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Effects of increased UV-B radiation on the lichen *Cladonia arbuscula* spp. *mitis*: UV-absorbing pigments and DNA damage

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**EFFECTS OF INCREASED UV-B RADIATION ON THE
LICHEN *Cladonia arbuscula* ssp. *mitis*: UV-ABSORBING
PIGMENTS AND DNA DAMAGE**

Roberta Buffoni Hall

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LIST OF PAPERS

The following papers are included in the thesis. They are referred to by their Roman numeral.

- I** Rozema, J., Björn, L. O., Bornman, J. F., Gaberscik, A., Häder, D.-P., Trost, T., Germ, M., Klisch, M., Gröniger, A., Sinha, R. P., Lebert, M., He, Y.-Y., Buffoni Hall, R. S., de Bakker, N. V. J., van de Staaij, J., Meijkamp, B. B. 2002. The role of UV-B radiation in aquatic and terrestrial ecosystems – an experimental and functional analysis of the evolution of UV-absorbing compounds. *J. Photochem. Photobiol. B: Biol.* 66: 2-12
- II** Buffoni Hall, R. S., Bornman, J. F., Björn, L. O., 2002. UV-induced changes in pigment content and light penetration in the fruticose lichen *Cladonia arbuscula* ssp. *mitis*. *J. Photochem. Photobiol. B: Biol.* 66: 13-20
- III** Buffoni Hall, R. S., Paulsson, M., Duncan, K., Tobin, A. K., Widell, S., Bornman, J. F., 2002. Water- and temperature-dependence of DNA damage and repair in the fruticose lichen *Cladonia arbuscula* sp. *Mitis* exposed to UV-B radiation. *Physiol. Plant.* (submitted)

ABBREVIATIONS

CFCs	chlorofluorocarbons
CPDs	cyclobutane pyrimidine dimers
DNA	deoxyribonucleic acid
HL	high light
MAAs	mycosporine-like amino acids
PAR	photosynthetically active radiation (400-700 nm)
PSII	photosystem II
UV-A	ultraviolet-A radiation (315-400 nm)
UV-B	ultraviolet-B radiation (280-315 nm)
UV-B _{BE}	biologically effective ultraviolet-B radiation
UV-C	ultraviolet-C radiation (100-280 nm)

PREFACE

Taci. Su le soglie
del bosco non odo
parole che dici
umane; ma odo
parole piu' nuove
che parlano gocciole e foglie
lontane.

Ascolta. Piove
dalle nuvole sparse.
Piove su le tamerici
salmastre ed arse,
piove su i pini
scagliosi ed irti,
piove su i mirti
divini, su le ginestre fulgenti
di fiori accolti,
su i ginepri folti
di coccole aulenti,
piove su i nostri volti
silvani,
piove su le nostre mani
ignude,
su i nostri vestimenti
leggieri,
su i freschi pensieri
che l' anima schiude
novella,
su la favola bella
che ieri
t' illuse, che oggi m' illude,
o Ermione.

From "La pioggia nel pineto", in "I libri delle Laudi: Alcyone" by
Gabriele D' Annunzio.

Wherever we cast our eyes we can find signs of life. We can perceive the slow flowing and humming of life in all the innumerable beings that surround us. However, paradoxically, we tend to forget that we too are part of nature.

Human beings, throughout their evolutionary history, have changed from being integral components of ecosystems into controllers of ecosystems. The development of agriculture, at the expense of natural forests, gave rise to a long history of ever-increasing exploitation of resources and marked the beginning of the modification of the earth's surface and atmosphere. Later, the advent of the Industrial Revolution brought about, together with food security and wealth, new agents of environmental change.

Stratospheric ozone depletion, accumulation of greenhouse gases, acid pollution, deforestation accompanied by soil erosion and loss of biodiversity are just some of the many environmental changes which present and future generations have and will have to deal with should *Homo sapiens* continue the heavy exploitation of the environment.

During my life, ever since I was a child, the experience of climbing up the mountains overlooking the blue immensity of the Mediterranean sea, of listening to the chant of cicadas hidden in the deep green of the pine trees in the hot, still air of a summer afternoon, and running after fireflies in the evenings in the poppy-rich meadows behind my grandmother's house – all this has given me an instinctive joy of living. Now, being an adult and a natural scientist, this joy has only grown and has become love and care for our wonderful Planet Earth.

1. INTRODUCTION

Solar UV radiation travels virtually unaltered from the sun to the earth's atmosphere. Once in this atmosphere, absorption and scattering by various gases and particles modify the radiation profoundly, removing much of the wavelength region most harmful to organisms. The earth's atmosphere is composed mainly of two gases: nitrogen and oxygen (78 and 21% respectively). The existence of free oxygen is of particular importance for the formation of the ozone molecule (O₃). Ozone is the only gas in the atmosphere that effectively absorbs part of the UV-B radiation (280-315 nm). Therefore, a decrease in ozone concentration is most likely to cause an increase in UV-B radiation at the earth's surface, especially of wavelengths shorter than 290 nm.

Deleterious and other effects of UV-B radiation on human health, animals, plants, microorganisms, materials and air quality have been well documented (UNEP 1998). In plants, direct absorption of UV-B radiation may cause damage to cellular components such as DNA, lipids and proteins. Further damaging effects of UV-B may be mediated by the generation of reactive oxygen species. However, not all the effects of UV-B on plants involve damage. A wide range of plant responses have been described as protective measures that ameliorate or prevent UV-B stress. In particular, the synthesis of UV-absorbing protective compounds, the induction of DNA repair enzymes such as DNA photolyases, and various morphological alterations (e. g. increases in leaf thickness, reduced leaf area, etc) are believed to be protective mechanisms.

