequent relaxation of NPQ for 8 min after switching off the light. Figures are given as ± SE, n = 10.

Species	Collection locality	α	$rETR_{MAX}$	lk (µmol m^{-2} s^{-1})	NPQ after 11 min	NPQ_{fast} (qE)	NPQ_{slow} (qI)	% NPQ relaxed after 2 min
Xanthoparmelia conspersa	Shade	0.35 ± 0.01	23.2 ± 1.2	67 ± 3	1.65 ± 0.13	0.85 ± 0.14	0.79 ± 0.09	16
	Sun	0.31 ± 0.00	36.6 ± 1.8	120 ± 7	0.84 ± 0.10	0.31 ± 0.09	0.55 ± 0.08	7
Heterodermia leucomela	Shade	0.31 ± 0.00	20.3 ± 1.9	66 ± 6	0.45 ± 0.04	0.17 ± 0.04	0.28 ± 0.05	-3
	Sun	0.26 ± 0.01	15.8 ± 1.4	62 ± 6	0.40 ± 0.06	0.19 ± 0.05	0.21 ± 0.04	15
Parmelia perlata	Shade	0.26 ± 0.02	9.0 ± 0.5	35 ± 2	1.10 ± 0.10	0.72 ± 0.06	0.39 ± 0.06	23
	Sun	0.27 ± 0.01	12.9 ± 2.3	49 ± 9	0.83 ± 0.10	0.44 ± 0.08	0.37 ± 0.04	14
Usnea undulata	Shade	0.24 ± 0.01	17.2 ± 0.8	72 ± 4	0.63 ± 0.09	0.36 ± 0.06	0.27 ± 0.04	27
	Sun	0.20 ± 0.01	70.6 ± 11.9	350 ± 54	0.22 ± 1.14	0.16 ± 0.02	0.06 ± 0.02	-12
Xanthoria parietina	Shade	0.26 ± 0.01	44.2 ± 2.4	171 ± 11	0.25 ± 0.02	0.09 ± 0.02	0.16 ± 0.01	4
	Sun	0.20 ± 0.01	70.6 ± 11.9	350 ± 54	0.22 ± 0.04	0.06 ± 0.01	0.14 ± 0.01	-12
Ramalina celastri	Shade	0.24 ± 0.01	12.9 ± 0.8	54 ± 4	1.42 ± 0.15	0.93 ± 0.13	0.49 ± 0.04	25
	Sun	0.25 ± 0.01	30.8 ± 2.4	105 ± 10	0.39 ± 0.05	0.31 ± 0.03	0.33 ± 0.03	-5
Mean	**Shade**	**0.28**	**21.1**	**77**	**0.92**	**0.52**	**0.40**	**15**
Mean	**Sun**	**0.25**	**34.7**	**143**	**0.32**	**0.25**	**0.28**	**3**

Figure 4.2 compares in detail the induction and relaxation of NPQ and the induction of rETR by light at 100 μmol m^{-2} s^{-1} in shade and sun collections. Differences between the induction of NPQ were rather variable, but NPQ was always higher in sun compared with shade collections, and only in *Xanthoria parietina* was the difference not significant (Table 4.2). On average, although there was considerably variation between species withinl sun and shade collections, after 11 min NPQ was almost three times as high in shade species (Table 4.1). In general, NPQ relaxed faster in shade than in sun collections, on average after 2 min dropping by 15% in shade and 3% in sun collections. This was confirmed by dividing NPQ into fast and slow relaxing components. In sun collections, NPQ_{slow} was on average c. 15% higher than NPQ_{fast}; by contrast, in shade collections NPQ_{slow} was about 25% less than that of NPQ_{fast}. Induction of rETR was rapid, and similar in sun and shade collections. Consistent with the measurements of $rETR_{MAX}$ from the rapid light curves, rETR induced after 11 min was higher in all sun collections except *Heterodermia leucomelos* (Fig. 4.2B). Generally the higher values of NPQ and lower values of rETR in shade collections observed in the induction/relaxation experiments (Fig. 4.2) were confirmed when NPQ and rETR were measured over a wider range of light levels for *Parmotrema perlatum* and *Ramalina celastri* (Fig. 4.3).

Table 4.2. Statistical analysis (two-way ANOVA, Microsoft Excel) of the effect on non-photochemical quenching (NPQ) and relative electron transport rates (rETR) of collection location (sun or shade) and time for six lichen species. An actinic light at 100 µmol photons m^{-2} s^{-1} was switched on for 11 min, and then switched off and measurements taken for a further 8 min. * = $P < 0.05$, ** = $P < 0.01$.

	Xanthoparmelia conspersa		*Heterodermia leucomela*		*Parmelia perlata*		*Usnea undulata*		*Xanthoria parietina*		*Ramalina celastri*		df
	NPQ	rETR	NPQ	rETR	NPQ	rETR	NPQ	rETR	NPQ	rETR	NPQ	rETR	
Sun or Shade	**	**	*	**	**	**	**	**	0.10	**	**	**	1
Time	**	**	**	**	**	**	**	**	**	**	**	**	8
Interaction	**	0.16	0.63	0.21	0.28	0.10	**	**	0.15	0.42	**	**	8

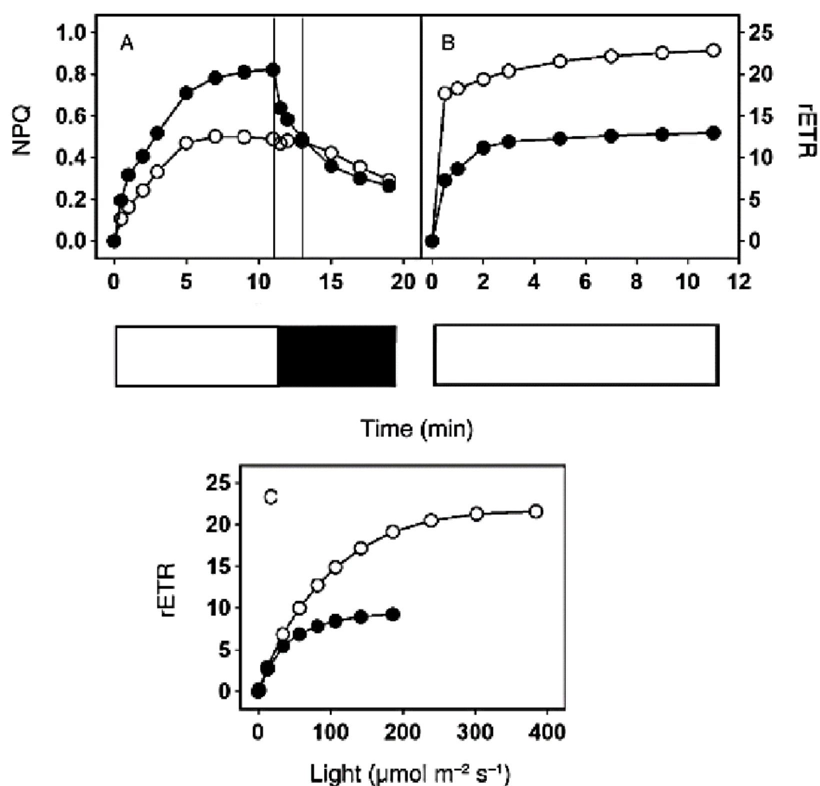

Figure 4.1: A. Induction and relaxation of non-photochemical quenching (NPQ) and B induction of relative electron transport rate (rETR) for five sun (open symbols) and five shade (closed

symbols) species of lichens in response to light at 100 µmol m^{-2} s^{-1}. C. ETR as a function of light intensity in five sun (open symbols) and five shade (closed symbols) species of lichens. Data taken from Beckett *et al*. (2021a).

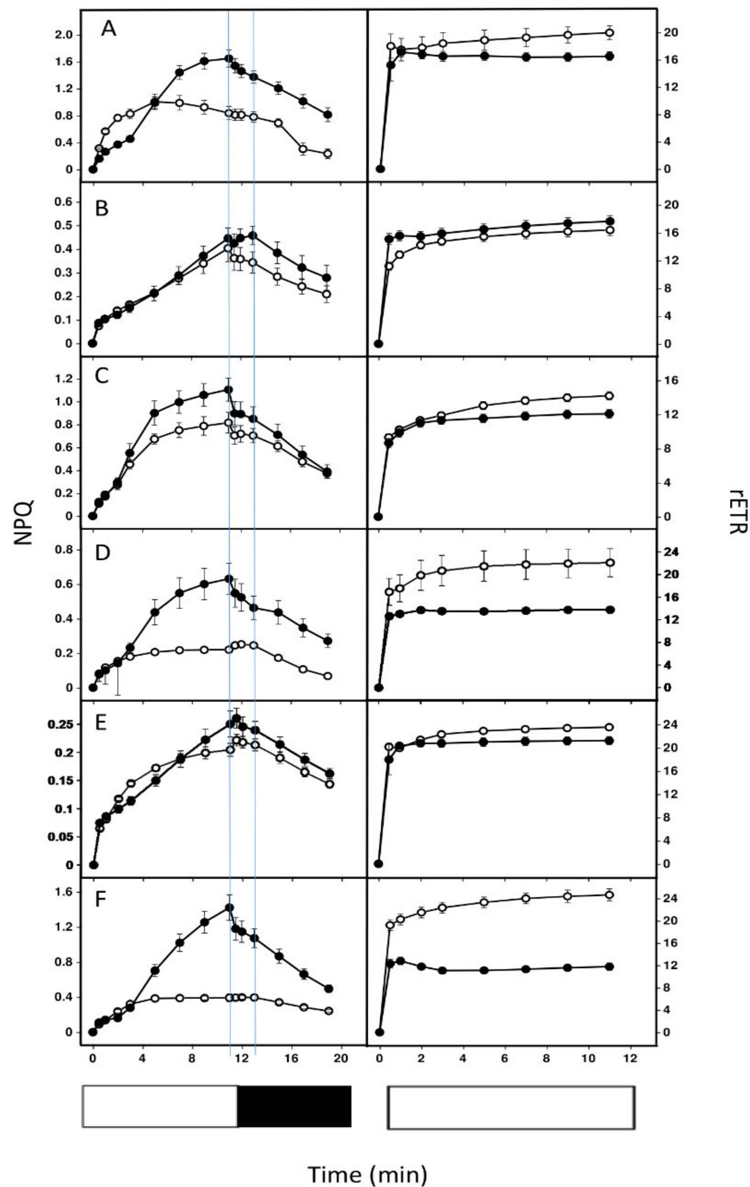

Figure 4.2: Induction and relaxation of non-photochemical quenching (NPQ), and induction of relative electron transport rate (rETR) in sun (open symbols) and shade (closed symbols) collections of lichens in response to light at 100 µmol m^{-2} s^{-1}. A, *Xanthoparmelia conspersa*; B, *Heterodermia leucomela*; C, *Parmelia perlata*; D, *Usnea undulata*; E, *Xanthoria parietina*; F,

