# LICHEN PHOTOBIOLOGY IN RELATION TO CLIMATE CHANGE: PROTECTION IN PELTIGERALEAN LICHENS AGAINST EXCESS ULTRAVIOLET (UV) RADIATION USING INDUCED MELANINS AND THE EFFECTS OF UV ON MELANIN SYNTHESIZING ENZYMES

**By**

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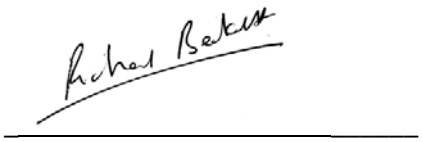
**Preface**

The experimental work described in this thesis was carried out by the candidate while based in the Discipline of Biological Sciences School of Life Sciences, of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg campus, South Africa from February 2014 to December 2015. Some of the work was carried out at the department of Ecology and Natural Resource Management (INA), Norwegian University of Life Sciences (NMBU), in June 2015. The research was financially supported by SANCOOP, the NRF (South Africa) and the Russian Foundation for Basic Research (grant no. 14-04-93962).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.



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I, Lusanda Patrick Herbert Matee declare that:

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(ii) This dissertation has not been submitted in full or in part for any degree or examination to any other university;

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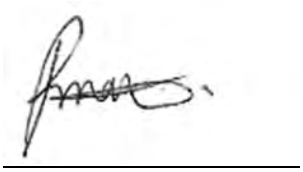
**Declaration 2: Publication**

**Publications from thesis**

**1.** Lusanda P Matee[1], Richard P Beckett[1], Knut A Solhaug[2] and Farida V Minibayeva[3] (*in press*) (2016)

Characterization and role of tyrosinases from the lichen *Lobaria pulmonaria* (L.) Hoffm*. The Lichenologist*



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**Abstract**

Depletion of the stratospheric ozone has allowed more solar ultraviolet (UV) radiation (100-400 nm) to reach the lower atmosphere. Despite the success of the continued implementation of Montreal protocol of 1987 and its amendments aimed at the protection of the ozone layer, it is possible that UV will remain elevated in some regions due to decreasing removal by clouds and aerosols. Strong interactions between ozone depletion and changes in climate induced by increasing greenhouse gases could result in increased UV radiation levels reaching the surface. Increased exposure to UV-B can cause significant damage to organisms including protein damage, inhibition of photosynthesis, formation of high energy reactive oxygen species (ROS), disruption of nitrogen fixation and induction of cell mutation and inhibition of growth. Lichens are composite organisms that arise from a symbiotic association between fungi and photoautotrophic algal partners, which may be microalgae and/or cyanobacteria. They have developed several protective strategies to protect themselves from excessive radiation. Lichens such as *Cladonia rangiferina*, *Lobaria pulmonaria* and *Umbilicaria rossica* are known to synthesize melanins in response to elevated levels of UV-B radiation. Synthesis of melanins is generally catalysed by enzymes that are involved in phenol coupling including P450 monooxygenases, ascorbate oxidases, peroxidases, laccases and tyrosinases (Nezbedová *et al*. 2001).

The work presented in this thesis investigated the enzymes involved in melanin biosynthesis in lichens from suborder Peltigerineae. We present a detailed characterization of redox enzymes from *Lobaria pulmonaria* and *Pseudocyphellaria aurata* with a special focus on tyrosinases. Furthermore, an evaluation of the response of *L. pulmonaria* to UV radiation was done. The role of the redox enzymes tyrosinases, laccases and peroxidases in melanin synthesis is elucidated by exposing *L. pulmonaria* thalli to various combinations of UV and PAR.

Laboratory experiments clearly indicated that *L. pulmonaria* and *P. aurata* have redox enzymes that are of similar nature to those of their free-living symbionts and other lichens. Tyrosinase from *L. pulmonaria* oxidized monophenolic compounds and was able to metabolize L-DOPA, thus showing characteristics of a typical fungal tyrosinase. L-DOPA can also be metabolized by peroxidases and laccase suggesting that peroxidases and laccases may participate in melanisation reactions. A field experiment showed that exposing shade-adapted *L. pulmonaria* to solar radiation in the field induces DOPA melanin synthesis. The synthesis of the brown cortical compounds occurred when lichen thalli were exposed to direct sunlight or placed under a wavelength neutral filter that marginally reduced overall light. Melanin synthesis was accompanied by an increase in laccase activity in lichens that were exposed to natural sunlight. By contrast, no changes in enzyme activity occurred in lichen thalli placed under the wavelength neutral filter. Placing lichen thalli under filters that removed both UV-A and UV-B prevent melanisation and was accompanied by an increase in tyrosinase activity. Filtering out UV-B had no effect on enzyme activity but slight browning occurred.

Results showed that laccases could be involved in melanin biosynthesis under some conditions, but provided no evidence for a role of tyrosinases in melanisation. However, it could be that melanisation requires tyrosinase, but rates of melanisation are controlled by the levels of melanin precursors such as tyrosine. No differences in maximal PSII efficiency and chlorophyll contents occurred between the radiation treatments indicating that no deterioration of photosynthetically apparatus occurred and the photobionts remained relatively healthy even when exposed to UV. Taken together these results suggest that further research in needed into determining the levels of melanin precursors, the roles of lichen redox enzymes in melanin synthesis and whether a transcriptional activation of these redox enzymes by UV radiation in lichens exists or not.

Furthermore, additional roles for tyrosinase need to be investigated, for example defence against pathogens.

**Acknowledgements**

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**List of abbreviations**

| | |
|---|---|
| 1, 3, 6, 8-THN | 1, 3, 6, 8-tetrahydroxynaphthalene |
| µl | Microliter(s) |
| ABTS | 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) |
| APOs | Aromatic peroxygenases |
| APxs | "Ascorbate peroxidases" |
| BRI | Browning reflectance index |
| *Ca* | Circa (approximately) |
| CcPs | "Cytochrome c peroxidases" |
| CfuCPO | *"Caldariomyces fumago* chloroperoxidase" |
| Chl | Chlorophyll |
| Chl *a* | Chlorophyll a |
| Chl *b* | Chlorophyll b |
| $CO_2$ | Carbon dioxide |
| Cu | Copper |
| DHN | Dihydroxynaphthalene |
| DNA | Deoxyribonucleic acid |
| DM | Dry mass |
| DMP | 2, 6-dimethoxyphenol |
| DMSO | Dimethyl sulfoxide |
| DOPA | 3,4-dihydroxyphenylalanine |
| Dopachrome | 2-carboxy-2, 3-dihydroindole-5, 6-quinone |
| DyPs | "Dye-decolorizing peroxidases" |
| ESRI | Environmental Systems Research Institute |
| $F_v/F_m$ | Maximal quantum yield of PSII |
| g | Grams |
| GHGs | Greenhouse gases |
| $H_20_2$ | Hydrogen peroxide |
| IPCC | Intergovernmental Panel on Climate Change |
| kDa | Kilodaltons |
| L-DOPA | L-dihydroxyphenylalanine |
| LsaPOX | "Lichen heme peroxidase" |

| | |
|---|---|
| MAAs | Mycosporines and mycosporine-like amino acids |
| MBTH | 3-Methyl-2-benzothiazolinone hydrazine |
| Min | minute(s) |
| ml | Millilitre(s) |
| mM | Millimolar(s) |
| Native PAGE | Non-denaturing gel electrophoresis |
| nm | Nanometer(s) |
| NOR | Norway |
| PAR | Photosynthetically active radiation |
| PKS | Polyketide synthases |
| POX | Peroxidase. |
| RSA | Republic of South Africa |
| RH | Relative humidity |
| ROOH | Organic hydroperocxide |
| ROS | Reactive oxygen species |
| Rpm | Revolutions min-1 |
| RGR | Relative growth rate |
| SDS | Sodium dodecyl sulphate |
| UNEP | United Nations Environment Programme |
| UV | Ultraviolet radiation |
| UV-A | Ultraviolet radiation of wavelengths in the 315 to 390 nm range |
| UV-B | Ultraviolet radiation of wavelengths in the 280 to 315 nm range |
| UV-C | Ultraviolet radiation of wavelengths in the 252 to 278 nm range |

**CHAPTER 1: Literature review**

**1.1 Climate change and potential effects of increased UV radiation**

Reduction of the stratospheric ozone has allowed more solar ultraviolet (UV) radiation to reach the lower atmosphere of the earth, this is specifically UV-B (280-315 nm) and to a lesser extent UV-A (315-400 nm) (Buffoni Hall *et al*. 2002). The conclusion of studies reviewed by Nybakken *et al*. (2004) is that increased UV-B radiation as a result of a depletion of the ozone layer might be an important factor impacting on natural ecosystems in the future. Fortunately, the continued implementation of Montreal protocol of 1987 and its amendments aimed at the protection of the ozone layer through a cutback of the production of harmful chlorofluorocarbons and other ozone depleting substances is working. However, it is possible that UV will remain elevated in some regions due to decreases removal by clouds and aerosols (Paul, 2000). Furthermore, a decrease in the stratospheric ozone may still occur as a result of stratospheric cooling caused by an increase in atmospheric $CO_2$ levels (Krapivin *et al*. 2012). Greater uncertainty regarding future surface UV-B radiation than future ozone exists as UV-B radiation is affected by climate change (UNEP, 2006). In the past, climate change may have directly affected UV radiation levels by altering the amount of ozone, UV-B absorbing tropospheric gases, aerosols and clouds in the atmosphere (Solomon, 2007). This climate change induced change in UV-B levels could also happen in future due to existing strong interactions between ozone depletion and changes in climate induced by increasing greenhouse gases (GHGs). It has been suggested that amount of stratospheric ozone can also be affected by the increases in the concentration of GHGs, which lead to decreased temperatures in the stratosphere and accelerated circulation patterns (McKenzie *et al*. 2011). Increased exposure to UV-B can affect biological and chemical processes in a detrimental manner thus an increase in UV-B radiation reaching the earth might prove problematic for organisms. For instance, UV-B can lead to protein damage, inhibition of photosynthesis, formation of high energy reactive oxygen species (ROS), disruption of nitrogen fixation and induction of cell mutation and inhibition of growth (Vincent & Neale, 2000).

**1.2 Lichens**

Lichens are composite organisms that arise from a symbiotic association between fungi and photoautotrophic algal partners, microalgae and/or cyanobacteria (Hawksworth, 1988; Stocker-Wörgötter, 2008). One-fifth of all fungi are in a lichen symbiosis, including more than 40% of ascomycetes (ca. 13500 species) (Hawksworth & Hill 1984). This lichenized lifestyle association allows lichens to tolerate extreme environmental conditions that would be otherwise unfavourable for the individual organisms that form the lichen symbiosis, owing to the evolution of a gene pool with wide-ranging synthetic pathways that allow for the production of a range of lichen phenolic compounds that protect against increasing environmental stress (De Vera *et al*. 2004). They dominate approximately 8% of terrestrial ecosystems, particular in Artic to Antarctic regions, as well as mountains and dryland crusts (Beckett *et al*. 2013; Russo *et al*. 2008). Lichens can be classified into three main morphological growth forms; the fruticose (branched tree-like shrubby form) crustose (crust-like) and foliose (leaf-like) (Büdel *et al*. 2008; Hawksworth *et al*. 1996). A fourth type exists but is restricted to a few cyanobacterial lichens, which is the gelatinous growth form (Nash, 1996). The thallus development that leads to each growth form is mainly determined by the fungal component of the lichen, with the exception of a few lichens where it is determined by the photobiont (Nash, 1996).

Lichens possess a number of physiological and chemical adaptations that enable them to tolerate severe environmental stresses such as temperature extremes, desiccation, rapid rehydration, and high UV radiation intensities (Kranner *et al*. 2008). Most studies that dealt with the harmful effects of UV-B radiation and the response of terrestrial ecosystems to this phenomenon focused on higher plants, and there is a need for studies that deal with the response of lichens to high UV-B.

### 1.2.1 How lichens respond to UV radiation: Lichen UV-protectant secondary metabolites

Lichens can produce a number of UV screening compounds such as anthraquinones, shikimic acid derivatives (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). The UV-absorbing nature of lichen metabolites was first investigated by Hale (1956) then by Gream and Riggs (1960) and later by Rao (1967). Compounds found on the upper cortex of lichens such as melanin, usnic acid and parietin have been suggested to protect against excess damaging radiation (Nybakken & Julkunen-Tiitto, 2006; Nybakken *et al*. 2004; Solhaug *et al*. 2003). A number of studies reported that lichen substances such as melanins, scytonemin, calycin, rhizocarpic acid, usnic acid, mycosporines and mycosporine-like amino acids have some photoprotective capabilities (Millot *et al*. 2012). Solhaug & Gauslaa (2012) suggested that melanins are more efficient light screening substances compared to the other above mentioned substances, in particular usnic acid

### 1.2.1 Mycosporines and mycosporine-like amino acids (MAAs)

Mycosporines and mycosporine-like amino acids (MAAs) are water soluble, low molecular weight compounds that absorb UV radiation (Büdel *et al*. 1997; Nguyen *et al*. 2013). They were first detected in the 1960's and a few years later the first chemical structure of a MAA from fungi was recorded Arpin & Curt (1979). These compounds have been reported in a number of organisms that have been exposed to high light, from microalgae, cyanobacteria, yeast and lichens from genera such as *Peltigera*, *Collema*, *Gonohymena* and *Peltula*. They share an analogous chromophore unit that corresponds to a cyclohexenimine ring or cyclohexanone and the presence of a wide variety substitutes located at different positions. A number of studies have demonstrated that mycosporines and MMAs display efficient absorption in both the UV-A and UV-B range and they have a high photostability (Nguyen *et al*. 2013). Mycosporines have UV absorption at 310-320 nm, in contrast to MAAs that have absorption maxima between 310 and

360 nm (Torres *et al*. 2004). Publications on the biosynthesis of mycosporines and MAAs are very limited due to their complexity (Nguyen *et al*. 2013).

### 1.2.2 Scytonemin

Scytonemin is a yellow-brown lipid-soluble UV screening compound reported in a number of cyanobacteria species and cyanolichens from the genera *Collema*, *Gonohymena* and *Peltula* (Garcia-Pichel & Castenholz, 1991; Nguyen *et al*. 2013; Richter *et al*. 2006). This compound was first detected in cyanobacteria in 1849 and later given the name scytonemenin (Nägeli 1849; Nägeli & Schwenderer, 1877). It is a dimeric molecule with indolic and phenolic subunits with a molecular weight of 544 kDa (Dillon & Castenholz, 1999; Proteau *et al*. 1993). A number of studies have reported the UV radiation screening properties of this compound (Büdel *et al*. 1997; Cockell & Knowland, 1999; Garcia-Pichel *et al*. 1992). Scytonemin has absorption maxima at 386 nm, corresponding to UV-A zone, but it can also significantly absorb in both the UV-C (252,278 nm) and UV-B zones (c. 300 nm) (Chivkunova *et al*. 2001; Dillon & Castenholz, 1999; Garcia-Pichel *et al*. 1992; Rastogi and Sinha, 2009).

