The effects of ultraviolet-B deficient conditions on antioxidant metabolism and polyamine accumulation in lettuce (*Lactuca sativa* L.)

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Abstract

All light from the sun contains radiation in the ultraviolet (UV) range, wavelengths between 200 and 400 nm that may be harmful to living organisms. Exposure of plant tissues to UV-B radiation (200-400 nm) may result in the formation of reactive oxygen species (ROS), highly reactive chemical species that are capable of damaging biological macromolecules such as DNA, proteins and the lipids of cellular membranes. To detoxify ROS and prevent damage from occurring, plants maintain a battery of antioxidants and associated enzymes. Polyamines are small, aliphatic amines that are found in plants that also function in the stress response by protecting DNA, stabilizing cellular macromolecules and aiding the dissipation of excess energy in photosystem II (PSII). Antioxidants and polyamines are also important in human metabolism and may play a role in preventing the development of several chronic diseases including cancer, diabetes and cardiovascular disease. As antioxidant compounds and polyamines are accumulated in plants under conditions of UV-B stress, there is scope to increase the nutritional value of plant foods by exposing crops to UV-B. The aim of the current experiments was to investigate the effects of UV-B on the activity of antioxidants and polyamine accumulation in lettuce (*Lactuca sativa* L.), a leafy vegetable commonly grown in horticultural set-ups that reduce UV-B exposure. The ability of plant material from different UV-B environments to protect human colon cells from oxidative injury was also investigated. Exposure to UV-B increased the activity of the antioxidative enzymes superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase and glutathione reductase as well as increasing the accumulation of the low-molecular weight antioxidants ascorbate and glutathione. Oxidative damage in UV-B exposed plants was reflected in increases in protein carbonyl and lipid hydroperoxide contents, and in increased oxidation of cellular ascorbate and glutathione pools. However, plants acclimatized to UV-B as the experiment progressed with markers of oxidative damage decreasing after one week of exposure. The response of lettuces to UV-B radiation also varied between varieties, the red-leafed cultivar 'Red Salad Bowl' having lower levels of oxidative damage and recovering more fully than other cultivars.

Polyamines were also accumulated in response to UV-B radiation, especially free and conjugated forms putrescine and spermidine. Accumulation of spermine however, increased as UV-B exposure progressed and a higher proportion of spermine was accumulated as bound-spermine than was the case for other polyamines. Two pathways for polyamine

biosynthesis exist in plants, starting from either ornithine decarboxylase (ODC) or arginine decarboxylase (ADC). ODC activity was not altered by UV-B. ADC activity was up-regulated in UV-B exposed plants, and the localization of this enzyme in chloroplasts suggests a role for polyamines in stabilizing PSII during UV-B stress. The accumulation of antioxidants and polyamines in UV-B exposed lettuces shows there is considerable scope for the nutritional quality of this crop to be improved through horticultural practices that expose lettuces to UV-B, especially if plants are allowed sufficient time for acclimation to UV-B stress before harvest.

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Abbreviations

6-4 PP – pyrimidine (6-4) pyrimidinone dimer

8-oxodGuo – hydroxy-2,8-deoxyguanosine

ABA – abscisic acid

ADC – arginine decarboxylase

AIH – agmatine iminohydrolase

APox – ascorbate peroxidase

AsA – ascorbic acid

Bch – Buttercrunch

CAT – catalase

CPA – N-carbmoylputrescine aminohydrolase

CPD – cyclobutane pyrimidine dimer

cv. – cultivar

DAO – diamine oxidase

DBTB – 5,5-dithiobis(2-nitrobenzoic acid)

dcSAM – decarboxylated S-adenosylmethionine

dGuo - deoxygaunosine

DHA – dehydroascorbate

DHAR – dehydroascorbate reductase

DMEM – Dulbrecco's modified Eagle's medium

DTPA – diethylenetriaminepentaacetic acid

DWFH – Drunken Woman Fringed Head

FBS – fetal bovine serum

GABA – γ-aminobutyric acid

GPox – glutathione peroxidase

GR – glutathione reductase

GSH – glutathione (reduced)

GSSG – glutathione disulfide

HBSS – Hank's balanced salt solution

IAA – indole-3-acetic acid

IEC – intestinal epithelial cell

MANOVA – multivariate analysis of analysis

MDA – monodehydroascorbate

MDHAR – monodehydroascorbate reductase

ODC – ornithine decarboxylase

PA – polyamine

PAO – polyamine oxidase

PAR – photosynthetically active radiation

PSII – photosystem II

PUFA – polyunsaturated fatty acid

Put – putrescine

ROL – Red Oak Leaf

ROS – reactive oxygen specie(s)

RSB – Red Salad Bowl

SAMDC – S-adenosylmethionine decarboxylase

SOD – superoxide dismutase

Spd – spermidine

Spm – spermine

TCA – trichloroacetic acid

UV – ultraviolet

vtc1 *– Arabidopsis thaliana vitamin C -1*

1 Introduction

Plants are dependent on light received from the sun for their continued growth and survival. Solar radiation is thus an essential factor in maintaining life on earth. However, alterations in the amount and spectral composition of radiation received from the sun can have important implications for the functioning of a plant's photosynthetic systems, and can impact on a plant's growth and development (Ordidge *et al.*, 2010). Radiation in the ultraviolet (UV) range (200-400nm) is particularly known for its adverse effects on plant growth and development. While the most severely damaging UV radiation (UV-C, 200-280nm) is attenuated by the earth's atmosphere, some damaging radiation of longer wavelengths (UV-B, 280-320nm and UV-A, 320-400nm) does manage to reach the earth's surface (Frohnmeyer and Staiger, 2003). UV-B radiation in particular can have adverse effects on the growth health of living organisms, including plants. The amount of UV-B radiation that reaches the earth's surface is not constant – it varies geographically, with altitude and latitude, and temporally, with the photoperiod and season. Additionally, concentrations of stratospheric ozone – the main component of earth's atmosphere responsible for UV attenuation – have decreased as the result of human activities resulting in an increase in UV-B radiation reaching the earth's surface (McKenzie *et al.*, 2007). Enhanced UV-B levels as a result of ozone depletion at higher latitudes are likely to continue for well into the 21st century, possibly as late as 2050 (Taalas *et al.*, 2000). Understanding the effects UV radiation has on the growth and functioning of organisms is thus a chief concern of science.

1.1 Ultraviolet radiation and plants

1.1.1 Molecular targets of UV-B radiation

A variety of biological molecules may be directly or indirectly altered by UV-B radiation, including DNA, proteins and lipid membranes. Direct absorption of UV-B radiation by DNA results in the formation of various photoproducts, most commonly cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidinone dimers (6-4 PPs). Proteins involved in the photosynthetic apparatus may also be directly affected and degraded by UV-B exposure. Indirectly, DNA and other biological molecules may be damaged by the formation of reactive oxygen species (ROS) as a result of UV-B radiation and there is evidence that exposure to UV-B alters patterns of gene expression. In the worst case scenarios, exposure to UV-B may inhibit cell functioning and lead to cell death (Bjorn *et al.*, 1999; Frohnmeyer and Staiger, 2003; Jansen *et al.*, 1998)

Photoproducts formed in by the absorption of UV-B radiation by DNA change the base pairing properties of the DNA strand, halting transcription and replication of DNA and preventing DNA and RNA polymerases from reading through the photoproduct (Frohnmeyer and Staiger, 2003; Strid *et al.*, 1994). DNA strands damaged in this way may be repaired by photolyases using energy from blue light and UV-A radiation, an effective system as radiation in these wavelengths accompanies all naturally occurring UV-B light. Cyclopyrimidine dimers and pyrimidine (6-4) pyrimidinone dimers – account for the vast majority of UV-B induced photoproducts, and although the latter are less common they may be more damaging through conversion to Dewar isomers which are less able to be repaired by photolyases (Glas *et al.*, 2010). DNA strands damaged in this way, or containing other lesions induced by oxidative damage (see below) may be repaired through light-independent processes (Frohnmeyer and Staiger, 2003). Failure to repair damage to DNA induced by exposure to UV-B results ultimately in cell death (Bjorn *et al.*, 1999).

UV-B radiation also has a direct effect on elements of the photosynthetic system. Direct exposure to even low fluencies of UV-B results in damage to photosystem II (PSII), and higher levels of exposure can result in PSII inactivation. Damage to the PSII reaction centre occurs primarily to the water-oxidising magnesium cluster, which results in the inactivation of the electron transport chain. Other targets for UV-B damage in PSII include the quinine electron acceptors, tyrosine electron donors and the reaction centres of the D1 and D2 protein. UV-B damaged D1 and D2 proteins may be degraded and replaced by *de novo* protein synthesis as part of a repair mechanism (Sicora *et al.*, 2006; Wu *et al.*, 2011). Degradation of these proteins is synergistically accelerated with PAR, and reaches its maximal rate at wavelengths of 300 nm, wavelengths shorter than this having less of an effect. Failure to repair damaged reaction centres results in the inactivation of PSII through the accumulation of damaged reaction centres, decreased oxygen evolution and reduced variable fluorescence (Fig. 1.1, Jansen *et al.*, 1998).

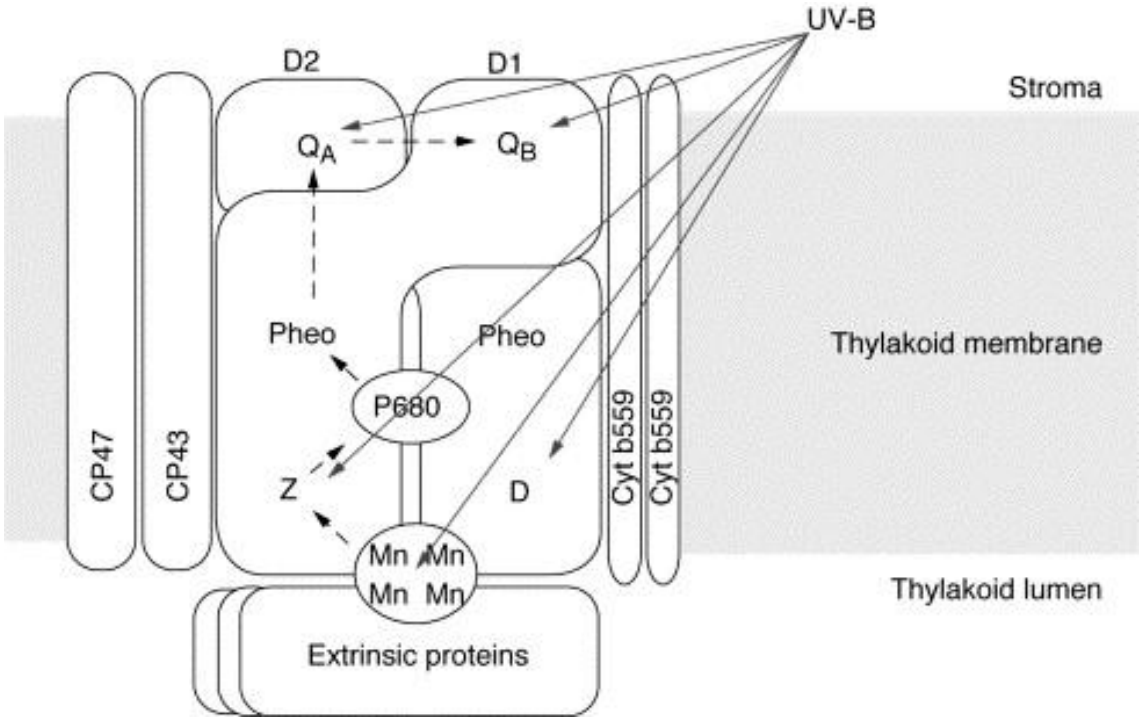

Figure 1.1 Schematic representation of photosystem II (PSII), indicating photosenstizers proposed to be involved in its UV-B mediated inactivation. P680 is the primary electron donor of PSII. Z and D are redox active tyrosines located on the D1 and D2 proteins, respectively; Z normally serves as the electron donor to P680. Electrons originate from water, the splitting of which is catalysed by a cluster of four magnesium atoms. Extrinsic proteins are involved in stabilising this reaction. On the acceptor side, a pheophytin serves as the primary electron acceptor. The plastoquinones, Q_A and Q_B, are the secondary electron acceptors. Photosensitizers that absorb in the UV-B range and that have been proposed to play a role in PSII inactivation and/or D1-D2 degradation are marked with arrows (figure and caption from Jansen *et al.*, 1998).

1.1.2 Formation and effects of reactive oxygen species

Damage to a range of metabolic systems may also result from the formation of ROS. Interactions between UV-B and molecular oxygen in animal and bacterial cells results in the formation of ROS and increased levels of oxidative stress suggest the operation of a similar process in UV-B exposed plant cells, although the precise pathway through which this occurs is not known (DeLong and Steffen, 1998; Gao and Zhang, 2008; Strid *et al.*, 1994). Radicals produced from molecular oxygen include singlet oxygen ($^1O_2$) and superoxide ($O_2\cdot^-$), both of which are highly reactive. Superoxide may undergo further reduction to produce peroxide ions (O_2^{2-}), and/or protonation to form the perhydroxyl radical ($HO_2\cdot$) or hydrogen peroxide (H_2O_2). The latter is particularly damaging as a source of highly reactive hydroxyl radicals ($OH\cdot$) via the Fenton reaction (Gill and Tuteja, 2010).

At low levels, ROS are important for cellular processes such as signal transduction and the maintenance of inter-membrane ion concentrations. However, in excess concentrations ROS

may disrupt cellular functioning through interactions with nucleic acids, proteins and cellular membranes (Bjorn *et al.*, 1999; Gill and Tuteja, 2010; Strid *et al.*, 1994). Interactions between ROS and nucleic acids are the most serious, as the oxidation of DNA results in the formation of lesions capable of inducing mutation, disrupting transcription, and can affect patterns of gene regulation and reducing protein synthesis (Britt, 1999; Cooke *et al.*, 2003). Oxidative damage to DNA is thus capable of having profound impacts on cell growth and development that may have serious consequences for the whole organism.

Cellular proteins may be oxidised through covalent modification by ROS or other by-products of oxidative stress (Gill and Tuteja, 2010). Most types of protein oxidation are irreversible and extensive protein oxidation can be energetically expensive for the cell to repair (Moller *et al.*, 2007). Limited oxidation of sulphur-containing proteins however, can be reversed and it is possible the oxidation products of the amino acids cystine and methionine are used as signalling molecules (Bigelow and Squier, 2011). Oxidative damage to proteins that cannot be repaired may result in breakages in polypeptide chains and conformational changes leading to protein degradation by proteases. In cases of widespread protein damage, autophagy of cellular compartments may be necessary to prevent the accumulation of dysfunctional proteins culminating in the eventual death of the cell (Dean *et al.*, 1997; Moller *et al.*, 2007).

Damage to lipid membranes via lipid peroxidation is one of the most serious effects of ROS, as it not only damages cellular compartmentalization and functioning but also results in the propagation of lipid radicals (ROO·) that may go on to cause further oxidative damage in the cell (Mano *et al.*, 2009). Lipid peroxidation proceeds through a three-step process of initiation, propagation and termination (Fig. 1.2). In the initiation phase, the hydrogen atoms from the unsaturated fatty acyl chains of polyunsaturated fatty acid (PUFA) residues are abstracted to ROS, principally OH·, forming a lipid alkyl radical (R·, [1]). During propagation the resulting R· are then oxidised in the presence of O_2 to form lipid peroxy radicals (ROO·, [2]), which can further react with a neighbouring PUFA to form a lipid hydroperoxide (ROOH) and another R· (3). The R· may be available for further reactions while ROOH spontaneously degrades to give a wide variety of lipid-derived radicals. The process may be terminated through the formation of dimers between the various radical species (Fig. 1.2 (4), Gill and Tuteja, 2010). If lipid peroxidation becomes widespread, it results in decreased membrane fluidity, membrane disruption and increased leaking of solutes across the membrane (Moller *et al.*, 2007).

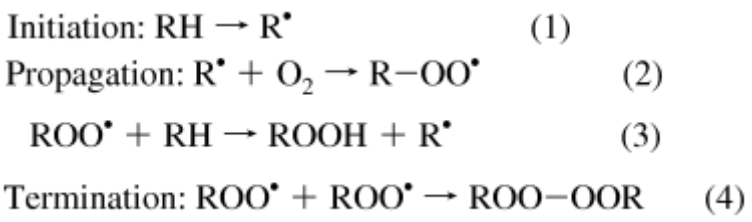

Figure 1.2 Steps in lipid peroxidation reaction (Figure from Guo *et al.*, 2009).

1.1.3 Effects on growth and development

A number of other responses have been recorded in plants exposed to UV-B. Many plants exposed to UV-B exhibit symptoms such as reduced height, increased auxiliary branching and shorter internode lengths (Jansen *et al.*, 1998; Li *et al.*, 2010). The area of the leaf surfaces may be reduced and the leaves themselves may thicken, allowing chloroplasts to move away from the leaf surface. Increased production of epicuticular waxes and other UV-B protectants may also occur as the plant attempts to screen cellular systems from the harmful effects of UV-B radiation (Jansen *et al.*, 1998).

Many of the changes seen at the level of the whole plant are thought to be the result of alterations or damage to the metabolic systems responsible for regulating normal growth and development. Indolacetic acid (IAA) is a hormone responsible for the control of shoot elongation and auxiliary branching and increased oxidation of IAA in the presence of UV-B may allow for the increased development of lateral buds and may reduce the overall height of the main stem (Huang *et al.*, 1997). Some authors have been reluctant to class these responses occurring from UV-B exposure as 'damage', suggesting instead that they may exhibit a protective function by shading the plant from direct exposure to UV-B containing radiation (Frohnmeyer and Staiger, 2003; Jansen *et al.*, 1998). A similar principle may apply to reductions in total biomass commonly associated with UV-B exposure, especially in herbaceous plants. Traditionally viewed as 'damage' resulting from photoinhibition and reduced carbon fixation (Krizek *et al.*, 1998), it has been suggested that biomass reductions may simply be a result of increased investment in protective systems (see below), a view given weight by observations that decreases in total biomass are not always associated with photoinhibition (Li *et al.*, 2010; Tsormpatsidis *et al.*, 2008)

The accumulation of epicuticular waxes and the morphological responses of leaves are also considered as protective strategies to avoid UV-B induced damage to cellular systems. The increased development of cuticular waxes that has been observed in some plant systems exposed to UV-B exhibits a protective function by reflecting or absorbing UV-B. The effectiveness of this protective shield may be increased through the incorporation of UV-B

absorbing compounds such as flavonoids and ferulic acids co-polymerized with lignin (Jansen *et al.*, 1998). Leaf thickening is a morphological response thought to help increase the screening capacity of the epidermis and allow for the movement of chloroplasts away from the leaf surface, where they may be susceptible to UV-B damage. Other responses, such as leaf curling, reductions in leaf surface area and increased tilting of leaves are thought to be mechanisms aimed at reducing the surface area exposed to potentially damaging radiation (Jansen *et al.*, 1998; Li *et al.*, 2010).

The production of various classes of phenolics is stimulated by exposure to even low fluences of UV-B. This primarily involves the induction or up-regulation of genes involved in the production of flavonoids and sinapic esters (Park *et al.*, 2007). The phenolics accumulated under UV-B exposure function as protectants, being concentrated in upper epidermal cell layers and absorbing radiation maximally in the 280-340 nm range. The flavonoids accumulated under UV-B exposure are also suspected of exhibiting an antioxidant function, helping to mitigate the oxidative stress experienced under UV-B and possibly conferring cross-tolerance to other stressors (Jansen *et al.*, 2008). However, up-regulation of flavonoid production is a complex process that may be overridden by other stressors such as high temperature and fungal attack (Strid *et al.*, 1994). As such some studies have suggested that sinapic esters may be a more important class of UV-B protectants (Landry *et al.*, 1995).

1.2 Antioxidant metabolism

During normal metabolism plants maintain a steady-state production of ROS, which are utilized in various ways including in the transmission of electrons in signalling pathways and in the photosynthetic electron transport chain (Noctor and Foyer, 1998). This results in a balance between reduction and oxidation reactions within the plant cell, which may be disrupted by a variety of stress conditions, leading to the overproduction of ROS and oxidative stress. To assure the maintenance of steady-state levels of ROS necessary for normal functioning, plants maintain a system of ROS-scavenging antioxidants which contains both enzymatic and non-enzymatic components. Redundancy and multi-functionality are major characteristics of plant ROS-scavenging systems, allowing for the maintenance of a greater degree of antioxidant power than might otherwise be possible (Mittler, 2002; Van Breusegem *et al.*, 2008).

1.2.1 Antioxidative Enzymes

1.2.1.1 Superoxide dismutase

Superoxide dismutase (SOD, EC 1.15.1.1) is one of the most important and widespread antioxidative enzymes in plant cells. Found in most cellular compartments, it is responsible for catalyzing the dismutation of $O_2^{\cdot -}$ to H_2O_2 as an initial step in ROS detoxification (Fig. 1.3). Regulation of SOD activity is likely to be signaled by rising levels of $O_2^{\cdot -}$ itself, and rapid rises in SOD activity are recorded as part of the initial response to several abiotic stressors including UV-B (Gill and Tuteja, 2010; Han *et al.*, 2009; Jain *et al.*, 2003; Selvakumar, 2008). The role of SOD is to provide immediate protection from the most severe effects of oxidative stress at the site of ROS production before other antioxidant defense mechanisms become operational. The rapid rise in SOD activity in response to oxidative stress is thus often a transient phenomenon: (Dawar *et al.*, 1998) recorded that SOD activity in excised chloroplasts peaked at 2.4 times its ordinary level only three hours after UV-B exposure, decreasing to only 1.3 times the usual levels thereafter.

1.2.1.2 Catalase

Catalases (CAT, EC 1.11.1.6) are found in almost all cells exposed to oxidative stress (Gill and Tuteja, 2010). Catalases in plant cells are most commonly localized to peroxisomes and are involved in moderating oxidative stress by catalyzing the conversion of H_2O_2 to water and molecular oxygen (Noctor and Foyer, 1998). CAT activity varies in response to different abiotic stressors, frequently being reduced after UV-B irradiation (Selvakumar, 2008; Zancan *et al.*, 2008) although increases have been recorded, especially at warmer temperatures and in combination with other stressors (Ambasht and Agrawal, 2003; Han *et al.*, 2009; Yang *et al.*, 2007b). A study by Berli *et al.*, (2010), showed CAT activity in grape vine (*Vitis vinifera* L.) leaves to be increased by abscisic acid (ABA) application but not by UV-B stress. ABA is induced by a variety of stressors including increased temperature, which is significant as plants with increased CAT activity were better able to withstand oxidative stress, indicating that cross-tolerance to UV-B may be conferred by factors acting downstream of the usual UV-B signaling pathway.

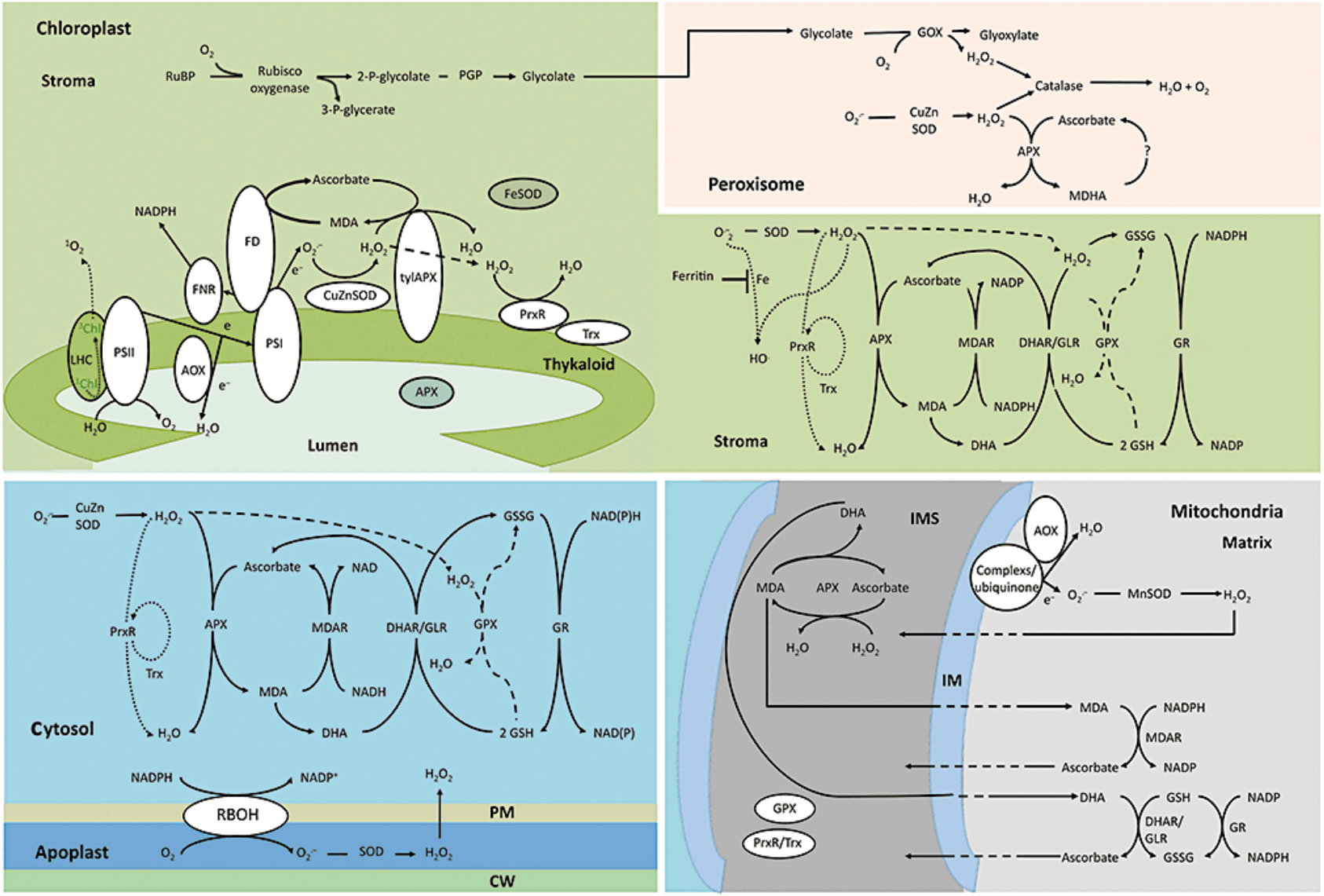
Chloroplast
Stroma
RuBP
Rubisco oxygenase
2-P-glycolate
PGP
Glycolate
3-P-glycerate
Ascorbate
FeSOD
MDA
NADPH
FD
FNR
CuZnSOD
tylAPX
PrxR
Trx
PSII
PSI
AOX
LHC
APX
Thykaloid
Lumen
Glycolate
GOX
Glyoxylate
Catalase
CuZn SOD
Ascorbate
APX
MDHA
Peroxisome
SOD
Ferritin
Fe
HO
PrxR
Trx
APX
Ascorbate
NADP
MDAR
DHAR/GLR
GPX
GR
GSSG
NADPH
MDA
NADPH
DHA
2 GSH
NADP
Stroma
CuZn SOD
Ascorbate
NAD
PrxR
Trx
APX
MDAR
DHAR/GLR
GPX
GR
GSSG
NAD(P)H
MDA
NADH
DHA
2 GSH
NAD(P)
Cytosol
NADPH
NADP+
RBOH
PM
Apoplast
SOD
CW
DHA
IMS
Mitochondria
Matrix
AOX
Complexs/ ubiquinone
MnSOD
MDA
APX
Ascorbate
IM
MDA
NADPH
MDAR
Ascorbate
NADP
GPX
PrxR/Trx
DHA
GSH
DHAR/ GLR
NADP
GR
Ascorbate
GSSG
NADPH

Figure 1.3 (previous page) ROS and scavenging pathways in plant cells. Abbreviations: AOX = alternative oxidase, PrxR = peroxiredoxin, GPX = glutathione peroxidase, LHC = light harvesting complex, APX = ascorbate peroxidise, RBOH = respiratory burst oxidase homolog, CW = cell wall, FD, ferredoxin, FNR = ferredoxin NADPH reductase, GLR – glutaredoxin, GOX = glycolate oxidase, IM = inner membrane, IMS = IM space, PGP = phosphoglycolate phosphotase, PM = plasma membrane, RuBP = ribulose-1,5-biphosphate, Rubisco = RuBP carboxylase oygenase, Trx = thioredoxin, tyl = thylakoid (Figure from Miller *et al.*, 2010).

