Induction of Cyclobutane Pyrimidine Dimers and 6-4 Photoproducts by Desiccation in the Moss *Ceratodon purpureus*

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Abstract

DNA damage in the form of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) has been well documented to be caused by ultraviolet-B (UV-B) radiation. This study presents the first evidence to suggest that these so called "photoproducts" can occur during desiccation, in the absence of UV-B. CPDs and 6-4PPs were measured using the ELISA technique in the moss *Ceratodon purpureus* which was subjected to desiccation (withdrawal of water) under differing light conditions. Both CPDs and 6-4PPs were found to accumulate in this moss in the dark in the absence of UV-B radiation. DNA damage in the form of 6-4PPs was also found to occur in *C. purpureus* desiccated in the light. A "threshold" model of DNA damage and repair during desiccation is proposed and its implications discussed.

C. purpureus and *Arabidopsis thaliana* were treated with the free-radical generator, methyl viologen, in order to determine if the accumulation of CPDs and 6-4PPs can occur as a result of oxidative stress. Additionally, ascorbate-deficient mutants of *A. thaliana* were studied in order to determine if ascorbate plays a role in the prevention of CPD and 6-4PP accumulation. It was found that ascorbate plays little or no role in preventing DNA damage during desiccation.

Samples of *C. purpureus* were taken over a 24 hour period from Arctic, Antarctic and Scottish field sites from ambient conditions and from under UV-B-screening films. CPD and 6-4PP accumulation was measured in these samples in order to determine how much DNA damage fluctuates throughout the day in this species and if UV-B plays a critical role in DNA damage accumulation. Some evidence was found to suggest that water content of *C. purpureus* is linked to DNA damage in this species under field conditions. No relationship was found between UV-B and DNA damage in the field.

Declaration

I, Kirsty Hannah Duncan, hereby certify that this thesis, which is approximately 40, 000 words in length, has been written by me and it is the record of work carried out by me and it has not been submitted in any previous application for a higher degree.

Kirsty Duncan
September 2003

Statement

I was admitted as a research student in October 1999 and as a candidate for the degree of Doctor of Philosophy in October 1999; the higher study for which this is a record was carried out in the University of St Andrews between 1999 and 2003.

Kirsty Duncan
September 2003

Certificate

I hereby certify that the candidate has fulfilled the conditions for the degree of Doctor of Philosophy in the University of St Andrews and that the candidate is qualified to submit this thesis in the application for that degree.

Dr AK Tobin

September 2003

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Kirsty Duncan
September 2003

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Abbreviations

A.....Absorbance
ANOVA.....Analysis of Variance
ASA.....Ascorbate
BER.....Base Excision Repair
CA.....Cellulose Acetate
CFC.....Chlorofluorocarbon
CPD.....Cyclobutane Pyrimidine Dimer
DHA.....Dehydroacsorbate
DNA.....Deoxyribonucleic Acid
DTT.....Dithiothreitol
dwt.....Dry Weight
ELISA.....Enzyme-Linked Immunosorbent Assay
FAD.....Flavin Adenine Dinucleotide
fwt.....Fresh Weight
g.....Relative Centrifugal Force
LST.....Lysis Storage and Transportation
MDHA.....Monodehydroascorbate
NADH.....Nicotinamide Adenine Dinucleotide (Reduced Form)
NEM.....N-ethylmaleimide
NER.....Nucleotide Excision Repair
PAR.....Photosynthetically Active Radiation (400-700nm)
PBS.....Phosphate Buffered Saline
PPFD.....Photosynthetic Photon Flux Density
PSI.....Photosystem I
PSII.....Photosystem II
rau.....Relative Absorbance Units
RNA.....Ribonucleic Acid
TOMS.....Total Ozone Mapping Spectrometer
UV.....Ultraviolet
UV-A.....Ultraviolet-A

UV-B.....Ultraviolet-B

UV-C.....Ultraviolet-C

v/v.....Volume/Volume

wt.....Wild Type

w/v.....Weight/Volume

6-4PP.....6-4 Photoproduct

Chapter 1

Introduction

1.0 Interaction of Stresses

Organisms are rarely exposed to any one single environmental stress, and it is much more likely that several interacting stresses will have an effect. For example, a plant that is exposed to a high level of sunlight will encounter an ultraviolet (UV) stress. Additionally, a number of other stresses may be acting on the plant such as heat stress and desiccation or, in the case of plants in polar regions, high light can be accompanied by freezing temperatures. The net response of the plant will be to the combined effects of these stresses.

Production of DNA damage lesions induced by ultraviolet-B radiation has been well documented (Tevini *et al.*, 1993; Britt, 1995, 1996). The available literature does not document the production of these particular lesions solely under UV-B.

1.1 Ultraviolet Radiation

Plants, by their very nature, are sessile organisms, and therefore they have to tolerate the light regime in which they live. The sun emits a continuous spectrum of light and of this the ultraviolet (UV) fraction can be split into four groups: Vacuum UV (40-200nm), UV-C (200-280nm), UV-B (280-320nm) and UV-A (320-400nm). Atmospheric particles absorb wavelengths below 290nm, and therefore it is only wavelengths of 290-1500nm that reach the surface of the Earth (Davies, 1995).

It is the shorter, higher-energy wavelengths of light that are more damaging to living organisms. Pyrimidine and purine bases have an aromatic π-electron system, which causes nucleic acids to have a strong absorbance near 260nm (Tevini *et al.*, 1993), that is in the UV-C region. However, there is no biologically significant UV-C (<280nm) radiation present at the Earth's surface, and therefore it is the small proportion of UV-B and the greater amount of UV-A that are the most important sources of epidermal damage in plants and animals (Britt, 1996).

UV-A radiation is less energetic than UV-B, and so it is less likely to interact directly with proteins or DNA (Wilson *et al.*, 2001). However, there is at least a 10-15-fold greater daily fluence of UV-A in the solar spectrum, and it is able to penetrate to greater depths than UV-B in leaves (Wilson *et al.*, 2001). UV-A is

more likely to reach the mesophyll cells of leaves where there is a large concentration of potential photosensitizing compounds (Wilson *et al.*, 2001). UV-A is able to interact with these compounds directly to produce active oxygen species via photosensitization reactions (Wilson *et al.*, 2001). Significant oxidative damage to photosynthetic membranes and protein complexes may occur, although it is not clear whether the amount of oxidative damage produced is significant in comparison with the baseline level of oxidative damage produced by normally functioning organelles (Britt, 1996).

Although there is more UV-A than UV-B in the solar spectrum, UV-B is more likely to cause direct damage to macromolecules such as DNA. Due to ozone layer depletion, there has recently been a significant increase in the level of UV-B reaching the surface of the Earth, and therefore the damaging effects of UV-B have raised considerable interest.

It should also be noted that evolution has occurred under UV radiation, and therefore all organisms have some method to tolerate and/or repair damage that might occur under natural levels. Furthermore, UV radiation is known to have some beneficial effects, for example, the catalysis of vitamin D3 from 7-dehydrocholesterol, which in animals regulate calcium and prevent rachitic (Tevini *et al.*, 1993).

1.2 Molecular Damage Caused by Ultraviolet Radiation

The effects of UV-B radiation on organisms fall into two groups: physiological responses and direct molecular damage to biological macromolecules, e.g., DNA, RNA, proteins and lipids (Taylor et al., 1996).

When cells are irradiated in their natural physiological environment, DNA damage induced by the direct absorption of photons is the most important source of mutations and cytotoxic effects (Davies 1995). Ultraviolet-B radiation can induce a number of damage products involving DNA, such as alkylation damage, DNA-protein cross-links and pyrimidine photohydrates (Fisher *et al.*, 1976; Tevini *et al.*, 1993; Davies 1995). However, the most biologically effective UV-induced photoreactions occur at the pyrimidine base, thymine (Tevini *et al.*,

1993). Ninety-eight percent of UV-B-induced damage is in the form of pyrimidine dimers (Mitchell and Karentz, 1993). Of these, cyclobutane pyrimidine dimers make up 75% of the total number of photoproducts, and (6-4) photoproducts the remaining 25% (Mitchell and Nairn, 1989). It has not yet been firmly established which of these photoproducts is the most cytotoxic (Mitchell, 1988; Mitchell and Nairn, 1989).

1.2.1 Cyclobutane Pyrimidine Dimers

In pyrimidine adducts either two thymine monomers, or a thymine and a cytosine monomer form a dimer (Tevini *et al.*, 1993). Cyclobutane pyrimidine dimers (CPDs) arise from a [2+2] cycloaddition of the C_5-C_6 double bonds of adjacent pyrimidine bases (Ravanat et al., 2001). Six diastereomers may be generated from thymine depending on the position of the pyrimidine moieties with respect to the cyclobutane ring (*cis/trans* stereochemistry) and on the relative orientation of the two C5-C6 bonds (*syn/anti* regiochemistry) (Cadet et al., 1985). Due to steric constraints, only *syn* isomers can be generated within DNA and oligonucleotides (Ravanat *et al.*, 2001 see figure 1.1). If left unrepaired, CPDs can kill cells, initiate carcinogenesis and cause mutations (Harm, 1980; Sage, 1993).

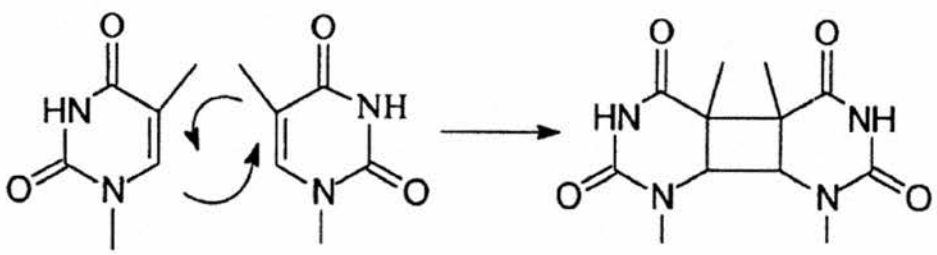


Figure 1.1: Formation of the Cyclobutane Pyrimidine Dimer

From Ravanat *et al.*, 2001

1.2.2 (6-4) Photoproducts

A competing reaction upon UV-B irradiation can cause another form of covalent link between adjacent pyrimidine bases to occur (Cadet and Vigny, 1990). (6-4) Photoproducts (6-4PPs) are formed in a reaction involving singlet excited oxygen. They arise from a [2+2] cycloaddition between the C5-C6 double bond of the 5'-

end pyrimidine and the C4 carbonyl group of the 3'-end thymine (Ravanat *et al.*, 2001). An oxetone or azetidine precursor then rearranges to give a stable (6-4) photoproduct (Davies, 1995), with a yield of approximately 0.1-0.5 times that of CPDs (Cadet and Vigny, 1990; Mitchell *et al.*, 1991). Upon further UV-B irradiation, the (6-4) photoproduct may form its Dewar isomer. This reaction is photoreversible, and irradiation with UV-C will cause Dewar isomers to turn back into 6-4PPs (Ravanat *et al.*, 2001). UV-irradiated DNA will normally contain a mixture of Dewar isomers and 6-4PPs (Ravanat *et al.*, 2001).

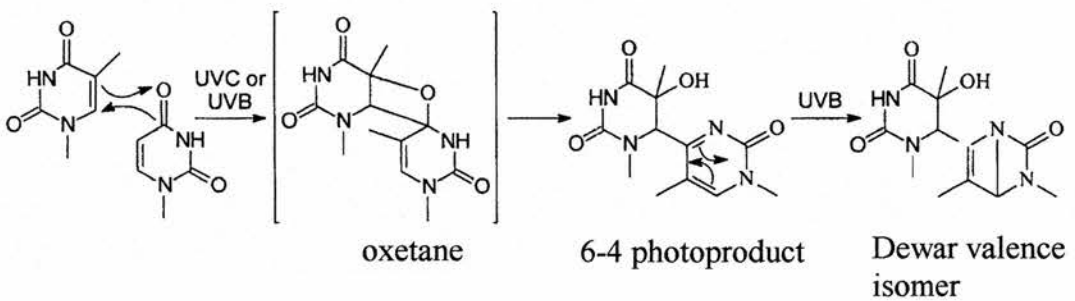


Figure 1.2: Formation of the (6-4) photoproduct and its corresponding Dewar isomer (From Ravanat *et al.*, 2001).

1.2.3 Other (minor) photoproducts

CPDs and 6-4PPs make up 98% of DNA damage induced by UV-B. However, of the remaining 2%, a minor photoproduct which is resistant to removal by DNA repair mechanisms may pose a greater threat to the viability of an organism than a photoproduct which is rapidly repaired (Davies, 1995). For example, cytosine photohydrates (formed by addition of water to the double bond) have a very low quantum yield in comparison to CPDs and 6-4PPs (10^{-3}mol.einstein^{-1}), making them minor photoproducts. However, if they persist in the cell, they can cause transition mutations through their deamination and subsequent hydration to uracil (Fisher et al., 1976). Therefore although this work has focussed on the two major types of photoproduct, it should still be taken into consideration that other minor photoproducts may be causing significant damage to an organism.

1.3 Oxidative Damage

Molecular oxygen is not particularly reactive, but it can become transformed by metabolic activity into reduced or electronically excited states (Smirnoff, 1995). Superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals are

collectively known as active oxygen (Halliwell and Gutteridge, 1989). They are produced as by-products of normal metabolism in organelles by ionizing radiation (Lu et al., 2001), and are common factors in the response of plants to major environmental extremes (Smirnoff, 1993). For example, very low water contents can hinder antioxidative defences, leaving the tissue exposed to oxidative deterioration (Smirnoff, 1993). Physical disruption of the cell structure can be caused by freezing and desiccation, leading to oxidative and free radical damage (McKersie *et al.*, 1990). Dessiccation, even in tolerant plants, induces oxidative stress (Dhindsa and Matowe 1981; Smirnoff 1993; Csintalan *et al.* 2001).

The major sources of activated oxygen in the cell are the organelles; reactions in both the chloroplast and the mitochondrion frequently misdirect electrons to oxygen, generating superoxide (Britt, 1996). All species of active oxygen are reactive and potentially damaging, causing lipid peroxidation, inactivation of enzymes, and oxidative DNA damage (Smirnoff, 1993).

Oxidative DNA damage is a major source of mutation load in living organisms (Halliwell and Gutteridge, 1989), although bases in an intact double helix are shielded from attack by hydroxyl radicals by their stacked interior conformation (Britt, 1996). The reactive components of DNA to free radicals are the four bases and the sugar component, deoxyribose (phosphate is relatively unreactive towards free radicals). For this reason, a relatively large fraction of oxidative damage occurs at the sugar phosphate backbone, leading to single-strand breaks (Britt, 1996). Bases near a nick are more likely to be exposed to oxidative attack (Britt 1996).The bases can be oxidized or reduced by the radicals, producing many different forms of oxidative DNA damage (Simic and Jovanovic, 1986). It should be noted that the DNA dimers produced by UV irradiation are not amongst the damage lesions caused by oxidative damage. Reactions of free radicals with deoxyribose can lead to loss of a base (mechanisms not fully understood), a strand break (C4 composition) or internal cyclization with a base attached to it. Pyrimidine radicals are particularly reactive at the 5,6 bond, and disappear rapidly in reactions with each other (Simic and Jovanovic, 1986; Lu *et al.*, 2001). It is well established that the main oxidative reaction of DNA gives rise to 8-oxo-dihydro-2'-deoxyguanosine (8-xodGwo) (Ravant *et al.*, 2001).

ROS	**Formation**	**Main Type of Damage Caused**
Superoxide (O_2)	Autoxidation of electron transport chain components	Leads to formation of H_2O_2 and $OH^{\cdot}$
Hydrogen Peroxide (H_2O_2)	Oxidases and the dismutation of superoxide	Inactivation of Calvin Cycle enzymes
Hydroxyl Radical ($OH^{\cdot}$)	Oxidation of H_2O_2 Iron-catalysed Harber-Weiss Reaction	Lipid peroxidation Inactivation of enzymes
Singlet Oxygen($^1O_2$)	Change in spin restriction from parallel to antiparallel Transfer of energy from photosensitisers, eg. chlorophyll	Lipid peroxidation Oxidation of nucleic acids

Figure 1.3: Formation and Effect of Reactive Oxygen Species
(Information taken from Smirnoff, 1993, 1995 and 1998).

1.4 Repair Mechanisms

It has already been mentioned that plants are obliged to be exposed to the sun in order to photosynthesize, and therefore they are constantly exposed to the threat of damage by UV radiation. However, plant interons are small, and the plant transcriptional unit is one quarter of the size of those of animals. The probability of a dimer occurring within a transcriptional unit is therefore reduced, and the intrinsic resistance of plant cells to UV-B is enhanced (Britt, 1995). However, it has also been shown that plants lacking in the normal repair mechanisms are extremely vulnerable to UV radiation (Britt *et al.*, 1993). Pyrimidine dimers cannot effectively base pair with other nucleotides, and therefore although they are not directly mutagenic, they can act as blocks to DNA replication and a single dimer can prevent expression of a transcriptional unit. Life evolved under the pressure of environmental stresses such as desiccation and UV radiation and

therefore all organisms have either tolerance or repair mechanisms to deal with damage caused to the DNA.

1.4.1 Photorepair

Photorepair of DNA lesions is rapid and effective and provides the bulk of protection against UV-B-induced DNA damage. The enzyme, photolyase, binds specifically to either CPDs or 6-4PPs to form an enzyme-substrate complex in a slow (dark) step. The second stage, excision of the dimer, is catalyzed by saturating light in the region of 300-500nm (Kim and Sancar, 1993). The photoreactivating light has to occur immediately after UV damage in order for there to be effective removal of the lesions (Tevini, 1993). Photolyase has two chromophores. The first (either methenyltetrahydrofolate or 8-hydroxy-deazaflavin) absorbs the photoreactivating light and transfers energy to the other chromophore, a fully reduced flavin adenine dinucloetide (FAD). The excited FAD^* then transfers an electron to the dimer, inducing its reversal (Yasui *et al.* 1994; Kleiner *et al.* 1999). The efficiency of photoreactivation is extremely high, and approximately one dimer is split for every blue light photon absorbed (Britt, 1996). Photoreactivation of CPDs is a common repair mechanism in most plants, however, photolyases specific to 6-4PPs appear to be rarer. Chen *et al.* (1994) determined a light-dependant pathway for the removal of 6-4PPs in Arabidopsis. This repair pathway does not require induction by prior exposure to visible light, as with CPD-specific photolyase, and neither does it require the *uvr1* gene product which is essential for the dark repair of 6-4PPs (Britt, 1995). Takahashi *et al.* (2002) observed that there were diurnal changes in the *CsPHR* transcript level for CPD photolyase in cucumber. The transcript level increased for three hours at the beginning of the photoperiod, and then decreased during the rest of the light period. A similar, but smaller pattern was seen in the dark, suggesting that whilst CsPHR transcription is controlled mainly by light, it is also moderately controlled by intrinsic rhythms. Three hours after the transcription level peaked, photorepair activity was optimal. The time-lag is suggested to result from the duration of the translation or activation processes (Berrocal-Tito *et al.*, 2000). As a result of this time lag effect, the oscillation of photorepair activity is synchronised with the intensity of solar UV-B (Xiong and Day 2001).

1.4.2 Excision Repair

Rather than direct reversal of damage lesions, dark repair pathways replace the damaged DNA with new, undamaged nucleotides (Britt, 1995). This requires approximately 30 different gene products (Moné *et al.*, 2001). Excision repair pathways can be divided into two types.

Base Excision Repair (BER) involves the removal of a single damaged base through the action of one of many lesion-specific glycosides, which leaves the DNA sugar-phosphate backbone intact. Nucleotide excision repair (NER) differs from BER in two ways. Firstly, the repair complex initiates removal of the damage by generating nicks on the damaged strand (Britt, 1996). Nicks are initiated at a specific distance both 3'and 5' of the lesion, and the oligonucloetide containing the DNA damage is excised through the action of helicose (Britt, 1995). Secondly, NER recognises a broad spectrum of DNA damage products and will, with varying efficiencies, cleave almost any abnormality in the DNA structure from nondistortion lesions to bulky adducts. It is unlikely that a plant would produce a separate repair protein for every possible DNA damage product, and therefore NER exists as a general repair pathway.

NER appears to be the primary source of removal of 6-4PPs in plants, but this type of repair has been reported absent for CPDs in some species (Trosko and Mansour, 1968, 1969; Swinton and Hanawalt, 1973). This may be due to the changing roles of excision and photorepair (see section 1.4.4).

1.4.3 Recombinational "Repair"

Tolerance pathways also exist in organisms, which allow the replication of chromosomes, despite the persistence of a damage lesion. It is unknown whether these pathways occur in plants, although it is known that wild-type *A. thaliana* can continue to grow despite the persistence of CPDs at a frequency of one dimer per 42 000 bases, suggesting that some form of tolerance mechanisms is in place. It is also possible that selective repair may occur in the critical regions of the genomes, as has been observed in mammalian systems (Bohr *et al.*, 1985; Thomas *et al.*, 1989).

1.4.4 Interaction of Repair Mechanisms

When attempting to determine the relative importance of photorepair and excision repair, the changing roles of excision and photorepair must be taken into account. For example (Takayanagi *et al.*, 1994) planted alfalfa seedlings in UV-free environments, and in un-filtered sunlight. Both sets of seedlings had the same amount of CPDs, and when they were given a further dose of UV-B, more dimers were found in the indoor seedlings that in sunlight ones. This suggests that the UV transparency and the repair capacity of higher plants are altered substantially in response to the ambient levels of UV and visible radiation (Britt, 1995).

Quaite *et al.* (1994) observed that in alfalfa, at low levels of UV damage, photoreactivation rapidly repaired CPDs, whereas excision repair was undetectable. At higher levels of damage, both photoreactivation and excision repair occurred. The initial rate of photoreactivation of CPDs in alfalfa was seen to increase as a linear function of dimer level. Quaite *et al.* (1994) suggested that this differential repair response could reflect an efficient and beneficial repair strategy. Photoreactivation requires only external energy in the form of visible photoreactivating light, and photoreversal of dimers is error-free and non-mutagenic. The excision process required nicks to be formed in the DNA which can trigger a UV stress response in a plant, resulting in cessation of macromolecular synthesis and growth (Kaston *et al.*, 1991; Nelson and Kastan, 1994). In addition, excision repair requires cellular energy in the form of ATP, and the products of approximately 30 genes. This results in a much higher rate of errors during repair. As a result, at low levels of UV (as a plant would be exposed to during the day under normal circumstances), photorepair would directly reverse any damage caused in an error-free fashion, and bypass the interruption to growth. At higher damage levels, the capacity of photorepair could become saturated either due to light limitation or limited photolyase molecules, and therefore it would be beneficial to a plant to use excision repair (Quaite *et al.*, 1994).

1.5 Plant Resistance to Oxidative Stress

A common effect of many environmental stresses, including exposure to UV-B and desiccation, is oxidative damage. Plants therefore have antioxidant systems in place to minimise damage caused by active oxygen species (Alscher *et al.* 1997).

These systems can be divided into two groups; systems which react with active forms of oxygen and keep them at a low level for example, superoxide dismustase (SOD), catalyse, peroxidases, ascorbate, α-tocopherol, and systems which regenerate oxidised antioxidants such as glutathione, glutathione reductase, ascorbate and mono- and dehydroascorbate reductases (Smirnoff 1993). Mosses contain many unique flavonoids that are beyond the cope of this thesis.

1.5.1 Ascorbate

Ascorbic acid, or vitamin C, has a variety of critical roles in both plants and animals. It is the most abundant soluble antioxidant in chloroplasts, (Smirnoff 2000), and it is able to directly detoxify active oxygen species and to act as a reductant in enzymatic reactions (Conklin *et al.*, 1996). In photosynthesis alone, ascorbate has no less than five functions:

i) Hydrogen peroxide scavenging catalysed by ascorbate peroxidase.

ii) Direct scavenging of superoxide, hydroxyl radicals and singlet oxygen.

iii) Regeneration of alpha-tocopheryl radicals and singlet oxygen.

iv) Electron donation to photosystem II by luminal ascorbate.

v) Co-factor of violaxanthin de-epoxidases involved in zea-xanthin-dependent dissipation of excess excitation energy (a component of non-photochemical quenching) (Smirnoff 2000).

Primary oxidation of ascorbate forms the monodehydroascorbate radical (MDA) which disproportionates to ascorbate and dehydroascorbate (DHA). Under normal circumstances, the ascorbate pool in leaves and chloroplasts is 90% reduced. DHA is unstable, particularly at the pH of illuminated stroma, and will readily regenerate. Ascorbate regeneration systems include direct reduction of MDA by PSI, NADH-dependent MDA reductase and glutathione-dependent dehydroascorbate reductase (see figure 1.4) (Smirnoff 2000).

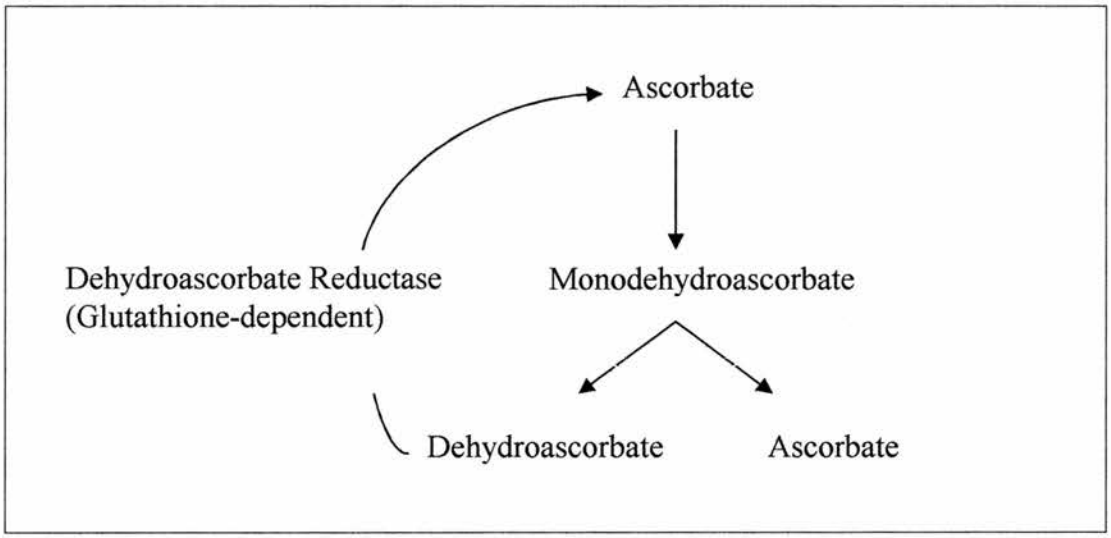


Figure 1.4: The Ascorbate / Dehydroascorbate cycle
For a more detailed cycle, see Smirnoff (1995), Smirnoff and Wheeler (2000) and Griffiths and Lunec (2001).

Total concentration of ascorbate in leaves is light-dependent, and growth at high light intensities produces leaves with higher ascorbate content than at low light intensity (Smirnoff and Pallanca, 1996; Grace and Logan 1996; Logan *et al.* 1996). The readjustment of ascorbate concentration from plants grown at high light and moved to low light is relatively slow, and a steady state is achieved after a few days (Eskling and Åkerlnd 1998). The biosynthetic pathway for the production of ascorbate differs in plants and in animals. The proposed pathway in plants indicates L-galacto-1,4-lactone as a direct precursor of ascorbate in a two-step oxidation catalysed by L-galactose dehydrogenase and L-galactono-1,4-lactone dehydrogenase (Østergaard et al. 1997; Wheeler et al 1998) (see figure 1.5).

The importance of ascorbate as an antioxidant can be seen using *Aradidopsis thaliana* mutants deficient in ascorbate production. Conklin *et al.* (1996) used screens for ozone-sensitive *A. thaliana* mutants, two of which contained only 25-30% of wild type ascorbate. Conklin *et al.* (2000) then went on to EMS-mutagenize two-week old *A. thaliana* seedlings, and found five mutant lines deficient in ascorbate. The vtc3 mutant, used in this study, was found to have reduced ability to convert mannose to ascorbate (Conklin *et al.* 2000).

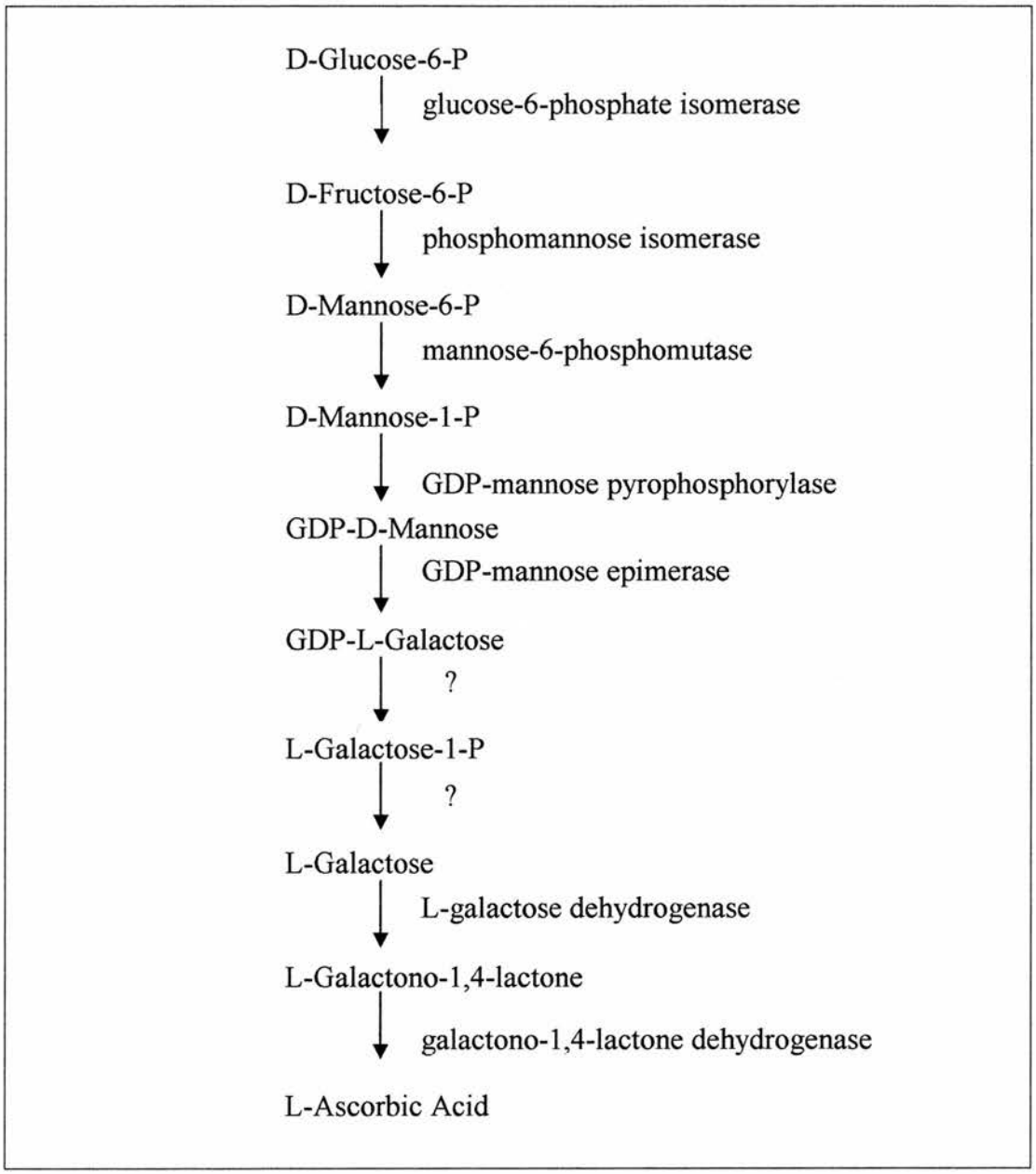


Figure 1.5: Proposed Pathway for Ascorbic Acid Synthesis in Higher Plants
Taken from Wheeler *et al.* 1998

1.6 Study Plants

Ceratodon purpureus is a desiccation-tolerant moss with a widespread distribution. It is found all over the world, from the tropics to the poles. *C. purpureus* can be air-dried and rehydrated within days, which, along with its abundance in the wild, makes it the ideal plant for a study on desiccation and DNA damage. *Sphagnum auriculatum* is a desiccation-intolerant species of moss

which is commonly found in boggy habitats. Its common distribution and desiccation intolerance made it the ideal comparison to *C. purpureus*.

Ascorbate is the most abundant antioxidant found in plant cells (Smirnoff and Wheeler 2000). As desiccation has been shown to induce oxidative stress, a vtc3 ascorbate-deficient mutant of *Arabidopsis thaliana* was used in this study to determine if lower ascorbate makes *A. thaliana* more susceptible to DNA damage caused by desiccation stress. For the purpose of this study, I define desiccation as water loss. Plants may therefore experience a gradient of desiccation states as they dry out.

1.7 Aims

The aims of this thesis were to:

1. Determine if there is an interaction between UV-B–induced DNA damage and desiccation in the moss *Ceratodon purpureus*.

Antioxidant measures employed by *C. purpureus* to withstand desiccation stress may also protect this species against UV-B radiation. A study was conducted in order to determine of this is the case.

2. Investigate the role of ascorbate as a means of protection against DNA damage induced by desiccation.

If DNA damage is attributable to free-radical generation during desiccation, then ascorbate, a powerful antioxidant, may help to negate that damage. A study was conducted to determine if raising the level of ascorbate could counteract the effect of desiccation.

3. To determine the natural fluctuation of DNA damage in C. purpureus under field conditions in the Arctic, Antarctic and in Scotland.

DNA damage may naturally fluctuate throughout the day. A study was conducted to determine if the amount of damage observed during desiccation under laboratory conditions is significant in comparison to natural daily changes.

Chapter 2

Materials and Methods

2.0 Materials

All chemicals were obtained from Sigma Chemical Company Ltd., Poole, UK, and were of Analytical Grade, unless otherwise stated. The components of all complex solutions mentioned are fully listed in the Appendix. Unless stated otherwise, all experimental procedures were carried out at 25°C.

2.1 Plant Material and Growth Environments

2.1.1 *Ceratodon purpureus* and *Sphagnum auriculatum*

C. purpureus and *S. auriculatum* were collected from a woodland site (grid reference NO 515 167) in St Andrews by gently pulling them away from the soil and leaf litter. Before any experimental work was carried out, both species were acclimatised for 7 days at approximately 100 μmol m^{-2} s^{-1} (PPFD) natural light and 25°C. The moss was sprayed twice a day with water to maintain a humid environment. High light (560 μmol m^{-2} s^{-1}) and low light (100 μmol m^{-2} s^{-1}) conditions were supplied by discharge lamps (6 tubes HQI-TS 250W/NDL, Sanyo-Gallenkamp, Loughborough, UK) with a photoperiod of 16hrs at 25°C. Dark conditions were achieved by placing a black photographic sack over the moss. Only the green gametophytic tissue or "leaves" were used in these experiments. Moss was kept in 12cm x 22cm plastic trays .

2.1.1.2 Irradiation of *C. purpureus*

Biologically effective UV-B (based on Caldwell, 1971) generalized plant damage action spectrum normalised to 300nm) treatment was supplied by 4 x 115cm UV-B lights (TL40/12: Starna Ltd, Romford, UK.). Cellulose acetate (Warne & Co. Ltd. London, UK) 0.13mm thick was used to cover the lights to eliminate the penetration of UV radiation below 292nm. The acetate was changed before each irradiation. *C. purpureus* was irradiated with UV-B in the dark for one hour under "high" (0.56 Wm^{-2}) or "low" (0.47 Wm^{-2}) UV-B.

2.1.1.3 Desiccation of *C. purpureus* and *Sphagnum auriculatum*

Desiccation of the plants was achieved by withdrawing water. The moss was laid on top of granules of silica gel wrapped in fine muslin and allowed to dry out. The silica gel prevented the moss from obtaining water from its surroundings.

2.1.1.4 Rehydration of *C. purpureus* and *S. auriculatum*

Following the six day desiccation period, the silica gel was removed, and both species were sprayed with distilled water twice every hour for five hours. Rehydration occurred under the same light and temperature conditions as dehydration.

2.1.2 *Arabidopsis thaliana*

Wild type (*wt*) and ascorbate- deficient mutant (*Vtc3*) seeds of *Arabidopsis thaliana* were kindly donated by Dr Nick Smirnoff, University of Exeter. Both wild type and *Vtc3* lines were derived from the Columbia (Col -0) ecotype (Conklin *et al.*, 2000). Seedlings were grown in a greenhouse at 560 μmol m^{-2} s^{-1} PPFD, in Levington's M2 medium nutrient potting compost (Levington Horticulture Ltd, Ipswich, UK). Seedlings were planted in 12cm x 22cm trays, with 8 plants to a tray. When the seedlings were four weeks old, they were transported to a growth cabinet (Consort Services, Dundee, UK) and water was withdrawn for either 6 or 7 days. At the end of the desiccation period, plants were rehydrated by watering the compost thoroughly. Light (560μmol $m^{-2}s^{-1}$ PPFD) was supplied in the growth cabinets (8 tubes Osram L58W/27-965) with a 16 hr photoperiod, and a constant temperature of 25°C. Dark conditions were achieved by covering the plants with black photographic sacks.

2.1.3 Light Measurements

Visible light, or the photosynthetic photon flux density (PPFD 400-700nm), was measured using a quantum photometer (Macam Photometrics Ltd., Livingston, UK). UV-B was measured using a UVX Radiometer (CA, USA (200-400nm).

2.2 Physiological and Morphological Measurements

2.2.1 Photosynthesis Measurements

2.2.1.1 Leaf Disk Electrode

The effect of methyl viologen on photosynthesis in *C. purpureus* was measured using an oxygen electrode (Hansatech leaf disk electrode (LD-1), Kings Lynn,

UK). The oxygen electrode measures the O_2 concentration in air within a sealed water-filled chamber and directly outputs to a chart recorder. Water from a water bath (Grant Cambridge Ltd, Hertfordshire, UK) was circulated around a jacket containing the O_2 electrode to give a constant temperature of 25°C.

2.2.1.2 Calibration

To calibrate the O_2 electrode, a small amount (approximately 2.0 mg) sodium dithionite was added to the chamber to remove all of the oxygen. The position of the pen on the chart recorder was then set to zero. The chamber was then washed thoroughly, and 1ml of air-saturated water added. Given that 1.0 ml of air-saturated water at 25°C contains 0.253µmoles of oxygen (Walker, 1986), the chart recorder was calibrated and the oxygen evolution of the samples was measured.

2.2.1.3 Inhibition of Photosynthesis in *C. purpureus* by Methyl viologen

C. purpureus leaves (0.02g) were soaked for 16 hrs in the dark in a range of methyl viologen concentrations between 0 and –1.0 mM. The leaf samples were then blotted dry and placed in the O_2 electrode chamber with 1ml of 50mM Tris Buffer (pH7.0) containing 0.1mM sodium bicarbonate. The rate of respiration (oxygen depletion) was then measured over a 5 min period in the dark at 25°C. Light was provided and the rate of photosynthesis (oxygen evolution) was measured over a 5min period.

2.2.1.4 Chlorophyll Fluorescence

Plant tissue was dark-adapted for one minute, and the Fv/Fm measured using a Fluorescence Monitoring System (Hansatech Instruments Ltd., Norfolk, UK).

2.2.2 Measurement of Leaf Area

Cellulose acetate was used to cover *A. thaliana*, and a leaf area outline of the whole plant was traced onto it. This outline was then coloured in and an image captured onto an AnalySIS™ image-analyser using a monochrome CCD camera (Norfolk Analytical Ltd, Hilgay, UK). The area of leaves exposed to light was measured using the AnalySIS computer package (Software Imaging System, Münster, Germany).

2.2.3 Dry Weights

Leaf tissue (approximately 1g fresh weight) was weighed and then dried at 67°C for 7 days, and weighed again. The percentage water content of the tissue was then calculated.

2.3 Collection of *C. purpureus* in the Field

2.3.1 Comparison of Collection Method

Due to the difficulties of transporting liquid nitrogen, another method of storing plant material until analysis could be carried out in the laboratory had to be found. Muralidharan and Wemmer (1994) have used a Lysis, Storage and Transportation buffer (LST) to store animal tissue for DNA analysis. In order to determine if this buffer could also be used for storage of plant tissue, the following experiment was conducted. Three 2cm x 2cm patches of *C. purpureus* were irradiated with $0.5638 Wm^{-2}$ biologically effective UV-B for 1 hour in order to induce DNA damage. Control plants were kept in the dark for 1 hour. Approximately 2g of the green leaf tissue was then cut from the moss, and was either added to 5ml LST buffer or it was frozen in liquid nitrogen and stored at -80°C. Prior to DNA extraction, LST buffer was stored at room temperature. After 1 month, DNA damage was assayed as in section 2.4. Samples stored in the LST buffer were drained before DNA extraction. Samples were then ground in liquid nitrogen as in section 2.4.1. The results showed that there was no difference in cyclobutane pyrimidine dimer (CPD) or 6-4 photoproduct (6-4PP) accumulation in *C. purpureus* stored in either medium (2-way ANOVA 6-4PPs: $F = 0.45$, $p = 0.502$, $n = 184$; CPDs: $F = 0.28$, $p = 0.596$, $n = 184$), and therefore LST buffer was used as a storage medium for material collected in the field.