### 1.2.3 Melanins

Melanins are a highly diverse group of polymophorous and multifunctional pigments composed of polymerized phenolic and/or indolic compounds (Henson *et al*. 1999; Ito & Wakamatsu, 2003). Due to the fact they remain poorly characterized it is difficult to precisely define what melanins are (Nosanchuk & Casadevall, 2003). These compounds are widely distributed in a number of organisms in numerous biological kingdoms (Nosanchuk & Casadevall, 2003). Several lichens produce melanins in the upper cortex of their thalli (Gauslaa &Solhaug, 2001). Melanins are multifunctional, providing protection against environmental stress such as UV light, ionizing radiation and oxidizing radiation (Eisenman & Casadevall, 2012).

#### Biosynthesis of melanins

In the DHN melanin biosynthesis synthesis starts with a polyketide synthase (PKS) using acetate as a precursor and melanin is generated via the polymerization of a double-ringed

structure known as a 1, 8-dihydroxynaphthalene (DHN) (Butler *et al*. 2001). The first product produced in the first step of this pathway is 1, 3, 6, 8-tetrahydroxynaphthalene (1, 3, 6, 8-THN) which is synthesized from Acetyl-CoA or malonyl CoA by a polyketide synthase (PKS) (Eisenman & Casadevall, 2012). This is followed by successive reduction and dehydration reactions, which produce scytalone, 1, 3, 6, 8-tetrahydroxynaphthalene, vermelone and finally the polymerization of DHN to melanin, which is catalyzed by a laccase (Bloomfield & Alexander, 1967).

**L-3, 4-dihydroxyphenylalanine melanin biosynthesis**

The fungal constituent of lichens may synthesize melanin via an L-DOPA pathway, which is similar to that of mammalian biosynthesis although some details differ (Bell & Wheeler, 1986; Eisenman & Casadevall, 2012). In this pathway, laccase catalyses the initial one-step oxidation of L-DOPA to dopaquinone and subsequent steps are believed to occur spontaneously to produce dihydroxyindole or dihydroxyndole-2-carboxylic acid intermediates that are polymerized into melanin (Land & Riley, 2000; Williamson 1997; Eseinman *et al*. 2011). Alternatively tyrosinase catalyses the oxidation L-tyrosine to L-DOPA which in turn is converted to dopaquinone and subsequent steps occur spontaneously to produce melanin (Eisenman & Casadevall, 2012; Langfelder *et al*. 2003). L-DOPA as suggested by the name of this pathway is an important cofactor in both reactions (Pomerantz & Warner, 1967).

Peroxidases can also mediate the oxidation of DOPA and other polyphenols to melanin (Maehly & Chance, 1954). A number of histochemical studies have demonstrated the peroxidase-dependent oxidation of tyrosine to melanin in the presence of DOPA as a substrate however; a great number of these were only done with plants and mammalian peroxidases (Okun *et al*. 1970a; Okun *et al*. 1971; Okun *et al*. 1970b; Okun *et al*. 1970c). It has also been suggested in electrophoretic studies that peroxidases can mediate the synthesis of melanin from tyrosine acting alone (Okun *et al*. 1971). A study by (Patel *et al*. 1971) on purified plant and mammalian peroxidases showed that peroxidases can mediate melanin biosynthesis from tyrosine with DOPA and dopachrome being intermediates in the proposed pathway. Similar results were shown in a

study by Takahama & Yoshitama (1998) on *Vicia faba*; these workers showed that horseradish peroxidase can oxidize DOPA to melanin, and that this reaction is enhanced by hydroxycinnamic acid esters. This suggests that the role of peroxidase in this pathway is still quite unclear; however, this enzyme may be involved in the synthesis of eumelanin**.**

**DHN melanin biosynthesis**

In the DHN melanin biosynthesis synthesis starts with a polyketide synthase (PKS) using acetate as a precursor and melanin is generated via the polymerization of a double-ringed structure known as a 1, 8-dihydroxynaphthalene (DHN) (Butler *et al*. 2001). The first product produced in the first step of this pathway is 1, 3, 6, 8-tetrahydroxynaphthalene (1, 3, 6, 8-THN) which is synthesized from Acetyl-CoA or malonyl CoA by a polyketide synthase (PKS) (Eisenman & Casadevall, 2012). This is followed by successive reduction and dehydration reactions, which produce scytalone, 1, 3, 6, 8-tetrahydroxynaphthalene, vermelone and finally the polymerization of DHN to melanin, which is catalyzed by a laccase (Bloomfield & Alexander, 1967).

**1.3 Redox enzymes**

Redox enzymes in lichens are typically made up of tyrosinases, peroxidases and laccases (Beckett *et al*. 2013). In general, lichens belonging to the order Peltigerales display strong redox activity (Beckett *et al*. 2013). Due to their ability to both metabolize and produce reactive oxygen species (ROS) these enzymes are thought to have more than one function in lichen biology (Beckett *et al*. 2005). Little information exists about the synthesis of lichen melanins; however, the different pathways of melanin biosynthesis mentioned above have been studied extensively in free-living fungi. Although tyrosinase is mostly commonly considered the key enzyme involved in L-DOPA melanin biosynthesis, all three of the above mentioned redox enzymes have been implicated in melanin synthesis (Butler & Day, 1998; Butler *et al*. 2001; Eisenman & Casadevall, 2012; Langfelder *et al*. 2003; Mastore *et al*. 2005; Takahama, 2004; Liers *et al*. 2011).

**Phenol oxidases: Laccases and tyrosinases**

Laccases (EC 1.11.1.14) and tyrosinases (EC 1.14.18.1) belong to a diverse group of multicoper oxidases that mediate one-electron oxidation of substrates and the associated reduction of molecular oxygen to water (Solomon *et al*. 2001, Mayer & Staples 2002). These enzymes can act on various substrates of both phenolic and non-phenolic nature (Sherif *et al*. 2013). They can co-occur in the same species and have overlapping substrate specifities (Beckett *et al*. 2013). These redox enzymes are involved in a number of reactions including pigment biosynthesis, phenolic metabolism, delignification and the degradation of humus (Zavarzina & Zavarzin, 2006).

**Tyrosinases**

Tyrosinases appeared probably due to a change in the chemical nature of the earth's atmosphere from a reducing one to one that is oxidising as a result of photosynthesis (Plonka & Grabacka, 2006). They are a family of copper containing enzymes with a universal distribution in nature, from bacteria to mammals (Claus & Decker, 2006; Durán *et al*. 2002). These copper containing enzymes are known to play important roles in the activation of oxygen, which is essential for the oxidation of a great range of substrates (Laufer *et al*. 2006a). Tyrosinases use molecular oxygen to catalyse the ortho-hydroxylation of monophenols to *o*-diphenols (cresolase activity of menophenolase) and the succeeding oxidation of the *o*-diphenols to *o*-quinones (catecholase activity or diphenolase) (Claus & Decker, 2006; Laufer *et al*. 2006; Selinheimo *et al*. 2007). The produced o-quinones successively polymerize non-enzymatically with various nucleophiles to form melanins (Selinheimo *et al*. 2007).

Lichen tyrosinases were initially detected owing to the ability of lichen thalli or leachates to metabolize L-DOPA and tyrosine in the absence of $H_2O_2$ (Laufer *et al*. 2006a; Zarvazina & Zarvazin 2006). Recent findings have shown that high tyrosinase activity occurs in lichens from the order Peltigerales, although high tyrosinase has been found in a non-Peltigeralean lichen *Dermatocarpon miniatum*. However, tyrosinase in this non-Peltigeralean lichen differs from that

of Peltigeralean lichens in its cellular location substratum specificity, stability and pH optimum (Beckett *et al*. 2012). The molecular masses of tyrosinases from a number of Peltigerean lichens were 60 kDa, something they share with some free-living fungi (Laufer *et al*. 2009; Van Gelder *et al*. 1997; Halaouli *et al*., 2005; Marusek *et al*. 2006). These tyrosinases, like those of free-living fungi, are strongly activated by SDS probably due to conformational change brought about by binding of SDS to latent tyrosinase forms which make up approximately 60-90% of tyrosinases in plants and fungi (Beckett *et al*. 2013; Moore & Flurkey, 1990, Van Gelder *et al*. 1997; Mayer 2006). Most of tyrosinase activity in lichens is intracellular, which is comparable to the location of fungal tyrosinase (Laufer *et al*. 2006a; Rast *et al*. 2003).

**Laccases**

Laccases are a family of metalloproteins, which have an active site containing one to four copper atoms (Valderrama *et al*. 2003). They are probably one of the oldest and most widely researched enzymes; this is due to their involvement in many physiological processes of ecological and economic importance. Their presence was first shown by Yoshida in 1883, in the exudates of the Japanese lacquer tree, *Rhus vernicifera*, this is where the name originated (Thurston, 1994). Laccases can be found in various fungi, certain bacteria, insects and plants (Piscitelli *et al*. 2011). Laccases share the ability to oxidase aromatic compounds (Nakamura & Go, 2005). These enzymes catalyse the reduction of molecular oxygen to water, bypassing hydrogen peroxide formation and catalyse the oxidation of aromatic compounds to produce a free radical (Morozova *et al*. 2007). They oxidise monophenols, p-diphenols, o-diphenols, aminophenols and diaminoaromatic compounds; they are generally non-specific in terms of their reducing substrates and the substrates oxidised tend to vary between the different laccases (De Vries *et al*. 1986; Thurston, 1994).

Laccases in lichens were first detected owing to the ability of lichen thalli or water leachates to oxidise 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 2, 6-dimethoxyphenol in the absence of $H_2O_2$ (Laufer *et al*. 2006b; Zavarzina & Zavarzin 2006). High

laccase activity has been detected in Peltigeralean lichens; lower laccase activity has been however detected in the lichens from the order Lecanorales (Beckett *et al*. 2013b). Lichen laccases have large molecular weight (135-200 kDa and up to 300-350 kDa) (Laufer *et al*. 2009; Lisov *et al*. 2007). Peltigeralean lichens can contain laccases of both a large (di- and tetrameric) and a monomeric nature, with the large forms being most prevalent. Large laccases have low oxidation potentials and are generally less resistance to high temperature, whilst the nature and physico-chemical properties of monomeric laccases remain largely unknown (Lisov *et al*. 2012).

**Peroxidases**

Peroxidases are a class of hemoproteins or heme-containing enzymes that catalyse the oxidation of a variety of substrates using hydrogen peroxide ($H_2O_2$) or organic hydroperoxides (ROOH) as an electron acceptor (Saunders, 1973; Welinder, 1992). These enzymes metabolise a similar range of phenolic substrates as laccases, however they have higher affinities and redox potentials due to the presence of hydrogen peroxide as an electron acceptor in their reactions. Peroxidases are classified within different superfamilies, i.e. animal and non-animal peroxidases. Three subclasses classes of peroxidases belonging to the non-animal peroxidase superfamily have been proposed the basis of sequence similarity: Class I intercellular peroxidases (found in most living organisms except animals), Class II secretory fungal peroxidases and Class III peroxidases (POX, EC 1.11.1.7) (found in all land plants) (Hofrichter *et al*. 2010). Class I peroxidases can be divided into three distinct groups; catalase–peroxidases (CPs; EC 1.11.1.6), cytochrome c peroxidases (CcPs; EC 1.11.1.5) and ascorbate peroxidases (APxs; EC 1.11.1.11). Class II peroxidases can be divided into manganese peroxidases (EC 1.11.1.13), lignin peroxidases (EC 1.11.1.14), and versatile peroxidases (EC 1.11.1.16) (Cosio & Dunand, 2009; Ruiz-Duenas *et al*. 2001). Some fungi and bacteria produce heme-thiolate peroxidase such as the classical *Caldariomyces fumago* chloroperoxidase (CfuCPO) and aromatic peroxygenases (APOs) found in agaric basidiomycetes and dye-decolorizing peroxidase (DyPs) (Hofrichter *et al*. 2010).

The first purified lichen heme peroxidase (LsaPOX) came from the thallus of *Leptogium saturninum* (suborder Collematineae). This peroxidase occurs as a dimer with a molecular mass of 80 kDa, but a 40 kDa monomeric form also occurs (Liers *et al*. 2011). Other reports of peroxidases come from the studies of Plat *et al*. (1987) who reported a putative bromoperoxidase in the lichen *Xanthoria parietina*. Kranner *et al*. (2003) and Laufer *et al*. (2006a) demonstrated the ability of a number of lichens to metabolize guaiacol in the presence of $H_2O_2$ at low rates. Recent data has also indicated that some lichens from the order Peltigerales also display peroxidase activity although this is not the case for all species from this order (Beckett *et al*. 2013; Liers *et al*. 2011). Before these reports, it was thought that similar to nonlichenized ascomycetes, lichen peroxidases were very low or non-existent (Morgenstern *et al*. 2008).

### 1.4 Introduction to present study

Peltigeralean lichens are geographically widespread (Miadlikowska & Lutzoni, 2004). These lichens have strong oxidoreductase enzyme activity. Work by Laufer *et al*. (2006a) showed the wide presence of the multicopper oxidases tyrosinase and laccase in the cell walls of lichens from the Peltigerales. A later study by Beckett *et al*. (2013) showed that strong peroxidase activity also occurs in the Peltigeralean genera *Lobaria, Pseudocyphellaria* and *Sticta.* Gauslaa & Solhaug (2001) showed that depending on the long-term level of solar radiation in its natural environment, the Peltigeralean lichen *Lobaria pulmonaria* deposits brown melanic pigments in its upper cortex. The pigments are generated by the fungal constituent. High levels of exposure to solar radiation resulted in browner thalli, which reflected increased pigmentation. This increased pigmentation resulted in reduced cortical transmission of UV and shortwave visible (Gauslaa & Solhaug, 2001). These findings supported earlier hypotheses suggesting that melanin has a photoprotective function in lichens.

The main aims of the work presented here was to study the potential effects of climate change on the health of lichens by testing out the effects of UV and PAR levels projected to occur in the near future. We also aim to understand the enzymatic processes involved in UV induced

melanin synthesis by studying role of redox enzymes tyrosinases, laccases and peroxidases in the synthesis of melanin produced in response to high light and UV stress in the Peltigeralean lichens *Lobaria pulmonaria* and *Pseudocyphellaria aurata*. We also aim is to test the prediction that the major lichen clade of Peltigeralean lichens synthesise eumelanins while other lichen clades synthesis allomelanins. These lichens are shade adapted (Gauslaa & Solhaug, 2001; Lange *et al*. 2004), cephalolichens lichens, consisting of green algal photobionts and nitrogen fixing cyanobacteria *Nostoc* sp. (Jordan, 1973; Lange *et al*. 2004). They were collected from montane regions of South Africa and a coastal regions of Norway. The work initially involved studying the enzymes involved in melanin biosynthesis. The basic properties of these enzymes, such as response to desiccation and rehydration, pH optima and thermostability, and substrate specificity were characterized. The activities of these enzymes in lichens exposed to various combinations of PAR and UV were then studied, and at the same time melanin synthesis, growth, and photosynthesis were measured. The overall aim was to test how lichens may respond to changes in visible light and UV radiation following climate change.