In many polar and subpolar ecosystems, where the largest relative decreases in ozone concentration are occurring, lichens are the dominant autotrophs. Lichens are symbiotic organisms composed of a fungal partner, called a "mycobiont", and of one or more photosynthetic partners ("photobiont") that may be either a green alga or a cyanobacterium. Although the nature of the symbiotic relationship between the two bionts is still under debate, it is recognised that the photobiont provides nutrients in the form of carbohydrates to the heterotrophic mycobiont,

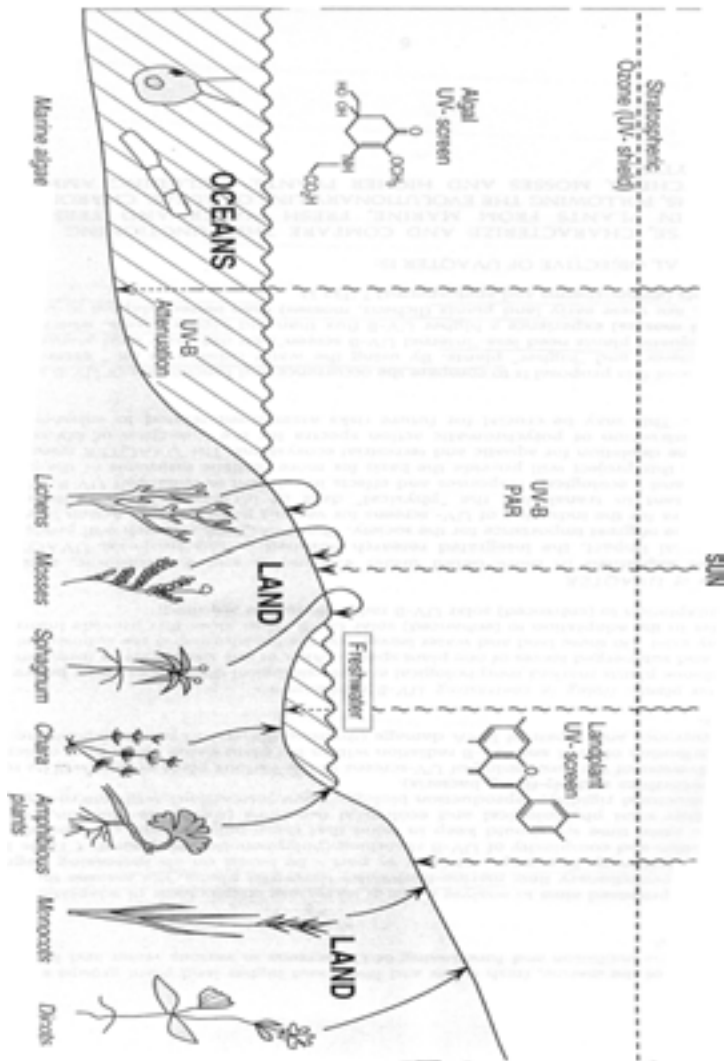
whereas the latter may constitute a physical shield protecting the photobiont from potentially harmful environmental stresses. Lichens constitute an ancient group of organisms that evolved early in times when the stratospheric ozone was still under formation. Thus, it is likely that they have experienced higher UV-B fluxes than at present. In order to survive, lichens must have developed adaptive and protective mechanisms, the study of which can give us clues as to how they coped with this form of stress and the extent of the biological impact that future increases of UV-B radiation might have on these organisms.

2. AIMS AND SCOPE OF THE PROJECT

This thesis is an integral component of a multinational project called UVAQTER, funded by the European Commission: Environment and Climate Research section. The general objective of the UVAQTER project was to evaluate the effectiveness of UV-B absorbing compounds along the evolutionary line: marine-freshwater-terrestrial plants (Fig. 1). The present thesis includes experiments on the accumulation of UV-absorbing compounds under various conditions, distribution of radiation within the lichen tissue using optical fibres, and the occurrence and significance of DNA damage.

Paper I provides a survey of the induction and functioning of UV-screening pigments in lower and higher plants. As a part of the main project, this thesis focusses on the effects of enhanced UV-B radiation on lichens, one of the groups of organisms believed to have been early colonisers of terrestrial habitats. The possibility of having experienced higher UV-B fluxes than at present in early times of plant life history, leads to the question whether these organisms are better adapted to UV-B than higher plants. To respond to this question, changes in UV-absorbing pigment content have been related to changes in light penetration in thalli of lichens exposed to enhanced UV-B radiation (paper II). In paper III the influence of enhanced UV-B radiation in combination with water and temperature stress on the incidence and repair of DNA damage is described.

Fig. 1. Marine, fresh water and lower and higher plant groups studied in the UVAQTER project.



3. LICHENS

3.1 General background

Lichens represent a very successful symbiosis between two partners, namely a fungal partner, the mycobiont, and a photosynthetic partner, the photobiont, which may be either a green alga or a cyanobacteria or both. The lichen symbiosis has often been interpreted as an example of controlled parasitism where the mycobiont, constituting the dominant part of the lichen thallus, acquires its carbon nutrition from the photosynthetic activity of the photobiont (Richardson, 1993). The diversion of photosynthate to the mycobiont poses a limitation to the growth rate of the photobiont, which is usually lower than that achieved in a free-living state (Raven, 1993). However, the nature of the lichen symbiosis could also be seen as that of a mutualistic relationship if one considers that the fungal partner provides the necessary physical matrix for the photobiont (Rikkinen, 1995). Without the protection afforded by the fungus, the photobiont could probably not survive in many of the harsh environments in which lichens are found (Hawksworth, 1994). The fungus, for example, contributes to the modification of the quality and the quantity of the light that reaches the photobiont layer. This undoubtedly represents a benefit for the photobiont, which is sensitive to high irradiances (Demmig-Adams et al. 1990).