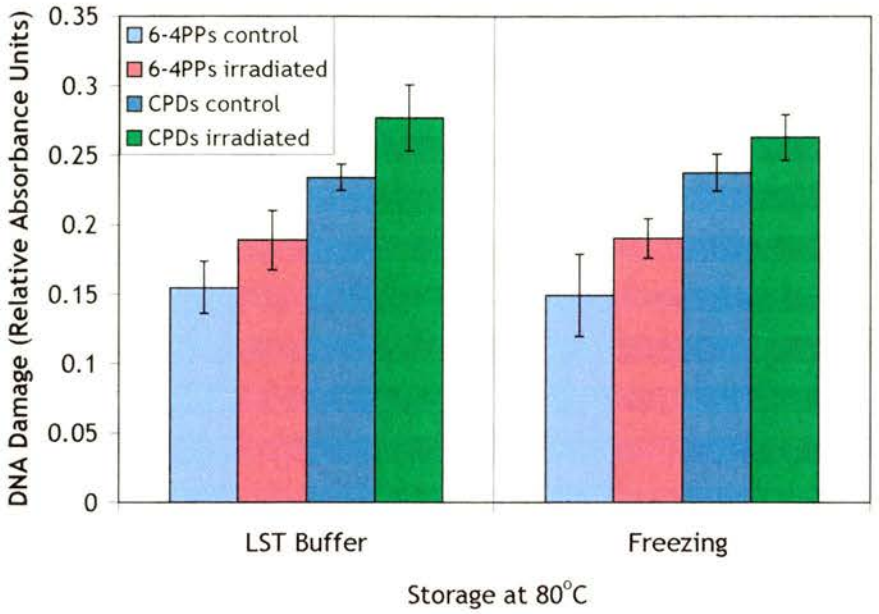


Figure 2.1: Comparison of methods for storage of plant tissue

DNA damage analysis of irradiated (0.5638Wm^{-2} biologically effective UV-B for 1 hour) and control (unirradiated) *C. purpureus* is unaffected by storage in LST buffer. Levels of both CPDs and 6-4PPs are similar in samples that have been stored at -80°C and in samples that have been stored in LST buffer

2.3.2 Antarctic Samples

Samples of *C. purpureus* were collected from Leonie Island (67 degrees 35', 68 degrees 20'W) on the 16th December 2001 by Dr Charles Cockell of the British Antarctic Survey. The study site was composed of dark soils, where *C. purpureus* was the only species present. A 1m x 1m mylar screen was erected at 2.5cm above ground level 2 hours before collection of the first sample. Three samples of *C. purpureus* from ambient conditions and from under the mylar screen were taken hourly during daylight, and every two hours during darkness. PAR and UV-B measurements were taken at ground level from ambient conditions and from under the screen. Temperature was measured using a Hobo Data Logger (Onset Computers, Massachusetts, USA). UV-B and PAR were measured using a Quantum Meter (Apogee Instruments, Logan UT) and a UVR Radiometer (UV Products, UK) respectively.

2.3.3 Arctic Samples

Samples of *C. purpureus* were collected from the Arctic field site (75 degrees 24.336 min N, 89 degrees 49.496 minW) on the 17th and the 21st-22nd July 2002 by Dr Charles Cockell. Field collection was carried out as in section 2.3.2, and samples were additionally collected from under a cellulose acetate screen which was also erected 2.5cm above the ground.

2.3.4 Scottish Samples

Field collection of *C. purpureus* was carried out in Dunlop, Ayrshire (grid reference NS 408 495) on the 5th August 2002. The study site was a domestic lawn where *C. purpureus* was the dominant species, but was growing amongst grass. Sample collection was carried out as in sections 2.3.2 and 2.3.3. Additionally, a 1m x 1m area was kept moist throughout the experiment by spraying it with water every hour during the day so that it was kept damp, but not soaked. PAR and UV-B measurements were taken at ground level from ambient conditions and from under the screens. Visible light was measured using a quantum photometer (see section 2.1.3), and UV-B was measured using a UVX Radiometer (CA, USA (200-400nm). Temperature and humidity were measured using a cable-free weather station (BAR 928, Oregon Scientific, USA).

2.4 Measurement of DNA Damage

2.4.1 Isolation of DNA

The following method was modified from Junghans and Metzlaff (1990). Approximately 50mg of frozen leaf tissue was ground in liquid nitrogen. The ground tissue was re-suspended in 1.5ml of Lysis Buffer (see Appendix) and the mix was kept in the dark (to prevent photorepair) at room temperature for 15 minutes. The sample was then aliquoted into two 1.5ml eppendorf tubes and 300µl of phenol (Tris stabilised to pH 7.5) was added to each. The solution was vortex-mixed and incubated at room temperature for two minutes. Chloroform (300µl) was added, the samples vortex-mixed and then centrifuged for three minutes at 10 000g in an MSE Micro-Centaur centrifuge (Scotlab, Coatbridge, UK) at room temperature. The supernatant was added to 600µl of phenol/chloroform (1:1 v/v). The sample was then vortex-mixed and centrifuged once more. Chloroform/isoamyl alcohol (600µl 24:1 v/v) was added to the

resulting supernatant, and the sample was vortex-mixed and centrifuged once more. The chloroform/isoamyl step was repeated to remove any traces of phenol from the sample. Ice-cold propan-2-ol (600µl) was added to the resulting supernatant, and the samples were held for at least one hour at –20°C. The samples were then centrifuged for 10 minutes at 10 000g, and the supernatant removed to reveal a pellet consisting of RNA and DNA. The pellet was washed in ice-cold 70% (v/v) ethanol and re-suspended in 400µl TNE (see Appendix). The preparation was left overnight at 4°C to allow the pellet to dissolve.

RNAase (50µg/ml) was added to each sample and the preparation was incubated at 37°C for 30 minutes. The RNAase was removed by one phenol/chloroform (600µl 1:1 v/v) and one chloroform (300µl) extraction. The DNA was precipitated by adding 1ml ethanol (96% v/v) and incubating for at least one hour at –20°C. Following centrifugation for 15 minutes at 10 000g at room temperature the supernatant was removed to reveal the DNA pellet. The DNA was washed by trickling ice-cold 70% (v/v) ethanol over it. This was removed and then the pellet was re-suspended in 100µl TE buffer (see Appendix). The sample was then left overnight at 4°C to allow the pellet to dissolve.

2.4.2 Quantification of DNA

An aliquot (20 µl) of each DNA extract solution (section 2.4.1) was diluted in 980µl of sterile distilled water and placed in a quartz cuvette. Each sample was placed in a spectrophotometer (Unicam Ltd., Cambridge, UK) and the absorbance of each sample at 260nm (A_{260})and 280nm (A_{280}) was read against a blank of distilled H_2O. The ratio of A_{260}:A_{280} was calculated (Vision softwear 1995, Unicam Ltd., Cambridge, UK). This ratio gives an indication of the purity of the DNA sample. Pure DNA gives a ratio of 1.8 while pure RNA produces a ratio of 2.0 (Sambrooke *et al.* 1989). Any sample with a wavelength ratio of under 1.6 or above 1.9 was discarded.

The amount of DNA in each preparation was then calculated using the formula:

A_{260} x 2500 = ng DNA per µl sample (A_{260} = absorbance at 260nm). Following this, the samples were diluted in TE to give 10ng of DNA per µl.

2.4.3 DNA Damage Analysis (ELISA Technique)

(For all solutions see Appendix). Thirty µl of each preparation (at a concentration of 10ng DNA per µl in TE) was added to each of eight wells of a 96-well microtitre (ELISA) plate (Corning Inc. New York, USA). This gave eight sub-samples of each preparation. Eight wells on each plate were loaded with the calf thymus standard (300ng DNA per well). Once the DNA had been added to the ELISA plates, they were left to dry with the lids slightly displaced for 48 hours at 37°C. The plates were then washed four times in PBS-T (100µl each wash) to remove non-absorbed DNA. Blocking solution (100µl) was added to each well and the plates were then left in the dark for 2 hours at 25°C. Each well was washed three times in 100µl PBS-T, and 50µl of either the 64M-2 (0.1% v/v in TE) or the TDM-2 (0.05% v/v in TE) antibody was added. These antibodies bind specifically to the (6-4) photoproduct (6-4PP), and the cyclobutane pyrimidine dimer (CPD) respectively, and they were a gift from Professor O. Nikaido (Kanazawa, Japan). The plates were left for 90 minutes in the dark, washed 5 times in PBS-T, 50µl of the secondary antibody (affinity purified goat anti-mouse IgG, 0.001% (w/v) in TE; Cambridge Biosciences, Cambridge, UK) added, and then left in the dark for a further 90 minutes. The plates were washed 5 times in PBS-T, and 50µl of a streptavidin-bound enzyme added (peroxidase conjugated streptavidin, 0.001%; Cambridge Biosciences, Cambridge, UK). Once again, the plates were kept in the dark for 90 minutes. The plates were then washed three times in PBS-T, once in sterile distilled water (100µl), and once in 50µl citrate buffer. Developer solution (50µl see appendix) was added, and the plates left in the dark for 30 minutes. The developing was then stopped by the addition of H_2SO_4 solution (25µl of 0.04% v/v). Absorbance of the samples was measured in a microtitre plate reader (Easy Reader 400, SLT Labinstruments, Austria) with the measuring wavelength at 492nm.

2.4.4 Preparation of Standards for DNA Damage Measurement

An assumption of this project is that UV-B has a damaging effect on DNA, producing CPDs and 6-4PPs. It is therefore to be expected that the greater the irradiance with UV-B, the more damaged the DNA will become. This assumption was tested by irradiating single-stranded calf thymus DNA (Sigma, Missouri, USA) with UV-B and measuring the damage as follows:

Calf thymus DNA (1ml of 10ng/µl in PBS) was placed in an open petri dish in a light-proof chamber and irradiated at 0.5638Wm^{-2} biologically effective UV-B for time intervals between 0 and 45 seconds at +5°C. An ELISA was then carried out to determine 6-4PP and CPD accumulation as described above and a standard curve obtained (primary antibody concentrations at 0.1% and 0.05% for 64M-2 and TDM-2 respectively).

The results showed that irradiance time, or the amount of UV-B, is directly related to DNA damage in the form of both 6-4PPs and CPDs (Figure 2.2). The assumptions of this project are therefore correct.

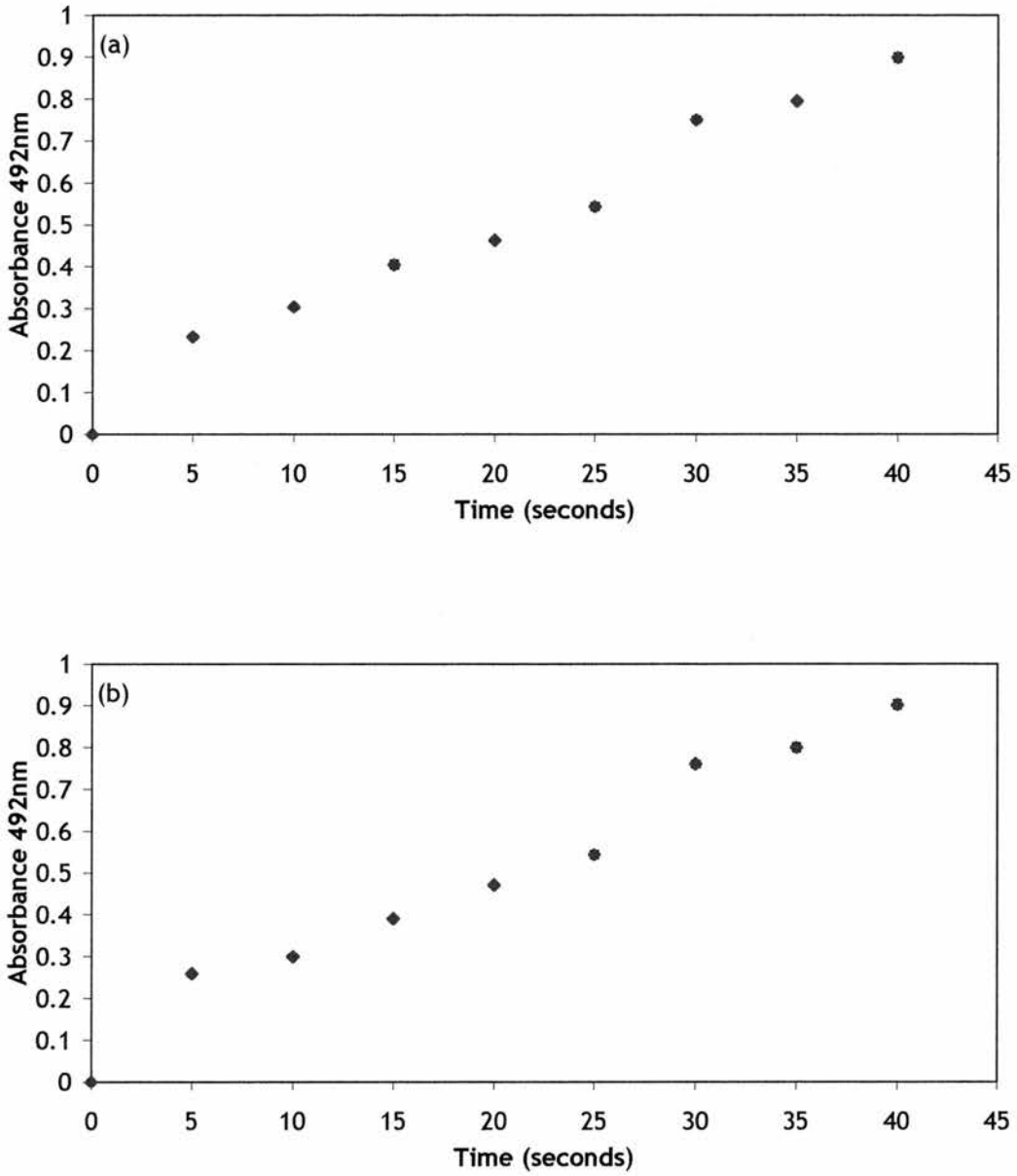


Figure 2.2: Irradiance of calf thymus DNA with UV-B

Both 6-4PPs (a) and CPDs (b) have a linear relationship with increasing UV-B irradiance. Each data point represents the mean of 4 samples (8 sub-samples per sample). Errors are standard errors of the mean.

2.4.5 Characterisation of the Antibodies

An ELISA was carried out using a range of concentrations of the 64M-2 and the TDM-2 antibodies from 0.025% (w/v) to 0.33% on the calf thymus DNA standard (300ng DNA per well) to determine the optimal concentration for each antibody.

The results show that at high concentrations of the primary antibody, there is non-specific binding resulting in an increased absorbance. The optimal concentration for the primary antibodies was determined as 0.1% and 0.05% for 64M-2 and TDM-2 respectively (figure 2.3).

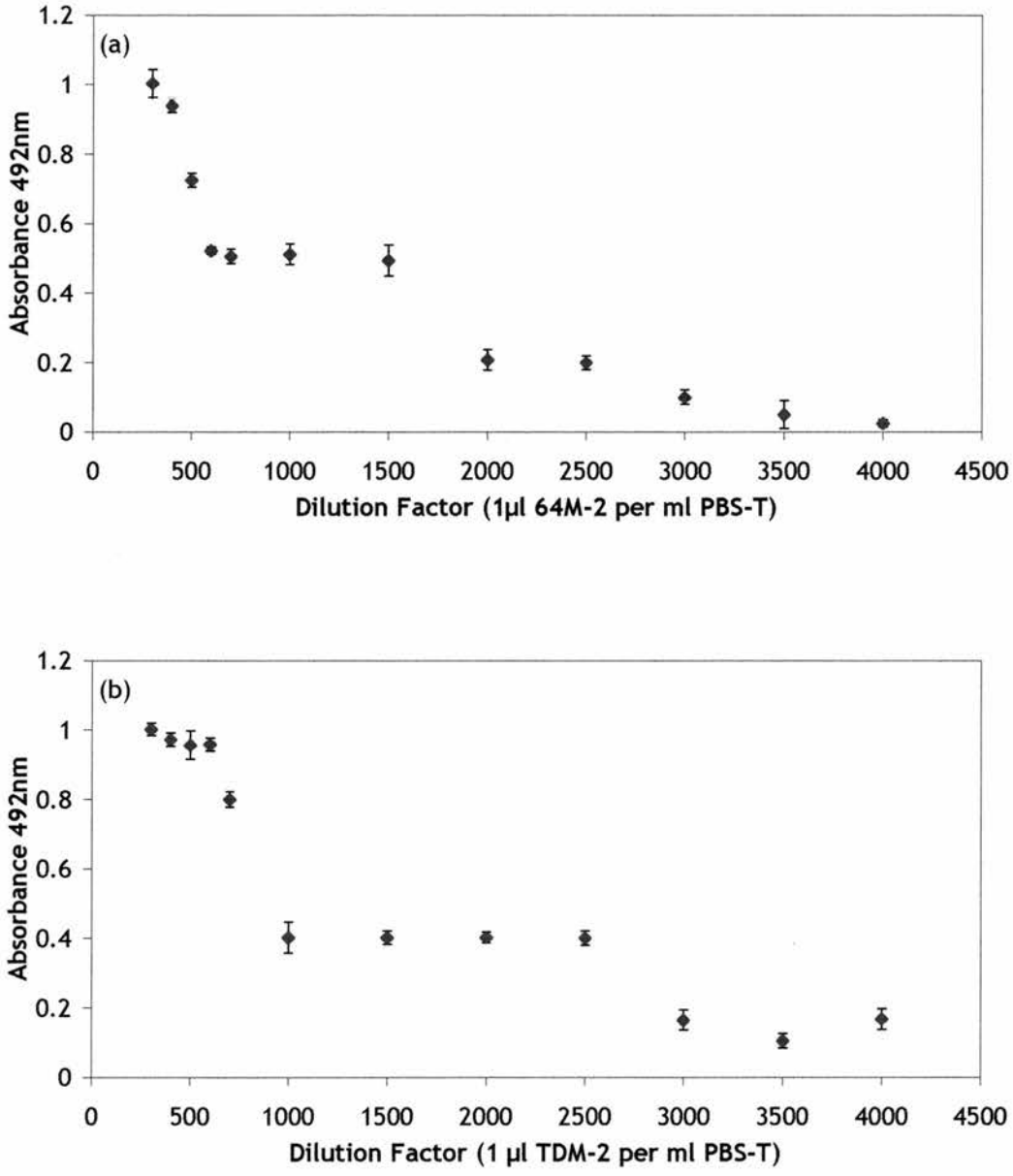


Figure 2.3: Characterisation of the primary antibodies, 64M-2 (a) and TDM-2 (b)

A dilution series of the 64M-2 and TDM-2 antibodies using a range of concentrations from 0.025% (w/v) to 0.33% on the calf thymus DNA standard (300ng DNA per well) to determine the optimal concentration for each antibody.

Each data point represents the mean of 4 samples (8 sub-samples per sample). Errors are standard errors of the mean.

2.4.6 Reproducibility of Results

In order to determine the minimum number of samples that could be used to obtain reliable results, an ELISA was carried out on 10 DNA extracts from *C. purpureus*. The cumulative standard error was calculated and the results showed that the optimal number of DNA extractions per treatment was 4 (Figure 2.4). This optimal number of treatments is where the standard error reaches a value where an increase in number of extracts will not have any significant effect on the standard error, and hence reproducibility of the experiment. Every procedure studying DNA damage therefore used at least 4 DNA extractions per treatment, and treatments were repeated at least twice.

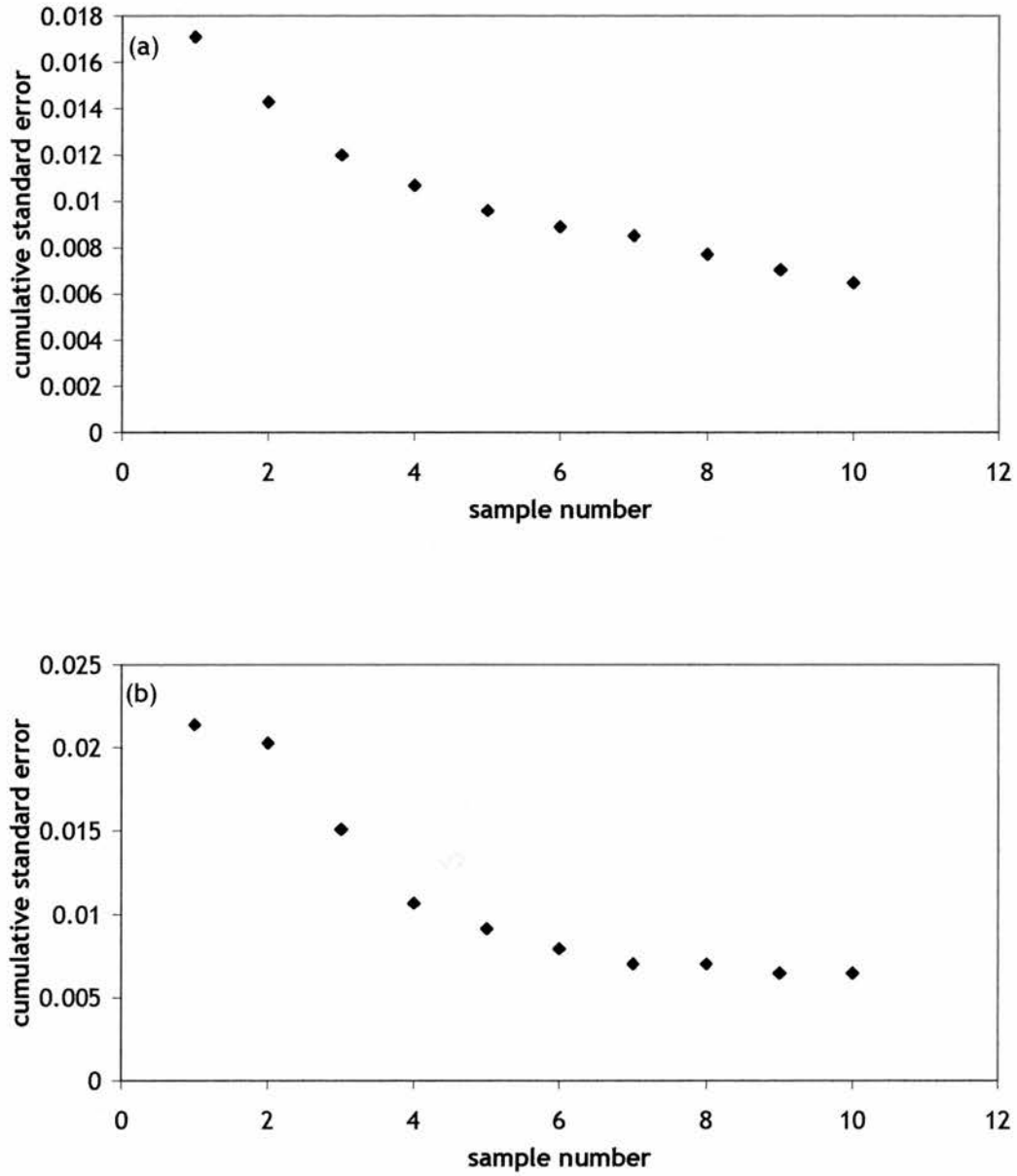


Figure 2.4: Cumulative standard error of DNA damage analysis carried out on *C. purpureus* DNA.

Each sample, or DNA extraction, consists of 8 sub-samples. The figure shows that in the case of both 6-4PPs (a) and CPDs (b), the optimal number of samples is 4.

2.4.8 Addition of Oxidant

C. purpureus was soaked with 0, 20 and 40μM methyl viologen in the dark and at 560μmol $m^{-2}s^{-1}$ light for 16 hours in order to induce free radicals. An ELISA was carried out in order to determine 6-4PP and CPD accumulation as in section 2.4.

2.4.8.1 Incubation of Methyl Viologen with the Primary Antibodies

In order to make certain that methyl viologen was not reacting with the primary antibodies, and hence giving a 'false positive' on the ELISAs, TDM-2 and 64M-2 were incubated for 16 hours with 40μM methyl viologen prior to carrying out an ELISA (section 2.4.3) on the UV-irradiated calf thymus DNA standard.

2.4.8.2 Incubation of Un-Damaged DNA with Methyl Viologen

Undamaged calf thymus DNA (10ng μl^{-1}) was incubated with 40μM methyl viologen at 560μmol $m^{-2}s^{-1}$ light for 16 hours.

2.5 Antioxidant Determination

2.5.1 Catalase Activity

The following method is taken from Mazza *et al.* (1999). Leaf tissue (100mg fresh weight) was homogenized in a mortar and pestle in 1ml of 50mM HEPES extraction buffer and centrifuged at 9500g for 20min at 4°C. Catalase (CAT) activity was measured spectrophotometrically by following the decrease in absorbance at 240nm (due to the degradation of H_2O_2) in a reaction mixture containing 0.96ml potassium phosphate (concentration) buffer (pH 7.0), 3μl H_2O_2 and 40μl of the extract. The absorbance change was recorded for 90 seconds, following the addition of the extract, and at the point where the rate was at its maximum.

2.5.2 Analysis of UV-B Absorbing Compounds

Three 0.5g (fresh weight) samples of *C. purpureus* leaves from each light treatment were taken at the same time on each day of the desiccation treatment. The samples were frozen in liquid N_2 and ground in 1ml methanol containing 1% (v/v) HCl. The resulting extract was centrifuged for 10mins at 10 000g at room temperature, the supernatant was removed and made up to a final volume of 1ml

with the methanol/HCl solution. The sample was then placed in a 1ml quartz cuvette (BDH, Poole, UK) and the absorbance read in a spectrophotometer by scanning between 280 and 320nm in 1nm increments. The total area below the scan trace (cm^2) was determined (Vision Software 1995, Unicam Ltd., Cambridge, UK) and the relative content of total UV-B absorbing compounds expressed as scan area per fresh weight of tissue (cm^2mg^{-1}).

2.5.3 Anthocyanin Content

Samples were extracted in 1ml methanol containing 1% (v/v) HCl as above. The supernatant was made up to 1ml with the extract solution, and the absorbance determined at 535nm on a spectrophotometer. Anthocyanin content was calculated from the equation derived from Beer's law, as given below, where the extinction coefficient for anthocyanins is taken as 35 000.

Concentration (g $1000ml^{-1}$) = absorbance/extinction coefficient

Anthocyanin (μg g^{-1} tissue) = A_{535} /35 000) x (10^6/100)/Fresh Weight (g)

2.5.4 Assay for Ascorbate and Dehydroascorbate

The following method is based on Kampfenkel *et al.*, (1995). Leaf tissue (0.2g) was frozen in a mortar with liquid nitrogen and ground to a powder. One ml of 6% (v/v) Trichloroacetic acid (TCA) was added and the resulting homogenate was transferred to a 1.5ml eppendorf tube. The homogenate was then centrifuged at (10 000g) for 2min and 20μl of the supernatant was added to six wells in a 96-well microtitre plate. Ten μl of dithiothreitol (DTT) solution and 10μl of 0.4M phosphate buffer were added to the wells. The plate was then incubated for 15 min at 42°C, and 10 μl of N-ethylmaleimide (NEM) was added. The plate was incubated for a further minute at room temperature, and then 80 μl of colour reagent was added. The plate was incubated for 45minutes at 42°C, and the absorbance measured in a microtitre plate reader (Easy Reader 400, SLT Labinstruments, Austria) at 550nm.

2.5.5 Assay for Ascorbate Only

Leaf tissue was homogenised and the supernatant obtained as in section 2.5.4. Twenty μl of 0.4M phosphate buffer, 20μl of the supernatant and 80μl of the colour reagent were added to six wells of a 96-well plate. The plate was incubated

at 42°C for 45mins and then the absorbance was read in a mictotitire plate reader (Easy Reader 400, SLT Labinstruments, Austria) at 550nm.

To obtain a reading for dehydroascorbate (DHA) only, ascorbate (ASA) results were subtracted from ASA + DHA results.

2.5.6 Preparation of Standards

A range of ascorbate concentrations (0-50nM) were prepared in 6% (w/v) TCA and the assays for ascorbate and dehydroascorbate carried out as above. A standard curve was obtained and used to calculate the ascorbate and dehydroascorbate concentration in the sample extracts (Figure 2.5).

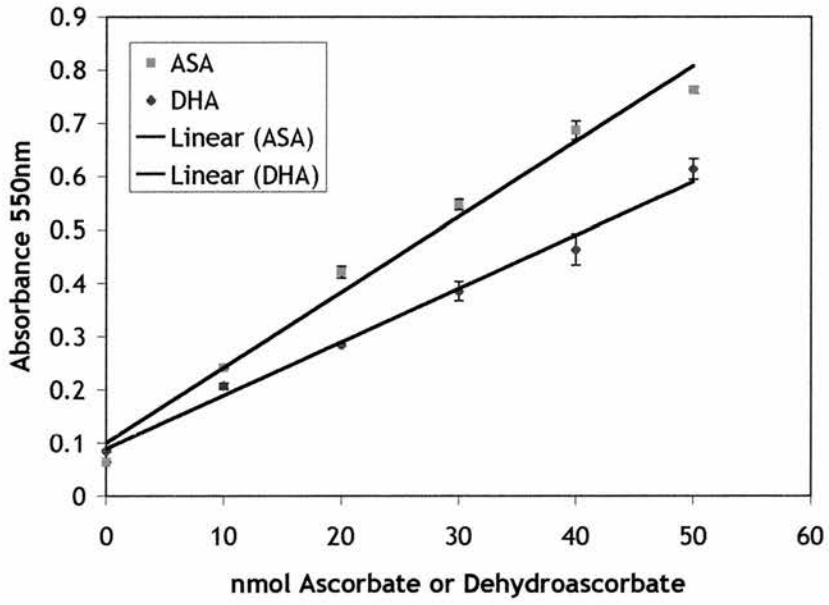


Figure 2.5: Standard curve for ascorbate and dehydroascorbate Assay

2.5.7 Feeding of *C. purpureus* with L-Galactono-1,4-Lactone

An experiment was carried out to determine whether feeding of L-galactono-1,4-lactone (the direct precursor to ascorbate) to *C. purpureus* could increase the ascorbate concentration in the moss. A solution of L-galactono-1,4-lactone (0, 10 and 20mM) and 0.1M silwet L-77 (Lehle Seeds, USA) was brushed onto 4cm x 4cm clumps of moss. The moss was sealed in plastic bags to retain moisture, and incubated at 25°C at 560 μmol m^{-2} s^{-1} 16 hours. Three samples of 0.2g of leaves

per treatment were then used to determine ascorbate or DHA content as in section 2.5.5. The results showed that "feeding" *C. purpureus* with 20mM L-galactono-1,4-lactone significantly increased the ascorbate content from 0.01nM per gram (fresh weight) to 1.36nM per gram (fresh weight) (Figure 2.6).

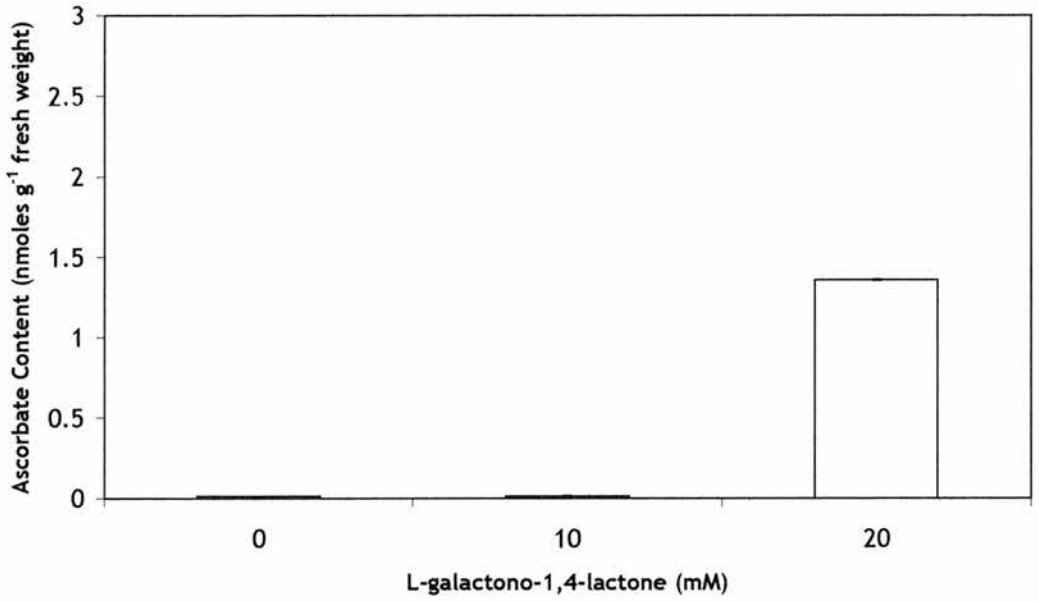


Figure 2.6: Increase in Ascorbate content of *C. purpureus* following pre-treatment with L-galactono-1,4-lactone

Brushing *C. purpureus* with 20mM L-galactono-1,4-lactone caused an increase in ascorbate content ($t = -5.82$, $n = 12$, $P < 0.05$).

2.5.8 Feeding of *A. thaliana* with L-Galctono-1,4-Lactone

Four-week old *Vtc3* and *wt A. thaliana* plants were brushed on both sides of their leaves with 20mM L-galactono-1,4-lactone containing 0.1mM Silwet L-77 (Lehle Seeds, USA). The plants were then covered with Saran Wrap and incubated at 560µmol $m^{-2}s^{-1}$ light for 16hrs at 25°C. Three samples of two leaves per plant were then used to determine ASA content as in section 2.5.5. The results showed that feeding of *A. thaliana* with 20mM of L-galactono-1,4-lactone significantly increased the ascorbate concentration from 1.16nmoles g^{-1} fresh weight to 2.84 nmoles g^{-1} fresh weight ($t = -7.46$, $n = 12$, $p < 0.05$) in *wt* plants, and from 0.8685nmoles g^{-1} fresh weight to 1.199nmoles g^{-1} fresh weight in *Vtc3*.

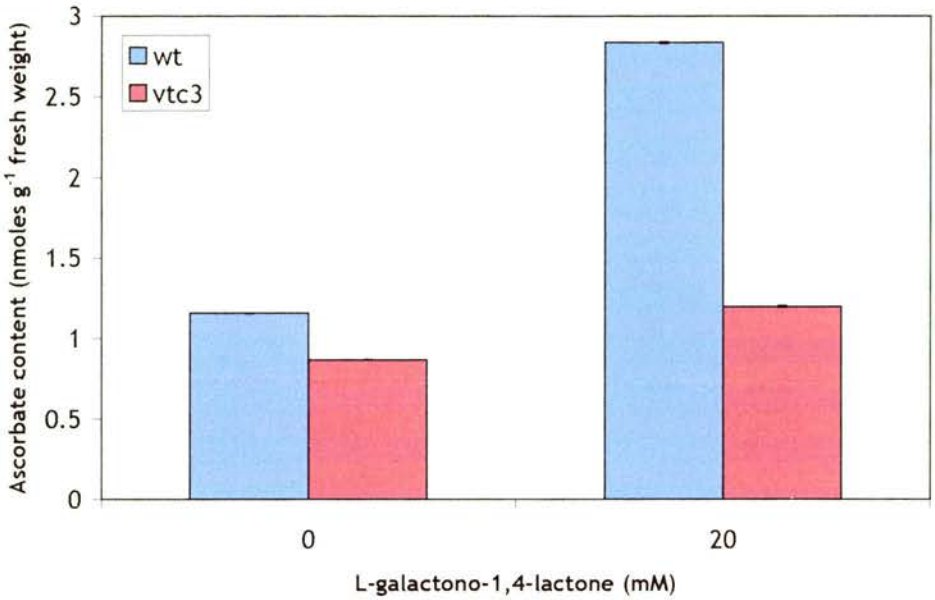


Figure 2.7: Increase in ascorbate content of *A. thaliana* leaves following pre-treatment with L-galactono-1,4-lactone

Brushing *A. thaliana* with 20mM L-galactono-1,4-lactone caused an increase in ascorbate content of plants ($t = -4.4$, $n = 12$, $p < 0.05$).

2.6 Determination of Protein Content

Protein content was determined using the Bradford (1976) method. Several dilutions of a thyroglobulin protein standard were prepared containing between 0 and 20µg ml^{-1} protein in distilled water. The protein standard (500µl) was added to 400µl H_2O and 100µl of BioRad Reagent (Coomassie Brilliant Blue G-250). The absorbance was read at 595nm in a spectrophotometer (Unicam Ltd., Cambridge, UK) 5mins after the addition of the BioRad Reagent. A standard curve was constructed (Figure 2.8) and this was used to calculate the amount of protein in leaf extracts used in the CAT assay procedure and the ASA and DHA assays (sections 2.5.1 and 2.5.4).

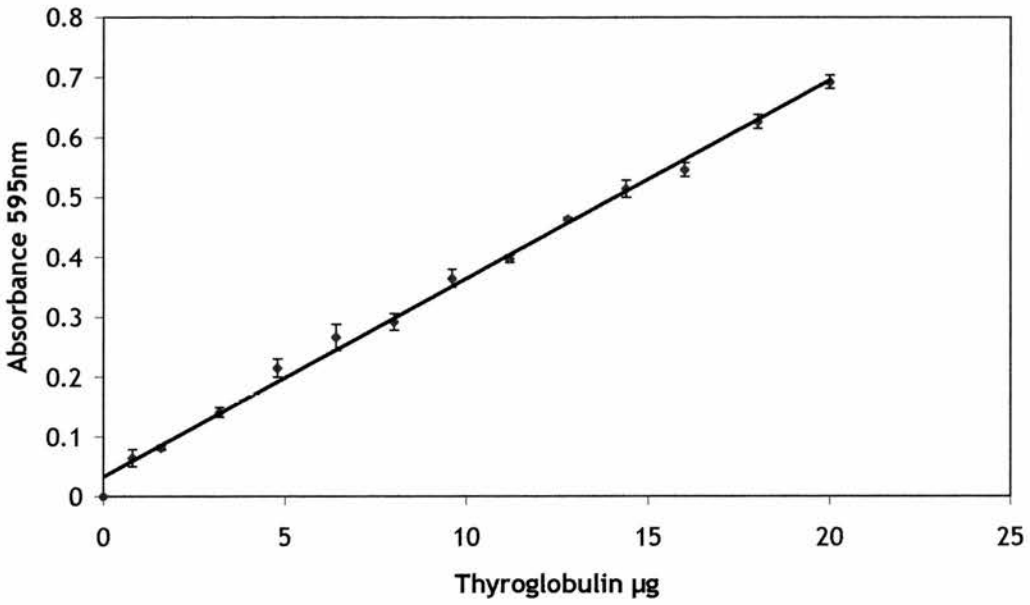


Figure 2.8: Thyroglobulin Standard Curve

Absorbance (595nm) of thyroglobulin standards using the Bradford (1976) method.

2.7 Statistical Analysis

All the data sets were correlated to confirm normal distribution, and statistical analysis performed by a t-test for paired comparisons, and the Tukey (95% confidence limit) ANOVA model for multi-comparisons, using the MINITAB™ programme (Minitab Inc, USA). No within-sample variation was found using this technique.

Chapter 3

Desiccation and DNA Damage

3.0 Introduction

As discussed in chapter one, DNA damage in the form of CPDs and 6-4PPs has been found to be induced by UV-B radiation. In fact there are no studies, to date, which show the formation of these photoproducts under any condition other than UV-B. Two of the greatest stresses affecting plants are desiccation stress and photodamage. Given that these can induce similar tolerance responses, for example induction of flavonoid biosynthesis, it might be predicted that an increase in plant defences against one of these stresses would help to defend the plant against the other stress (Csintalan *et al.*, 2001). The initial aim of this experimental work was therefore to determine if there is an interaction between desiccation stress and UV-B-induced DNA damage in *C. purpureus*. The hypothesis was that exposure to desiccation would reduce the susceptibility of the tissue to UV-B damage. However, the results of this study showed that under desiccation, *C. purpureus* accumulates CPDs and 6-4PPs in the absence of UV-B. This provides the first evidence of photoproduct accumulation in the absence of UV-B exposure.

3.1.1 Bryophytes are Ideal Plants for Desiccation Studies

Plants are sessile photosynthetic organisms, and as such, are obliged to expose themselves to the sun, which makes injury by desiccation stress and ultraviolet-B radiation a constant hazard. Bryophytes are particularly vulnerable to desiccation because of their morphology. They have no vascular tissue to carry water from the soil to the aerial parts of the plant. A matt of moss is made up of several plants. Lacking roots, each plant grips the substratum with elongate cells or cellular filaments called rhizoids (Watson, 1964). Mosses maintain equilibrium between atmospheric humidity and the hydrature of their protoplasm (they are poikilohydric). They may therefore be subjected to many drying/wetting cycles within a single day. The ability of bryophytes to withstand repeated desiccation and rehydration has led to a remarkable number of species that are desiccation-tolerant. For example, *Tortula ruralis* can be reduced to 20% of its original weight in two hours and even after prolonged periods (10 months) of desiccation resumes metabolic activity within minutes of receiving an adequate water supply (Bewley, 1972). Malta (1921; cited in Crawford, 1989) describes the moss *Grimia pulvinata* which survived 70 years in the dry state and still retained the capacity to

generate and grow. Spores of *Ceratodon purpureus* have remained viable even after drying for 16 years (Schofield, 1985).

3.1.2 Study Species

3.1.2.1 *Ceratodon purpureus*

C. purpureus, the species used in this study, is a low-growing moss that forms tufts or sometimes cushions. Its leaves are short and hair-like, spreading when moist to become folded or twisted when dry. The species is the primary colonizer of disturbed sites and when mature, the sporophytes are flame-red, hence its nick-name, the "fire moss". *C. purpureus* is found on a wide range of substances including soil, rock, wood, humus, slate and sand (Somero and Shaw, 1994). *C. purpureus* can grow in broad ranges of pH, and is able to tolerate a much higher pollution level than other mosses (Schofield, 1985). These two factors ensure that *C. purpureus* can survive in both urban and natural environments. The species has a very broad geographical range that includes all the major continents (Crum and Anderson, 1981). With its ability to grow heterotrophically in the dark, (Zeidler *et al.*, 1999) *C. purpureus* has proven useful in photobiological studies, particularly in studies of phytochrome control of phototropism (Sineschchekovv *et al.*, 2000; Esch *et al.*, 1999; Brucker *et al.*, 2000) and gravitropism (Kern and Sack, 2001; Kuznetsov *et al.*, 1999). The cosmopolitan distribution of *C. purpureus*, and its ability to dehydrate and rehydrate within hours make it an ideal organism for a study of the interaction of desiccation and UV-B radiation.