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## CHAPTER 2: Materials and methods

### 2.1 Lichen material

Figures 1.1 - 1.3 show maps of the different collection localities, and Table 1 lists the lichen species used and their collection sites. Thalli collected were cleaned thoroughly, air dried in plastic containers, between layers of filter paper for a period of 2 d and stored in a freezer until they were used for a laboratory experiment. A few days before the beginning of each experiment thalli were gradually rehydrated in plastic containers with three layers of filter paper. These containers were placed in a low temperature (10$^{o}$C) germination growth chambers (Labcon (PTY) Ltd., Krugersdorp, RSA) and sprayed with deionised water at the beginning and sprayed daily; when fully hydrated enzyme activity in Peltigeralean lichens is optimal (Beckett *et al*. 2014). Full thallus hydration was determined by running preliminary hydration experiments whereby maximal photosystem II efficiency ($F_v/F_m$) and redox enzyme activity was measured in dry material and gradually hydrating lichens while measuring at intervals until 13 d after the start of the experiment.

Table 1: Species from the suborder Peltigerineae used in this thesis and their respective collection localities

| **Species**<br><br>**Suborder *Peltigerineae*** | **Collection localities** |
|---|---|
| *Pseudocyphellaria aurata* (Ach.) Vain. | Nottingham Road ; Drakensberg , RSA |
| *Lobaria pulmonaria* (L.) Hoffm. | Porsgrunn , NOR ; Cape Town ,RSA |

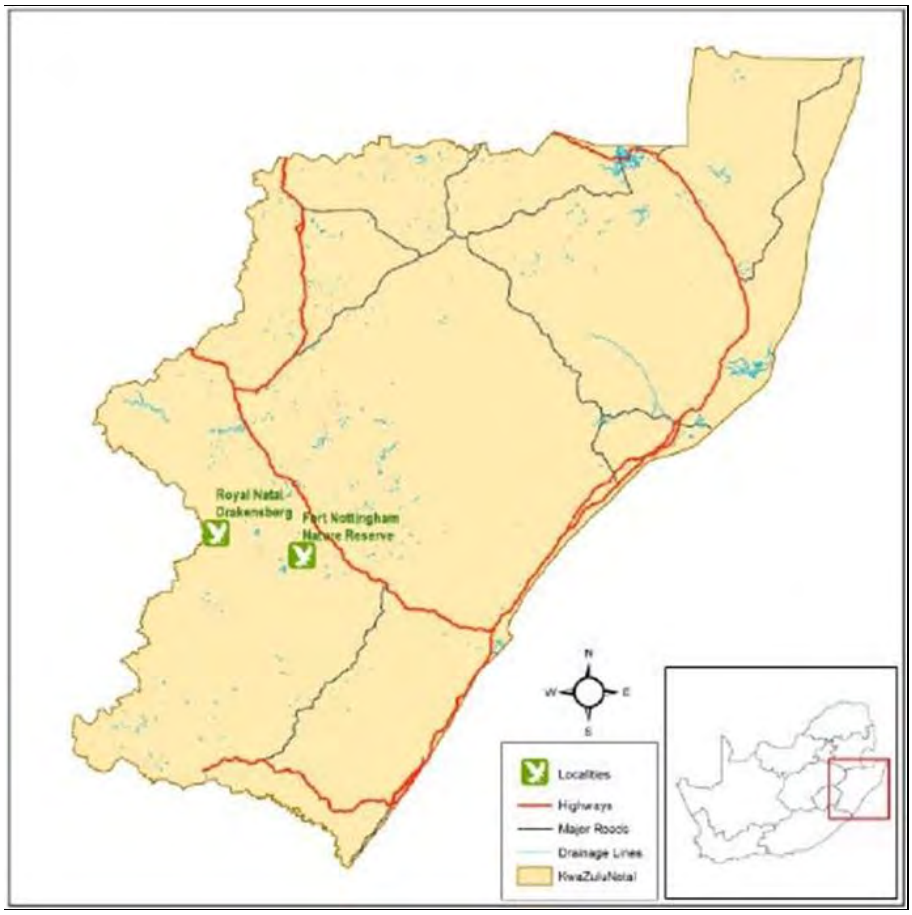


**Figure 1.1**: Map of collection localities where *P. aurata* was collected in Nottingham Road; Drakensberg, RSA. Map created using ArcGIS 10.3 software. (Environmental Systems Research Institute (ESRI), Redlands, CA, USA).

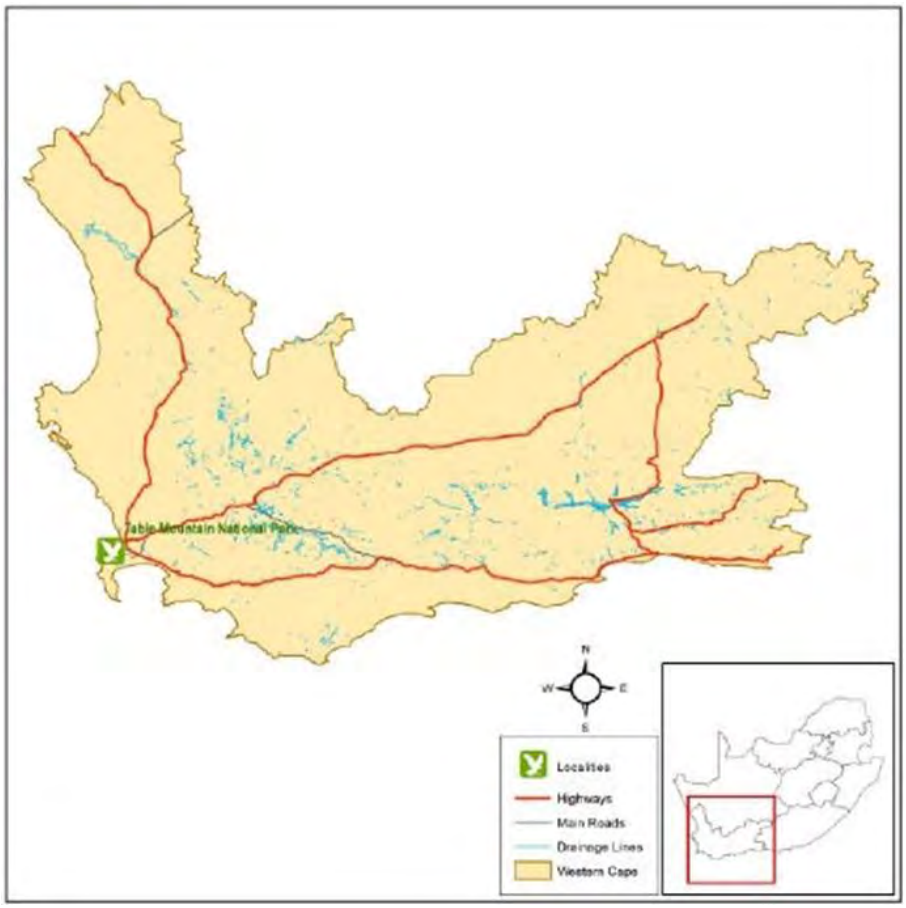


**Figure 1.2**: Map of collection locality where *L. pulmonaria* was collected in Cape Town, RSA. Map created using ArcGIS 10.3 software. (Environmental Systems Research Institute (ESRI), Redlands, CA, USA).

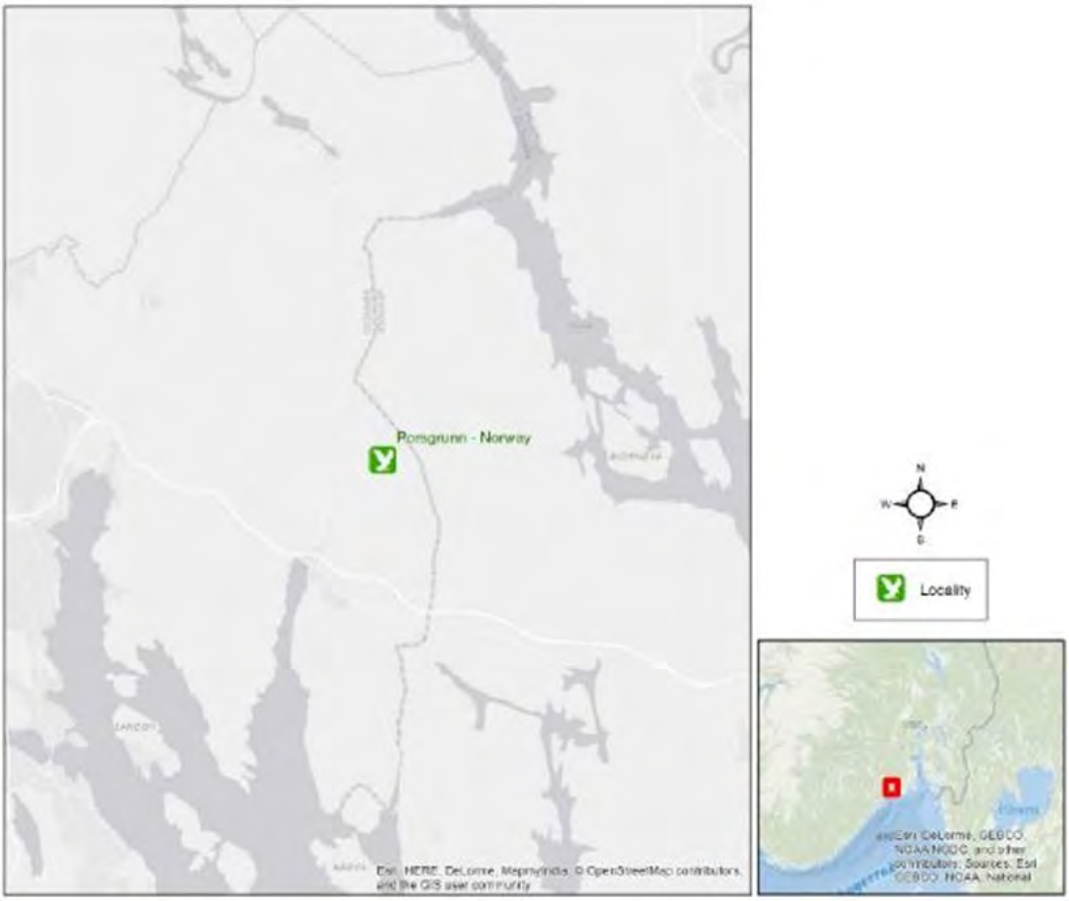


**Figure1.3**: Map of collection locality where *L. pulmonaria* was collected in Porsgrunn, NOR. Map created using ArcGIS 10.3 software. (Environmental Systems Research Institute (ESRI), Redlands, CA, USA).

**2.2 Preparation of lichen discs or thalli**

After each collection trip, material was cleaned and the healthiest thalli were chosen from specimens having a diameter greater than 5cm. To reduce variability in the material was cut into either discs or fragments of the same size. For experiments using discs, lichen material was cut up into 5 mm discs (the central area and extreme margin were avoided when doing so) and 5 discs were weighted collectively and used as one sample (ca 0.2 g). For experiments that used fragments, material was cut up into fragments of similar size and weight (*ca* 0.5 g of fresh mass). For the testing enzyme activity after exposure to radiation treatment whole thalli were used for the enzyme assays (two whole thalli were used for/represented 1 sample) (*ca.* 0.5 g)

**2.3 Preparation of crude extracts**

Crude extracts for the determination of enzymatic activities were prepared by lichen thoroughly grinding lichen thalli (*ca*. 10 g fresh mass) in 50 mM phosphate buffer pH 7 solution using a mortar and pestle. This was followed by the centrifuging of the extracts at 5 000 × *g* for 20 min, and the supernatant was collected and centrifuged at 1000 *g* for 15 min. The supernatant was then transferred into 2 ml safe-lock Eppendorf tubes and placed into an ice-bucket for further analysis.

**2.4 Enzymatic assays**

**L-Dopa assay for tyrosinase**

Tyrosinase activity was determined from the oxidation rate of 2 mM l-dihydroxyphenylalanine (DOPA, Sigma) to 2-carboxy-2, 3-dihydroindole-5, 6-quinone (dopachrome; Horowitz *et al*. 1970) in 50 mM phosphate buffer pH 6. 200 µl of 10mM DOPA was mixed with 100 µl extract and 50 mM phosphate buffer pH 6 to give a final volume of 1 ml. For species where activity was low, an additional reagent was added, 100 µl of 20 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH). This compound traps dopaquinone formed by the oxidation of L-DOPA, and makes the assay more sensitive (Winder &Harris, 1991). Reagents were pipetted into suitable plastic cuvettes, mixed by inversion and absorbance measured by using an Agilent Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, USA). The colorimetric assay gave a pink product and the extinction coefficient of the product was measured at $A_{475}$ is 3·6 $mm^{-1}$ $cm^{-1}$. As an additional test for the presence of latent forms the effect of 2 mM sodium dodecyl sulphate (SDS, Promega) on enzyme activity was sometimes used (Moore & Flurkey, 1990). For all assays, 3 replicates were found adequate to validate results obtained.

**ABTS assay method for laccase and peroxidase activity**

Laccase activity was determined from the oxidation of 0.3 mM 2,2- azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS, Sigma) in sodium acetate buffer 100 mM, pH 4.5 (Eggert *et al*. 1996). Extract (100 µl) was pipetted into plastic cuvettes with 50 µl of 6 mM ABTS and 100

mM phosphate buffer pH 5.0 to give a final volume of 1 ml. Solutions were mixed by inversion and absorbance measured by using an Agilent Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, USA). The extinction coefficient of the product was measured at $A_{420}$ is 36 $mM^{-1}$ $cm^{-1}$. Peroxidase activity was estimated as the rate of increase in absorbance following the addition of 0.1 mM $H_2O_2$) (Liers *et al*. 2011).

**Metabolism of monophenols by tyrosinase**

Monophenolase activity was determined for phenol, tyrosine and tyramine using a method based on the coupling reaction between 3-methyl-2-benzothiazolinone hydrazone (MBTH) and the quinone products of the oxidation of monophenols in the presence of polyphenol oxidase (Espín *et al*. 1997). Samples (100 µl) were incubated in 10 mM MBTH and 50 mM phosphate buffer, pH 6, and 2 mM of substrate (monophenol). Extinction coefficients of the products were from L-tyrosine, $\varepsilon_{476}$= 20.7 $mM^{-1}$ $cm^{-1}$ (Garcia-Molina *et al*. 2007); from tyramine, $\varepsilon_{476}$= 33.0 $mM^{-1}$ $cm^{-1}$ (Rodriguez-Lopez *et al*. 1994) and from phenol $\varepsilon_{476}$= 30.5 $mM^{-1}$ $cm^{-1}$ (Ciregna, 2013)

**2.5 Electrophoretic studies**

The presence of specific oxidoreductases was tested electrophoretically in crude extracts of the thalli of *L. pulmonaria* as described by Laufer *et al*. (2006a). A modified method of Laemmli (1970) was followed using SDS- and native PAGE (5% and 12%). Tyrosinase activity was visualized by incubating gels in 10 mM L-DOPA (Sigma) in 0.1 M phosphate buffer pH 6. To test for the metabolism of L-DOPA by peroxidases, $H_2O_2$ (2 mM) was added, and the gels left to stain for 12 h. Peroxidase and laccase activity was visualized by incubating gels in sodium acetate buffer (0.25 M, pH 5) containing glycerol (10%), and *o*-dianisidine (1 mM) with or without $H_2O_2$ (1 mM).