1.2.1.3 Ascorbate Peroxidase

Ascorbate peroxidase (APox, EC 1.11.1.11) is an antioxidant enzyme found in the organelles of plant cells, as well as in the cytosol and apoplast (Fig. 1.3, Noctor and Foyer, 1998). Its role is to detoxify H_2O_2 by catalyzing the transfer of protons from ascorbic acid (AsA). APox activity is increased by exposure to UV-B, increased H_2O_2 formation possibly being an important signal in increasing the transcription of genes coding for APox (Garg and Manchanda, 2009; Morita *et al.*, 1999; Pekker *et al.*, 2002). Experiments on potato (*Solanum tuberosum* L.) plants exposed to UV-B radiation indicated the increased expression of several isoforms of APox that were only minimally expressed in plants not exposed to UV-B. The induction of several APox isoforms not found in plants exposed only to photosynthetically active radiation was also recorded (Santos *et al.*, 2004). The up-regulation of APox activity has been demonstrated even in plants where APox activity is low due to nutrient deficiency (Zancan *et al.*, 2008). Whilst some studies suggest that APox activity may become limited under conditions of extreme oxidative stress (Jain *et al.*, 2003), it seems likely that the regulation of APox activity is a key dimension in the response of plants to UV-B exposure.

1.2.1.4 Glutathione Peroxidase

Glutathione peroxidase (GPox, EC 1.11.1.9) is an enzyme found in the membranes of several cellular compartments including chloroplasts, mitcochondria and peroxisomes as well as in the cytosol and apoplast (Fig. 1.3, Noctor and Foyer, 1998). Similarly to APox, GPox is responsible for catalyzing the reduction of H_2O_2 to H_2O, although utilizing the reduced form of glutathione (GSH) as a substrate. In lipid membranes GPox is also able to detoxify ROS generated through lipid peroxidation (Garg and Manchanda, 2009). Perhaps because GPox operates further down the chain of antioxidant responses, increases in GPox activity are been less commonly reported in response to abiotic stress compared to other antioxidant enzymes (Dixon *et al.*, 1998). However, some increases in GPox activity resulting from UV-B exposure have been reported, including in soybean (*Glycine max* [L.] Merr.) plants and in cucumber cotyledons (Jain *et al.*, 2003; Xu *et al.*, 2008), indicating a possible role for GPox in the UV-B stress response. The biosynthesis of GPox is suspected to be induced by

oxidative stress, and over-expression of GPox is known to enhance stress tolerance in transgenic plants (Gill and Tuteja, 2010).

1.2.1.5 Glutathione Reductase

Whilst it is not directly involved in detoxifying ROS, glutathione reductase (GR, EC 1.8.1.7) is an important component in the functioning of antioxidant systems in plants. GR catalyzes the reduction of glutathione from its oxidized (GSSG) to it's reduced (GSH) form, using proton transfer from NAD(P)H to break the disulfide bridge between glutathione molecules (Fig. 1.3; Gill and Tuteja, 2010). Insufficient GR activity results in the oxidation of the glutathione pool and may impair the operation of enzymes reliant on GSH as a substrate, leading to a diminished ability of peroxidases to detoxify H_2O_2. Whilst GR is considered to play an important role in enzymatic defenses against oxidative stress, not all studies report increasing GR activity after exposure to stress (Noctor and Foyer, 1998). However, significant increases in GR activity (alongside that of GPox) have been recorded in (Xu *et al.*, 2008)'s study of soybean plants exposed to solar UV-B radiation, and were also enhanced in cucumber cotyledons placed under additional oxidative stress supplied by the AAPH· radical (Jain *et al.*, 2003). Such results suggest that increasing GR activity may be a strategy aimed at dealing with extreme oxidative stress, perhaps operating in conditions where the activity of other enzymes in the antioxidant response system is inhibited.

1.2.2 Non-enzymatic antioxidants

1.2.2.1 Ascorbate

Ascorbate functions in antioxidant metabolism as a substrate for the action of APox (see above) and also acts to directly detoxify ROS (Garg and Manchanda, 2009; Noctor and Foyer, 1998). Ascorbate in plant cells may thus exhibit a range of oxidation states. Ascorbic acid (AsA) is the reduced form that is most productive of antioxidant activity. Partial oxidation of AsA, often catalyzed by the action of APox, results in the formation of monodehydroascorbate (MDA) as a single proton is donated from an AsA molecule to ROS. In order to regain full antioxidant capacity, MDA must be regenerated to AsA through one of two routes. In the chloroplasts, ferrodoxin from photosystem I may catalyze the reduction of MDA to AsA as part of the water-water cycle. Outside of the chloroplasts, MDA is reduced through the action of a special enzyme, monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), which acts utilizing NAD(P)H as a substrate (Mittler, 2002). If MDA is not regenerated, it quickly disproportionates into AsA and dehydroascorbate (DHA). DHA cannot be further oxidized and must be regenerated for the further functioning of ascorbate in antioxidant metabolism. This is achieved through the action of the enzyme dehydroascorbate

reductase (DHAR, EC 1.8.5.1) in the ascorbate-glutathione cycle, utilizing reduced glutathione as a substrate (Fig. 1.3, Garg and Manchanda, 2009).

Ascorbate is accumulated in response to several abiotic stressors (Gill and Tuteja, 2010; Jansen *et al.*, 1998) and its importance to defence against UV-B induced oxidative stress was demonstrated in an experiment by (Gao and Zhang, 2008). *Arabidopsis thaliana vitamin c -1* (*vtc1*) mutants deficient in the biosynthesis of ascorbate were exposed to enhanced UV-B and exhibited decreased chlorophyll content, decreased maximal efficiency of PSII (ΦPSII), and increased lipid peroxidation and H_2O_2 levels compared to wild-type plants, indicating greater susceptibility to oxidative stress. Non-phytochemical quenching and the de-epoxidation state of xanthophylls also decreased dramatically in *vtc1*. As AsA is also a co-factor in the biosynthesis of the enzyme violaxanthin de-epoxidase, it is possible that reduced thermal dissipation of excess photons contributed to the over-reduction of electron transport systems, the overproduction of ROS and oxidative stress (Gao and Zhang, 2008; Noctor and Foyer, 1998). (Agarwal, 2007) reported increases in AsA content of 160 and 113% in *Cassia auriculata* L. seedlings exposed to 7.5 and 15.0 kJ m^{-2} UV-B respectively, and AsA accumulation after UV-B irradiation has also been reported in both wild and crop legumes in India and in wheat (Selvakumar, 2008; Yang *et al.*, 2007b).

Exposure to oxidative stress, including that caused by UV-B, also has an effect on the oxidation state of the ascorbate pool. Increases in DHA accumulation during Agarwal's, (2007) experiment on *Cassia* seedlings was greater than AsA accumulation, the difference between DHA and AsA accumulation being greater at the higher UV-B level. MDA accumulation has been reported in grape vine (*Vivia faba*) leaves after only ten minutes UV-B exposure, accumulation of MDA also being higher at higher UV-B doses although MDA accumulation appears inhibited at very high doses of UV-B (Hideg *et al.*, 1997). Longer term exposure to UV-B, however, may result in an increase in the AsA pool skewing the ratios of AsA/MDA and AsA/total ascorbate in favour of AsA. Hence the AsA/DHA ratio in wheat plants increased after 10 days exposure to UV-B, provided that growth occurred at adequate temperatures and AsA increased disproportionately compared with total ascorbate in the Indian legumes *Vigna unguiculata* and *Crotalaria juncea*, possibly as a result of increased MDAR activity (Selvakumar, 2008; Yang *et al.*, 2007b). A notable exception is the case of *Arabidopsis vtc1* mutants, where a much greater proportion of ascorbate was in its oxidised MDA form, possibly attributable to much quicker exhaustion of the smaller pools of AsA present in *vtc1* than in wild type plants – indicating that the oxidation of ascorbate does not necessarily operate in balance with its regeneration (Gao and Zhang, 2008).

1.2.2.2 Glutathione

Glutathione is another non-enzymatic antioxidant that occurs in both an oxidized (GSSG) and reduced (GSH) forms. In its reduced state, glutathione may be used to donate protons to ROS either directly or via enzymatically catalyzed reactions (Dixon *et al.*1998; Gill and Tuteja 2010; Noctor and Foyer 1998). The resulting oxidation of GSH forms a disulfide bridge between two glutathione molecules forming glutathione disulfide (GSSG). GSSG exhibits no antioxidant power and must be regenerated to GSH for the continued effectiveness of antioxidant systems involving glutathione, a reaction that is catalyzed by the enzyme GR (see section 1.2.1.5 above).

The accumulation of glutathione in plant systems as a result of abiotic stresses including UV-B has been reported by several workers (Gill and Tuteja, 2010). Increased synthesis of GSH under abiotic stress may be a strategy to counteract the effects of oxidation on the glutathione pool and maintain the functioning of antioxidant pathways (Noctor and Foyer, 1998). GSH and GSSG both increased in *Cassia auriculata* seedlings after just 100 minutes of exposure, with the GSH/GSSG ratio increasing enhanced at levels of UV-B less than 15 kJ m^{-2} (Agarwal, 2007). The GSH/GSSG ratio in wheat exposed to UV-B followed a similar pattern to that of AsA/DHA, increasing when the temperature was adequate (Yang *et al.*, 2007a). However, *Arabidposis vtc1* mutants displayed a reduced GSH/GSSH ratio compared to wild type *Arabidopsis* (Gao and Zhang, 2008), indicating that other components of a plants antioxidant metabolism may have an effect on the GSH/GSSG cycle.

1.2.2.3 α-Tocopherol

α-Tocopherol is a fat-soluble antioxidant involved in protecting cellular membranes against oxidative damage (Gill and Tuteja, 2010; Jansen *et al.*, 2008). Its ability to protect against oxidative stress induced by UV-B radiation was shown in experiments by (DeLong and Steffen, 1998). Excised thylakoid membranes of spinach plants (*Spinacia oleracea* L.) supplied with exogenous α-tocopherol were protected from damage associated with UV-B exposure, exhibiting less oxidative damage than untreated controls. However, α-tocopherol content declined after UV-B exposure in the non-supplemented thylakoids. Similar declines in α-tocopherol activity have been observed following UV-B exposure in other crop plants including maize (*Zea mays*) seedlings and cucumber cotyledons (Carletti *et al.*, 2003; Jain *et al.*, 2003), suggesting that α-tocopherol is a less important component of antioxidant metabolism during UV-B induced oxidative stress than other antioxidants such as ascorbate, which are known to be preferential scavengers of oxygen radicals.

1.2.3 Genotypic differences in antioxidant metabolism

Plants of different species and varieties differ constitutively in their ability to withstand stress, including UV-B stress. These differences are determined by genetic factors that delimit a plant's ability to respond to biotic and abiotic stimuli, and may reflect a greater or lesser degree of adaptation to a stressful environment or artificial selection for a particular trait (Frohnmeyer and Staiger, 2003; García-Macias *et al.*, 2007). As various environments differ in the amount of UV-B radiation that they receive, so it is possible to detect differences in the response of various plant species and crop cultivars to UV-B. Amongst tolerant genotypes, plants with different morphological forms may adopt different strategies to successfully deal with the challenges posed by a high UV-B environment. The woody structures of many trees and shrubs may afford a degree of protection from UV-B, so these plants may be able to withstand UV-B exposure with minimal perturbation of physiological systems (Li *et al.*, 2010). Such was the case with the UV-B tolerant poplar species *Poplus kandingensis*, which displayed no variation in biomass, leaf area or APox activity when grown under supplemental UV-B (Ren *et al.*, 2010). It is otherwise with shorter-lived herbaceous plants, which must complete their life-cycle in a single season and may be more reliant on physiological mechanisms to confer UV-B tolerance. Hence a UV-B tolerant cultivar of buckwheat (*Fagopyrum esculentum*) from the Quinghai-Tibet plateau displayed greater increases in APox acidity and AsA accumulation when subjected to a supplemental UV-B than where observed in UV-B sensitive cultivars (Yao *et al.*, 2008). Better functioning of the antioxidant system (including increased AsA regeneration) is the likely source of increased tolerance in this instance.

In the physiological response to UV-B, plants of a UV-B tolerant genotype may be able to increase the functioning of antioxidant pathways in a more effective manner than non-tolerant genotypes. The response of UV-B tolerant genotypes usually differs from that of sensitive genotypes in the magnitude of the response rather than its direction, although non-tolerant varieties may increase the functioning of less effective components of the antioxidant pathway. Thus SOD, APox, GR activity and AsA content all increased in both wild and domestic legumes studied by Selvakumar (2008), but increases in SOD and GR were greater in the more UV-B tolerant wild species while the UV-B sensitive domesticated legume was characterized by a greater increase APox activity and AsA content. In a like manner, under UV-B stress conditions the tolerant barley (*Hordeum vulgarae* L.) cultivar 'Golden Promise' accumulated MDA and DHA less than sensitive cultivars, indicating a greater ability to maintain antioxidant functioning under stress (Hideg *et al.*, 2006).

The patterns of gene expression underlying the response of tolerant and sensitive genotypes to UV-B exposure was investigated by Fang *et al.* (2009) in a study of two cultivars of rice (*Oryza sativa* L.). UV-B treatment of the UV-B tolerant Lemont cultivar resulted in the up-regulation of genes involved in signal transduction, DNA repair and of several genes coding for biosynthesis of thylakoid-bound APox, molecular chaperones and enzymes involved in the synthesis of UV-B protectant flavonoids. A gene coding for the universal stress protein was also up-regulated. UV-B treatment of the UV-B sensitive cultivar Dular, however, resulted in the down-regulation of the universal stress protein and in the up-regulation of fewer genes involved in thylakoid-bound APox synthesis, DNA repair and signal transduction. Simultaneously APox activity, antioxidant metabolism and biomass were all greater in plants of the UV-B tolerant cultivar, suggesting that differences in gene expression between cultivars may have important impacts on the ability of crops to withstand environmental stressors.

1.3 Polyamine metabolism

Polyamines (principally spermidine [Spd] and spermine [Spm]) are small, aliphatic amines found in all living organisms (Table 1.1, Bouchereau *et al.*, 1999; Kusano *et al.*, 2008; Alcazar *et al.*, 2010). Polyamines (PAs) and the diamine putrescine (Put) are essential for the continuation of life-sustaining processes. Polyamines in plants perform a large number of functions, being involved in regulation of DNA replication, the transcription of genes, cell elongation and organ development, leaf senescence, fruit ripening and biotic and abiotic stress responses (Bouchereau *et al.*, 1999).

Table 1-1 Names and structures of the principal polyamines found in plants (table adapted from Hunter and Burritt, 2011).

Name	Structure
Putrescine	$NH_2(CH_2)_4NH_2$
Spermidine	$NH_2(CH_2)_3NH(CH_2)_4NH_2$
Spermine	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

1.3.1 Polyamine biosynthesis and catabolism

Polyamines in most plants (except *Arabidopsis*) may be synthesised via either of two pathways, beginning with the production of putrescine from ornithine or arginine and progressing in a stepwise manner to the larger polyamines spermidine and spermine (Alcazar *et al.* 2010; Liu *et al.* 2007). In the ornithine pathway, biologically occurring ornithine is converted directly to Put through the removal of the carboxylic acid attached to the delta

carbon atom, a reaction catalysed by the enzyme ornithine decarboxylase (Bagni and Tassoni, 2001). For the synthesis of Put from arginine, a longer route is required. The decarboxylation of arginine (by arginine decarboxylase [ADC], EC 4.1.1.19) results in the formation of agmatine. The enzyme agmatine iminohydrolase (AIH, EC 3.5.3.12) then acts using agmatine and H_2O as substrates, to remove an amine group from the alpha carbon atom to form N-carbamoylputrescine, which is converted to putrescine through the action of N-carbmoylputrescine amidohydrolase (Alcazar *et al.*, 2010).

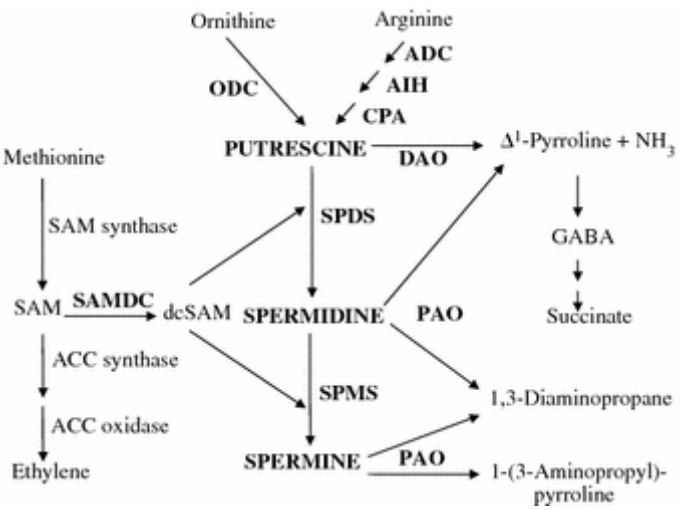

Figure 1.4 Polyamine biosynthesis and catabolism (Figure from Alcazar *et al.*, 2006).

Putrescine may be converted into longer-length polyamines (Spm and Spd) through the addition of amino groups from decarboxylated S-adenosylmethionine (dcSAM). The first reaction in this pathway is the addition of an aminopropyl group from dcSAM to Put, forming Spd. This reaction is catalysed by spermidine synthases (EC 2.5.1.16, (Alcazar *et al.*, 2010)). The addition of another aminopropyl goup from dcSAM results on the formation of Spm, with spermine synthases (EC 2.5.1.16) catalysing this reaction (Kusano *et al.*, 2008). The dcSAM required for these reactions is synthesised in plant cells from methionine, via S-adenosylmethionine, utilizing components of a pathway also involved in ethylene evolution. Consequently, decreases in ethylene evolution have been frequently recorded accompanying polyamine accumulation (Bouchereau *et al.*, 1999). Two enzymes, S-adenosylmethionine synthase (EC 2.5.1.6) and S-adensylmethionine decarboxylase (SAMDC, EC 4.1.1.50) are required to complete dcSAM synthesis, SAMDC being responsible for catalysing the shift away from ethylene evolution and towards polyamine accumulation (Tiburcio *et al.*, 1994).

Polyamines may be catabolised in reactions catalysed by polyamine oxidases (PAO, EC 1.5.3.3). Catabolism of polyamines by PAOs may be terminal or may result in degradation to lower-molecular weight polyamines, depending on the class of PAOs involved (Alcazar *et al.*,

2010). Terminal catabolism of Spd and Spm results in the formation of 4-aminobutanal or (3-aminopropyl)-4aminobutanal and 1,3-diaminopropane and H_2O_2. Reaction with water and oxygen (catalyzed by PAO) oxidises Spm to Spd, 3-aminopropanal and H_2O_2. Spd may undergo a similar reaction, being converted by PAO to Put, 3-aminopropanal and H_2O_2 (Kusano *et al.*, 2008). The catabolism of Put utilizes a different enzyme, diamine oxidase (DAO, EC 1.4.3.6) as a catalyst and H_2O and O_2 as additional substrates, the products of Put catabolism being H_2O_2, NH_3 and 4-aminobutanal, which cyclises Δ-pyrroline. Δ-Pyrroline is converted in plant cells to the signalling molecule γ-aminobutyric acid (GABA), and may be eventually incorporated into the Krebs cycle through eventual conversion to succinate (Alcazar *et al.*, 2010; Kusano *et al.*, 2008).

1.3.2 The role of polyamines in stress tolerance

Alterations in polyamine levels occur in response to a wide variety of stress situations (Alcazar *et al.*, 2010). The accumulation of Spd and Spm has been recorded to confer tolerance to a range of stressors, including elevated temperature, salinity, drought and environmental pollutants and accumulation of Put has been associated with tolerance to chilling and hypoxia (Alcazar *et al.*, 2010; Bouchereau *et al.*, 1999).

Stress tolerance associated with the accumulation of Spd and Spm is likely to occur through the stabilization of phospholipid membranes and cellular metabolites under stressful conditions. The polyamine pools under cells stressed by elevated temperature shift from Put to Spd and polyamines with higher molecular weight, including caldine, pentamine and caldopentamine and the Spm isomer thermospermine (Bouchereau *et al.*, 1999; Kusano *et al.*, 2008). The conjugation of polyamines (through the action of transglutiminases) to proteins may help ensure correct folding and protect against protein denaturation, and incorporation of bound and conjugated polyamines into cellular membranes may inhibit transbilayer movement of phospholipids and help stabilize light-harvesting complexes in the thylakoids (Alcazar *et al.*, 2010; Alcazar *et al.*, 2006; Ioannidis and Kotzabasis, 2007; Sfichi-Duke *et al.*, 2008). Accumulation of Spd and Spm in stomatal guard cells promotes guard cell closure through effecting Ca^{2+} accumulation and the production of nitric oxide (NO), a key part of the ABA-signalling system involved in stomatal closure. H_2O_2 produced from Put, Spd and Spm oxidation may also have similar effects (Alcazar *et al.*, 2010). Spd and Spm are effective in blocking K^+ and Ca^{2+} channels, playing an important role in the maintaining cation homeostasis during osmotic stress. Polyamine accumulation has been implicated in directing biochemical pathways away from ethylene evolution and towards greater polyamine biosynthesis, inhibiting the ethylene-induced leaf senescence that is encouraged by salinity

and drought (Tiburcio *et al.*, 1994). Additionally, polyamines may play a role as antioxidants in cellular membranes and thermospermine is essential for continued protein synthesis at higher temperatures (Bouchereau *et al.*, 1999).

Similarly to Spm and Spd, Put may become covalently bound to certain amino acids allowing polyamines to become bound to proteins, including antioxidative enzymes. Polyamine-bound antioxidants display greater membrane permeability, allowing for better penetration to the sites of oxidative stress than is the case for unbound antioxidants. A covalently bound Put-SOD complex in mammalian cells, for example was found to be twenty times more membrane permeable than SOD alone and facilitated greater antioxidant protection than unbound SOD (Bouchereau *et al.*, 1999). A similar mechanism is proposed to operate in plant cells. The conjugation of Put to other molecules (principally hydrocinnamic acids) in the cell has been shown to be effective in promoting antioxidant functioning (Bagni and Tassoni, 2001). A Cu-Put-pyrimidine complex was discovered to promote the oxidation of glutathione and was able to directly scavenge $O_2 \cdot^-$. Put accumulation is also thought to play a role in proline accumulation, a key aspect of salt-tolerance – possibly by allowing the greater production of GABA. The connection of Put and proline through GABA, and the incorporation of GABA into the citric acid cycle is suggestive of a further function of Put as a reserve of organic nitrogen that may be utilized during stress and recovery (Bouchereau *et al.*, 1999).

1.3.2.1 Polyamine accumulation and UV-B radiation

The polyamine content of plant cells may decrease in response to periods of increased UV-B irradiation. Decreases in Spd and Spm content in *Arabidopsis* were recorded when Put accumulation slowed under exposure to high and very high levels of UV-B radiation, suggesting that a sufficient Put pool is necessary for the maintenance of higher-molecular weight polyamines under conditions of UV-B stress (Rakitin *et al.*, 2008a), suggesting that perhaps these larger molecules may be degraded after UV-B exposure. Another possibility is that UV-B induced ethylene evolution impacted Spd and Spm accumulation through retarding the accumulation of Put (Rakitin *et al.*, 2009). Decreases in the content of free Spd and Spm associated with decreased Put content were also recorded by (Smith *et al.*, 2001). However, the polyamine accumulation on UV-B exposure has been recorded to be dependent on the availability of photosynthetically active radiation (PAR), with evidence suggesting that lower levels of PAR result in decreased polyamine content upon exposure to UV-B, with more moderate levels of PAR resulting in increased polyamine content (Kramer *et al.*, 1992; Smith *et al.*, 2001).