Figure 3.0 Photograph of *C. purpureus* Taken in the St Andrews field site

3.1.2.2 *Sphagnum auriculatum var. inundatum*

The genus Sphagnum is restricted to bogs where water shortages hardly ever exist (Crawford, 1989). Sphagnum forms large cushions or clumps in wet and boggy habitats. Every part of the moss is permeated with a system of delicate capillary tubes, having the effect of a very fine sponge. The cells readily absorb water and retain it. Water can be squeezed out, but the moss does not collapse, and is ready to take on fluid again. The absorbent abilities of Sphagnum have led to it being used widely as a rooting medium for orchids, stable litter and in surgical dressings (Schofield, 1985). Its common distribution and its desiccation-intolerance make *S. auriculatum* the ideal choice for comparison to *C. purpureus.*

3.1.3 Desiccation

Photooxidative damage is common under conditions when the light absorbed by plants is in excess of their capacity to utilise it in photosynthesis. An example of such a condition would be when a plant is subjected to desiccation stress (Lovelock *et al.,* 1995). Cellular damage induced by desiccation is often only manifested upon rehydration. For example, leakage of the cellular ions is often used as a measure of the extent of the membrane damage resulting from a wet/dry/wet cycle (Bewley, 1979; Bewley and Kronchko, 1982; Gaff, 1980). Other symptoms include misshapen chloroplasts and mitochondria that have lost their internal organization (Oliver and Bewley, 1984).

Desiccation-tolerant plants can be defined as those which can survive rehydration from the air-dried state. Although desiccation-tolerant plants are represented in most major classes of plants, the majority belong to the algae, bryophytes and lichens (Oliver, 1996). There are two main types of desiccation-tolerant plants:

1. Desiccation-tolerant plants whose internal water content rapidly equilibrates to the water potential of the environment.
2. Modified-desiccation-tolerant plants that employ physiological and morphological mechanisms to retard and control the rate of water loss (Oliver, 1996).

C. purpureus appears to employ both of these strategies. Although the internal water content of an isolated sample may rapidly equilibrate to the water potential of the environment, the species also grows in clump structures which may maintain some local humidity, thus preventing water loss (Alpert, 1987). Bewley (1979) concluded that there are three criteria which a plant or plant structure must meet in order to survive severe loss of water. These are:

1. Limitation of the damage incurred to a repairable level.
2. Maintenance of physiological integrity in the dried state.
3. Mobilization of repair mechanisms upon rehydration.

Bewley and Oliver (1992) simplified these criteria into a working hypothesis that desiccation tolerance can be achieved by mechanisms that are based on the protection of cellular integrity or are based on the repair of desiccation- (or rehydration-) induced cellular damage.

The protective mechanisms of tolerance appear to involve two major components, sugars and proteins, both of which are postulated to be involved in maintaining cellular integrity during the drying phase (Bewley *et al.*, 1993; Crowe *et al.*, 1992; Dure, 1993; Leopold *et al.*, 1992). Sucrose is the major free sugar available for cellular protection in desiccation-tolerant mosses (Bewley *et al.*, 1978; Smirnoff, 1992; Willis, 1964), although stachyose has been found in the Antarctic desiccation-tolerant moss *Bryum pseudotriquetrum* (Robinson *et al.*, 2000) and there may be other free sugars available. Dehydration and re-hydration of membranes causes them to pass through their phase transition temperature. During rehydration this leads to solute leakage at junctions between liquid crystalline and gel phases. Non reducing sugars such as sucrose which are present in tolerant cells at high concentrations can associate with the phospholipids head groups and prevent phase transitions (Crowe and Crowe, 1992). Sugars protect cytoplasm and high concentrations of sucrose (in desiccation resistant cells) along with oligosaccharides such as raffinose and stachyose, allow vitrification of the cytoplasm at low water content which prevents protein denaturation, crystallization and minimises spurious reactions (Leopold *et al.*, 1992). The gametophytic cells of the moss, *Tortula ruralis*, contain approximately 10% sucrose per dry weight (Strauss and Hauser, 1986). The concentration of sucrose

does not alter in *T. ruralis* when desiccation occurs in the dark and the light (Bewley *et al.*, 1978), and the lack of an increase in soluble sugars during drying seems to be a common feature of desiccation-tolerant mosses (Smirnoff, 1992), suggesting that they are permanently in a state of readiness for desiccation.

Gas exchange and chlorophyll fluorescence measurements have shown that recovery of photosynthesis in bryophytes after desiccation can be remarkably rapid, and appears to leave little time for repair processes requiring synthesis of proteins or other cell components on any substantial scale (Tuba *et al.*, 1996; Csintalan *et al.*, 1999; Marschall and Proctor, 1999). Proctor and Smirnoff (2000) determined that the photosystems of the desiccation-tolerant mosses *Racomitrium lanuginosum*, *Anomodon viticulosus* and *Rhytidiadelphus loreus* survive a drying and rewetting episode essentially intact, and return to a functional state with remarkable rapidity. Proctor and Smirnoff (2000) added protein synthesis inhibitors to rehydrated moss, and found that their addition had no effect on initial recovery rates of photosynthesis, suggesting that there is no need for rapid protein synthesis following desiccation. Oliver *et al.*, (Oliver 1991, 1996; Bewley and Oliver 1992; Oliver and Bewley 1997; Oliver *et al.*, 1998) suggest that desiccation tolerance in bryophytes is essentially repair-based. They propose that repair of damage caused during desiccation (or rehydration) is linked with the synthesis of proteins called rehydrins which are preferentially synthesised following rehydration. Proctor and Smirnoff's results (2000) indicate that protein synthesis is not immediately necessary upon rehydration, and suggest that the repair process is likely to be a matter of reassembly rather than resynthesis. Their results indicate that the requirement for protein synthesis during recovery is brought about by photo-oxidative damage (Foyer *et al.*, 1994).

3.1.4 Desiccation-Induced DNA Damage

Active oxygen is a common factor in the response of plants to the major environmental extremes (Smirnoff, 1993). A low water content can hinder antioxidant defences and leave tissue exposed to oxidative deterioration. Along with oxidative DNA damage, desiccation in mosses can lead to oxidation of the protein sulphydryl groups (Smirnoff 1993) and lipid peroxidation (Dhindsa and

Matowe, 1981; Seel *et al.*, 1992). Oxidised proteins or lipids can in turn react with DNA to form adducts (Esterbauer *et al.*, 1990).

Oxidative damage in more tolerant mosses is known to be less severe if the mosses are desiccated slowly (Smirnoff, 1993). Slow drying may be less damaging either because it allows acclimation to occur before metabolism ceases or because the rapid water movement during fast drying is physically disruptive (Smirnoff, 1993). A large decrease in water content can change enzyme and substrate concentrations as well as any association between enzymes which usually allow substrate channelling (Hrazdina and Jensen 1992). The result could be misdirection of electrons followed by increased superoxide formation.

DNA is a relatively simple system in terms of reaction with free radicals. Its reactive components are the four bases, the sugar deoxyribose, and phosphate. Phosphate is relatively unreactive towards free radicals, but the bases are readily oxidised or reduced. Reactions of free radicals with deoxyribose are particularly significant, leading to either loss of a base (the mechanism is not fully understood), a strand break (C-4 position), or internal cyclization with a base attached to it (Simic and Javonoic 1986). The 5,6 double bond of pyrimidines is particularly vulnerable to hydroxyl radicals (Lu, 2001). Pyrimidine radicals are very reactive and disappear rapidly in reactions with each other. However, if the pyrimidine radicals are part of a DNA macromolecule, the damage is less severe due to the impaired mobility of macromolecule radicals (Simic and Javnoic 1986). Due to their high reactivity, the hydroxyl radical and the hydrogen atom are the species most damaging to DNA (Simic and Javonoic 1986). There are no reports of oxidative damage resulting in the formation of CPDs or 6-4PPs.

3.1.5 UV-B-Induced DNA Damage

As already discussed in chapter 1 (section 1.2), CPDs and 6-4PPs are the two main DNA damage lesions produced as a result of UV-B irradiation. Other minor photoproducts may also be produced, such as cytosine photohydrates, but CPDs and 6-4PPs are far more common (Fisher and Johns, 1976). The two main repair pathways for these lesions are photorepair and excision repair. Photorepair is thought to be the major repair pathway for CPDs. The light-dependent enzyme,

photolyase, binds to the damage lesion to directly reverse the damage (Kim and Sancar, 1993). Excision repair is the primary repair mechanism for 6-4PPs, and this type of repair has been reported absent for CPDs in some species (Trosko and Mansour, 1968, 1969; Swinton and Hanawalt, 1973). Excision repair can take place in the dark, and the process involves removal of the damaged nucleotide followed by its replacement. This can involve as many as 30 different gene products, along with requiring energy in the form of ATP (Britt, 1995; Moné *et al.*, 2001).

3.1.6 Aim of Chapter:

1. To determine if there is an interaction between desiccation stress and DNA damage induced by UV-B.
2. To determine if desiccation stress, in the absence of UV-B, results in formation of CPDs and 6-4PPs in *C. purpureus* and in *S. auriculatum.*

3.1.7 Review of Materials and Methods

In order to determine if desiccation had any effect on the response of *C. purpureus* to UV-B, *C. purpureus* was kept in either a hydrated or a desiccated state before being subjected to UV-B (See methods section 2.1.1.2 and 2.1.1.3) and DNA damage analysis was carried out as in section 2.4. The results of this study showed that DNA damage had accumulated by the end of the desiccation period in the absence of UV-B. In order to determine when damage occurs during desiccation, *C. purpureus* was monitored during desiccation using chlorophyll fluorescence as an indication of the state of desiccation (section 2.2.1.4). The above studies were all carried out at 100μmoles m^{-2} s^{-1} PAR where photorepair may have been active. *C. purpureus* was desiccated for 6 days in the absence of UV-B under 0, 100 and 560 μmoles m^{-2} s^{-1} light in order to determine of light was having an effect of DNA damage accumulation. Daily samples were taken for water content (section 2.2.3), chlorophyll fluorescence (section 2.2.1.4) and DNA damage (section 2.4). Following the six day desiccation period, *C. purpureus* was rehydrated under the three light regimes (section 2.1.1.4). DNA damage and Fv/Fm was measure five hours after rehydration.

In order to determine if DNA damage occurs during desiccation in a species other than *C. purpureus*, *Sphagnum auriculatum* was desiccated in the dark for six days as described in methods section 2.1.1.3. Samples were taken at day 0 and day 6 for DNA damage analysis (section 2.4).

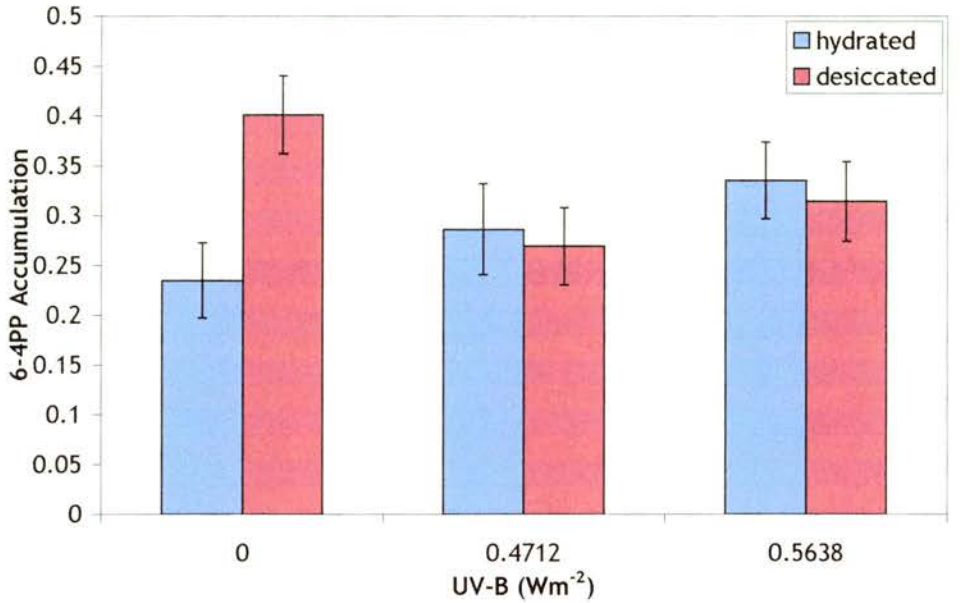
3.2 Results

3.2.1 Interaction of UV-B Irradiation and Desiccation on DNA Damage in *C. purpureus*

Figure 3.3a shows that 6-4PP accumulation significantly increases with increased UV-B in fully hydrated moss (ANOVA $F_{2,1}=13.65$, $p<0.05$). Desiccated plants, in the absence of UV-B, were found to have significantly more 6-4PPs than hydrated plants ($t=-7.71$, $df=143$, $p=<0.05$). However, desiccated samples which were irradiated with UV-B had lower levels of 6-4PPs than desiccated plants in the absence of UV-B ($t=4.94$, $df=143$, $p<0.05$).

CPDs were also seen to increase in hydrated plants with an increase in UV-B ($F=85.37$, $p<0.05$ figure 3.3b). Desiccation caused an overall significant decrease in CPD accumulation ($F=145.04$, $p<0.05$), although the level of CPDs was not significantly different in hydrated and desiccated plants at each level of UV-B radiation. There was a slight decrease in Fv/Fm with increasing UV-B irradiation, although this was not significant ($F=1.4$, $p=0.27$).

a)



b)

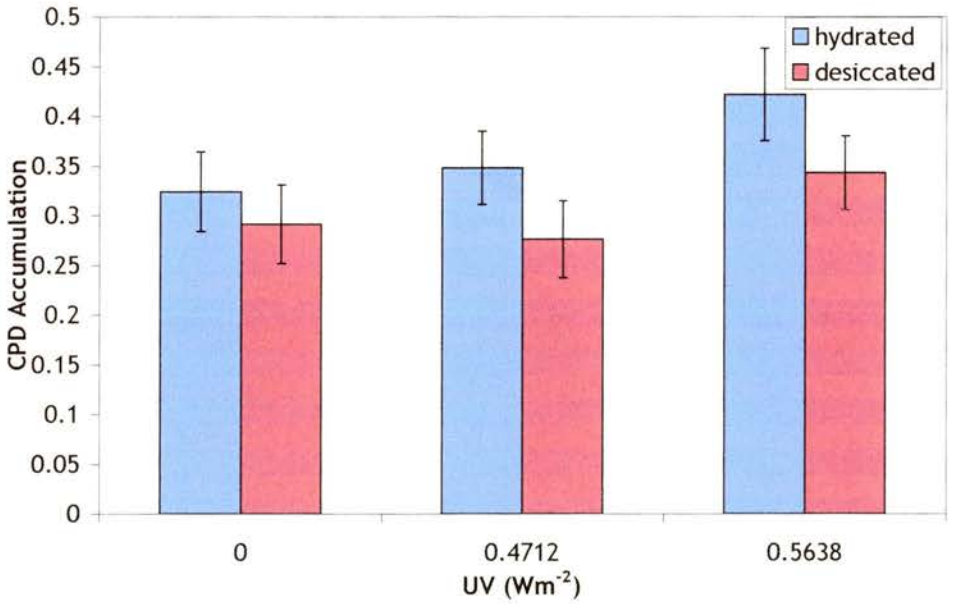


Figure 3.3: Interaction of UV-B and desiccation on 6-4PP (a) and CPD (b) accumulation in *C. purpureus*

C. purpureus was either kept in a hydrated condition or desiccated in 100 µmol m^{-2} s^{-1} light conditions until the Fv/Fm dropped to below 0.1. The hydrated and desiccated samples were then exposed to 0 Wcm^{-2}, 0.4712 Wcm^{-2} or 0.5638 Wcm^{-2} UV-B for one hour. 6-4PP and CPD accumulation was measured as relative absorbance units (see methods sections 2.4.3). Data show means of 8 individual DNA extractions. Errors are standard error of the mean.

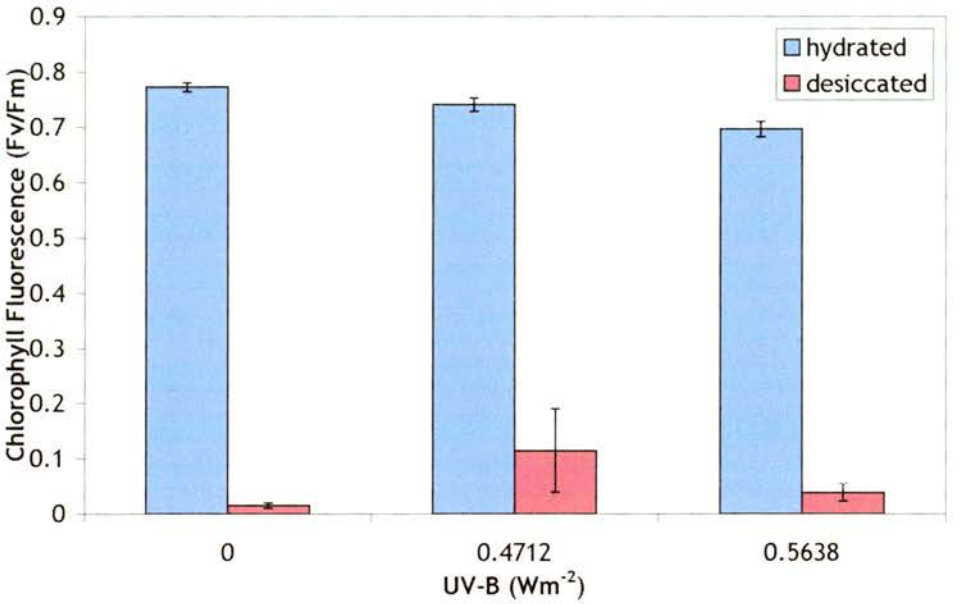


Figure 3.4: Interaction of UV-B and desiccation on chlorophyll fluorescence in *C. purpureus*

C. purpureus was treated as described in figure legend 3.3. Fv/Fm was measured at the beginning and the end of the desiccation period. Data show the means of 8 Fv/Fm measurements. Errors are standard error of the mean.

3.2.2 DNA Damage in *C. purpureus* During the Desiccation Period

The results of the previous section showed that DNA damage had accumulated by the end of the desiccation period. The aim of this section of work was to determine when the damage occurs during desiccation. *C. purpureus* was monitored during desiccation using chlorophyll fluorescence in light conditions of 100 μmol m^{-2} s^{-1}. When the Fv/Fm reached 0.753, 0.227, 0.058 and 0, samples were taken for DNA damage analysis. The results show that, as before, there is an accumulation of 6-4PPs (figure 3.5 $F=97.58$, $p<0.05$) during desiccation. There is no significant increase in CPDs during the course of desiccation ($t=-1.92$, $df = 71$, $p=0.059$).

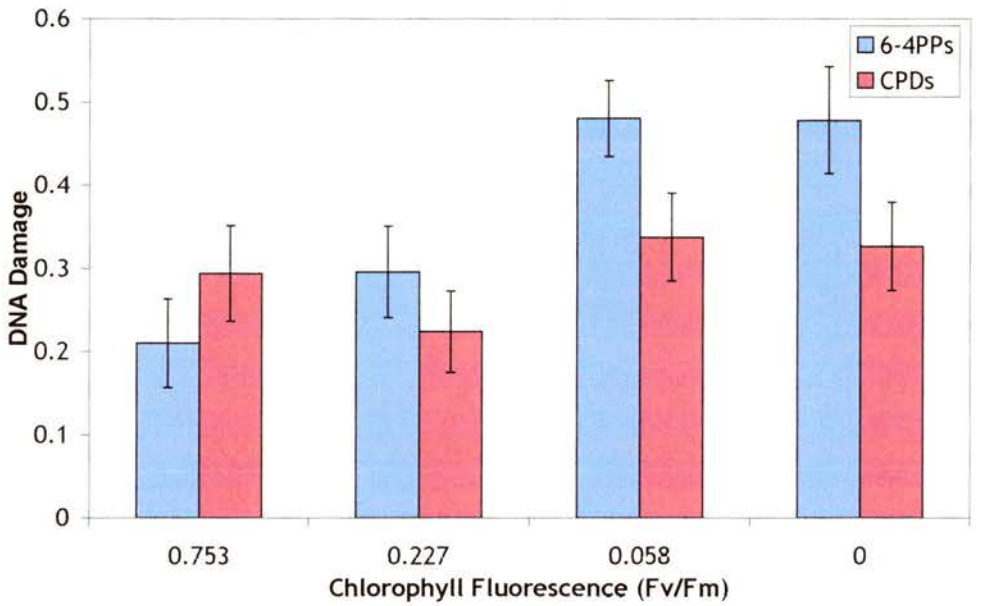


Figure 3.5: Accumulation of CPDs and 6-4PPs in *C. purpureus* at various stages of desiccation

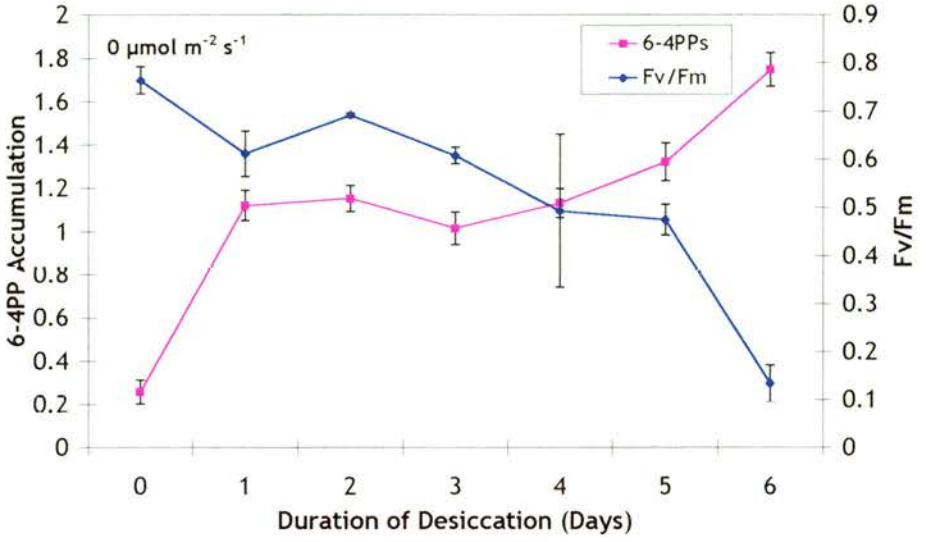
Chlorophyll fluorescence (Fv/Fm) was used as an indicator of the extent of desiccation. Samples were taken for DNA damage analysis (as described in methods section 2.4) when the Fv/Fm reached 0.753, 0.227, 0.058 and 0. Data shown are the means of DNA extractions from 9 individual samples of *C. purpureus*. Errors are standard error of the mean.

3.2.3 DNA Damage in *C. purpureus* Desiccated Under Three Light Regimes

The previous two sections looked at DNA damage in *C. purpureus* desiccated under 100μmoles m^{-2} s^{-1} (PAR). As photorepair may have been active under these conditions, *C. purpureus* was desiccated for six days at three different light intensities 0, 100 and 560 μmol m^{-2} s^{-1} (PAR) in order to determine if light was having an effect on DNA damage accumulation. Chlorophyll fluorescence, water content and DNA damage were measured daily. Figures 3.7 to 3.12 show that there is an increase in 6-4PPs with decreasing Fv/Fm and water content in *C. purpureus* desiccated in the dark. 6-4PPs increase significantly with decreasing water content ($F=16.9$, $p<0.05$), and this response is unaffected by light ($F=1.95$, $p=0.143$). It should be noted that during desiccation at each of the three light regimes, 6-4PP accumulation increased during the first few days and was followed by a decrease in damage. A further increase in 6-4PP accumulation was then observed.

Figures 3.13-3.18 show that there is an increase in CPDs with a decrease in water content and Fv/Fm in dark and 100 μmol m^{-2} s^{-1} light conditions. Desiccation under the three light regimes has a significant effect on CPD accumulation with desiccation in the light resulting in fewer CPDs. (Light: $F=77.62$, $p<0.05$; Water content: $F=6.62$, $p<0.05$). At day 6 of desiccation, *C. purpureus* desiccated at 560 μmol m^{-2} s^{-1} had lower levels of CPDs than *C. purpureus* desiccated at 100 and 0 μmol m^{-2} s^{-1}.

a)



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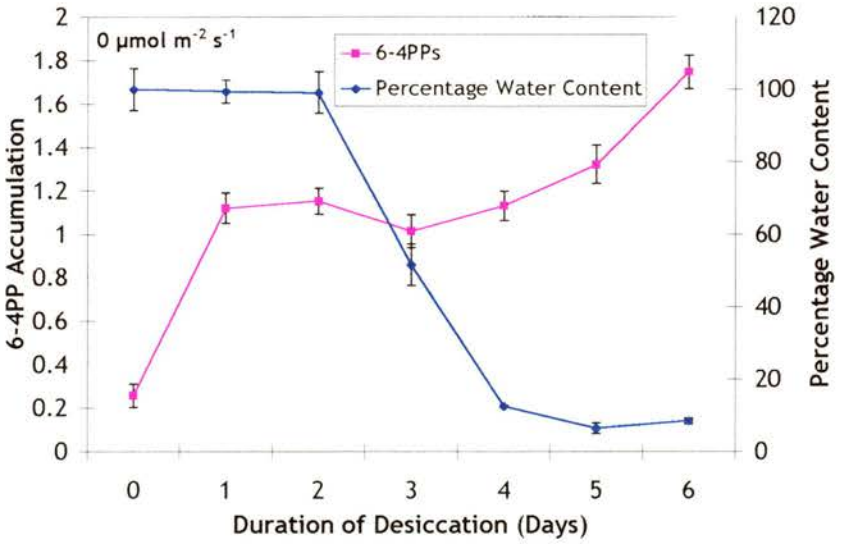
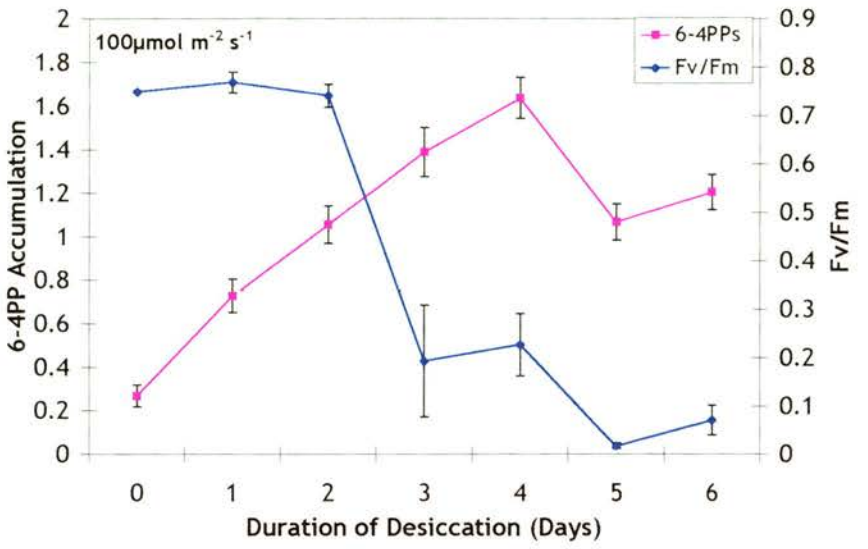


Figure 3.6: 6-4PP accumulation in *C. purpureus* desiccated in the dark for six days with Fv/Fm (a) and water content (b)

C. purpureus was desiccated in the dark as described in methods section 2.1.1.3. Daily samples were taken for analysis of DNA damage, water content and chlorophyll fluorescence (Fv/Fm) (see methods sections 2.4.1, 2.2.3 and 2.2.1.4 respectively). DNA data show the means of six individual DNA extractions. Fv/Fm and water content data show the means of six samples. Errors are standard error of the mean.

a)



b)

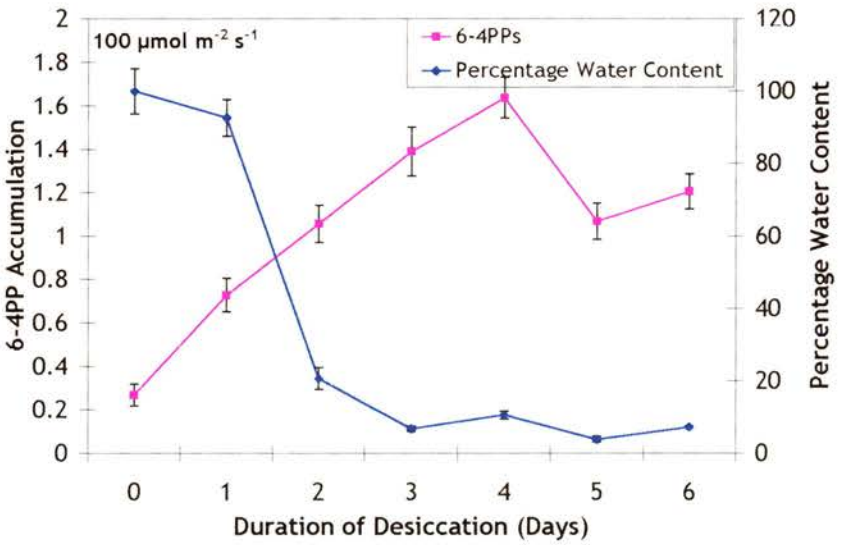
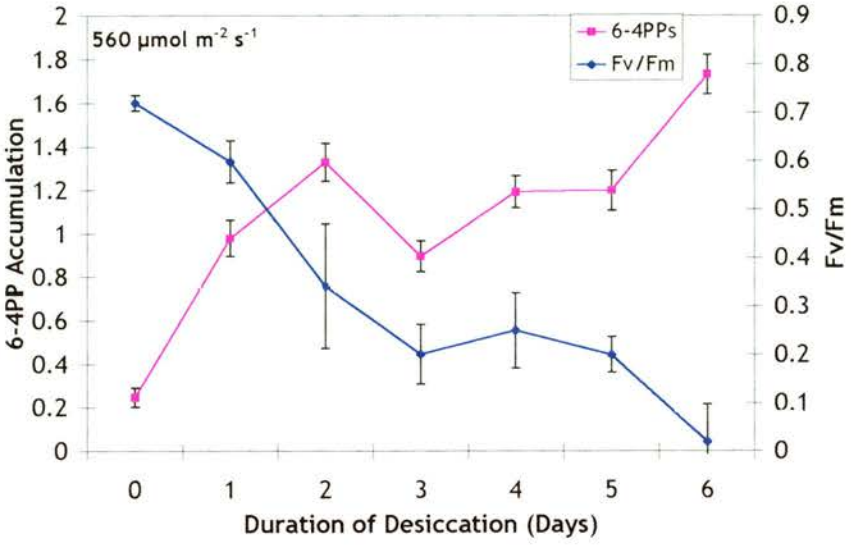


Figure 3.7: 6-4PP Accumulation in *C. purpureus* desiccated in 100 µmol m^{-2} s^{-1} with Fv/Fm (a) and water content (b).

C. purpureus was desiccated in 100 µmol m^{-2} s^{-1} light as described in figure legend 3.6.

a)



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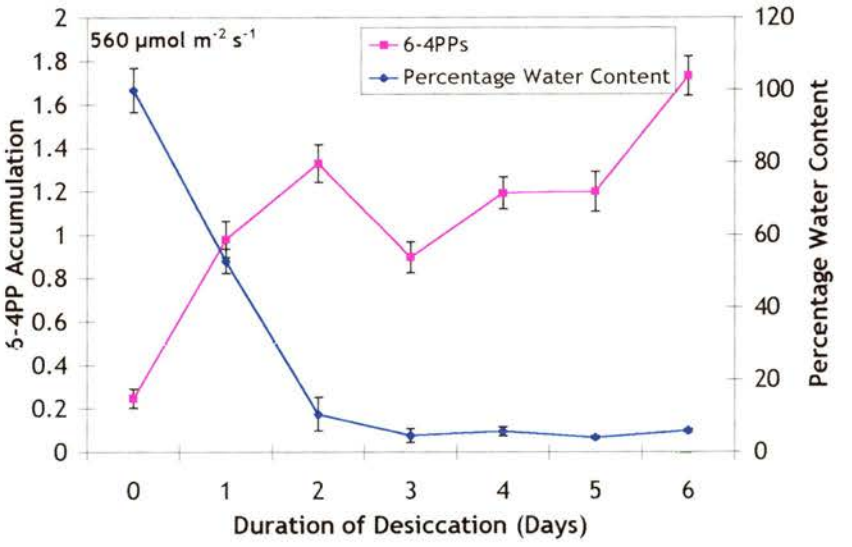
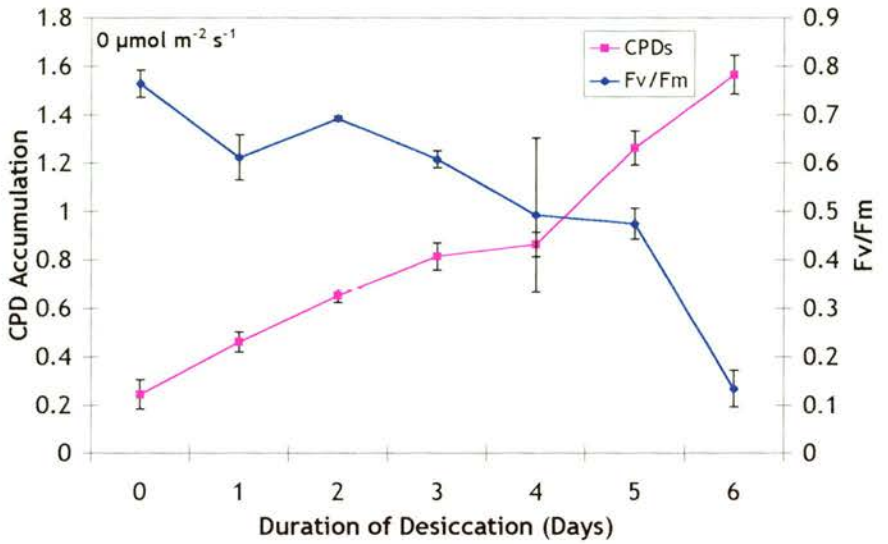


Figure 3.8: 6-4PP Accumulation in *C. purpureus* desiccated in 560 µmol m^{-2} s^{-1} with Fv/Fm (a) and water content (b).

C. purpureus was desiccated in 560 µmol m^{-2} s^{-1} light as described in figure legend 3.6.

a)



b)

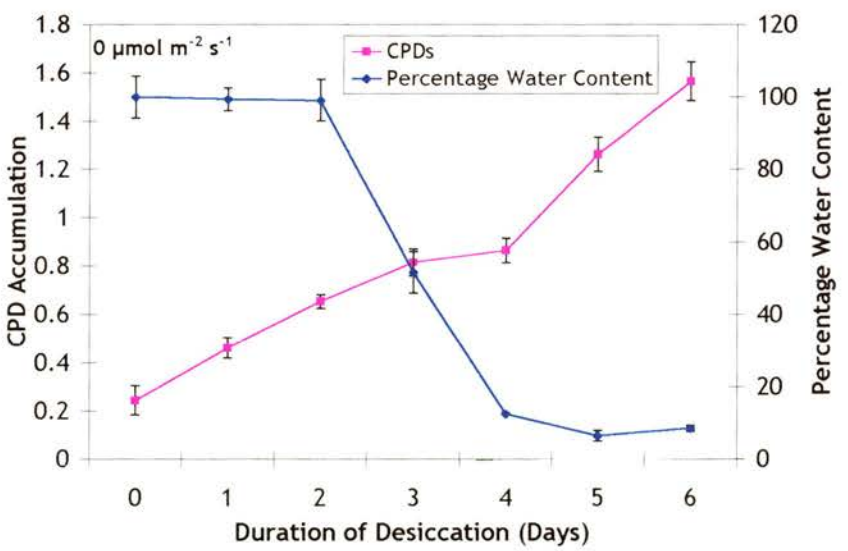
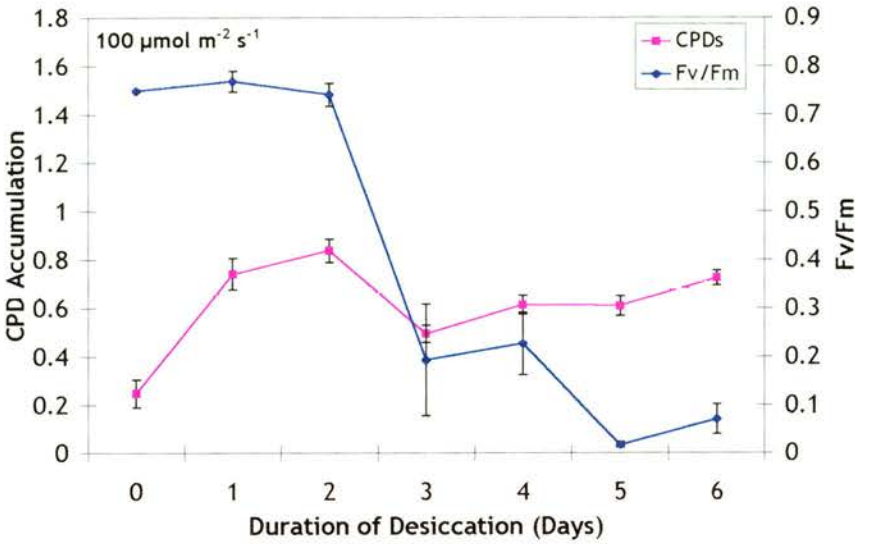


Figure 3.9: CPD accumulation in *C. purpureus* desiccated in the dark for six days with Fv/Fm (a) and water content (b)

C. purpureus was desiccated in the dark as described in methods section 2.1.1.3. Daily samples were taken for analysis of DNA damage, water content and chlorophyll fluorescence (Fv/Fm) (see methods sections 2.4.1, 2.2.3 and 2.2.1.4 respectively). DNA data show the means of six individual DNA extractions. Fv/Fm and water content data show the means of six samples. Errors are standard error of the mean.

a)



b)

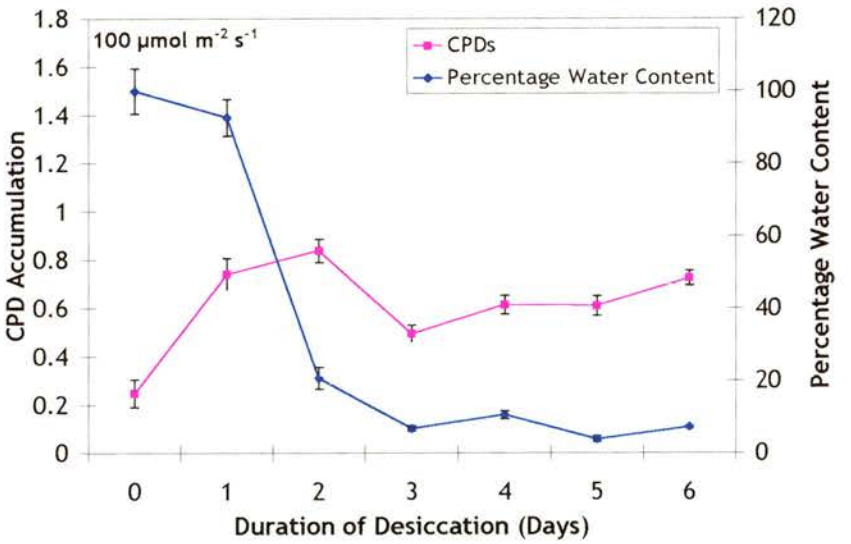
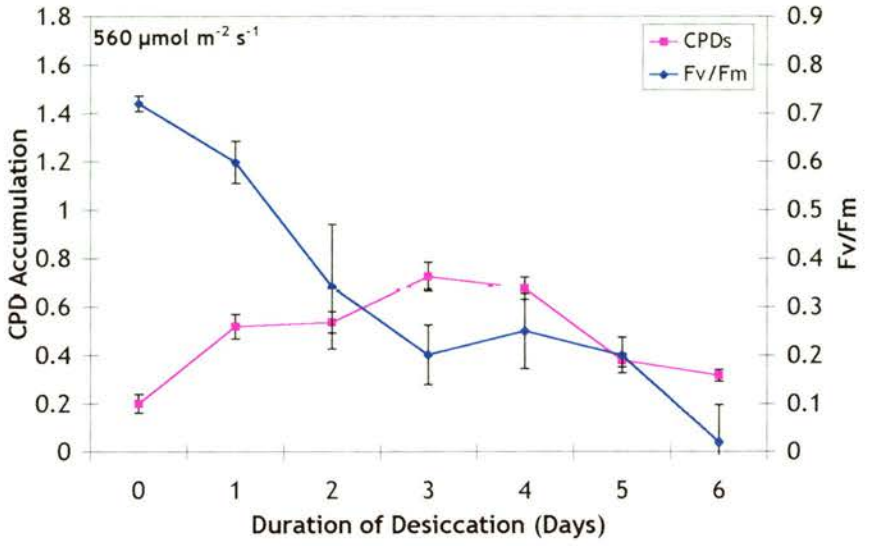


Figure 3.10: CPD Accumulation in *C. purpureus* desiccated in 100 µmol m^{-2} s^{-1} with Fv/Fm (a) and water content (b).