**2.6 Enzyme characterization**

**Thermostability**

The thermostability of tyrosinase from *L. pulmonaria* was tested using the L-DOPA assay as specified above. Enzyme activity was measured at time zero. Extracts were then immersed in a

thermostatically controlled water bath set at 40 ˚C. The experiment was carried out for 3 h; tubes were removed at intervals of 40 min, rapidly chilled in ice and activity measured at room temperature.

**pH optimum**

The effect of pH on enzyme activity was tested using 2 mM L-DOPA for tyrosinases as described above. For laccase and peroxidase 1 mM 2,6-dimethoxylphenol (DMP) was used as a substrate, and the production of coerulignone measured ($\varepsilon_{469}$ = 27.5 $mM^{-1}$ $cm^{-1}$) (Gómez-Toribio *et al*. 2009a, b) in the pH range from 3.0 to 8.0 in 50 mM citrate-phosphate buffer. *L*. *pulmonaria* was used as the test species for this investigation.

**Kinetic constants for L-DOPA metabolism by tyrosinase and DOPA-peroxidase**

The kinetic constants for L-DOPA metabolism by tyrosinase and DOPA-peroxidase were determined from the oxidation rate of L-DOPA concentrations ranging from 0.1- 1 mM in 50 mM phosphate buffer pH 6 in the presence or absence of 1 mM hydrogen peroxide (100 µl) in the assay solution and analysing the kinetic constants using the program “Hyper”.

**Effect of hydration on redox enzyme activity**

Lichens were collected and dried in the dark between 3 layers of filter paper for a period of 2 d, and then 1 cm discs cut from the thallus, the central area and extreme margin were avoided when doing so and the discs placed on wet non-cellulosic cloth in growth chambers at 10$^{o}$C in the dark. For each time point, three replicates comprising of five 1 cm discs corresponding to *ca* 0.1 g dry mass of were sampled, and the activities of laccase, peroxidase and tyrosinase measured. Activity was measured in dry material and at intervals until 13 d after the start of the experiment.

**2.7 Effect of UV light on melanisation and enzyme activity**

**Ultraviolet radiation cabinet experiment**

A growth cabinet experiment was carried out at the Centre for Plant Research in a controlled environment (SKP) at the Norwegian University of Life Science (NMBU) for a period of

15 d in September 2014. Lichen thalli were cut up into fragments *ca*. 0.5 g. Fragments from each species were rehydrated by spraying them with de-ionized water until they were fully hydrated and storing them in a freezer (10 $^0$C) for a period of 5 d. In addition, they were sprayed with de-ionized water once a day for the duration of the rehydration period (5 d). After a period of 5 d, 120 thalli were randomly selected from each species, 80 thalli for cultivation were taken and 10 control thalli which were placed aside for initial ($T_0$) measurements and 30 other thalli for each treatment were placed on 3 layers of filter paper in each of three open top plastic boxes and placed in growth chambers at 10 $^0$C. All thalli were sprayed with water twice a day in the morning at 9 AM and in the evening at 11 AM. Two chambers were used for the experiment; one was illuminated with white fluorescent tubes (Philips Master TL-D 36W/840, Ahlsell Norge AS, Moss, Norway) as a light source of photosynthetic active photon flux (PAR) with a density of 125 µmol photons $m^{-2}$ $s^{-1}$. The other was illuminated with a combination of photosynthetic active photon flux (PAR) and UV-B with a density of 1 Wm-2 (Q-panel UV 313, Largo, Gothenburg, Sweden), which was filtered through 0.15 mm thick cellulose di-acetate film (Rachow Kunststoff Folien GmbH, Hamburg, Germany). The daily photoperiod was 12 h for PAR; the UV-B light was on for 6 h in the middle of the photoperiod for all experimental days. UV-B intensities were measured with a UV-B sensor (model SKU 430, Skye Instruments) as described by Solhaug *et al.* (2003). At intervals of 5, 10, and 15 d, material was sampled. Measurements of reflectance spectra of the upper cortex, chlorophyll fluorescence, dry mass (DM), enzyme activity (laccase, tyrosinase, tyrosinase in the presence of SDS, and peroxidase) were taken at the beginning of the experiment and at every sampling interval.

**Field experiment**

Whole thalli (125 from South African and 125 from Norway) (N=15: Norway Growth 60, South African growth 60, Norwegian enzyme 50 analysis, South African enzyme analysis,50) were randomly selected, and three or four attached with cotton thread to wooden frames (15.9 × 15.9 $cm^2$) covered with nylon mesh netting. Frames were placed 5 cm under different types of 70 × 70

cm filters. Three replicate frames of the four light treatments were randomly arranged on an unshaded lawn in the grounds of the Norwegian University of Life Sciences, Ås, Norway for three weeks during June 2015. The weather was mainly clear and sunny with only a couple of rainy days. The treatments were first, no screen (normal ambient light); second, a wavelength-neutral acrylic screen that transmitted all light with a 15% reduction in intensity (Acrylic sunbed, 3 mm, Finn Løken A.S. Ås, Norway); third, a polyester screen that removed UV-B (PET, 0.175 mm, Nordbergs Tekniska AB, Vallentuna Sweden), and fourth a polycarbonate screen that removed both UV-A and UV-B (clear polycarbonate, 3 mm, Finn Løken A.S. Ås, Norway). The second treatment was included to test the effect of the physical presence of a screen. The thalli were sprayed with deionized water every morning *ca* 9:00 a.m. and evening after sunset to ensure that they were moist and photosynthetically active during the first part of the day. Parameters measured at the start and end of the experiment were the reflectance spectra of the upper cortex, maximal photosystem II efficiency ($F_v/F_m$), chlorophyll content, and the activities of the enzymes tyrosinase (with and without SDS), laccase and peroxidase. Relative growth rates were also measured.

**Fig 2**: Photographs showing the experimental set up for the field experiment and the field experiment.

**Growth rate measurement**

Material was collected and prepared as in the field experiment. Dry Mass (DM) for all thalli was recorded in the beginning and end of the study. Additional thalli were used to correct for differences in water content at start and end of the experiment due to differences in RH in the air when the thalli were air dried. Growth was measured as relative growth rate (RGR). RGR was expressed as (mg $g^{-1}$ $day^{-1}$) and calculated using the following equation (Evans 1972):

$$\frac{\left(\ln\left(\frac{DM2}{DM1}\right)\right) * 1{,}000}{\Delta t}$$

Where DM2– Dry Mass at the end

DM1- Dry Mass at beginning of experiment

Δt- number of days between times start and end at which DM (g) were measured

**Chlorophyll fluorescence**

All thalli were placed on 3 layers of filter paper in each of three open top plastic boxes and preconditioned in the hydrated state under low incandescent light for 48 h at 18 $^{o}$C. Before measurements were taken all thalli were dark adapted for a period of 15 min. Chlorophyll fluorescence for all thalli was measured with a portable, modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany) operated by a small portable laptop that calculated and stored the data. Measurements were taken in low-light-adapted room by gently placing the optical fibre of the fluorometer on the thalli being measured. The effective quantum yield of PS II ($F_v/F_m$) was calculated with the saturating light pulse at an intensity of 15 000 µmol $m^{-2}$ $s^{-1}$ and a duration of 0.6 s (Solhaug & Gauslaa, 1996).

**Measurement of chlorophyll content**

Chlorophyll was extracted from *L. pulmonaria* as described by Palmqvist & Sundberg (2002). Whole lichen thalli were ground to a fine powder in a ball mill. This powder was then transferred to an Eppendorf tube with 1.5 ml $MgCO_3$-saturated dimethyl sulfoxide (DMSO). Specimens were vortexed several times while being incubated at 60 $^{0}$C for 40 min using a hot water bath. After this, extracts were then centrifuged at 18000 rpm for 5 min and the absorbance of the supernatants was measured using a Shimadzu UV2001 PC spectrophotometer. Absorbance of chlorophyll content was measured at 647 and 664 nm and the amount of chlorophyll *a* and *b* was calculated according to the method of Wellburn (1994)

$$\text{Chlorophyll } a = 12.19 * (A665 - A750) - 3.45 * (A649 - A750)$$

$$\text{Chlorophyll } b = 21.99 * (\text{A649} - \text{A750}) - 5.32 * (\text{A665} - \text{A750})$$

**Reflectance spectra**

Reflectance spectra (450–900 nm) were recorded using an integrating sphere (ISP-50-REFL OceanOptics, Eerbeek, the Netherlands) placed directly on the upper surface of each thallus. A halogen lamp (DH2000, OceanOptics) illuminated the thallus via a 600 µm optical fibre, connected to the sphere. Reflectance was measured at one random position per thallus with a spectrometer (model SD2000, OceanOptics) connected to the output port of the sphere with a 400 µm-thick fibre. The percentage reflection was calculated on the basis of a dark spectrum and a reference spectrum from a white reference tile (WS-2, OceanOptics).

**Melanin determination**

Melanins from Norwegian replicate thalli from the field experiment were extracted at the end of the experiment using a modification of the method of Ellis & Griffiths (1974). Briefly, thalli were incubated overnight in 2 M NaOH, centrifuged at 4000 *g* for 20 min and the pellet discarded. The pH of the supernatant was adjusted to 1 using 5 M HCl, and melanins left to precipitate overnight. Samples were centrifuged at 4000 *g* for 20 min, and the supernatant discarded. The resulting pellet was washed in distilled water, chloroform, ethyl acetate and acetone (made up to 5 ml), and then dried overnight at 80 $^{0}$C. The C: N ratio of the melanins were then measured in 5 mg samples using the Vario Micro Cube (Elementar, Frankfurt). The browning reflectance index (BRI), calculated as BRI = (1/R550 – 1/R700)/R750) (Chivkunova *et al*. 2001).

**2.8 Data analysis**

Statistical analyses where necessary were performed using the software SPSS (SPSS Inc., Chicago, IL, USA). Effects of treatment on each investigated parameter were analysed by general linear model (GLM) univariate analysis of variance (ANOVA) where appropriate, least significant difference between means (three replicates) at $P < 0.05$ probability was used for inspection of differences between values. *Further* examination of all possible pairwise comparisons of treatments means were performed with a Duncan's multiple range post hoc procedure with $p < 0.05$ used as statistical indication of significance.

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## CHAPTER 3: Functional biochemical characterization of redox enzymes from the lichens *Lobaria pulmonaria* (L.) Hoffm. and *Pseudocyphellaria aurata* (Ach.) Vain

### Introduction

The work described in this chapter was carried out to characterize in detail the redox enzymes from *L. pumonaria* and *P. aurata* with a special focus on tyrosinase as we predicted that Peltigeralean lichens synthesize eumelanins and that tyrosinase may be the key enzyme initiating melanin biosynthesis in these lichens. Laboratory experiments were carried out to provide a detailed biochemical characterisation of the basic properties of redox enzymes compromised of heme-containing peroxidases and the copper-containing phenol oxidases tyrosinase and laccase in two lichen species from the order Peltigerales. This was done in order to test out the prediction that redox enzymes from Peltigeralean lichens are similar to those from fungal counterparts that synthesize L-DOPA melanins by virtue of their biochemical characteristics and reaction mechanisms and to increase our understanding of the roles of these enzymes in lichen biology and elucidate their possible role in melanin biosynthesis based on their biochemical properties and kinetic analyses. The exact roles of redox enzymes in lichen biology remain uncertain; however, it has been suggested that they may possibly include lignocellulose breakdown, saprophytic activities, humification, defence against pathogens and abiotic stress (melanin synthesis) amongst others (Beckett *et al*. 2013). Before carrying out a detailed biochemical characterization, we surveyed these enzymes in *L. pulmonaria* using native-PAGE gel electrophoresis where the proteins are separated in non-denaturing conditions. Characterization studies such as, effect of pH, effect of rehydration on redox enzyme activity and metabolism of monophenols by tyrosinase were carried out using *L. pulmonaria* as the test species, a species with a wide distribution in Europe, Asia, North America and Africa (Geiser & McCune, 1997). Thermostability of enzymes and kinetic constants for L-DOPA metabolisms were done for both *L. pulmonaria* and *P. aurata*. The latter is a cosmopolitan species with a wide distribution in North, South, and Central America, the West Indies, Europe, Asia, and Africa (Moore, 1968).

## Results

### Screening the oxidoreductases of *Lobaria pulmonaria* by native PAGE

Following electrophoresis and subsequent staining with L-DOPA as a substrate *L. pulmonaria* showed a single tyrosinase isoform with a molecular mass of 45 kDa (Fig. 3). Staining gels with o-dianisidine and $H_2O_2$ revealed a single peroxidase isoform with a molecular mass of 35 kDa, while using a 5% gel and adding o-dianisidine alone revealed a single laccase isoform with a molecular mass of 190 kDa. Adding $H_2O_2$ and incubating gels in L-DOPA followed by staining overnight indicated that both peroxidases and laccases can also metabolize L-DOPA.

### Recovery from desiccation

Our results show that peroxidase and laccase enzyme activity in desiccated lichen thalli was low but rehydration led to a rapid stimulation during rehydration with maximal enzyme activity being reached at 2-3 days, however after this activity slightly decreased with an increase in rehydration time. Tyrosinase activity on the other hand was quite high in even in in desiccated thalli. The maximal activity for laccases and latent tyrosinase (tyrosinase +SDS) was achieved within 2 days of rehydration while for peroxidase and tyrosinase was obtained after 3 days of rehydration (Fig. 4). Our findings show that rehydration of desiccated lichen induce activity of both laccase and peroxidase activity more significantly than it does for tyrosinases, i.e. although tyrosinase activity was also stimulated by hydration, this stimulation was not significant. When SDS was added to the reaction mixture with L-DOPA, stimulation of tyrosinase activity was much higher than when it was absent from the assay but rehydration only led to a small stimulation of activity.

### Effect of pH on enzyme activity

Tyrosinase from *L. pulmonaria* had a pH optimum of 6.5 using L-DOPA as a substrate, while L-DOPA oxidase had a pH optimum of 4.5 using the same substrate. When the effect of pH was tested on peroxidase and laccase activity using DMP as a substrate, we found that peroxidase

activity was optimal at pH was 4.5. Laccase activity on the other hand had a rather broad activity peak between pH 3.5 and 5.5 (Fig. 5).