Ramalina celastri. Error bars denote the standard error, n = 10–15. Vertical lines on the plots delimit NPQ during the first 2 min of darkness. White and black sections in the boxes at the base of the plots indicate the time periods when samples were exposed to light or darkness respectively.

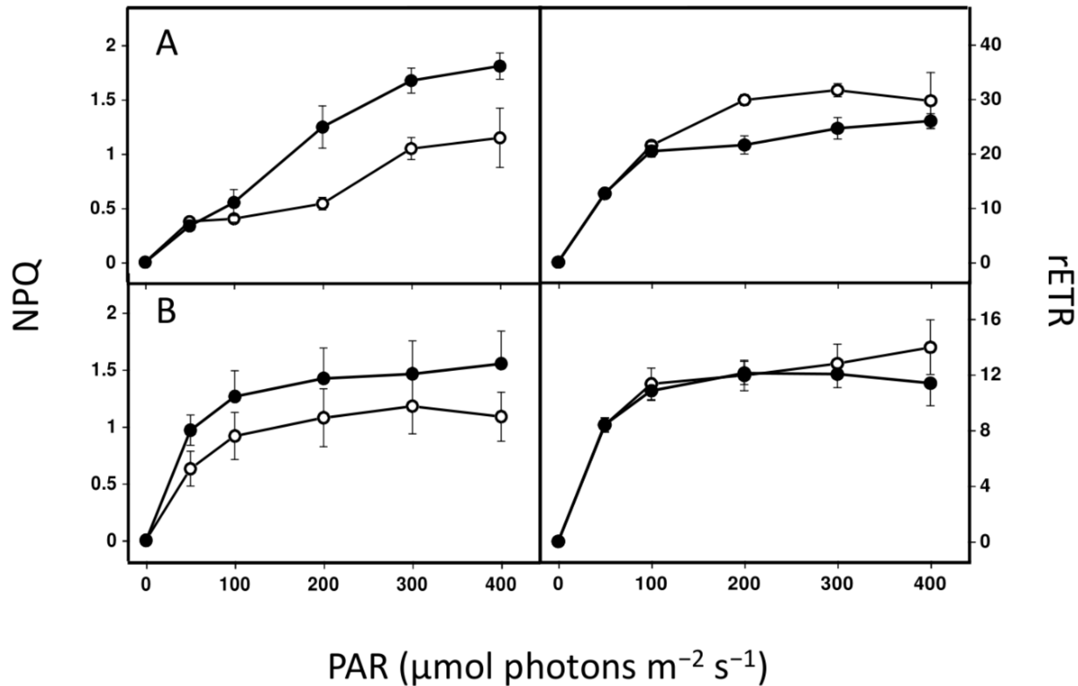

Figure 4.3: Non-photochemical quenching (NPQ) and relative electron transfer rate (rETR) as a function of light intensity in sun (open symbols) and shade (closed symbols) collections of A. *Parmelia perlata* and B. *Ramalina celastri*. Error bars denote the standard error, n = 10–15.

In the experiments with melanized and pale collections of shade lichens with *Symbiochloris* as the photobiont, the effective quantum yield of PSII electron transport under light limited conditions (α) was rather similar in melanized and pale collections (Table 4.3). $rETR_{MAX}$ and the PAR where saturation sets in (lk) were on average 22 and 18% higher respectively in melanized forms. In the induction of NPQ experiments (Fig. 4.4), for *Crocodia aurata* and *Lobaria virens* NPQ was considerably higher in melanized than pale forms, while in *L. pulmonaria* NPQ was

initially induced faster in the melanized forms but after 11 min both the pale and melanized forms displayed similar values of NPQ. For all three species, rETR was slightly higher after 11 min in melanized than pale thalli.

Table 4.3. Summary of the photosynthetic parameters of pale and melanized collections of the same lichen species. Rapid light curves were used to derive alpha (α), the maximal quantum yield of PSII electron transport under light limited conditions, the start of light saturation (lk) and the maximal relative electron transport rate ($rETR_{MAX}$). In separate experiments, dark-adapted lichens were illuminated with actinic light at 100 µmol m^{-2} s^{-1} for 11 min, and the light then switched off and thalli kept in darkness for a further 8 min; non-photochemical quenching (NPQ) was measured at intervals. Figures are given as ± SE, n = 10.

Species	Thallus Colour	α	$rETR_{MAX}$	lK (µmol m^{-2} s^{-1})	NPQ after 11 min	% NPQ relaxed after 2 min
Crocodia aurata	Pale	0.38 ± 0.01	12.9 ± 0.8	49 ± 2	0.85 ± 0.11	53
	Melanised	0.31 ± 0.01	14.7 ± 0.4	54 ± 4	1.51 ± 0.22	57
Lobaria pulmonaria	Pale	0.38 ± 0.02	9.5 ± 1.2	28 ± 5	2.38 ± 0.09	58
	Melanised	0.33 ± 0.10	11.4 ± 0.9	36 ± 4	2.40 ± 0.18	61
Lobaria virens	Pale	0.36 ± 0.02	6.5 ± 0.6	26 ± 8	2.15 ± 0.17	27
	Melanised	0.32 ± 0.10	9.0 ± 0.8	30 ± 4	2.98 ± 0.16	50
Mean	Pale	**0.37**	**9.6**	**34**	**1.79**	**46**
	Melanised	**0.32**	**11.7**	**40**	**2.29**	**56**

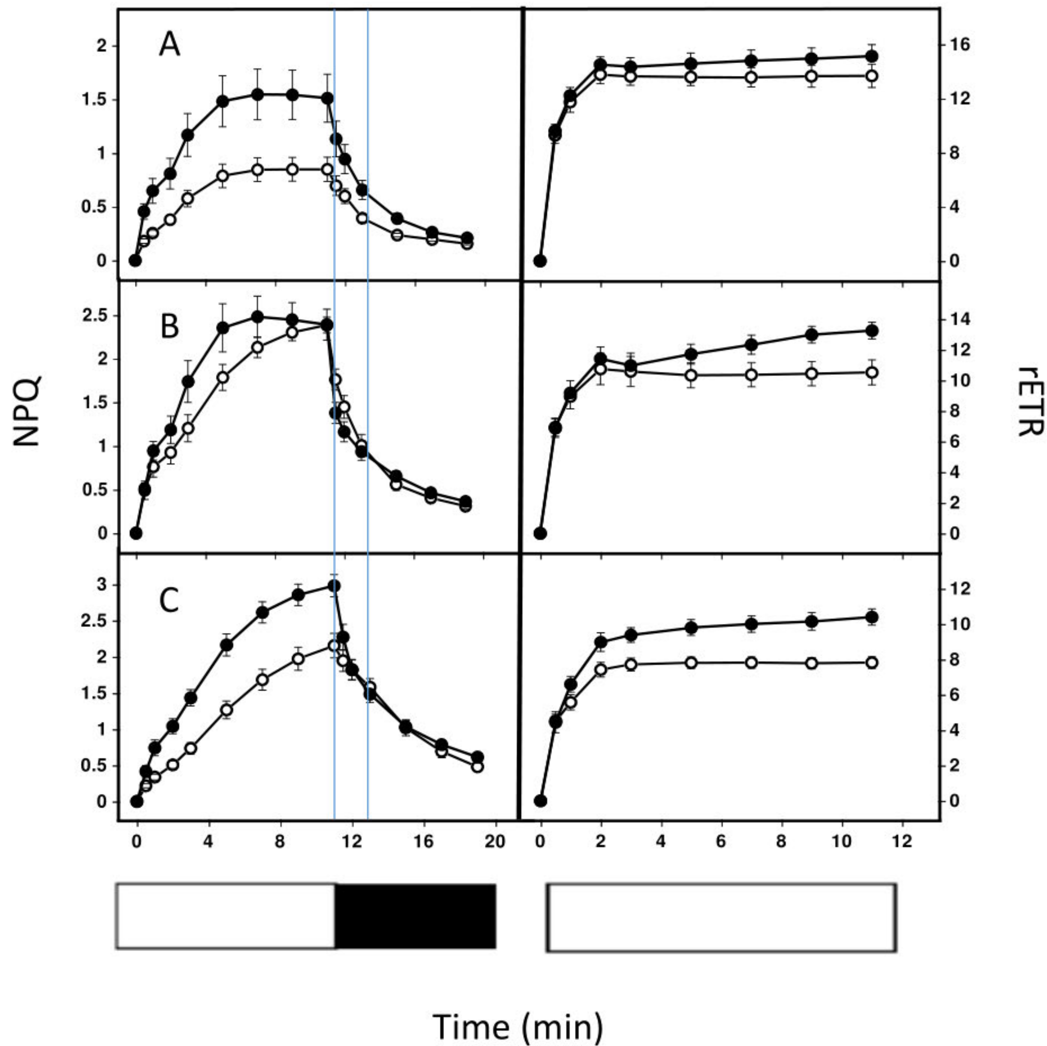

Figure 4.4: Induction and relaxation of non-photochemical quenching (NPQ), and induction of relative electron transport rate (rETR) in pale (open symbols) and melanised (closed symbols) collections of lichens in response to light at 100 μmol m^{-2} s^{-1}. A. *Crocodia aurata*; B. *Lobaria pulmonaria*; C. *Lobaria virens*. Error bars denote the standard error, n = 10–15. Vertical lines on the plots delimit NPQ during the first 2 min of darkness. White and black sections in the boxes at the base of the plots indicate the time periods when samples were exposed to light or darkness respectively.