Lichens are traditionally divided into three main morphological groups: the crustose, foliose and fruticose types. Crustose lichens are tightly attached to the substrate and cannot easily be removed from it without destruction. Foliose lichens are leaf-like, flat and only partially attached to the substrate. Fruticose lichens are hair-like, strap-shaped or shrubby and their thallus lobes may be flat or cylindrical. A more detailed description of the fruticose lichens will follow, since *Cladonia arbuscula* ssp. *mitis* - the material used for this thesis - belongs to this morphological group. The majority of fruticose lichens possess a radially symmetric thallus as opposed to foliose lichens in which a dorsiventral organisation is typically recognisable. The genus *Cladonia* develops a fruticose thallus verticalis that originates from primordia of the fruit body and results in

apothecia-bearing stalks termed podetia. Highly branched fruticose lichens have a high surface to volume ratio that results in a more rapid drying and wetting pattern compared to foliose lichens (Büdel et al. 1996). The majority of lichens develop internally stratified thalli in which the main subdivisions are into upper cortex, photobiont layer, medulla and lower cortex. The upper cortex is formed by fungal hyphae that are highly conglutinated to the extent that single hyphae are not usually distinguishable. Photobiont cells are generally excluded from this layer but occur just underneath the cortex in the so-called photobiont layer. In *Cladonia arbuscula* the upper cortex is not present and the photobiont layer immediately reaches the surface of the lichen thallus (Carlin, 1981). The photobiont layer is usually localised in the upper part of the medulla, which is the structure that occupies the major part of the internal thalline volume and is composed of loosely interwoven hyphae. This structural organisation allows the formation of internal cavities filled with air and is thus important for gas exchange (Büdel et al. 1996). The lichen cortex protects the photobiont layer from physical abrasion and from pathogenic organisms. In addition, cortical hyphae may be embedded in an extracellular gelatinous matrix composed of amorphous pigments and/or crystallised substances that may have a role in shielding the inner layers of the lichen from excessive radiation (Rikkinen, 1995). Moreover, due to their hydrophilic nature, these substances help in protecting the lichen against rapid desiccation. Hyphal cell walls of the medullary layers are often encrusted with crystallised secondary products, the nature of which is hydrophobic. Their possible role may be to prevent water loss from the medullary layer and to maintain gas exchange by preserving air cavities within the medulla (Greenhalgh et al. 1987).

3.2 Desiccation tolerance

Unlike vascular plants, that have developed the capacity to maintain their water content at a fairly constant level, lichens are poikilohydric organisms whose water status varies according to the surrounding environmental conditions. Lichens can use precipitation, fog, dew and high relative humidity as sources of water. The capacity of lichens to absorb wa-

ter from non-saturated atmosphere implies an inner water potential lower than that of the atmosphere. Accumulation of high concentrations of polyols seems to be the mechanism by which lichens can achieve such low water potentials (Farrar et al. 1976). The presence of high amounts of polyols is thought to have a role also in cold tolerance, since these substances reduce the likelihood of ice crystal formation (Nash III, 1996). Lichens typically undergo a diurnal cycling between the desiccated and hydrated state. In the air-dry state the photobiont cells lose their turgor, decrease in size and collapse (Büdel et al. 1991). No gas exchange occurs in this state and the dissociation of the light harvesting complexes from PSII has been observed (Lange et al. 1989). Loss of cell membrane integrity has been reported (Brown et al. 1990). Upon rehydration, lichens resume their normal metabolic functions with the re-establishment of membrane integrity, re-coupling of light harvesting chlorophyll complexes with PSII (Bilger et al. 1989), re-acquisition of green algal cell turgidity and an increase in photosynthetic activity. In a study with mosses, it was found that membrane disruption does not occur during drying but during subsequent rehydration (Platt et al. 1994). Oliver (1996) suggested that the ability of plants to tolerate desiccation resides more in their ability to repair the damage upon rehydration than in their capacity to prevent its occurrence during drying. Due to the cessation of metabolic activity in conditions of prolonged periods of desiccation, lichens in the air-dry state have been considered fairly resistant to both high light (Demmig-Adams et al. 1990) and to extreme temperatures (Kappen, 1988; Larson, 1983). However, recently their apparent hardiness in the desiccated state has been questioned. Evidence that lichens in the desiccated state might be susceptible to damage from high light has been reported (Kershaw et al. 1980; Gauslaa et al. 1999). In paper III, evidence that air-dry lichens are prone to UV-induced DNA damage will be presented.

4. STRATOSPHERIC OZONE

4.1 Role and destruction of the ozone layer

Ozone molecules, made up of three atoms of oxygen, comprise a thin layer of the atmosphere that protects life on earth from the harmful effects of the solar ultraviolet radiation. However, in primeval times, the earth's atmosphere lacked an ozone layer, since the atmosphere was composed mainly of nitrogen and carbon dioxide. Therefore, in such an environment where much higher UV-B fluxes than at present reached ground level, life was probably restricted to below water. The formation of an ozone layer is believed to have been of fundamental importance in allowing evolution of terrestrial forms of life (Caldwell, 1979).

Ozone molecules, found in a region of the stratosphere between 10 and 30 km above the earth's surface, completely absorb UV-C radiation (100-280 nm) and partially absorb UV-B radiation (280-315 nm). Therefore, any potential decrease in stratospheric ozone concentration is of great concern, as it would lead to an increase in the short, energy-rich wavelengths in the UV-B range. Since the first observation of a drastic reduction in total ozone over Halley Bay in Antarctica in 1985 (Farman et al. 1985), the yearly appearance of an ozone hole over the Antarctic and large decreases of ozone concentration at high latitudes in the northern hemisphere have become well-established (European Research in the Stratosphere 1996-2000). Apart from the natural production and breakdown of ozone in the stratosphere, man-made destruction derives from chemical processes that involve chlorine- and bromine-containing substances from anthropogenic activity. These substances are loaded into the troposphere but, due to the circulation dynamics of the atmosphere, transported to higher altitudes and toward polar regions. Here they are photolysed by UV radiation and the resulting products are highly reactive molecules that react with ozone in a catalytic cycle breaking down ozone into oxygen and regenerating themselves.

4.2 Recovery of the ozone layer

In 1987 the Montreal Protocol, the first international agreement on an environmental issue, aimed at a 50% cut-back in the production of chlorofluorocarbons (CFCs) for the protection of the ozone layer. Subsequently, the persistence and occurrence of further ozone decreases over Antarctica led to the realisation that the measures that had been taken were inadequate and further steps were required. Subsequent amendments have imposed stricter measures and phase-out deadlines also for developing countries. The concentration of CFCs is decreasing (Prinn, 2000) but the continual concern associated with these substances is related to their long lifetime, which for some compounds may extend up to 110 years. In addition, although financial aid has been promised to developing countries for producing alternatives to CFCs, the production of these substances is still increasing in these countries.