**Thermostability of tyrosinases**

Tyrosinase from both *L. pulmonaria* and had *P. aurata* moderately high thermostabilities at 40 $^{0}$C. *P. aurata* displayed a slightly higher thermostability, maintaining 75 % of its activity for approximately 50 minutes while in *L. pulmonaria* 50 % of activity was lost within the first 40 min of the experiment, but no further loss of activity occurred for the remaining duration of the experiment (Fig .6).

**Tyrosinase action on monophenols**

Tyrosinases from *L. pulmonaria* metabolized the monophenols tyrosine, tyramine and phenol at rates of 2.8, 2.2 and 1.9 µmoles $g^{-1}$ dry mass $min^{-1}$. Thus tyrosine was metabolized faster than the other two monophenols. Hydrogen peroxide was shown to display some inhibitory action on tyrosinase metabolism of all three monophenols as rates for tyrosine, tyramine and phenol were 1.9, 1.23 and 1.22 respectively in its presence.

**Kinetic constants for L-DOPA metabolism by tyrosinase and L-DOPA oxidase**

Tyrosinases from both *L. pumonaria* and *P. aurata* had a lower affinity for L-DOPA compared to L-DOPA oxidase. Michaelis-Menten analysis of L-DOPA metabolism by tyrosinase indicated that the Km was 0.104± 0.186 mM for *P. aurata* and for *L. pulmonaria,* it was 0.124 ± 0.277 mM (Fig. 7). The affinity of L-DOPA oxidase was much higher, with a Km 0.0292 ± 0.035 for *P. aurata* and 0.0301 ± 0.0206 mM for *L. pulmonaria*.

**Discussion**

Results presented here indicate that redox enzymes from *L. pulmonaria* resemble those from other lichens and free-living fungi. Comparison of tyrosinase from *L. pulmonaria* and *P.*

*aurata* using data obtained in this study and the findings of Laufer *et al.* (2006a) indicate that tyrosinases from Peltigeralean lichens share a number of similarities.

**Redox enzymes in L. pulmonaria**

Lichens produce a number oxidoreductases such as phenol oxidases tyrosinase and laccase, and heme peroxidases (Beckett *et al*. 2013). In our electrophoretic survey, tyrosinase was visualised as protein band corresponding to a molecular mass of 45 kDa. Although this was consistent with that of tyrosinases from free living fungi (Halaouli *et al*. 2005; Van Gelder *et al. 1997)*, it was different to earlier molecular masses reported by (Laufer *et al*. 2006a) in other Peltigera species such as *P. aurata*, *P. rufescens* and *P. malacea*. The most likely explanation for this disparity is that it is now known that tyrosinase compromises two parts (Mauracher *et al*. 2014). The main active unit is 45 kDa, but there exists a 15 kDa subunit that renders the enzyme "latent "when it binds to the main active unit. It is seems that in the species tested by (Laufer *et al*. 2006a) the complete enzyme reforms during electrophoresis and can for unknown reasons displays activity and can be visualised as a single protein band. In *L. pulmonaria* however, the main active unit does not associate with the small subunit. Laccase from *L. pulmonaria* has a molecular mass of 190 kDa which is similar to that of some free-living fungi and the lichen *Nephroma* belonging to the same suborder Peltigerineae (Edens *et al*. 1999; Laufer *et al*. 2006a). *L. pulmonaria* has a 36 kDa peroxidase which is also consistent with earlier findings in lichens belonging to the same order (Liers *et al*. 2011). Results from gels also indicated that peroxidases and laccases were quite capable of metabolising L-DOPA even at a pH that is higher their normal optima.

**Kinetics of redox enzymes**

Initial experiments on rehydration following desiccation showed *L. pulmonaria* display optimal redox enzymes activity after 2-3 d of rehydration, and all extracts used in all the kinetic experiments described here were prepared with lichens treated this way to obtain good activities. Rehydration of lichen thalli led to a large stimulation of laccase and peroxidase activity. By

contrast, desiccated lichen thalli possessed relatively high tyrosinase activity, and rehydration had very little effect on tyrosinase activity. This is consistent with results obtained in other Peltigeralean species suggesting that tyrosinases play little or no role in desiccation tolerance in Peltigeralean lichens (Beckett *et al*. 2012; Laufer *et al*. 2006a). Adding SDS to rehydrated lichen thalli extracts stimulated tyrosinase activity; this stimulation decreased with time of rehydration. The stimulation of tyrosinase activity by SDS has been reported before in lichens and further support the idea that tyrosinases exist as latent and active forms (Laufer *et al*. 2006a). These "latent" forms are strongly activated by SDS , due to a conformational change brought about by the binding of SDS to the latent forms thus rendering them active (Duarte *et al*. 2012; Moore & Flurkey, 1990). The existence of latent forms and their activation may allow fungi to increase tyrosinase activity rapidly in response to stress such as wounding (Laufer *et al*. 2006a). Peroxidase and laccase activity was stimulated in hydrated thalli during the first 4 d of rehydration, consistent with the results found in other lichens (Beckett *et al*. 2014). These results presented in this study do not support a role for peroxidases in ROS scavenging in lichens, as activities were low during the early stages of rehydration when most ROS are formed (Weissmann *et al*. 2005). These enzymes may produce ROS, which possibly play a role in facultative saprophytic activities that are beneficial during periods of limited photobiont photosynthesis (Beckett *et al*. 2014, 2015).

The pH optima of laccase and tyrosinase are similar to those reported in other Peltigeralean lichens and free-living fungi (Beckett *et al*. 2012; Krishnaveni *et al*., 2015; Laufer *et al*. 2006b). Peroxidases from *L. pulmonaria* had a distinct pH optimum of 4.5, contrary to the findings of in *Leptogium saturninum* which display increasing activity with decreasing pH (Liers *et al*. 2011). These results are however comparable with those found in free-living such as that of manganese- peroxidase (MnP) of *Lentinula edodes* (Boer *et al*. 2006). The pH optima of "DOPA oxidase" tested using a mixture of L-DOPA and $H_2O_2$ was identical to that of peroxidase measured with DMP and $H_2O_2$ suggesting that the same enzyme metabolises both substrates. The thermostability of tyrosinase was comparable with that of tyrosinase from free-living fungi; similar

results have been reported from free-living fungi such as *Lentinula boryana* (De Faria *et al*. 2007; Duarte *et al*. 2012; Liu *et al*. 2005). Peroxidases had a higher catalytic efficiency on L-DOPA than tyrosinase, seen by its 3-4 times higher affinity for DOPA measured as a Km of 0.029 mM in *P. aurata* and 0.03 mM in *L. pulmonaria* compared to that of tyrosinase which was 0.19 in *P. aurata* and 0.12 mM in *L. pulmonaria*. Efficient DOPA conversion has also been shown in other by the peroxidase of the lichen *Leptogium saturninum.* Results suggest a possible involvement of peroxidases in melanin biosynthesis in *L. pulmonaria* as thalli of species in the genera *Leptogium* are also heavily pigmented (Liers *et al*. 2011). There is currently no general agreement on the exact role of peroxidases in melanin synthesis, and further studies are needed to investigate this. However, the higher catalytic efficiency of peroxidase towards DOPA compared to tyrosinase shown here could suggest that peroxidase plays a critical role in later steps of eumelanin biosynthesis, while tyrosinase is the enzyme responsible for initiating it. Tyrosinase from *L. pulmonaria* was shown to be able to metabolise monophenols, a characteristic shared with most tyrosinases from free-living fungi (Selinheimo *et al*. 2007). This ability to metabolize monophenols has also been reported in other *Peltigerales* lichens (Beckett *et al*. 2012; Zavarzina & Zavarzin, 2006). Of all the monophenols investigated, tyrosinase had a slightly greater affinity for L-tyrosine as a substrate, consistent with results from free living fungi (Espín *et al*. 2000). Monophenol hydroxylase and diphenoloxidase activities of tyrosinase from *L. pulmonaria* taken together with the other results from the biochemical characterisation of redox enzymes from this lichen further suggest that *L. pulmonaria* tyrosinase may be the key enzyme initiating melanin biosynthesis, as L-tyrosine is considered the main monophenol precursor of L-DOPA melanin (Solano, 2014).

**Conclusion**

Taken together our results demonstrate that *L. pulmonaria* and *P. aurata* produce redox enzymes of comparable nature to those of free living-fungi by virtue of their biochemical characteristics and kinetic parameters. The existence of high enzyme activities suggests that they are there for a reason. Furthermore, these results indicated that although tyrosinase could be the

key enzyme involved in the melanin biosynthetic pathway of *L. pulmonaria* we may intuitively regard the probable involvement of all three redox enzymes in melanin synthesis as all three redox enzymes are capable of metabolising L-DOPA and thus may be involved in L-DOPA melanin synthesis. However, this suggestion was not supported by field experiments carried out in chapter 4 that showed that no simple correlation exists between melanisation in the field and redox enzyme activity (see next chapter). As discussed further in Chapter 5, further experiments are needed to determine the precise roles of theses enzymes in lichen Biology.

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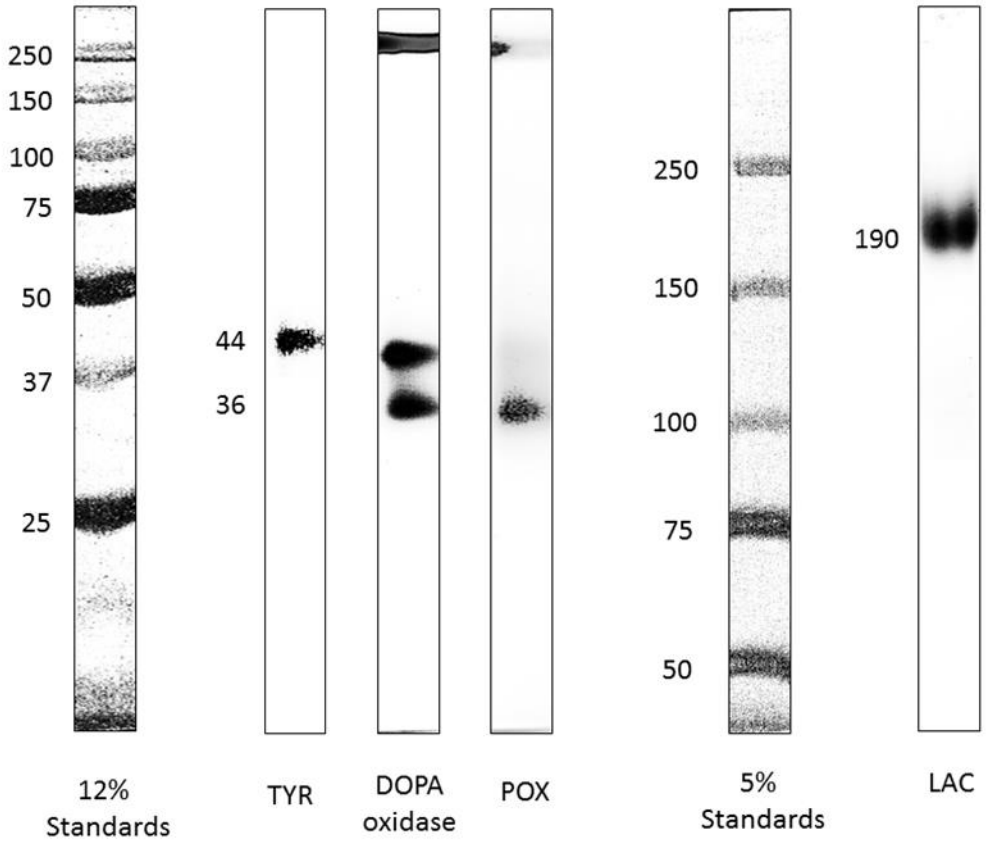


**Fig 3:** Native PAGE of crude extracts from *Lobaria pulmonaria*. Tyrosinase activity was visualized by incubation in 2 mM DOPA for 30 min, DOPA oxidase activity by 2 mM DOPA and 10 mM $H_2O_2$ for 12 h, peroxidase activity by 1 mM *o*-dianisidine and 10 mM $H_2O_2$ for 30 min, and laccase activity by *o*-dianisidine in the absence of $H_2O_2$. Molecular masses of standard proteins are indicated in kDa.

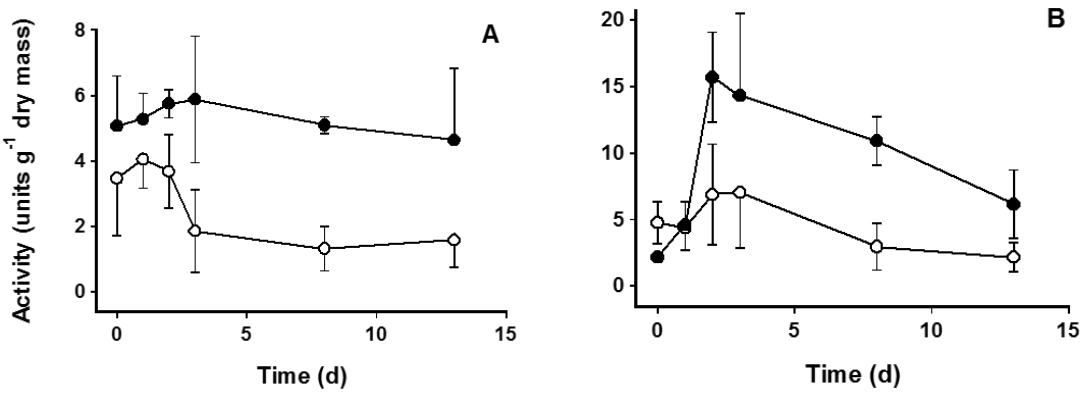


**Fig 4**. The effect of rehydration on the activities of A, tyrosinase activity (solid symbols) and latent tyrosinase activity (open symbols) and B, laccase activity (solid symbols) and peroxidase activity (open symbols) in *L. pulmonaria*. Activity was measured on 0 d, and then material transferred to moist filter paper. Activity was then measured at intervals for the next 13 d. 4 replicates were done per extract and each assay was replicated 3 times, In this and subsequent graphs and mean values are plotted ± 1 SE.

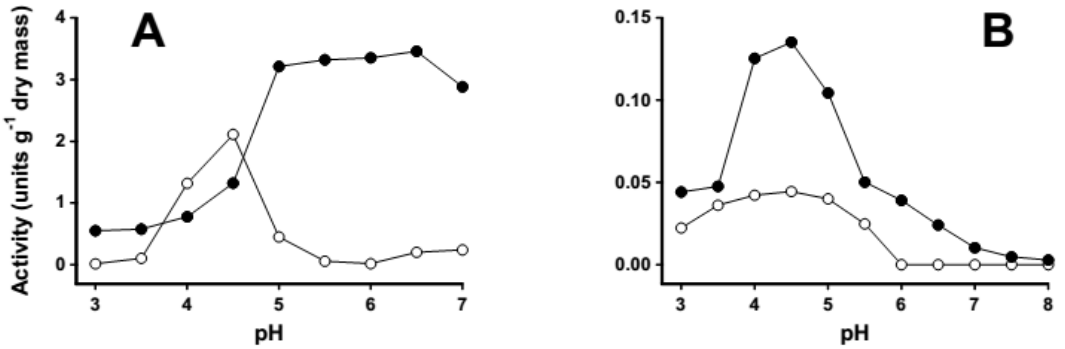


**Fig 5**. The effect of pH on enzyme activity in *Lobaria pulmonaria*. A. Tyrosinase (closed circles) and L-DOPA oxidase (open circles). B. Laccase (solid circles) and peroxidase (open circles), measured with DMP as the substrate.