4.4 Discussion

The main aim of the work presented here was to compare the patterns of NPQ induction and relaxation in collections of lichens from exposed microhabitats with those of the same species from more shaded sites. All species tested here normally grow in more exposed microhabitats, but it is relatively easy to undertake collections of more shaded thalli. Results showed that, generally, shade collections display higher but faster relaxing NPQ, and lower rates of rETR (Fig. 4.2, Table 4.1). The differences in NPQ and rETR resemble those we reported earlier for different species of sun and shade lichens (Beckett *et al*. 2021a), summarized in Fig. 4.1. Interestingly, the finding of generally higher values of NPQ in shade than sun forms differs from results usually obtained with higher plants (Demmig-Adams *et al*. 2020). The second part of the study compared collections of pale thalli from three species of shade lichens with melanized thalli of the same species growing nearby but in slightly more exposed habitats. Results showed that patterns of induction and relaxation of NPQ in the melanized forms resemble those from shade lichens (Fig. 4.4). It seems likely that melanins induced by temporary high light may have long-lasting effects; photobionts under a melanized upper cortex can adopt the characteristics of those from shade lichens. Taken together, results suggest that lichen photobionts can flexibly adjust the amount and type of NPQ and their rETR in response to light availability.

4.4.1 Rapid light curves

Rapid light curves enable comparison of the parameters of photosynthesis in sun and shade collections of the same species. First, the effective quantum yields of PSII electron transport under light limited conditions of dark-adapted sun and shade lichens are rather similar, whether estimated as α or F_V/F_M (Table 4.1). In general, both α and F_V/F_M tend to be slightly lower in sun than shade lichens, possibly reflecting some residual stress in the sun populations. These differences might

have disappeared if a recovery rehydration period longer than the standard 24 h had been used. Similar results have been found in higher plants (Greer 2022), probably because the efficiency of the light reactions is the same, irrespective of how much light has been received during growth. Second, except for *Heterodermia leucomelos*, the light intensity where saturation of photosynthesis sets in (lk) is lower in the shade collections than in the sun collections (averaging 77 compared with 143 μmol m^{-2} s^{-1}). Third, the average $rETR_{MAX}$, the maximal relative electron transport rate (reflecting the light saturated rate of photosynthesis), is lower in shade than sun collections (21.1 compared with 34.7). As discussed in the 'Introduction', downregulation of photosynthetic capacity in shade plants probably represents an adaptation to save energy on maintaining unnecessarily high levels of cytochromes and enzymes (Greer 2022). Although there are few comparable studies with lichens, Piccotto & Tretiach (2010) obtained similar results from a survey of a range of lichens from contrasting habitats (including some collections of the same species). In the present study, decisions on where to collect sun and shade material were based on careful visual inspection of the study sites and, in general, selections were reflected in the values of lk recorded. Possibly for *Heterodermia*, the differences in light availability of the sun and shade collections were less than visual inspection would suggest, although it is also possible that this species is inherently less plastic. However, in general, lk, or the PAR where saturation starts, appears to be a good quantitative measure of the light regimes of the collection sites of the lichens.

4.4.2 Shade forms display higher NPQ than sun forms

The photosynthetic parameters of the sun and shade collections of lichens differ mainly in their kinetics of induction and dark relaxation of NPQ (Table 4.1, Fig. 4.2). In some species, the induction of NPQ appears to be 'biphasic', with the induction kinetics from 0–3 min differing from those from 3–11 min. To understand the reason for these differences at a molecular level would

require a more sophisticated approach than the one used here. In all cases, NPQ induced after 11 min is higher in shade than sun collections (Fig. 4.2), on average two times higher (Table 4.1). In only one species (*Xanthoria parietina*) is the difference not significant (Table 4.2). This is interesting, because in *Xanthoria* the lk and $rETR_{MAX}$ data derived from the rapid light curves suggest that the bright orange and pale collections do not represent genuine sun and shade forms, respectively (Table 4.1). It may be relevant that the material of *Xanthoria* used here was the only collection from the generally less bright, cool temperate regions and had the lowest values of NPQ (Table 4.1). For the other (Afromontane) species used here, it seems likely that in shaded habitats sunflecks can cause light levels to increase very suddenly, potentially causing oxidative stress. Therefore, effective defence mechanisms must be constitutively in place. Higher NPQ in shade collections did not only occur at 100 µmol m^{-2} s^{-1} (the level used to measure induction kinetics), but also occurred when NPQ was measured over a wider range of light levels (Fig. 4.3). There are a small number of reports from other workers that lichens collected from more shaded habitats may have generally high NPQ (MacKenzie *et al*. 2002; Veres *et al*. 2020). In contrast to these results from lichens, in higher plants sun forms normally have higher NPQ than shade forms (Demmig-Adams *et al*. 2020). Similarly for lower plants, in both filmy ferns and bryophytes, sun forms and species have been reported to possess higher NPQ than those from more shaded sites (Proctor 2003; Proctor & Smirnoff 2015). Furthermore, growing free-living algae under increasing light intensities increases NPQ, probably due to higher pools of xanthophyll cycle pigments (Blommaert *et al*. 2021). The reasons for the differences between lichens and other photosynthetic organisms remain unclear. However, there are reports that suggest that plants growing in shade do not always display low NPQ. For example, Griffiths & Maxwell (1999) found rather similar NPQ in deep shade and sun species of epiphytic bromeliads. As discussed in the Introduction, the typical

microhabitats where shade lichens grow are characterized by sunflecks, and high NPQ provides photoprotection from sudden increases in light levels. A further reason for the generally low NPQ of sun forms could be the presence of lichen substances in the upper cortex. Although none of the lichens tested here, except *Xanthoria parietina*, are pigmented, Ndhlovu *et al*. (2022) showed that even unpigmented lichen substances can increase the tolerance of lichens to photoinhibition, apparently by increasing reflectance. It seems likely that sun collections of lichens contain higher concentrations of substances than those growing in shade (Solhaug & Gauslaa 2012), reducing their need for NPQ.

4.4.3 NPQ in shade forms relaxes faster than in sun forms

In addition to shade forms displaying more NPQ than sun forms, a further difference is that NPQ in shade forms tends to relax faster (Fig. 4.2, Table 4.1). On average, 15% of NPQ relaxed during the first two minutes of darkness in shade forms, compared with only 3% in sun forms. NPQ has a fast-relaxing component (NPQ_{fast}), corresponding approximately to qE, and a slow-relaxing component (NPQ_{slow}) corresponding to ql (Kalaji *et al*. 2017). The fast-relaxing component relaxes during the first few minutes of darkness and is related to xanthophyll cycle activity, while the slow-relaxing component is caused by photoinhibition and a variety of other processes and takes longer to relax (Gilmore 2004). In sun forms, on average more than half of the NPQ is attributable to qI (Table 4.1). While shade forms also display significant qI, on average they possess more than double the fast-relaxing component compared to sun forms (Table 4.1). Unfortunately, there have been few studies that have measured qI and qE in sun and shade populations of the same lichen species to compare with results presented here. However, NPQ has been suggested to play both positive and negative roles in ensuring optimal plant productivity in environments where light levels are rapidly changing (Murchie & Ruban 2020). On the positive side, NPQ delays the onset

of photoinhibition by reducing ROS production. On the negative, while not affecting photosynthesis in high light, NPQ can greatly reduce the quantum yield of photosynthesis at lower light levels. In other words, under low light a lichen 'expressing' high NPQ will require a higher irradiance to achieve the same photosynthetic rate as one without it. The implication for lichens could be that, as discussed above, while shade forms growing in habitats subjected to rapidly changing light levels will benefit from high NPQ, they will also benefit from NPQ that relaxes rapidly following transition to low light. Possession of rapidly relaxing NPQ will enable lichens to efficiently utilize the lower light levels available after a sunfleck has passed.

Interestingly, in *Xanthoparmelia* the induction of NPQ during the transition from darkness to light occurs more rapidly in sun compared to shade forms (and to a lesser extent in *Xanthoria*) (Fig. 4.2). Intuitively, it could be predicted that when growing in a habitat characterized by sunflecks there may be some advantage in rapidly inducing NPQ. We can offer no obvious explanation for the rapid induction of NPQ in sun forms of *Xanthoparmelia*. However, even in this species, shade forms display higher overall NPQ, and fast relaxing NPQ comprises a greater proportion of the total (52% for shade compared to 37% for sun, that is NPQ_{fast} / total NPQ after 11 min illumination) (Table 4.1).