Studies into the combined effects of UV-B and PAR suggest that not only the intensity, but also the spectral quality of PAR has an effect on polyamine accumulation during UV-B responses. Whilst soybeans exposed to less intense PAR (300 μmol m^{-2} s^{-1}) accumulated less Put and Spd in response to UV-B than those exposed to more moderate doses (600 μmol m^{-2} s^{-1}), altering the spectral composition of the PAR resulted in different patterns of polyamine accumulation and a reduction in UV-B protection. Put and Spd accumulated more under lamps deficient in blue light, whilst Spm accumulated under red/far-red lamps (Kramer *et al.*, 1992). Since the spectral quality of PAR is also known to affect the biosynthesis of common conjugates of polyamines such as anthocyanins and phenolics, it is possible that the higher accumulation of Spm under red/far-red light acts in concert with these molecules to provide greater UV-B protection (Kramer *et al.*, 1992; Smith *et al.*, 2001).

The effect of UV-B exposure on Put accumulation is less clear. Several studies have pointed to an increased Put accumulation in the period immediately following UV-B exposure, the rate of Put accumulation slowing thereafter (Smith *et al.*, 2001; An *et al.*, 2004). The period of increasing accumulation may vary between plant species: Put accumulation increased for 12 days before declining in cucumber leaves (An *et al.*, 2004), while maximum accumulation in *Arabidopsis* occurred only one day after exposure to UV-B. Put accumulation in UV-B exposed plants is not necessarily correlated with the UV-B dose received by the plant. Accumulation of Put in *Arabidopsis* is recorded as occurring maximally at moderate (6-9 kJ/m^2) rather than higher UV-B doses (Rakitin *et al.*, 2008b). This suggests that Put accumulation is a deliberate strategy adopted by the plant to deal with UV-B stress rather than a result of UV-B damage.

Other studies have recorded decreases or no significant change to the levels of free Put in response to UV-B exposure. The free Put content of *P. vulgaris* was decreased by 50 % after 14 days exposure to UV-B (Smith *et al.*, 2001), and Tegelberg *et al.* (2006) detected no significant variation in the levels of free Put in willow (*Salix* sp.) trees subjected to both drought and enhanced UV-B, despite the fact that Put was increased by drought stress alone. However, most studies into the effects of UV-B on polyamine accumulation have focused on free polyamines, including Put, and further research is needed to establish what the effects are on conjugated and bound polyamines.

The general pattern of polyamine accumulation in response to UV-B radiation is thus a decrease in the levels of free Spd and Spm, often accompanied by an increase in free Put levels. This may be the result of increased conjugation of polyamines under conditions of

UV-B stress, or it may be mediated via the reconversion of Spm and Spd to Put, which may itself be conjugated with other molecules associated with UV-B stress. Conjugated and bound polyamines may assist in maintaining the redox status and membrane stability under stress, although further research into the presence and action of conjugated polyamines in UV-B exposed plants is required.

1.4 Antioxidants, polyamines and human health

Antioxidants from plant foods are important for the maintenance of good health in human beings and many other animals. ROS have been linked to the development of a large number of chronic diseases, including cancer, diabetes, cardiovascular disease and neurodegenerative diseases. Consumption of foods high in antioxidants may thus help in the prevention of disease (Fang *et al.*, 2002; Jansen *et al.*, 2008). Ascorbate is a particularly important factor in human nutrition, as human beings lack the enzymes necessary for ascorbate synthesis and are thus reliant on dietary sources (Halliwell, 2006; Olmedo *et al.*, 2006). Ascorbate also plays important roles in collagen synthesis, iron absorption and in regulating the function of the immune system (Marcil *et al.*, 2011; Olmedo *et al.*, 2006). Glutathione is also an important antioxidant in human cells and may be increased through dietary sources (Halliwell, 2006). Other phytochemicals with antioxidant capacity such as phenolics, are also speculated to play a role in reducing oxidative stress and the development of chronic diseases (Galleano *et al.*, 2010; Jansen *et al.*, 2008; Poiroux-Gonord *et al.*, 2010).

Polyamines are important regulators of cell maturation and proliferation, and as such are important for the maintenance of general health and trauma recovery. They are particularly important in intestinal metabolism where intestinal epithelial cells are undergoing constant cell division and differentiation (Majumdar, 2003). Polyamines have also been recorded to play an important role in the regulation of cellular adhesion and inflammation, key factors in the development of cardiovascular disease (Soda *et al.*, 2005). Dietary polyamines have been shown to contribute to maintaining levels of Put, Spm and Spd throughout the body (Bardocz *et al.*, 1998), and are particularly important for the maintenance of health in older people, as the body's ability to synthesise polyamines decreases with age. Polyamines also function in the regulation of calcium influx/efflux and polyamine depletion is suspected to play a role in the development of neurodegenerative diseases (Hunter and Burritt, 2011). Insufficient polyamine uptake is also though to be linked to food hypersensitivity and allergy development (Larque *et al.*, 2007). Their function as enhances of cell proliferation, however, means that foods with a high polyamine content should be avoided by those suffering from cancer and other proliferative diseases (Larque *et al.*, 2007; Saunders and Wallace, 2007).

1.5 Applications: UV-B and tunnel house crops

Leafy green vegetables are a potential dietary source of these phytonutrients, especially antioxidants. One of the most important leafy green crops grown worldwide is lettuce (*Lactuca sativa* L.) (Hunter and Burritt, 2005). When growing crops for human consumption, horticultural practices are influential in altering the nutritional quality of the product (Zhao *et al.*, 2007b). Lettuce production in temperate climates is increasingly occurring under the protection of tunnel houses (Lamont, 2009), which are typically covered in plastic materials known to modify the spectral characteristics of light reaching the lettuce plants. Commonly, a portion of the UV-B light will be filtered out by the plastic covering (García-Macias *et al.*, 2007; Ordidge *et al.*, 2010). In recent years, the development of plastic films of differing UV-B transparencies has allowed researchers to study the effects of growth under UV-B blocking film (Tsormpatsidis *et al.*, 2008). Plants grown in tunnels using UV-B opaque films are usually larger, have a greater yield and a longer growing season than those grown in the open air (Rader and Karlsson, 2006; Sonsteby *et al.*, 2009). Evidence from experiments on lettuce plants suggests that these effects are due at least in part, to the attenuation of natural UV-B light by the plastic film. In previous studies, the mean dry weight of lettuce plants growing under a standard commercial film with only low transmission of UV-B radiation was 34 % lower than the dry-weight of plants grown under a film that completely blocked UV-B and UV-A radiation. Additionally, plants grown under a plastic film completely transparent to UV-B and UV-A had dry weights that were 42 % lower than those grown under UV blocking films. Lettuces growing under UV blocking films also had a greater number of leaves than those grown under UV transmitting films (García-Macias *et al.*, 2007; Tsormpatsidis *et al.*, 2008). This agrees well with the known effects of UV-B on plant productivity.

The secondary metabolite content of lettuces grown under UV-B attenuating plastics is usually lower than those grown in the open air or in UV-B transmitting environments. Quantitative and qualitative differences in the polyphenol content between lettuces greenhouse and open-air grown lettuces were recorded by (Romani *et al.*, 2002), with open-air grown lettuces having a higher polyphenol content and greater variation in polyphenol composition. Greater red colouration has been reported in lettuces exposed to UV-B (García-Macias *et al.*, 2007) and phenolic, anthocyanin, luteolin, and quercetin contents have been recorded to be higher in the red leaf lettuce cultivar Lollo Rosso grown under UV-B transmitting plastic films than in those grown under UV-B blocking films. However, no similar response to UV-B was recorded in a closely related green leaf variety (Tsormpatsidis *et al.*, 2008; Ordidge *et al.*, 2010). Reduced levels of chlorogenic acid, rutin and quercetin

glycosides have also been reported in the red leaf cultivar 'Red Sails' and the green romaine cultivar 'Kalura' under protected cultivation compared with the open field, although effects are not equal across all cultivars (Zhao *et al.*, 2007a). Increased content of UV-B-absorbing compounds has also been recorded in the leaves of tomato plants grown under natural light conditions (as opposed to UV-B deficient conditions), providing further evidence that UV-B exposure may increase the phytonutrient content of crop plants (Cle *et al.*, 2008).

The increases in secondary metabolite content observed in lettuces grown under UV-B transmitting films correlate well with increases in the antioxidant capacity. Red-leaf Lollo Rosso lettuce grown under a film transparent to UV-B increased antioxidant capacity by 13 % over those grown under standard horticultural films, whilst total polyphenol content was 21 % higher. The antioxidant capacity of those grown under UV-B blocking films however, was only 52 % of those under standard horticultural films, with total polyphenols reaching only 55 % (García-Macias *et al.*, 2007).

Other factors related to spectral quality and affecting the phytonutrient content of lettuces and other crops include leaf position and season. Leaf position in head producing lettuces is known to alter phytonutirent content, with phenolics and ascorbate being higher in outer leaves and carotenoids being higher in inner leaves (Romani *et al.*, 2002). UV-B levels are also known to vary with season, and some studies of the effect of plastic coverings have pointed to seasonal effects. In experiments by (Zhao *et al.*, 2007b), early summer field grown pak choi had a greater antioxidant capacity than those grown in high tunnels, but this difference disappeared in the mature stage of late summer grown pak choi. No difference in antioxidant capacity between field and greenhouse vegetables at late summer was also recorded for both red and green lettuce (Zhao *et al.*, 2007b). Other studies have also found greater variation between the phenolic contents of high tunnel and field grown lettuce earlier in the season than in summer (Zhao *et al.*, 2007a), although (Tsormpatsidis *et al.*, 2008) recorded the secondary products of red leaf lettuce being significantly higher later in the season.

Opportunities exist for the extension of current studies into the effects of UV-B blocking films on lettuces. Most studies to date have focused on the increased production of secondary metabolites under UV-B deficient conditions and little or no investigation has been made of several important phytonutrients obtained from lettuce, including ascorbate and α-tocopherol. Whilst increased antioxidant capacity observed in UV-B transparent conditions has correlated well with increases in secondary metabolites, including phenolic compounds, studies to date

have tended to use only a single assay of antioxidant power. Studies using multiple biologically-relevant assays are able to better characterise the antioxidant dynamics of extracts, and further investigation using other assays could shed light on the role of non-phenolic compounds (Philpott *et al.*, 2009). The interactions between the various metabolites involved in defence against UV-B are complex, and examination of several variables at once could shed new light on the dynamics of UV-B defence. Particular room exists for investigation of the role of polyamine. Lastly, given that responses to UV-B differ between various cultivars, there is room for further investigation into the effects of UV-B exposure to include a greater number of cultivars. This is of particular interest as variation in cultivars responses may not be a straight-forward division between red and green varieties (Llorach *et al.*, 2008; Ordidge *et al.*, 2010).

1.6 Aims

The aims of this thesis are to compare the effects that different UV-B regimes have on various lettuce varieties. Specifically we investigated (1) the effects of UV-B on the kinetics of antioxidant metabolism including the activity of antioxidant enzymes and accumulation of ascorbate and glutathione; (2) the effects of UV-B polyamine accumulation; and (3) the effect of the UV-B regime on the ability of the lettuce extract to defend against oxidative damage in human cells.

2 Antioxidant metabolism

2.1 Introduction

In recent years ROS have been implicated as factors in the development of a range of chronic diseases, especially those commonly associated with ageing, including cancer, cardiovascular disease, stroke and diabetes (Floegel *et al.*, 2011; Jansen *et al.*, 2008; Psaltopoulou *et al.*, 2011). Plant-derived antioxidants have thus emerged as key factors in human health and nutrition. High dietary intakes of antioxidants such as ascorbate, tocopherols and carotenoids have been associated with reduced risk of disease, such as the reduced occurrence of markers of cardiovascular disease (Floegel *et al.*, 2011). The antioxidant rich nature of the Mediterranean diet has also been associated with reduced risk of chronic degenerative diseases including several types of cancer, in populations adhering to that diet (Gomez-Romero *et al.*, 2007; Itsiopoulos *et al.*, 2009; Psaltopoulou *et al.*, 2011). By reducing oxidative processes in metabolism such as lipid peroxidation, antioxidants play a role in the prevention of conditions that lead to disease such as atherosclerosis, and ascorbate was recently found to play a key role in regulating the growth of cancerous tumours (Floegel *et al.*, 2011; Kuiper *et al.*, 2010). Green leafy vegetables are a potentially valuable source of dietary antioxidants, especially as they are commonly eaten raw thereby preserving antioxidant activity that may be lost in processing and cooking (Natella *et al.*, 2010; Wolosiak *et al.*, 2011; Xu and Chang, 2011).

The antioxidant contents of lettuce leaves may vary with their position inside a lettuce head. In addition to being older, more fully expanded and representing a more advanced life stage, leaves at the outside of the head have a greater exposure to extreme environmental conditions than the inner-most leaves which are younger, less fully expanded and may be shielded from environmental extremes (Barg *et al.*, 2009; Paulwelyn *et al.*, 2011). The outermost leaves of a lettuce head may thus have a greater content of metabolites that function to shield the plant's metabolism or alleviate the negative effects of sudden changes in environmental conditions. In cases where these metabolites play an active role in human metabolism, the up- or down-regulation of these metabolites in leaves from different parts of the lettuce head could result in a product of differing nutritional quality for the consumer. Several authors have recorded decreased concentrations of some metabolites in the inner leaves of heading lettuces, including flavonoids, ascorbate and trace nutrients such as iron and calcium (Hohl *et al.*, 2001; Mou and Ryder, 2004). Decreased total phenolics in the inner leaves of crisphead lettuces have also been associated with decreased antioxidant capacity of extracts (Ozgen and

Sekerci, 2011). Further characterization of the variation in antioxidant metabolites between inner and outer leaves of the lettuce head and their response to stress conditions may be useful to give a more complete description of the plant's functioning and the factors affecting its nutritional value.

Metabolites important for human nutrition may also vary over time as a plant develops and responds to stressors in its environment. The production of any metabolite may vary at each growth stage as the physical and biochemical structure of a plant changes during its development, and also as the plant responds to stress (Jovanovic *et al.*, 2006). The response of a plant to any stressor is likely to be affected by its life history and developmental state, which in turn will affect its future growth and nutritional value to consumers. Studies on leafy green vegetables have found the antioxidant capacity of both Pac Choi and spinach to be increased in mature over immature plants grown in an open field in but not those grown in greenhouses (Zhao *et al.*, 2007b). While this did not hold for cultivars of romaine and leaf lettuce in the same study, decreases in ascorbate content have been recorded with increasing age at harvest in crisphead lettuce (Sorensen *et al.*, 1994) and it is probable that the antioxidant systems of lettuce are not static over time. (Jovanovic *et al.*, 2006) recorded differential activities of antioxidant enzymes in buckwheat at various developmental stages and temporal changes in the activity of various antioxidant enzymes and the accumulation of phenolics have also been recorded in a variety plants exposed to stress including rice, wheat, soybean and lettuce (Dai *et al.*, 1997; Dawar *et al.*, 1998; Romani *et al.*, 2002; Sharma *et al.*, 1998). The dynamics of antioxidant systems over extended periods of stress had been less often studied and could benefit from further description.

As detailed in chapter one, UV-B is a stressor that may affect the levels of metabolites in plant products that are important nutrients for human consumers. The activities of antioxidant enzymes, ascorbate and glutathione are usually increased after UV-B exposure, yielding a product with a higher antioxidant capacity (see chapter one). Conditions of cultivation are known to affect a crops exposure to UV-B, thereby having an impact on the nutritional quality of the product, the antioxidant capacity of plants grown under UV-B blocking covers being compared with those exposed to solar UV-B (García-Macias *et al.*, 2007). In this study, the aim was to further explore the effects of UV-B by describing the activity and concentration of various physiologically and nutritionally important metabolites in plants exposed to different UV-B regimes through growth under plastic covers of varying UV-B transparency.

2.2 Methods

2.2.1 End point experiments

Preliminary experiments were carried out in the winter of 2010. *Lactuca sativa* L. seeds (King's Seeds, Katikati; cv. 'Red Salad Bowl' and 'Buttercrunch') were germinated in a glasshouse and transplanted to 2.5 L pots after four weeks, once they had reached the third leaf stage. The substrate in which plants were grown was a 1:1 mixture of potting mix and horticultural sand. Transplants were allowed to acclimatize for one week before being subjected to UV-B treatment.

UV-B was provided by Philips TL 40W/12RS lamps (Philips, Eindhoven, the Netherlands) mounted above the plants and switched on for 4 hours each day (centered on the approximately the middle of the photoperiod), providing plants with a maximum dose of between 7.8 and 8.5 kJ m^{-2} UV-B_{BE}. The effect of UV-B on antioxidant activity was quantified by removing UV-B from the growth environment. UV-B exposure was varied through the use of two 1 m x 1 m x 1 m frames covered by plastic films of different UV-transparency. 0.13 mm Mylar-D® (DuPont, Dover, DE, USA) film was used to remove wavelengths below 320 nm providing a PAR plus UV-A treatment deficient in UV-B (UV-B–), and 0.13 mm cellulose diacetate film (Lonza-Flien, Weil am Rhein, Germany) was used to remove wavelengths below 295 nm, providing a PAR, UV-A and UV-B treatment (UV-B+).

Lettuce plants of each variety were randomly assigned to one of three experimental blocks, and to UV-B– and UV-B+ treatments within each block. The positioning of experimental blocks was randomly rearranged on a weekly basis to prevent the emergence of edge effects. Experimental treatment continued for four weeks before plants were destructively harvested over a period of a week.

Growth and photosynthetic parameters of each plant were measured immediately prior to harvesting. Above ground fresh weight was measured and a sample of leaves taken for dry weight analysis. Maximum photochemical efficiency (F_v/F_m) was measured by averaging readings from two leaves in the outer whorl using a Junior PAM (Waltz, Effeltrich, Germany) operated by WinControl-3 software. Leaf samples from the inner and outer whorls of each plant were then taken, frozen in liquid nitrogen and stored at -80 °C before analysis, or at – 70 °C when storage at – 80 °C was unavaliable.

2.2.2 Time-course experiments

A time-course experiment was carried out over the summer of 2010-11 to investigate the kinetics of antioxidant activity under UV-B irradiation. *L. sativa* seeds (King's Seeds,

Katikati) were germinated and transplanted into 2.5 L pots as in the preliminary experiment (above). Three leafy lettuce varieties of differing colouration were selected: a red ('Red Salad Bowl'), a green ('Royal Oak Leaf') and a speckled variety that was considered intermediate between red and green ('Drunken Woman Fringed Head').

UV-B treatment was as per the earlier end-point experiment and begun one week after transplanting. Greater space allowed for the inclusion of an additional control group that was neither covered by a plastic film nor subjected to radiation from UV-B lamps. Plants of each variety and treatment were destructively harvested in a stratified random manner after one week, two weeks and four weeks of UV-B treatment. Baseline measurements were provided by an additional set of three untreated plants of each cultivar that were harvested immediately before UV-treatment commenced (time = 0).

As before, growth and photosynthetic parameters of plants were measured immediately prior to harvest. Above-ground fresh weight, shoot height and the length of the longest leaf were measured and a sample of leaves taken for dry weight analysis. Maximum photochemical efficiency was also measured as before. As leaves of the inner whorl were sometimes very small, leaf samples from the outer whorl of each plant only were taken, frozen in liquid nitrogen and stored at - 80 °C prior to analysis.

2.2.3 Extraction and analysis of antioxidant enzymes and protein carbonyls

Tissue samples were ground to a fine powder using a motor and pestle chilled with liquid nitrogen. Protein carbonyls, SOD, CAT, GPox and GR were then extracted by homogenising 50 mg of tissue in 450 µl of ice cold extraction buffer containing 100 mmol potassium phosphate, 1 mmol K_2EDTA, 0.1% (v/v) Triton X-100 and 1% (w/v) Polyclar AT (SERVA Chemicals Ltd, Heidelberg, Germany). 5 mmol ascorbate was added to the buffer for the extraction of APox. Homogenates were then centrifuged at 20,800 x *g* at 4 °C for 15 minutes, the supernatant obtained being divided into 50 µl aliquots and stored at -80 °C prior to analysis.

Except where otherwise stated, assays were carried out using a PerkinElmer (Wallac) 1420 multilabel counter (Perkin Elmer, San Jose, CA, USA) controlled by a PC, and fitted with a temperature control cell set to 25 °C and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Prekin Elmer, San Jose, CA, USA).

2.2.3.1 Protein carbonyl assay

Samples from both experiments were analysed for protein carbonyl contents according to the method of Reznick and Packer (1994), modified for measurement in a microplate reader. A

Lil420 multilabel counter (Perkin Elmer, San Jose, CA, USA) fitted with a temperature control cell and an auto-dispenser, and controlled by a PC was used to determine levels of protein carbonyls by measuring absorbance at 370 nm and relating this to the extinction coefficient of DNPH at 370 nm (0.022 μmol^{-1} cm), corrected for the calculated path-length of the solution (0.6 cm). The protein content of the extracts was determined using a Lowry protein assay (Fryer *et al.*, 1986) and protein carbonyl content expressed as nmol protein carbonyl mg^{-1} protein.

2.2.3.2 Superoxide dismutase assay

SOD was assayed using the method of Banowetz *et al.* (2004) with minor modifications. Briefly, 50 µl of extract, diluted extract or standard (prepared from bovine liver SOD; Sigma-Aldrich, St. Louis, MO, USA) was mixed with 125 µl of freshly prepared reaction solution containing piperazine-1,4-bis(2-ethanesulfonic acid) (Pipes) buffer, pH 7.8, 0.4 mmol o-dianisidine, 0.5 mmol diethylenetriaminepentaacetic acid (DTPA) and 26 µmol riboflavin. One unit of the SOD standard corresponded to the amount of enzyme that inhibited the reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 and 25 °C. Absorbance at 450 nm (A_{450}) was measured immediately (t = 0 minutes) and samples were illuminated with an 18W fluorescent lamp placed 12 cm above the plate for 30 minutes (t = 30 minutes) and the A_{450} measured again. A regression analysis was used to prepare a standard line relating SOD activity to the change in A_{450} and SOD activities of the extracts were calculated with reference to the standard line and expressed as units of SOD mg^{-1} total protein.

2.2.3.3 Catalase assay

The chemoiluminescent method of Maral *et al.* (1977), as modified by Janssens *et al.* (2000) was used to assay catalase activity in microplates. 50 µl of extract, dilute extract or purified bovine liver CAT (Sigma-Aldrich, St. Louis, MO, USA) was mixed with 100 µl of 100 mM phosphate buffer (pH 7.0, containing 100 mM NaEDTA and 10^{-6} mol H_2O_2) and incubated at 25 °C for 30 minutes. 50 µl of a solution containing 20 mM luminol and 11.6 units ml^{-1} of horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was injected into each well and the resulting light emission was measured. The intensity of the light emission was proportional to the amount of H_2O_2 remaining, and a regression analysis was used to prepare a standard line relating CAT activities of the bovine liver standard to those of extracts, and expressed as µmol of H_2O_2 consumed mg^{-1} total protein.

2.2.3.4 Glutathione peroxidase assay

Glutathione peroxisdase activity was determined according to the method of Ursini *et al.* (1985) as modified by Contreras *et al.*, (2005) and adapted for microplates. The reaction mixture contained 0.1 mol phosphate buffer (pH 7.0), 200 mmol H_2O_2, 90 mmol NADPH and 1 U of GR (Sigma-Aldrich, St Louis, MO, USA). The oxidation of NADPH was monitored after its addition to the mixture by following absorbance at 340 nm for 3 minutes, and the activity of GPox calculated using the extinction co-efficient of NADPH (6.2^{-1} mM cm^{-1}), corrected for the pathlength of the solution.

2.2.3.5 Glutathione reductase assay

Glutathione reductase was assayed according to the method of Cribb *et al.* (1989) with minor modifications. 50 µl of extract, diluted extract of standard (wheat germ GR in homogenization buffer, Sigma-Aldrich, St. Louis, MO, USA) was mixed with 150 µl of 100 nmol sodium phosphate buffer (pH 7.6) containing 0.1 nmol 5,5-dithiobis(2-nitrobenzoic acid) (DNTB) and 10 µl NADPH (10 mg^{-1} ml, 12 nmol). The reaction between DNTB and GSH converted from GSSG was begun through the injection of 10 µl of GSSG (1 mg^{-1} ml; 3.25 mmol) and absorbance at 415 nm (A_{415}) was measured every 30 seconds for 3 minutes, the plate being automatically shaken before each reading. The rate of increase in A_{415} per minute was calculated and regression analysis used to prepare a standard line relating GR activities to A_{415}. GR activities in extracts were calculated with reference to the standard line and expressed as nmol of GSSG reduced per min per mg of total protein.

2.2.3.6 Ascorbate peroxidase

APox activity was determined by following the decrease in absorbance at 290 nm as ascorbate disappeared Rao *et al.* (1996). The 1 ml reaction mixture contained 100 mmol potassium phosphate (pH 7.0), 0.5 mmol ascorbate, 0.2 mmol H_2O_2 and up to 50 µl of extract. APox activity (µmol^{-1} min) was calculated using an extinction coefficient of 2.8/mmol/cm. The assay was conducted using a Pharmacia Ultrospec 3000 spectrophotometer (Pharmacia, Uppsala, Sweden) fitted with a temperature controlled cell. The instrument was controlled and data acquired using a PC running the Swift (enzyme kinetics) software package (Pharmacia).

2.2.4 Lipid hydroperoxide extraction and determination

50 mg of ground tissue was homogenized in 0.3 ml of a methanol:chloroform (2:1, v/v) mixture and left to stand on ice for one minute. 0.2 ml of chloroform and then 0.2 ml of water were added, mixing for 30 seconds after each step. Phases were separated by centrifugation at 12,000 x *g*, lipid hydroperoxides being contained in the chloroform phase which was

transferred to a new tube and stored at -70 °C before analysis. Lipid hydroperoxide content of samples was determined using the ferric thiocyanate method of (Mihaljevic *et al.*, 1996), adapted for measurement in a microplate reader. Absorbance at 480 nm was measured, compared against a calibration curve generated by measuring the A_{480} of t-butyl hydroperoxide and lipid hyrdoperoxide content expressed as nmol lipid hydroperoxides g^{-1} FW.

2.2.5 Ascorbate extraction and determination

Frozen leaf tissue was ground using a motor and pestle pre-chilled with liquid nitrogen to prevent oxidation of ascorbate via ascorbate oxidase prior to analysis. Ground tissue was homogenized in 250 µl of 5 % (w/v) metaphosphoric acid (5 ml g^{-1} fresh weight). Homogenates were centrifuged at 20,800 x *g* for 15 minutes at 4 °C and the supernatant divided into aliquots and stored at - 80 °C prior to analysis. Oxidized and total ascorbate was determined using the microplate-adapted colorimetric method described by (Gillespie and Ainsworth, 2007).