C. purpureus was desiccated in 100 µmol m^{-2} s^{-1} light as described in figure legend 3.9.

a)



b)

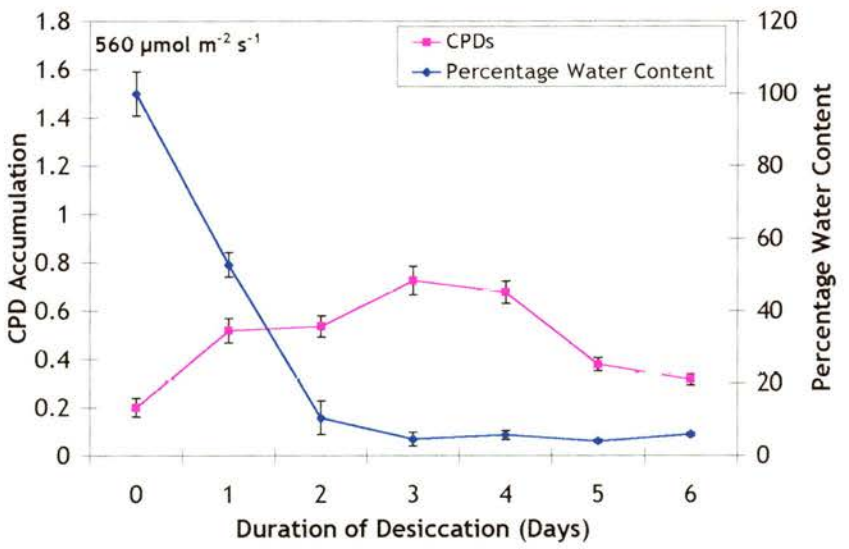


Figure 3.11: CPD Accumulation in *C. purpureus* desiccated in 560 µmol m^{-2} s^{-1} with Fv/Fm (a) and water content (b).

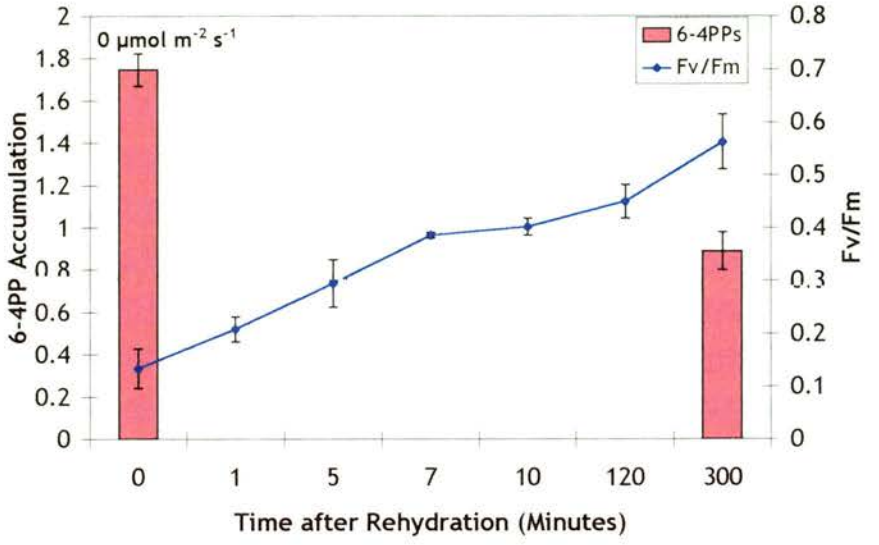
C. purpureus was desiccated in 560 µmol m^{-2} s^{-1} light as described in figure legend 3.9.

3.2.4 DNA Damage Following Rehydration in *C. purpureus*

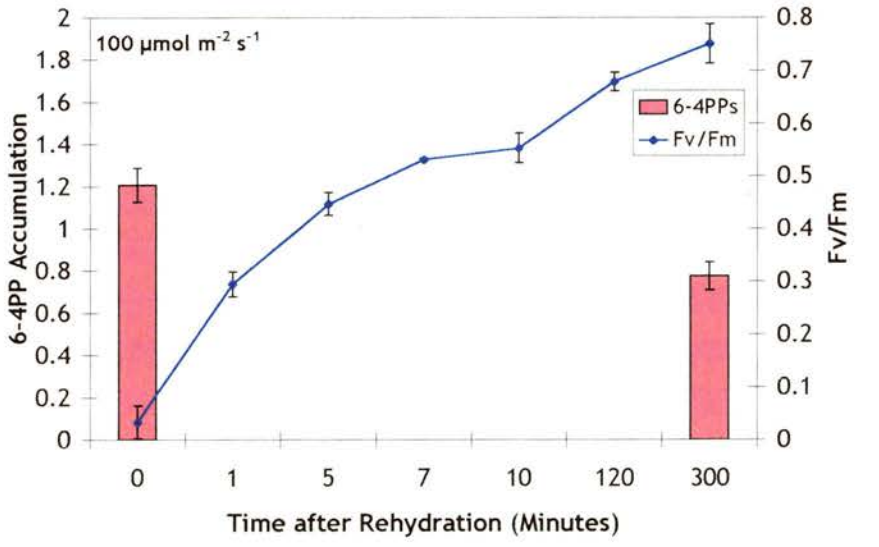
Following the six day desiccation period, *C. purpureus* was rehydrated by spraying with distilled water (see methods section 2.1.1.4). Chlorophyll fluorescence measurements and DNA damage analysis was carried out during this rehydration period. Figure 3.12 shows that in each of the three light regimes the accumulation of 6-4PPs decreased with increasing Fv/Fm. The decrease in 6-4PPs was significant for all three light regimes (dark: $t=10.09$, $n=6$, $p<0.05$; 100 μmol m^{-2} s^{-1}: $t=10.10$, $n=6$, $p<0.05$; 560 μmol m^{-2} s^{-1}: $t=15.14$, $n=6$, $p<0.05$). Recovery of Fv/Fm was greatest in *C. purpureus* desiccated at 100 μmol m^{-2} s^{-1} ($F_{2,12}=19$, $p<0.05$).

Figure 3.13 shows that CPDs significantly decrease with increasing Fv/Fm at 0 and 100 μmol m^{-2} s^{-1}light, but do not decrease significantly at 560 μmol m^{-2} s^{-1} (dark: $t=55.9$, $n=6$, $p<0.05$; 100 μmol m^{-2} s^{-1}: $t=-101.86$, $n=6$, $p=<0.05$; 560 μmol m^{-2} s^{-1}: $t=-0.03$, $n=6$, $p=0.975$). Recovery of Fv/Fm upon rehydration is highest in *C. purpureus* kept at 100 μmol m^{-2} $s^{-1}$and lowest at 560μmol m^{-2} s^{-1}.

a)


0 µmol m-2 s-1
6-4PPs
Fv/Fm
6-4PP Accumulation
Fv/Fm
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0.1
0.3
0.5
0.7
0
1
5
7
10
120
300
Time after Rehydration (Minutes)

b)


100 µmol m-2 s-1
6-4PPs
Fv/Fm
6-4PP Accumulation
Fv/Fm
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0.1
0.3
0.5
0.7
0
1
5
7
10
120
300
Time after Rehydration (Minutes)

c)

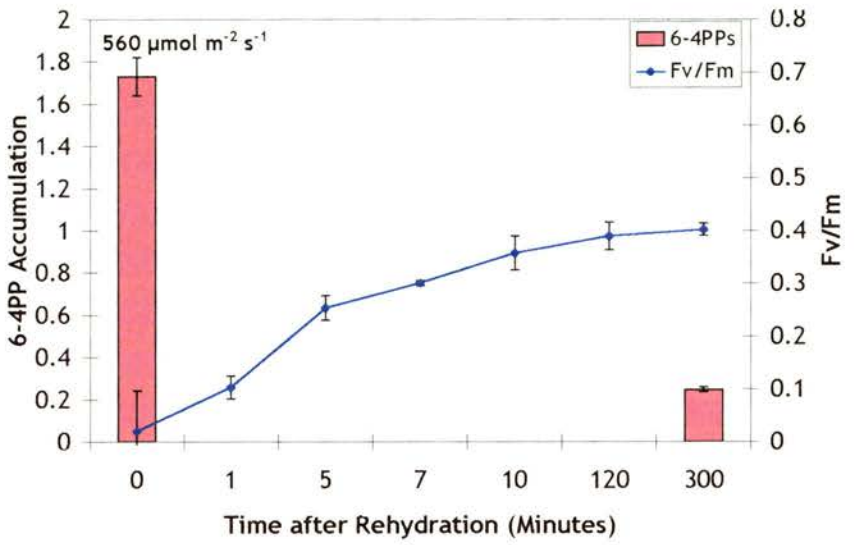
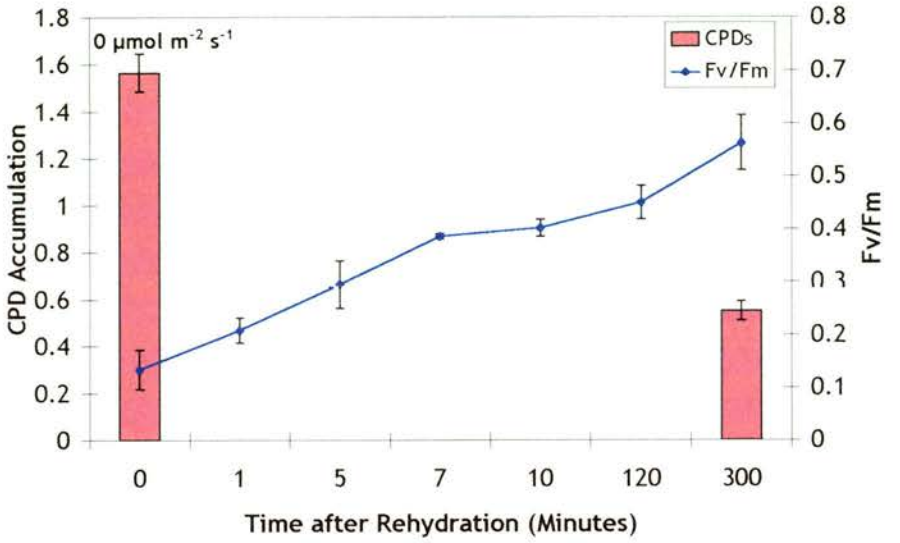


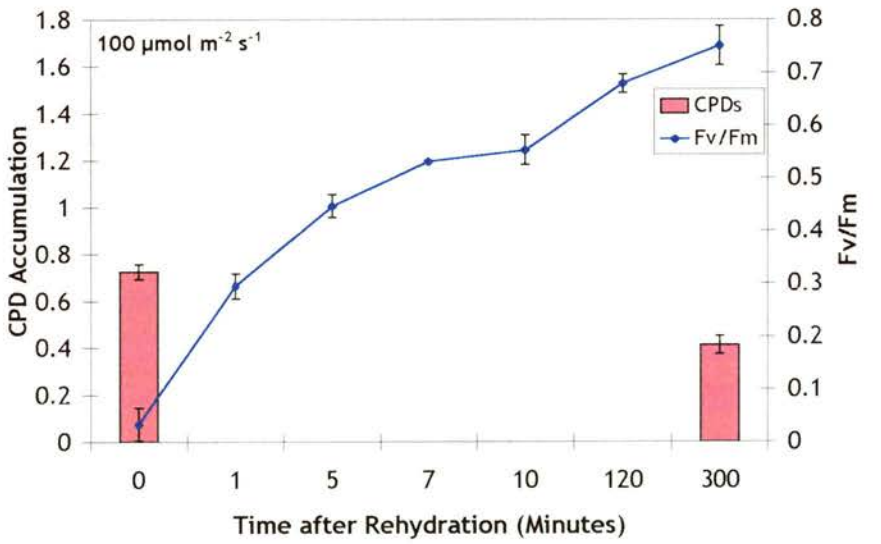
Figure 3.12: 6-4PP Accumulation following rehydration in *C. purpureus*

Following desiccation for six days under the three light regimes, *C. purpureus* was sprayed with distilled water, and kept moist in this way for five hours at 25°C at 0 (a), 100 (b) and 560 (c) µmol m^{-2} s^{-1} light (see methods section 2.1.1.4). The Fv/Fm was recorded at intervals throughout this "recovery" period, and a sample was collected at the end of this treatment for DNA damage analysis. DNA data show the means of six individual DNA extractions. Fv/Fm data show the means of six samples. Errors are standard error of the mean.

a)


0 µmol m-2 s-1
CPDs
Fv/Fm
CPD Accumulation
Fv/Fm
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0.1
0.3
0.5
0.7
1
5
7
10
120
300
Time after Rehydration (Minutes)

b)


100 µmol m-2 s-1
CPDs
Fv/Fm
CPD Accumulation
Fv/Fm
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0.1
0.3
0.5
0.7
1
5
7
10
120
300
Time after Rehydration (Minutes)

c)

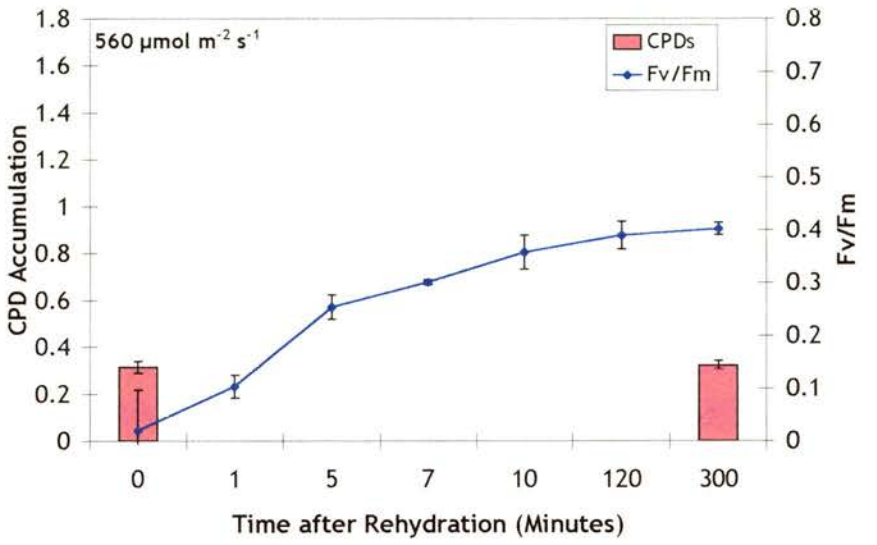


Figure 3.13: CPD Accumulation following rehydration in *C. purpureus*

Following desiccation for six days under the three light regimes, *C. purpureus* was sprayed with distilled water, and kept moist in this way for five hours at 25°C at 0 (a), 100 (b) and 560 (c) µmol m^{-2} s^{-1} light (see methods section 2.1.1.4). CPD Accumulation and Fv/Fm were then measured as described in figure legend 3.12

3.2.5 DNA Damage with Desiccation in Sphagnum *auriculatum*

In order to determine if desiccation results in DNA damage in species other than C. purpureus, S. *auriculatum* was desiccated in the dark for six days as described in the methods section 2.1.1.3. Figure 3.14 shows that there is a significant decrease in 6-4PP accumulation with desiccation in *S. auriculatum* (t=2.55, n=32, p=0.016). In contrast, there is a significant increase in CPD accumulation (t=-2.01, p=0.048, n=40).

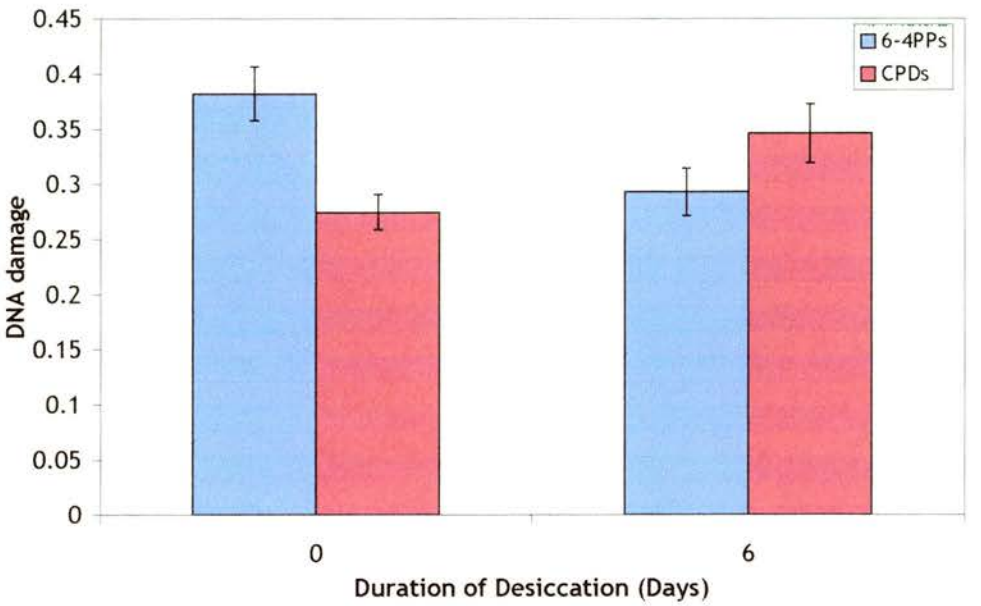


Figure 3.14: Accumulation of 6-4PPs and CPDs in Sphagnum after 0 and 6 days desiccation.

S. *auriculatum* was desiccated in the dark at 25°C for six days as described in section 2.1.1.3. Samples were taken at day 0 and day 6 for DNA damage analysis. Data are means of six individual DNA extractions. Errors are standard errors of the mean.

3.3 Discussion

3.3.1 Desiccation in *C. purpureus* Induces 6-4PPs and CPDs in the Absence of UV-B

As CPD and 6-4PP accumulation are well documented to occur with UV-B radiation, the observed increase in both these photoproducts with increasing UV-B radiation was expected. However, what is surprising is that desiccation in the absence of UV-B radiation resulted in a significant increase in 6-4PP and CPD accumulation. The former occurred in all three light regimes, whereas CPDs only increased significantly in the dark and under 100 μmol m^{-2} s^{-1} light. This increase in DNA damage with desiccation may be because either the enzymes involved in the repair processes are not fully hydrated, and therefore have lost activity, or it could be that desiccation stress causes damage to DNA- either directly or indirectly. Remarkably, the results firmly indicate that the repair systems in *C. purpureus* are operating under extremely low (4% RWC) water contents. Owing to the presence of light, it cannot be determined if this repair is excision or photorepair. The increase in 6-4PPs, during desiccation in the absence of UV-B, observed therefore suggests that desiccation is causing the damage.

Perhaps the most likely explanation for the increase in DNA damage with desiccation is that desiccation results in the formation of active oxygen species which damage the DNA (Lu, 2001; Simic and Javonoic, 1986). CPDs and 6-4PPs have not been reported to occur under any condition other than irradiation with UV-B. In response to the results of figure 3.3, this chapter was devoted to determining if CPDs and 6-4PPs do indeed occur as a result of desiccation. Desiccation of *C. purpureus* was therefore carried out under the three light regimes in order to determine the patterns of DNA damage accumulation with differing repair responses. Photorepair is unable to occur in the dark, and therefore desiccation in the dark prevents simultaneous photorepair (Hada *et al.*, 2000; Nakajima *et al.*, 1998; Chen *et al.*, 1994). However in dark conditions, excision repair may still occur (Taylor *et al.*, 1996; Britt, 1995, 1996; Sancar and Tang, 1993; Sancar and Sancar 1999). With one repair process inhibited in the dark, it was expected that if desiccation did cause DNA damage, then more damage would accumulate during desiccation in the dark than during desiccation in the

light. Alternatively, photorepair may become enhanced by a high light intensity and therefore it was expected that less DNA damage would be found in samples desiccated under high light. These hypotheses depend upon *C. purpureus* being able to repair DNA damage whilst in the desiccated state.

3.3.2 Repair of Damage in the Desiccated State

Oliver *et al.*, (Oliver, 1991, 1996; Bewley and Oliver, 1992; Oliver and Bewley, 1997; Oliver *et al.*, 1998) suggested that desiccation-tolerant plants repair any damage caused by desiccation upon rehydration. Proctor and Smirnoff (2000) contested this, and proposed that desiccation-tolerant plants are in a continual state of readiness for desiccation, therefore preventing damage during desiccation, making repair upon rehydration unnecessary. The current results suggest that, at least for *C. purpureus*, DNA damage caused during desiccation can be repaired whilst the plant is still in the desiccated state. *C. purpureus* desiccated in 560 μmol m^{-2} s^{-1} light, where photorepair is possible (Kim and Sancar, 1993), accumulated lower levels of CPDs than samples desiccated in the dark. *C. purpureus* desiccated at 100 μmol m^{-2} s^{-1} light, had less 6-4PPs on day 5 of desiccation than on day 4.

3.3.3 The Differential Repair Response

Although repair does appear to be possible in desiccated plants, CPDs and 6-4PPs may not be repaired in the same way. Quaite *et al.*, (1994) found that alfalfa (*Medicago sativa* L.) seedlings preferentially used photorepair when there was a low level of DNA damage. At higher levels of CPDs, the plants then used both excision and photorepair. Quaite *et al.*, (1994) suggest that this differential repair response may provide an efficient and beneficial strategy. Excision repair is not an error free process. The nicks induced during "repair" can trigger a UV stress response that can result in the cessation of protein synthesis, thus halting growth (Kastan *et al.*, 1991; Nelson and Kastan, 1994). Excision repair requires approximately 30 different gene products (Moné *et al.*, 2001) and thus there is much potential for errors to occur. Photorepair is, however, inherently error free, non mutagenic and requires only external energy in the form of photoreactivating light. Hence, it might benefit a plant to preferentially use photorepair when DNA damage levels are low, and, when the damage levels are higher, to use both photo-

and excision repair (Quaite *et al.*, 1994). In the light of these results, I support the theory suggested by Quaite *et al.*, (1994), and furthermore propose that DNA damage may need to reach some sort of "threshold" level before repair is induced.

3.3.4 DNA damage thresholds

3.3.4.1 6-4PPs

In the light of my results and Quaite *et al.*'s (1994) theory of a differential repair response, I propose a model for a DNA damage threshold (figure 3.15). DNA damage in *C. purpureus* appears to accumulate to a critical, or "threshold" level before repair is induced. If further damage exceeds the repair capacity of the plant, then a further increase in DNA damage will result.

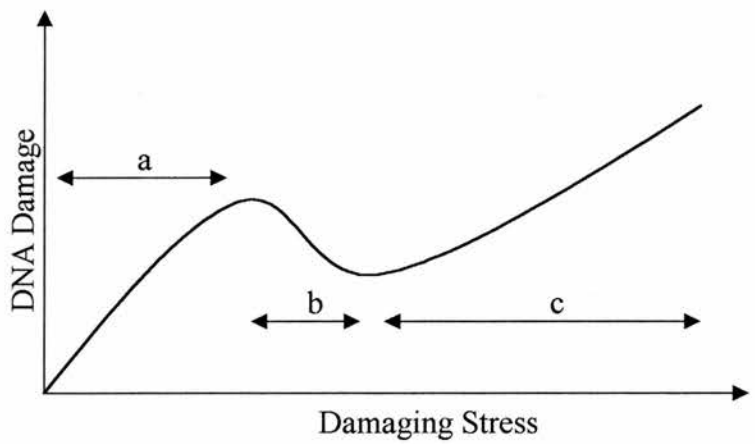


Figure 3.15: Proposed Model for a DNA Repair Threshold.

The model (based on results presented in this chapter) shows net accumulation of DNA damage (CPDs or 6-4PPs) with time. In section (a), any damage accumulated is tolerable to the plant, and little or no repair takes place. In section (b), the level of damage is higher than tolerable, and therefore repair occurs, resulting in a decrease in damage. Section (c) shows DNA damage accumulating faster than it can be repaired, resulting in an increase in damage. Every point on the graph represents a net balance between DNA damage and repair. A decrease in damage may be the result of a repair processes being promoted, rather than activated for the first time.

6-4PPs in *C. purpureus* desiccated under each of the three light regimes appear to accumulate to a critical level before repair is induced. Once the repair process(es) are activated, a decrease in 6-4PP accumulation is observed. Upon further desiccation, the level of damage exceeds the repair capacity of the plant, resulting in a further rise in 6-4PP accumulation. The level of damage reached before there is a decrease is different under the three light regimes. In samples desiccated in the dark, the lowest level of 6-4PP accumulation occurs on day two of the desiccation treatment. *C. purpureus* desiccated under 100 μmol m^{-2} s^{-1} light does not show a decrease in the number of 6-4PPs until after day four. The absorbance measurements of the 6-4PP damage (i.e., the amount of damage), the Fv/Fm and the water content of *C. purpureus* are all different in the three light regimes at the point when the damage threshold is reached. This indicates that water content alone is not directly responsible for the accumulation of damage, and the light environment does also play a role (probably by activating repair).

In the dark, it is impossible for the plant to undergo photorepair, as no photoreactivating light is available (Hada *et al.*, 2000; Chen *et al.*, 1994; Roza *et al.*, 1991). However, under 100 μmol m^{-2} s^{-1} light conditions, there may be the correct wavelengths of light present for photorepair (Kim and Sancar 1993). The delay in the decrease of damage may therefore be because photorepair is occurring. Once the level of damage reaches some form of damage threshold it may then initiate excision repair to work alongside photorepair. The following increase in damage would then be because the damage induced by desiccation exceeds the repair capacities of both photorepair and excision repair. *C. purpureus* desiccated under 560 μmol m^{-2} s^{-1} light showed a decrease in 6-4PP earlier than plants desiccated at 100 μmol m^{-2} s^{-1} (day two compared to day four). If 6-4PPs and CPDs are caused by oxidation, then it is possible that at 560 μmol m^{-2} s^{-1} light, *C. purpureus* becomes photooxidised. There may be too much excitation energy coming from the 560 μmoles m^{-2} s^{-1} light, causing further oxidation, and therefore DNA damage. The damage exceeds the repair capacity, and on day six of the desiccation, *C. purpureus* desiccated at 560 μmoles m^{-2} s^{-1} is seen to have approximately the same amount of 6-4PPs as plants desiccated in the dark,

suggesting that the level of damage is high, as it is possible for both repair systems to be working.

3.3.4.2 CPDs

In plants desiccated in the dark, an increase in CPD accumulation was observed. 6-4PP accumulation in the same samples showed the "threshold" pattern of a rise in damage, followed by a decrease, and another increase. The pattern of CPD accumulation in dark samples showed an almost steady increase. It is thought that photorepair is the primary repair mechanism for CPDs (Britt, 1996; Davies, 1995; Chen *et al.*, 1996; Taylor *et al.*, 1996). Excision repair of CPDs has been reported to be absent in some plants (Trosko *et al.*, 1968, 1969; Swinton and Hanawalt, 1973). However, during desiccation in the dark, the rate of damage accumulation slows down between days three and four. If the damage threshold argument is correct, then this slight decrease in the rate of damage accumulation could be due to excision repair of CPDs. As mentioned in section 1.4.2, excision repair of CPDs may have been reported absent in some plants if the plants were not damaged enough to repair the CPDs. Nothing is known about the relative toxicity of CPDs as compared to 6-4PPs in plants (Britt, 1996). It could be the case that CPDs are less toxic than 6-4PPs. The plant may then be able to tolerate a certain amount of DNA damage before repair takes place.

The high rate of repair of CPDs at 560 μmol m^{-2} s^{-1} differs from the high rate of 6-4PP accumulation found in the same samples. This differential response in photoproducts could be for a number of reasons. Either photorepair is more effective at removing CPDs than 6-4PPs, or CPDs are more toxic to the plant than 6-4PPs, and therefore damage must be repaired rather than tolerated. *C. purpureus* may not have photolyase specific for 6-4PPs.

3.3.5 Rehydration Following Desiccation Results in DNA Damage Recovery

C. purpureus rehydrated at 100_μmol m^{-2} s^{-1} light recovers Fv/Fm fully, whereas plants rehydrated in the dark and at 560 μmol m^{-2} s^{-1} light only recovered half of their potential Fv/Fm. It may be that the samples in 560 μmol m^{-2} s^{-1} light conditions could be experiencing photoxidation, particularly as it is generally thought that on rehydration following desiccation, plants may experience an

"oxidative burst" (Minibayeva and Beckett, 2001; Mayaba *et al.*, 2002). There is a decrease in 6-4PPs and CPDs following rehydration in all samples, except for CPDs in *C. purpureus* kept at 560 μmol m^{-2} s^{-1} light. In this case at the end of the desiccation period, CPD accumulation was already similar to levels in fully hydrated plants. Rehydration therefore cannot be expected to produce a decrease in CPDs. In some ways, it is surprising that rehydration results in a decrease in DNA damage. If desiccation is causing the DNA damage by oxidation, then an oxidative burst during rehydration could be expected to increase the number of photoproducts rather than reduce them. DNA damage was only assayed five hours after the plant was rehydrated. It is possible that an oxidative burst caused a sudden increase in damage, thus inducing the repair systems (which were no longer inhibited by lack of water). The decrease in damage observed after five hours might therefore be the result of a sharp increase in damage followed by a period of ferocious repair.

3.3.6 Desiccation in *S. auriculatum* induces CPDs in the absence of UV-B

Samples of *S. auriculatum* that were desiccated in the dark for six days showed a significant increase in CPDs. This proves that *C. purpureus* is not the only plant to accumulate photoproducts upon desiccation. A decrease in the amount of 6-4PP accumulation was seen in Sphagnum desiccated in the dark. In the light of these results, this does not indicate that desiccation does not induce 6-4PPs in Sphagnum. On the contrary it suggests that 6-4PPs are induced, and then excision repair has removed the lesions so that the level of 6-4PPs in desiccated plants is in fact lower than the level of 6-4PPs in hydrated plants. This, however, is only a suggestion and taking samples of Sphagnum throughout the desiccation period would reveal the pattern of DNA damage.

3.4 Conclusion

The results of this study are expected to be highly controversial. CPDs and 6-4PPs are the precursors of skin cancer in humans and hundreds of studies have assumed UV-B to be fundamental to their induction. However, major questions remain. No alternative technique has been used to study desiccation-induced photoproducts, and it may be that a rise in photoproducts with desiccation is simply an artefact of the ELISA technique. However, the complex patterns of DNA damage and the

results of chapter four make this unlikely. Additionally, the results suggest that repair of DNA damage may be possible in *C. purpureus* that has a very low water content. As both types of repair require enzymes, there must be some protective mechanism in place to protect these proteins during desiccation, for example, an increase in sugars, or heat shock proteins. There is a need for further study into a number of topics, in particular:

1. To determine if an increase in DNA damage in the absence of UV-B can be detected using a different technique that is not dependant on specific binding of antibodies (for example, the HPLC technique used by Davies, 1995).
2. To determine how *C. purpureus* can repair DNA damage at low water contents.

The main conclusions of this chapter are as follows:

1. Irradiation with UV-B results in an increase in CPDs and 6-4PPs in hydrated samples of *C. purpureus*
2. Desiccation results in an increase in 6-4PPs in *C. purpureus* desiccated in the dark at 100 and 560 μmol m^{-2} s^{-1} light.
3. Desiccation results in an increase in CPDs in *C. purpureus* desiccated in the dark and at 100 μmol m^{-2} s^{-1} light, but not in plants desiccated at 560 μmol m^{-2} s^{-1} light.
4. Desiccation results in an increase in CPDs, but not 6-4PPs in Sphagnum desiccated in the dark.
5. There is differential repair of the two types of damage. CPDs and 6-4PPs do not undergo repair at the same level of stress, i.e., at the same point in desiccation.
6. DNA damage that occurs during desiccation in *C. purpureus* is repaired upon rehydration.

Chapter 4

Oxidation and DNA Damage

4.0 Introduction

The results of chapter 3 indicated that DNA damage in the form of 6-4PPs and CPDs can be induced in *C. purpureus* during desiccation in the absence of UV-B. As these so called "photoproducts" are not documented to occur under any condition other than UV-B radiation, the current chapter will put forward the case of oxidative stress, a condition which occurs during desiccation, as a causative factor in the production of 6-4PPs and CPDs. In order to determine if oxidative stress does indeed cause 6-4PPs and CPDs to accumulate, samples of *C. purpureus* were treated with methyl viologen, a free-radical generating herbicide.

Additionally, this chapter focuses on plant antioxidative systems. If oxidation is causing DNA damage, then it would be expected that an increase in antioxidants would curb any damage. *C. purpureus* was therefore treated with L-galactono-1,4-lactone, the pre-cursor for the important antioxidant ascorbate (Smirnoff, 1996), with the hypothesis that an increase in ascorbate will negate the effects of desiccation on DNA damage. *Arabidopsis thaliana* plants were studied to determine if this species also experiences an increase in DNA damage with desiccation. DNA damage during desiccation was studied in the *A. thaliana Vtc3* mutant, deficient in the production of ascorbate (Conklin *et al.*, 2000). If antioxidants do counteract the effects of DNA damage caused by desiccation, then it is expected that the *Vtc3* mutant will incur more DNA damage than the wild type plants. *Vtc3* plants were treated with the L-galactono-1,4-lactone precursor for ascorbate in order to determine if any damage caused during desiccation could be restored to levels similar to that of wild type plants.

4.1 Methyl Viologen

Methyl viologen (1,1'dimethyl-4,4'-bipyriminium dichloride) is a herbicide widely used in agriculture as it is highly effective, and is readily rendered inert by its absorption into soil and by bacterial action (Babbs *et al.*, 1989). Methyl viologen acts by accepting an electron from PSI in chloroplasts to produce the methyl viologen radical. This in turn is rapidly oxidised by molecular oxygen to regenerate methyl viologen, with concomitant reduction of oxygen to superoxide. The methyl viologen molecule is then ready for another cycle of reduction and oxidation. Some of the superoxide produced is

converted to hydrogen peroxide. Superoxide and hydrogen peroxide can then undergo the Fenton reaction to produce hydroxyl radicals (Farrington *et al.*, 1973; Halliwell and Gutteridge, 1984; Vaughan and Duke, 1983; Babbs *et al.*, 1989). Treatment of plants with methyl viologen results in the greatest production of hydroxyl radicals observed in biological systems (Babbs *et al.*, 1989). Methyl viologen is known to cause oxidative damage to DNA, resulting in oxidised pyrimidines and strand breaks (Petrovsk and Dusinská, 1999), but there are no reports of oxidative damage producing 6-4PPs or CPDs. As methyl viologen reacts mainly by accepting electrons from PSI, its effects are therefore more severe in the light. *C. purpureus* treated with methyl viologen was studied in the light and in the dark, which effectively give two separate levels of oxidative stress.

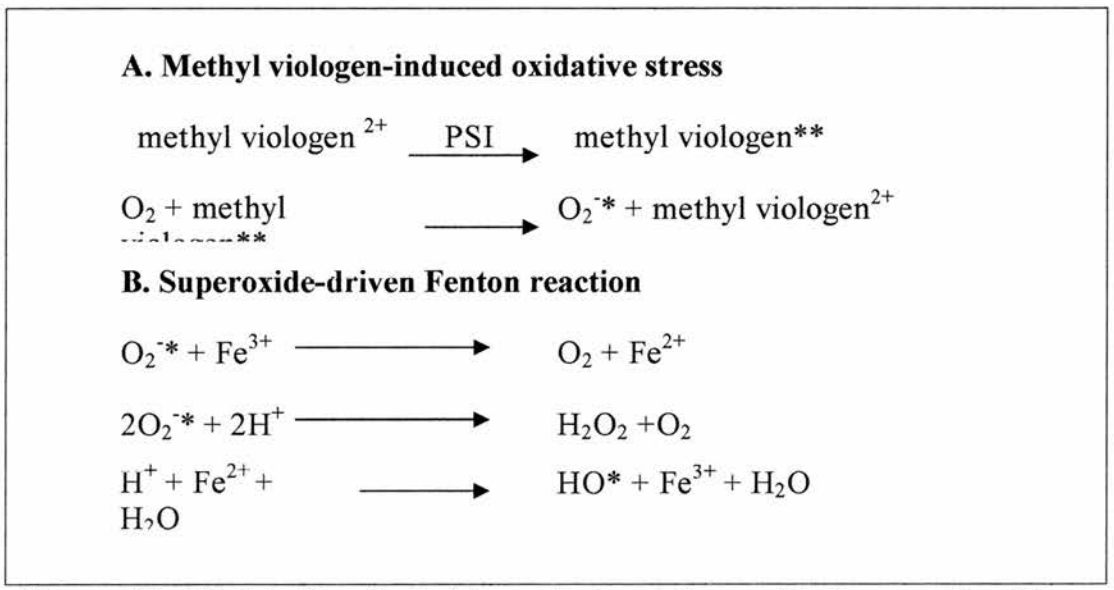


Figure 4.1: Model of methyl-viologen-induced free radical generation (from Babbs *et al.*, 1989).

4.1.2 Plant Antioxidant Systems

In response to environmental stresses, plants are known to have a number of antioxidant systems in place in order to minimise any damage caused by active oxygen (Alscher *et al.*, 1997). If 6-4PPs and CPDs can be caused by active oxygen, then it is likely that antioxidant systems will play an important role in reducing the extent to which they accumulate. This chapter will focus on two of the most common plant methods of dealing with free radicals, flavonoids and ascorbic acid.

4.1.2.1 Flavonoids

The majority of UV-B-absorbing compounds are a class of phenolic derivatives known as the flavonoids (Harborne, 1984). The flavonoids are ubiquitous plant compounds and consist of ten subclasses including anthocyanins, flavonols and chalcones. The accumulation of flavonoids during UV-B radiation has been well documented (Schmelser *et al.*, 1998; Rozema *et al.*, 2002; Stapleton and Walbot, 1994). Flavonoids can absorb up to 99% of the UV-B radiation reaching the surface of the leaf without affecting the transmittance of the photosynthetically active radiation to the chloroplasts, thus protecting the DNA without compromising photosynthesis (Koostra 1994; Caldwell *et al.*, 1983; Wilson and Greenberg 1993; Reuber *et al.*, 1996). Flavonoids are often present in the epidermal cell layers of leaves and in tissues that are susceptible to UV light, such as the apical meristem (Winkel-Shirley 2002).

In addition to their light-shielding properties, flavonoids are also known to scavenge UV-B-induced free radicals to render them less harmful to other cell constituents (Landry *et al.*, 1995; Hideg and Vass 1996). Ryan *et al.*, (2002) found that UV light induces the synthesis of flavonols with high hydroxylation levels in Petunia. Hydroxylation does not affect the UV-absorbing properties of these compounds, but does increase their antioxidant activity, suggesting that flavonols are involved in the general stress response to oxidative stress.

If oxidative stress is contributing to the accumulation of DNA damage during desiccation, as observed in chapter 3, it might be expected that flavonoids would accumulate under these conditions. Flavonoids and anthocyanins were therefore measured in *C. purpureus* in order to test this hypothesis.

4.1.2.2 Ascorbate and Dehydroascorbate

Ascorbate has a number of critical roles in plants and animals. It has the capacity to directly eliminate several different reactive oxygen species, maintain alpha-tocopherol in the reduced state and act as a substrate for ascorbate peroxidase. Ascorbate can also preserve the activity of a number of different enzymes by maintaining prosthetic group metal ions in the reduced

state (Smirnoff, 1996). The theory behind the experiments presented in this chapter assumes that an abundant and multifunctional antioxidant such as ascorbate will be critical in the prevention of oxidative damage. If this is the case, then an ascorbate-deficient *A. thaliana* mutant would be expected to become more damaged as a result of an oxidative stress such as desiccation. It has already been determined that desiccation can cause DNA damage in *C. purpureus*. Assuming that oxidative stress is the cause of this damage, *A. thaliana* mutants deficient in ascorbate production should display higher levels of DNA damage than wild type plants. Ascorbate-deficient mutants have been found to function normally until exposed to oxidative stress (Smirnoff, 2000), implying that wild type plants must permanently carry a high ascorbate load in order to deal with periodic exposure to oxidative stress. The *Vtc3* mutant of *A. thaliana* has been found to have a reduced ability to convert mannose to ascorbate. At two weeks of age, the *Vtc3* mutant has only 33% and at full maturity 50% of the wild type level of ascorbate (Conklin *et al.*, 2000). Due to the critical roles of ascorbate as an antioxidant and cellular reductant, it is suggested that ascorbate levels lower than 33% of the wild type levels would be lethal to *A. thaliana*.

The product of the reaction of ascorbate with reactive oxygen species is monodehydroascorbate (MDHA) which disproportionates to form dehydroascorbate (DHA) and ascorbate. DHA is unstable at physiological pH, and therefore MDHA and DHA are converted rapidly back to ascorbate by the three enzymes, monodehydroascorbate reductase, GSH-dependent dehydroascorbate reductase and glutathione reductase (Smirnoff, 1995; Noctor and Foyer, 1998; Asada 1999). Under normal circumstances, the ascorbate pool is at least 90% reduced. Ascorbate pool size is determined by its rate of synthesis, its rate of turnover and by the light intensity at which the plants are grown. A high pool size may inhibit ascorbate synthesis (Pallanca and Smirnoff 2000) and pool size is larger in the leaves of plants grown at high light intensities (Grace and Logan 1996; Smirnoff and Pallanca 1996).

The direct pre-cursor to ascorbate, L-galactono-1,4-lactone, can be readily converted to ascorbate when supplied to intact tissue. This can elevate the

ascorbate pool up to 10 fold within a few hours (Smirnoff 2000). Treating *Vtc3* mutants with L-galactono-1,4-lactone should increase the ascorbate concentration to at least wild type levels. In the present study both *Vtc3* mutants and wild type *A. thaliana* plants were treated with L-galactono-1,4-lactone in order to determine if a high ascorbate concentration can negate any effects that desiccation might have on DNA damage.

Throughout this chapter, the term "treated" will refer to plants treated with L-galactono-1,4-lactone, as described in methods sections 2.5.7 and 2.5.8.

4.2 Aims

The aims of this chapter were to:

1. Determine if oxidation causes DNA damage in *C. purpureus*.
2. Determine if *C. purpureus* increases antioxidant defences during desiccation.
3. Determine if *A. thaliana* accumulates DNA damage during desiccation, and if the *Vtc3* ascorbate-deficient mutant accumulates more damage than wild type plants.
4. Determine if ascorbate plays a critical role in the protection of DNA.