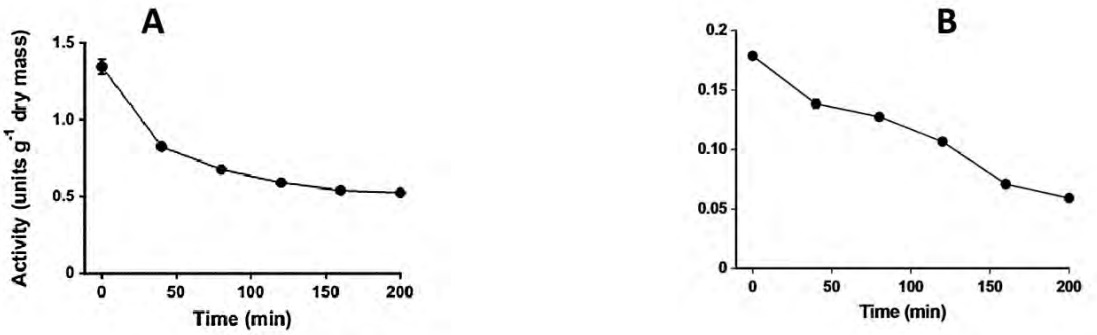


**Fig 6**: Thermostability of tyrosinase at 40 $^{0}$C measured with L-DOPA as the substrate in A. *L pulmonaria* and B. *P. aurata*

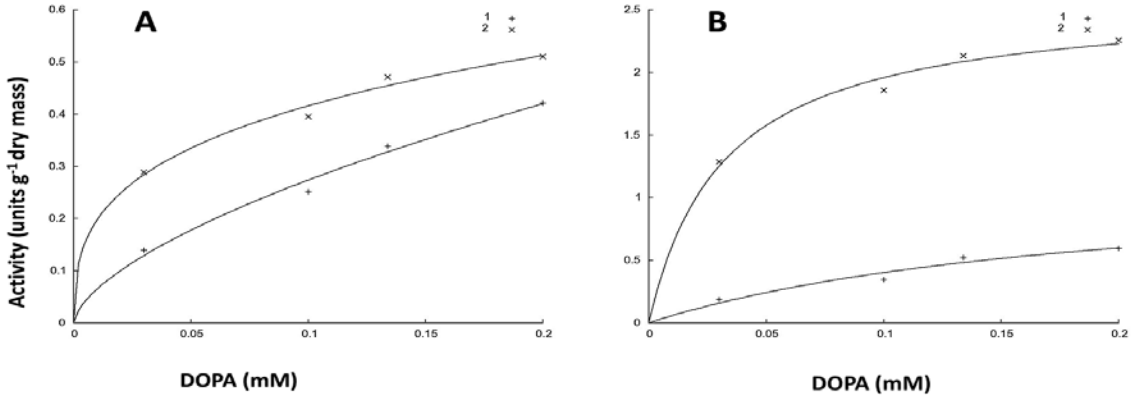


**Fig 7**: Data for Vmax/Km values, which represent catalytic efficiency of L-DOPA metabolism by tyrosinase (+) at pH 6.0 and L-DOPA oxidase (x) at pH 4.5 in a crude extract from A) *P. aurata* and B) *L. pulmonaria*. The fitted lines were derived from a Lineweaver-Burke transformation of the raw data. The Km for tyrosinase from *P. aurata* was 0.104 ± 0.186 mM and for L-DOPA oxidase it was 0.0292 ± 0.035. For *L. pulmonaria* the Km for tyrosinase was 0.125 ± 0.278 and for L-DOPA oxidase it was 0.0301- ± 0.0206.

## CHAPTER 4: UV-induced melanin synthesis in the lichen *Lobaria pulmonaria*

### Introduction

The ability of *L. pulmonaria* to synthesize melanin as a sun-screening polymer in response to exposure to harmful solar radiation has been reported in a number of publications (Gauslaa & Solhaug, 2001, 2004; McEvoy *et al*. 2007; Nybakken *et al*. 2007). Melanin protects the photobiont from excessive radiation (Gauslaa & Solhaug, 2001). Although several studies have been done on the induction of melanin in *L. pulmonaria*, the enzymes responsible for melanin synthesis and the chemical composition of these melanin have not been documented. Most fungi synthesize melanin using either the DHN or the L-DOPA pathway (Bell & Wheeler, 1986; Butler & Day, 1998; Eisenman & Casadevall, 2012; Langfelder *et al*. 1998; Langfelder *et al*. 2003; Nosanchuk & Casadevall, 2003; Nosanchuk *et al*. 2004; Solano, 2014)( see Introduction for more details). Although in the laboratory it is easy to show that lichen tyrosinases, laccases and peroxidases can readily oxidise L-DOPA into dark pigments and therefore participate in melanisation, it is unknown which enzymes are involved in melanisation in the field. Some fungi such as the black yeast *Phaeococcomyces sp.* and the filamentous ascomycete *Verticillium dahliae* can synthesize melanin via the L-DOPA pathway when supplied with exogenous substrates L-DOPA or tyrosine, but synthesize DHN melanin under natural conditions (Bell & Wheeler, 1986; Butler *et al*. 1989; Wheeler *et al*. 1978). Therefore, a combination of both field and laboratory experiments are needed to determine the role of lichen redox enzymes in melanin biosynthesis.

In the work presented in this chapter, a combination of laboratory and field experiments were used to elucidate the role of Peltigeralean lichen redox enzymes in melanin biosynthesis in order to test out the prediction that Peltigeralean lichens which are rich in the redox enzymes, tyrosinases, laccases and peroxidases synthesise eumelanins using the L-DOPA pathway while lichens from other clades synthesize allomelanins using DHN pathway. We also aimed at understanding how melanins may affect lichen functioning by studying the presence of melanins on the growth, photosynthesis and energy balance in Peltigeralean lichens. A growth chamber

experiment was carried out with both *L. pulmonaria* and *P. aurata,* while the field experiment was carried out using *L. pulmonaria*. In the northern hemisphere *L. pulmonaria* displays UV-induced melanin synthesis (Solhaug *et al*. 2003, Nybakken *et al*. 2007). To test the role of redox enzymes, we induced melanin synthesis by exposing thalli to various combinations of PAR and UV light and then measured the activities of tyrosinase, laccases and peroxidase. To check whether the induced melanins were of the DOPA type, the C/N ratio (C:N) was measured; DOPA melanins typically contain a N content of c. 10 % , compared with 1 % for the other types (Loganathan & Kalyanasundaram, 1999). The dry mass of all thalli was recorded at the beginning and end of the study, and the relative growth rate calculated (RGR, mg $g^{-1}$ $day^{-1}$). We also investigated the relationship between radiation screening and photosynthetic light use efficiency in lichens exposed to UV radiation by measuring parameters such as chlorophyll a, b, total chlorophyll, and chlorophyll fluorescence.

**Results**

**Growth Chamber Experiment**

Under the experimental conditions in the growth chambers, no melanisation was observed. Data obtained showed that thalli receiving UV radiation had reduced photochemical efficiencies and slower growth rates. In addition, no clear pattern upregulation or downregulation of enzyme activity was observed in response to UV treatment; enzyme activity fluctuated in an irregular manner (data not shown). We therefore opted to repeat the experiment under field conditions.

**Field experiment**

All *L. pulmonaria* material collected from South Africa experienced severe chlorophyll bleaching within a few days of exposure to radiation in the field and was discarded.

**Effects of ultraviolet irradiation (UV) on screening compound synthesis**

Melanin content, expressed as BRI, significantly varied between the different radiation regimes. Thalli exposed to normal ambient light with no screen had the highest BRI, followed by

thalli under a wavelength-neutral screen that transmitted all light with a 15% reduction in radiation intensity, which had a statistically comparable BRI. Filtering UV-B greatly reduced melanisation, while melanisation was prevented by removing both UV-A and UV-B (Fig. 8 & 9). The reflectance curves expressing mean visible reflectance showed the same pattern as the BRI, with the lowest visible reflectance occurring in the thalli exposed to UV radiation. Thalli exposed to UV radiation, which were highly melanised, reflected on average ± 20 % less light than thalli exposed only to PAR (Fig 10). Like BRI, the reflectance of thalli exposed to exposed to normal ambient light with no screen and those that were under the wavelength neutral filter were relatively similar. Similarly, melanin formation seen visually by the dark brown cortical melanic compounds was in agreement with both the BRI and reflectance curves. When we extracted and analysed melanins from the most heavily melanised thalli we found that the C: N ratio was 10.4 ± 0.3 (n=3), typical for DOPA melanin ("eumelanin").

**Effect of UV radiation on redox enzyme activity in lichen**

In lichen thalli exposed to natural ambient light without a filter, the activity of laccase was strongly induced, while there was a small non-significant increase in peroxidase activity (Fig. 11). Lichens placed under a wavelength-neutral filter displayed no changes in in enzyme activity. Enzyme activity changed slightly from the initial activity values when UV radiation was filtered, removing UV-B and removing both UVA and UV-B significantly increased the activity of active tyrosinase (Fig. 11A), but not of latent forms (Fig. 11B).

**Effects of ultraviolet irradiation (UV) on photosynthetic pigments**

In general, the various radiation regimes had no significant effect on the health of the photobiont, assessed by comparing the photosynthetic capacity, photochemical efficiency and chlorophyll content of the lichen thalli receiving various radiation treatments with that at the start of the experiment start of the experiment.

**Chlorophyll fluorescence**

There were no significant differences in maximal PSII efficiency between the radiation treatments. Exposure to UV radiation had no significant effect on maximal photosystem II activity ($F_v/F_m$) (Fig. 12C).

**Chlorophyll content**

The chlorophyll a/b ratio (Fig. 12A) and the total chlorophyll content (Fig. 12B) did not vary between all radiations treatments.

**Effects of ultraviolet irradiation (UV) on relative growth rate (RGR)**

The average RGR significantly differed between the radiation treatments. Lichens in the open treatment displayed a negative growth rate, while the growth rate of lichens from which both UV-A and UV-B was excluded, was significantly higher than other treatments (Fig. 13).

**Discussion**

Melanins are usually dark brown or black pigments of high molecular mass that are synthesized through the oxidative polymerization of phenolic compounds (Polacheck *et al*. 1990). Lichens synthesize brownish and amorphous melanic compounds in their upper cortex which are probably responsible for screening high radiation (Gauslaa & Solhaug, 2001; Nybakken *et al*. 2004). When we conducted growth chamber experiments on the effect of UV on *L. pulmonaria* thalli, we did not observe any melanisation, possibly to due to the limitations and difficulties involved in supplying solar radiation that is realistic when compared to what might be expected in the field. Radiation regimes used in the growth chamber experiment were different from what one would find in the field, as the UV-A and PAR were 15-25 times lower than in solar radiation while the UV-B was quite close to maxim UV-B during summer in Norway. Therefore, the absolute amounts and ratios of radiation regimes used were different from what lichens would normally receive in the field. Other limitations could also have been related to differences in RH and evaporation between field and laboratory experiments as air temperature fluctuates in the field, but further investigations are needed to confirm this.

When exposed to different radiation regimes in the field UV, *L. pulmonaria* from South Africa experienced severe chlorophyll bleaching within a couple of days and no melanins were formed. This was expected, as melanisation requires an active metabolism (Gauslaa *et al*. 2006), thus thalli that became severely bleached would have been unable to synthesize new compounds. The chlorophyll bleaching that occurred in the South African material could be because the material was collected in Skeleton Gorge, Cape Town, South Africa. Anecdotal observations suggest that these lichens normally experience include very low ambient light, and therefore they were presumably more shade adapted than the Norwegian material. Many studies have shown that shade-adapted lichens can be highly susceptible to photoinhibition during high light stress (Coxson, 1987; Demmig-Adams *et al*. 1990; Manrique *et al*. 1993). Transplanting the South African material collected during winter to Norway in the field during summer meant that they were exposed to different conditions in terms of ambient light and the length of the photoperiod. It seems likely that South African material could not adapt quickly enough to the abrupt changes in ambient light exposure and photoperiodic conditions, which will need to be considered in future trans-international transplant experiments.

Our results from the *L. pulmonaria* collected from Norway showed that cortical melanins in *L. pulmonaria*, quantified by BRI, are synthesised within 3 wks. Melanisation occurred when shade-adapted thalli were transplanted to an open area without a filter or to under a wavelength-neutral filter that transmits both PAR and UV. Similar to the results of Solhaug *et al*. (2003), filtering both UV-A and UV-B prevented melanin synthesis (Fig. 8). This clearly indicates that UV is the signal that triggers melanisation, as all treatments received similar levels of PAR. Limited melanisation also occurred in lichens receiving UV-A but not UV-B. The brown reflectance index showed the same results as the pictures taken in that lichen thalli that were heavily melanised also happened to have the highest BRI observed and had the lowest reflectance (Fig. 8 & 9). This means that melanic thalli absorb more solar energy than pale thalli. Analysis of the chemical content of the material revealed a low C:N ratio of the melanins formed (10:1) , indicating that

they are synthesized via the L-DOPA melanin synthesis pathway rather than the hydroxylated naphthalene derived molecules that produce melanins with a C:N ratio close to 100:1 (Loganathan & Kalyanasundaram, 1999; Solano, 2014).

Measurements of total chlorophyll content, the ratio of chlorophyll a to b, and chlorophyll fluorescence give important information about the state of the photosynthetic apparatus, changes in photochemical efficiency, photobiont tolerance, light adaptation and heat dissipation in photosynthetic organisms (Maxwell & Johnson, 2000). Recording these parameters in the present experiment was useful in determining the sensitivity of the lichens to UV radiation. Similar to the results of Solhaug *et al*. (2003), all thalli exposed to the different radiation regimes remained healthy, assessed by measurements of maximum photochemical efficiency of dark-adapted thalli ($F_v/F_m$). No differences in the chlorophyll content (a/b ratio and total chlorophyll) were also found between the radiation treatments (Fig. 12). Results suggest that melanins in the fungal cortex provides the first line of defence against UV radiation for the photobionts and their physiological processes (Solhaug *et al*. 2003). These results were in agreement with earlier findings in lichens where UV-B radiation led to only a small reduction or was without effect on photosystem II efficiency (Bjerke *et al*. 2005; Solhaug *et al*. 2003).