Apart from CFCs, increases in the amounts of carbon dioxide, methane, nitrous oxide and other greenhouse gases, especially in the last two decades, have led to a warming of the troposphere and a cooling of the stratosphere, conditions favouring ozone breakdown and which are likely to contribute to the delay in the recovery of the ozone layer.

According to the latest fully-coupled chemistry-climate models, the disappearance of the Antarctic ozone hole should occur in the second half of the 21st century; considerably later than was predicted a few years ago (European Research in the Stratosphere 1996-2000).

5. UV-B RADIATION

5.1 UV-B radiation effects on plants and lichens

UV-B radiation induces a wide range of direct and indirect effects on plants. Many macromolecules are potential targets for direct UV-B absorption, in particular DNA, membrane lipids and proteins (Jansen et al. 1998). Pyrimidine dimer formation (paper III), peroxidation of unsatu-

rated membrane lipids (Kramer et al. 1991), increased free radical production (Hideg and Vass, 1996; Szilágyi et al. 2002), damage to photosystem II (PSII) (Teramura et al. 1994), disruption of thylakoid membranes (Teramura et al. 1994) are some of the direct effects of UV-B radiation at the molecular and cellular levels. Other effects at a physiological and morphological level are the decrease in the efficiency of photosynthesis (Bornman, 1989; Strid et al. 1990), the accumulation of pigments such as flavonoids in higher plants (paper I; Teramura et al. 1994), other phenolic compounds in lichens (paper II) and increases in leaf thickness (Cen et al. 1993).

Many of the effects induced by UV-B radiation allow for at least a duality of interpretations. The difference between damage, repair and acclimation can be subtle and it is not always possible to identify one particular mechanism as the explanation underlying a given phenomenon. For example, the UV-B induced degradation of the D1 protein of PSII can be seen either as a damage or as a part of a repair mechanism leading to the substitution of the damaged components of PSII (Jansen et al. 1998).

In this thesis, one of the potentially significant effects of an enhanced level of UV-B radiation, namely pyrimidine dimer formation in DNA, will be discussed (paper III). Among the protective mechanisms against UV-B radiation, attention will be focused on repair of DNA damage (paper III) and on the accumulation and distribution of UV-absorbing compounds (paper I and II).

5.2 Limitations of growth chamber studies

Most of the data on plant response to UV-B radiation have been obtained from experiments conducted in laboratories or greenhouses (Caldwell et al. 1994). The conditions in these latter facilities rarely approach those in a natural environment and often the magnitude of the effects detected in indoor studies is much greater than that obtained from field studies (Searles et al. 2001).

Artificial conditions differ from natural conditions in many respects: the spectral composition of the light used is different from the natural sunlight, unrealistically high UV-B/PAR (400-700 nm) ratios are often applied, and other environmental stresses such as water deprivation, temperature, etc., are usually absent (Caldwell et al. 1994). To maintain a realistic balance between UV-A (315-400 nm), visible light and UV-B applied in indoor experiments is very important, although usually technically difficult. In many indoor studies, too low visible light and /or UV-A radiation, as compared to that of sunlight, might lead to an overestimation of the effects of UV-B radiation due to the fact that both visible light and UV-A play a role in inducing protective mechanisms in plants (Takeuchi et al. 1996; Warner et al. 1983).

Plants in nature are seldom affected by only a single stress factor such as UV-B radiation. Instead, plants are subjected to a combination of environmental stresses and their overall response to them can be very different from that induced by a single stress. For example, the effectiveness of UV-B radiation can be ameliorated (Teramura et al. 1990) or in some cases aggravated (Dube' et al. 1992) depending on the plant species and on the nature of the stress factor interacting with UV-B radiation (Bornman and Teramura, 1993; Caldwell et al. 1998). However, experiments in controlled environments are often necessary, providing valuable information on specific targets and mechanisms during relatively short time periods. They are also useful as predictive indicators for organism response in natural environments, but they should be verified as much as possible under field conditions.

5.3 Experimental exposure to UV-B radiation

There are basically two ways of conducting UV-B experiments in outdoor studies: exclusion of UV-B radiation from sunlight and UV-B supplementation (Caldwell et al. 1994; McLeod 1997).

Outdoor exclusion experiments involve removing a part or all of the UV radiation from natural sunlight by using UV-absorbing filters. The most

common filters available effectively remove the radiation below 290 nm (cellulose diacetate) or below 320 nm (Mylar foils) resulting in the exclusion of UV-C, UV-C + UV-B radiation, respectively. The exclusion method is relatively easier to use than the UV-supplementation method, since it is less expensive and less burdened with problems connected to the use of complex UV-B lamp equipment. Results obtained from such experiments provide indications on the degree of the effects that current UV-B radiation has on plants but they cannot be used directly to draw conclusions about the effects of future ozone depletion. This is due to the fact that, in these studies, controls consist of samples exposed to unfiltered ambient UV-B radiation. The results described in paper I have been obtained from fairly long-term UV-exclusion combined with UV-supplementation experiments in such a way that current UV-B and enhanced UV-B radiation effects on lichens could be studied.

For the UV-supplementation experiment, extra UV-B was added to the naturally occurring solar UV-B. Controls in these studies were comprised either of samples exposed to ambient solar UV-B radiation or samples exposed to lamps where the UV-B component was filtered out. The latter arrangement allows for the assessment of the effects of an increased UV-A radiation above natural levels. This is necessary since the lamps used in enhanced UV-B experiments emit also UV-A radiation although the level is usually considered negligible compared to the high solar UV-A level. The UV-C part of the emitted light is completely removed by using cellulose diacetate filters placed under the lamps. In the long-term outdoor experiment conducted on lichens, described in paper II and in the many experiments contributing to paper I (J. van de Staaij et al. 2002; M. Germ et al. 2002; A. Gaberscik et al. 2002), a stepwise enhancement of daily UV-B was used. In this method UV-B lamps were automatically switched on and off in a time-delayed fashion for a fixed period around noon and the irradiation period was changed at two-week intervals to follow seasonal changes in the natural course of UV-B. The enhancement of UV-B was designed to give an ozone-depletion scenario of between 15% (paper II) and 20% (J. van de Staaij et al. 2002). The disadvantage with this method is that it does not take into account variations in ambient solar UV-B radiation caused by, for example, cloudiness. As a

result, during cloudy periods, the extra UV-B radiation added by the lamps simulates higher ozone depletion than desired.