4.2.1 Review of Materials and Methods

The DNA damage observed during desiccation may be a result of oxidation. In order to determine if oxidation also induces "photoproducts", *C. purpureus* was incubated in the dark and in the light with 0-40μM methyl viologen (a known free-radical generator). Oxygen evolution was measured in *C. purpureus* incubated in 0-40μM methyl viologen in the light and dark using an oxygen electrode in order to determine the concentration of methyl viologen that has a significant effect on photosynthesis and respiration (section 2.2.1.3). *C. purpureus* was then incubated in the dark and at 560 μmole $m^{-2}s^{-1}$ PAR with 0,20 and 40μM methyl viologen (methods section 2.2.1.3.1) and DNA damage was measured (section 2.4). To make certain that methyl viologen was not reacting with the primary antibodies, TDM-2 and 64M-2 were incubated for 16 hours with 40μM methyl viologen in the dark prior to carrying out an ELISA on UV-irradiated calf thymus (section

2.4.8.1). Undamaged calf thymus DNA was also treated with 40μM methyl viologen (section 2.4.8.2).

UV-B absorbing compounds and anthocyanin content was measured each day for six days during desiccation in *C. purpureus* under 0, 100 and 560 μmole $m^{-2}s^{-1}$ light (sections 2.5.2 and 2.5.3).

In order to determine if increasing the ascorbate content of *C. purpureus* negated the effects of desiccation on DNA damage, *C. purpureus* was brushed with 20mM L-galactono-1,4-lactone and desiccated at 0, 100 and 560 μmole $m^{-2}s^{-1}$ light (section 2.5.7). Ascorbate content and DNA damage were measured at the beginning and end of the 6 day desiccation period (section 2.5.5 and 2.4).

Wild type and Vtc 3 *A. thaliana* plants were either treated or untreated with L-galactono-1,4-lactone and desiccated for 6 days in the dark and at 560 μmole $m^{-2}s^{-1}$ light. Water content, leaf area and Fv/Fm were measured each day (see methods sections 2.2.3, 2.2.2 and 2.2.1.4). Mutant and wild type *A. thaliana* plants were either treated or untreated with L-galactono-1,4-lactone and desiccated for 6 or 7 days in the dark and at 560μmole $m^{-2}s^{-1}$ light. The plants were then rehydrated (section 2.1.2) and Fv/Fm was measured.

Ascorbate and dehydroascorbate was then measured daily in mutant and wild type *A. thaliana* plants that were treated and untreated with L-galactono-1,4-lactone and desiccated for 6 days in the light and in the dark (section 2.5.5-2.5.7). DNA damage was also measured in these plants (section 2.4).

4.3 Results

4.3.1 Effects of Methyl Viologen on Respiration, Photosynthesis and DNA Damage in *C. purpureus*

Figure 4.2 shows that both photosynthesis (oxygen evolution in the light) and respiration (oxygen uptake in the dark) are inhibited by methyl viologen concentrations above 10 μM, with almost complete inhibition occurring at 40μM methyl viologen.

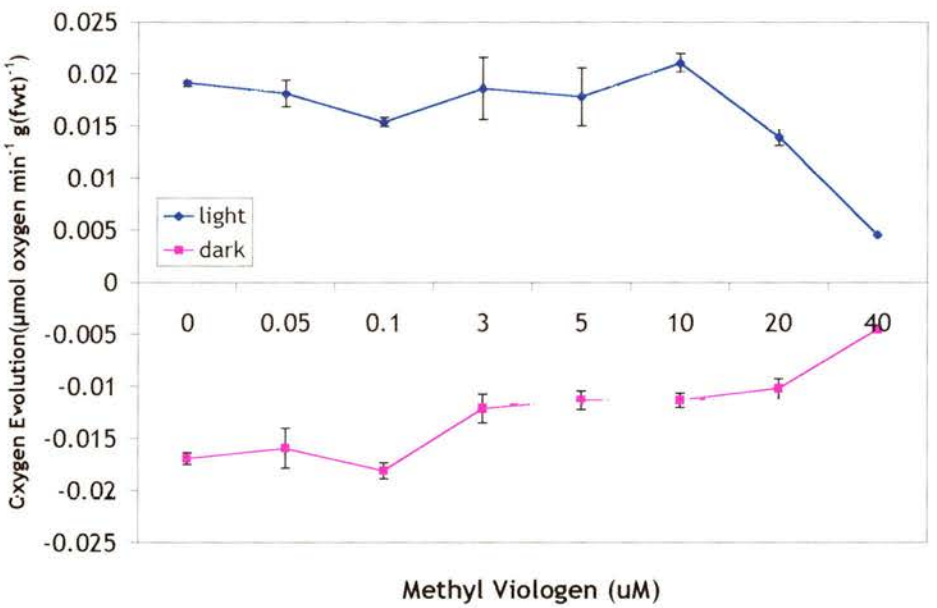
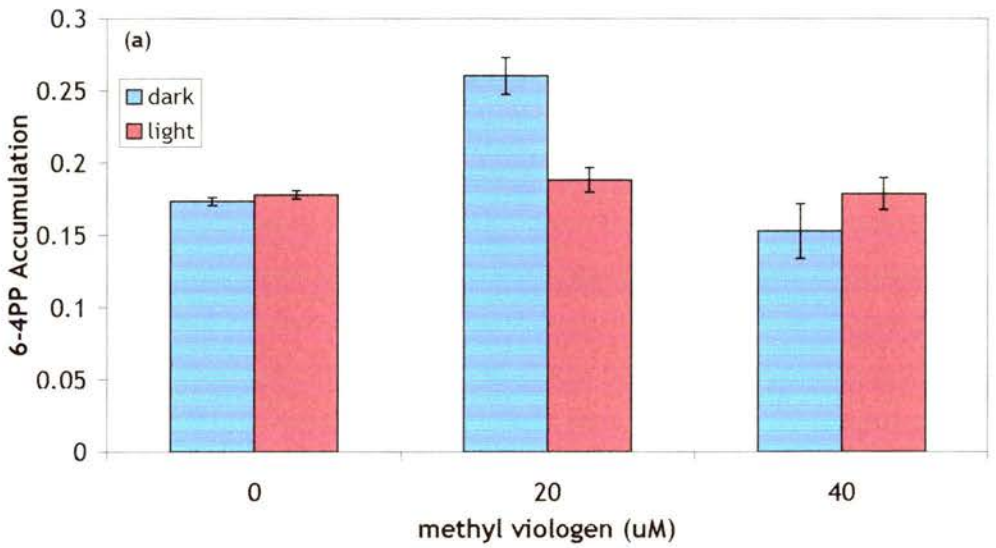


Figure 4.2: Oxygen Evolution of *C. purpureus* incubated with methyl viologen
Oxygen evolution was measured in *C. purpureus* incubated in 0-40μM of methyl viologen in the light and dark in order to determine the concentration of methyl viologen that has a significant effect on photosynthesis and respiration. Oxygen depletion (respiration) and evolution (photosynthesis) was measured using an oxygen electrode (see methods section 2.2.1.3). Data show the means of three samples and errors are standard error of the mean.

Figure 4.3a shows that methyl viologen has a significant effect on 6-4PP accumulation ($F_{2,524}$ = 16.14, $p<0.05$), with the greatest accumulation of 6-

4PPs occurring in the dark. CPDs also significantly increase with methyl viologen concentration (Figure 4.3b: $F_{2,524} = 61.82$, $p<0.05$). Light does not have a significant effect on either 6-4PP or CPD accumulation in the presence of methyl viologen (6-4PPs: $F_{1,524} = 2.9$, $p=0.089$; CPDs: $F_{1,524} = 2.69$, $p=0.102$).



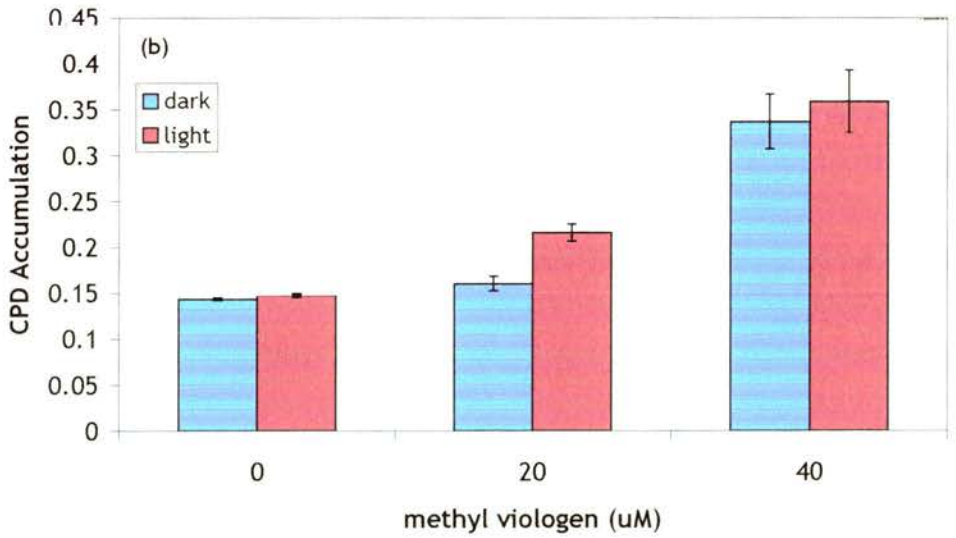


Figure 4.3: 6-4PP (a) and CPD (b) accumulation in *C. purpureus* incubated with methyl viologen

To test the effect of methyl viologen on DNA damage, *C. purpureus* was incubated in the dark and at 560µmol $m^{-2}s^{-1}$ PAR with 0, 20 and 40µM methyl viologen (methods section 2.2.1.3.1). Data represent the means of 6 DNA extractions. Errors are standard error of the mean.

Figure 4.4 confirms that pre-incubation with methyl viologen did not affect the response of either of the primary antibodies (6-4PPs: t=-0.44, n=48, p=0.663; CPDs: t=-1.49, n=48, p=0.144). To further test the effect of methyl viologen it was incubated for 8 hours with undamaged calf thymus DNA. Figure 4.5 shows that methyl viologen does not induce the accumulation of 6-4PPs or CPDs on DNA *in vitro* (6-4PPs: t=4.21, n=96, p=0.94; CPDs: t=0.22, n=96, p=0.83).

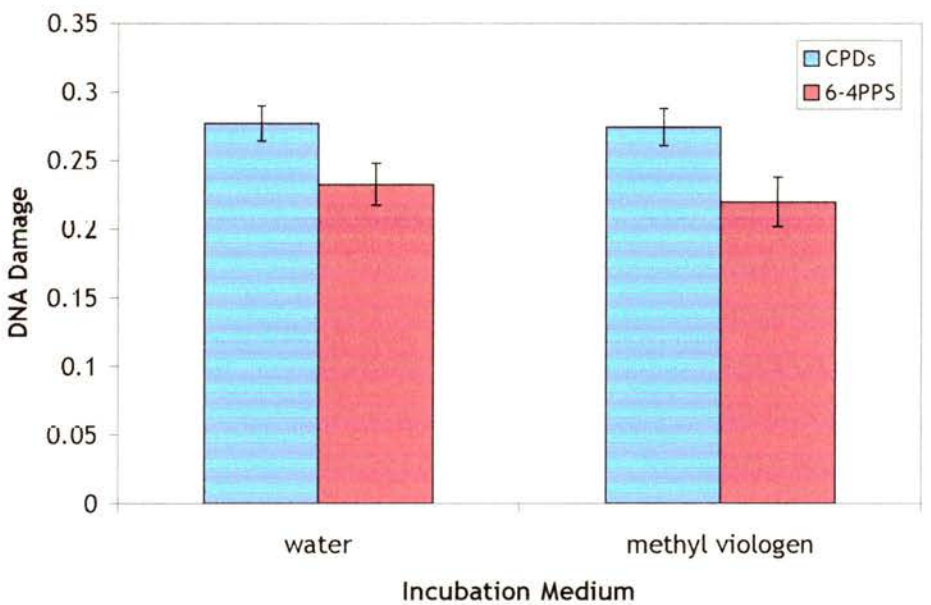


Figure 4.4: Incubation of methyl viologen and water with the primary antibodies

In order to make certain that methyl viologen was not reacting with the primary antibodies, and hence giving a 'false positive' on the ELISAs, TDM-2 and 64M-2 were incubated for 16 hours with 40µM methyl viologen prior to carrying out an ELISA on UV-irradiated calf thymus DNA (methods section 2.4.8.1). Data show the means of three incubation events. Errors are standard error of the mean.

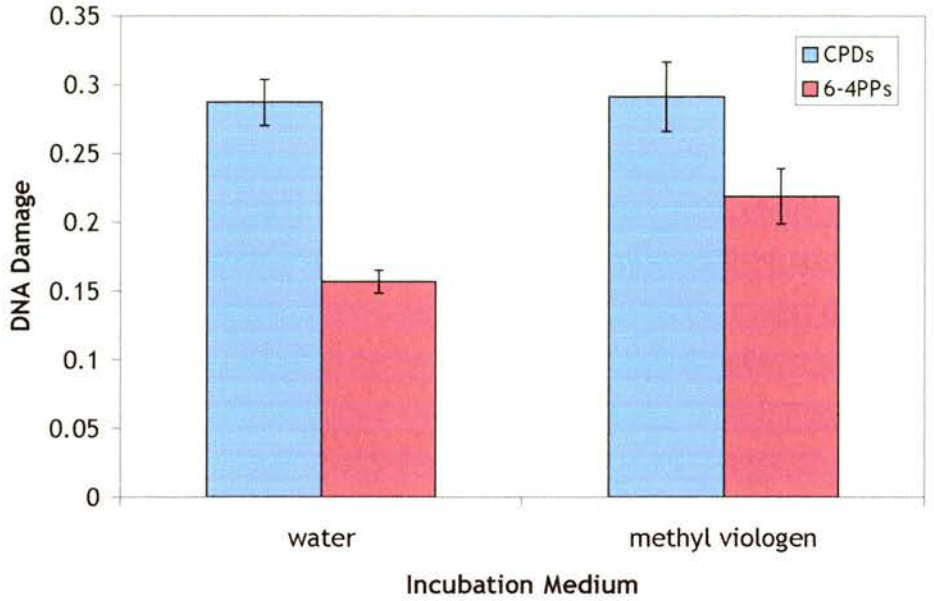


Figure 4.5: Incubation of undamaged calf thymus DNA with methyl viologen and water

To further test the effect of methyl viologen, undamaged calf thymus DNA was treated with 40μM methyl viologen (methods section 2.4.8.2). Data show the means of six incubation events. Errors are standard errors of the mean

4.3.2 Accumulation of Antioxidants during Desiccation in *C. purpureus*

Both the UV-B-absorbing compounds and anthocyanin content increased in *C. purpureus* during desiccation (UV-B absorbing compounds (figure 4.6a): $F_{2,180}=116.82$, $p<0.05$; anthocyanin content (figure 4.6b): $F_{2,180}=137.19$, $p<0.05$). Additionally, light had a significant effect on accumulation of UV-B-absorbing compounds and anthocyanins, with most accumulation occurring under high light (560μmol $m^{-2}s^{-1}$) conditions (UV-B-absorbing compounds: $F_{6,180}=172.02$, $p<0.05$; Anthocyanins: $F_{6,180}=221.26$, $p<0.05$).

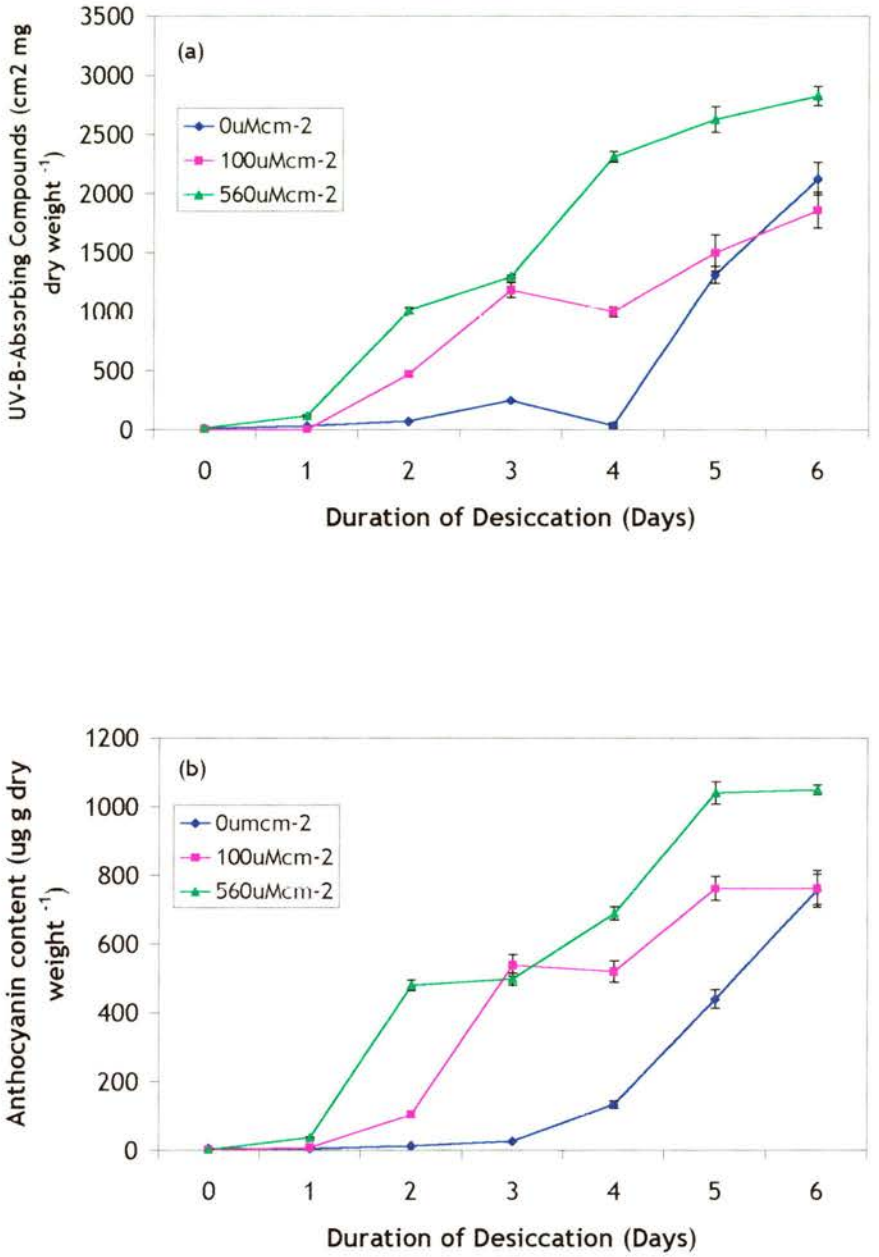


Figure 4.6: Accumulation of total UV-B-absorbing compounds (a) and anthocyanins (b) during desiccation of *C. purpureus*.

Throughout the six-day desiccation period, UV-B-absorbing compounds (a) and anthocyanin content (b) were measured in *C. purpureus* desiccated under at 0, 100 and 560 µmol $m^{-2}s^{-1}$ light (see methods sections 2.5.2 and 2.5.3 respectively). Data show the means of 10 individual extractions and 2 desiccation events. Errors are standard error of the mean.

4.3.3 Effect of Ascorbate on Desiccation-Induced DNA Damage in *C. purpureus*

Feeding L-galacto-lactone to *C. purpureus* resulted in an increase in ascorbate concentration only in samples kept at 560µmol $m^{-2}s^{-1}$ light (Figure 4.7: t=-4.42, n=28, $p<0.05$). This was also the only treatment that led to a change in ascorbate content during desiccation, with a decrease from 6.778 to 2.59 nmoles g^{-1} (fwt) during the desiccation period (t=-5.82, n=12, $p<0.05$). Feeding L-galacto-lactone to *C. purpureus* had no effect on either 6-4PP (figure 4.8a) or CPD (figure 4.8b) accumulation under the same desiccation conditions (6-4PPs: $F_{1,236}=1.27$, p=0.301; CPDs: $F_{1,236}=0.36$, p=0.557).

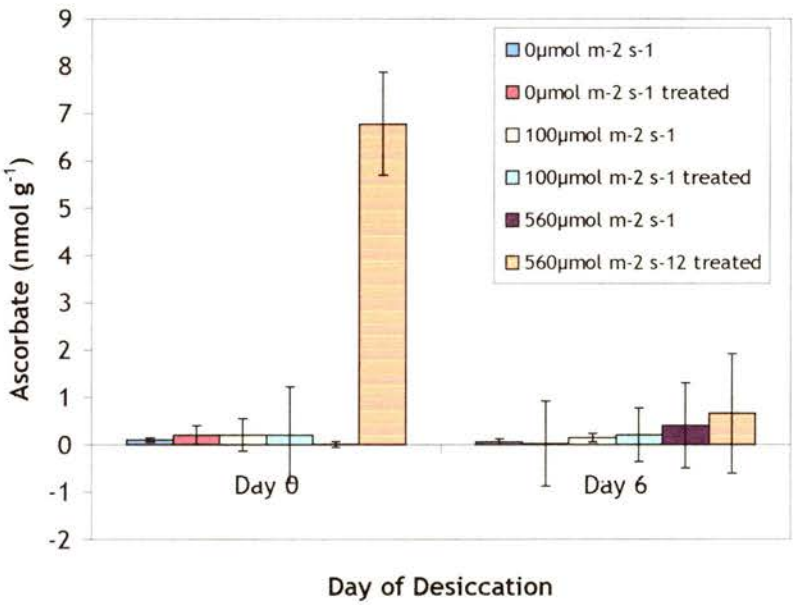
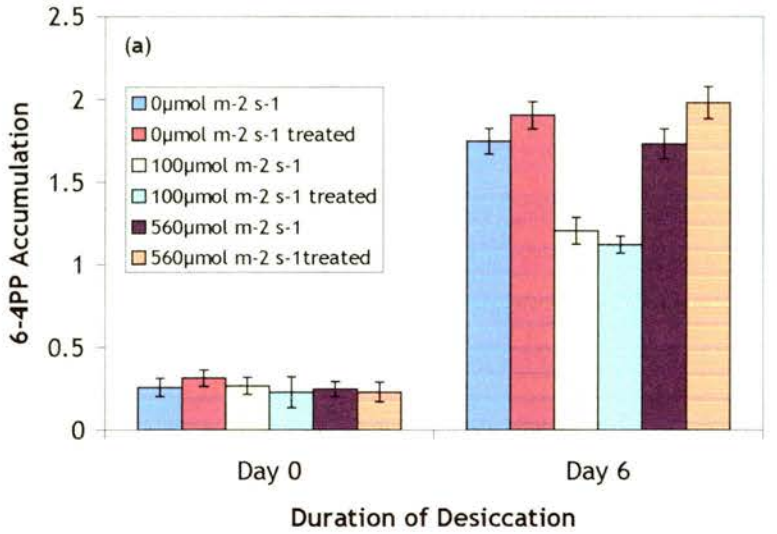


Figure 4.7: Ascorbate concentration in *C. purpureus* on day 0 and day 6 of the desiccation period.

C. purpureus was brushed with 20mM L-galactono-1,4-lactone, and then desiccated at 0, 100 and 560 µmol $m^{-2}s^{-1}$ light (methods section 2.5.7). Data show the means of ascorbate assays over two desiccation periods. Errors are standard errors of the mean.



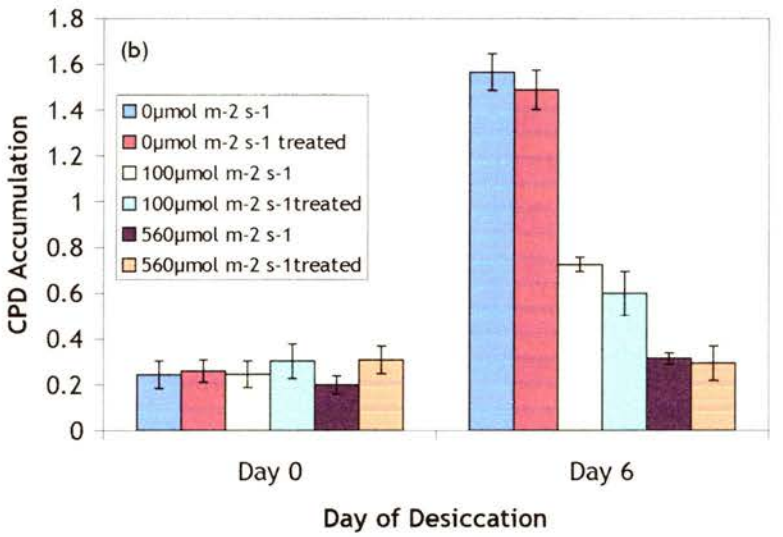


Figure 4.8: 6-4PP (a) and CPD (b) accumulation in control *C. purpureus* and *C. purpureus* treated with L-galactono-1,4-lactone at day 0 and day 6 of desiccation.

C. purpureus was brushed with 20mM L-galactono-1,4-lactone, and then desiccated for six days at 0, 100 and 560 µmol $m^{-2}s^{-1}$ light (methods section 2.5.7). Samples were taken at the beginning and the end of the desiccation period for DNA damage analysis (methods section 2.4). Data show the means of twelve DNA extractions taken over two desiccation events. Errors are standard error of the mean.

4.3.4 Effects of Desiccation on *A. thaliana*

4.3.4.1 Water Content of *A. thaliana* During Desiccation

The water content in the leaves of both mutant and wild type *A. thaliana* decreased during the six day desiccation period in plants desiccated in the dark (figure 4.9a) and in the light (figure 4.9b) ($F_{5,135}=16.26$, $p<0.05$). Plants desiccated in the light lost more water from their leaves than plants desiccated in the dark ($F_{1,135}=37.84$, $p<0.05$).

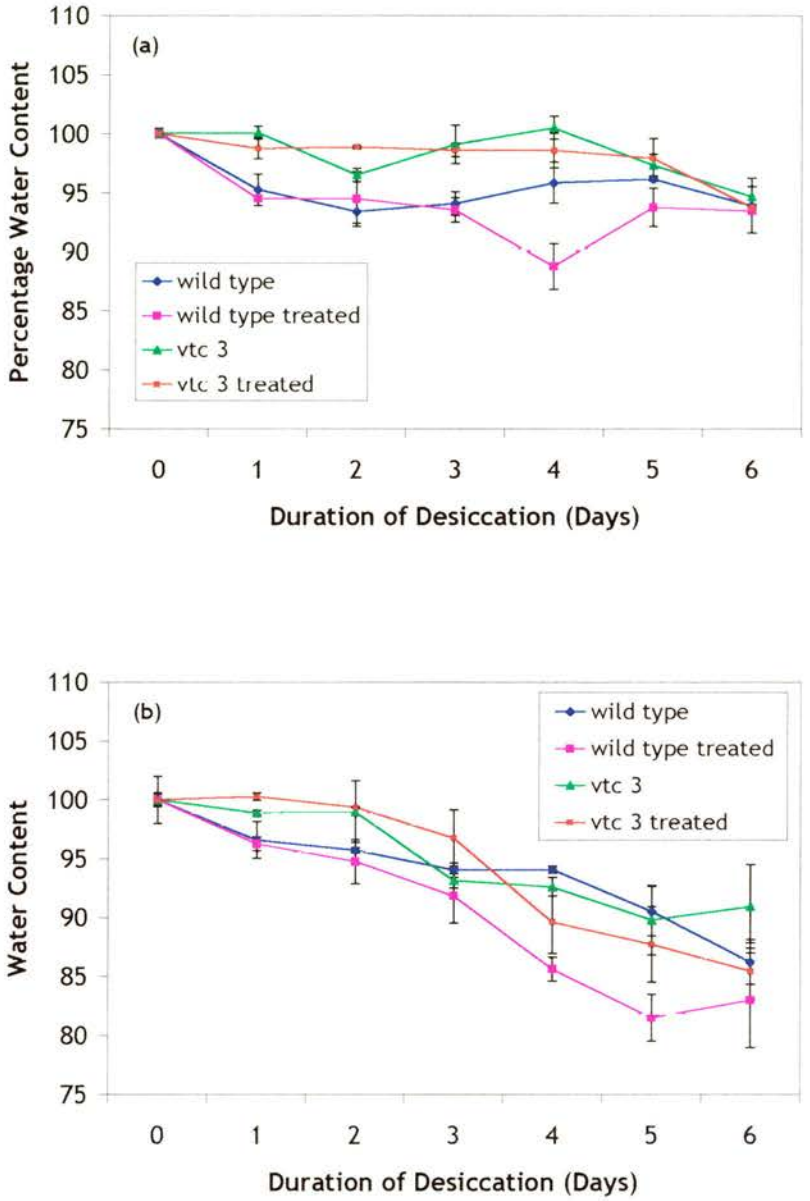


Figure 4.9: Water content of the leaves of *A. thaliana* desiccated in the dark (a) and light (b).

Wild type and *vtc3 A. thaliana* were desiccated for six days as in the methods section (2.1.2). Samples were taken each day for water content analysis (section 2.2.3) Data represent the mean of six leaf samples taken over two desiccation events.

4.3.4.2 Leaf Area of *A. thaliana* During Desiccation

There was no change in mean leaf area of wild type or *Vtc3* mutants of *A. thaliana* desiccated in the dark for six days (figure 4.10a $F_{1,47}=0.46$, $p=0.567$). There was, however, a significant increase in leaf area of wild type and mutant *A. thaliana* plants desiccated in the light for six days (figure 4.10b $F_{1,95}=11.16$, $p<0.05$). The *Vtc3* mutants had a significantly smaller leaf area than wild type plants under all conditions ($F_{1,95}=18.29$, $p<0.05$).

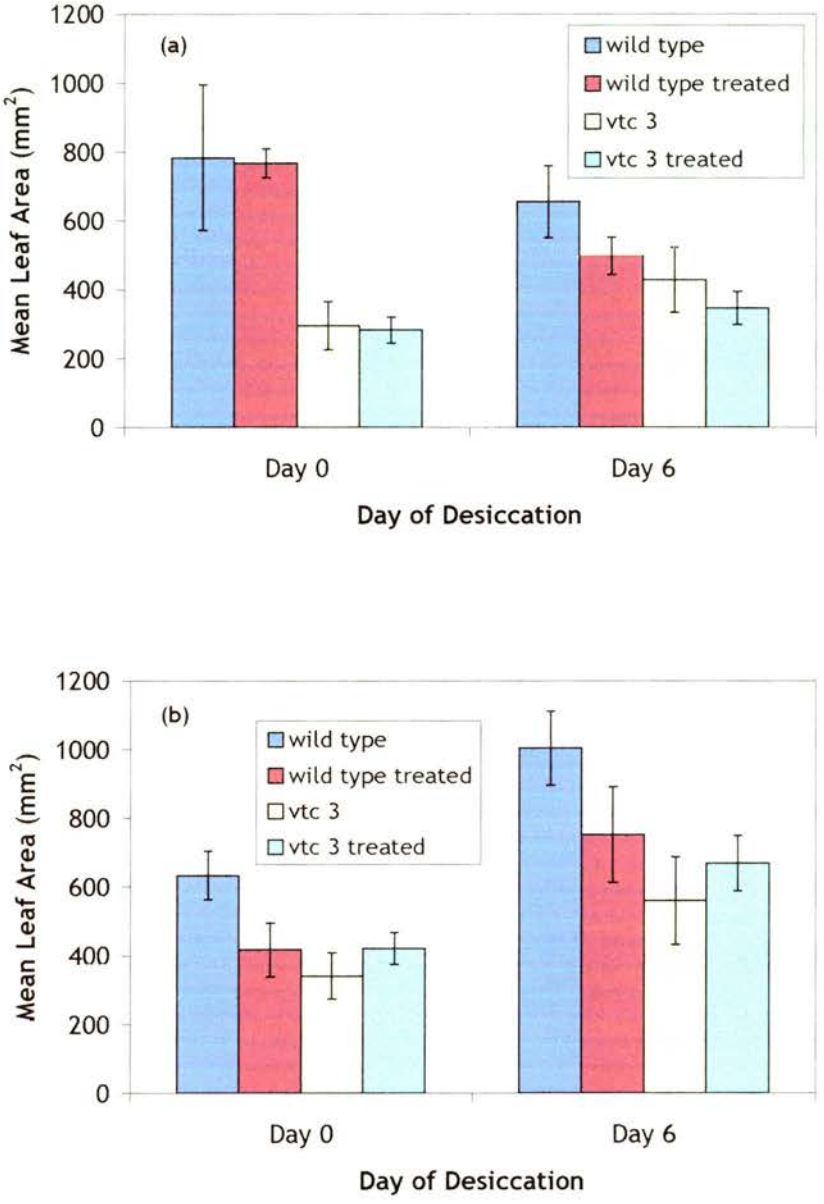


Figure 4.10: Leaf area of *A. thaliana* leaves of plants desiccated in the dark (a) and light (b).

Vtc 3 mutant and wild type *A. thaliana* plants were desiccated for 6 days in the light and in the dark as described in methods section (2.1.2).. Samples were taken on day 0 and day 6 of the desiccation period for leaf area analysis (methods section 2.2.2). Data represent the mean of six plants measures over two desiccation events. Errors are standard error of the mean.

4.3.4.3 Chlorophyll Fluorescence of *A. thaliana* During Desiccation and Rehydration

The Fv/Fm of *A. thaliana* desiccated in the dark showed a steady decrease throughout the desiccation period (figure 4.11a: $F_{6,134}=7.43$, $p<0.05$). The Fv/Fm of samples kept in the light did not show any sign of decreasing until day 7 (figure 4.11b: $F_{1,134}=240.69$, $p<0.05$). The Fv/Fm of the *Vtc3* mutants did not differ significantly from that of the wild type plants ($F_{1,134}=0.08$, $p=0.779$). The action of feeding both wild type and mutant plants with L-galactono-1,4-lactose did not alter the Fv/Fm ($F_{1,134}=0.01$, $p=0.926$).

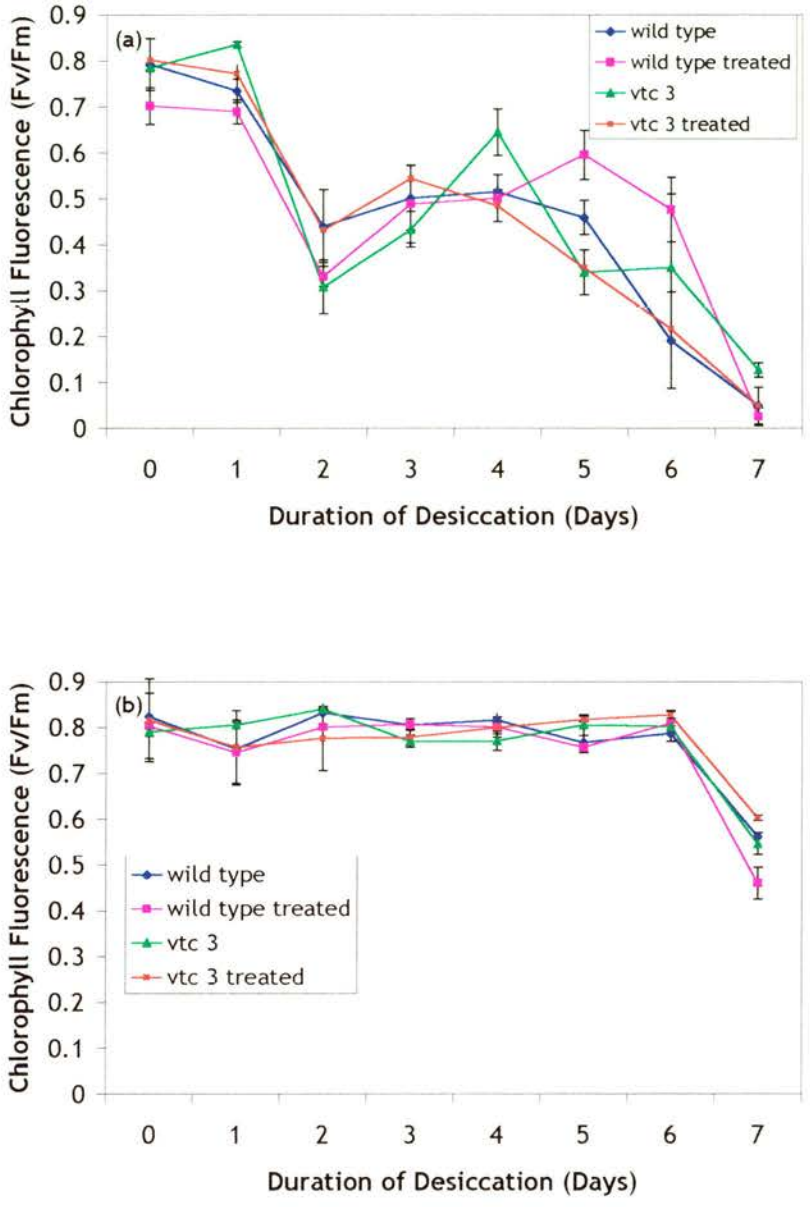
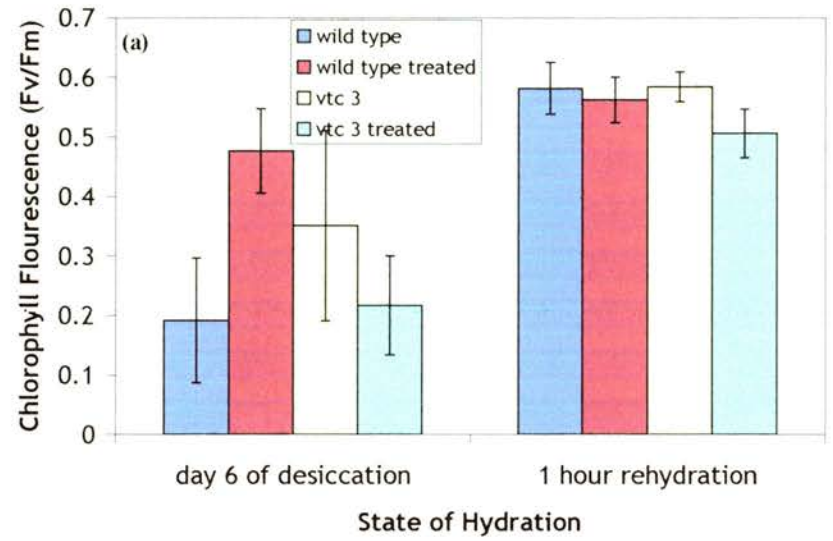


Figure 4.11: Fv/Fm of *A. thaliana* during desiccation in the dark (a) and light (b)

Chlorophyll fluorescence (Fv/Fm) was measured in mutant and wild type *A. thaliana* plants that had either been treated or not-treated with L-galactono-1,4-lactone during a 7 day desiccation period (see methods sections 2.2.1.4 and 2.5.8). Data are means of 12 measurements taken over two desiccation periods. Errors are standard error of the mean.

A. thaliana was desiccated for either six days or seven days before it was rehydrated by watering the compost thoroughly. Plants desiccated in the dark for six days showed significant recovery one hour after rehydration (figure 4.12a: $F_{1,43}$=4.08, p=0.05), but plants desiccated in the dark for seven days did not recover pre-desiccation Fv/Fm levels (figure 4.12b F1,43=0.81, p=0.372). *A. thaliana* desiccated in the light for six days did not show any significant change in Fv/Fm with desiccation, and therefore rehydration had little effect (figure 4.13a: F1,43=0.51,p=0.481). *A. thaliana* desiccated in the light for seven days showed a significant decrease in Fv/Fm one hour after rehydration (figure 4.13b: F1,20=100.59 p<0.05).



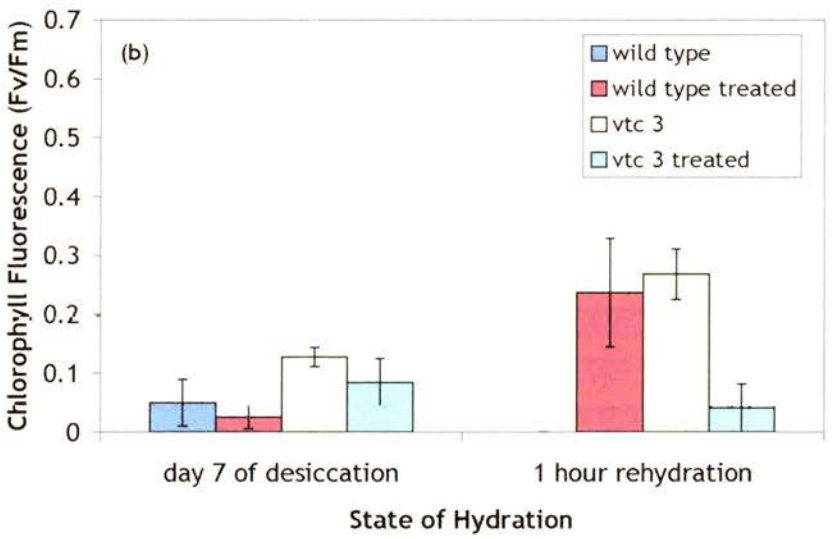
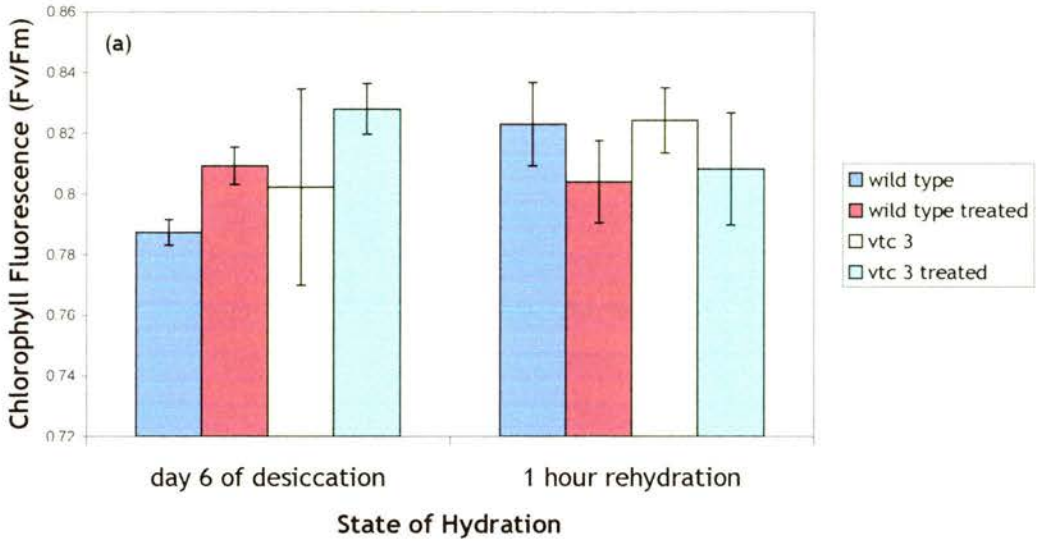


Figure 4.12: Chlorophyll fluorescence of *A. thaliana* desiccated in the dark for six days (a) and seven days (b), and rehydrated for one hour.