2.2.6 Glutathione extraction and analysis

Ground tissue was homogenized in 5 % (w/v) sulfosalicyclic acid (5 ml g^{-1} fresh weight) and homogenates centrifuged at 20,800 x *g* at 4 °C for 15 minutes. Supernatants were then divided into aliquots and stored at -80 °C prior to analysis. Reduced and total glutathione levels were determined from 50 µl aliquots using the enzymatic recycling methods for microtitre plates described by Rahman *et al.* (2006).

2.2.7 Extraction and determination of photosynthetic pigments

Chlorophyll a, chlorophyll b and carotenoids from samples from the final harvest (4 weeks) were extracted using the method of Hiscox and Israelstam, (1979), with modifications. 20 mg of ground tissue was added to 7 ml of dimethyl sulfoxide and extracted for 48 hours at room temperature in the dark. Extracts were centrifuged at 668 x *g* for 15 minutes and the absorbance of the supernatant at 470 nm, 648 nm and 664 nm (A_{470}, A_{648} and A_{664}) measured using a Ultrospec 2000 UV/visible spectrophotometer (Pharmacia). Pigment concentrations were calculated as follows and expressed as µg ml^{-1} extract (Chappelle *et al.*, 1992):

$$\text{Chlorophyll a} = 12.25A_{664} - 2.79A_{648}$$

$$\text{Chlorophyll b} = 21.50A_{648} - 5.40A_{664}$$

$$\text{Carotenoids} = (1000A_{470} - 1.82 \text{ chlorophyll a} - 85.02 \text{ chlorophyll b}) / 198$$

2.2.8 Extraction and determination of anthocyanins

Anthocyanins from samples of the final harvest (4 weeks) were extracted using the method of (Li and Kubota, 2009), with modifications. 200 mg of ground tissue was added to 5 ml of 2 % HCl in methanol for 24-48 hours. Extracts were centrifuged at 1446 x *g* for 15 minutes and the supernatant used for spectrophotometric determination of the total anthocyanin content with a Ultrospec 2000 UV/visible spectrophotometer (Pharmacia) as per the pH differential method of Giusti and Wrolstad (2001).

2.2.9 Statistical methods

Statistical analyses were performed using SPSS 19 for Windows XP. For data from the first end-point experiment differences between means were detected using a repeated measures analysis of variance, where measurements from inner- and outer-whorl leaves of the same individual constituted the repeated measures. For data from the time course experiments, separate ANOVAs were performed to detect any difference between time = 0 samples from controls (untreated samples at t = 1, 2 and 4 weeks), and to detect differences between controls and treated (UV-B+ or UV-B-) samples. One plant of the cultivar 'Royal Oak Leaf' at t = 0 did not yield sufficient material for all analyses, so replication for the assays of lipid hydroperoxides and ascorbate was disproportionate, the appropriate modifications to the ANOVA being made automatically by SPSS. Where possible, differences between varieties were distinguished using Tukey's HSD test, and differences between treatments and controls distinguished using Dunnett's *t* test.

2.3 Results

2.3.1 End-point experiment

2.3.1.1 Growth and photosynthetic parameters

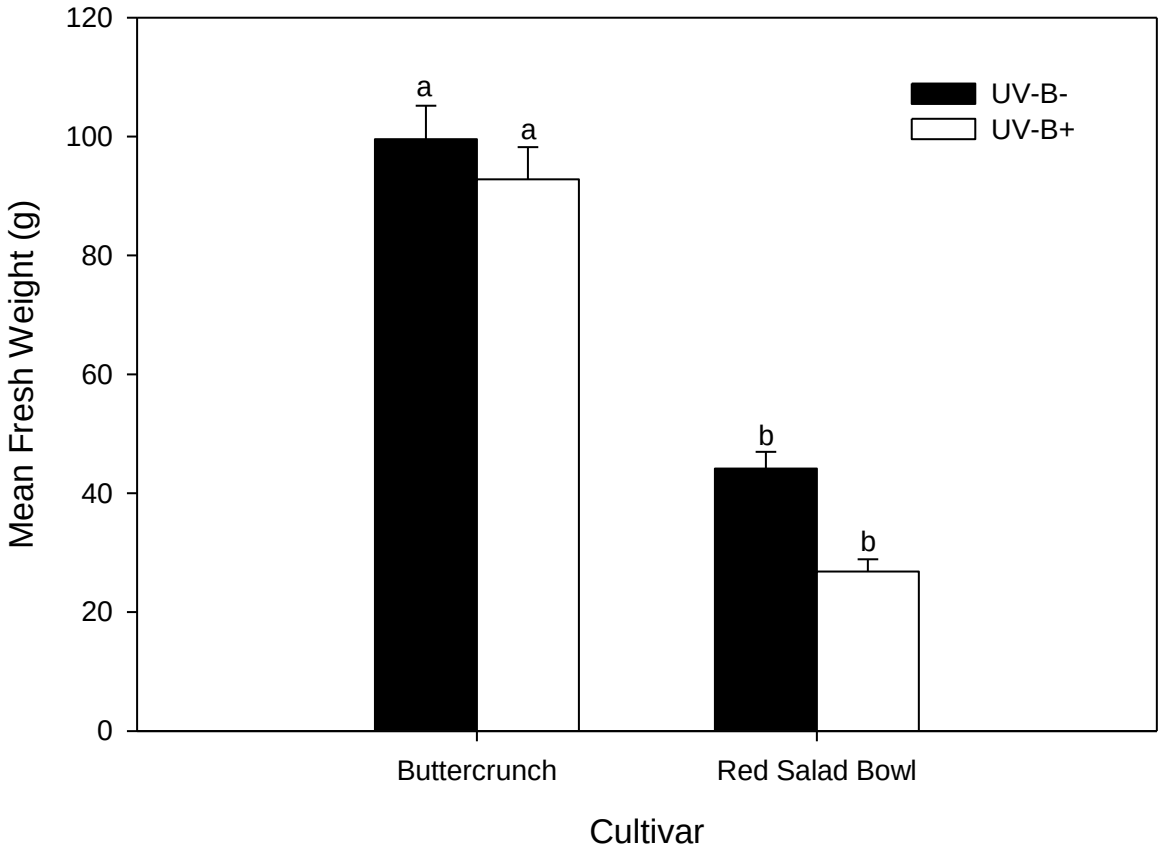

Figure 2.1 Mean above-ground fresh weight of lettuces after 4 weeks UV-B treatment ± 1 standard error. Bars with different letters are significantly different (n = 12, α = 0.05).

In this experiment, the effect of UV-B exposure on *L. sativa* was investigated by comparing mature plants of two cultivars growing under protective plastics of differing UV-B transparency. Biomass accumulation as measured by the above-ground fresh weight of plants was not significantly affected by UV-B treatment regime (p = 0.135), although it did differ between cultivars (p = 0.004; Fig. 2.1).

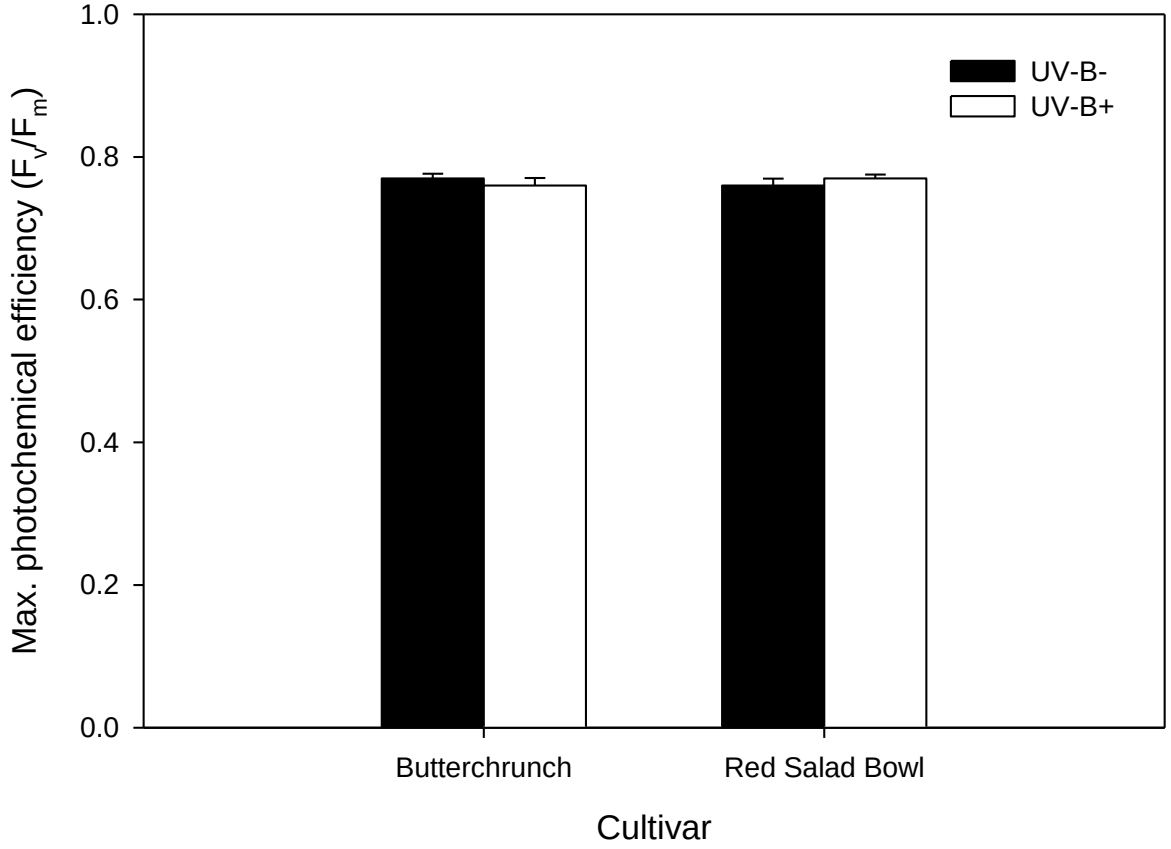

Figure 2.2 Mean (± 1 standard error) maximum photochemical efficiency of lettuce plants exposed to varying UV-B conditions for 4 weeks. Bars with different letters are significantly different (n = 12, α = 0.05).

Maximum photochemical efficiency (F_v/F_m) provides a proxy for the efficiency of photosynthesis by quantifying the proportion of damaged reaction centres. The plants in this experiment displayed no significant variation in the values of F_v/F_m, suggesting that the levels of UV-B exposure in this experiment were not sufficient to cause photoinhibiton (Fig. 2.2)

2.3.1.2 Protein carbonyl contents

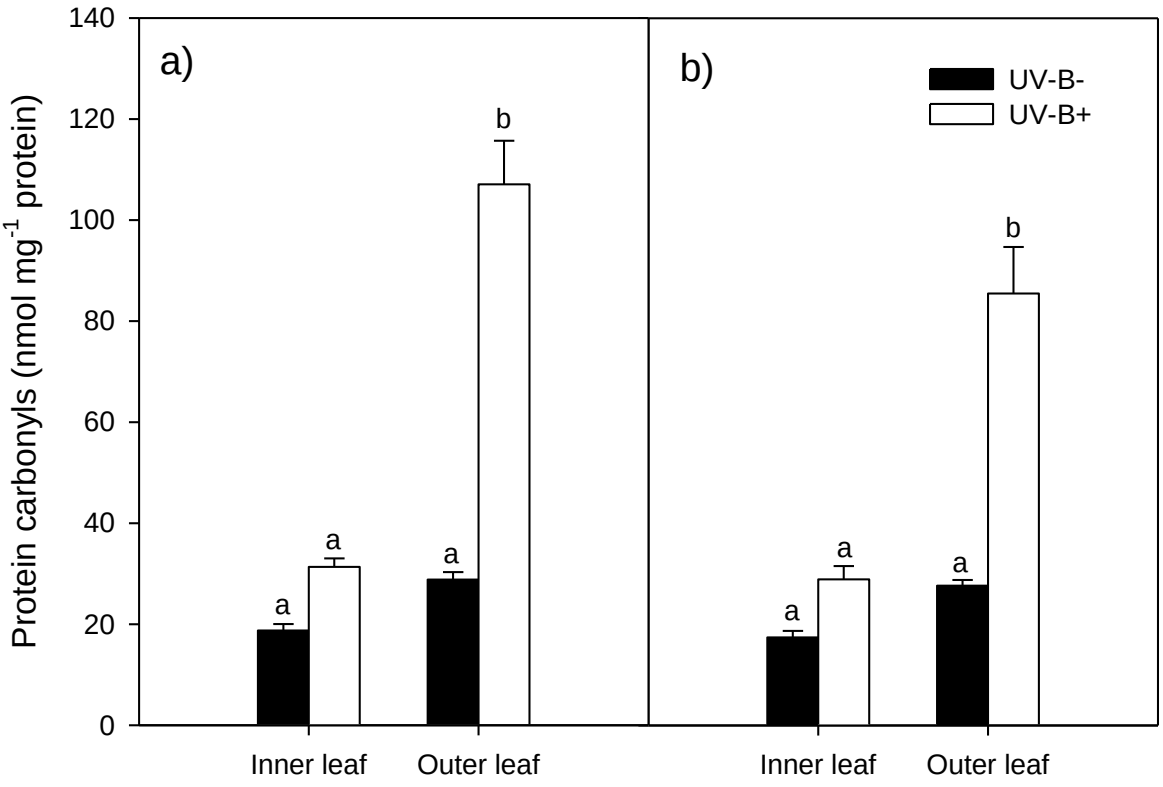

Figure 2.3 Mean protein carbonyl contents after 4 weeks UV-B exposure in a) cv. 'Buttercrunch' and b) cv 'Red Salad Bowl'. Bars with different letters are significantly different (n = 12, α = 0.05) and error bars are ± 1 standard error.

The protein carbonyl content was increased significantly by exposure to UV-B ($p < 0.001$), with an interactive effect of UV-B and leaf position within plants ($p < 0.001$), indicating substantive oxidative damage to proteins. Protein carbonyl content was higher in outer-whorl leaves than inner-whorl leaves, maximum values occurring in the outer-whorl leaves of UV-B exposed plants. No significant difference in the protein carbonyl content was detected between cultivars (Fig. 2.3a, b).

2.3.1.3 SOD activity

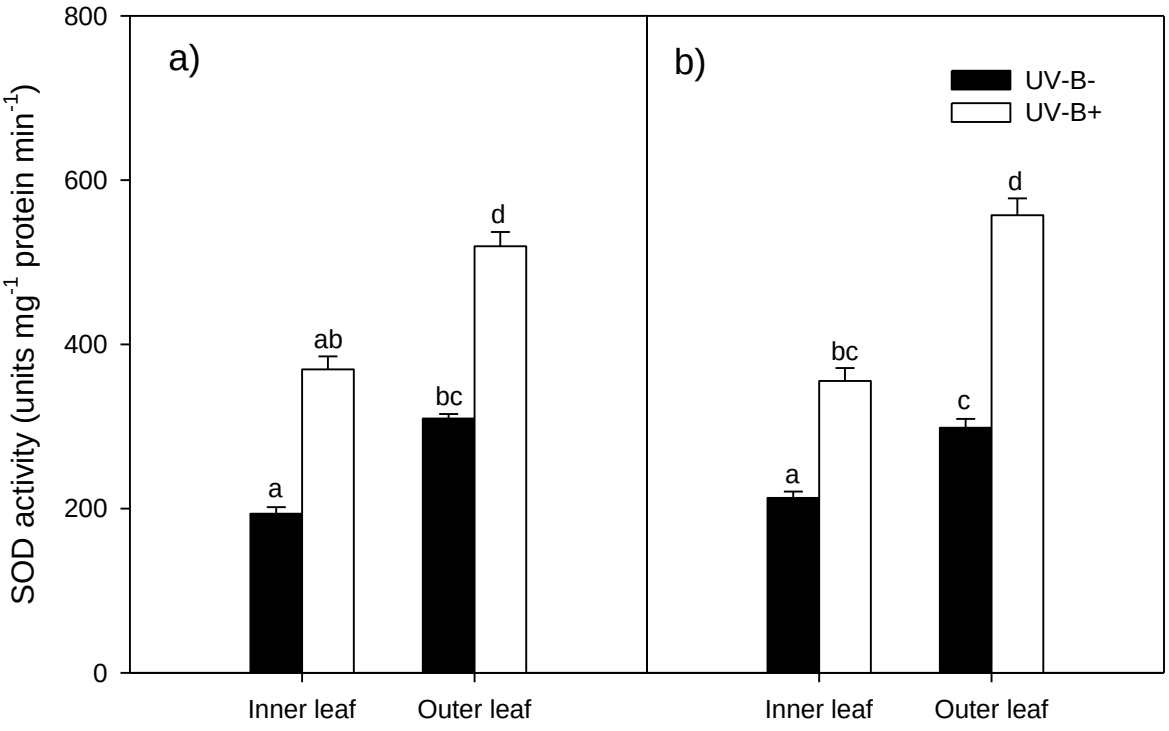

Figure 2.4 SOD activities after 4 weeks UV-B exposure in a) cv. 'Buttercrunch' and b) 'Red Salad Bowl'. Bars with different letters are significantly different (n = 12, α = 0.05) and error bars are ± 1 standard error.

The activity of SOD in UV-B exposed plants was significantly higher than that in unexposed plants ($p < 0.001$, Fig. 2.4a, b). There was an interactive effect of leaf position and UV-B treatment ($p = 0.003$), outer-whorl leaves having higher SOD activities than inner-whorl leaves, SOD activity in outer-whorl leaves being increased by UV-B to a greater extent than it was in inner leaves.

2.3.1.4 CAT activity

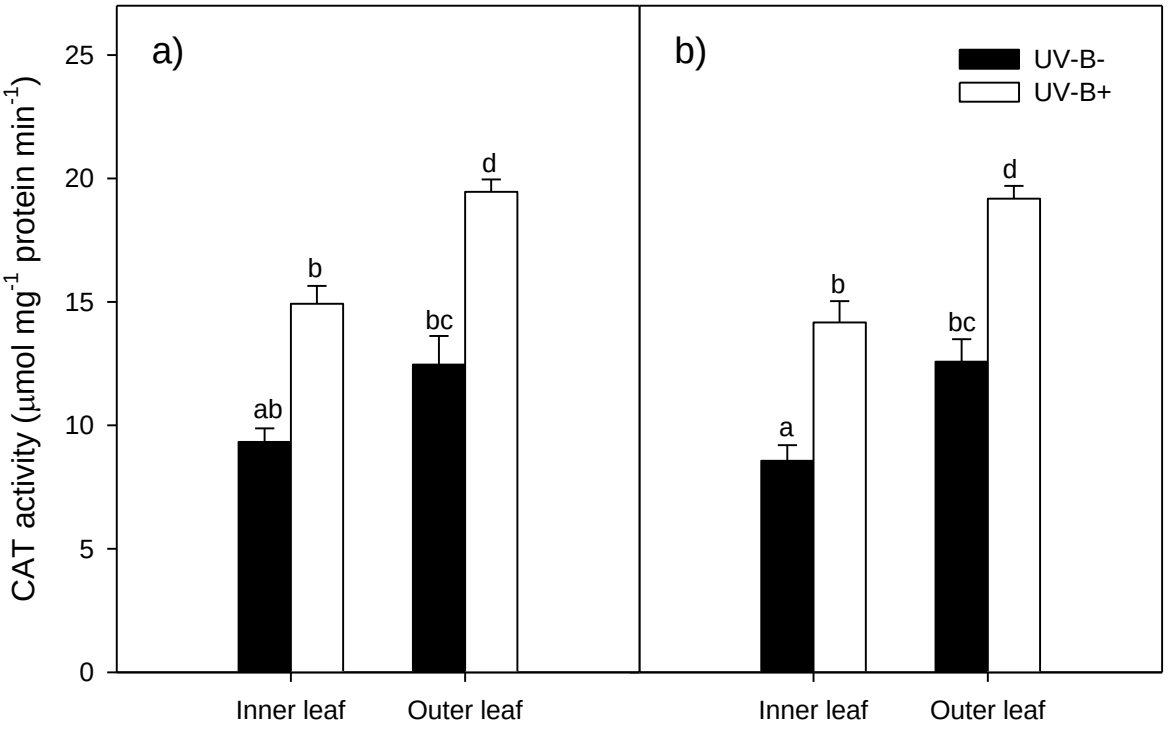

Figure 2.5 Mean CAT activites after 4 weeks UV-B exposure in a) cv 'Buttercrunch' and b) cv. 'Red Salad Bowl'. Bars with different letters are significantly different (n = 12, α = 0.05) and error bars are ± 1 standard error.

CAT activities varied significantly between UV-B exposed and unexposed plants ($p < 0.001$), and also between inner-whorl and outer-whorl leaves of the same plant ($p < 0.001$), but not between cultivars. CAT activity was highest in outer leaves for all plants, and was uniformly increased by exposure to UV-B radiation (Fig. 2.5a, b).

2.3.1.5 GPox activity

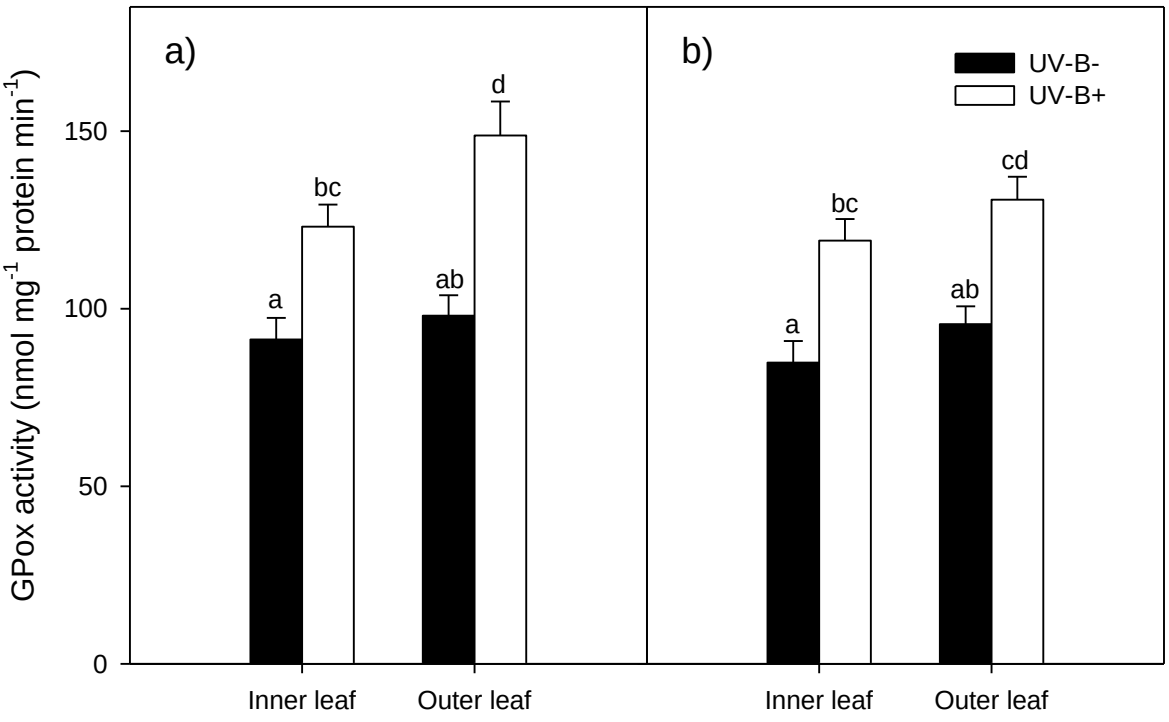

Figure 2.6 Mean GPox activities after 4 weeks UV-B exposure in a) cv. 'Buttercrunch' and b) cv. 'Red Salad Bowl'. Bars with different letters are significantly different (n = 12, α = 0.05) and error bars are ± 1 standard error.

GPox activities were significantly increased in the UV-B exposed plants growing under the cellulose diacetate film ($p < 0.001$). Leaf position within each plant also had a significant effect ($p < 0.001$), GPox activity being greater in the outer whorls. No significant variation between cultivars was detected and leaves of the inner and outer whorls responded UV-B in a similar manner (Fig. 2.6a, b).

2.3.1.6 GR activity

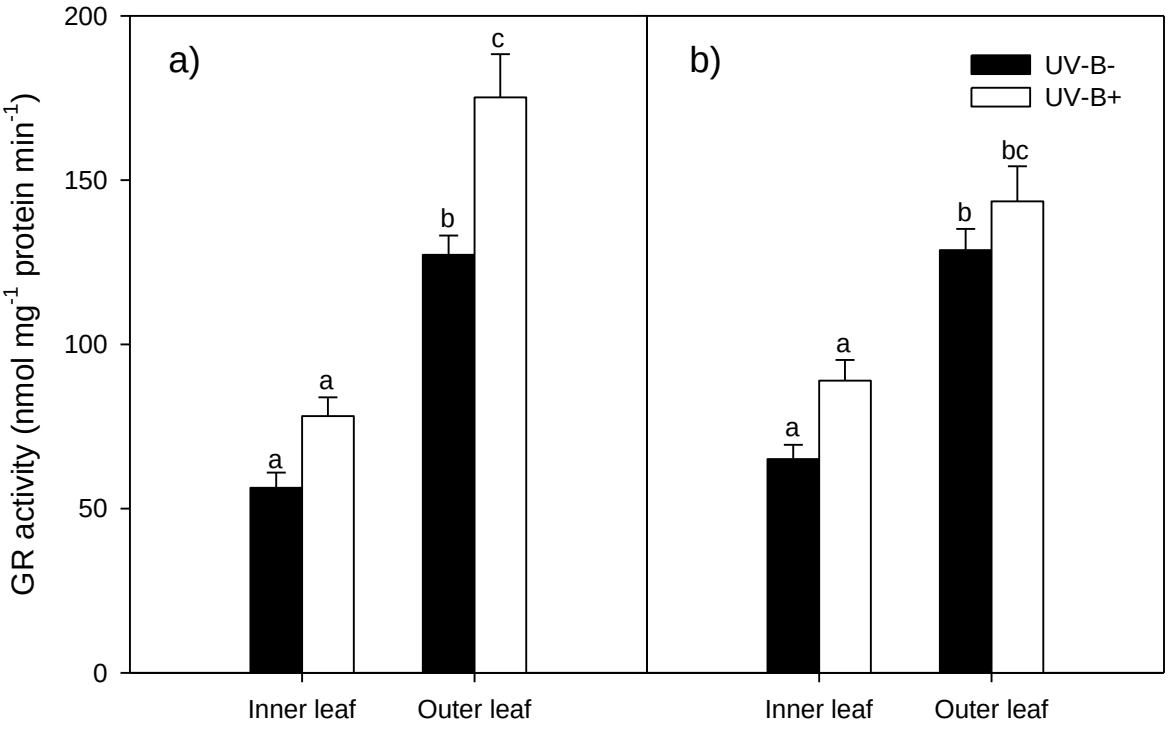

Figure 2.7 Mean GR activity after 4 weeks UV-B exposure in a) cv. 'Buttercrunch' and b) cv. 'Red Salad Bowl'. Bars with different letters are significantly different (n = 12, α = 0.05) and error bars are ± 1 standard error.