In the indoor experiments presented in papers II and III, lichens were exposed to relatively high levels of UV-B radiation ($13 \text{ kJ m}^{-2} \text{ day}^{-1} \text{ UV-B}_{\text{BE}}$ and $7.4 \text{ kJ m}^{-2} \text{ day}^{-1} \text{ UV-B}_{\text{BE}}$) and compared to UV-B free controls. This experimental design, although presenting some limitations as discussed in the previous section, is considered a useful way to investigate UV-B radiation targets such as DNA and UV-induced mechanisms such as DNA photorepair and accumulation of UV-absorbing compounds. The results from indoor and outdoor experiments support each other (paper II).

6. RESPONSE TO UV-B RADIATION

6.1 Evolution and function of UV-absorbing compounds

UV-absorbing compounds are widespread and are found in lower and higher plants, including aquatic and terrestrial life forms. One of the many roles of these compounds appears to be the protection of organisms from harmful effects of UV-B radiation by means of their direct absorption of these wavelengths. However, recent evidence suggests that some of the phenolic compounds may contribute to the decrease in active oxygen species by acting as antioxidants (Husain et al. 1987; Foyer et al. 1994; Markham et al. 1998; Olsson et al. 1998; Ryan et al. 2002). Although almost omnipresent, UV-absorbing molecules are chemically very different in the various plant groups, since, for instance, their degree of polymerisation and complexity decreases from higher plants to lower plants to cyanobacteria (paper I).

Cyanobacteria are believed to have been among the first photosynthetic organisms to have populated the earth some time around three billion years ago. These organisms were confined below water, thus partially avoiding the high fluxes of solar UV-C and UV-B radiation that reached the earth's surface more or less unaltered due to the lack of O_2 and O_3 in

the primeval atmosphere (Lowry et al. 1980; Canuto et al. 1982; Caldwell, 1979). However, proximity to visible light in order to carry out photosynthesis was a necessity for these organisms, as it still is. Therefore, they had to face the problem of avoiding the life-threatening UV radiation that accompanied visible light at fluxes much higher than at present. In cyanobacteria some of the simplest and evolutionary oldest screening pigments occur, namely scytonemin and mycosporin-like amino acids (MAAs) (paper I). It is hypothesised that, due to their absorption in the UV-C range in addition to UV-B and UV-A (Klisch et al. 2002), scytonemins may have allowed cyanobacteria to survive and later colonise more exposed shallow-water and terrestrial habitats (paper I). MAAs are very effective absorbers of UV-A in addition to UV-B radiation and they also occur in phytoplankton and macroalgae. Interestingly, indications that MAAs may be located in the chloroplast have led to the suggestion that these molecules were inherited by eukaryotic algae via the endosymbiont hypothesis (Whatley, 1981). It has been demonstrated that MAAs synthesis and accumulation can be induced by exposure to UV-B radiation (Klisch et al. 2002) showing that these compounds constitute an important defence against UV-B radiation in aquatic organisms.

Flavonoids, synapic esters and anthocyanins are among the main UV-absorbing pigments found in many plants. The role of flavonoids as compounds protecting plants against UV-B radiation has been well demonstrated, especially in studies where mutants lacking flavonoids have been compared to their corresponding wild type (Li et al. 1993; Lois et al. 1994; Reuber et al. 1996). In many species, flavonoid synthesis is stimulated by exposure to UV-B radiation (Tevini et al. 1981; van de Staaij et al. 1995; Olsson et al. 1998), which seems to act at the gene level by increasing the expression of the enzymes of the phenylpropanoid pathway (Hahlbrock et al. 1989; Kubasek et al. 1992) such as *Chs*, which encodes the enzyme chalcone synthase, and phenylalanine ammonia lyase (PAL). UV-B induced flavonoids are localised mainly in the upper epidermis of leaves and by absorbing the incident UV-B radiation they limit its penetration within the tissue (Olsson et al. 1999). In addition, certain of these compounds appear to protect the plant by scavenging free radicals resulting from oxidative stress caused by UV-B radiation

(Hideg et al. 1996). In many plant species exposed to UV-B radiation (paper I and Meijkamp et al. 2001; Reuber et al. 1996; Olsson et al. 1999), flavonoids with

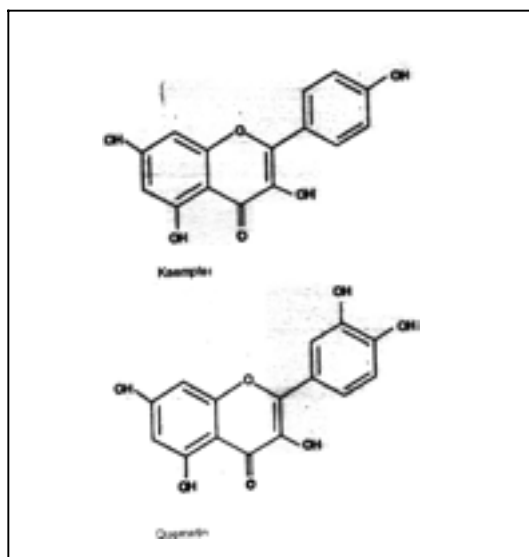


Fig. 2. Molecular structure of kaempferol (above) and quercetin (below) found in some terrestrial and aquatic higher plants.

additional hydroxyl groups on the B-ring, e.g. of quercetin (Fig. 2), have been shown to accumulate more than those compounds lacking the extra groups, e.g. kaempferol. The additional hydroxyl groups on the flavonoid skeleton increase the antioxidant capacity of the compounds and may be part of the explanation for their selective induction upon exposure to UV-B radiation (Larson, 1988). Recent studies further confirm this (Ryan et al. 2002).