Both mutant and wild type *A. thaliana* plants were either treated or untreated with L-galactono-1,4-lactone and desiccated for 6 (a) or 7 (b) days in the dark as in methods sections 2.5.8 and 2.1.2. The Fv/Fm was measured following the desiccation period and one hour after rehydration. Data are means of 12 measurements taken over two desiccation periods. Errors are standard error of the mean.



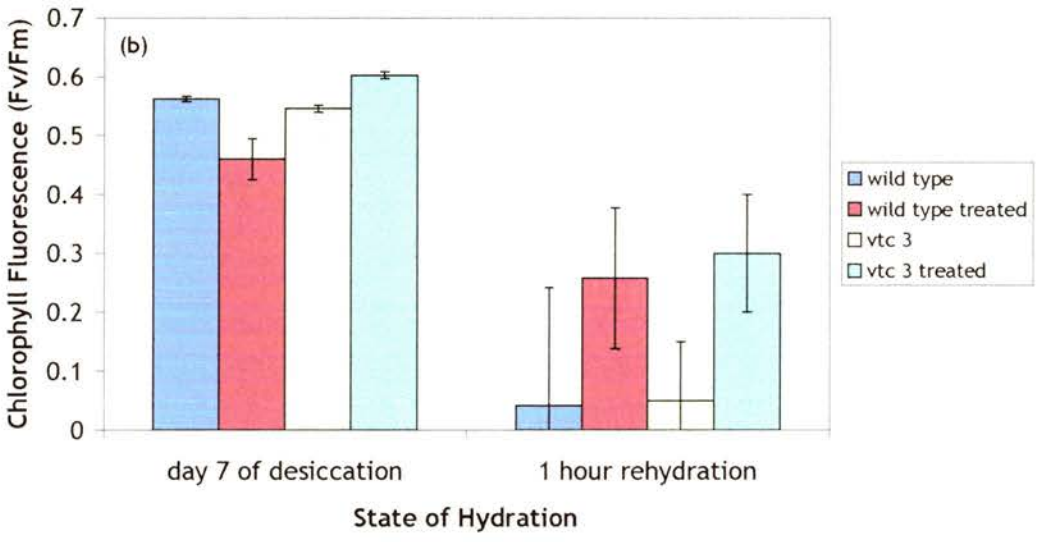


Figure 4.13: Chlorophyll fluorescence of *A. thaliana* desiccated in the light for six days (a) and seven days (b), and rehydrated for one hour.

Mutant and wild type *A. thaliana* was both treated and un-treated with L-galacono-1,4-lactone, and desiccated at 560µmol $m^{-2}s^{-1}$ for either 6 or 7 days as in methods sections 2.5.8 and 2.1.2. Chlorophyll fluorescence (Fv/Fm) was measured following the desiccation period and 1 hour rehydration. Data are means of 12 measurements taken over two desiccation periods. Errors are standard error of the mean.

4.3.5.1 Ascorbate Concentration during Desiccation in *A. thaliana*

Figure 4.14 shows the ascorbate concentration in *A. thaliana* during desiccation in the dark (figure 4.14a) and in the light (figure 4.14b). Treating ascorbate-deficient vtc3 *A. thaliana* mutants in the presence of light with L-galactono-1,4-lactone does increase the ascorbate content to levels similar to that of wild type plants (fig 30, $t = -11.25$, $n=12$, $p<0.05$). During desiccation in the dark, there is a peak in ASA levels in all samples of *A. thaliana* on day 4 of desiccation (wild type: $t=-26.35$, $n=12$, $p<0.05$; wild type fed: $t=-12.62$, $n=12$, $p<0.05$; *Vtc3*: $t=-29.66$, $n=12$, $p<0.05$, *Vtc3* fed: $t=-14.17$, $n=12$, $p<0.05$). This is followed by a decline in ASA levels between days 4 and 6 (wild type: $t=25.17$, $n=12$, $p<0.05$; wild type fed: $t=7.47$, $n=12$, $p<0.05$; *Vtc3*: $t=-19.68$, $n=12$, $p<0.05$; *Vtc3* fed: $t=11.59$, $n=12$, $p<0.05$). ASA levels in the light peak on day 4 for wild type plants, wild type plants that have been fed with L-galactono-1,4-lactone, and *Vtc3* plants ($t=-21.87$, $n=12$, $p<0.05$; $t=-13.15$, $n=12$, $p<0.05$; $t=-0.6101$, $n=12$, $p<0.05$ respectively). ASA levels in *Vtc3* plants fed with L-galactono-1,4-lactone peak on day 3 ($t=-25.02$, $n=12$, $p<0.05$) (figure 4.14b). Feeding with L-galactono-1,4-lactone does not appear to cause any significant differences in ASA concentration in any of the plant treatments throughout the course of desiccation ($F1,279=1.07$, $p=0.301$).

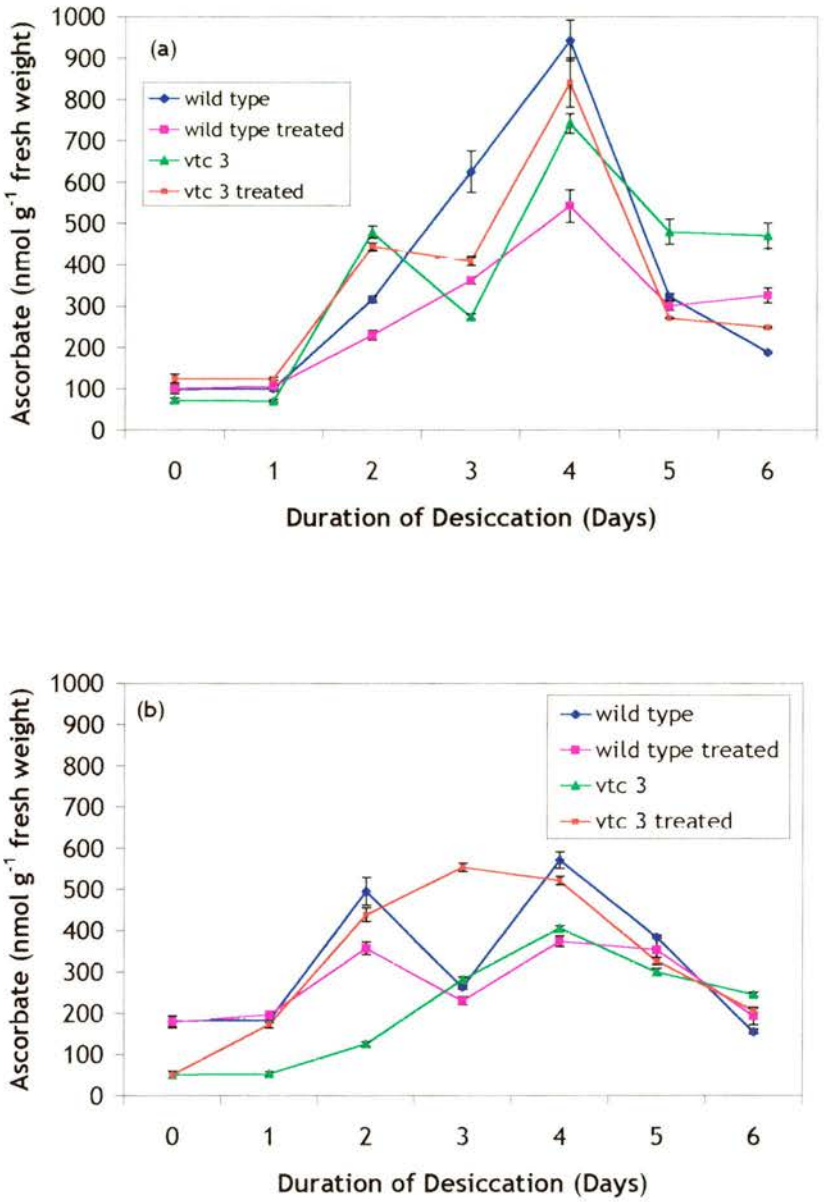


Figure 4.14: Ascorbate concentration in *A. thaliana* desiccated in the dark (a) and in the light (b).

Wild type and mutant *A. thaliana* were either treated or not treated with L-galactono-1,4-lactone and then desiccated in the dark (a) and or the light (b) for six days. Samples were taken daily for analysis of ascorbate content (see methods section 2.5.5). Data represent the means of twelve extractions and two desiccation periods. Errors are standard error of the mean.

4.3.5.2 Dehydroascorbate Concentration during Desiccation in *A. thaliana*

Concentrations of DHA reach their lowest on day 4 for wild type, *Vtc3* and *Vtc3* fed plants desiccated in the dark(figure 4.15a), and for all plants desiccated in the light (figure 4.15b). Desiccation significantly affects DHA concentration ($F_{5,279}=128.92$, $p<0.05$). Feeding wild type and mutant plants with L-galactono-1,4-lactone does not affect DHA concentration ($F_{1,279}=3.04$, $p=0.082$). There is no significant difference in DHA concentration between mutant and wild type plants ($F_{1,279}=0.35$, $p=0.557$). DHA concentrations are higher in plants desiccated in the light ($F_{1,279}=6.04$, $p<0.05$).

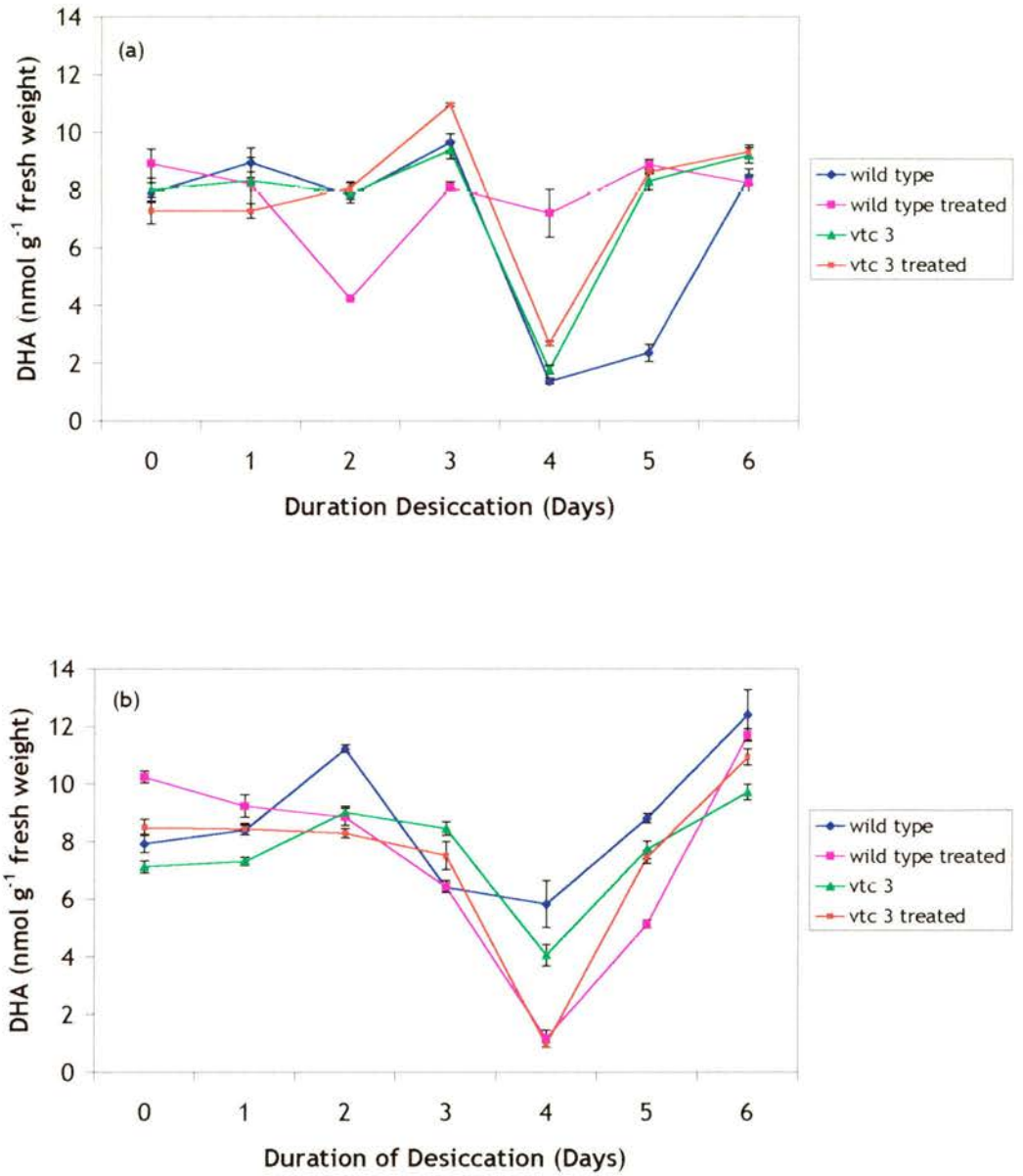


Figure 4.15: Dehydroascorbate concentration in *A. thaliana* desiccated in the dark (a) and in the light (b).

Wild type and mutant *A. thaliana* was treated as in figure legend 4.14. Samples were taken daily for the analysis of DHA content (see methods section 2.5.4). Data represent the means of twelve extractions and two desiccation periods. Errors are standard error of the mean.

4.3.6 Desiccation and DNA Damage in *A. thaliana*

4.3.6.1 6-4PP Accumulation in the Dark

There was a significant increase in 6-4PP accumulation in wild type *A. thaliana* desiccated in the dark (figure 4.16a $F_{5,162}=2.44$, $p<0.05$). Wild type *A. thaliana* that had been fed with L-galactono-1,4-lactone showed a significant increase in 6-4PP accumulation between days 0 and 3 ($t=-2.76$, $n=28$, $p<0.05$). This was followed by a significant decrease in 6-4PP accumulation between days 3 and 5 ($t=-4.41$, $n=28$, $p<0.05$) and a further increase between days 5 and 6 ($t=-1.55$, $n=28$, $p=0.132$).

The *A. thaliana Vtc3* mutant showed an insignificant increase in 6-4PP accumulation between days 0 and 2 of the desiccation period ($t=-1.08$, $n=28$, $p=0.29$). This was followed by a significant decrease between days 3 and 4 ($t=-6.59$, $n=28$, $p<0.05$). The *Vtc3* mutant that had been fed with L-galactono-1,4-lactone showed a significant decrease in 6-4PP accumulation between days 2 and 3 ($t=4.06$, $n=28$, $p<0.05$). This was followed by a significant increase between days 4 and 5 ($t=-12.33$, $n=28$, $p<0.05$) (figure 4.16b).

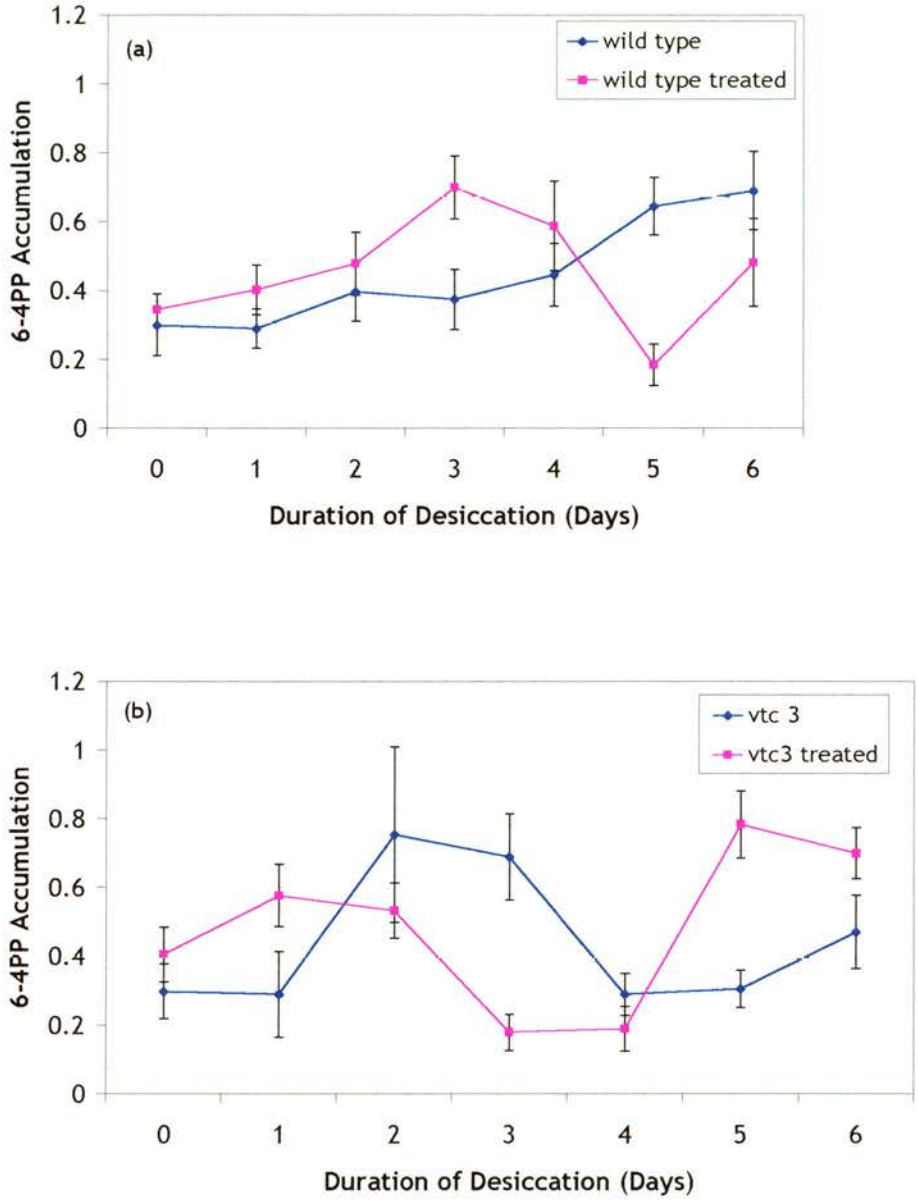


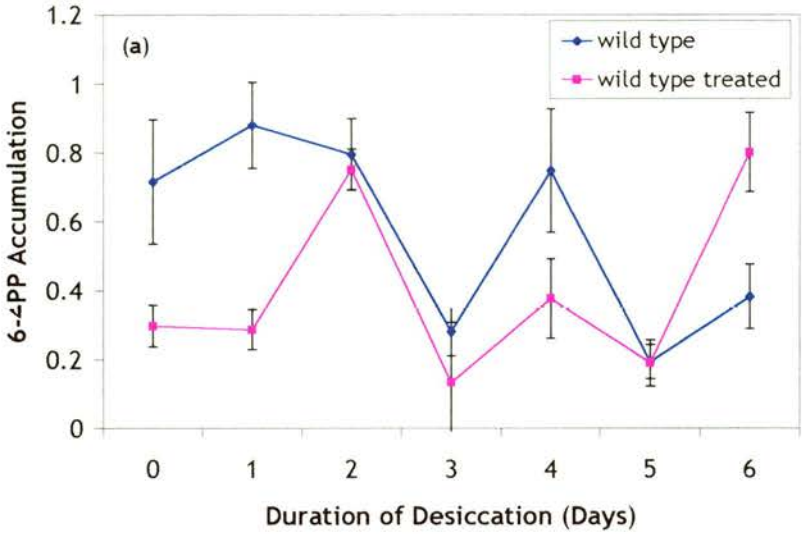
Figure 4.16: 6-4PP accumulation in wild type (a) and *Vtc3* (b) *A. thaliana* during desiccation in the dark.

Wild type (a) and *vtc3* (b) *A. thaliana* was either treated or left untreated with L-galactono-1,4-lactone as described in methods section 2.5.8. The plants were then desiccated for 6 days in the dark (methods section 2.1.2) and samples were taken daily for DNA damage analysis (methods section 2.4). Data are means of 12 DNA extractions and two desiccation events. Errors are standard error of the mean.

4.3.6.2 6-4PP Accumulation in the Light

On day 0 of desiccation, wild type plants had more 6-4PP accumulation than wild type plants that had been fed with the L-galactono-1,4-lactone precursor ($t=-2.74$, $n=28$, $p<0.05$). Wild type plants showed a decrease in 6-4PP accumulation between days 2 and 3 ($t=3.83$, $n=28$, $p=0.001$), and an increase between days 3 and 4 ($t=t=2.9$, $n=28$, $p<0.05$). *A. thaliana* that had been fed with L-galactono-1,4-lactone showed an increase in 6-4PP accumulation between days 1 and 2 ($t=-5.84$, $n=28$, $p<0.05$). There was a decrease in 6-4PP accumulation in these plants between days 2 and 3 ($t=5.7$, $n=28$, $p<0.05$) and an increase between days 5 and 6 ($t=-4.18$, $n=28$, $p<0.05$). On day 6 of desiccation, wild type plants that had been fed with the L-galactono-1,4-lactone precursor had a higher 6-4PP accumulation than wild type plants that had not been administered the precursor ($t=-5.32$, $n=28$, $p<0.05$) (figure 4.17a).

There was a significant increase in 6-4PP accumulation in the *Vtc3* mutant plants between days 4 and 5 ($t=7.05$, $n=28$, $p<0.05$). *A. thaliana Vtc3* mutant plants that had been fed with the L-galactono-1,4-lactone precursor showed a decrease in 6-4PP accumulation between days 1 and 4 ($t=-8.77$, $n=28$, $p<0.05$), an increase between days 4 and 5 ($t=-4.23$, $n=28$, $p<0.05$) and a further decrease between days 5 and 6 ($t=-3.61$, $n=28$, $p<0.05$) (figure 4.17b).



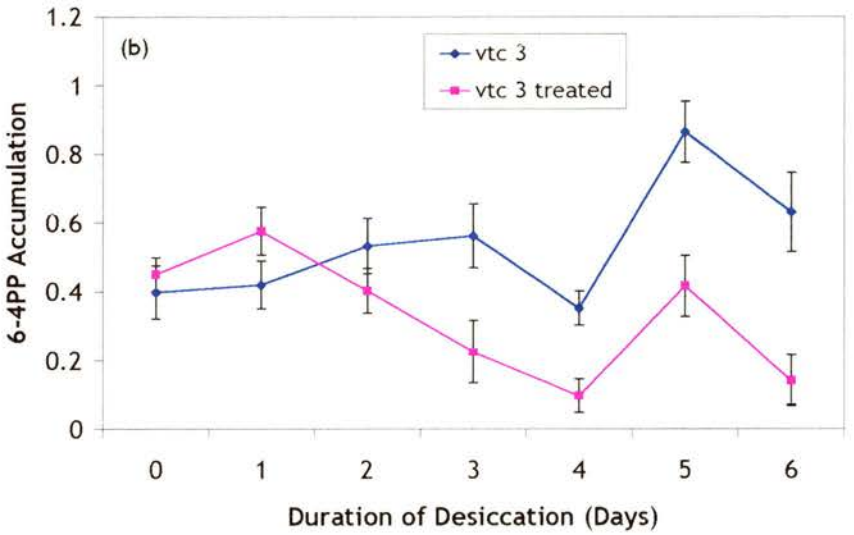


Figure 4.17: 6-4PP accumulation in wild type (a) and *Vtc3* (b) *A. thaliana* during desiccation in the light.

A. thaliana plants were treated as in figure legend 4.16, but desiccated at 560μmol $m^{-2}s^{-1}$ light. Data are means of 12 DNA extractions and two desiccation events. Errors are standard error of the mean.

Overall, the results showed that desiccation significantly affected 6-4PP accumulation ($F_{5,1335}=6.41$, $p<0.05$). Additionally, feeding the plants had a significant effect ($F_{1,1335}=5.98$, $p<0.05$). There was no overall difference in 6-4PP accumulation between plants desiccated in the dark or the light ($F_{1,1335}=0.43$, $p=0.514$) and the *Vtc3* mutant plants did not differ significantly from the wild type plants in 6-4PP accumulation ($F_{1,1335}=6.41$, $p=0.121$).

4.3.6.3 CPD Accumulation in the Dark

Wild type *A. thaliana* desiccated in the dark showed a decrease in CPD accumulation between days 1 and 2 of desiccation ($t=11.85$, $n=28$, $p<0.05$) followed by an increase between days 3 and 5 ($t=2.88$, $n=28$, $p<0.05$). Wild type plants that had been fed with the L-galactono-1,4-lactone precursor showed an increase in CPD accumulation between days 0 and 2 ($t=3.22$, $n=28$, $p<0.05$), an insignificant decrease between days 2 and 3 ($t=-0.84$, $n=28$, $p=0.405$) and a further increase between days 3 and 6 of the desiccation period ($t=0.06$, $n=28$, $p<0.05$) (figure 4.18a).

The *Vtc3* mutant desiccated in the dark showed an increase in CPD accumulation between days 0 and 2 ($t=-4.40$, $n=28$, $p<0.05$), a decrease between days 2 and 3 ($t=-7.16$, $n=28$, $p<0.05$) and an increase between days 3 and 4 ($t=-4.43$, $n=28$, $p<0.05$). The *Vtc3* mutant that had been fed with L-galactono-1,4-lactone showed a decrease in CPD accumulation between days 2 and 3 ($t=6.22$, $n=28$, $p<0.05$), an increase between days 3 and 4 ($t=-3.22$, $n=28$, $p<0.05$) and a further decrease between days 4 and 5 ($t=-2.75$, $n=28$, $p<0.05$) (figure 4.18b).

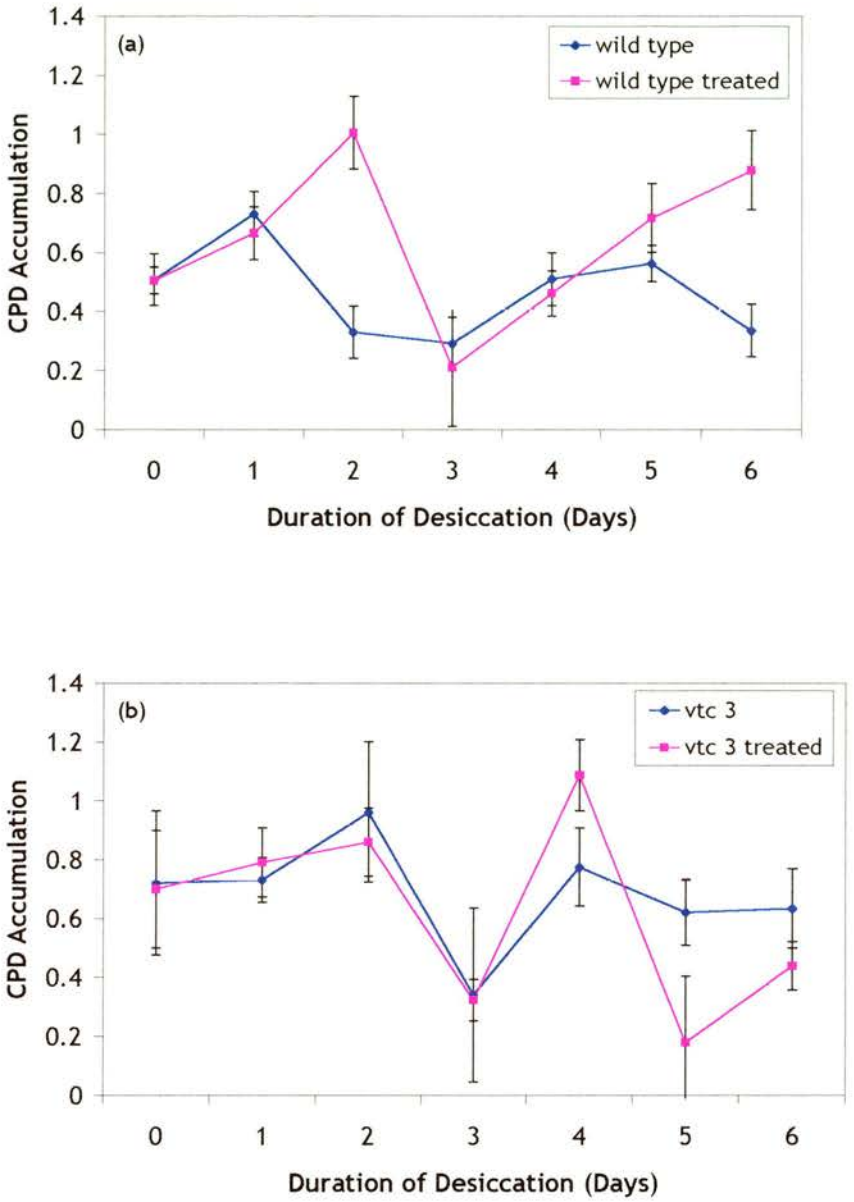


Figure 4.18: CPD accumulation in wild type (a) and *Vtc3* (b) *A. thaliana* during desiccation in the dark.

Wild type (a) and vtc3 (b) *A. thaliana* was treated as in figure legend 4.16. Data are means of 12 DNA extractions and two desiccation events. Errors are standard error of the mean.

4.3.6.4 CPD Accumulation in the Light

Wild type *A. thaliana* desiccated in the light showed a decrease in CPD accumulation between days 1 and 3 of desiccation ($t=5.58$, $n=28$, $p<0.05$) and an increase between days 3 and 4 ($t=2.86$, $n=28$, $p<0.05$). Wild type plants that had been fed with L-galactono-1,4-lactone showed a significant increase in CPDs between days 1 and 2 ($t=-2.87$, $n=28$, $p<0.05$), a decrease between days 2 and 4 ($t=-4.79$, $n=28$, $p<0.05$) and an increase between days 4 and 5 ($t=7.15$, $p<0.05$) (figure 4.19a).

A. thaliana Vtc3 mutant plants desiccated in the light showed no significant change in CPD accumulation throughout the desiccation period ($F5, 43= 0.9$, $p=0.398$). *Vtc3* mutants fed with L-galactono-1,4-lactone showed an increase in CPD accumulation between days 3 and 4 ($t=-4.27$, $n=28$, $p<0.05$), a decrease between days 4 and 5 ($t=-8.09$, $n=28$, $p<0.05$) and an increase between days 5 and 6 ($t=-7.14$, $n=28$, $p<0.05$) (figure 4.19b).

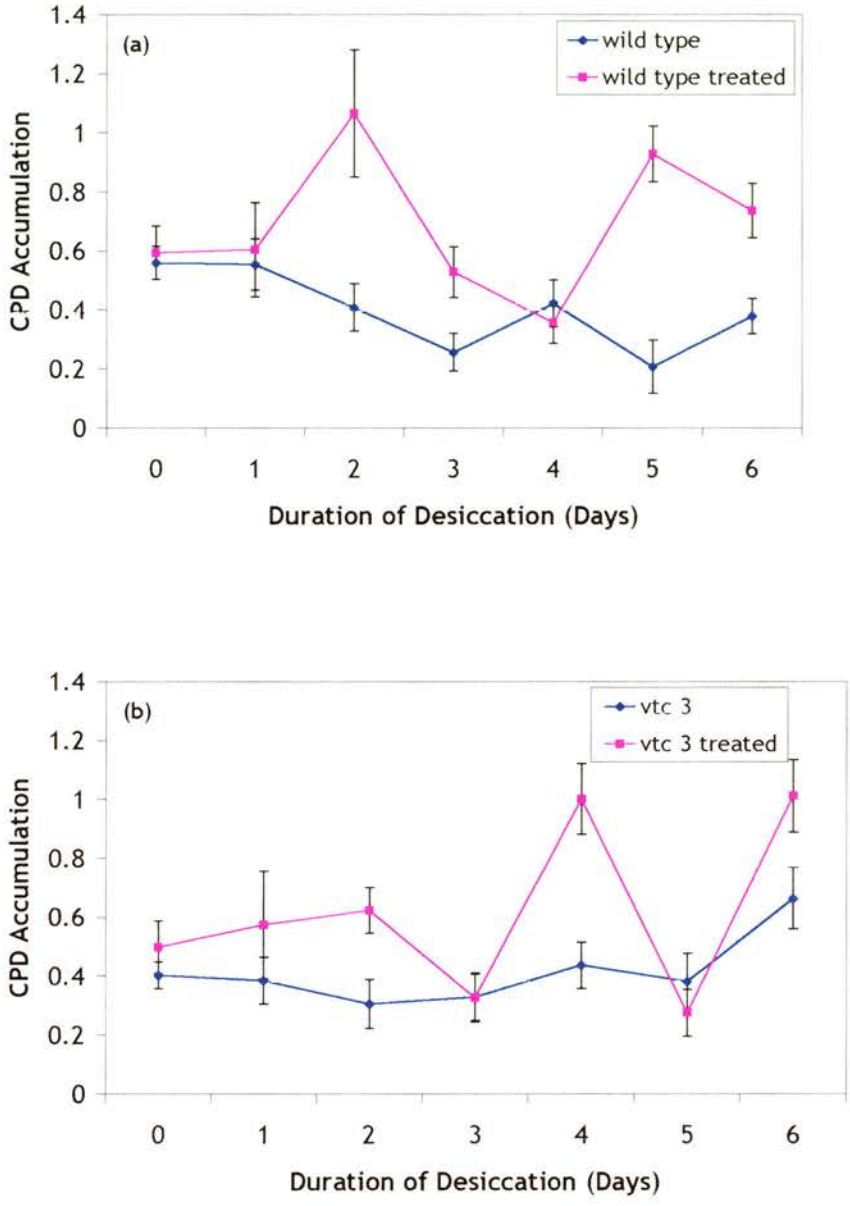


Figure 4.19: CPD accumulation in wild type (a) and *Vtc3* (b) *A. thaliana* during desiccation in the light.

A. thaliana plants were treated as in figure legend 4.16, but desiccated at 560µmol $m^{-2}s^{-1}$ light. Data are means of 12 DNA extractions and two desiccation events. Errors are standard error of the mean.

4.4 Discussion

4.4.1 Oxidative-Induced DNA Damage in *C. purpureus*

Treating *C. purpureus* with radical-generating methyl viologen results in accumulation of DNA damage in the form of 6-4PPs and CPDs. Accumulation of CPDs is seen to increase with increasing methyl viologen concentration. 6-4PPs are seen to accumulate in *C. purpureus* treated with 20μM methyl viologen in the dark, but not in samples kept in the light. As discussed in section 4.0, methyl viologen generates free radicals by accepting electrons from the electron transport chain of photosynthesis, and therefore it is more toxic in the light (Babbs *et al.*, 1989). Methyl viologen is a powerful electron-acceptor and therefore it is possible that it may accept electrons from sources other than PSI in the dark. If ROS is responsible for generating DNA damage then it would be predicted that more damage will occur when methyl viologen is added in the light. However, there was no significant change in 6-4PP accumulation of samples kept in the light. In this instance, photorepair may be active, therefore reducing the net accumulation of 6-4PPs. The reduction of 6-4PP accumulation in *C. purpureus* treated with 40μM methyl viologen in the dark may be due to activation of excision repair. Evidence from chapter 3 suggests that *C. purpureus* may be able to tolerate a certain level of damage before activating excision repair.

In the case of CPDs, DNA damage increased with methyl viologen concentration. Although methyl viologen is thought to be more toxic in the light, the levels of CPD accumulation did not differ significantly in the dark and in the light. This again suggests that photorepair may be active in *C. purpureus* kept in the light.

Methyl viologen did not react directly with the primary antibody, nor did it have any effect on calf thymus DNA *in vitro*. These results suggest that methyl viologen requires a living organism from which to accept electrons resulting in a chain effect of damage. This result provides more evidence that oxidation is causing DNA damage in the form of 6-4PPs and CPDs and that this damage can occur in the absence of UV-B. This is consistent with the

hypothesis, presented in the previous chapter, that damage caused to DNA during desiccation is likely to be as a result of oxidation.

4.4.2 Accumulation of Flavonoids in *C. purpureus*

The above results indicate that the DNA damage which accumulates in *C. purpureus* is likely to occur as a result of oxidative stress. Total UV-B-absorbing compounds (flavonoids and anthocyanins) were seen to accumulate during desiccation in *C. purpureus*. It is probable that these compounds accumulate in order to reduce the oxidative effects of desiccation. The highest levels of both flavonoids and anthocyanins were found in *C. purpureus* desiccated at high light. Desiccation at high light is likely to result in photooxidative stress and therefore it would appear that *C. purpureus* accumulates greater quantities of flavonoids under these conditions. Desiccation has been found to result in the accumulation of pigments. For example, some resurrection plants accumulate anthocyanins in exposed surfaces in response to severe dehydration (Farrant, 2000). Balakumar *et al.* (1993) and Yang *et al.* (2000) found that flavonoid concentration increased in response to desiccation and UV-B radiation in cowpea and cucumber respectively, but not relative to desiccation alone. However, the current results firmly indicate that *C. purpureus* does accumulate UV-B-absorbing compounds and anthocyanins during desiccation in the absence of UV-B.

The results of chapter 3 indicated that, in general, plants desiccated at high light accumulate fewer CPDs than plants desiccated in the dark or under low light conditions. This could be for two reasons. Firstly, high light conditions could activate photorepair, resulting in a net reduction in damage. Secondly, flavonoid production is favourable under high light conditions; therefore *C. purpureus* desiccated under high light may have higher protection against oxidative damage than *C. purpureus* desiccated under low light or in the dark. To determine which of these hypotheses is the most likely, flavonoid concentration could be enhanced in *C. purpureus* desiccated in the dark and under low light conditions using another "feeding" method. If DNA damage is lower in plants treated with flavonoids, then it is likely that the antioxidant

function of flavonoids is sufficient to negate the effects of desiccation-induced DNA damage.

4.4.3 Ascorbate and DNA Damage in *C. purpureus*

Ascorbate levels in *C. purpureus* were observed to be very low. Treatment of the moss with L-glactono-1,4-lactone resulted in an increase in ascorbate under high light conditions, but not under low light. By day 6 of desiccation, the enhanced ascorbate concentration in treated moss had decreased significantly, but still remained higher than in control samples. However, there was no difference in DNA damage accumulation between treated and untreated moss. The low ascorbate concentration of *C. purpureus* suggests that ascorbate is generally not an important antioxidant in this particular species. The lack of effect of ascorbate on DNA damage accumulation supports this hypothesis. It was therefore decided to use *A. thaliana* as a model plant for this experiment, as ascorbate is known to be an important antioxidant in this species (Smirnoff 2000).

4.4.4 The Effect of Desiccation on Chlorophyll Florescence, Leaf Area and Water Content of *A. thaliana*

Chlorophyll fluorescence, leaf area and water content measurements were carried out on *A. thaliana* in order to determine the length of a desiccation period that would be sufficiently stressful, but would not kill the plants. The Fv/Fm of dark-desiccated plants dropped steadily throughout a 7-day desiccation period, although the Fv/Fm did not alter in light-desiccated plants until day 7. Rehydration experiments showed that *A. thaliana* desiccated for 6 days could recover following one hour of rehydration, but plants rehydrated for one hour following 7 days of desiccation had much lower Fv/Fm values. It has been reported that an oxidative burst can occur during rehydration, which can sometimes be more damaging than desiccation (Neill *et al.*, 2002). This oxidative burst is probably responsible for the low Fv/Fm values in rehydrated plants. The water content of leaves of all types of *A. thaliana* (wild type and *Vtc3* mutants both treated and

untreated with L-galactono-1,4-lactone) grown in the light and in the dark had significantly decreased following 6 days of desiccation. It was therefore decided that a 6-day desiccation period was sufficient to stress the plants without proving lethal.

The leaf area analysis showed that the leaves of the *Vtc3* mutants were smaller than wild type plants. There was no change in leaf area for plants desiccated in the dark, but leaf area actually increased in plants desiccated in the light. This suggests that despite a loss of water from the leaves, the plants were able to grow. It is possible that the leaves grew during the first part of the desiccation period when water was not too limiting, and then stopped growing and lost water during the second part of the desiccation period. However, the water content results show a steady decrease in water content throughout the desiccation period. Analysis of leaf area on each day throughout the desiccation period would have given a clearer indication of when the leaves were growing.

4.4.5 DNA Damage in Wild Type and Ascorbate-Deficient *A. thaliana*

The DNA damage results for *A. thaliana* are interesting in a number of ways. The erratic patterns of damage are perhaps the most striking feature of the results. It is only in light of the results from chapter 3 that the current results seem to make any sense. The wild fluctuations in accumulation of damage could in fact be patterns of damage and repair.