GR activity was significantly higher in UV-B exposed plants than unexposed plants ($p < 0.001$) and was affected by an interaction between the effects of variety and leaf position ($p = 0.024$). GR activity appeared to increase both inner- and outer-whorl leaves of UV-B exposed plants of the cultivar 'Buttercrunch', but increased substantially in only the inner-whorl leaves of the cultivar 'Red Salad Bowl' (Fig. 2.7a, b).

2.3.1.7 APox activity

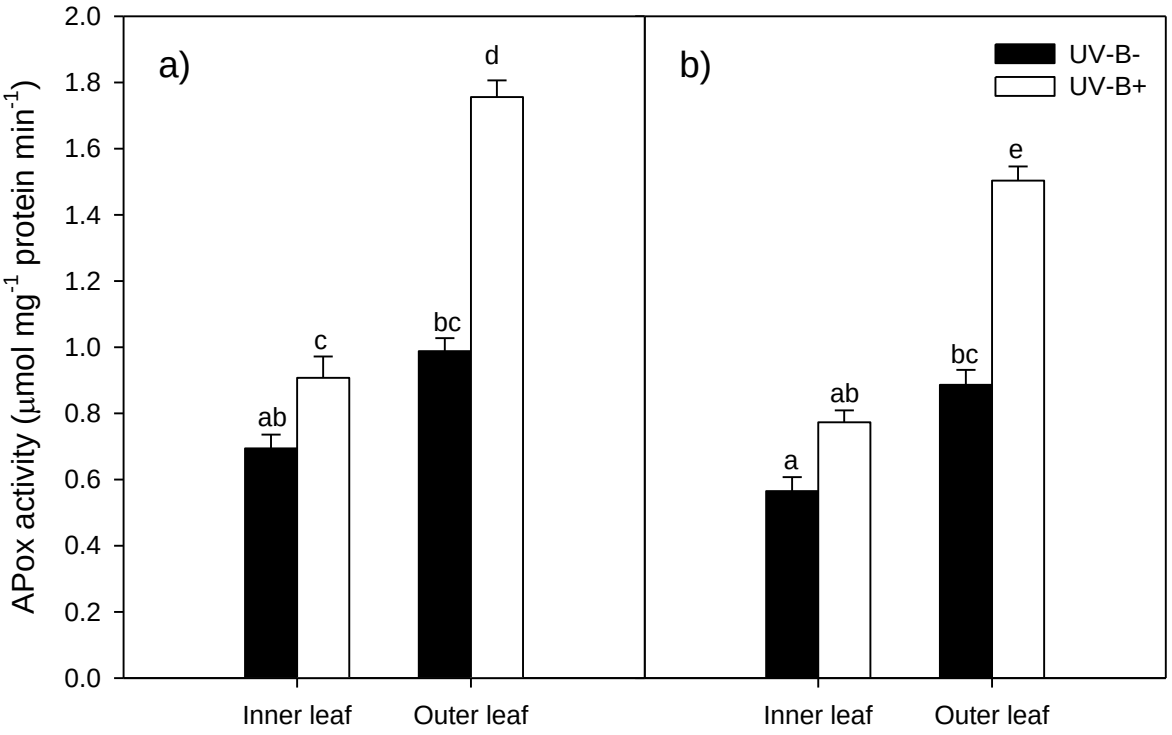

Figure 2.8 Mean APox activity after 4 weeks UV-B exposure in a) cv. 'Buttercrunch' and b) cv. 'Red Salad Bowl'. Bars with different letters are significantly different (n = 12, α = 0.05) and error bars are ± 1 standard error.

APox activity was significantly affected by UV-B treatment ($p < 0.001$), being higher in UV-B exposed plants and also varied between cultivars ($p < 0.001$), generally being higher in the cultivar 'Buttercrunch' than 'Red Salad Bowl'. There was also a significant interaction between the effects of leaf position and UV-B treatment ($p < 0.001$), the APox activity of outer-whorl leaves being increased by UV-B exposure to a greater degree than that of inner-whorl leaves (Fig. 2.8a, b).

2.3.2 Time-course experiment

2.3.2.1 Plant growth parameters

The above-ground fresh weight of lettuce plants increased at each successive harvest, being affected by a three-way interaction between the effects of UV-B treatment, time and cultivar ($p = 0.002$). The cultivars 'Royal Oak Leaf' and 'Drunken Woman Fringed Head' appeared to increase in weight faster than the cultivar Red Salad Bowl, with little difference between UV-B treatments. Tukey's HSD tests were in most cases unable to determine which samples differed, although four weeks after exposure began, the growth of both covered (UV-B+ and

UV-B–) plants of the cultivar Red Salad Bowl had been exceeded by uncovered controls that had attained weights similar to those of other cultivars (Fig. 2.9a, b, c).

As expected, the length of the longest leaf increased over time being affected by significant interactions between time and treatment ($p = 0.005$) and cultivar and treatment ($p = 0.006$). Tukey's HSD test however, was unable to determine differences between cultivars and time points, although leaves tended to be longer in treated (UV-B+ and UV-B–) plants compared with controls, the difference being most marked at four weeks. At the end of the experiment leaves of treated plants of the cultivar 'Royal Oak Leaf' appeared to be longest, followed by those of treated plants of the cultivar 'Drunken Woman Fringed Head', leaves of untreated plants and the cultivar 'Red Salad Bowl' appearing to be the shortest (Fig 2.10a, b, c)

Maximum photochemical efficiency (F_v/F_m), varied significantly but only slightly with an interactive effect of cultivar and time ($p = 0.013$). Tukey's HSD tests however, were unable to determine which varieties differed, although photochemical efficiency appeared to generally increase slightly with length of treatment (Fig 2.11a, b, c).

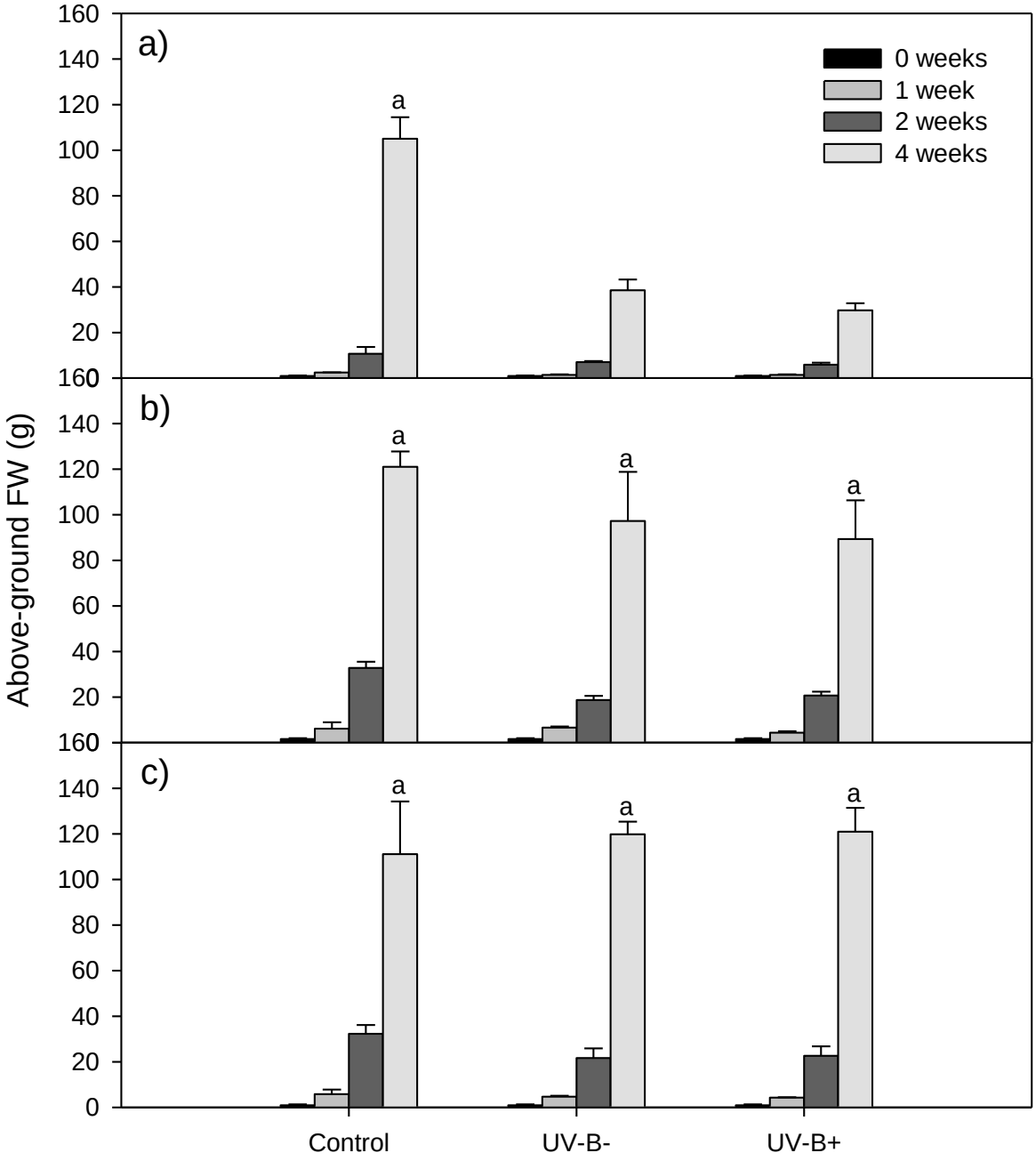

Figure 2.9 Mean above-ground fresh weight of a) cv. 'Red Salad Bowl, b) cv. 'Drunken Woman Fringed Head' and c) cv. 'Royal Oak Leaf' at each harvest. Tukey's HSD test was in most cases unable to determine which samples differed, although bars marked with the letter 'a' fall into a group distinct from all others ($n = 3$, $\alpha = 0.05$). Error bars are one standard error.

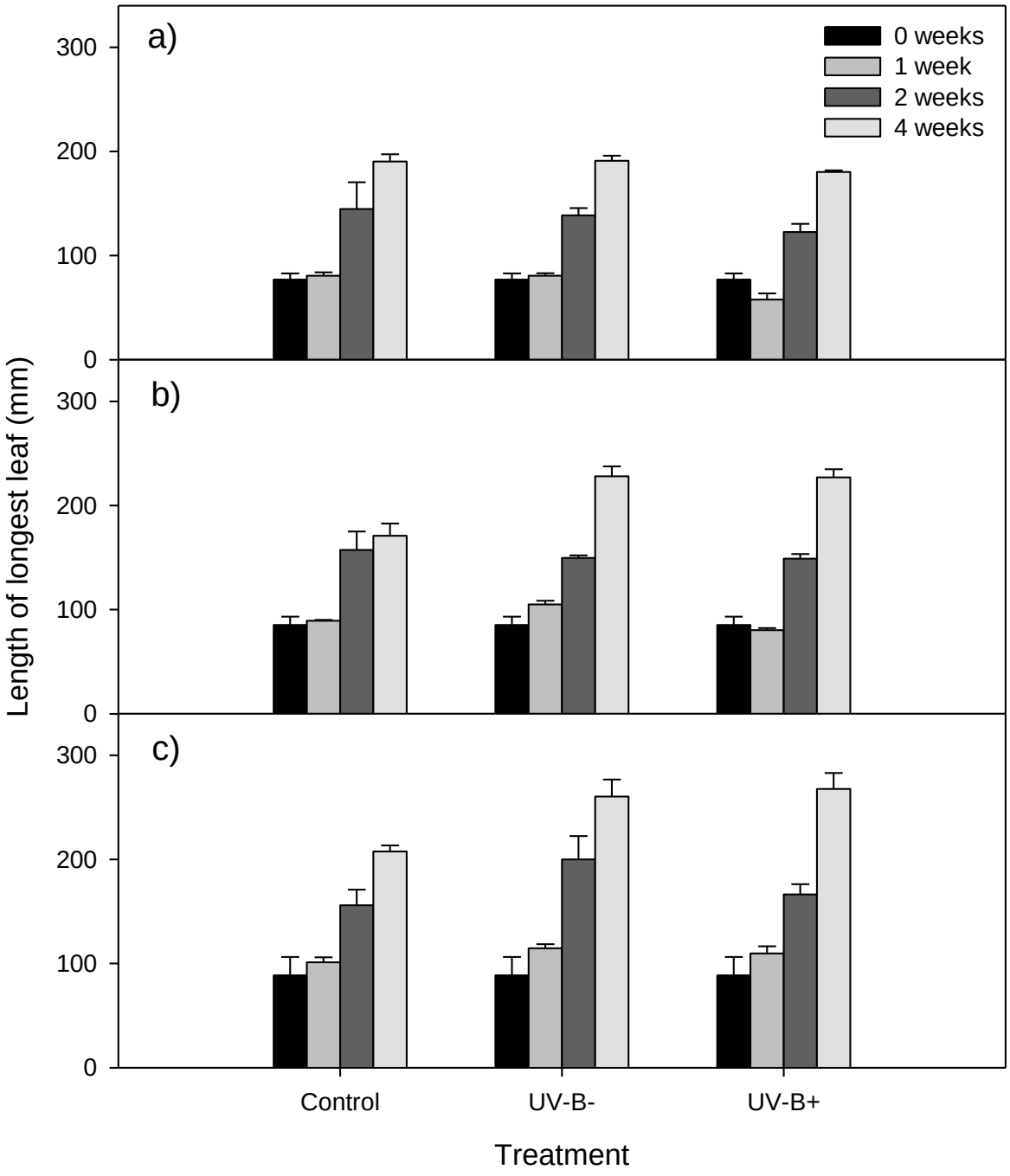

Figure 2.10 Length of the longest leaf of a) cv. 'Red Salad Bowl', b) cv. 'Drunken Woman Fringed Head' and c) cv. 'Royal Oak Leaf' at each harvest point. Although differences did exist between samples, Tukey's HSD test was unable to determine which samples differed ($n = 3$, $\alpha = 0.05$). Error bars are ± 1 standard error.

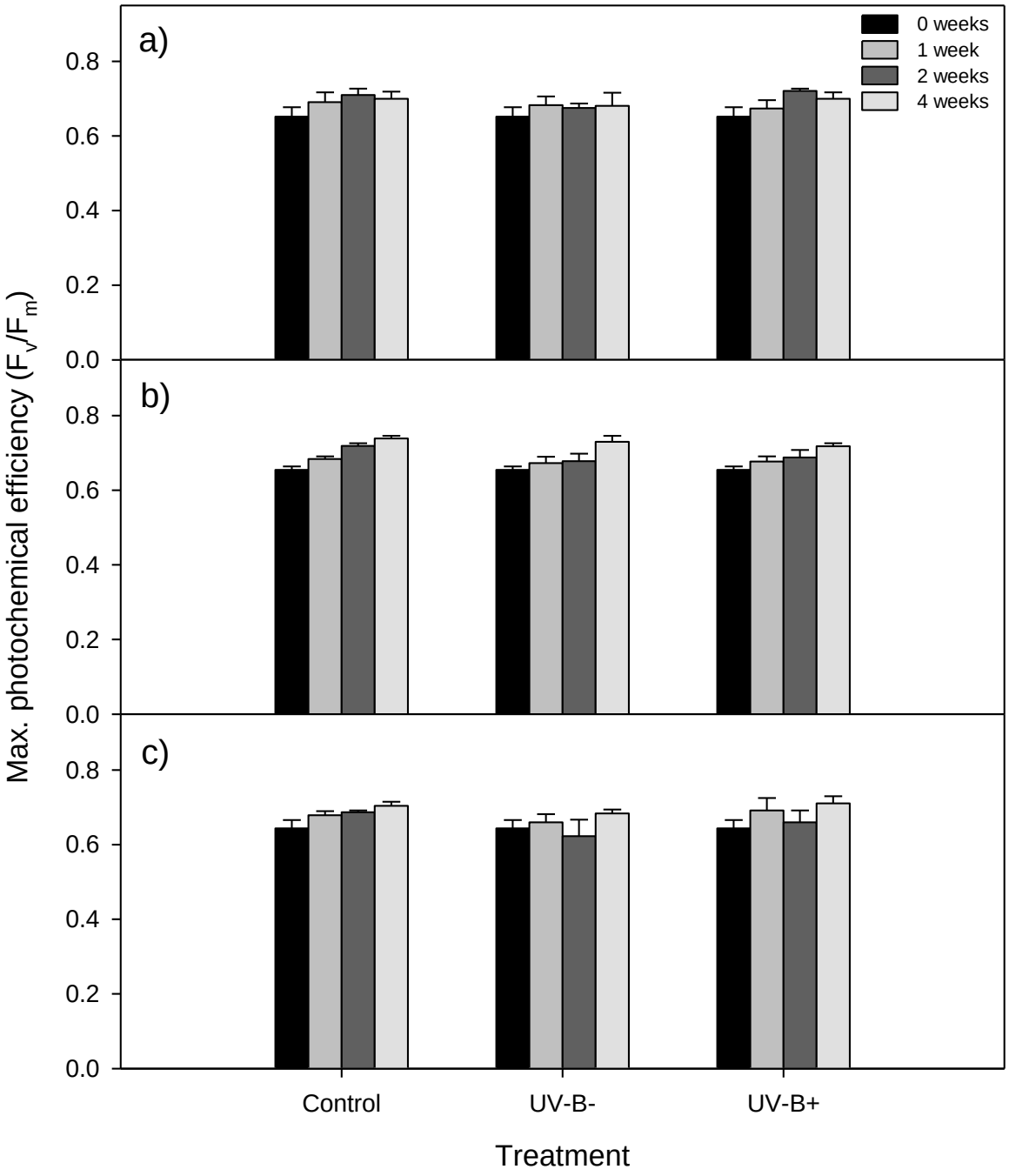

Figure 2.11 Mean maximum photochemical efficiency of a) cv. 'Red Salad Bowl', b) cv. 'Drunken Woman Fringed Head and c) cv. 'Royal Oak Leaf at each harvest. Tukey's HSD tests produced only poor resolution of the differences between samples (n = 3, α = 0.05), error bars are ± 1 standard error.

2.3.2.2 Photosynthetic pigments and anthocyanins

Chlorophyll a content at the end of the time-course experiment varied significantly with an interactive effect of treatment and cultivar (p = 0.021), Tukey's HSD showing chlorophyll a content to be greater in the cultivar 'Royal Oak Leaf' than in other cultivars, and to be generally greater in controls than treated plants (Fig 2.12a). Chlorophyll b content varied

significantly with treatment ($p = 0.002$) and cultivar ($p = 0.050$), controls having higher levels of chlorophyll b than treatments and the cultivar 'Royal Oak leaf' having higher levels of chlorophyll b than the cultivar 'Drunken Woman Fringed Head' (Tukey's HSD, Fig 2.12b). Carotenoid concentration also varied significantly with an interactive effect of treatment and cultivar ($p = 0.028$), varying in a similar manner to chlorophylls with the highest levels in controls and plants of the variety 'Royal Oak Leaf' (Tukey's HSD, Fig. 2.12c).

Anthocyanins at the end of the experiment varied significantly with cultivar ($p < 0.001$) but not treatment, concentrations in the cultivar 'Red Salad Bowl' being significantly higher than in other cultivars (Tukey's HSD, Fig 2.13).

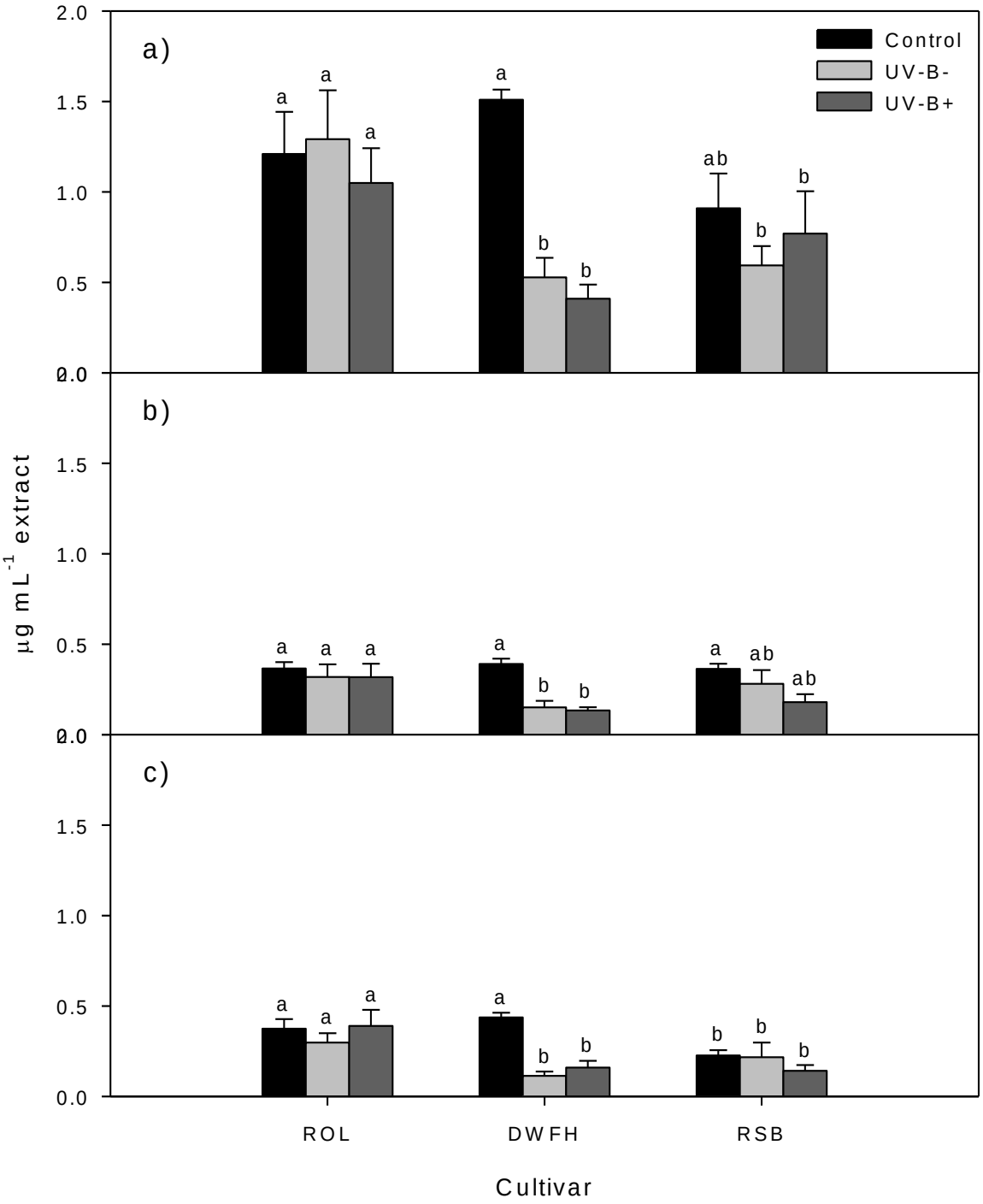

Figure 2.12 Mean concentrations of a) chlorophyll a, b) chlorophyll b and c) carotenoids after 4 weeks UV-B treatment. Bars with different letters are significantly different (n = 3, α = 0.05) and error bars are ± 1 standard error.

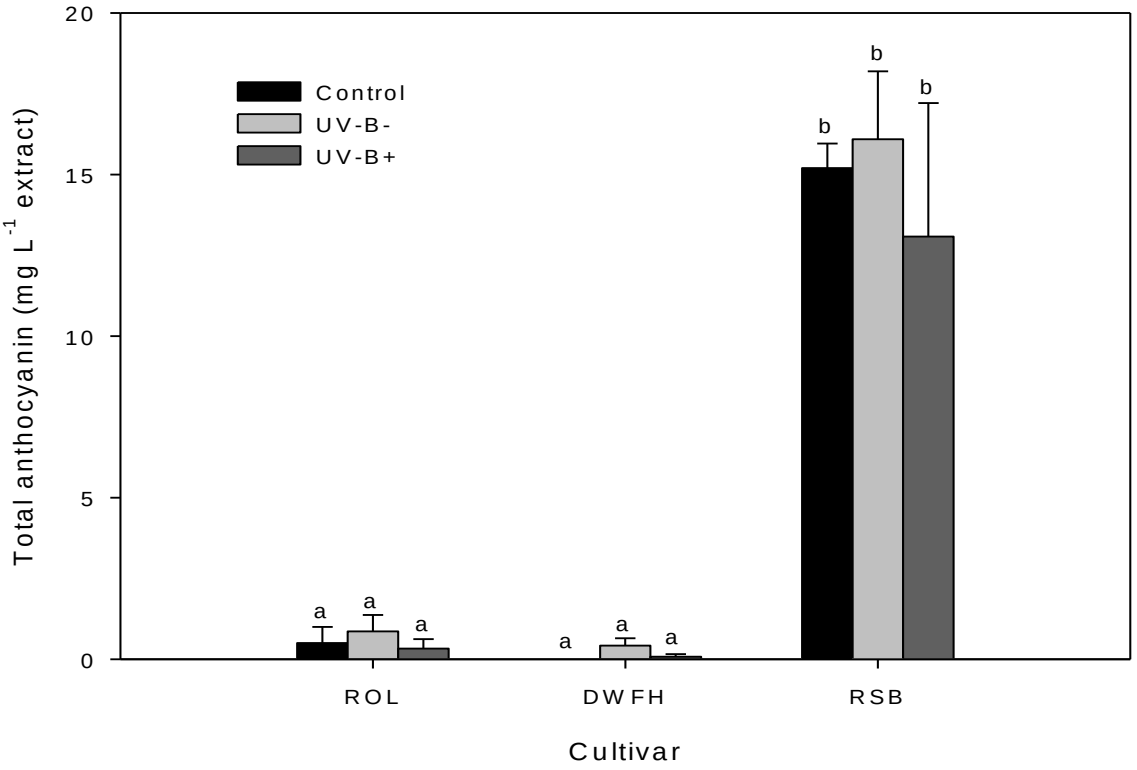

Figure 2.13 Mean total anthocyanins after 4 weeks UV-B treatment. ROL = 'Royal Oak Leaf', DWFH = 'Drunken Woman Fringed Head' and RSB = 'Red Salad Bowl'. Bars with different letters are significantly different (n = 3, α = 0.05) and error bars are ± 1 standard error.