Since lichens are a symbiosis between a fungal and a green algal or cyanobacterial component, it would be reasonable to suppose that lichens would contain scytonemins and/or MAAs. A variety of organisms have been found to contain MAAs including lichens (Karentz et al. 1991;

Büdel et al. 1997), whereas in the lichen species so far screened, scytonemins appear to be present in those containing cyanobacterial photobionts (Büdel et al. 1997). Lichens lack flavonoids, although they produce a unique range of secondary metabolites with aromatic structures. Secondary metabolites in lower organisms have been seen as relics of an early biochemical evolution (Zähner et al. 1983). It has been suggested that terrestrialsation of life on earth could only have been possible after the development of a symbiotic relationship between semi-aquatic green algae and fungi (Wright, 1990). In this view, some of the earliest fungal organisms on land might have been, in fact, lichens (Hawksworth 1994). The capacity to tolerate desiccation and the protection afforded by UV-absorbing secondary metabolites may have helped lichens to live successfully in terrestrial habitats (Green et al. 1994).

Table I. Total phenolic content in ‘tips’ and ‘stems’ of *Cladonia arbuscula* ssp. *mitis* measured (as described in Material and Methods: paper II) after 1-week irradiation with high light (HL) with or without additional UV-B radiation (HL+UV-B). Controls were from the greenhouse. % increase = the percentage increase from the control value. Data are means of 3 independent experiments \pm SEM. Values followed by different letters were significantly different at $P < 0.05$.

Treatment	Tips		Stems		Tips/Stems
	Area (mg DW) ⁻¹	% increase	Area (mg DW) ⁻¹	% increase	
Control	1157.2 \pm 38.6 a		451.2 \pm 41.8 c		2.6
HL	1478.6 \pm 37.9 b	28	474.1 \pm 36.5 c	5	3.1
HL+UV-B	1488.4 \pm 99.9 b	29	1145.0 \pm 97.3 a	154	1.3

There is increasing evidence that the function of many of the lichen phenolic compounds is to act as filters for UV radiation (Galloway, 1988; Solhaug et al. 1996; Bachereau et al. 1997). Further evidence in this direction comes from studies on the fruticose lichen *Cladonia arbuscula* ssp.

mitis that was exposed to enhanced UV-B radiation in short-term, indoor and in long-term, outdoor experiments (paper II). In both experiments an increased amount of phenolic compounds was found in samples that had been exposed to UV-B radiation as compared to non-exposed controls (Tables I and II). Although the highest amounts of phenolic compounds were found in the apical regions of the lichen thallus that were directly exposed to the incoming UV-B, and arbitrarily called “tips”, the largest increases, found in UV-B exposed samples, occurred in a more central region of the thallus, designated as “stems” (HL+UVB: Table I; UVA+UVB: Table II).

Table II. Total phenolic content of ‘tips’ and ‘stems’ measured (as described in Material and Methods: paper II) in samples grown for 90 days outdoors under sunlight without UV-B radiation (No UV-B) or with additional UV-A + UV-B radiation (+UV-A+UV-B). % increase = percentage increase from the “No UV-B” value. Data are means of 3 independent experiments \pm SEM. Values followed by different letters were significantly different at $P < 0.05$.

Treatment	Tips		Stems		Tip/Stem
	Area (mg DW) ⁻¹	% increase	Area (mg DW) ⁻¹	% increase	
+UV-A +UV-B	1326.7 \pm 79.5 a	13	919.5 \pm 63.8 c	48	1.4
No UV-B (controls)	1169.6 \pm 38.5 b		620.5 \pm 19.8 d		1.9

The central part of the thallus showed also the lowest content of phenolic compounds in non-irradiated control samples (control; Table I and II). In nature, the central parts of fruticose lichens are partly or totally shaded by the higher parts of the thallus and the low content of UV-absorbing pigments might reflect a lower requirement of these portions for protection. It has been suggested that lichen secondary metabolites, due to their chemical structure and similarly to flavonoids, may contrib-

ute to the antioxidant defence system of lichens. Many of these substances, especially depsides and depsidones, bear carbonyl groups (CHO) and one or more orthohydroxyl groups in the same benzene ring (Hidalgo et al. 1992; Hidalgo et al. 1994). Thus conditions leading to the development of free radicals, such as high visible light and/or UV-B radiation, may also induce the accumulation of phenolic compounds as a protective strategy, as has been reported for higher plants. This could explain the increase in phenolic compounds found in samples of *Cladonia arbuscula* exposed to high light only (HL, 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$) (HL: Table I).

6.2 Penetration and measurement of radiation within tissues

In recent years the use of the fibre optic technique has become a powerful tool for studying the internal UV microenvironment of vascular plant tissues (Bornman and Vogelmann, 1988; Bornman et al. 1991; Reuber et al. 1996; Olsson et al. 1999). The technique consists of inserting quartz optical fibres into leaves or other tissues and measuring the radiation at different depths. This allows for an assessment of the penetration and distribution of UV radiation within that tissue. If this is correlated with the content of UV-absorbing compounds, measured as the absorbance of extracts of the tissue under examination, information on the localisation of UV-screening pigments within the tissue can be obtained. The fibre optic technique was used to gain insight into aspects of light attenuation in lichen thalli (paper II).

Light penetration measurements were conducted on the central part of the lichen thallus ("stem") of samples grown for three months outdoors under natural solar radiation where the UV-B part of the spectrum had been removed (No UVB: Fig. 3) or where supplemental UV-B radiation had been added (UVA+UVB: Fig. 3) (paper II). The penetration of UV-B radiation (280 nm) throughout the whole thallus was much lower in

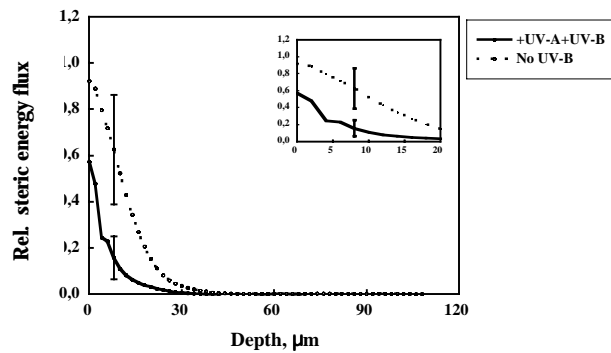


Fig. 3. Penetration of 280 nm radiation within the thallus of lichens grown outdoors either with additional UV-A and UV-B, or without UV-B radiation. Curves are means of 6 measurements taken from 2 stem pieces of each lichen sample (paper II, Material and Methods). At certain thallus depths, curves at 280 nm were significantly different ($P < 0.001$).

lichens grown under supplemental UV-B radiation (Fig. 3). This was especially pronounced in that part of the thallus just below the upper surface, suggesting a localisation of the UV-absorbing pigments at the surface of the outer medullary layer. It is known that in both plants and lichens, protective pigments are located primarily towards the adaxial surfaces of horizontally inclined structures such as leaves or foliose thalli. In lichens, the great majority of substances are deposited as crystals or as amorphous substances on the outer surface of the fungal hyphae in the medulla, or in the cortex in those lichens possessing such tissue (Rundel, 1978).