4.4.4 rETR induces rapidly in both sun and shade forms

In contrast to NPQ, rETR is induced very rapidly in all species (Fig. 4.2). However, there are some suggestions of a biphasic induction of rETR, and some shade forms appear to activate rETR slightly more quickly than sun forms. Presumably, rapid activation is an advantage for populations of lichens that receive much of their solar radiation as sunflecks. In higher plants, after dark-adapted leaves are illuminated, several minutes are required for PSII and PSI to be synchronized for O_2 evolution, NADP reduction and ATP synthesis. Synchronization is associated with several

processes at the molecular level (e.g. the phosphorylation of light harvesting complex II) (Kalaji *et al*. 2017). While the differences appear relatively small, there could be some merit in using more sophisticated approaches than the one used here to compare the rates of induction of rETR in sun and shade forms.

4.4.5 Induction and relaxation of NPQ in pale and melanized thalli

Comparisons of the induction and relaxation of NPQ in melanized and pale thalli of members of the same species show that melanized forms generally have more NPQ than pale forms (Fig. 4.4, Table 4.3). In all species, NPQ increases faster in melanized forms than pale forms. After 11 minutes, NPQ was considerably higher in melanized than pale *Crocodia aurata* and *Lobaria virens*, while in *L. pulmonaria* NPQ was similar. Thus, in general, the induction and relaxation of NPQ in the photobionts of the melanized thalli resemble more closely those of the shade rather than the sun collections in the first part of this study (Fig. 4.2). However, in all three species, rETR was slightly higher after 11 min in melanized than in pale thalli, and $rETR_{MAX}$ was c. 20% higher (Table 4.3). Furthermore, lk was c. 20% higher in melanized than pale thalli. This suggests, for rETR and lk, the photobionts in melanized thalli resemble more closely those of the sun forms described in the first part of this study. Based on growth measurements, Gauslaa & Goward (2020) suggested that in *Lobaria pulmonaria* melanic pigments may adjust the light received by the photobiont beneath the screening upper cortex to rather uniform levels, for example across a gradient in tree canopy openness. The implication would be that photosynthetic parameters, for example NPQ, should not differ between pale and melanic thalli, which was clearly not observed here. However, Gauslaa & Goward (2020) also point out that melanin formation is rapid under inducing conditions (Solhaug *et al*. 2003), but it is unknown how fast fungal melanins are removed when pigmented thalli experience lower light levels. It might be relevant that the study of Gauslaa

& Goward (2020) was carried out in inland British Columbia, corresponding to the 'boreal' climatic zone, while the lichens used here were collected from a nemoral boreal region in Norway or South African sub-tropical Afromontane vegetation. Possibly, in the regions we collected our lichens, the light levels of sunflecks are higher, resulting in melanization that may be excessive for some of the year.

4.5 Conclusions

The main study here compared the induction and relaxation of NPQ in collections of the same lichen species from exposed and more shaded locations. The lichen species all have trebouxioid photobionts and normally grow in more exposed microhabitats but can readily be collected from more shaded locations. Although there are some differences between species, results gave a rather consistent picture. Shade forms display generally higher NPQ, presumably to protect lichens from occasional rapid increases in light that occur during sunflecks. However, the NPQ of shade forms relaxes quickly when light levels are reduced (relatively more NPQ is qE), presumably to ensure efficient photosynthesis can occur after a sunfleck has passed. Results are rather at variance with data from other photosynthetic organisms, where more usually the NPQ of sun forms is higher than that of shade forms. Interestingly, the *Symbiochloris* photobionts of melanized shade-adapted lichens behave as if they are adapted to relatively lower light levels than pale forms, suggesting that in some locations melanization induced during a temporary period of high light might reduce photosynthesis later in the growing season. While a recent study suggested that lichen photobionts are rather poor in adapting to temperature shifts (Nelsen *et al*. 2022), results presented here suggest that photosynthetic responses to light may be more plastic.

4.6 Acknowledgements

RB thanks the Research Fund of the University of KwaZulu-Natal and the Kazan Federal University Strategic Academic Leadership Program (PRIORITY-2030) for financial support. KM thanks Deutscher Akademischer Austauschdienst (DAAD) for a Ph.D. bursary and FM thanks the Russian Science Foundation, grant no. 18-14-00198, and the Russian government assignment of FRC Kazan Scientific Center of RAS for financial support. We are grateful to Yngvar Gauslaa and Knut Solhaug for help with collecting *Lobaria*.

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CHAPTER 5

Role of glutathione in desiccation tolerance of pale and melanised thalli of the lichen *Lobaria pulmonaria*

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Formatted for: South African Journal of Botany

Abstract

Some lichenized ascomycetes can synthesize cortical melanins, either as part of normal development or in response to UV stress. The main role of melanins is often assumed to be reducing UV-B or light stress in general. However, melanins also possess strong antioxidative properties, suggesting that they may participate more widely in defence against stress-induced reactive oxygen species (ROS). In many organisms, glutathione (GSH) is often considered to be one of the main cellular non-enzymatic antioxidants responsible for ROS scavenging. The aim of the present investigation was to test the role of melanins in stress tolerance and GSH metabolism in the lichen *Lobaria pulmonaria*. Initial analyses showed that melanised thalli of *L. pulmonaria* contain only c. 70% the GSH of pale thalli. Exogenous applications of the pro-oxidant hydrogen peroxide (H_2O_2) caused similar proportional breakdown of GSH and inhibition of PSII activity in pale and melanised thalli. Similarly, subjecting thalli to a desiccation / rehydration cycle caused similar proportional changes in GSH levels in both thallus types. Estimation of the GSH half-cell reduction potential ($E_{GSSG/2GSH}$) showed that melanised thalli maintain similar potentials as pale thalli during stress with much smaller absolute levels of GSH. Taken together, our results suggest that melanins may supplement the ROS scavenging role of normal cellular antioxidants, and therefore may be involved in the tolerance of lichens to stresses other than UV and high light.

Keywords: Reactive oxygen species (ROS), desiccation, glutathione (GSH), hydrogen peroxide (H_2O_2)

5.1 Introduction

In their natural habitat, lichens frequently experience drying and wetting cycles, and can survive extreme desiccation (Kranner et al., 2008). While most lichens are desiccation tolerant (DT), desiccation nevertheless increases ionic strength and changes cytoplasmic pH causing reactive oxygen species (ROS) production, which can lead to lipid peroxidation and protein denaturation (Kranner et al., 2008). Although our knowledge of DT mechanisms in lichens is fragmentary, it appears that they possess both constitutive and inducible mechanisms. These mechanisms include the activation of enzymatic and non-enzymatic antioxidants, and in addition the expression of specific proteins such as late-embryogenesis abundant (LEA) proteins or dehydrins, and high levels of intracellular polyols (Carniel et al., 2021). Enzymatic antioxidants include superoxide dismutase (SOD) and catalase (CAT) (Weissman et al., 2005). Lichens also contain a unique class of peroxidases that are likely to assist in ROS scavenging during desiccation (Liers et al., 2011). Non-enzymatic antioxidants such as glutathione (GSH), ascorbic acid, and tocopherols also play crucial roles in ROS scavenging during desiccation and rehydration (Kranner, 2002). Interestingly, Mayaba and Beckett (2001) showed that the activities of antioxidant enzymes are typically very low during the early stages of rehydration, exactly the time when ROS production is highest (Beckett and Minibayeva, 2007). This suggests that non-enzymatic antioxidants may be more important than enzymatic oxidants in protecting lichens from ROS produced during desiccation and rehydration.

Probably the most important non-enzymatic antioxidant is the low molecular mass thiol glutathione (GSH, γ-glutamyl-cysteinyl-glycine), which has long been studied as an important antioxidant during stresses such as desiccation and photoprotection in lichens (Cempírková and Večeřová, 2017). In unstressed tissues, GSH is usually maintained in a reduced state by NADPH-

dependent glutathione reductases (GR), but under stressful conditions such as desiccation, it is oxidized to GSSG (oxidized glutathione, Kranner, 2002). Thus, there is much evidence that DT is associated with an ability to use GSH to remove ROS during dehydration and then reduce GSSG upon rehydration (Kranner and Grill, 1997). The effectiveness of GSH depends on factors such as the total pool size of GSH, the ratio of oxidized to total glutathione (GSSG/GSH), and the activity of GR (Cempírková and Večeřová, 2017; Vráblíkova et al., 2005). Lichens contain significant amounts of GSH, and interestingly intact lichens contain about 30% more than the sum of its content in the isolated partners (Kranner et al., 2005).

While ROS scavenging is a common way to reduce the effects of stress, lichens also use a variety of other mechanisms to avoid excessive ROS production. In response to long-term high light stress, some lichens produce dark brown melanins in the upper cortex (Matee et al., 2016; Choudhury et al., 2017). Melanins strongly absorb PAR and UV-B and will thereby reduce ROS formation in the photobiont and medullary mycobiont tissues of lichens. The roles of melanins in tolerance to other abiotic stresses remain unclear (Mafole et al., 2017). In addition to being simply screening pigments, a common property of biological pigments in general is their ability to neutralize exogenous free radicals (McGraw, 2005). In some cases, fungal melanins act as powerful antioxidants, and can for example contribute to virulence of a pathogen by neutralizing the oxidative burst of host phagocytic cells (Schnitzler et al., 1999). Fungal melanins can also protect cells from pro-oxidants such as hypochlorite, permanganate and hydrogen peroxide (Jacobson et al., 1995). However, it is worth noting that a recent study indicated that in the lichen *Cetraria aculeata,* highly melanised thalli showed greater membrane oxidation than pale thalli following heat stress (Chowaniec et al., 2023). Increased oxidation was apparently not accompanied by increases in membrane damage, assessed by ion leakage. Nevertheless, it does

appear that melanisation is not always associated with increased tolerance to abiotic stress in lichens.