Relative growth rate (RGR) is a simple and useful parameter for assessing the influence of various environmental factors and stressor on the health and performance of lichens (Bidussi *et al*. 2013). When we assessed RGR in lichen thalli exposed to the various radiation treatments, additional thalli were used to correct for differences in water content at start and end of the experiment due to differences in RH in the air when the thalli were air-dried. Results indicated UV may have been harmful to the mycobiont, for example, UV is known to damage lichen DNA (Ünal & Uyakanikgil, 2013). Lichen thalli in the open treatment displayed a negative growth rate, while the highest growth rate occurred in thalli from which UV-A and UV-B were both filtered, i.e. lichens were exposed to PAR only (Fig. 13). The lower growth rates in lichens exposed to UV-A suggests that normal, ambient UV-A may directly harm the mycobiont, even if a thallus becomes melanised.

Our transplant experiment showed that no simple correlations exist between melanisation in the field and tyrosinase activity. Melanised lichen thalli did not display increased tyrosinase activity, whereas filtering out UV radiation lead to a significant increase in tyrosinase activity suggesting that these enzymes may not be responsible for melanisation (Fig 11). Interestingly, lichens in the open treatment (no filter) displayed increased laccase activity. It may be tempting to speculate that laccase were responsible for melanin synthesis, because, for example, laccases are thought to be the enzyme catalysing L-DOPA melanins in certain fungi such as *L. edodes* (Nagai *et al*. 2003). However, lichens placed under a wavelength-neutral filter became melanised (Fig. 8), but displayed no increased laccase activity (Fig 11). The increase in laccase activity in lichens in the open treatment may have been rather a general stress response. Placing a wavelength-neutral screen over lichens caused several subtle differences in the environment compared to lichens in the open treatment. While the Perspex screen reduces light intensities by c. 15% across all wavelengths, more significantly observations suggest that the screens tends to reduce dew formation, and will therefore reduce the time that lichens are metabolically active. This will effectively reduce the time that hydrated, active thalli are exposed to UV radiation, particularly early in the morning. There is no obvious explanation for the increase in tyrosinase of lichens placed under the filters that remove both UV-A and UV-B. However, taken together these results suggest that increased tyrosinase activity is not essential for melanin synthesis.

**Conclusion**

Work is currently underway to purify and sequence lichen tyrosinase so that they can be compared with their counterparts from free-living fungi at the molecular level. Intuitively, based on their substrate specificity determined in the laboratory, the main role of lichen tyrosinase is melanin synthesis. However, the absence of correlation between tyrosinase activity and melanisation reported here suggest that further studies are needed to determine the levels of melanin precursors and also the role of other redox enzymes. Our biochemical characterization described in chapter 3 indicated that laccases and peroxidases may be involved in melanin

biosynthesis, although their role appears less critical than that of tyrosinase. Additional roles for tyrosinase need to be investigated, for example defence against pathogens. For instance, infection of the mushroom *A. bisporus* by a pathogenic bacterium causes discoloration of the cap, and is accompanied an induction of fungal tyrosinase (Soler-Rivas *et al*. 2000). Furthermore, tyrosinase may be needed to synthesize quinones needed for radical-generating extracellular redox cycling that has been reported to occur in the lichen *Usnea* (Beckett *et al*. 2015). It must also be noted that no publications have demonstrated UV activation of a tyrosinase promoter in fungi or lichens. Possibly in *L. pulmonaria* tyrosinases are constitutively expressed, and substrate levels (tyrosine or L-DOPA) control the rate of melanin synthesis.

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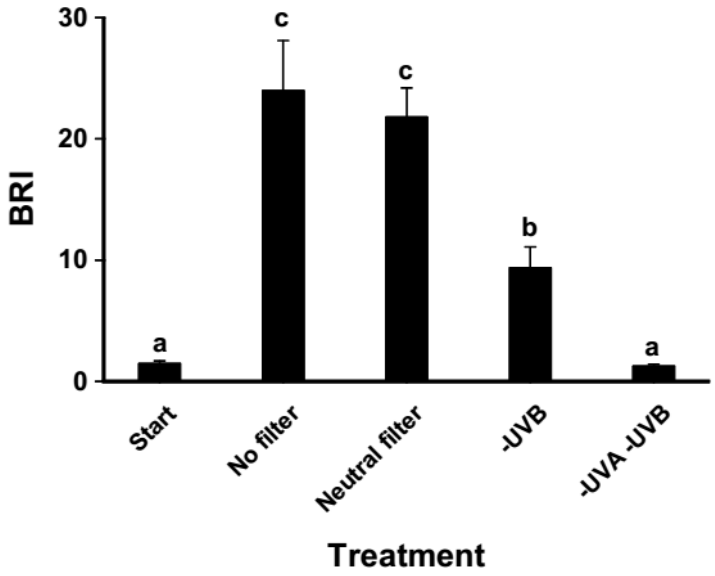


**Fig. 8**: Effect on melanisation of transferring shade-adapted *L. pulmonaria* to an exposed site under no filter, a neutral filter, a filter that removes UV-B and a filter that removes both UV-A and UV-B. Melanisation was measured as the browning reflectance index (BRI, see text for details). Bars (±SE) with different letters are significantly different ($p < 0.05$) according to Duncan's multiple range tests.

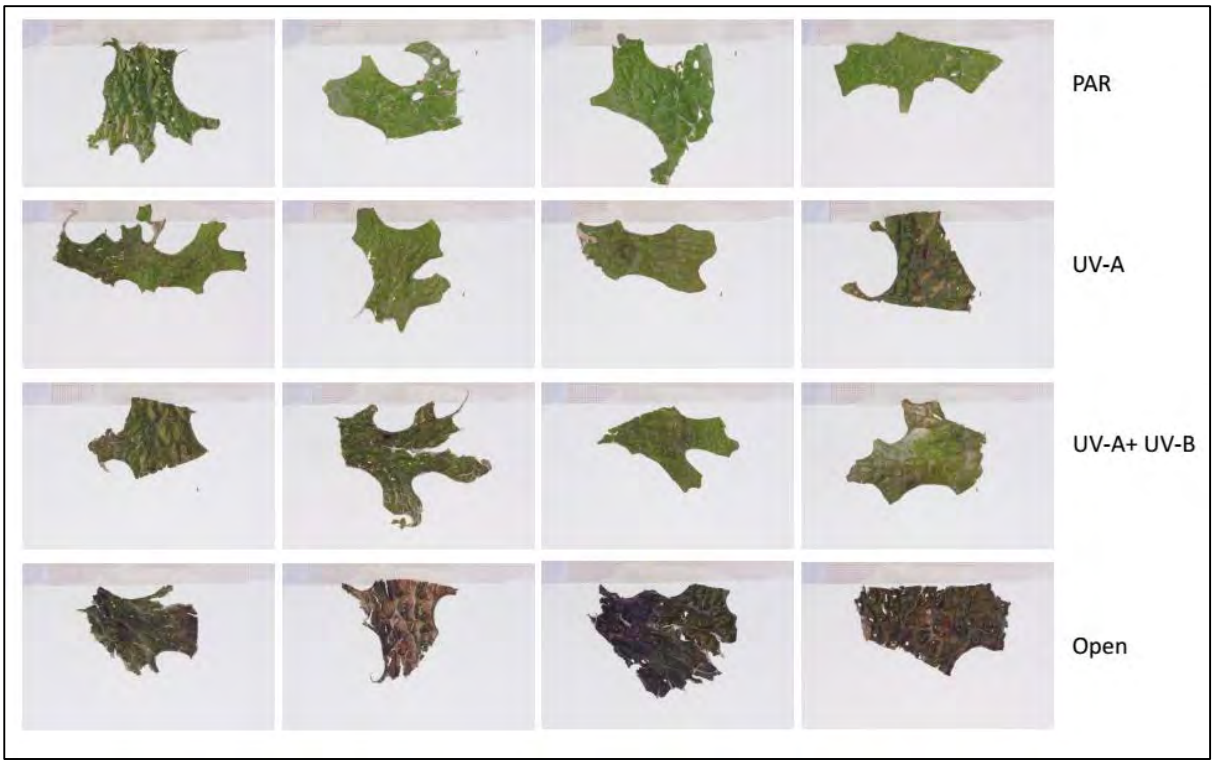


**Fig. 9**: Photograph showing desiccated *L. pulmonaria* thalli after being transferred to an exposed site under no filter, a neutral filter, a filter that removes UV-B and a filter that removes both UV-A and UV-B treatments.

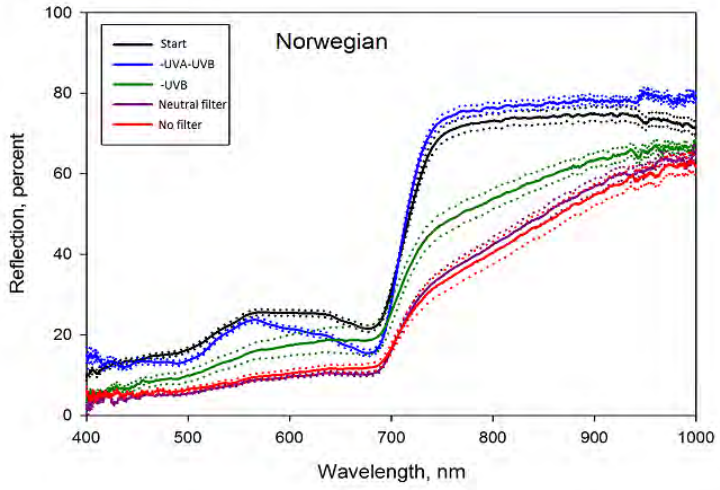


**Fig. 10**: Mean percentage reflectance spectra from the upper surface of *L. pulmonaria* thalli collected from Norway before (start) and after transfer to an exposed site under no filter, a neutral filter, a filter that removes UV-B and a filter, that removes both UV-A and UV-B treatments.

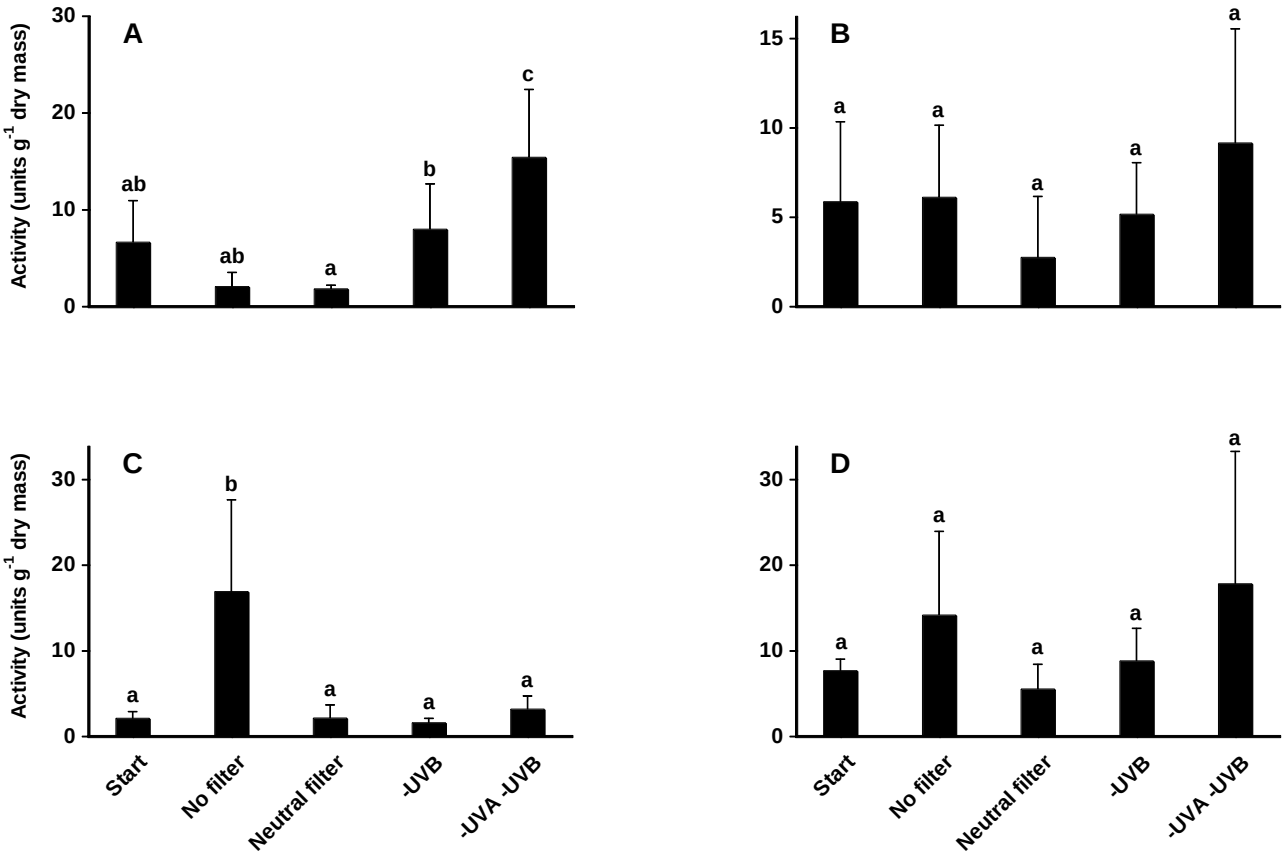


**Fig. 11**: Effect on the activity of redox enzymes of transferring shade-adapted *L. pulmonaria* to an exposed site under no filter, a neutral filter, a filter that removes UV-B and a filter that removes both UV-A and UV-B. Values are mean ± 1 SE, and columns with the same letters on top do not differ significantly ($P < 0.05$). A, tyrosinase activity (active forms); B, stimulation of tyrosinsase activity by SDS (latent forms); C, laccase activity; D, peroxidase activity.

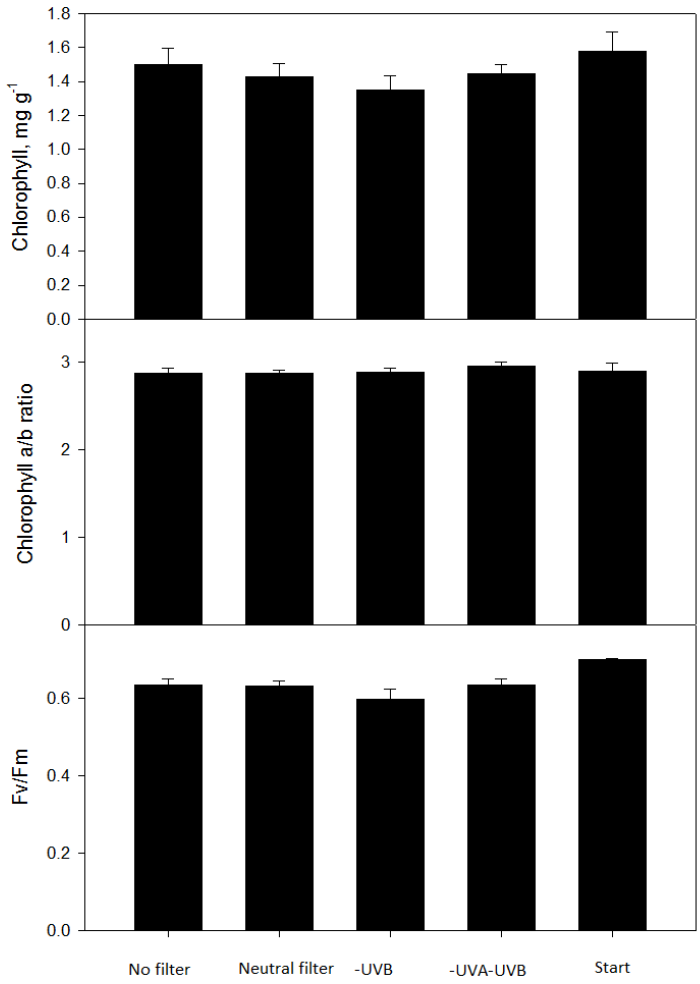


**Fig. 12:** Effects on the A. Chlorophyll content (mg $g^{-1}$), B. chlorophyll a: b ratio, C. maximal photosystem II activity ($F_v/F_m$) of transferring shade-adapted *L. pulmonaria* to an exposed site under no filter, a neutral filter, a filter that removes UV-B and a filter that removes both UV-A and UV-B treatments. Duncan's multiple range tests indicated that there were no significant differences between the treatments for these three parameters.