Apart from pigment accumulation, attenuation of potentially damaging solar UV-B radiation may also be achieved by scattering and reflecting the radiation by means of cuticular projections (papillae, trichomes), or cuticular and epicuticular substances deposited on the leaf surface of

many plants (Vogelmann 1993; Karabourniotis et al. 1999). However, in most plants that have been studied, this strategy does not seem to be of primary importance. A study conducted on several species showed that less than 10% of the incident UV radiation is reflected (Gausman and Allen, 1973; Gausman et al. 1975; Robberecht et al. 1980). The most efficient mechanism of UV exclusion still seems to be the accumulation of UV-screening phenolics. In lichens, light reflectance seems to be a more effective mechanism than in plants. Gauslaa (1984) showed that pale fruticose lichens such as *Cladonia stellaris* are efficient reflectors, although in this study measurements did not include the UV range of the spectrum but only PAR and infra-red regions. However, pale fruticose lichens in the air-dry state showed as much as 40% reflectance in the wavelength interval of 400-700 nm. Hydration of the thalli resulted in a decrease in reflectance especially in the visible light part of the spectrum.

6.3 DNA damage and repair

DNA is considered to be the primary cellular target of UV-B radiation. DNA strongly absorbs in the UV-C and UV-B regions of the spectrum, with the peak absorption for unshielded DNA being around 260 nm and the maximum absorption of DNA in intact plants near 280 nm (Quaite et al. 1992). However, also longer wavelengths (UV-A, 315-400 nm) have been shown to play a role in the formation of lesions, although to a lesser extent (Quaite et al. 1992).

UV-B-induced DNA damage can be classified into two major categories: cyclobutane pyrimidine dimers (CPDs), which make up approximately 75% of the damage and pyrimidine-(6-4)-pyrimidinone photoproducts [(6-4)-photoproducts], which make up the rest (Mitchell et al. 1993). CPDs covalently link two adjacent pyrimidines on the same DNA strand and they are very stable photoproducts unlike the 6-4 photoproducts, which tend to isomerise into Dewar photoproducts in the presence of UV-A radiation (Fig. 4) (Matsunaga et al. 1993). Both types of dimers block DNA replication and transcription, and if not removed, they can lead to the death of the cell.

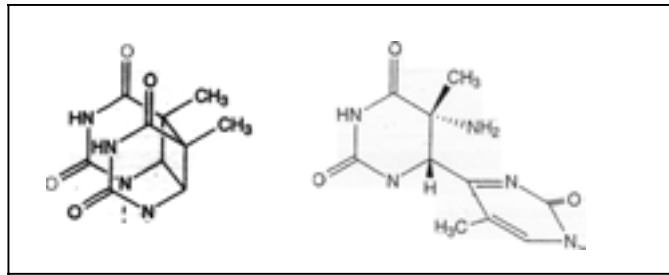


Fig. 4. Two common UV-induced photoproducts: a cyclobutane pyrimidine dimer (to the left) and a pyrimidine (6-4) pyrimidinone photoproduct (to the right).

Plants can use two broad strategies to limit the amount of damage caused by UV-B radiation: decrease the penetration of UV-B radiation within the tissue and repair the damage eventually caused by the radiation that succeeded in penetrating into the tissue.

One way of decreasing the penetration of UV-B radiation in the tissue is by the accumulation of UV-absorbing pigments, as described in the previous section. Evidence that UV-absorbing pigments are important for the protection of the DNA come from various studies in which plants that are genetically deficient in these pigments were shown to be much more sensitive to UV-induced DNA damage than their respective wild types (Li et al. 1993; Stapleton et al. 1994). In the present study, UV-B-induced CPD accumulation was detected in air-dry thalli of the lichen *Cladonia arbuscula* ssp. *mitis* exposed for 1, 2 and 7 days to UV-B radiation together with PAR ($800 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Fig. 5) (paper III). Interestingly, the presence of PAR before, during and after UV-B irradiation did not reverse the accumulation of CPDs.

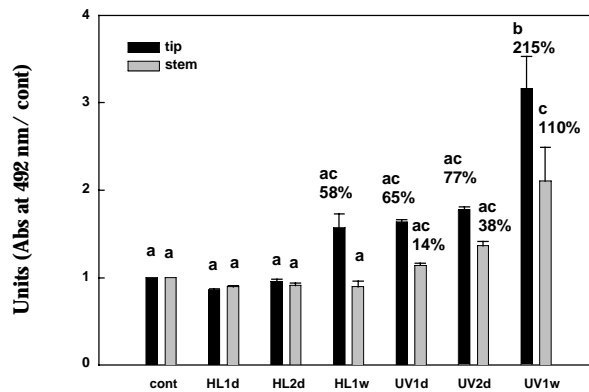


Fig. 5. CPDs detected separately in “tips” and “stems” of lichens treated with relatively HL ($800 \mu\text{mol m}^{-2} \text{s}^{-1}$) or HL+UV-B radiation ($+13 \text{ kJ m}^{-2} \text{ day}^{-1}$ biologically effective UV-B, UV-B_{BE}) (referred to as HUV) for 1, 2, or 7 days. Controls (cont) refer to greenhouse conditions. Values are normalised to the control value. Data are means from 3 independent experiments \pm SEM. Data sets followed by different letters were significantly different at $P < 0.05$.