4.4.6 6-4PP Accumulation in *A. thaliana*

Wild type *A. thaliana* desiccated in the dark for six days shows accumulation of 6-4PPs in the absence of UV-B. This result is similar to that of *C. purpureus* observed in chapter 3. It can therefore be concluded that desiccation does cause accumulation of DNA damage in the absence of UV-B in *A. thaliana*. Wild type plants that have been treated with L-galactono-1,4-lactone display the familiar threshold pattern of damage with an increase in damage, followed by a period of repair, followed by a further increase where perhaps damage is outweighing repair. This suggests that either the process of treatment with L-galactono-1,4-lactone actually causes damage to the plant,

or that un-treated plants are severely damaged and are constantly repairing, thus giving the appearance of a steady increase in damage. The latter hypothesis is unlikely, and it is more likely that the process of feeding the plant (which involved the application of silwet which provides cuticular penetration of plant surfaces) actually caused some stress to the plants. However, wild type plants desiccated in the light had a higher amount of damage than wild type plants treated with L-galacton-1,4-lactone. Rather than confirming that the process of treating with L-galactono-1,4-lactone does not harm the plants, it could be possible that stress caused by the treatment induces repair. This argument could only be resolved by measuring free radical generation and DNA damage simultaneously.

Both *Vtc3* plants and *Vtc3* plants treated with L-galactono-1,4-lactone and desiccated in the dark showed an increase-decrease-increase pattern in damage. Wild type treated and un-treated plants desiccated at high light showed similar fluctuations in 6-4PP accumulation. *Vtc3* plants desiccated in high light again showed accumulation of 6-4PPs with desiccation. *Vtc3* plants treated with L-galatono-1,4-lactone and desiccated at high light had less DNA damage than untreated plants suggesting either that the treatment sufficiently increased ascorbate levels to protect the plant against damage, or that the treatment had damaged the plants sufficiently to induce repair. Analysis of all the 6-4PP results showed that desiccation is having an effect on DNA damage, but that wild type and mutant plants do not differ in their DNA damage response to desiccation.

4.4.7 CPD Accumulation in *A. thaliana*

There are similar problems in interpreting the results for CPD accumulation in desiccated *A. thaliana.* CPD accumulation fluctuates wildly, and there appear to be no strict rules as to whether or not *Vtc3* plants are more damaged than wild type plants or less damaged, or whether treatment with L-galactono-1,4-lactone is damaging or protecting. Both treated and untreated wild type plants desiccated in the dark show fluctuation in CPD accumulation during desiccation, with treated plants accumulating more damage than untreated plants by the end of the desiccation period. Under

high light conditions, wild type plants show virtually no change in CPD accumulation, suggesting that photorepair is keeping damage low, meanwhile CPD accumulation fluctuates in wild type plants treated with L-galactono-1,4-lactone.

Overall, analysis of the CPD results indicates that CPD accumulation is not linked to desiccation in *A. thaliana*. Furthermore *Vtc3* and wild type plants do not differ in their overall response to desiccation, and treatment with L-galactono-1,4-lactone has no significant effect. However, the erratic fluctuation in the levels of both 6-4PPs and CPDs clearly shows that something is happening in these plants. The changing roles of excision and photorepair make it almost impossible to determine what factors are causing the oxidative damage. In order to prove a direct link between oxidation and DNA damage, free radicals would have to be measured simultaneously with DNA damage. Another possible method of clarifying whether or not photorepair is occurring would be to study photolyase activity.

4.4.7 Ascorbate and Dehydroascorbate in *A. thaliana* During Desiccation

The ascorbate pool in plants is generally thought to be at least 90% reduced. If the plant is subjected to oxidative stress, the ascorbate pool will become at least partially reduced (Law *et al.*,1983). A peak in ascorbate concentration should therefore correspond with a trough in DHA concentration. Additionally, total concentration of ascorbate in leaves is light-dependent. Growth at high light intensities has been found to produce leaves with higher ascorbate concentration than at low light intensity (Smirnoff and Pallanca 1996; Grace and Logan, 1996; Logan *et al.*, 1996). The readjustment of ascorbate content in plants moved from high to low light intensities, or *vice versa*, is relatively slow and may take several days to occur (Eskling and Åkerlund 1998). A surprising result, therefore, is that *A. thaliana* desiccated in the light had less ascorbate than plants desiccated in the dark. Plants desiccated in the light may be experiencing a higher photo-oxidative stress than plants in the dark. A high level of DHA would therefore be expected to correspond with a low ascorbate concentration in light-desiccated plants. A significant difference was found in the DHA levels of plants desiccated in the

light and in the dark, with light-desiccated plants having slightly higher levels of DHA. Light-desiccated plants must therefore have been experiencing higher photo-oxidative stress than plants desiccated in the dark.

During desiccation in the light, the *Vtc3* mutants had the lowest ascorbate levels. Treating the *Vtc3* mutant with L-galactono-1,4-lactone restored the ascorbate level back to that of the wild type plant. However, by day 2 of desiccation, levels of ascorbate in *Vtc3* plants treated with L-galactono-1,4-lactone were no different to that of *Vtc3* plants. This suggests that treatment with L-galactono-1,4-lactone does not have a lasting effect. Ascorbate must be continually synthesised by the plant (Smirnoff, 2000). Treatment with L-galactono-1,4-lactone is therefore not expected to negate the effects of oxidative damage over a long term such as in the present study. In order to determine if raising the ascorbate level of *A. thaliana* can negate oxidative damage to DNA, then studies would have to be carried out using an instant oxidative stress such as exposure to methyl viologen or UV-B radiation.

Wild type plants treated with L-galactono-1,4-lactone and desiccated in the light and in the dark did not have a higher ascorbate concentration than wild type plants. However, L-galactonor-1,4-lactone-treated wild type plants had an increased level of DHA at the beginning of the desiccation period (in both dark-and light-desiccation), suggesting that the excess ascorbate was oxidised to DHA. As with the *Vtc3* plants treated with L-galactono-1,4-lactone, the increased ascorbate/DHA appears to disappear within a few days.

In both dark and light desiccated plants there is a peak in ascorbate concentration and a trough in DHA concentration on day 4 of desiccation. A possible explanation is that the plants are able to synthesise ascorbate until they reach a critical water potential, whereupon the enzymes involved in ascorbate synthesis become inactive, and ascorbate production ceases. If this is the case, then it is expected that the level of ascorbate would increase once more upon rehydration.

4.5 Conclusion

The results of the present study confirm that oxidation plays a critical role in the production of 6-4PPs and CPDs, and that desiccation in the absence of UV-B can result in 6-4PP accumulation in wild type *A. thaliana* desiccated in the dark. However, no evidence was found to suggest that the presence of antioxidants reduce oxidative damage to DNA. The major problem with this study was in determining if a reduction in DNA damage can be accounted for by an increase in repair or by a lack of damaging stress. Similar difficulties lie in attributing an increase in damage to either an increase in damaging stimuli or a reduction in repair. It is suggested that this problem could be solved by directly measuring free radical stress or by measuring the activity of photolyase. Both these proposals are in themselves problematic owing to the difficulties associated with direct measurement of free radicals (Mackerness *et al.*, 1999; Smirnoff 1993; Smirnoff 2000; Halliwell and Gutteridge, 1989) and the hydrating effects of the buffers used in a photolyase assay. Additionally, the method of increasing the ascorbate concentration by treatment of the plants with L-galactono-1,4-lactone failed to maintain an increased ascorbate concentration for more than a few days. In order to determine if ascorbate can negate the effects of oxidative-induced DNA damage, then a study should be carried out using a shorter-term stress, such as exposure to methyl-viologen or UV-B radiation.

If exposure to oxidative stress results in DNA damage, then it is important to determine whether conditions in the field could also result in damage to DNA. The following chapter will therefore focus on DNA damage in *C. purpureus* under field conditions in the Arctic and Antarctic.

Chapter 5

Natural Variance

5.0 Introduction

The third chapter of this thesis provided evidence that desiccation in the absence of UV-B can cause DNA damage in the form of 6-4PPs and CPDs in *Ceratodon purpureus*. This raised the question as to how much DNA damage fluctuates throughout the day under field conditions. Many studies have looked at DNA damage in Polar Regions and attributed this to UV-B (Day *et al.*, 2001). However, results from chapter 4 suggest that a number of environmental conditions could contribute to damage to DNA, particularly those that pose an oxidative stress such as desiccation. Most of the water in the Arctic and the Antarctic is locked up as ice and snow, and is not available to plants (Crawford, 1989). The Antarctic and Arctic field sites were chosen for further study on the basis that they might provide a means of comparing drought and UV-B stresses.

5.1 Ozone Depletion

The detrimental effects of increased solar UV-B have been known for several years (Smith *et al.*, 1992; Ryan, 1992 and Weiler *et al.*, 1994). Ozone (O_3) depletion is most pronounced over the Antarctic continent. The NASA Total Ozone Mapping Spectrometer (TOMS) website shows ozone levels declining by more than 70% during late winter and early spring (http://jwocky.gsfc.nasa.gov/TOMSmain.html). A reduction in ozone by one-half can result in a doubling of solar UV-B reaching the Earth's surface (Fredrick and Lubin, 1994 and Booth *et al.*, 1988).

Chlorofluorocarbons (CFCs) such as CCl_3F and CCl_2F_2 were developed as inert, non-toxic compounds that could be safely used as refrigerants, foaming agents, solvents and in aerosol sprays. The inert nature of the CFCs enables them to escape unchanged through the troposphere and into the stratosphere where UV-C causes photochemical decomposition, yielding free chlorine atoms that catalyse the destruction of ozone (Figure 1.1).

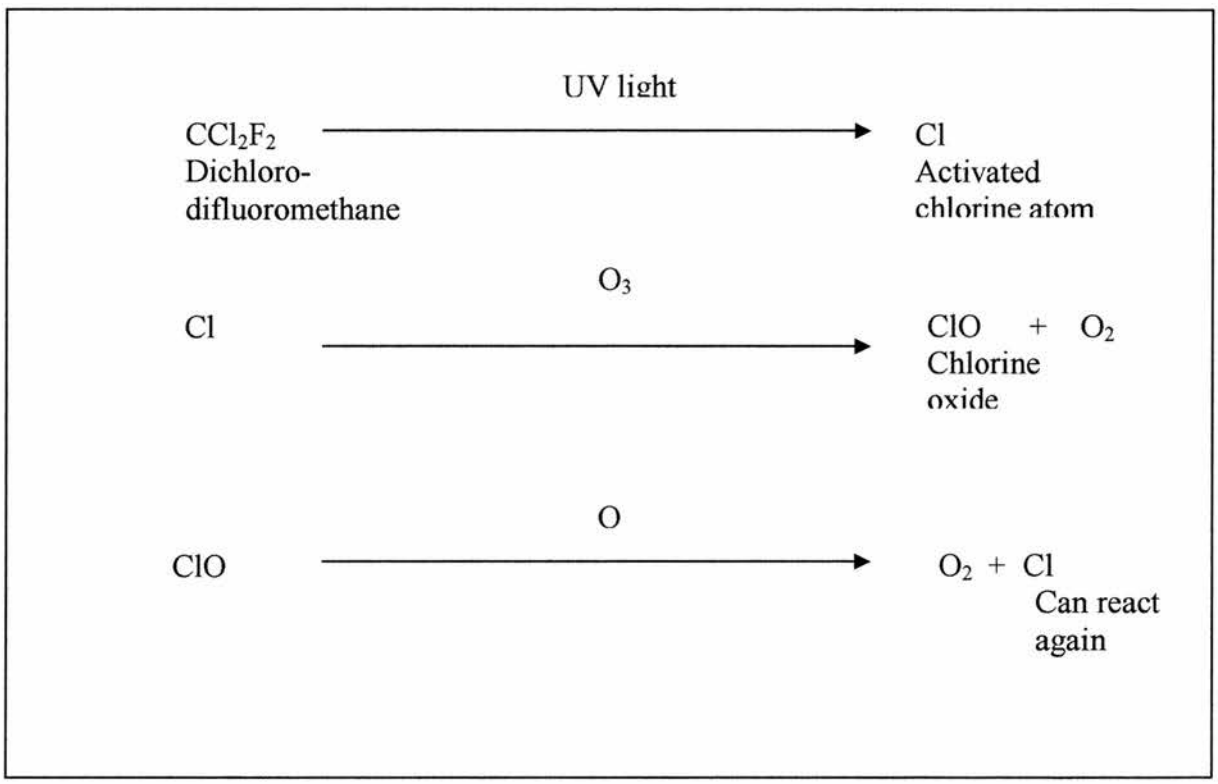


Figure 1.1: Depletion of ozone as catalysed by dichlorodifluoromethane (MacKenzie, 1987)

Although ozone depletion is more prominent over Antarctica, there is a less severe thinning taking place over corresponding latitudes in the North (Madronich *et al.*, 1995 and Shindell *et al.*, 1998).

The detrimental effects of UV-B have been reported in many studies, although the effects are found to vary across and between species (Jordan, 1996). There have been few studies on the interaction of UV-B with water content, especially in natural systems. Furthermore, studies into the effect of UV-B on Antarctic biota have only recently begun.

The influence of UV-B on Antarctic organisms is of particular relevance since ozone depletion and therefore increases in solar UV-B are most pronounced in Antarctica (Madronich *et al.* 1998). The growing ozone hole in the Arctic is

increasing the need for study in this area. Virtually nothing is known about the consequences of ozone depletion and increased solar UV-B on natural ecosystems located outside of Antarctica. The majority of studies on Antarctic biota have been on marine phytoplankton. Solar UV-B levels in Antarctica have been found to depress photosynthesis, resulting in reductions in marine productivity of 5-20% (Smith *et al.*, 1992 and Prezelin *et al.*, 1994).

5.1.1 The Effects of UV-B on Antarctic Angiosperms

Day *et al.* (1999, 2001) and Ruhland and Day (2000) have used screens to reduce the ambient level of UV-B over populations of the only two Antarctic angiosperms, the Hair Grass (*Deschampsia antarctica*) and the cushion-forming *Colobanthus quietensis*. Their findings indicated that solar UV-B reduces the vegetative growth of both species, resulting in their leaves becoming denser with higher concentrations of photosynthetic and UV-B-absorbing compounds. Xiong and Day (2001) determined that rates of photosynthetic gas exchange in these species were not affected by exposure to UV-B. They hypothesize that under UV-B the increase in photosynthetic pigments in both plants allows them to maintain their photosynthetic rates per unit leaf area at rates similar to plants under reduced UV-B levels. Rozema *et al.* (2002) administered increased UV-B to *D. antarctica*, and reported reduced leaf growth, with increased number of shoots, and increased leaf thickness. However, this study did not find a significant increase in UV-B-absorbing compounds under increased UV-B. The effects of UV-B on Antarctic angiosperms are therefore unclear, and may be influenced by other environmental factors, such as water availability.

5.1.2 Effects of UV-B on Morphology of Bryophytes

The influence of UV-B on bryophytes has not been well studied. The ability of bryophytes to retain nutrients makes them critical components of many polar and subpolar ecosystems (Oechel and Van Cleave, 1986). Gehrke (1999) reported reduced growth of the subarctic mosses *Hylocomium splendens* and *Polytrichum commune* over a 3 year period, although Phoenix *et al.* (2001) found that some growth parameters of *H. splendens* increased over a longer term study. *Sphagnum fuscum*, a hummock-forming species which is commonly found to be the dominant species in many peat bog communities in the Northern Hemisphere, was

found to have reduced height under supplemental UV-B administered over two growing seasons. However, as with *Deschampsia antarctica*, the biomass production of *S. fuscum* was not affected (Gehrke, 1998).

5.1.3 UV-B-Induced DNA Damage

Many studies provide indirect, correlative evidence to suggest that plant growth inhibition caused by UV-B may be related to DNA damage (Hidema *et al.*, 1997; Ballaré *et al.*, 1996 and Mazza *et al.*, 1999). For example, Rousseax *et al.* (1999) observed an inhibition in leaf growth of *Gunnera magellanica* growing in Southern South America (under the ozone hole) with solar UV-B. They found that when the ozone hole passed overhead, CPD density in *G. magellanica* leaves increased by 65%. Additionally, a correlation was found between CPD load at midday and the effective UV dose integrated over the morning hours (Rousseaux *et al.*, 2001). However, Lud *et al.* (2002) found that there was no correlation between UV-B and either CPD load or photosynthetic activity in the bryophyte *Sanionia uncinata* growing at field sites both in the Antarctic and the Arctic. The authors suggest that the apparent lack of damage may be due to an increase in repair, thus underlining that fact that at any one point in time, repair processes may be active and therefore any DNA damage observed is 'net' rather than 'gross' damage.

5.2 Aims

The main aims of this chapter were to:

1. Determine how much DNA damage fluctuates throughout the day in *C. purpureus*.
2. Determine if this fluctuation varies globally.

Additionally, the opportunity was taken to erect UV screens, and to take samples of *C. purpureus* for water content analysis, thus providing secondary aims:

3. To determine to what extent accumulation of DNA damage is caused by UV-B.

4. To determine if there is a correlation between water content of *C. purpureus* and accumulation of DNA damage.

5.2.1 Review of Materials and Methods

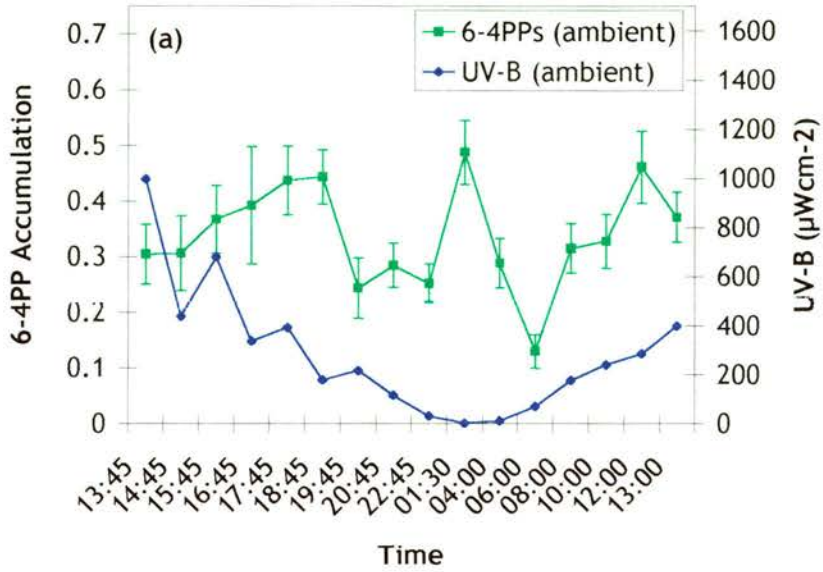
Full details of the materials and methods for this chapter can be found in the methods sections 2.3.2 to 2.3.4.

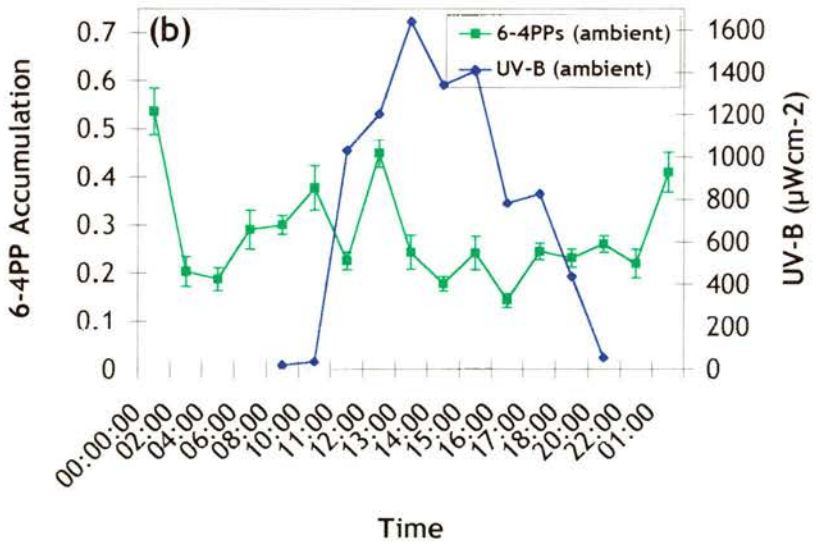
5.3 Results

Statistical analysis was performed using Pearson Correlations (Minitab). Statistical significance is taken as $p \leq 0.05$. If a correlation was 100% significant, then the factors being tested would show identical trends throughout the period of sampling.

5.3.1 Ambient DNA damage in *C. purpureus* with ambient UV-B flux

DNA damage in the form of 6-4PPs and CPDs is seen to fluctuate throughout the day in samples of *C. purpureus* taken from all three field sites. The accumulation of 64PPs and CPDs does not appear to coincide with UV-B at any of the three field sites (figures 5.2 and 5.3). Patterns of CPD and 6-4PP accumulation do not coincide, except in the Antarctic where there is a 51.8% correlation.


(a)
6-4PPs (ambient)
UV-B (ambient)
6-4PP Accumulation
UV-B (µWcm-2)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1600
1400
1200
1000
800
600
400
200
0
13:45
14:45
15:45
16:45
17:45
18:45
19:45
20:45
22:45
01:30
04:00
06:00
08:00
10:00
12:00
13:00
Time


(b)
6-4PPs (ambient)
UV-B (ambient)
6-4PP Accumulation
UV-B (µWcm-2)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1600
1400
1200
1000
800
600
400
200
0
00:00:00
02:00
04:00
06:00
08:00
10:00
11:00
12:00
13:00
14:00
15:00
16:00
17:00
18:00
20:00
22:00
01:00
Time

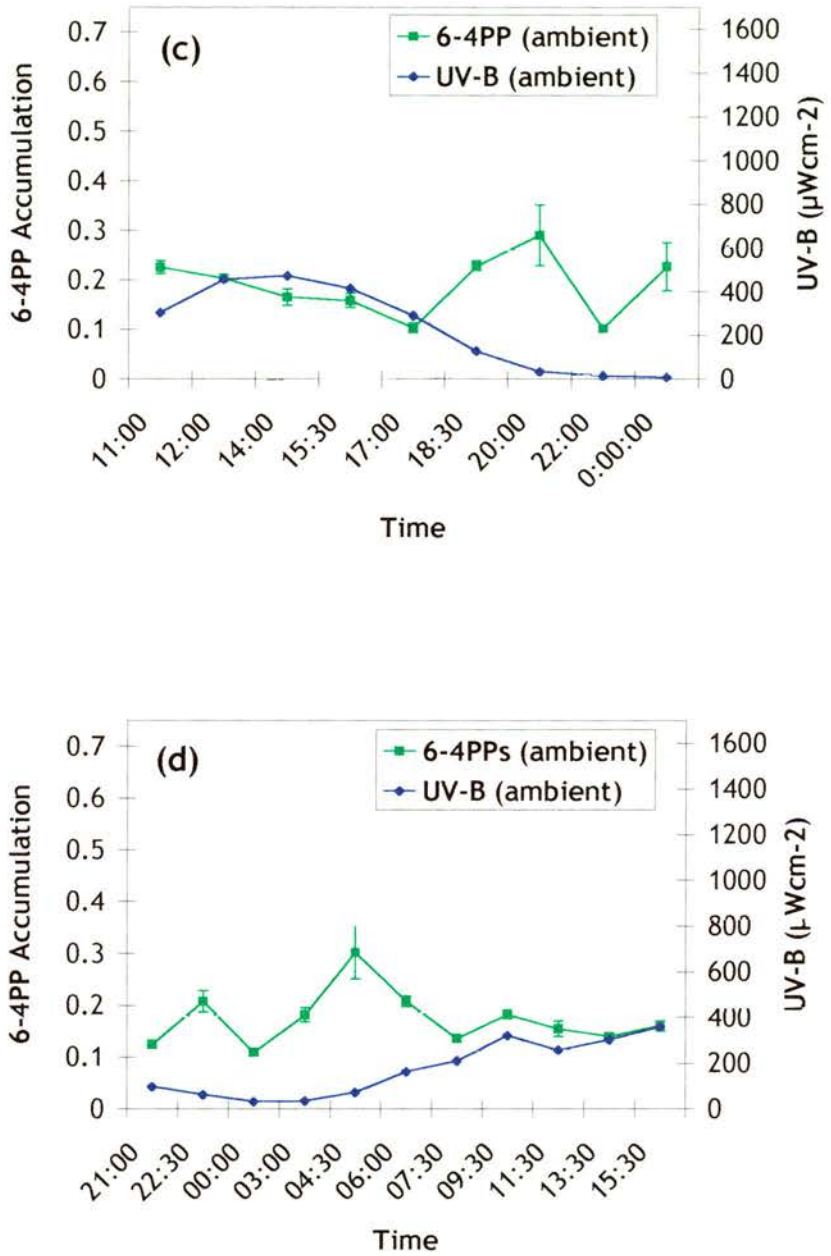
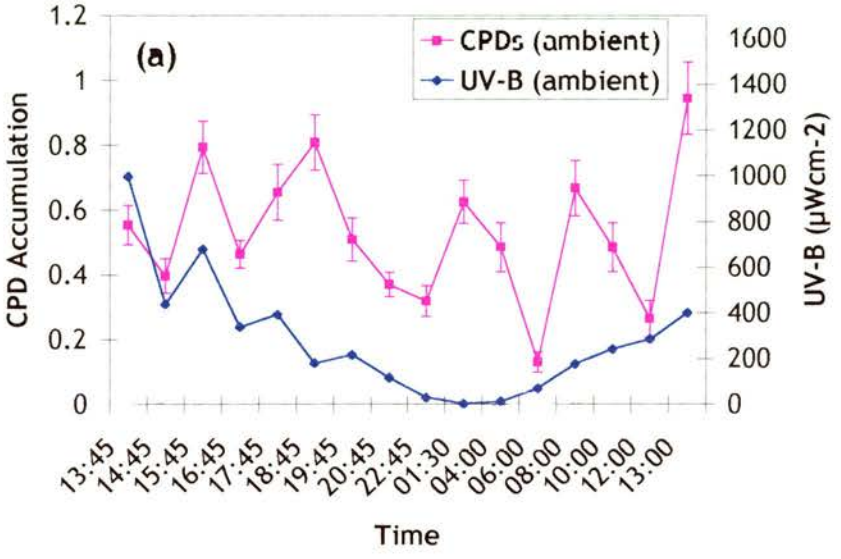


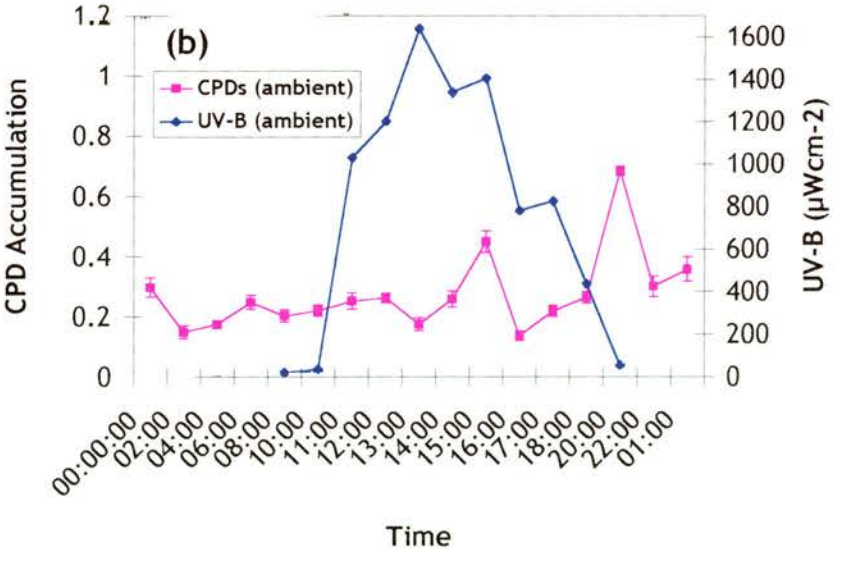
Figure 5.2: Accumulation of 6-4PPs in C. purpureus with ambient UV-B radiation at the Antarctic (a), Scottish (b) and Arctic (c-d) field sites.

Ambient field samples of *C. purpureus* were collected as described in methods sections 2.3.2 and 2.3.3. Antarctic samples (a) were collected from Leonie Island (67 degrees 35', 68 degrees 20'W) on the 16th December, 2001. Scottish samples (b) were collected from Dunlop, Ayrshire (grid reference NS 408 495) on the 5th August, 2002. Arctic samples were collected on the 17th (c) and 21st-22nd (d) of

July 2002 at 75 degrees 24.336 min N, 89 degrees 49.496 min W. DNA damage analysis was carried out (methods section 2.4) in the laboratory.

Data points represent the mean of 3 individual DNA extractions and 8 sub-samples. Error bars show ± one standard error from the arithmetic mean





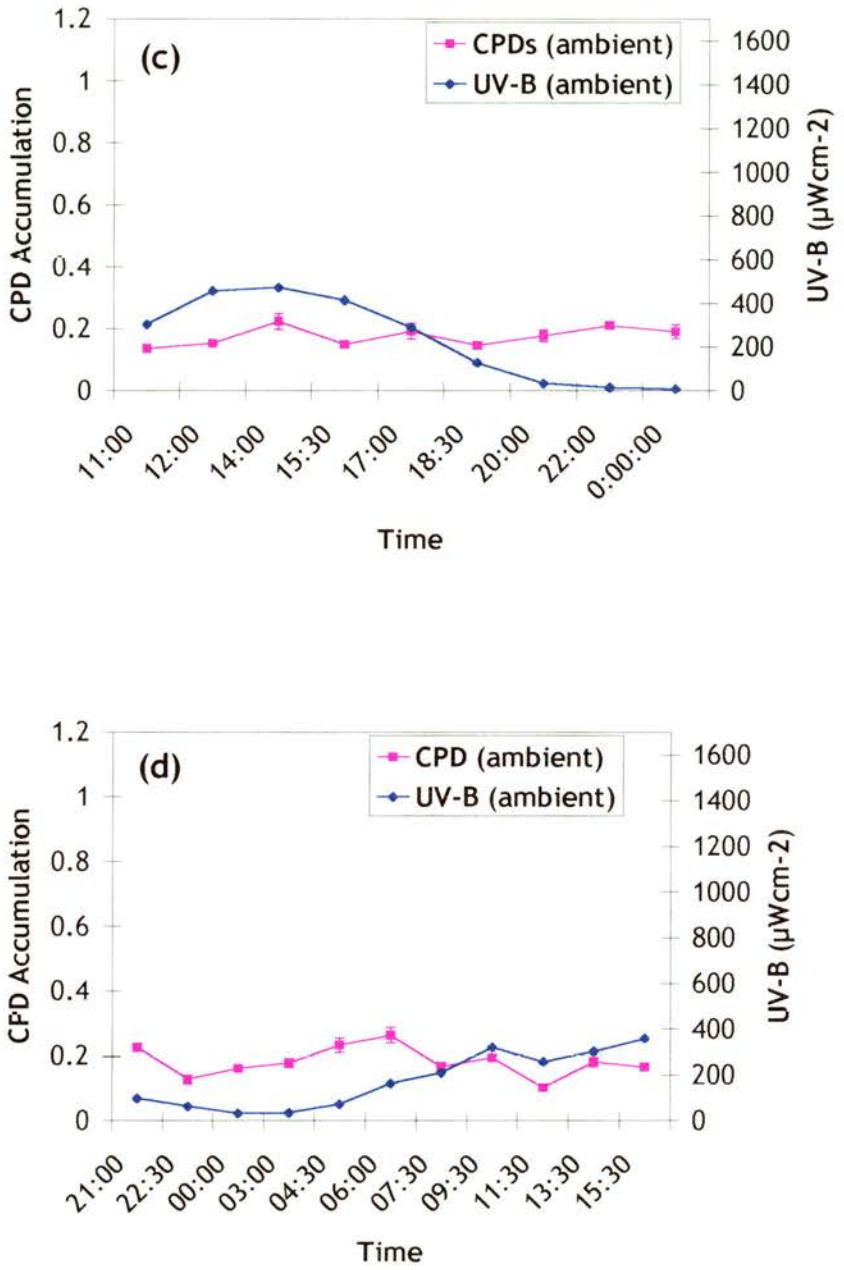


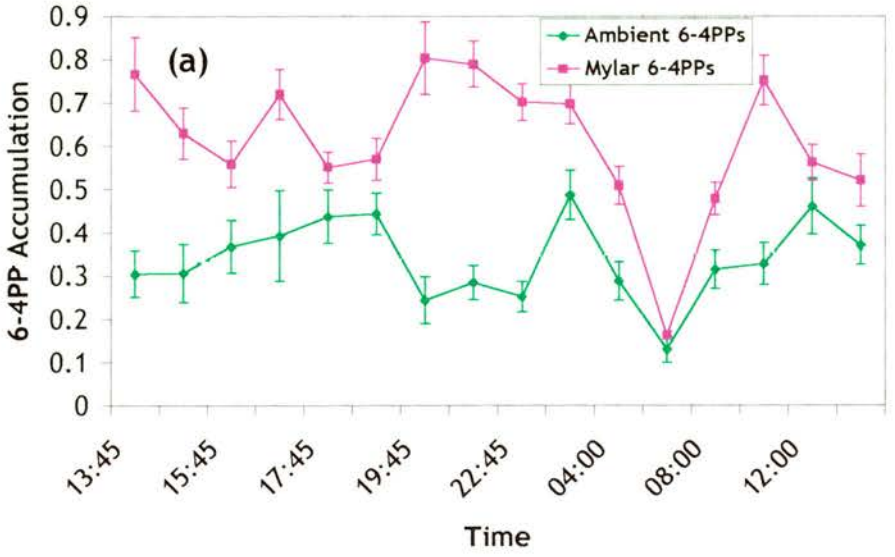
Figure 5.3: Accumulation of CPDs in *C. purpureus* with ambient UV-B radiation at the Antarctic (a), Scottish (b) and Arctic (17th July 2002 (c), 21-22nd July 2002 (d)) field site.

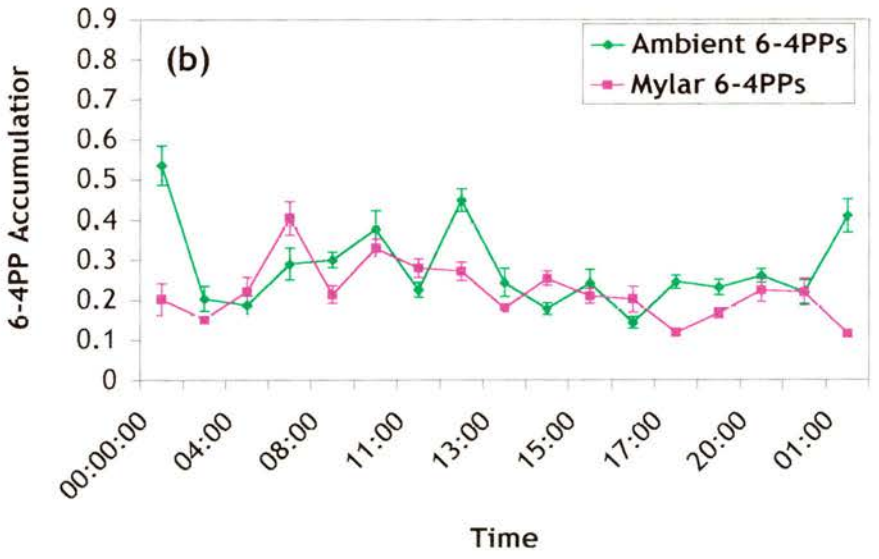
Samples were collected and the DNA analysed as described in figure legend 5.1. Data points represent the mean of 3 individual DNA extractions and 8 sub-samples. Error bars show ± one standard error from the arithmetic mean

5.3.2 DNA damage in *C. purpureus* under ambient and reduced UV-B conditions

DNA damage in *C. purpureus* screened with a UV-B-blocking mylar filter (opaque to UV below 380nm) was not less than that of *C. purpureus* left at ambient conditions. This was true for *C. purpureus* at all three field sites. DNA damage in the form of 6-4PPs fluctuated throughout the day. 6-4PP accumulation significantly coincides in ambient and UV-B-reduced samples from the Arctic site (86.8%). At the Antarctic and Scottish site, the correlation was not significant (25.2% and 9.6%, respectively).

The patterns of CPD accumulation in samples taken from under the UV-B screen were similar to those in samples from ambient conditions. The patterns of accumulation were significantly similar in samples from the Antarctic and from samples taken on the 17th of July 2002 from the Arctic (52.7% and 72.4% respectively). Patterns of CPD accumulation in samples taken on the 21st-22nd of July from the Arctic site and those taken from the Scottish site were not significantly similar to those taken from under Mylar screens (32.6% and 8% respectively) (figures 5.4 and 5.5).


(a)
Ambient 6-4PPs
Mylar 6-4PPs
6-4PP Accumulation
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
13:45
15:45
17:45
19:45
22:45
04:00
08:00
12:00
Time


(b)
Ambient 6-4PPs
Mylar 6-4PPs
6-4PP Accumulatior
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
00:00:00
04:00
08:00
11:00
13:00
15:00
17:00
20:00
01:00
Time

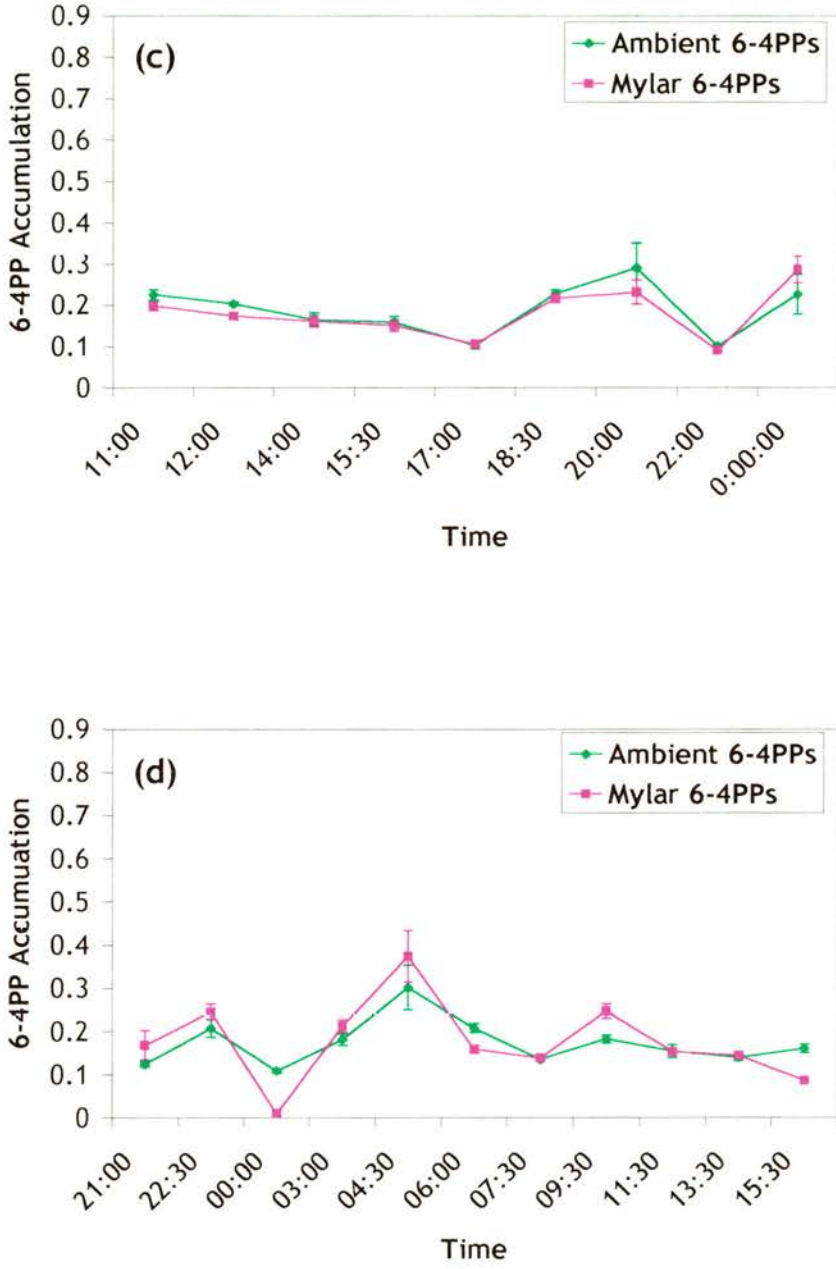
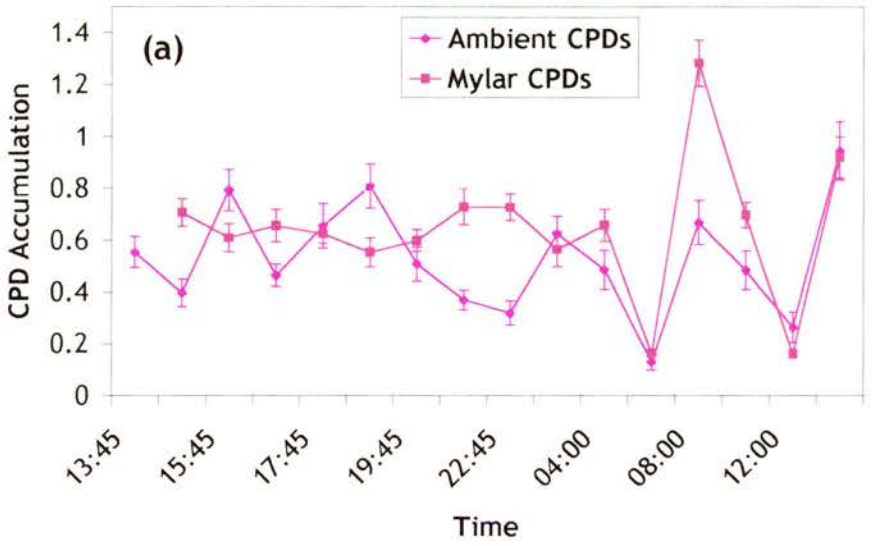
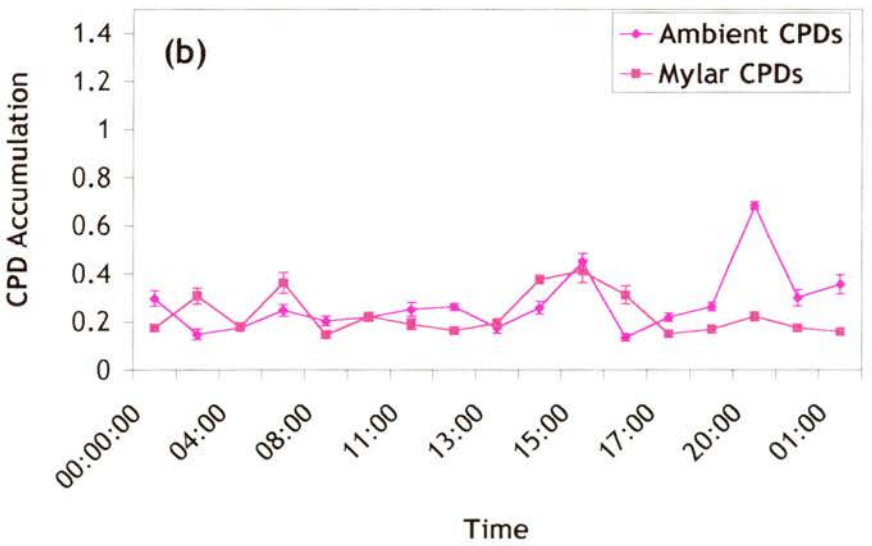


Figure 5.4: Accumulation of 6-4PPs in *C. purpureus* under ambient and reduced UV-B radiation (Mylar screened) at the Antarctic (a), Scottish (b) and arctic (c-d) field sites.