2.3.2.3 Oxidative damage to proteins and lipids

Protein carbonyl contents was significantly affected by interactions between UV-B treatment and time ($p = 0.014$) and UV-B treatment and cultivar ($p = 0.014$). Samples at time = 0 did not differ significantly from controls. Protein carbonyls were generally elevated in UV-B+ treated plants over controls (Dunnett's t, $p < 0.001$), reaching maximum values one week after treatment began before declining somewhat. The level of protein carbonyls in the cultivar Red Salad Bowl peaked at a lower level than in other cultivars (Tukey's HSD), before decreasing to levels similar to that of the controls and UV-B- treatments (Fig. 2.14a, b, c).

Lipid peroxide contents were also affected by an interaction between the effects of UV-B treatment and time ($p < 0.001$) and UV-B treatment and cultivar ($p < 0.001$). As with protein carbonyls, time = 0 samples did not significantly differ from controls in lipid hydroperoxide contents. Lipid peroxide levels were generally elevated in UV-B+ treatments over controls (Dunnett's t, $p < 0.001$) and were lower in the cultivar Red Salad Bowl than the other cultivars tested (Tukey's HSD, Fig 2.15a). Lipid peroxide levels appeared to display the same temporal pattern as that of protein carbonyls being elevated one week after exposure began in

UV-B+ treated plants and decreasing thereafter (Turkey's post hoc tests), although remaining elevated above controls in the cultivars 'Royal Oak Leaf' and 'Drunken Woman Fringed Head' (Fig. 2.15b, c).

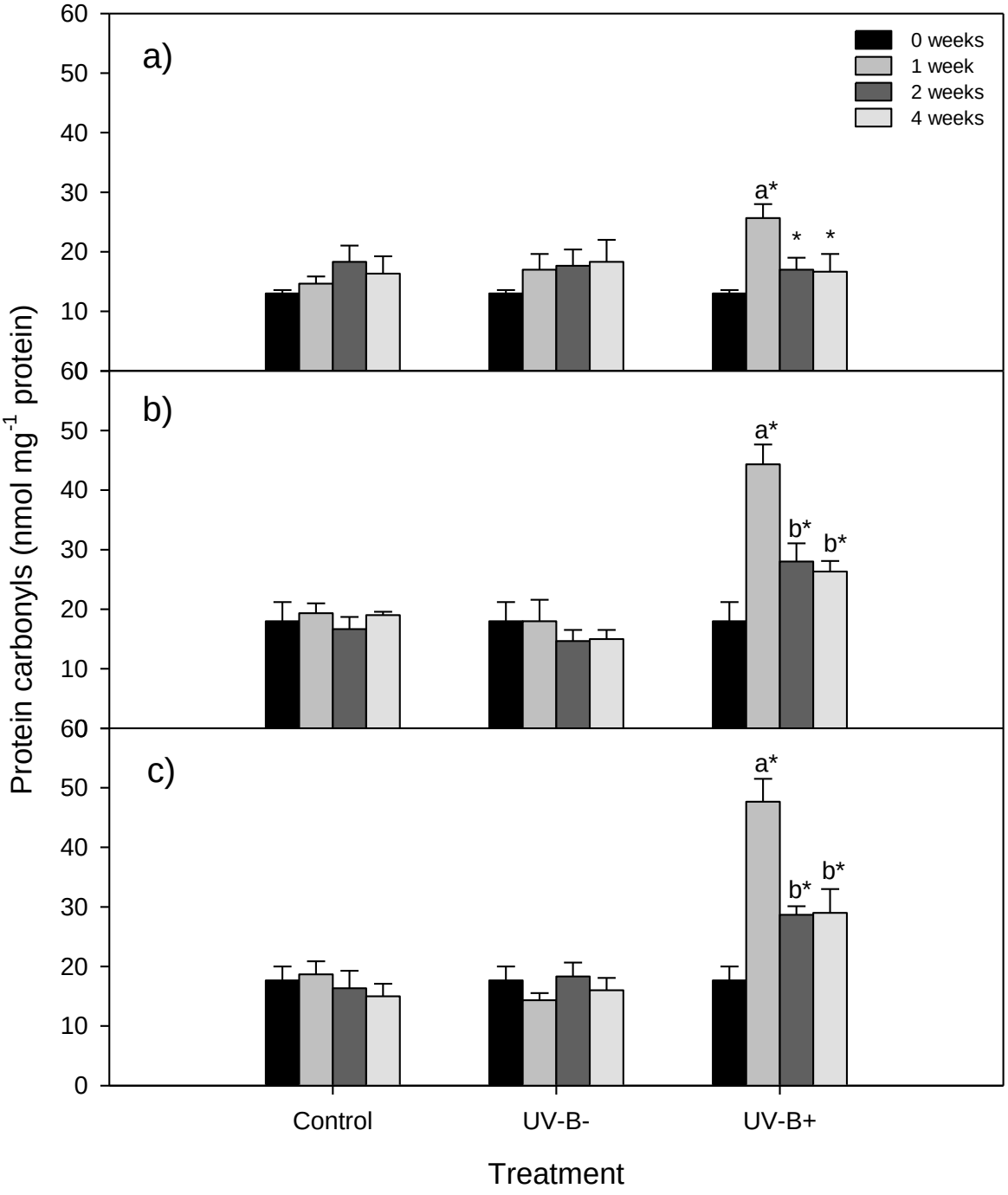

Figure 2.14 Protein carbonyl contents of a) cv. 'Red Salad Bowl', b) cv. 'Drunken Woman Fringed Head' and c) cv. 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls, those marked with '*' differ between varieties (n = 3, α = 0.05) and error bars are ± 1 standard error.

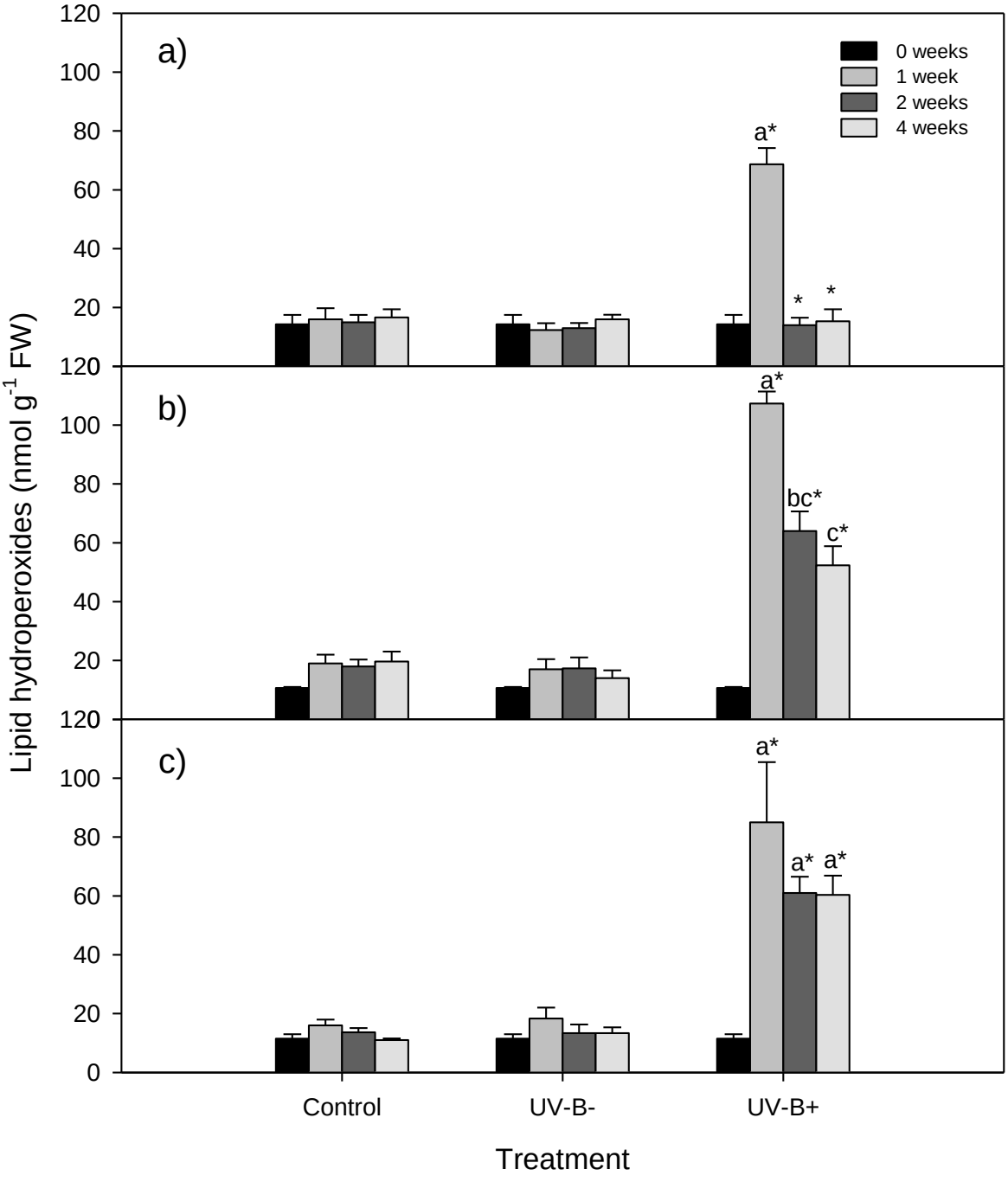

Figure 2.15 Lipid hydroperoxide contents of a) cv. 'Red Salad Bowl,' b) cv. 'Drunken Woman Fringed Head' and c) cv. 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

2.3.2.4 SOD activity

SOD activity was affected by interactive effects of UV-B treatment and time ($p = 0.014$) and UV-B treatment and cultivar ($p = 0.014$). Time = 0 samples did not differ significantly from controls. SOD activity in the variety 'Red Salad Bowl' appeared less than that of other cultivars, although this could not be confirmed by post hoc tests (Fig. 2.16a). SOD activities in UV-B+ treated plants were generally elevated over controls (Dunnett's *t*, $p < 0.001$), rising to a peak two weeks after treatment began and appearing to decline or remain steady thereafter (Tukey's HSD test, Figs. 2.16b, c).

2.3.2.5 CAT activity

CAT activity was affected by an interaction between UV-B treatment and time ($p = 0.048$). There was no significant difference between CAT activity samples at time = 0 and controls. UV-B+ treated plants were generally displayed higher CAT activities than controls (Dunnett's *t* test, $p < 0.001$), the level of activity appearing to increase over time being higher at t = 4 weeks than at other time points (Tukey's HSD; Figs. 2.17a, b, c).

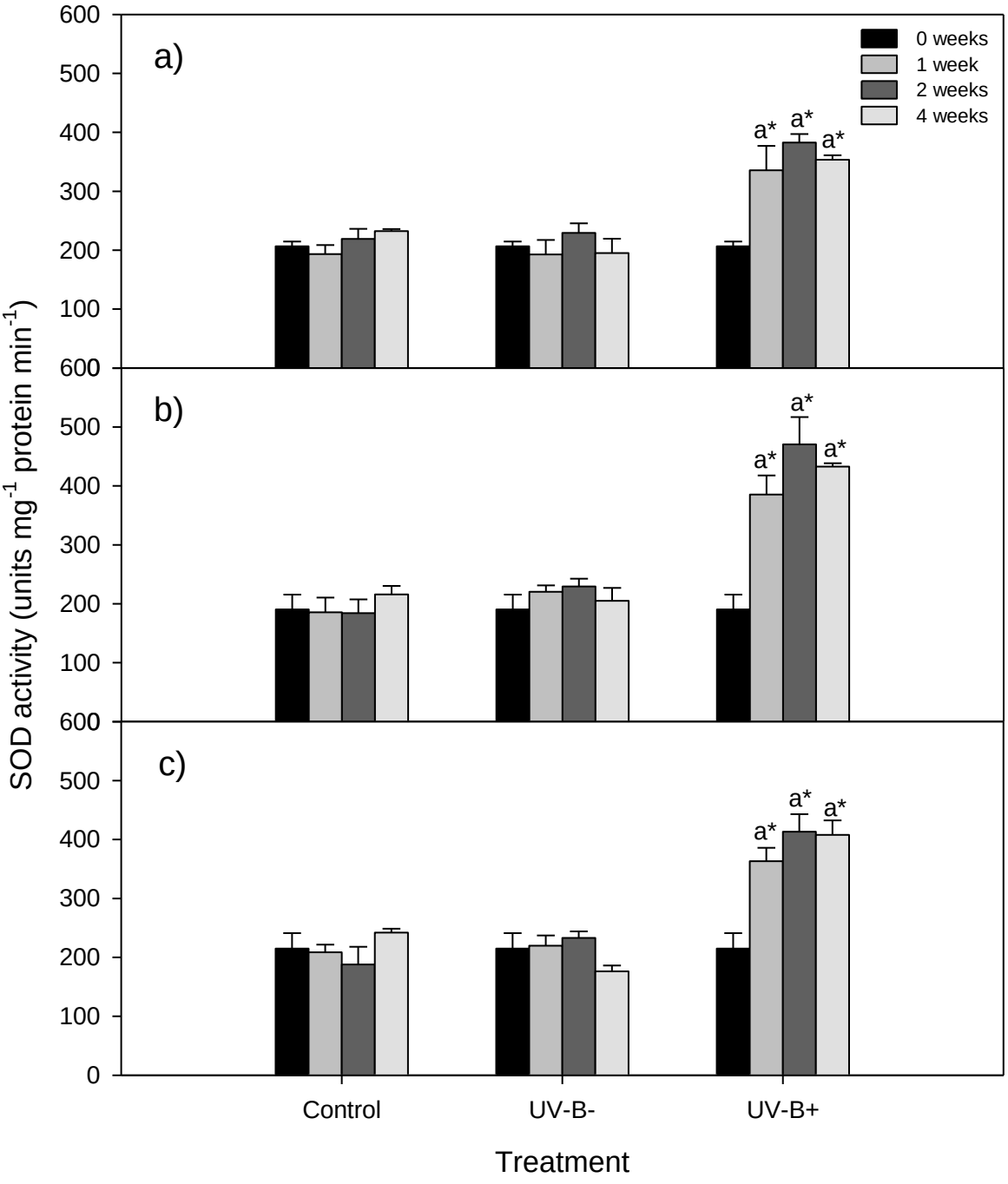

Figure 2.16 Mean SOD activity of a) cv. 'Red Salad Bowl', b) cv. 'Drunken Woman Fringed Head' and c) cv. 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

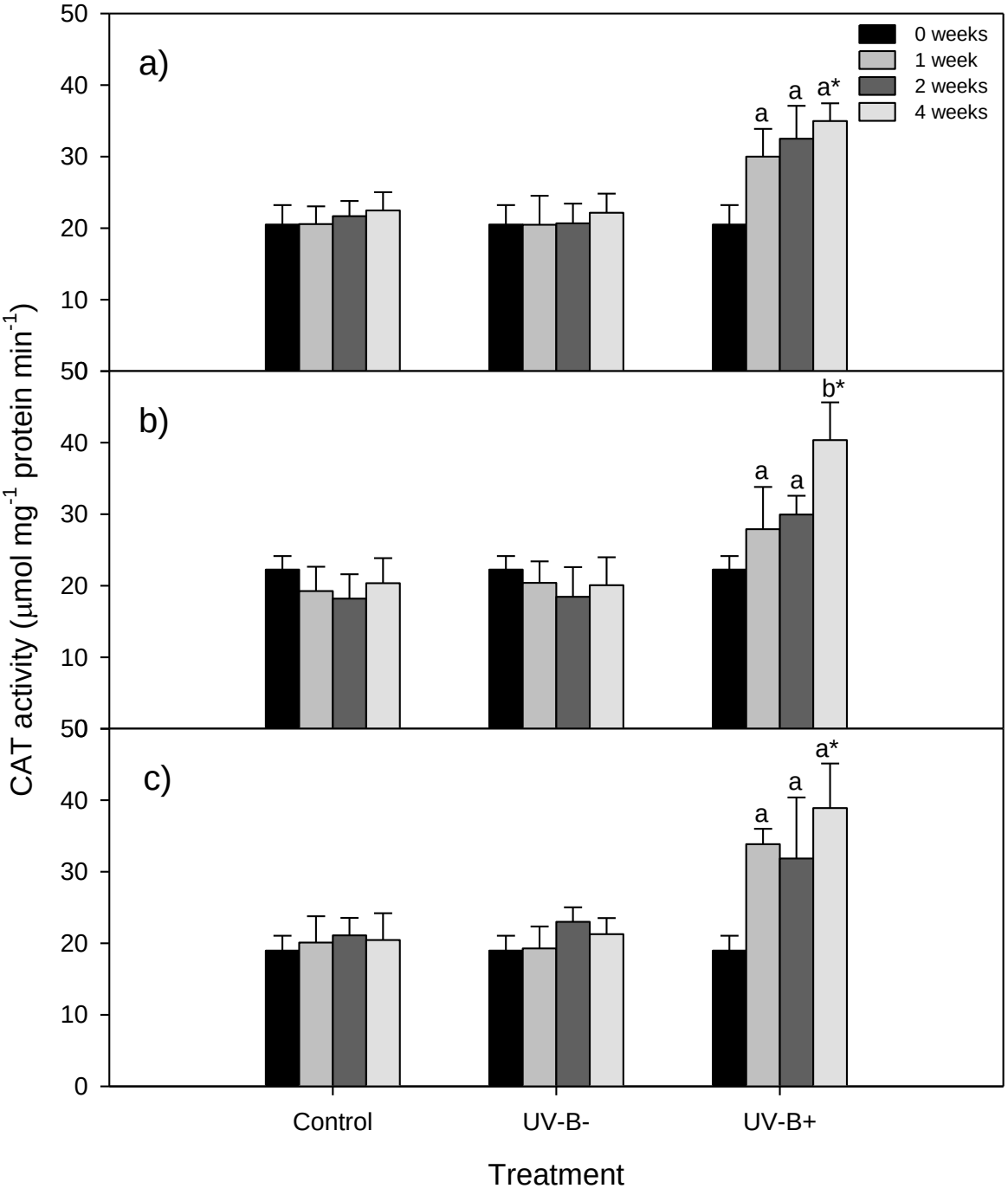

Figure 2.17 Mean CAT activities of a) 'Red Salad Bowl', b) 'Drunken Woman Fringed Head' and c) 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

2.3.2.6 GR activity

GR activity was affected by an interaction between UV-B treatment and cultivar ($p = 0.016$). Samples at t = 0 did not differ significantly from controls. UV-B+ treated plants displayed GR activities that were significantly elevated over controls (Dunnett's t, $p < 0.001$), although

GR activity was significantly lower in the cultivar 'Red Salad Bowl' than in the cultivar 'Drunken Woman Fringed Head' (Tukey's HSD; Figs. 2.18a, b, c).

2.3.2.7 GPox activity

GPox activities were affected by main effects of variety ($p = 0.048$) and treatment ($p < 0.001$). There was no significant difference between time = 0 samples and controls. GPox activities in UV-B+ treated plants were significantly higher than in controls (Dunnett's t, $p = 0.000$) while those in UV-B- treatments were not. GPox activities in UV-B exposed plants of the cultivar 'Red Salad Bowl' appeared similar to controls and were also significantly lower than those of 'Royal Oak Leaf' (Tukey's HSD; Fig, 2.19a, b, c),.

2.3.2.8 APox activity

APox activities were affected by a three-way interaction between the effects of UV-B treatment, cultivar and time ($p = 0.048$). Samples at time = 0 did not differ from controls. In general, APox activities were increased above those in control plants by the UV-B+ treatment (Dunnett's t, $p < 0.001$). APox activity appeared to peak spike two weeks after UV-B+ exposure began in the cultivar 'Royal Oak Leaf', declining thereafter while it rose slightly or remained stable through the whole of the experimental period in the cultivars 'Drunken Woman Fringed Head' and 'Red Salad Bowl' (Fig. 2.20a, b, c).

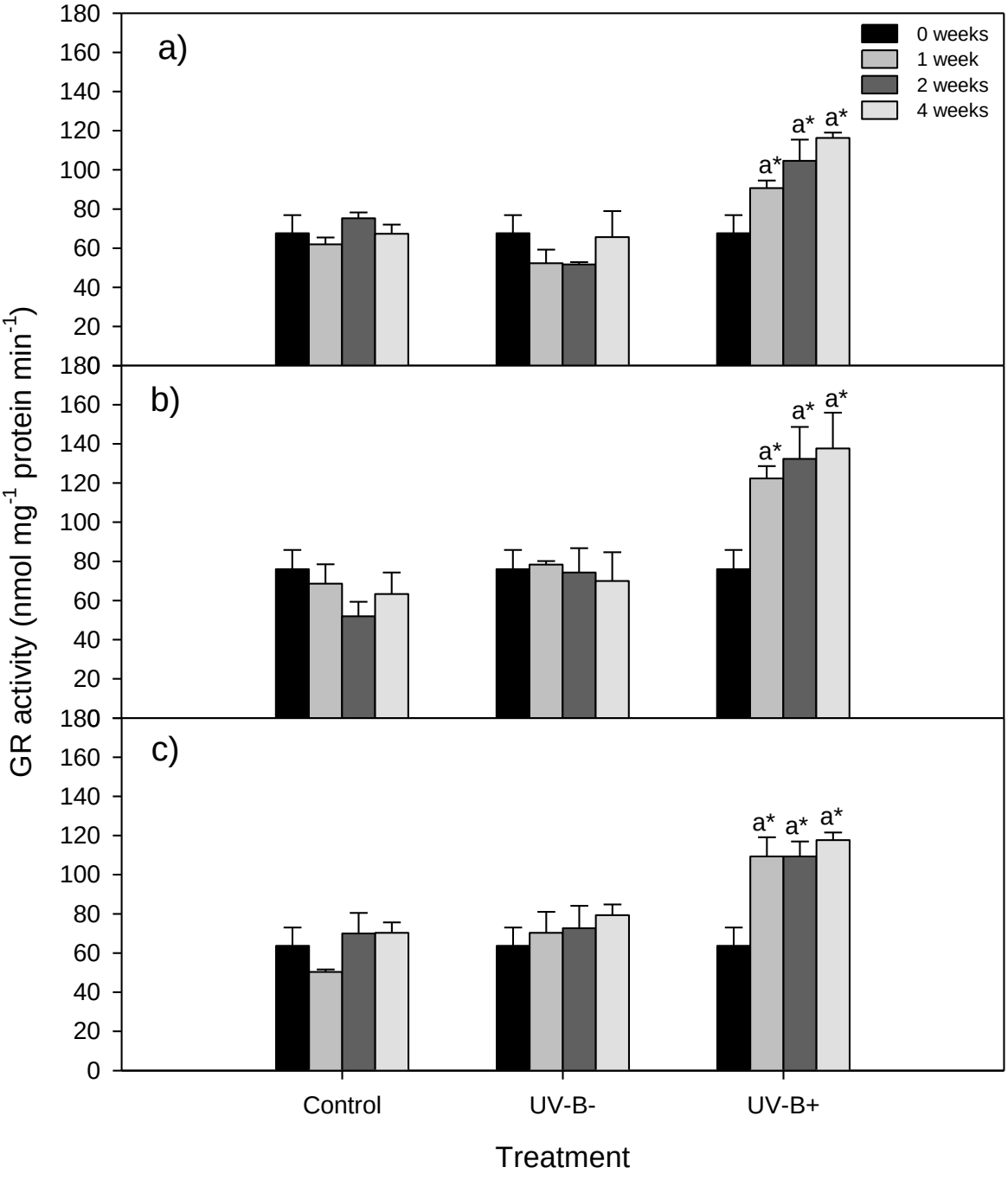

Figure 2.18 Mean GR activities of a) 'Red Salad Bowl, b) 'Drunken Woman Fringed Head' and c) 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