Surprisingly, there have been no attempts to test if melanins can contribute to desiccation tolerance in lichens. In the free-living fungus *Cenococcum geophilum*, inhibiting melanin synthesis increases susceptibility to osmotic stress and desiccation (Fernandez and Koide, 2013). In these fungi, it was suggested that melanins may increase DT by scavenging desiccation-induced ROS, strengthening cell walls, or by reducing the rate of drying by decreasing cell wall porosity. It is a common observation that free-living fungi (e.g., *Leptosphaeria maculans*, *Hortaea werneckii*) and lichens growing in extremely exposed habitats are often heavily melanised (Gostinçar et al., 2012), suggesting melanisation may promote DT. The tripartite cephalolichen *Lobaria pulmonaria* has been widely used as a model species for studying stress tolerance in lichens (di Nuzzo et al., 2022). *L. pulmonaria* is an epiphytic lichen which grows on the trunks of old trees in temperate forests (Geiser and McCune, 1997). This species exhibits a wide distribution across various regions and shows a broad range of tolerance to environmental stress (Shirazi et al., 1996). It readily synthesises melanins in the upper cortex in response to UV light (Matee et al., 2016). In the present study we tested the hypothesis that melanins may reduce oxidative stress following the application of pro-oxidants and during wetting and drying cycles in *L. pulmonaria*, and that such reductions may be reflected in smaller changes in GSH levels and cellular redox status. We also tested the ability of melanin to scavenging exogenously applied hydrogen peroxide (H_2O_2) by measuring GSH levels and the activity of PSII.

5.2 Materials and Methods

5.2.1 Lichen material

Pale and melanized thalli of *Lobaria pulmonaria* (L.) Hoffm. were collected from the bark of poplar trees growing in the outskirts of Syktyvkar, Komi Republic, Russia (latitude 61.034’ N, longitude 50.033’ E). Lichen material was cleaned, slowly dried at room temperature overnight, and then stored at −20°C for up to four weeks. Before each experiment, 1 cm discs were prepared, cut in half and stored moist in a growth chamber at 20 µmol m^{-2} s^{-1} at 12°C on wet filter paper for up to 48 h to recover from any field and storage stress.

5.2.2 Effect of H_2O_2 on vitality, PSII activity and GSH content

To test the effect of H_2O_2 on vitality and photosystem II (PSII) activity, pale and melanised thalli (15 half discs of each) were immersed in H_2O_2 at 0, 1, 10 and 100 mM and then gently shaken for 6 h. Disks were then rinsed with distilled water to remove traces of H_2O_2, blotted and then transferred to a solution of 1% nitroblue tetrazolium (NBT, Sigma, Darmstadt, Germany). After 16 h disks were transferred to petri dishes. After a dark adaptation period of at least 10 min, an imaging PAM (Walz, Effeltrich, Germany) with a red LED was used to measure the maximal efficiency of photosystem II (PSII; F_V/F_M), where F_M = maximum fluorescence and F_V = variable fluorescence or ($F_M – F_O$), with F_O = minimal fluorescence yield of the dark-adapted state.

To measure the effect of H_2O_2 on GSH content, samples of pale and melanised thalli consisting of five replicates each comprising five half discs were gently shaken in the same range of H_2O_2 concentration as above for 6 h in beakers. After incubation, samples were placed in 2 mm Eppendorf tubes and then immediately emersed in liquid nitrogen for 5 - 20 min and stored at -80°C. Samples were freeze-dried for 72 h before GSH analyses.

5.2.3 Effect of desiccation and rehydration on GSH content

Thalli were rehydrated overnight on wet filter paper, and their mass after 24 h taken as their turgid mass. Thalli were then desiccated over silica gel for 7 d and slowly rehydrated for 24 h at 100% relative humidity in a growth chamber at 20 µmol m^{-2} s^{-1} at 12°C. Thalli were sampled after 0, 0.25, 2, 6 and 24 h of rehydration. At each time point, five replicates each comprising five half disks (c. 50 mg dry mass) were sampled as above. The mass of thalli dried for 7 d on silica gel was taken as the dry mass. Water content (WC) as percentage of fresh mass was calculated using:

$$\text{WC (\%FM)} = \frac{\text{FM}-\text{DM}}{\text{FM}} \times 100$$

where FM = fresh mass and DM = dry mass.

5.2.4 GSH and redox potential analysis

GSH analysis was carried out as described by Kranner (1998). Briefly, 50 mg of freeze-dried samples and an equal amount of PVPP was ground to powder using 2 x 5 mm agate beads for 2 min at 30 Hz (bead mill, Tissue Lyser II, Qiagen, Hilden, Germany) followed by 15 sec at 25 Hz in the presence of 0.1 M HCl. This precipitates proteins and prevents the oxidation of GSH to GSSG during extraction (Kranner, 1998). Samples were then separated into two, to measure the oxidized glutathione (GSH blocked with N-ethylmaleimide) and total glutathione (i.e. the sum of GSH + GSSG, with disulphides reduced to thiols). Low-molecular-weight thiols were separated on a RP-18 column and detected fluorometrically (excitation: 380 nm; emission: 480 nm).

The concentration of GSH was calculated by subtracting the GSSG concentration from the total amount of GSH/GSSG. The glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) was calculated from the molar concentrations of thiols and disulphides considering the leaf water content and taking into account deviations from standard conditions in terms of pH (7.3) and

temperature using the Nernst equation, as outlined in Schafer and Buettner (2001). This equation, as presented below, incorporates constants such as the gas constant (R = 8.314 J K^{-1} mol^{-1}), temperature (T) in Kelvin, the number of transferred electrons (n = 2), and the Faraday constant (F = 9.6485 x 10^4 C mol^{-1}).

$$E_{GSSG/2GSH} = E^{0pH} - \frac{RT}{nF} \; In \; \frac{[GSH]^2}{GSSG}$$

5.2.5 Data analysis

Data was initially assessed for normality using the Kolmogorov-Smirnov test, and any data not meeting the assumptions were log-transformed. Two- and three-way ANOVA tests were conducted to evaluate the effect of hydrogen peroxide concentration on F_V/F_M, total glutathione, and half-cell reduction potential ($E_{GSSG/2GSH}$), as well as the effect of desiccation and rehydration on total glutathione and half-cell reduction potential ($E_{GSSG/2GSH}$) in both pale and melanized thalli of *Lobaria pulmonaria.* Where applicable, Tukey's HSD post-hoc tests were conducted to determine significant differences between the melanized and pale thalli at each time interval. All analyses were carried out using RStudio.

5.3 Results

5.3.1 Effect of H_2O_2 on F_V/F_M

Figs. 5.1 and 5.2 illustrate the effects of H_2O_2 on the maximal efficiency of PSII (F_V/F_M) in pale and melanised thalli of *L. pulmonaria*. The inhibition of PSII activity significantly increases with time and H_2O_2 concentration, and melanised thalli were slightly but significantly ($P < 0.001$) more sensitive to H_2O_2 than pale thalli (Fig. 5.1; Table 5.1).

Table 5.1

Statistical analysis (Three -Way Repeated Measure ANOVA) of the effect of hydrogen peroxide concentration [H_2O_2] on the maximal efficiency of PSII (F_V/F_M) in pale and melanized thalli of *Lobaria pulmonaria*. *n* = 25.

	F_V/F_M	df
Melanisation	**	1
[H_2O_2]	***	3
Time	***	3
Treatment x [H_2O_2]	**	3
Treatment x Time	n.s	3
[H_2O_2] x Time	***	9
Treatment x Time x [H_2O_2]	n.s	9

n.s = not significant, * = P < 0.05, ** = P < 0.01, *** = P < 0.001

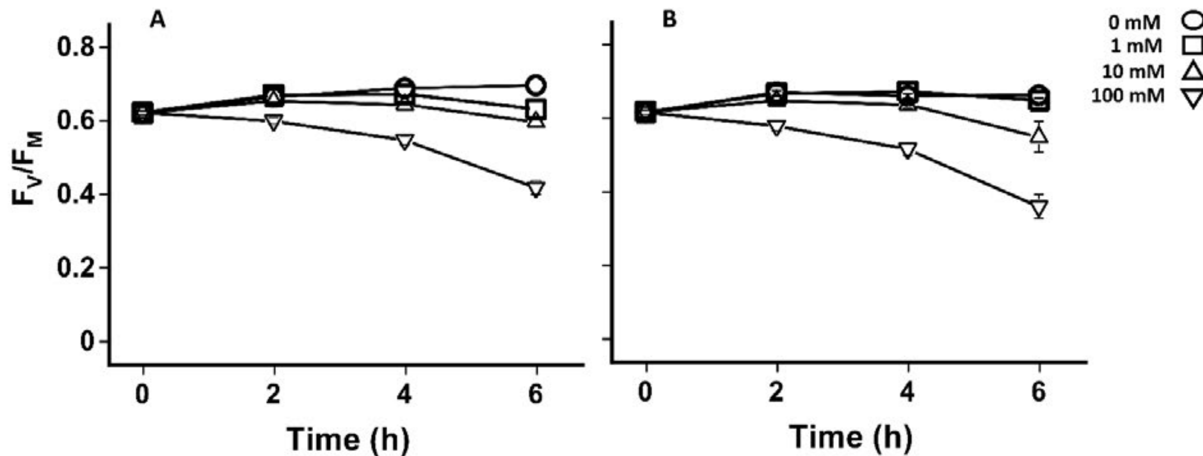

Fig. 5.1. Maximal efficiency of photosystem II (F_V/F_M) in *Lobaria pulmonaria* treated with different concentrations of hydrogen peroxide (H_2O_2) for 6 h. (A), pale thalli and (B), melanised thalli.