Samples were collected as described in figure legend 5.1.

Data points represent the mean of 3 individual DNA extractions and 8 sub-samples. Error bars show ± one standard error from the arithmetic mean


(a)
Ambient CPDs
Mylar CPDs
CPD Accumulation
1.4
1.2
1
0.8
0.6
0.4
0.2
0
13:45
15:45
17:45
19:45
22:45
04:00
08:00
12:00
Time


(b)
Ambient CPDs
Mylar CPDs
CPD Accumulation
1.4
1.2
1
0.8
0.6
0.4
0.2
0
00:00:00
04:00
08:00
11:00
13:00
15:00
17:00
20:00
01:00
Time

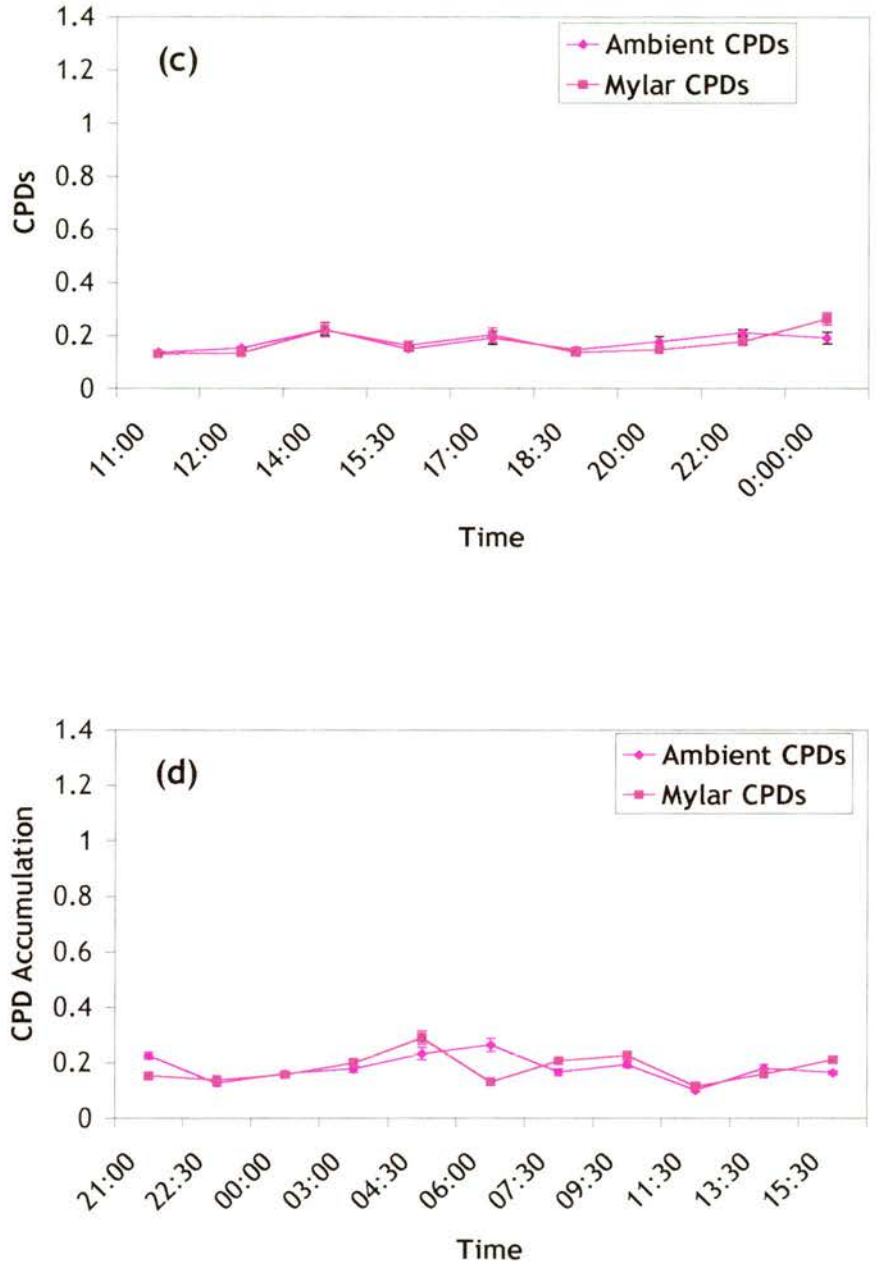


Figure 5.5: Accumulation of CPDs in *C. purpureus* under ambient and reduced (Mylar screened) UV-B radiation at the Antarctic (a), Scottish (b) and Arctic (c-d) field sites.

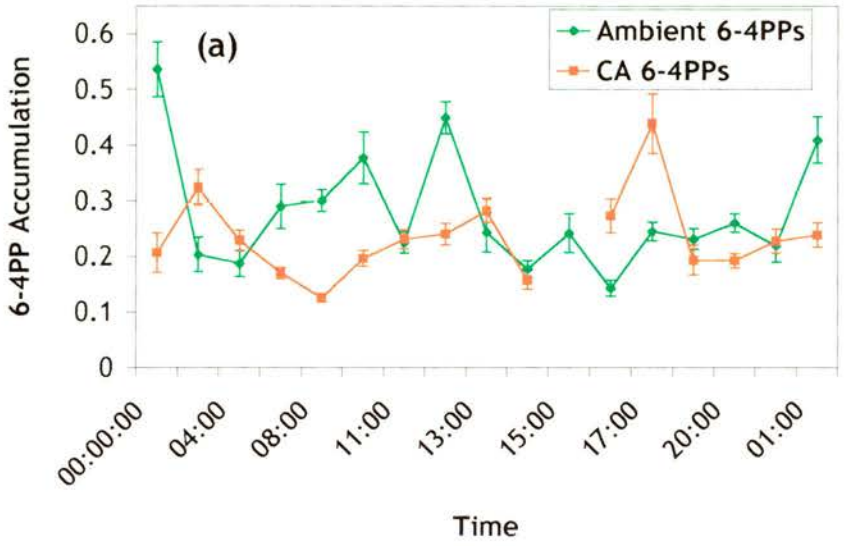
Samples were collected as described in figure legend 5.1.

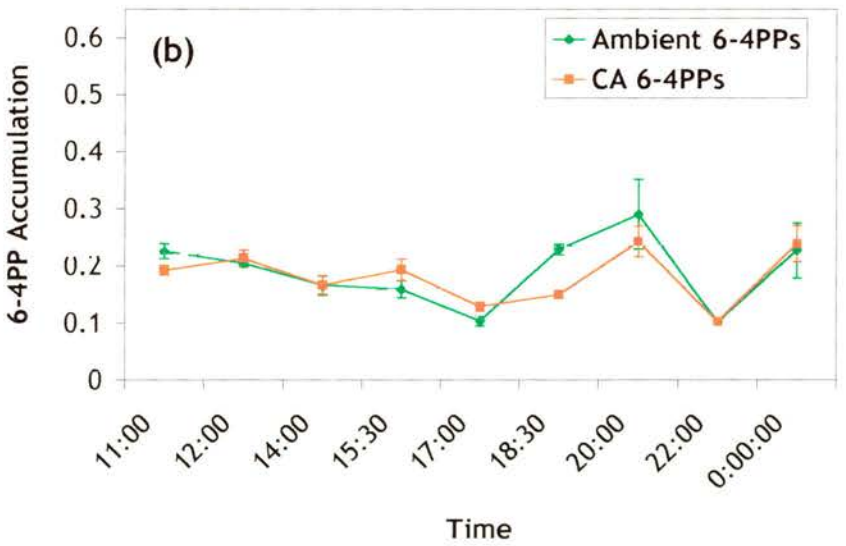
Data points represent the mean of 3 individual DNA extractions and 8 sub-samples. Error bars show ± one standard error from the arithmetic mean

5.3.3 DNA damage in *C. purpureus* under cellulose acetate screens

In samples taken from the Arctic, patterns of DNA damage accumulation were similar in samples from ambient conditions and samples from under a cellulose acetate (CA) screen (which does not filter UV-B, but acts as a control to the mylar screen). These patterns are significantly related for 6-4PPs on both experimental days (17th July 2002: 80.4%; 21st July 2002: 64.7%). The patterns of CPD accumulation are similar, but not significant (17th: 55.3%; 21st: 20%).

In samples taken from Scotland (Dunlop, Ayrshire), patterns of DNA damage accumulation in ambient conditions were different from those found in samples taken from under the CA screen (6-4PPs: -15.4%; CPDs: -8.5%) (figures 5.6 and 5.7).


(a)
Ambient 6-4PPs
CA 6-4PPs
6-4PP Accumulation
0.6
0.5
0.4
0.3
0.2
0.1
0
00:00:00
04:00
08:00
11:00
13:00
15:00
17:00
20:00
01:00
Time


(b)
Ambient 6-4PPs
CA 6-4PPs
6-4PP Accumulation
0.6
0.5
0.4
0.3
0.2
0.1
0
11:00
12:00
14:00
15:30
17:00
18:30
20:00
22:00
0:00:00
Time

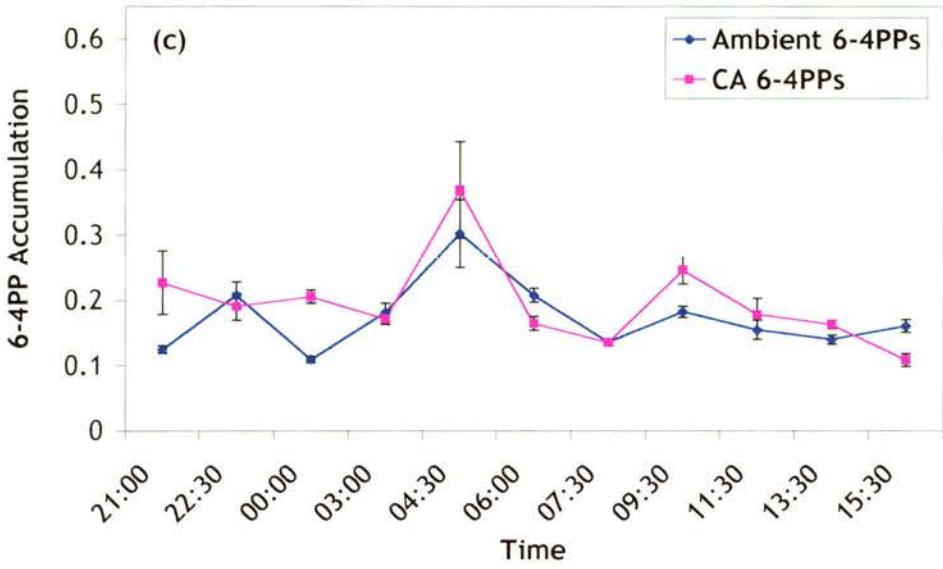
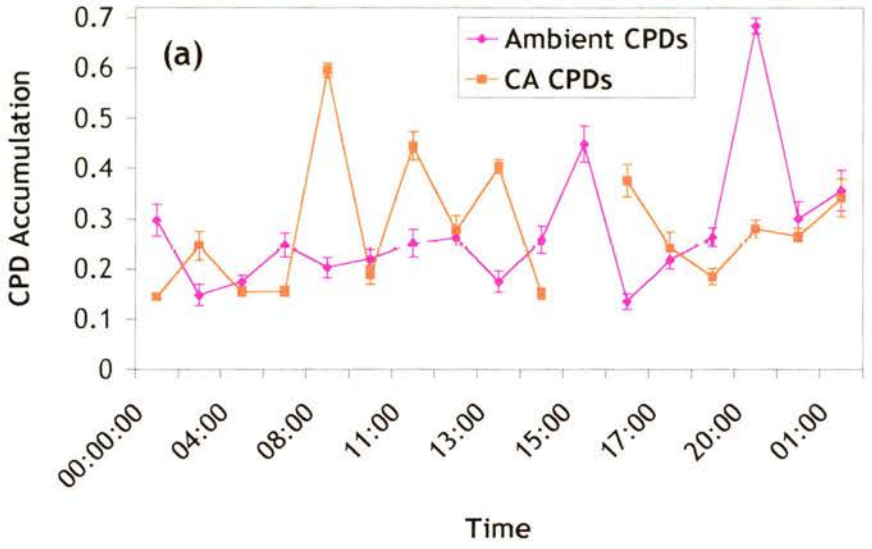
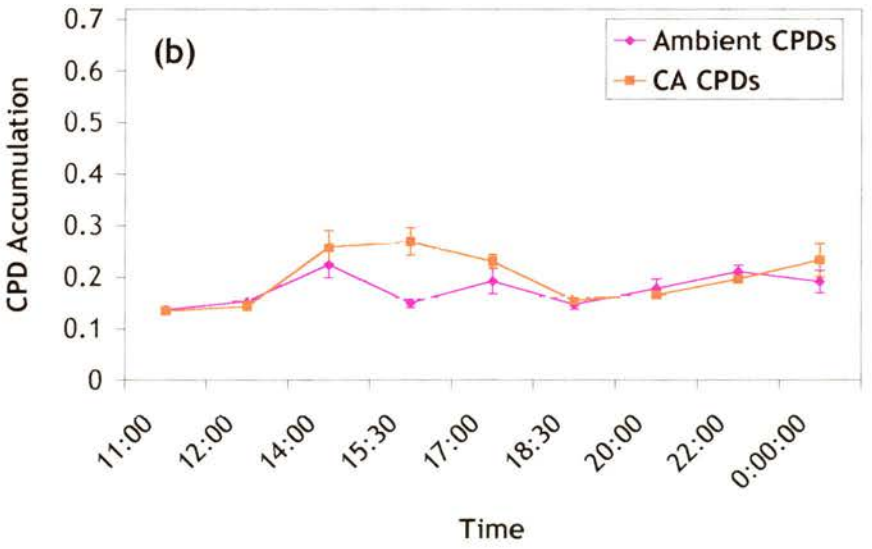


Figure 5.6: Accumulation of 6-4PPs in *C. purpureus* under ambient and control (Cellulose acetate-screened (CA)) conditions at the Scottish (a) and Arctic field site on the 17th July 2002 (b) and on the 21st of July 2001 (c).

Both ambient samples of *C. purpureus* and samples from under a cellulose acetate screen were collected as described in figure legend 5.1. Data points represent the mean of 3 individual DNA extractions and 8 sub-samples. Error bars show ± one standard error from the arithmetic mean


(a)
Ambient CPDs
CA CPDs
CPD Accumulation
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
00:00:00
04:00
08:00
11:00
13:00
15:00
17:00
20:00
01:00
Time


(b)
Ambient CPDs
CA CPDs
CPD Accumulation
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
11:00
12:00
14:00
15:30
17:00
18:30
20:00
22:00
0:00:00
Time

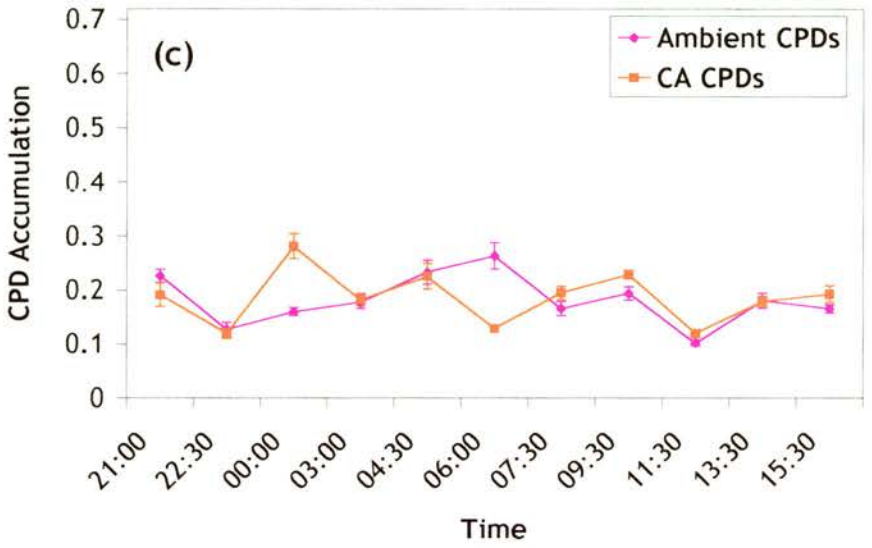


Figure 5.7: Accumulation of CPDs in *C. purpureus* under ambient and control (Cellulose acetate-screened (CA)) conditions at the Scottish (a) and Arctic field site on the 17th July 2002 (b) and on the 21st of July 2001 (c).

Both ambient samples of *C. purpureus* and samples from under a cellulose acetate screen were collected as described in figure legend 5.1. Data points represent the mean of 3 individual DNA extractions and 8 sub-samples. Error bars show ± one standard error from the arithmetic mean

5.3.4 DNA damage and water content

In moss from the Scottish site there is no significant correlation between either 6-4PP, or CPD accumulation and water content of *C. purpureus* (figure 5.8). However, although the relationship is not significant, there does seem to be an inverse trend in CPD accumulation with water content (-29.4%). The water content of *C. purpureus* is high during the night, and the moss was seen to dry out during the day, from 73% water at midnight to 12% water at 16:00hrs. CPD accumulation increased from 0.2976 relative absorbance units (rau) at midnight to 0.6844 rau at 20:00hrs.

C. purpureus which was kept moist (between 90-98% water content) by spraying it with water (as described in methods section 2.3.4) did not have less DNA damage than ambient samples. No relationship was found between ambient samples, and those with higher water contents (figure 5.9: 6-4PPs: -12.9%; CPDs: -0.05%).

5.3.5 DNA damage and temperature

No relationship was found between DNA damage and temperature at any of the sites (data not shown)

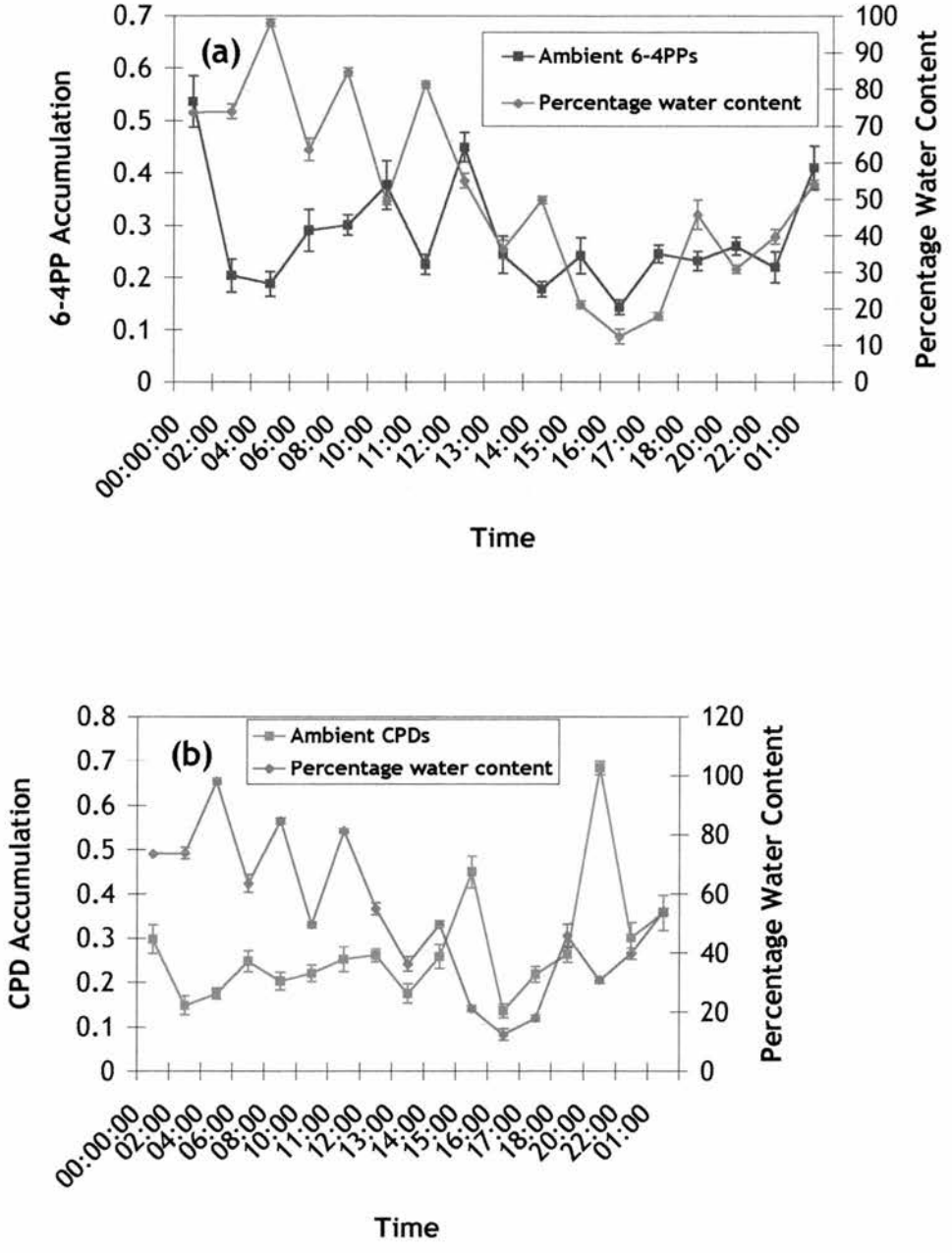


Figure 5.8: Accumulation of 6-4PPs (a) and CPDs (b) with water content in *C. purpureus* taken from the Scottish field site.

Samples of *C. purpureus* were collected (methods section 2.3.4) and analysed for water content (methods section 2.2.3) and DNA damage (methods section 2.4). Data points for DNA damage represent the mean of 3 individual DNA extractions

and 8 sub-samples. Data points for water contents represent the means for three separate samples. Error bars show ± one standard error from the arithmetic mean

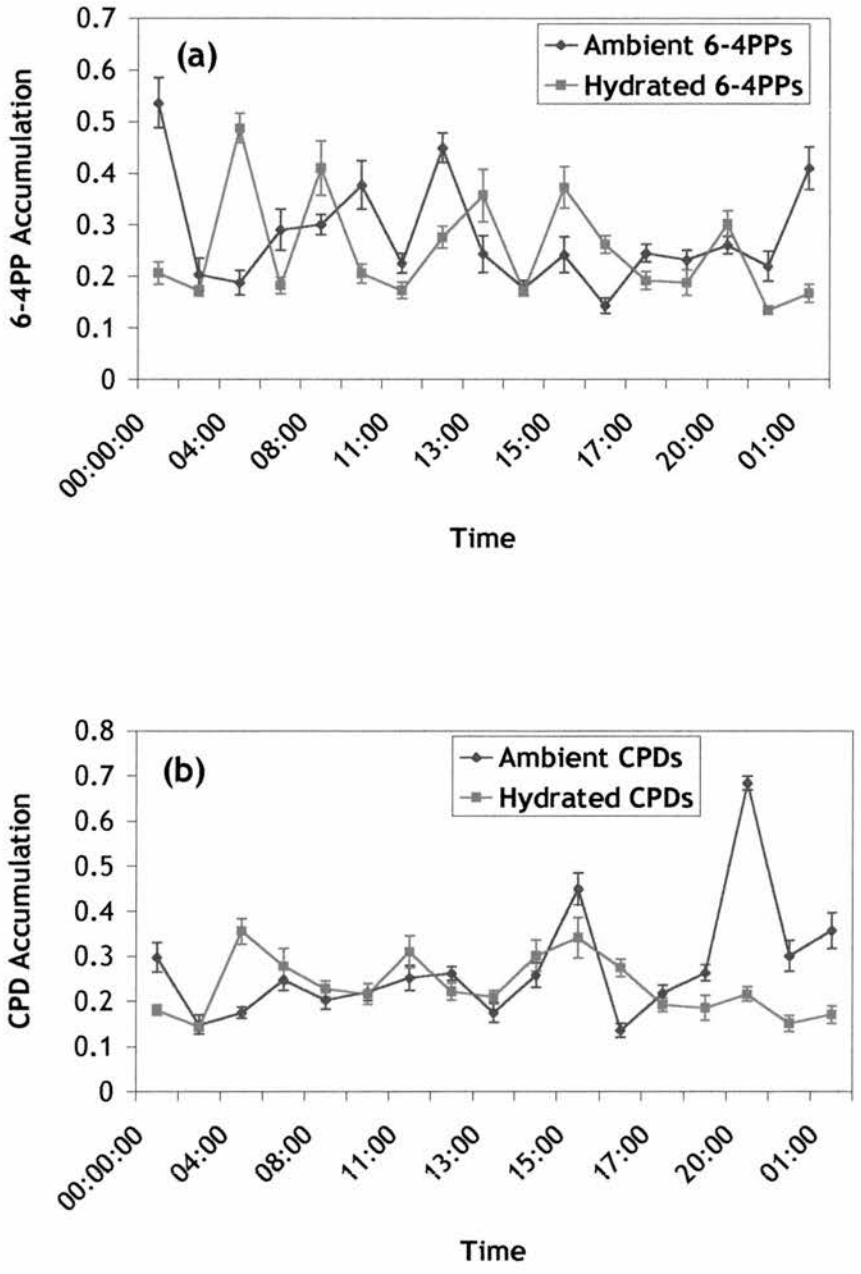


Figure 5.9: Accumulation of 6-4PPs (a) and CPDs (b) in *C. purpureus* under ambient and hydrated conditions at the Scottish field site.

Samples of *C. purpureus* were collected as described in figure legend 5.8. Hydrated C. purpureus was kept moist by spraying with water, as described in methods section 2.3.4. Data points represent the mean of 3 individual DNA

extractions and 8 sub-samples. Error bars show ± one standard error from the arithmetic mean

5.4 Discussion

5.4.1 Daily Variation in DNA Damage

A major aim of this chapter was to determine the daily fluctuation in CPDs and 6-4PPs of *C. purpureus* growing in field conditions. Understanding how DNA damage varies throughout the day will enable us to determine whether the DNA damage accumulation with desiccation seen under laboratory conditions is unusually high, or a relatively normal change in damage load. Setting the maximum change in DNA damage under desiccation in the dark (determined for *C. purpureus* in chapter 3) to 100% it is possible to compare the change in DNA damage in *C. purpureus* under field and laboratory conditions.

C. purpureus from the Antarctic was seen to have the largest fluctuation in CPDs, with 51% of the change seen under desiccation in laboratory conditions. The highest fluctuation in 6-4PPs was observed in the Scottish samples, where it reached 26.4% of that found under desiccation in laboratory conditions. *C. purpureus* from the Arctic (on the 17th July 2001) had least fluctuation in DNA damage ranging from only 6.6% of CPDs to 13% of 6-4PPs compared to laboratory samples. The results show that DNA damage in *C. purpureus* not only fluctuates daily, but also globally. *C. purpureus* under field conditions had less damage than that of *C. purpureus* under desiccation conditions in the laboratory, suggesting that the DNA damage determined under desiccation is a genuine stress to the plant, and that desiccation stress is perhaps more harmful to *C. purpureus* than UV-B radiation. This assumes that the field samples were not experiencing desiccation. It was not possible to carry out water content analysis of Arctic and Antarctic samples. Results from the Arctic over separate days show that daily patterns of UV-B at the same field site were similar, although patterns in DNA damage in *C. purpureus* were not.

In order to determine how DNA damage load affects the physiological status of the plant, a long term study should be set up to monitor photosynthetic parameters along with DNA damage under field conditions.

5.4.2 DNA Damage and UV-B Radiation

The results clearly show that there is no direct relationship between UV-B radiation and DNA damage in *C. purpureus* under field conditions. In ambient samples of *C. purpureus*, DNA damage did not increase throughout the day with increasing UV-B radiation. Decreasing the UV-B radiation using UV-B-filtering (Mylar and CA) screens did not reduce the DNA damage observed at any of the three field sites. Owing to the general amount of fluctuation in the damage, it seems unlikely that the peaks of 6-4PP accumulation are due to a build up of damage several hours after the peak in UV-B radiation.

Patterns of DNA damage under ambient conditions and mylar screens did not always converge. This suggests that some factor other than UV-B is causing the fluctuation in damage. For example at midnight at the Scottish site, *C. purpureus* under ambient conditions had a relatively high level of 6-4PP accumulation. At the same time, *C. purpureus* under the mylar screen had much less damage. The damage in *C. purpureus* under ambient conditions cannot be attributed to UV-B radiation, as no UV-B radiation was present at midnight. Similarly, the moss was found to have a high water content at that time, and therefore desiccation stress cannot be attributed to the accumulation of 6-4PPs under these conditions. In contrast, at midnight at the Scottish site, CPD levels are not particularly high, suggesting that some factor is affecting 6-4PP accumulation, but not CPD accumulation. Following the peak of 6-4PP accumulation, there is a decrease in accumulation. The "threshold" model of chapter 3 may be able to explain these patterns of damage. At midnight at the Scottish field site, photorepair was not possible due to the lack of light. Excision repair (the primary repair pathway for 6-4PPs) is energy-dependent, and therefore DNA damage may reach a threshold before a plant uses excision repair. The high 6-4PP accumulation observed at midnight at the Scottish site may be approaching the damage threshold, hence the decrease in damage at 02:00hrs. The initial cause of the damage is, however, still unknown.

The apparent lack of a relationship between UV-B radiation and DNA damage in *C. purpureus* could be due to this species being UV-B-tolerant. This has been

reported before for the bryophyte, *Sanionia uncinata* (Lud *et al.*, 2002) which does not appear to be affected by UV-B radiation under either Arctic or Antarctic conditions.

Newsham *et al.* (2002) found that the daily dose of unweighted UV-B at Rothera Point on the Western Antarctic Penisula (67 degreesS, 68 degreesW) was a predictor of concentrations of total carotenoids and UV-B-screening pigments extracted from the bryophytes *Cephaloziella varians* (a liverwort) and *Sanionia uncinata* (a moss). Concentrations of carotenoids and UV-B screening pigments were significantly and positively associated with ozone-dependant irradiance parameters. Furthermore, no associations between Fv/Fm and ozone-dependent parameters were found, suggesting that the production of carotenoids and UV-B-screening compounds protected photosynthetic function in these two species. An increase in UV-B-absorbing compounds could also protect plants against DNA damage caused by UV-B. Lud *et al.* (2001) used UV-screens to reduce UV-B, and also used UV-lamps to increase UV-B at their field site on Leonie Island, Antarctica. In contrast to the results of Newsham *et al.* (2002), Lud *et al.* (2001) found no relationship between UV-B and carotenoid and UV-B-absorbing compounds in *D. antarctica* (Poaceae). However, Xiong and Day (2001) did find that leaves of *D. antarctica* exposed to UV-B had higher concentrations of photosynthetic and UV-B-absorbing compounds. They suggest that the development of thicker leaves containing more photosynthetic and screening compounds allowed the plants to maintain their photosynthetic rates per unit leaf area. Carotenoids and UV-B-absorbing compounds were not measured in the present study. It is possible that an increase in these compounds protected *C. purpureus* against DNA damage. A further study of this kind would need to include pigment and growth analysis, although Arctic and Antarctic bryophytes grow very slowly and growth ananlysis can be tricky for mosses of this type.

5.4.3 DNA damage and water content

Lud *et al.* (2002) acknowledge that in Polar Regions, low temperatures limit the availability of liquid water, thus rendering Antarctic and Arctic plants particularly prone to desiccation. Similar to UV-B radiation, desiccation can cause oxidative stress (Takacs *et al.* 1999). Takacs *et al.* (1999) propose that some protective

mechanisms against oxidative stress are constitutive. It might be expected then that an increase in desiccation-tolerance results in an increase in tolerance to UV-B radiation. Supposing that *C. purpureus* from the polar sites is exposed to more desiccation stress than that from the Scottish site, it might then be expected that samples from the Polar sites would have a higher tolerance to oxidative stress and therefore also to UV-B radiation.

In the Scottish study, it was found that where water content was high in ambient samples, CPD levels were low, and vice versa. This result does agree with previous experiments (as discussed in Chapters 3 and 4) and although, in the Scottish field study, this relationship was not significant (Pearson Correlation 29.4%, p=0.252) it is a stronger relationship than that of UV-B and DNA damage (Pearson Correlation 0.085%, p=0.746). However, keeping the moss moist by spraying it with water did not seem to reduce DNA damage. It is possible that the hydrated samples were kept too moist, and that in itself led to damage. Monitoring the physiological status of the moss by chlorophyll fluorescence may help in future studies, to determine if hydrating the moss is causing damage to the plant. There were some peaks of 6-4PP accumulation present in hydrated samples but not in ambient samples. This suggests again that there are many factors that determine the level of CPD and 6-4PP accumulation in *C. purpureus* at any one time. The results from chapter 4 suggest that oxidative stress causes DNA damage, and therefore there may be more than a single environmental factor causing damage at any one time.

It was only possible to measure water contents in *C. purpureus* from the Scottish field site. Additionally, there was only enough moss under the screens to take samples for DNA damage analysis, and not water content analysis. It is thought that due to the strong relationship between desiccation and DNA damage found in chapter 3, desiccation might indeed play a role in the patterns of DNA damage in *C. purpureus* taken from the Polar field sites. Additionally, it would be useful, in a future experiment, to analyse the water content of *C. purpureus* from under the UV-screens.

5.4.4 Effects of screens

A mylar screen was used to reduce the level of UV-B, but this may have had effects on temperature, humidity and therefore water content of the moss. A cellulose acetate screen, which filters out UV-C, but not UV-B, was therefore used as a control against the effects of the screens. It was found that the DNA damage levels under the CA screen did not necessarily correspond with the ambient conditions. Although the relationship between CA and ambient conditions was strong in Arctic moss, the weakest correlations between DNA damage under the ambient and CA screens were found at the Scottish site. The temperature under both the Mylar and the CA screen was increased by 1-2°C. Temperature did not seem to have any effect on DNA damage accumulation, and therefore this cannot be the reason for the differences in DNA damage load between ambient and screened conditions. It is unclear, therefore, what effect the CA screen had on DNA damage in these experiments. It is possible that the water status of *C. purpureus* under the screens was different to that of *C. purpureus* at ambient conditions. Including water content analysis of future experiments might help to negate this problem.

5.5 Conclusion

Previous field studies into 6-4PP and CPD accumulation have only taken into account UV-B as a source of these photoproducts. This research has shown that other factors may also cause an increase in damage, such as desiccation and exposure to oxidative stress (Chapters 3 & 4). Although no strict correlation was observed between water content of *C. purpureus* and DNA damage, the relationship between DNA damage and UV-B was even weaker. In order to determine if water content of *C. purpureus* plays a role in accumulation of DNA damage, then a longer term study must be carried out over several field seasons. In addition to manipulating the level of UV-B in the field, so must the water content of the moss be measured in order to determine which environmental condition is most important in the formation of 6-4PPs and CPDs. Pigment analysis, growth and physiological status of *C. purpureus* would also help in our understanding of photoproduct accumulation in this species. Additionally, this

field study should be repeated using a non-immunological method to determine absolute numbers of photoproducts (Boelen *et al.* 2000; van de Poll *et al.*, 2001) in order to compare the results with other, similar studies.

Chapter 6

Summary

Summary

6.1 DNA Damage in the Absence of UV-B

Chapter 3 focused on the finding that desiccation induced DNA damage in the form of CPDs and 6-4PPs in the absence of UV-B in the moss, *Ceratodon purpureus*. Table 6.1 summarises the conditions under which 6-4PPs and CPDs were found in *C. purpureus*, S. *auriculatum* and *A. thaliana.* This is overwhelming evidence to suggest that desiccation does indeed initiate DNA damage, although this study has still to be carried out using another method of measuring DNA damage to ensure that these results are not a peculiarity of the ELISA technique. *C. purpureus* was found to be able to repair any damage induced during desiccation upon rehydration. Evidence was found to suggest that repair can occur in *C. purpureus* even whilst it is still in the desiccated state. The "threshold" model of DNA damage and repair was proposed.

6.2 Oxidation-Induced DNA Damage

Chapter 4 concentrated on determining if the damage induced during desiccation was a result of oxidative stress. *C. purpureus* was incubated with the free-radical generating herbicide, methyl viologen, and 6-4PPs and CPDs were seen to accumulate. This provided powerful evidence to suggest that DNA damage is indeed a result of oxidative stress. Ascorbate-deficient *A. thaliana* mutants were studied along with wild type plants in order to determine if ascorbate plays a role in defending the species against oxidative-induced DNA damage. Ascorbate content was temporarily increased in *A. thaliana* and *C. purpureus* by incubation with the pre-cursor, L-galactono-1,4-lactone. No evidence was found to suggest that the presence of ascorbate reduces oxidative damage to DNA in either *A. thaliana* or *C. purpureus*.

6.3 Natural Fluctuation in DNA Damage in *C. purpureus*

In order to determine if the DNA damage produced during desiccation in this study was biologically significant, chapter 5 looked at the natural fluctuation in DNA damage in *C. purpureus* from Arctic, Antarctic and Scottish field sites. Previous studies into 6-4PP and CPD accumulation have only taken into account

UV-B as a source of the photoproducts. The relationship between UV-B and DNA damage was seen to be weaker than the relationship between water content of *C. purpureus* and DNA damage.

6.4 Conclusion

This study provides powerful evidence to suggest that 6-4PPs and CPDs can accumulate in the absence of UV-B radiation. It is likely that oxidation is the cause of these so called "photoproducts". The use of a method other than the ELISA technique to measure DNA damage following desiccation is a crucial piece of further work. A number of alternative techniques are available that do not require specific binding of antibodies to DNA damage, for example HPLC (Davies, 1995) and electrophoresis of DNA that has been cleaved by endonucleases at the site of dimers (Quaite *et al.*, 1994).

(a) 6-4PPs	dark	100μmol $m^{-2}s^{-1}$	560μmol $m^{-2}s^{-1}$
C. purpureus	+	+	+
S. auriculatum	-	not tested	not tested
A. thaliana	+	not tested	-

(b) CPDs	dark	100μmol $m^{-2}s^{-1}$	560μmol $m^{-2}s^{-1}$
C. purpureus	+	+	-
S. auriculatum	+	not tested	not tested
A. thaliana	-	not tested	-

Figure 6.1: Summary of results for 6-4 photoproduct (a) and cyclobutane pyrimidine dimer (b) formation during desiccation in the absence of UV-B

The symbols indicate an increase (+) or no change (-) in photoproduct formation with desiccation under 0, 100 and 560μmol $m^{-2}s^{-1}$ (PAR) light.

Appendix

Appendix

Unless stated otherwise, all solutions were made up using sterile distilled water

Solutions for DNA extraction method

Lysis Buffer: 50mM Tris HCl pH7.6
100mM NaCl
50mM EDTA
0.5% (v/v) SDS
10mM 2-mercaptoethanol

TNE solution: 10mM Tris HCl pH 8.0
100mM NaCl
1mM EDTA

TE solution: 10mM Tris-HCl pH 7.6
100mM EDTA pH 8.0

Solutions for ELISA method

5 x PBS (phosphate buffered saline):
0.8M Disodium hydrogen orthophosphate (anhydrous)
0.2M Sodium dihydrogen orthophosphate
1M Sodium chloride
Sterile water

PBS-Tween: 1 x PBS plus Tween 20 at dilution of 100µl per 500ml PBS

Blocking Buffer: 1 x PBS plus 5% (w/v) dried milk (fat free)

Sodium Citrate Buffer:

0.2M Citric Acid

0.1M Sodium dihydrogen orthophosphate pH 5.0

Developing solution:

0.002g 1,2-phenylenediamine in 50ml sodium citrate buffer plus 20µl H_2O_2

2.5N Sulphuric Acid: 69.4ml conc. Sulphuric acid in 500ml water

Solutions for Ascorbate and Dehydroascorbate Assay

0.4M Phosphate Buffer, pH 7.4:

$Na_2HPO_4.12H_2O$

$NaH_2PO4.1H_2O$

10mM DTT:

16mg dithiothreitol (DTT) in 10ml 0.2 NA phosphate buffer pH7.4

NEM 0.5%:

50mg N-ethylmaleimide (NEM) in 10ml water.

Colour Reagent:

A – 4.6% (w/v) TCA

15.3% (w/v) H_3PO_4

0.6% (w/v) $FeCL_3$

B- 4% (w/v) dipyridyl in 70% ethanol

Final working colour reagent:

2.2ml A: 0.8ml B

Solutions for CAT assay

Extraction Buffer:

50mM HEPES

0.1mM EDTA

Potassium Phosphate Buffer:

50mM K_2H2PO_4

50mM $K_2HPO_4.3H_2O$ (pH 7.0)

LST Buffer for Field Collection

100mM Tris HCl (pH 8.3)

0.5M KCl

4.5% (v/v) Nonidet

4.5% (v/v) Tween

1% (w/v) Sodium Azide

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