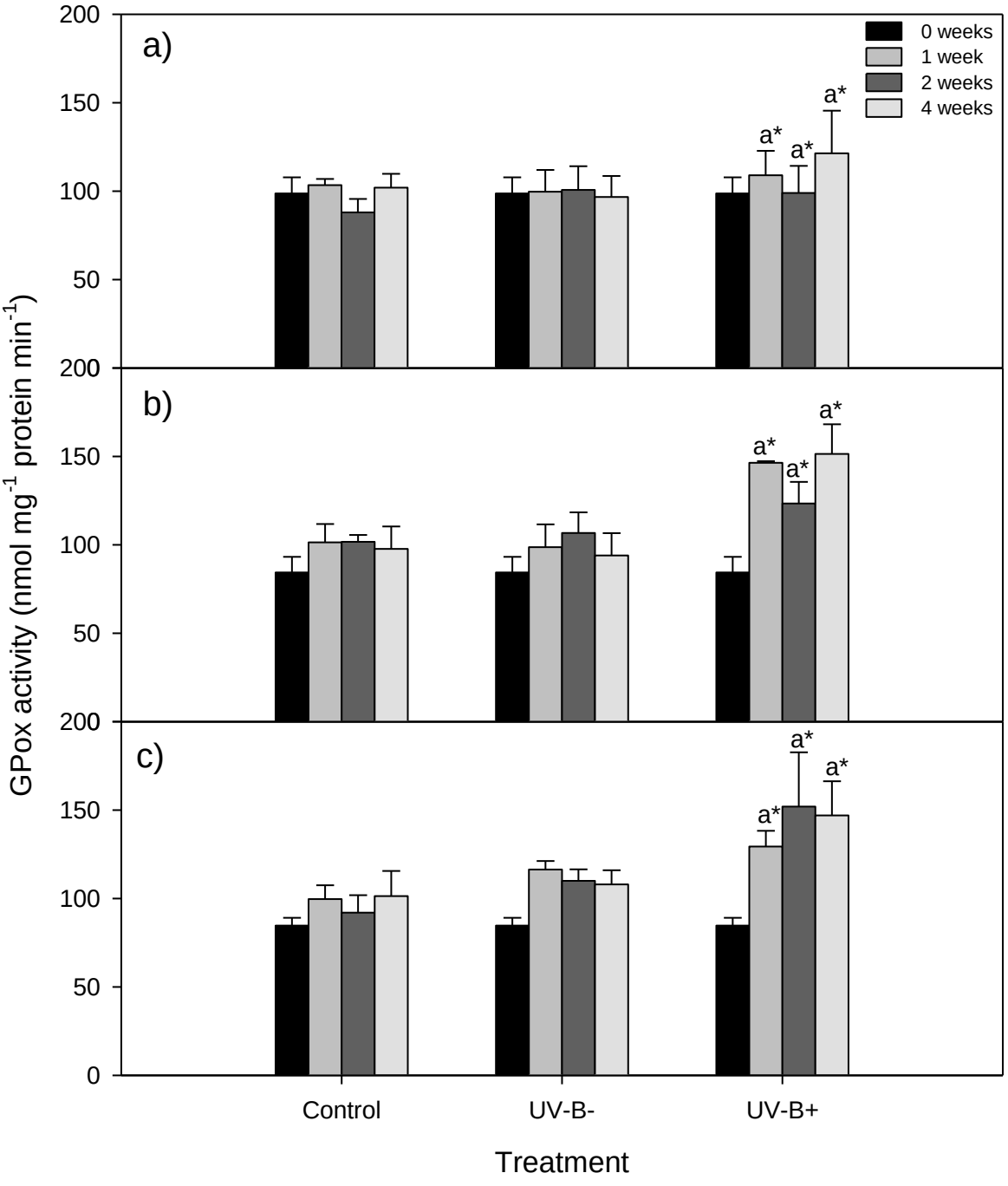

Figure 2.19 Mean GPox activities of a) 'Red Salad Bowl', b) 'Drunken Woman Fringed Head' and c) 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

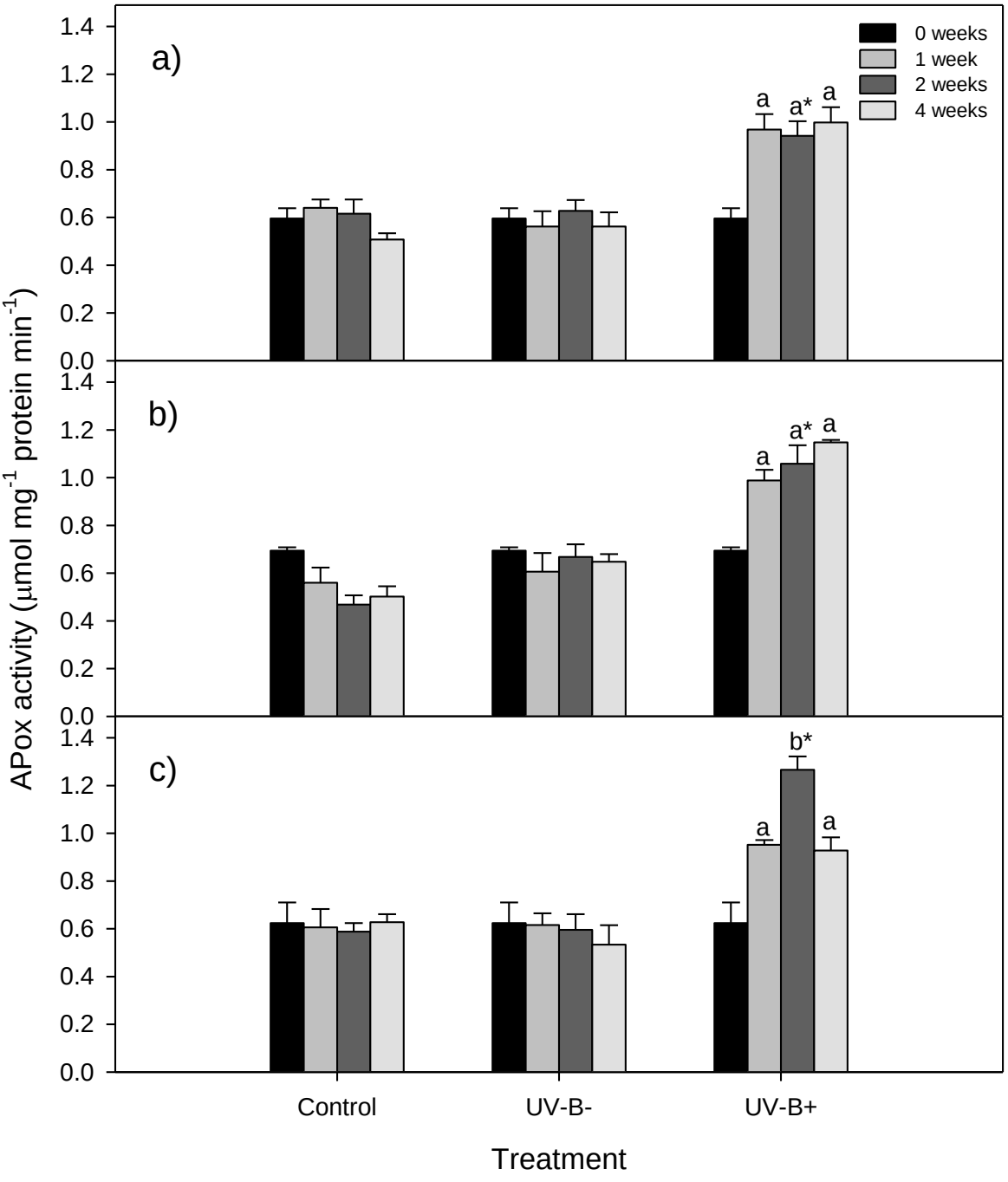

Figure 2.20 Mean APox activities of a) 'Red Salad Bowl' b) 'Drunken Woman Fringed Head' and c) 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

2.3.2.9 Ascorbate

Total ascorbate was affected by an interactive effect of UV-B treatment and cultivar (p = 0.024) and cultivar and time (p = 0.026). Samples at time = 0 did not differ significantly from controls. Total ascorbate was generally increased by in the UV-B+ treatment over controls (Dunnett's t, $p < 0.001$) and was increased at t = 2 and t = 4 (Turkey's HSD). The cultivars 'Royal Oak Leaf' and 'Drunken Women Fringed Head' displayed the highest levels of total ascorbate; although Tukey's HSD test could detect no significant difference between the cultivars (Fig. 2.21a, b, c).

The ratio between oxidized ascorbate (MDA+DHA) and total ascorbate was affected by a three-way interaction between UV-B treatment, time and cultivar (p = 0.038). Samples at time = 0 did not differ significantly from controls. The proportion of oxidized ascorbate appeared not to be elevated at in the cultivar 'Red Salad Bowl' (Fig. 2.22a), and was increased in UV-B+ treated plants of the cultivars 'Royal Oak Leaf' and 'Drunken Woman Fringed Head' at t = 1, but decreased to control levels by t = 2 (Fig. 2.22b, c).

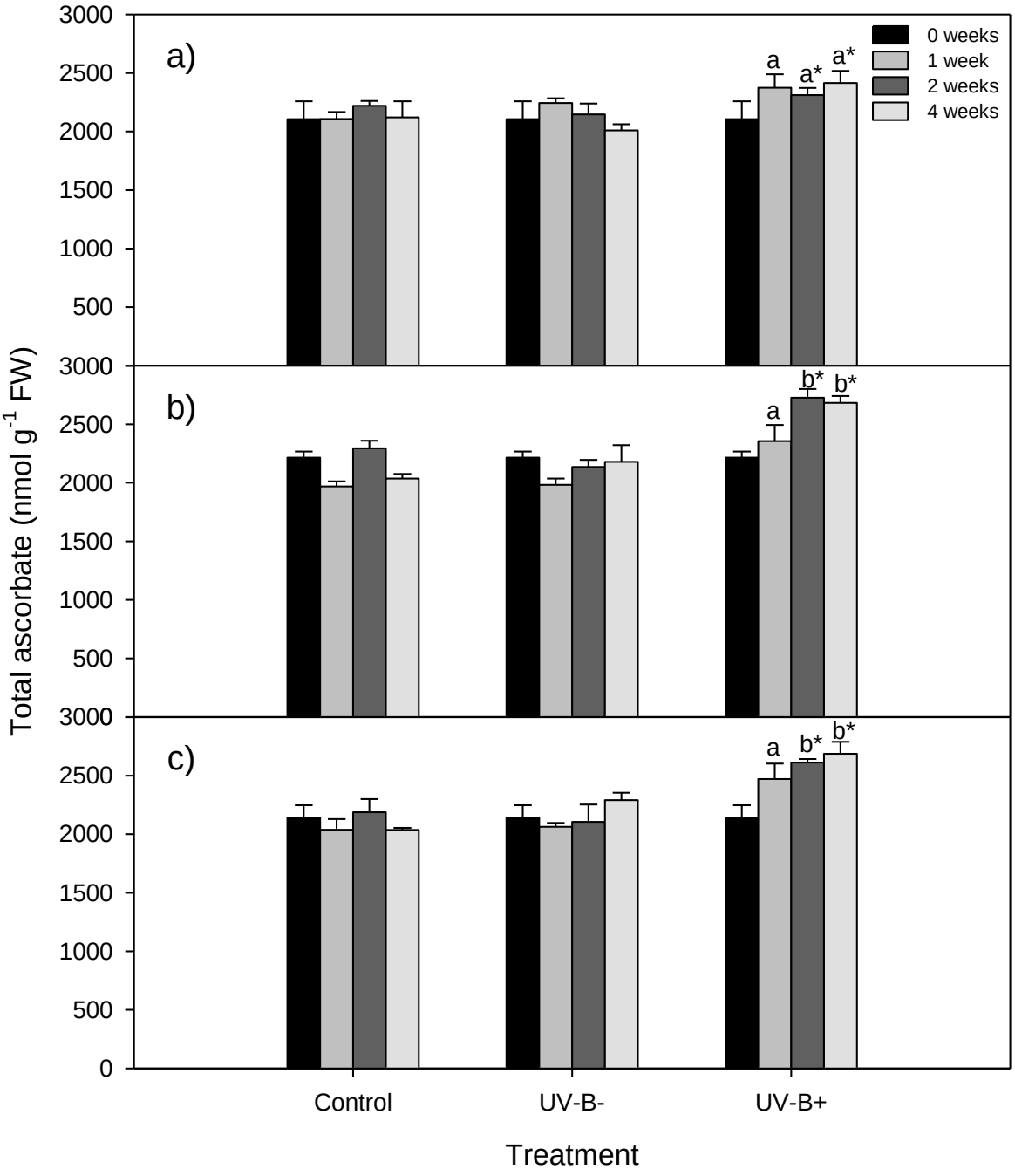

Figure 2.21 Total ascorbate of a) 'Red Salad Bowl, b) 'Drunken Woman Fringed Head' and c) 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

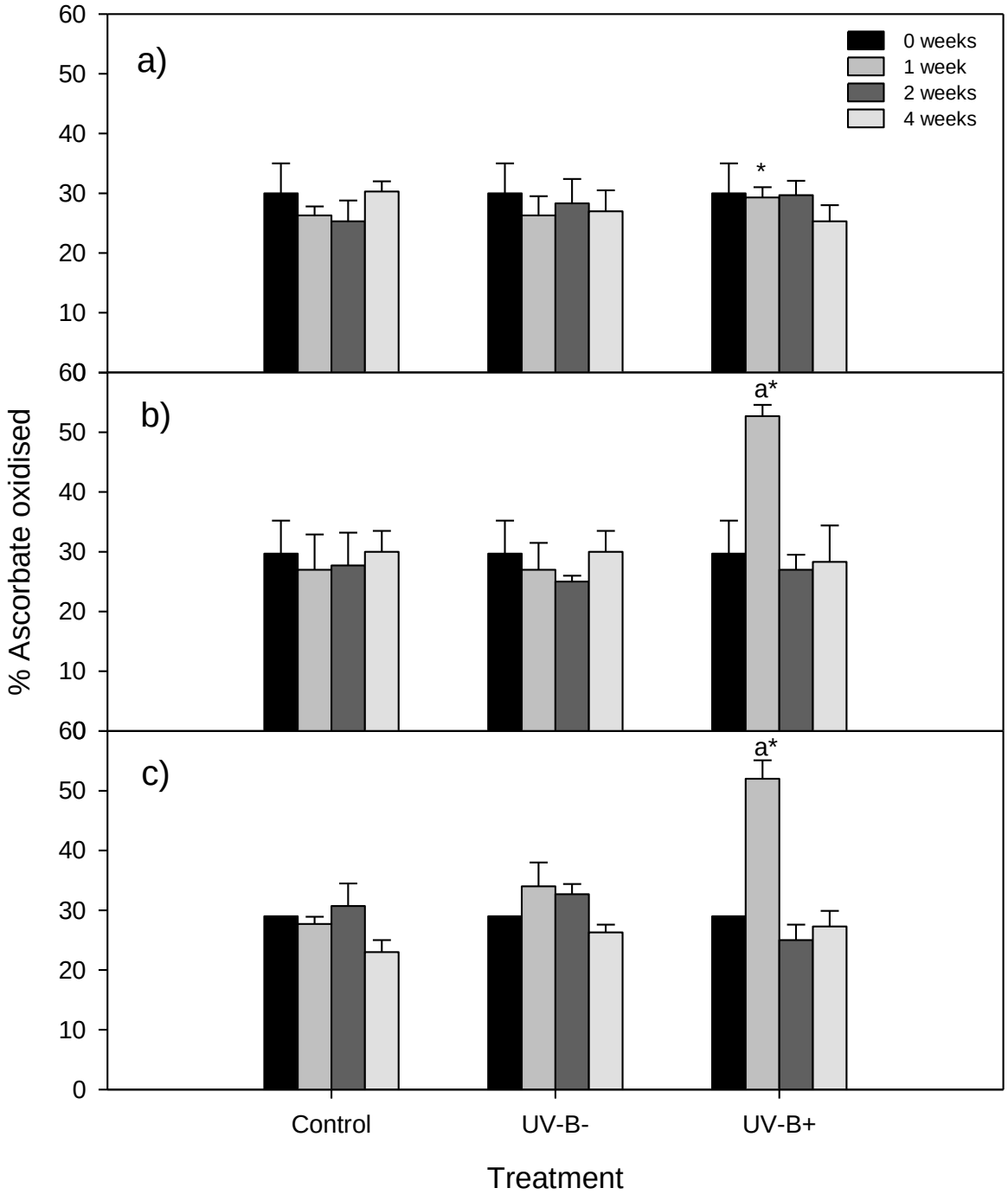

Figure 2.22 % Ascorbate oxidised in a) 'Red Salad Bowl,' b) 'Drunken Woman Fringed Head' and c) 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

2.3.2.10 *Glutathione*

Total glutathione was affected by significant interactions between variety and treatment ($p < 0.001$) and treatment and time ($p = 0.017$). UV-B treated samples at time = 0 did not differ significantly from controls. UV-B+ treated plants of all varieties displayed elevated levels of glutathione compared to controls (Dunnett's *t* test, $p < 0.001$), but the increase appeared less

in the variety ‘Red Salad Bowl’ than in the other varieties (Fig. 2.23a). Total glutathione appeared to increase with time in UV-B+ treated plants, although this could not be confirmed by Turkey’s HSD test (Fig 2.23b, c).

The ratio of oxidized to total glutathione was affected by a three-way interaction between the effects of cultivar, harvest time and treatment ($p = 0.248$). UV-B treated samples at time = 0 did not differ from controls. Like the oxidation state of the ascorbate pool, oxidation of glutathione occurred in UV-B+ treated plants but was less in the variety ‘Red Salad Bowl’ than other cultivars (Fig. 2.24a). In the cultivars ‘Royal Oak Leaf’ and ‘Drunken Woman Fringed Head’ oxidation of glutathione was extensive at t = 1, but fell to levels comparable to the controls by t = 2, remaining at low levels thereafter (Fig. 2.24b, c).

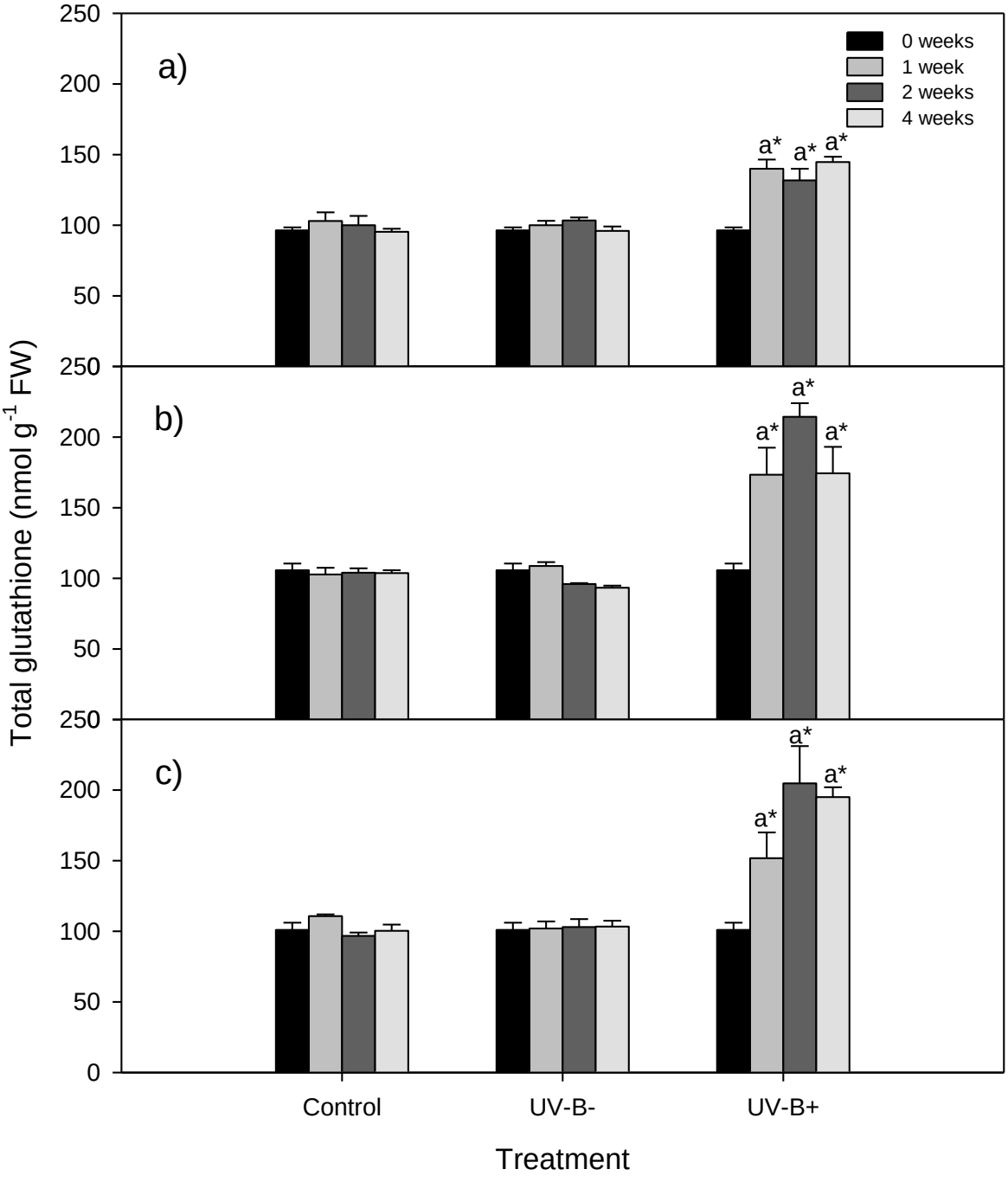

Figure 2.23 Total glutathione of a) 'Red Salad Bowl', b) 'Drunken Woman Fringed Head and c) 'Royal Oak Leaf' at each harvest. Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

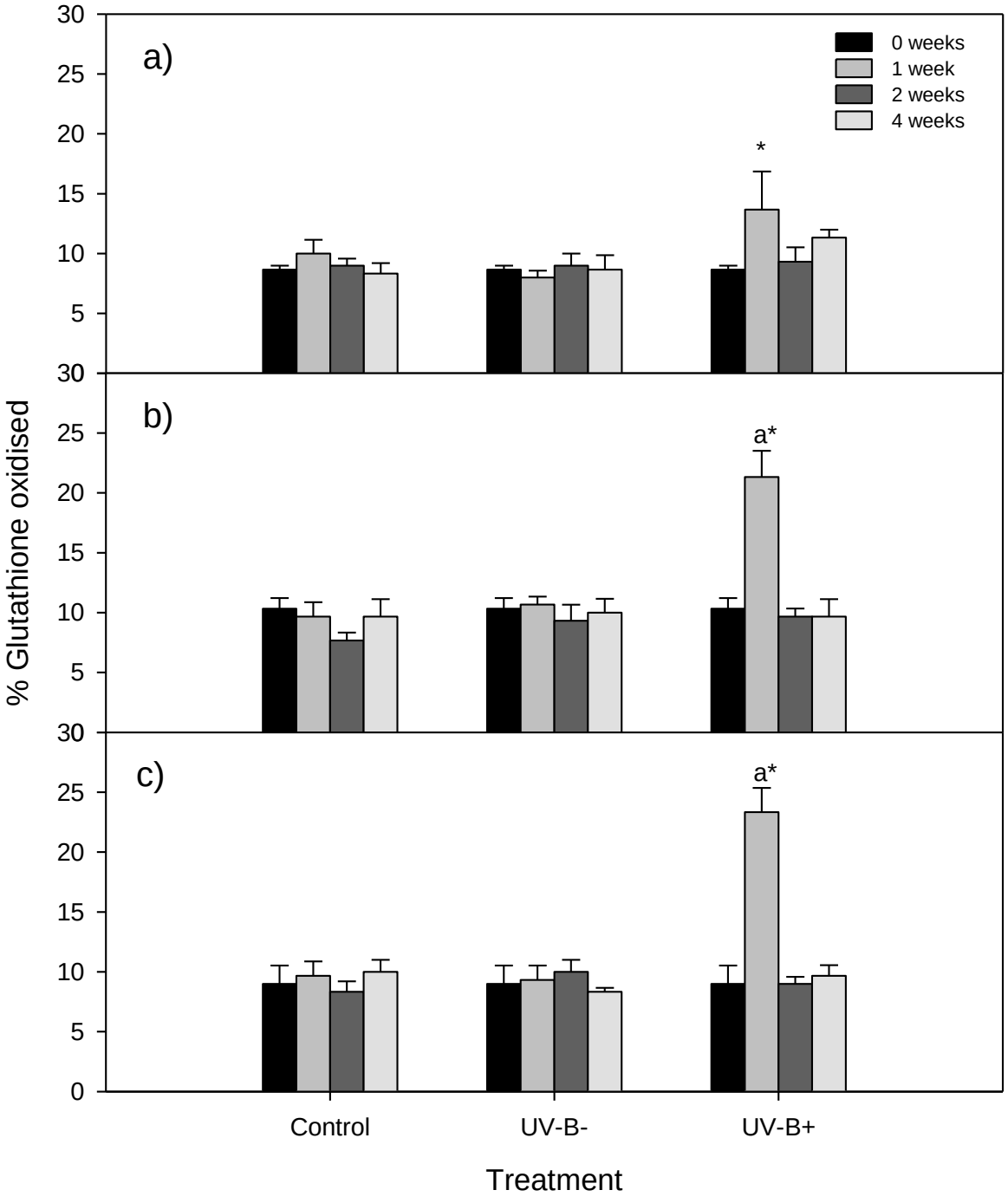

Figure 2.24 The % of glutathione oxidised in a) 'Red Salad Bowl', b) 'Drunken Woman Fringed Head and c) 'Royal Oak Leaf at each harvest (0, 1, 2 and 4 weeks). Bars with different letters are significantly different from controls and those marked with '*' differ between varieties (n = 3, α = 0.05). Error bars are ± 1 standard error.

2.4 Discussion

Plants grown in the absence of UV-B radiation are often larger, have higher levels of photosynthetic pigments and display greater phytochemical efficiency than those exposed to UV-B (García-Macias *et al.*, 2007; Krizek *et al.*, 1998). This was not generally the case in the

current study, where plant growth parameters were usually similar between UV-B+ and UV-B- treatments, and pigment levels were depressed in covered plants. Reduced growth in UV-B exposed plants is often attributed to photoinhibition occurring during UV-B stress as damage to the plastiquinone electron accepters and the D1 protein in PSII lowers the quantum yield of photosynthesis thereby reducing resources available for growth (Gao and Ma, 2008; Lesser, 2008). However, there was no evidence of photoinhibition in the current experiment, suggesting that lettuce plants were able to sufficiently acclimate to UV-B. An alternative hypothesis holds that reduced growth under UV-B is the result of resources being diverted away from growth and towards defense responses, including the production of protective pigments. Protective pigments such as anthocyanins may limit growth by absorbing light required for photosynthesis (García-Macias *et al.*, 2007). The lower growth of the anthocyanin-rich cultivar 'Red Salad Bowl' in the current experiment lends some support to this hypothesis although reductions in the growth of 'Red Salad Bowl' occurred in both UV-B+ and UV-B– treatments and anthocyanins were not significantly up-regulated by UV-B, suggesting that other factors (possibly the overall light levels, or exposure to UV-A) also played a role in limiting the growth of 'Red Salad Bowl'.