Wavelengths between 370 to 450 nm (UV-A and PAR) are known to be the energy source of a class of flavoproteins known as photolyases that catalyse the direct reversal of pyrimidine dimers to monomers (Sancar, 1994). This repair mechanism is known as photoreactivation. In some plant species, irradiation with visible light has been shown to lead to almost complete removal of thymine dimers within a few hours (Taylor et al. 1997). Hydrated thalli, kept at 25°C , were capable of removing thymine dimers after exposure to PAR following UV-B radiation treatment, where a decrease in CPD accumulation occurred; an almost complete removal of CPDs was seen when PAR was given during the UV-B treatment (paper III). Hydrated thalli kept at low temperature (2°C)

lacked the capacity to repair DNA damage, since UV-B-induced CPD accumulation was not reduced by PAR given during and/or after the UV-B irradiation (paper III). In conclusion, these data suggest that the water content of lichen thalli is of major importance with regard to the accumulation and repair of DNA damage, since dry thalli are sensitive to UV-induced DNA damage and they show reduced repairing capacity as compared to hydrated thalli. Temperature is also an important factor since low temperatures slow down the activity of many enzymes (Takeuchi et al. 1996).

6.4 “Tips” versus “Stems”

The apical parts (“tips”; paper II) of pale fruticose lichens such as *Cladonia arbuscula* ssp. *mitis*, are directly exposed to the solar radiation and thus they are exposed to the UV-B part of the spectrum as well. These upper parts are also subjected to a more rapid drying and wetting pattern compared to the subapical parts of the thallus (“stems”; paper II). Therefore the apical parts of the thallus may afford physical and physiological protection to the stem parts, which are characterised by lower levels of UV radiation and longer periods of moistening. Data resulting from outdoor and indoor experiments have shown that tips of lichens contain the highest amounts of UV-protective pigments in both non-treated controls and UV-irradiated samples (Tables I and II). The relatively high amount of phenolic compounds seen in pre-experimental samples (controls) suggests that these substances accumulate in natural conditions almost to near saturation, probably as a result of the constant need of the apical parts for protection (tips, controls: Table I). However, when UV-B radiation is enhanced it might exceed the absorbing capacity of the apical regions and might reach also the normally shaded central parts of the thallus causing an increase in the production of UV-absorbing pigments (HL+UVB: Table I). The larger increase in phenolic content observed in stems as compared to tips (Table I) may not only be due to a lower initial level of these pigments but also to the longer periods of metabolic activity ensured by prolonged retention of moisture.

Stems accumulated less DNA damage and showed a higher degree of DNA damage repair than tips in both dry and wet thallus conditions (paper III). In addition, photolyase extracted from stems was able to repair CPDs of a standard UV-C irradiated DNA to a greater extent than photolyase extracted from tips (Fig. 6). Since tips are the growing parts of a fruticose lichen thallus and are characterised by small average cell sizes with high turnover rates (Honegger, 1996), the results lead to the suggestion that photolyase expression is under developmental control. In plants some indications of a similar pattern come from studies on *Arabidopsis thaliana* and wheat (Pang et al. 1991; Taylor et al. 1997).

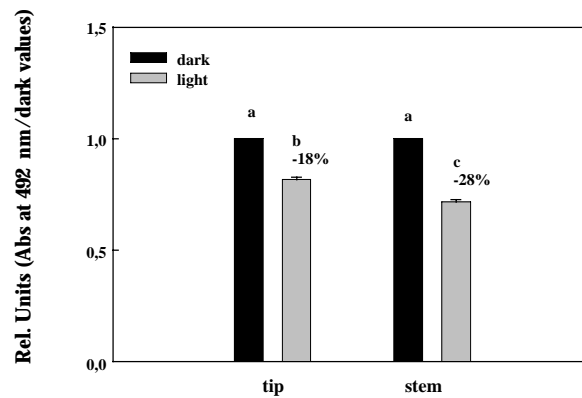


Fig. 6. Photolyase activity in control lichen “tips” and “stems” determined as the percentage reduction of CPDs in light-treated samples (PAR, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 3 h) as compared to dark-treated samples (dark). Data are means of 3 independent experiments \pm SEM. Data sets are normalised to dark values. Values followed by different letters were significantly different at $P < 0.05$.

7. CONCLUDING REMARKS

The lichen *Cladonia arbuscula* ssp. *mitis* responded to enhanced UV-B radiation by increasing the content of UV-absorbing pigments. The increase in phenolic content occurred in apical parts of the podetial branches (tips) as well as in the more central regions of the thallus (stems), although in the latter the increase was much more pronounced. The accumulation of phenolic compounds decreased the penetration of UV-B radiation to the underlying photosynthetic algal layer, which is immediately below the surface in this species. The UV-absorbing pigments may have also protected the stems from the accumulation of UV-induced DNA damage in the form of CPDs, since the amount of DNA damage was found to be consistently lower in stems than in tips. However, it is also likely that the amount of incident UV radiation on stems may be lower due to the natural shading of the upper parts of the thallus.

Air-dry lichen thalli were more susceptible to UV-induced DNA damage and were less capable of repairing pyrimidine dimers than hydrated thalli. However, at low temperatures, hydrated thalli were sensitive to UV-induced DNA damage and were not able to repair it. Thus, water content and temperature represent two key factors for the capacity of lichens to respond to UV-B radiation.

Tips, as compared to stems, are the lichen parts most susceptible to damage caused by UV-B radiation, since they accumulated the highest amount of pyrimidine dimers and had the least repair capacity. In addition, they showed a lower relative increase in phenolics than stems. Therefore, the apical parts are likely to be the most adversely affected parts of the lichen thalli by UV-B radiation. This might have consequences at the whole lichen level, since the apical parts are also the growing and photosynthesising regions of the lichen thallus.

The effects of UV-B radiation on DNA damage and repair in lichens in natural environments are not known and require further research. Furthermore, lichens are usually regarded as individuals, although they are a symbiotic entity involving at least two partners. Further investigations

are required on the effects of UV-B radiation on the DNA of the single symbiotic partners in order to assess whether the fungus constitutes an effective shield protecting the photobiont against DNA damage.

To return to the main objectives of this thesis and to address the question of whether lichens are better adapted to UV-B than higher plants the results obtained seem to suggest that at least the lichen under study was not better than many higher plants in avoiding and/or repairing damage caused by UV-B radiation.

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PUBLICATIONS