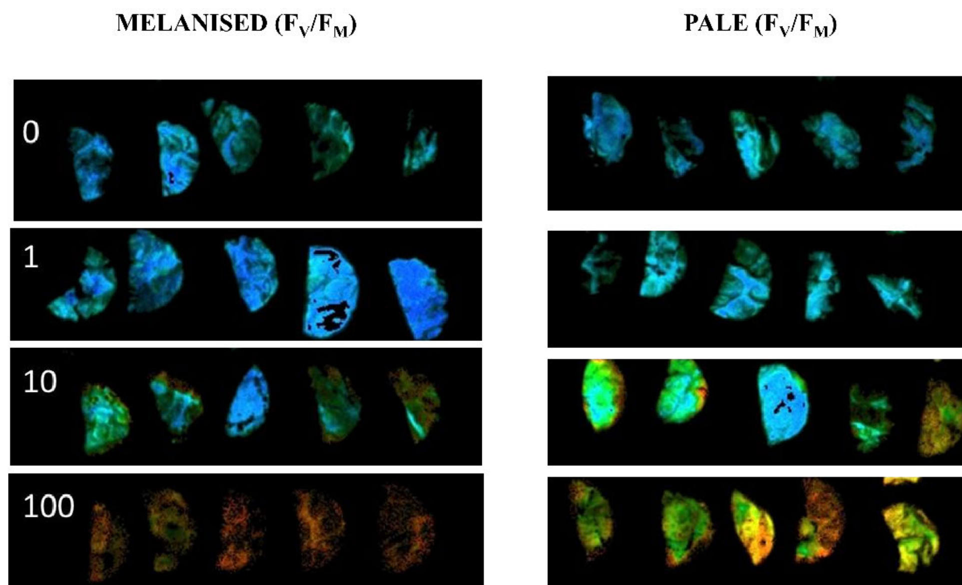

Fig. 5.2. The effect of H_2O_2 on F_V/F_M in melanised and pale thalli of *Lobaria pulmonaria*. Red = near dead (<0.15), green = stressed (ca. 0.3), blue = healthy (>0.6).

5.3.2 Effect of H_2O_2 on GSH content and redox potential

Before treatment of thalli with H_2O_2 (at time zero in the experiment), the melanised forms had c. 70% of the total GSH compared to pale forms (Fig. 5.3), while the glutathione redox couple was slightly higher (more oxidized) in melanised than pale forms (Fig. 5.4). While 1 mM H_2O_2 had little effect on GSH levels, 10 and 100 mM significantly reduced the total amount of GSH while the redox couple shifted to slightly more positive (oxidising) values ($P < 0.001$). Total amount of GSH remained significantly higher in pale compared to melanised forms ($P = 0.005$; Table 5.2) Both pale and melanised forms responded in the same way to higher concentrations of H_2O_2.

Table 5.2

Statistical analysis (Two-Way ANOVA) of the effect of hydrogen peroxide concentration [H_2O_2] on the total glutathione and half-cell reduction potential ($E_{GSSG/2GSH}$) in pale and melanized thalli of *Lobaria pulmonaria*. *n* = 25.

	Total GSH	$E_{GSSG/2GSH}$	df
Melanisation	**	***	1
[H_2O_2]	***	***	3
Melanisation x [H_2O_2]	n.s	*	3

n.s = not significant, * = P < 0.05, ** = P < 0.01, *** = P < 0.001

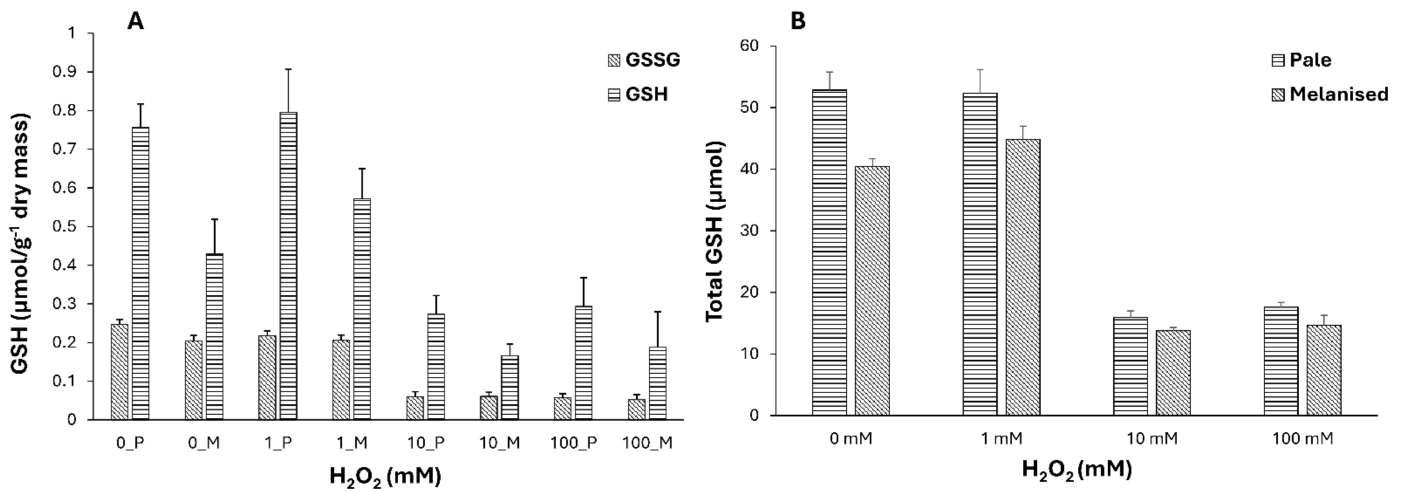

Fig. 5.3. The effect of a 6 h treatment with H_2O_2 on GSH and GSSH levels in *Lobaria pulmonaria*. A, values of GSH and GSSG plotted separately, B, total GSH amount. Letters M and P on x-axis represent melanised and pale thalli respectively.

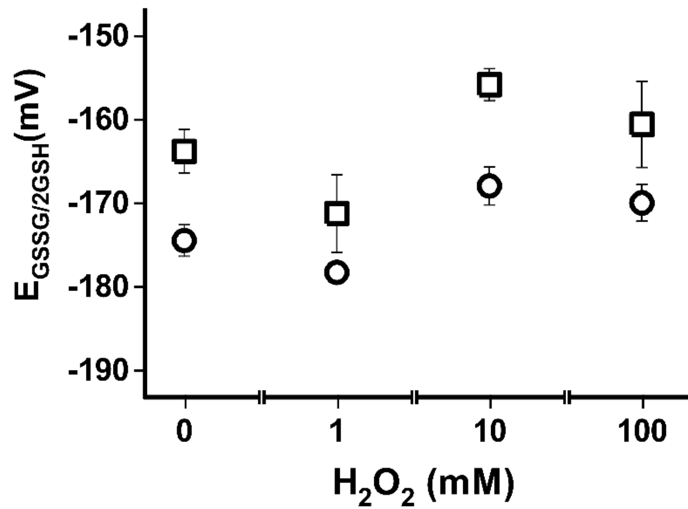

Fig. 5.4. The effect of H_2O_2 on the GSH redox potential ($E_{GSSG/2GSH}$) in melanised (open squares) and pale (open circles) thalli of *Lobaria pulmonaria* treated with varying concentrations of H_2O_2 for 6 h.

5.3.3 Effect of wetting and drying cycle on GSH content and redox potential

Desiccation of *L. pulmonaria* over silica gel for 7 d reduced the water content (WC) to less than 10% in both pale and melanised thalli (Fig. 5.5). When transferred to air at 100% RH, the WC increased rapidly for 2 h and slowly thereafter. In general, the water contents of the two types of thalli changed in a similar way, although the water content after rehydration for 6 h was slightly lower in the melanised thalli.

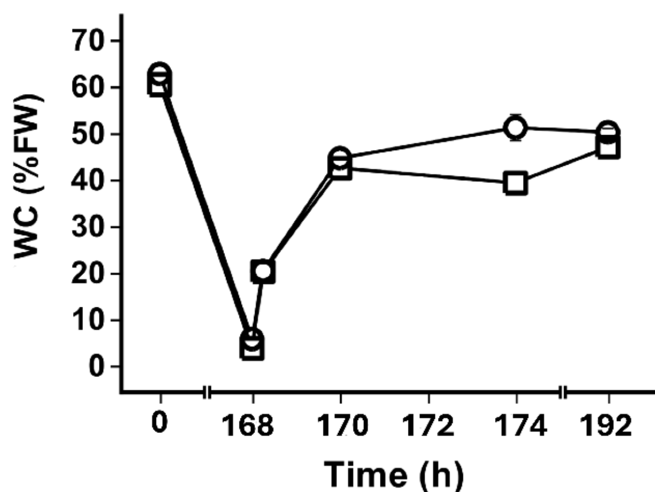

Fig. 5.5. The effect of a drying / wetting cycle on the percentage relative water content (WC) in *Lobaria pulmonaria* melanised (open squares) and pale (open circles) thalli subjected to 7 d desiccation over silica gel and 24 h rehydration.