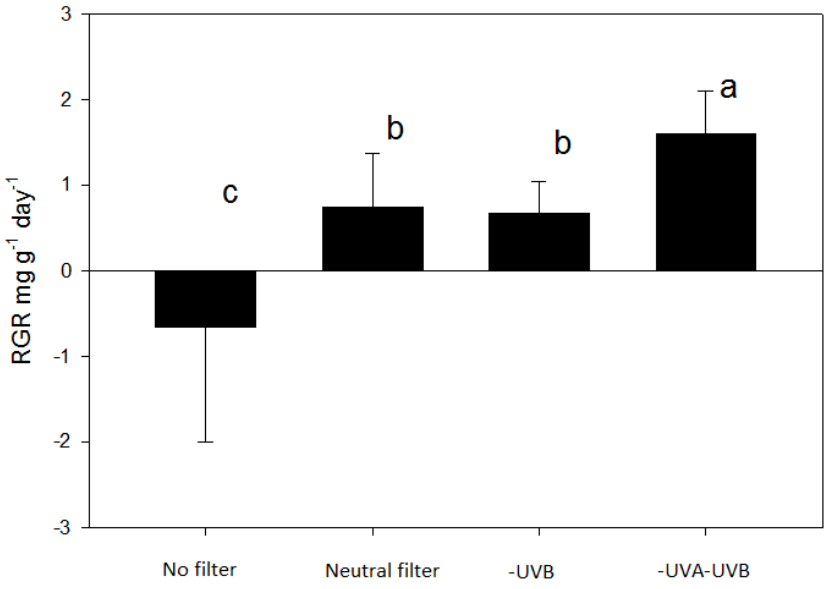


**Fig. 13**: Effects on the relative growth rate of transferring shade-adapted *L. pulmonaria* to an exposed site under no filter, a neutral filter, a filter that removes UV-B and a filter that removes both UV-A and UV-B treatments. Bars (±SE) with different letters are significantly different ($p < 0.05$) according to Duncan's multiple range tests.

## CHAPTER 5: General conclusion

### General conclusion

Discovery of the Antarctic ozone hole in the mid-1980s caused world-wide concern over the increased UV-B radiation reaching the earth's surface (Chipperfield *et al*. 2015; Farman *et al*. 1985). Excessive UV-B radiation is associated with a number of detrimental biological effects such as protein damage, inhibition of photosynthesis, formation of high energy reactive oxygen species (ROS), disruption of nitrogen fixation and induction of cell mutation and inhibition of growth (Vincent & Neale, 2000). The Montreal protocol has been successful in reducing concentrations of ozone-depleting substances in the atmosphere; however in some areas such as the Polar regions ozone holes still occur seasonally as the direct result of atmospheric circulation, which may indicate a more broader linkage to climate change (McKenzie *et al*. 2007). Stratospheric ozone is still much lower than in the 1950's and recovery to this state is not expected for several decades. Furthermore a decrease in the stratospheric ozone may still occur as a result of stratospheric cooling caused by an increase in atmospheric $CO_2$ levels (Krapivin *et al*. 2012). Due to interactions between climate change and ozone depletion, UV-B irradiance may continue to increase. The interaction between stratospheric ozone depletion and climate change may increase solar ultraviolet (UV) radiation reaching the earth's surface, particularly in in high-latitude regions, and this has led to an increase in publications on ecosystem consequences of enhanced UV-B radiation. However a large number of these studies focus on higher plants and bryophytes (Caldwell *et al*. 1998). This raises concern as there are fewer studies that outline the effects of increased UV on lichens as majority of lichens inhabit areas of higher latitude and altitude.

Lichens are symbiotic associations between photobionts and mycobionts, these being fungi, algae and/or cyanobacterium (Beckett *et al*. 2013; Russo *et al*. 2008). The mycobiont (fungi) and photobiont (algae/cyanobacterium) occur together in an integrated thallus; the photobiont is found extracellular to the fungi (Smith, 1979). Lichens are the earliest colonizers of land on planet

earth, and may have colonised the terrestrial biome about 600 million years ago (Beckett et al. 2013; Björn, 2007). Melanins are an example of cortical phenolic compounds in lichens that screen UV radiation; they have a wide distribution, occurring in almost all biological kingdoms (Eisenman & Casadevall, 2012). These phenolic compounds have been demonstrated to be present in lichens where they serve as protection against high light and temperature stress (Stepanenko *et al*. 2002). Lichens such as *Cladonia rangiferina* (Nybakken & Julkunen-Tiitto, 2006), *L. pulmonaria* (Solhaug *et al*., 2003) and *Umbilicaria rossica* (now called *Lasallia rossica* (*Dombr.*) (Stepanenko *et al*. 2002) synthesize melanins in response to elevated levels of UV-B radiation. Synthesis of melanins is generally catalyzed by enzymes that are involved in phenol coupling (Williamson *et al*. 1998). These enzymes include P450 monooxygenases, ascorbate oxidases, peroxidases, laccases and tyrosinases (Nezbedová *et al*. 2001). Previous work has shown that lichens from the order Peltigerales have high redox enzyme activity which include tyrosinases, laccases and peroxidases (Beckett *et al*. 2003; Beckett *et al*. 2013). Furthermore, the Peltigeralean lichen *L. pulmonaria* forms melanin in the outer layer of its cortex depending on how much solar radiation it receives (Gauslaa & Solhaug, 2001). In the work presented here, we determined that Peltogeralean lichens synthesize eumelanins or L-DOPA melanins. DOPA melanins are synthesized by the phenoloxidases laccase and tyrosinase. Tyrosinase catalyzes the hydroxylation of L-tyrosine to L-DOPA, which in turn is converted to dopaquinone. Alternatively, laccase catalyzes the one-step oxidation of L-DOPA to dopaquinone. The subsequent steps occur spontaneously, producing dihydroxyindole or dihydroxyndole-2-carboxylic acid intermediates that polymerize into melanin (Eisenman *et al*. 2007; Land & Riley, 2000; Williamson, 1997). Although currently there is no general agreement on the exact role of peroxidases in this pathway, a number of authors have suggested that this enzyme is involved in melanin synthesis (Mastore *et al*. 2005; Takahama, 2004) and the work we presented here suggests that due to the higher catalytic efficiency of peroxidase towards DOPA compared to tyrosinase shown by this enzyme in *L. pulmonaria* could

suggest that peroxidase plays a critical role in later steps of eumelanin biosynthesis, while tyrosinase is the enzyme responsible for initiating it.

DHN melanin is generated via the polymerization of a double-ringed structure known as a 1, 8-dihydroxynaphthalene (DHN) (Butler *et al*. 2001). The first product produced is 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) which is synthesized from either Acetyl-CoA or malonyl CoA by a polyketide synthase (PKS) (Eisenman & Casadevall, 2012). This is followed by successive reduction and dehydration reactions, which produce scytalone, 1,3,6,8-tetrahydroxynaphthalene, vermelone and finally the polymerization of DHN to melanin, which is catalyzed by a laccase (Bell & Wheeler, 1986; Bloomfield & Alexander, 1967; Butler & Day, 1998; Langfelder *et al*. 1998; Langfelder *et al*. 2003).

The main aims of the work presented here were to study the potential effects of climate change on the health of lichens, to understand the enzymatic processes involved the synthesis of UV induced melanins by studying the role of redox enzymes in the synthesis of melanin produced in response to high light and UV stress in the lichens *L. pulmonaria* and *P. aurata*. These lichens are shade adapted (Gauslaa & Solhaug, 2001; Lange *et al*. 2004), cephalo-lichens, consisting of green algal photobionts and nitrogen fixing cyanobacteria belonging to the genus *Nostoc* (Jordan, 1973; Lange *et al*. 2004). The work initially involved characterizing enzymes reported to be involved in melanin biosynthesis. The basic properties of these enzymes, such as response to desiccation and rehydration, pH optima and thermostability, and substrate specificity were characterized. The activities of these enzymes in lichens exposed to various combinations of PAR and UV were then studied, and at the same time melanin synthesis, growth, and photosynthesis were measured. The overall aim was to test how lichens may respond to changes in visible light and UV radiation following climate change. An understanding of the aspects of lichen response to UV stress, how UV-B radiation may influence lichens and the effect of UV-B on melanin synthesizing enzymes may provide insight on the biology melanin biosynthesis in lichens and

possibly give important knowledge that can prove useful in predicting the impact of climate change on lichen communities.

**Characterization of redox enzymatic activities**

Results presented in chapter three clearly show that Peltigeralean species *P. aurata* and *L. pulmonaria* produce redox enzymes that are of similar nature to those of their free-living symbionts and other lichens. Laccases and peroxidases from *L. pulmonaria*, like the corresponding enzymes from other lichens (Beckett *et al*. 2013), probably can produce ROS, which may play a role in facultative saprophytic activities that are beneficial during periods of limited photobiont photosynthesis as suggested by results obtained from rehydrating lichen thalli in the dark (Fig. 4). "Starvation", induced by long-term storage in the dark, strongly induced their activity. Tyrosinase from *L. pulmonaria* oxidized monophenolic and diphenolic compounds, and was able to metabolize L-DOPA, thus showing characteristics of a classical fungal tyrosinase. L-DOPA can also be metabolized by peroxidases and laccase suggesting that although tyrosinase could be the key enzyme involved in the melanin biosynthetic pathway of *L. pulmonaria*, peroxidases and laccases may participate in melanization reactions. Results from kinetics of DOPA metabolism by extracts from both *L. pulmonaria* and *P. aurata* showed that peroxidases oxidize L-DOPA more efficiently than tyrosinases (Fig. 7), suggesting that peroxidases may have a much more significant role in melanin biosynthesis than previously considered.

**Effects of UV on lichens**

In later work, growth chamber and field experiments were used to evaluate the effect of UV radiation on Peltigeralean lichens and to elucidate the role of redox enzymes in melanin biosynthesis. Lichen thalli were exposed to various combinations of UV and PAR, melanins were quantified, and redox enzyme activity measured. To determine whether there is a trade-off between radiation screening and photosynthetic light use efficiency in melanic lichens, we investigated the how UV and melanin synthesis affected the functioning of the photosynthetic

apparatus by measuring chlorophyll fluorescence and photosynthetic pigments. Relative growth rates were also recorded.

The growth chamber experiment showed the limitations of these kinds of investigations, and the difficulty of extrapolating results to field situations. We were unsuccessful in inducing melanins in lichen thalli probably because in chambers the ratio light of different wavelengths did not resemble those of a natural environment. Typically, chambers deliver unrealistically high ratios of UV-B to PAR (400-700 nm) (Caldwell *et al*. 1998; Paul & Gwynn-Jones, 2003). Our growth chamber experiment had the same limitation as low levels of UV-A and PAR were delivered in combination with relatively high UV-B levels. Low levels of UV-A and PAR limit photorepair of UV-B induced DNA damage (Caldwell & Flint, 1994). Data obtained from our growth chamber experiment was not included in this thesis as no useful information was obtained. Only results from field data are presented here.

In agreement with data in the literature, results from our field experiment using *L. pulmonaria* as a test species showed that melanin synthesis can be induced in 3 wk by transplanting material to an open area without any filter, or by placing it under a wavelength neutral filter. The induced melanins were synthesized from L-DOPA rather than naphthalene derived molecules as indicated by the low C: N ratio (10:1) of the melanins in comparison to the 100:1 C: N ratio that is usually found in naphthalene-derived molecule synthesized melanins. The signal for melanin synthesis was UV, as filtering both UV-B and UV-A prevented melanisation (Fig. 8).

There was no significant difference between treatments in $F_v/F_m$ and chlorophyll contents were not affected by radiation treatment, indicating that no deterioration of photosynthetic apparatus occurred due to a specific radiation treatment and the photobionts remained relatively healthy (Fig. 12). Lichens in the open treatment exhibited a negative growth rate while the highest growth rate occurred in thalli exposed only to PAR. While it is tempting to suggest that a trade-off between radiation screening and photosynthetic light use efficiency in melanic lichens might exist

based on these findings, this seems rather unlikely as similar relative growth rates occurred in lichens under the wavelength neutral filter and the lichen thalli that showed very little melanization under the filter that removes UV-B.

Melanized lichen thalli did not show any increased tyrosinase activity, suggesting that it may not be responsible for melanization (Fig. 12A). Laccase activity was upregulated in thalli receiving unfiltered light, while no changes in lichen in enzyme activity occurred in lichen thalli placed under the wavelength neutral filter (Fig 12C) suggesting that laccases may be involved in melanin biosynthesis under certain conditions. Despite the widespread occurrence of tyrosinases in lichen, and their comparable nature to those of free-living fungi where they have been suggested to have a key role in melanin biosynthesis, their roles in lichen biology remain largely unknown.

**Further studies**

Work is currently underway to purify and sequence lichen tyrosinases so they can be compared to those from free-living fungi. The absence of a simple correlation between tyrosinase activity and melanisation reported here suggests that further studies need to be done to determine whether melanisation is controlled by the levels of melanin precursors such as tyrosine. It may be useful to carry out further studies to investigate understand the mechanism(s) responsible for receptor activation by UV radiation in lichens. Furthermore, the role of other redox enzymes such as peroxidases and laccases in the biosynthesis of melanins need to be investigated. Future work should also investigate additional roles of tyrosinases. For instance infection of the mushroom *Agaricus bisporus* by the pathogenic bacteria *Pseudomonas tolaasii* causes discolouration of the cap which is accompanied by an activation of fungal tyrosinase activity (Soler-Rivas *et al*. 2000). Another additional role for tyrosinases may be to synthesize quinones needed for radical generation of extracellular redox cycling that has been reported to occur in the lichen *Usnea* (Beckett *et al.* 2015). A possible role of tyrosinase in detoxification of soil phenolic compounds also needs to be further investigated.

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