Fig. 5.6 illustrates the changes in the GSH and GSSG contents of pale and melanised thalli during a desiccation / rehydration cycle, while Fig. 5.7 illustrates the corresponding changes in the GSH redox potential. At the start of the experiment, the melanised forms contained c. 70% of the total GSH of the pale thalli. Desiccation almost doubled the total amount of GSH in both pale and melanised forms, while the GSH redox potential became significantly more negative (reducing) (Table 5.3). During rehydration in air at 100% RH, total GSH initially slightly increased and then slightly decreased, and remained significantly higher ($P < 0.001$) in pale compared to melanised thalli (Fig. 5.6 and Table 5.3), while the GSH redox potential became less negative (Fig. 5.7). However, although neither total GSH content nor the redox potential fully recovered to pre-desiccation values. During recovery from desiccation, the GSH redox couples were always significantly lower (less oxidised) ($P < 0.001$), in the pale thalli compared to those of the melanised thalli (Table 5.3).

Table 5.3

Statistical analysis (Two-Way ANOVA) of the effect of seven days desiccation followed by rehydration on the total glutathione and half-cell reduction potential ($E_{GSSG/2GSH}$) in pale and melanized thalli of *Lobaria pulmonaria*. n = 25.

	Total GSH	$E_{GSSG/2GSH}$	df
Melanisation	***	***	1
Time	***	***	5
Melanisation*Time	n.s	***	5

n.s = not significant, * = P < 0.05, ** = P < 0.01, *** = P < 0.001

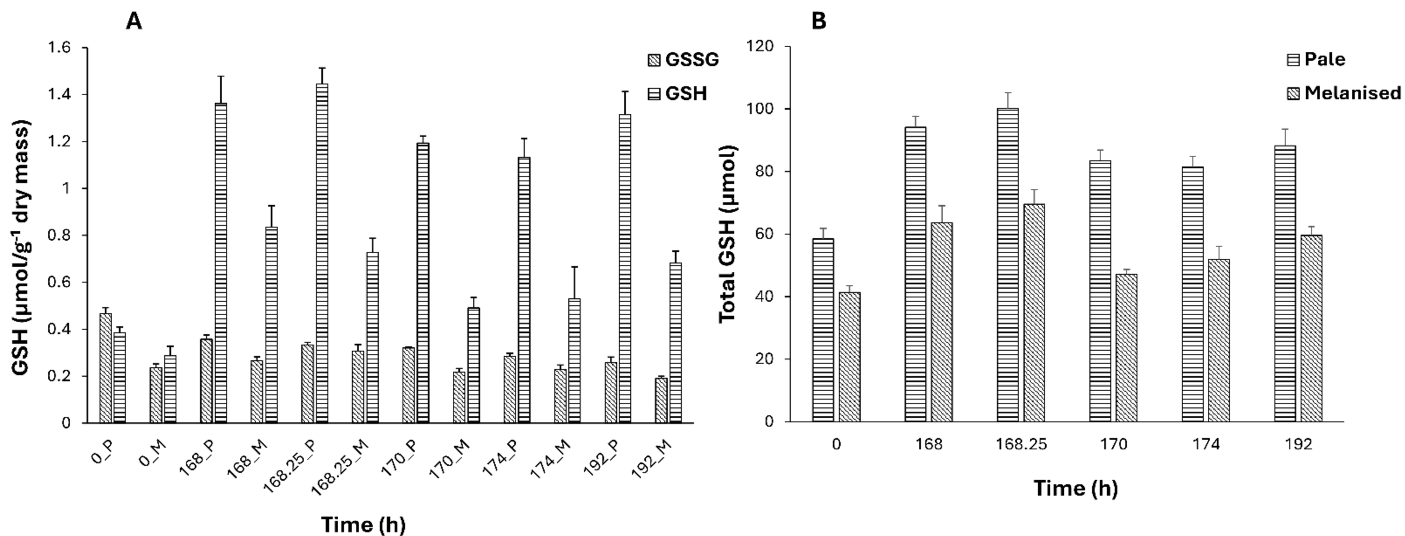

Fig. 5.6. The effect of a desiccation / rehydration cycle on GSH and GSSH levels in *Lobaria pulmonaria*. A, values of GSH and GSSG plotted separately, B, total GSH amount. Letters M and P on X-axis represent melanised and pale thalli respectively.

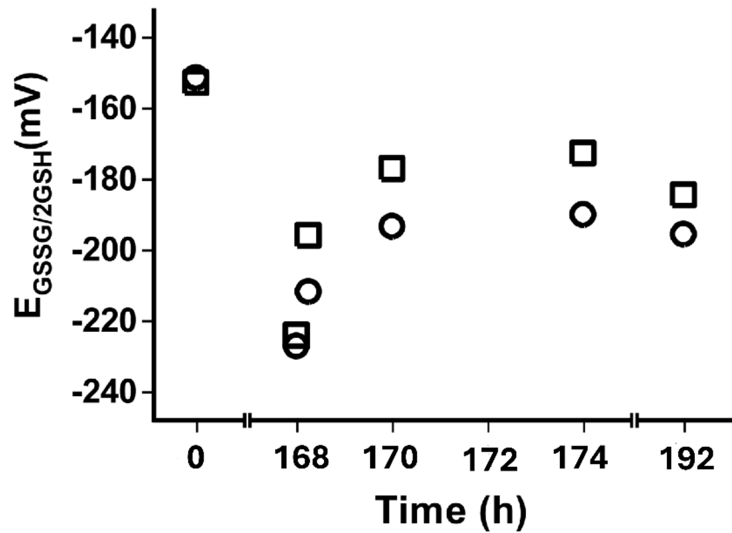

Fig. 5.7. The effect of a desiccation / rehydration cycle on the GSH redox potential ($E_{GSSG/2GSH}$) in melanised (open squares) and pale (open circles) thalli of *Lobaria pulmonaria* subjected to 7 d desiccation over silica gel and 24 h rehydration.

5.4 Discussion

Here we show that metabolism of the low molecular mass antioxidant GSH during stress differs between pale and melanised thalli of the lichen *L. pulmonaria*. Melanins are known to protect lichens from the harmful effects of high UV-B and PAR (Mafole et al., 2019), but their roles in the tolerance of lichens to other abiotic stresses remain largely unknown. We originally predicted that as melanins can act as strong antioxidants (Minibayeva et al., 2024), and as abiotic stresses almost invariably increase ROS formation, melanins may contribute to the maintenance of a favourable cellular redox potential during stress. Results consistently showed that the total levels of GSH were significantly lower in melanised than pale thalli (Figs. 5.3 and 5.6). While both exogenous applications of H_2O_2 and a desiccation rehydration cycle caused rather similar proportional effects, in melanised thalli the stresses had much smaller effects on actual GSH levels.

Melanised thalli appear to need lower GSH levels to buffer cellular redox during stress, suggesting melanins may contribute to ROS scavenging in addition to acting as light screens.

5.4.1 Levels of GSH in pale and melanised thalli

The concentrations of total GSH in the pale *L. pulmonaria* used here, collected from Siberia, were c. 1 µmol g^{-1} dry mass while those of Kranner (2002), collected from Slovenia, were more than double these values. This suggests that collection locality may have a significant effect on GSH levels. In the present study, results from both experiments suggest that melanised thalli have total GSH contents that are about 70% those of pale lichens (Figs. 5.3 and 5.6). This is probably not simply a result of melanised thalli having generally lower rates of metabolism than pale. For example, Mafole et al. (2017) showed that respiration rates of melanised *L. pulmonaria* are very similar to those of pale thalli, although photosynthetic rates are slightly lower presumably because of the light-screening effect of melanins in the upper cortex. The lower GSH values in the melanised thalli may be because when stressed the lichens will need lower concentrations of non-enzymatic antioxidants, as melanins can act as ROS scavengers.

5.4.2 Effects of exogenous H_2O_2 on pale and melanised thalli

Superficially, exogenous applications of H_2O_2 appear to have similar effects on pale and melanised *L. pulmonaria* (Figs. 5.1 and 5.2). Concentrations of 10 and 100 mM greatly reduced the total amount of GSH both thalli (Fig. 5.3) and shift the redox potential to slightly more positive (oxidising) values (Fig. 5.4). However, a more careful analysis of the results shows that the actual quantitative reductions in GSH were much smaller in melanised than pale thalli. Minibayeva et al. (2024) provided strong evidence that melanins extracted from *L. pulmonaria* can display strong ROS scavenging ability when exogenously applied to other organisms. Melanins were able to

moderate the harm to a variety of mammalian metabolic processes caused by addition of exogenous H_2O_2. It seems likely therefore that melanins are responsible for some of the H_2O_2 breakdown, resulting in the smaller reductions in GSH levels observed in melanised thalli.

5.4.3 The effects on desiccation on GSH levels in pale and melanised thalli

This study provides evidence that melanins play a role in protecting *L. pulmonaria* from the effects of desiccation stress. Melanised thalli had consistently slightly higher (more oxidising) redox potentials than pale thalli. Desiccating lichens over silica gel for 7 d significantly increases the total amount of GSH in both pale and melanised forms (Fig. 5.6). Interestingly, these results differ from the study of Kranner (2002), which reported that desiccating pale *L. pulmonaria* over silica gel for 2 d or 60 d caused small or more substantial reductions in the total GSH level respectively. Also, at variance with the results of Kranner (2002), results from the present study showed that at the end point of desiccation during the early stages of rehydration, the GSH redox potential tends to become less rather than more oxidizing following desiccation (Fig. 5.7). It appears that both pale and melanised *L. pulmonaria* from this collection respond to desiccation by increasing their GSH levels. They are also able to effectively prevent any reductions in the ratio of reduced GSH to oxidized GSSG, suggesting they possess more effective DT mechanisms than the material studied by Kranner (2002). While proportionally the effects of desiccation and rehydration are similar in pale and melanised thalli, melanised thalli can maintain or even improve their redox status during this stress with much smaller changes in the actual levels of GSH (Fig. 5.6). Consistent with the experiment with exogenously applied H_2O_2, results suggest that during desiccation stress melanins may act as antioxidants, reducing the dependence of the lichen on GSH to act as a cellular redox buffer.

5.5 Conclusion

The main roles of melanins in lichens are generally considered to be protection of the symbionts from the harmful effects of high UV-B and PAR (Mafole et al., 2019). However, given their biophysical properties, in particular their ability to act as scavengers of ROS, it has often been speculated that they may play roles in the tolerance of lichens to other stresses. Here we show that melanised thalli possess much lower levels of GSH than pale thalli, possibly indicating that melanins take over the roles of normal cellular antioxidants. Melanised thalli can maintain similar GSH redox couples to pale thalli using much smaller absolute changes in GSH levels. Future studies should compare the levels of other antioxidants in pale and melanised thalli, and test whether melanised thalli possess higher tolerance to other abiotic stresses.

5.6. Acknowledgements

The authors thank DAAD for a postgraduate research fellowship for KWG Mkhize.

5.7 References

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CHAPTER 6

General Conclusions

In extreme habitats lichens can be the dominant life form, and display resistance to a wide range of environmental stresses, with tolerances varying significantly between species. Morphological and physiological adaptations largely influence lichens dominance to harsh conditions that would be too stressful to be inhabited by either of the individual partners (Williams et al., 2017). Despite this, our knowledge of how lichens adapt to extreme environments remains fragmentary, and the significance of different protective mechanisms involved remains understudied and underreported.

Therefore, the present thesis aimed at quantifying mechanism of stress tolerance in lichens, specifically those mechanisms that enable them to withstand stresses such as light and desiccation. In particular, results presented here adds to our knowledge of how lichens are able to thrive in extreme rapidly changing environments and furthermore, provide a foundation for a transgenic approach that could be used to improve stress tolerance in crop species and therefore crop yield (Farrant and Hillhorst, 2022).

6.1 Overall conclusion

Lichen photobionts are often exposed to high light stress, which may cause a long-term reduction of photosynthesis and damage to the photosynthetic apparatus as a result of photoinhibition. Shade or sunfleck species often experience rapidly changing light during the day and are probably as vulnerable as sun adapted species. In chapter 2, for the first time we show that tolerance to photoinhibition is correlated with increase in NPQ in shade adapted lichens. *Crocodia aurata* increases its tolerance to photoinhibition following pretreatment with moderate light for 48 h while

hydrated. This increased tolerance is associated with enhanced NPQ, which dissipates excess energy as heat to prevent oxidative stress. However, this pretreatment does not increase tolerance in the cyanolichen *Sticta fuliginosa*, nor in the chlorolichen *Ramalina celastri* from sun-exposed habitats, suggesting species-specific responses and potential differences in pretreatment needs. Furthermore, it seems likely that in "sun" populations tolerance is fully expressed and cannot be further increased. These results provide good evidence that NPQ plays an important role in protecting photosynthetic organisms from photoinhibition by dissipating excess light energy as heat, especially in habitats subjected to rapidly changing light levels.

However, as mentioned in earlier Chapters, NPQ has both positive and negative roles in environments in which light levels are rapidly changing. Positively, NPQ delays the onset of photoinhibition by reducing ROS production and negatively, while not affecting photosynthesis in high light, NPQ can greatly reduce the quantum yield of photosynthesis at lower light levels (Kromdijk et al., 2016). Therefore, in the work described in chapter 3, a comparison was made of the induction and relaxation of NPQ in the dark, and photosynthetic induction in lichens from exposed and shaded habitats. These results showed that shade lichens have higher NPQ compared to sun lichens and that shade lichens relax NPQ more quickly than sun lichens. The rapid NPQ relaxation in shade lichens may enhance photobionts' ability to efficiently utilize the lower light levels for photosynthesis available after a sunfleck has passed.

In the work presented in chapter 4, a comparison was made of the induction and relaxation of NPQ and the induction of photosynthesis in collections of the same lichen species from exposed and from more shaded locations, all containing a trebouxioid photobiont. Results from this chapter revealed that shade populations generally have higher NPQ compared to the sun population of same species in addition to the rapid relaxation of NPQ, likely as protection against sudden light

increases during sunflecks. However, while consistent with the results obtained in chapter 3, results were at variance with data from other photosynthetic organisms, where generally sun species would have higher NPQ due to larger pools of xanthophyll cycle pigments compared to shade species. Additionally, and more consistent with literature data from other species, maximum rETR is lower in shade than sun collections, probably reflecting a downregulation of photosynthetic capacity to reduce energy costs. This study also compared pale and melanised thalli of shade lichens with *Symbiochloris* photobionts. Results revealed that NPQ behaviour in melanised thalli from slightly more exposed sites was similar to shade rather than sun forms of the trebouxioid lichens, suggesting that temporary high light-induced melanisation may be excessive and might hinder photosynthesis later. Overall, this indicates that melanin in lichens effectively shields the photobiont from excessive light, resulting in shade-adapted behaviour.

In addition to high light, the impact of melanin on other stresses, such as desiccation, was also investigated in chapter 5, enhancing our understanding of melanin's role in mitigating desiccation effects. This study was aimed to investigate the role of melanins in stress tolerance and GSH metabolism in the lichen *Lobaria pulmonaria*. Results showed that the total levels of GSH were significantly lower in melanised than pale thalli also during the desiccation / rehydration cycle. Furthermore, melanised thalli can maintain similar changes in the GSH redox couple during stress with much smaller absolute levels of GSH. Therefore, it seems likely that melanised thalli need lower GSH levels to buffer cellular redox during stress, suggesting that melanins may contribute to ROS scavenging in addition to acting as light screens.

6.2 Future recommendations

While this thesis provides evidence of ecophysiological responses of lichens to environmental stresses, further research is required to bridge the gap in understanding of tolerance mechanism that could improve crop species. This includes, but not limited to, the role of cyclic electron flow (CEF) and pseudocyclic electron flow (PCEF) in photoprotection, assessing the impact of inhibiting CEF, PCEF and NPQ upon photoinhibition; and the DNA repair mechanisms, specifically repair of ROS or light induced damage of D1 protein in lichens adapted to different habitats consisting of different photobionts. Furthermore, the levels of xanthophyll pigments and activity of epoxidases in sun and shade populations in response to light stress still requires further investigation. Another potentially informative line of investigation would be to study the effect of melanin on GSH content in response to UV stress, comparing both melanised and pale thalli, measuring GSH over different time intervals. A progressive decrease in GSH levels during melanisation would add support to the contention that melanins can take over the role of GSH as a cellular redox buffer.

While excess light can cause photoinhibition and damage to the chloroplast, photodamaged chloroplasts and proteins should be eliminated or repaired to avoid further cellular damage (Izumi et al., 2017). Therefore, photooxidative damage-induced chlorophagy is another critical mechanism used by photosynthetic organisms to prevent the buildup of ROS by degradation of damaged chloroplasts. Surprisingly, while chlorophagy and other autophagy processes have been documented in other photosynthetic organisms such as *Arabidopsis* plants (Izumi et al., 2017) and unicellular algae *Chlamydomonas* (Heredia-Martínez et al., 2018), they have not been studied in lichens. This can be studied using microscopy techniques such as Transmission Electron Microscopy (TEM) along with molecular studies of gene expression related to chlorophagy in

response to UV and high light stress comparing lichens of different photobionts. Moreover, although mycobionts are renowned for their protective mechanisms for photobionts, such as the production of secondary lichen compounds, their capacity to produce antioxidative enzymes remains relatively understudied. Performing transcriptome analysis of antioxidative enzymes like SOD and CAT in response to light stress in mycobionts could offer valuable insights into the extent of protection in an isolated state.

Crucially, as noted by Simkin et al. (2019), manipulating aspects of photosynthesis such as quantum yield and NPQ through genetic engineering could improve crop yield under rapidly changing environments. These biotechnological approaches would require more intense research on molecular pathways and gene expression or regulation of stress related and tolerance genes to produce crops that can adapt to rapidly changing environments. It is now realized that the lower leaves in the canopies of many crops experience fluctuating light. Therefore, it seems reasonable to assume that studying how "shade" lichens have evolved to successfully exploit habitats characterized by sunflecks may help improve photosynthesis in crops.

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6.3 References

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Appendix 1

GPS Coordinates of Colleting sites

Collection site	Grid coordinates
South Africa	
Fort Nottingham Nature Reserve, Kwa Zulu Natal	-29.420, 29.911
Umgeni Nature Reserve, Howick, KwaZulu Natal	-29.475, 30.239
Cumberland Nature Reserve, Kwa Zulu Natal	-29.512, 30.514
Queen Elizabeth Park Nature Reserve, KwaZulu Natal	-29.573, 30.328
Overseas sites	
Kazan (outskirts), Republic of Tatarstan, Russian Federation	55.832, 49.002
Syktyvkar (outskirts), Republic of Komi, Russian Federation	62.105, 50.588