Plants grown under the UV-B transmitting cellulose diacetate film displayed significantly more oxidative damage (as indicated by protein carbonyl and lipid peroxide levels) than untreated controls or plants growing under the UV-B blocking Mylar-D® film. Protein carbonyl and lipid peroxide levels peaked one week after treatment began before decreasing to a steady level. Increased oxidative damage is frequently reported in studies where plants are exposed to UV-B, although full recovery from oxidative stress is uncommon (Dai *et al.*, 1997; Sharma *et al.*, 1998; Singh *et al.*, 2010; Xu *et al.*, 2008). The cultivar 'Red Salad Bowl' experienced lower levels of oxidative damage than the others studied and was able to fully recover within two weeks after treatment began. Red lettuces are frequently reported as having higher concentrations of UV-B absorbing compounds and a greater antioxidant capacity than green cultivars (Liu *et al.*, 2007; Tomas-Barberan and Espin, 2001; Tsormpatsidis *et al.*, 2008), and it is possible that the reduced oxidative damage displayed by the cultivar 'Red Salad Bowl' was a result of internal shading from anthocyanins and other phenolics that reduced oxygen radical generation in the electron transport chain of PSII, a major source of ROS during UV-B stress (Tsormpatsidis *et al.*, 2010).

The activities of all antioxidant enzymes assayed were increased in UV-B exposed plants indicating a significant effect of UV-B on the functioning of the antioxidant system. Enzyme activities were generally higher in the outer-whorl leaves than in the inner-whorl leaves, and

increases in SOD and APox activity bought about in UV-B exposed plants was significantly greater in outer-whorl leaves than inner-whorl leaves. Increases in antioxidant enzyme activity have been previously recorded in response to high light. Other metabolites (flavonoids, ascorbate) functioning in the UV-B response are also recorded to be increased in the outer leaves of lettuce heads, so it seems likely that the lowered induction of stress-related metabolites in inner-whorl leaves compared with outer leaves is a result of their being shielded from direct exposure to UV-B (García-Macias *et al.*, 2007; Hohl *et al.*, 2001; Mou and Ryder, 2004; Zhou *et al.*, 2009). Nonetheless, the induction of most antioxidant compounds in the current study was less in the outer-whorl leaves of the red-coloured cultivar 'Red Salad Bowl' than in the green cultivar 'Buttercrunch'. The same was not generally true of leaves from the inner-whorl which was not so visibly red in colour, suggesting that the accumulation of UV-B protectant pigments may interfere with antioxidant metabolism. A study by Xu *et al.* (2008) of a flavonoid-rich soybean genotype similarly showed less activity of antioxidant enzymes after UV-B irradaiation than occurred in a flavonoid deficient mutant. As the activity of many antioxidant enzymes has frequently been considered to be up-regulated by the presence of ROS (Dawar *et al.*, 1998; Garg and Manchanda, 2009; Morita *et al.*, 1999), the prevention of ROS generation afforded by UV-B screening compounds could have an indirect effect on the induction of antioxidant enzyme activities.

Particularly important in the response to UV-B irradiation is the up-regulation of antioxidant enzyme activities by ROS in chloroplasts such as SOD, APox and GR (Foyer and Noctor, 2009; Garg and Manchanda, 2009; Mittler, 2002). In addition to oxidatively damaging cellular components, ROS participate in a complex web of signalling pathways and a delicate balance must be maintained in the cell between their production and their removal. Up-regulation of antioxidant enzymes is thought to originate from signals generated by ROS themselves, and the exact site and or ROS production and the species produced are important in determining the compartmentalization and kinetic patterns of enzyme up-regulation. Patterns of compartmentalization and the kinetics of enzyme up-regulation may differ between oxidative stressors of different origins (Foyer and Noctor, 2009). The primary ROS generated in response to UV-B are $O_2\cdot^-$ and H_2O_2, probably in the photosystems and thylakoids (Dawar *et al.*, 1998; Mackerness *et al.*, 2001). Increased generation of $O_2\cdot^-$ is involved in the up-regulation of SOD activity. The resulting increase in levels of H_2O_2 from the dismutation of $O_2\cdot^-$ and increased oxidative stress also contributes to the further up-regulation of the activities of other antioxidants including CAT and APox (Noctor and Foyer, 1998). APox acts to removed excess H_2O_2 using ascorbic acid (AsA) as a substrate, after

which AsA must be regenerated via the ascorbate-glutathione cycle (Mittler, 2002; Noctor and Foyer, 1998). The action of APox is a possible factor driving the oxidation of ascorbate and glutathione pools that occurs at certain points in the response to UV-B radiation.

Time-dependent increases the activity of antioxidant were discovered after the start of UV-B treatment in plants growing under the UV-B transparent cellulose diacetate film. The most common trend amongst antioxidant enzymes was one of continuous increase in activity as stress progressed, although the activity of some enzymes (SOD in all cultivars and APox in the cultivar 'Royal Oak Leaf') showed a pattern of peaking two weeks after exposure before declining again. Increases in antioxidant enzyme activities have been previously reported in many plant species including barley, *Arabidopsis*, rice, wheat and soybean. While few studies have elucidated the responses of the enzymatic antioxidant system over time, some shorter-term studies have yielded similar results to the present study (Fedina *et al.*, 2010). In Kubo *et al.*'s (1999) study of abiotic stress in *Arabidopsis*, SOD, APox and GR activities were extensively increased by UV-B after 7 days treatment, but CAT activity was increased less. Similar patterns were also reported by Dai *et al.* (1997) in a study of rice cultivars. SOD activity peaked 7 days after UV-B exposure was begun, before a peak in CAT activity 14 days after exposure. Dawar *et al.* (1998) suggested that rapid increases in antioxidant enzyme activities are a first response to oxidative stress initiated before other measures such as the synthesis of UV-B screening compounds are able to be synthesised. Other studies have reported mixed results: no effect of SOD on UV-B activities (Mazza *et al.*, 1999), or increases in SOD activity combined with a decrease in the activities of other antioxidant enzymes – most frequently CAT and APox – have been reported (Agrawal and Rathore, 2007; Jovanovic *et al.*, 2006).

Previous studies of the UV-B response in plants have highlighted the importance of redox-regulated systems and other elements that may also play a role in determining the up-regulation of antioxidant defences. A study by Fang *et al.* (2009) showed the up-regulation of a number of transcripts alongside those encoding thylakoid-bound APox, such as genes coding for the universal stress protein and transcripts encoding proteins involved in signal transduction, including protein kinase domain containing proteins. MAP-kinase cascades may be activated by H_2O_2 and could play an important role in activating the upregulation of antioxidant genes (Foyer and Noctor, 2009). The promoter regions of genes involved in the synthesis of antioxidant enzymes are known to contain sites specific to MAP-kinases and other redox-sensitive regulators (Lushchak, 2011). Recent work also points to the perception of UV-B through monomerization of the UV RESISTANCE LOCUS 8 (UVR8) protein,

which interacts with ubiquintin ligases involved in light signalling and the regulation of the majority of genes involved in the UV-B response, including those for the synthesis of flavonoids (Oravecz *et al.*, 2006; Rizzini *et al.*, 2011). However, the relationship between UV-B perception and the activation of the various antioxidant defence pathways is as yet unclear (Ulm and Nagy, 2005).

The levels of the non-enzymatic antioxidants ascorbate and glutathione in the current experiment were elevated in UV-B exposed plants growing under the cellulose diacetate film, oxidation of both ascorbate and glutathione peaking one week after UV-B treatment began in the cultivars 'Royal Oak Leaf' and 'Drunken Woman Fringed Head' before declining to control levels. Accumulation of ascorbate is an important strategy in defence against UV-B induced oxidative stress and has been previously reported in a diverse range of plants, including soybean, wheat, barley and mung bean (Agrawal and Rathore, 2007; Mazza *et al.*, 1999; Sharma *et al.*, 1998; Xu *et al.*, 2008). Ascorbate is considered the primary antioxidant in plant tissues and is responsible for the longer-term maintenance of redox equilibrium in plant tissues (Foyer and Noctor, 2009). Through regulating the action of phytohormones, including IAA, ascorbate also plays an important role in plant developmental processes. The oxidation state of ascorbate is particularly critical to its role as a signalling molecule (Lee *et al.*, 2011; Tyburski *et al.*, 2008). The ability to maintain the ascorbic acid pool in an appropriate redox state is an important quality in enabling plants to tolerate UV-B and other oxidative stressors. Alterations to the oxidation state of the oxidation state of the ascorbate pool could interfere with plant growth and development, and the ability to recover the oxidation state may play an important role in enabling growth to continue normally under UV-B stress. The regeneration of oxidized ascorbate is achieved through the action of the enzymes MDHAR and DHAR (see section 1.2.2.1) and glutathione is regenerated through the enzyme GR (section 1.2.1.5). Effectively up-regulating these enzymes is a key factor in conferring tolerance to UV-B (Selvakumar, 2008; Singh *et al.*, 2006).

Glutathione accumulation was increased in UV-B exposed plants growing under the cellulose diacetate film, especially in the cultivars 'Royal Oak Leaf' and 'Drunken Woman Fringed Head', and reached its highest levels two weeks after UV-B treatment was begun. Like ascorbate, glutathione has diverse roles in plant systems in addition to its function as an antioxidant (Gill and Tuteja, 2010). Through conversion to GSSG, glutathione is able to participate in signalling via thiol-disulfide exchange and changes in glutathione levels are involved in a variety of physiological events including germination, cell wall development, bolting and seed set (Foyer and Noctor, 2009). Glutathione is oxidised through its own action

as an antioxidant, in the process of AsA regeneration and directly by the ROS-scavenging enzyme GPox (Garg and Manchanda, 2009; Mittler, 2002). Oxidised glutathione is involved in the signalling of higher ROS levels and inducing the relevant antioxidant defences (Foyer and Noctor, 2009; Gill and Tuteja, 2010). The reduction of GSH to GSSG and its loss of function as an antioxidant serve to amplify signals leading to the up-regulation of antioxidant enzymes, reducing oxidative pressure and helping to maintain the balance of pro- and antioxidants in the cell. Synthesis of GSH is also known to increase in response to oxidative stress (Noctor and Foyer, 1998). In the current experiment, increased oxidation of glutathione was evident in all cultivars one week after the commencement of UV-B treatment after which the oxidation state of the glutathione pool recovered. Increases in the overall glutathione level coincided with the most intense period of oxidative damage. Synthesis of additional GSH provides a substrate for the action of the enzyme GPox and for the regeneration of AsA and the greater oxidative stress in the green cultivars 'Royal Oak Leaf' and 'Drunken Woman Fringed Head' may have necessitated the up-regulation of GSH synthesis in order to aid the action and regeneration of other parts of the antioxidant system. Lower oxidative stress in the cultivar 'Red Salad Bowl' due to the presence of protective pigments such as anthocyanins resulted in less glutathione oxidation in that cultivar, contributing to the lower up-regulation of antioxidant enzymes and reduced synthesis of glutathione.

In the current study, the redox status of ascorbate and glutathione had been restored to pre-stress levels within two weeks after the start of UV-B exposure, by which time both most antioxidants had reached levels close to their maximum resulting in a mature product that is likely to be more nutritionally valuable to human consumers. At earlier stages in the stress response, lower levels of antioxidants correlated with higher levels of potentially harmful compounds. High levels of ROS (indicated in the current study by the presence of protein carbonyls and lipid hydroperoxides) can lower the nutritional value of foodstuffs through the destruction of nutrients and promote the formation of undesirable compounds including carcinogens (Cheung *et al.*, 2007; Choe and Min, 2006). The action of antioxidant compounds in human metabolism is also an important factor to take into account when considering their potential protective effects. As DHA is unable to act as an antioxidant, ascorbate must be supplied in its reduced form as AsA if it is to be effective in human metabolism (Nualart *et al.*, 2003). In the current study the high level of oxidation of the ascorbate pool after one week of UV-B exposure indicates that the nutritional value of vegetable crops may be limited for a time after exposure to a stressor. This may particularly be a factor in the production of 'baby' vegetables, which are harvested before maturity and

potentially before the crop has fully acclimated to stressors in its growth environment. The need for acclimation, however, is not universal to all crops. The cultivar 'Red Salad Bowl' contained high levels of UV-B protectant pigments that appeared to alleviate oxidative damage associated with UV-B. From a nutritional standpoint, this cultivar displays fewer of the disadvantages associated with stress acclimation and so may be more suitable than other cultivars for cultivation as a baby lettuce variety or in conditions where there may be sudden fluctuations in the environment.

In summary, plants exposed to UV-B radiation the under the cellulose diacetate film experienced a short but period of intense oxidative damage and the oxidation of ascorbate and glutathione that prompted the up-regulation of enzymatic and non-enzymatic antioxidant defences. The up-regulation of antioxidant activities continued after all or most oxidative damage was repaired after about two weeks of UV-B exposure, including the synthesis of additional ascorbate and glutathione as exposure continued plants acclimatised to UV-B exposure. Oxidative stress and the up-regulation of antioxidant systems varied between cultivars that had different levels of anthocyanins, pointing to the important role of UV-B protectant compounds in reducing oxidative damage and mediating aspects of the stress response.

3 Polyamines

3.1 Introduction

As detailed in section 1.3, polyamines are small aliphatic amines that occur in all living tissues and are involved in a wide variety of metabolic processes, including all steps of DNA, RNA and protein synthesis, cell division and growth and various stress responses (Bouchereau *et al.*, 1999; Tassoni *et al.*, 2004). The role of polyamines and the diamine putrescine as promoters of cell proliferation has led in recent years to an examination of their role in abnormal cell growth and cancer pathologies, and consideration has been made as to the role that dietary polyamines play in disease epidemiology (Larque *et al.*, 2007). However, their roles in cell division and differentiation means that polyamines are also vital for the good health of tissues with rapid cell turnover such as the gastrointestinal tract, and they are also important regulators of inflammation and may aid in preventing neurological disorders (Hunter and Burritt, 2011; Majumdar, 2003; Soda *et al.*, 2005). Consideration here has been given to vegetable foodstuffs, which as sources of antioxidants may have health-promoting properties but which may contain relatively low concentrations of polyamines.

Polyamine concentrations in plant foodstuffs however, vary in accordance with a number of biological factors that affect polyamine biosynthesis and metabolism. The polyamine content of plant tissue varies between tissue types and developmental stage. Higher polyamine concentrations were recorded in the roots of apple trees than in leaves (Gao *et al.*, 2009), and the polyamine contents of young leaves being higher than that of fully expanded leaves. In fruits, polyamine levels tend to peak during the early stages of development, coinciding with the period of maximum cell division and decreasing as the fruit becomes fully ripe (Gomez-Jimenez *et al.*, 2010; Tassoni *et al.*, 2004). The proportion of various polyamine titers (free, conjugated and bound polyamines) may also be affected, bound and conjugated forms sometimes being recorded as being dominant at fruit set, the level of free polyamines increasing as the fruit develops (Colin *et al.*, 2002). In leaves, polyamine levels tend to be higher in younger, expanding leaves than in older fully developed ones (Fujihara and Yoneyama, 2001). The potential contribution that leafy vegetables such as lettuce make to the dietary intake of polyamines may thus vary on depending factors such as the maturity of the plant at harvest and the leaf tissues consumed.

Plant growth conditions also affect the polyamine content of its tissues. Polyamines play a role in several plant stress responses (Bouchereau *et al*. 1999). Studies by a number of authors have recorded increases in polyamine levels under stress conditions although the

increase is not always maintained as the duration of stress increases (Botella *et al.*, 2000; Liu *et al.*, 2007; Santa-Cruz *et al.*, 1997; Tonon *et al.*, 2004). Furthermore, the levels of the various polyamine titers (acid-soluble free polyamines, acid-soluble polyamines conjugated to small molecules and acid-insoluble polyamines bound to macromolecules) are known to be altered under stress conditions. Roussos and Pontikis (2007), for example, recorded that insoluble-bound forms of putrescine increased in concentration under salt stress. Longer length polyamines (Spd and Spm) are often recorded to be accumulated in response to stress, and bound and conjugated polyamines may increase in concentration, stabilizing cell membrane systems and increasing the effectiveness of antioxidant enzymes (Bouchereau *et al.*, 1999; Xu *et al.*, 2011). The two routes of polyamine biosynthesis in plants, via ADC and ODC, are differently sensitive to abiotic stressors. Increased ADC transcript levels being reported under diverse stresses such as salinity, drought and heavy metal toxicity indicates that the ADC route may be the more sensitive of the two pathways. (Groppa and Benavides, 2008; Liu *et al.*, 2006; Liu *et al.*, 2011; Urano *et al.*, 2004). SAMDC activities and transcripts have also been reported to be up-regulated under stress, perhaps indicating greater investment in production of Spd and Spm as membrane-stabilizing metabolites.

UV-B radiation is an abiotic stressor that may cause damage to the D1 and D2 proteins of PSII, damage DNA and generate ROS (Frohnmeyer and Staiger, 2003; Gill and Tuteja, 2010; Jansen *et al.*, 1998). Exposure to UV-B radiation has been reported to have various effects on polyamine accumulation, increasing putrescine and spermidine levels in some instances and decreasing putrescine levels in others (see section 1.3.2.1; (Radyukina *et al.*, 2010; Rakitin *et al.*, 2008a; Rakitin *et al.*, 2009; Smith *et al.*, 2001). The aim of the current experiments was to further investigate the effects of UV-B exposure on the polyamine metabolism of the commonly consumed vegetable *Lactuca sativa* (L.), through growth under UV-B blocking and UV-B transparent plastic coverings. In particular, the polyamine levels of inner-whorl leaves were compared with those of fully-expanded outer-whorl leaves. The kinetics of polyamine accumulation and the enzymes involved in polyamine biosynthesis were also examined during a period of UV-B exposure.

3.2 Methods

3.2.1 Plant material

Lettuce seeds of various cultivars were germinated and grown as detailed in sections 2.2.1 and 2.2.2. Inner and outer leaves were harvested from the end-point experiment as detailed in section 2.2.1 and outer leaves harvested from the time course experiment as detailed in

section 2.2.2. Harvested material was frozen in liquid nitrogen and stored at -70 °C prior to analysis.

3.2.2 Polyamine extraction

Polyamines were extracted using the method of Smith *et al.* (2001) with modifications. Frozen leaf tissue was ground to a fine powder using a pre-chilled mortar and pestle. 50 µg of ground tissue was then homogenised with 1 ml 5 % (w/v) trichloroacetic acid (TCA) and homogenates maintained at 4 °C for one hour. Extracts were then centrifuged at 20,800 x *g* at 4 °C for 20 minutes and supernatants removed for determination of free and TCA-soluble conjugated polyamines. Pellets of cell debris were then washed three times in 1 ml 5 % TCA, and re-suspended in 0.3 ml 5 % TCA for analysis of TCA-insoluble bound polyamines. Pellet suspensions and 0.2 ml aliquots of the original supernatants were then hydrolysed overnight with 0.3 ml 12 mol hydrochloric acid at 100 °C. Hydrolysates were then centrifuged at 20,800 x *g* at 4 °C for 20 minutes and 0.1 ml aliquots of the resulting supernatants dried *in vacuo* before being re-dissolved in 0.1 ml 5 % TCA.

3.2.3 Polyamine dansylation and HPLC analysis

Extracts were dansylated as per the method described by Smith *et al.* (2001) with modifications. A 0.1 ml aliquot of each extract was taken and added to 0.1 ml of saturated sodium carbonate and then 0.2 ml of dansyl chloride in acetone (7.5 g^{-1} ml) was added and the mixture incubated in the dark for 20 minutes at 60 °C. Excess dansyl chloride was eliminated by adding 0.1 ml proline (0.1 g^{-1} ml) and incubating the mixture in the dark for 15 minutes at room temperature. Dansylated polyamines were then extracted by adding 0.25 ml benzene, mixing for one minute and then centrifuging the mixture for one minute at room temperature at 25,000 x *g*. The benzene phase was removed and stored at -80 °C prior to analysis.

Polyamines were separated and quantified using reverse-phase HPLC using a PerkinElmer liquid chromatography system and fluorescence detector as per the methods of Marce *et al.* (1995) and Burritt (2008). Extracts were eluted at 25 °C through a 4.6 x 250 mm, 5 µm particle size reverse-phase (C_{18}) column (JASCO International Co., Ltd.). The solvent system was made of the following gradients: 68 % acetonitrile for 4 minutes, followed by a linear gradient to 100% acetonitrile over 1 minute, then 100 % acetonitrile for 5 minutes, and finally a linear gradient to 68 % acetonitrile over 1 minute. The rate of flow was a constant 1.5 mL min^{-1}. Data were analysed using a Delta chromatographic system (SGE Analytical Science Pty Ltd. Victoria, Australia). Dansylated standards of 50 nmol Put HCl, Spd HCl and Spm HCl were used, and hexanediamine was used as an internal standard.

3.2.4 Extraction and assay of enzymes involved in PA biosynthesis

3.2.4.1 ADC and ODC extraction

For enzymes involved in polyamine biosynthesis, the method of Biondi *et al.* (2001) was used, with modifications. Frozen leaf tissue was ground to a fine powder using a pre-chilled mortar and pestle. For ADC and ODC, 100 mg of frozen tissue was homogenized in 500 µl of ice-cold extraction buffer (100 mmol tris-HCl buffer, pH 8.5) containing 5 µmol pyridoxal-5-phosphate, 50 µmol EDTA and 500 µmol phenylmethylsulphonyl fluoride). Homogenates were then centrifuged at 20,800 x *g* at 4 °C for 20 minutes, and the supernatant removed for later analysis of ADC or ODC content. The pallet of cell debris was washed in 500 µl of extraction buffer and then re-suspended in 500 µl of extraction buffer. Supernatant and pallet suspensions were stored in 200 µl aliquots at -80 °C prior to analysis.

3.2.4.2 SAMDC extraction

For SAMDC, frozen leaf tissue was ground to a fine powder using a pre-chilled mortar and pestle and 100 mg of frozen tissue was homogenized in 500 µl of extraction buffer containing 100 mmol tris-HCl (pH 7.6), 25 µmol pyridoxal phosphate, 50 µmol EDTA and 500 µM phenylmethysulphonyl fluoride. Homogenates were centrifuged at 20,800 x *g* and the supernatant collected and stored in 200 µl aliquots at -80 °C prior to analysis.

3.2.4.3 Assay of enzymes involved in PA biosynthesis

ADC, ODC and SAMDC were determined by measuring the rate of $^{14}CO_2$ evolution from radioactively labeled arginine, ornithine and S-adenosyl-methionine, respectively, using the methods of Tiburcio *et al.* (1993) with modifications (Burritt, 2008). Reactions were carried out in sealed scintillation vials fitted with gas-tight caps. Each vial contained a filter paper disk 1.5 cm in diameter, transfixed to a steel pin and soaked in 2 M KOH to trap the $^{14}CO_2$ liberated during the reaction. For ADC, the reaction mixture contained 200 µl of enzyme extract, 2.98 kBq L-(U-^{14}C)-arginine and L-arginine to give a final concentration of 10 mmol Arg in a final reaction volume of 400 µl. The reaction mixture was contained within an Eppendorf tube that had the lid removed. Assays were run for 60 minutes at 30 °C, after which the reaction was terminated by injection of 0.2 ml 10% TCA into reaction vials. After 60 minutes further incubation, the reaction mixture was removed and 5 ml of scintillation cocktail added (Flo-Scint V, Packard Radiometric, Packard Instruments B.V. Chemical Operations, Gronignen, the Netherlands). Radioactivity was determined using a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland). Assays for ODC and SAMDC followed the same method, excepting the reaction mixture which for ODC contained 200 µl of enzyme extract, 8.07 kBq DL-(1-^{14}C) ornithine and L-ornithine to give a final concentration of 4

mmol ornithine in a final reaction volume of 400 µl; and for SAMDC contained 200 µl enzyme extract, 1.055 kBq adenosyl-methionine S-(carboxyl-^{14}C) and S-adenosyl-methionine to give a final concentration of 0.2 mmol S-adenosyl-methionine in a final reaction volume of 400 µl. Boiled enzyme preparations were used as controls and enzyme activity expressed as µmol $^{14}CO_2$ liberated mg^{-1} protein h^{-1}.

3.2.5 Statistical analysis

Statistical analysis was performed using SPSS 19 for Windows. The sphericity assumption was frequently violated, so multivariate analysis of variance (MANOVAs) was used to analyze data rather than repeated measures ANOVAs. In the time course experiments, separate MANOVAs were performed to detect any difference between samples at time = 0 and controls (untreated samples at t = 1, 2 and 4), and to detect differences between controls and treated (UV-B+ or UV-B–) samples. MANOVA analysis was followed up where applicable by univariate ANOVAs for each dependent variable, which were used alongside Dunnet's t tests to distinguish differences between controls and treatments, and Tukey's HSD tests to distinguish differences between time points and between cultivars.

3.3 Results

3.3.1 Total Polyamines

MANOVA performed on the data for total putrescine, spermidine and spermine in inner and outer leaves showed polyamine levels to be affected by a significant interaction between the effects of UV-B treatment and cultivar ($p < 0.001$). Univariate ANOVAs gave an insight to the dynamics of polyamine accumulation in inner and outer leaves. For inner leaves, UV-B+ treatment increased polyamine accumulation in both cultivars equally ($p < 0.001$ in all cases, Fig. 3.1a) whereas in outer leaves an interaction between treatment and cultivar resulted in a greater increase in polyamine accumulation under UV-B+ treatment in the cultivar 'Buttercrunch' than 'Red Salad Bowl' ($p = 0.002$, 0.000 and 0.003 for Put, Spd and Spm respectively; Fig 3.1b).

MANOVAs in the time course experiment showed total polyamines at time = 0 not to be significantly different from controls. They indicated that total polyamine levels were affected by a significant three-way interaction between treatment, cultivar and time ($p = 0.023$). Univariate ANOVAs showed putrescine levels to be affected by an interactive effect of treatment and variety ($p = 0.007$), putrescine levels in UV-B+ treated plants of 'Royal Oak Leaf' and 'Drunken Woman Fringed Head' appearing higher than in 'Red Salad Bowl', although Tukey's HSD test was unable distinguish between varieties (Figs. 3.2a-c). The level

of spermidine was dependent on a similar interactive effect of treatment and cultivar ($p < 0.001$), the cultivars 'Royal Oak Leaf' and 'Drunken Woman Fringed Head' accumulating higher levels of spermidine than 'Red Salad Bowl' (Figs. 3.2d-f). Spermine levels however, were dependent on a three-way interaction between treatment, cultivar and time ($p = 0.007$), being more rapidly elevated in 'Royal Oak Leaf' and 'Drunken Woman Fringed Head' than in 'Red Salad Bowl' (Figs. 3.2g-i).

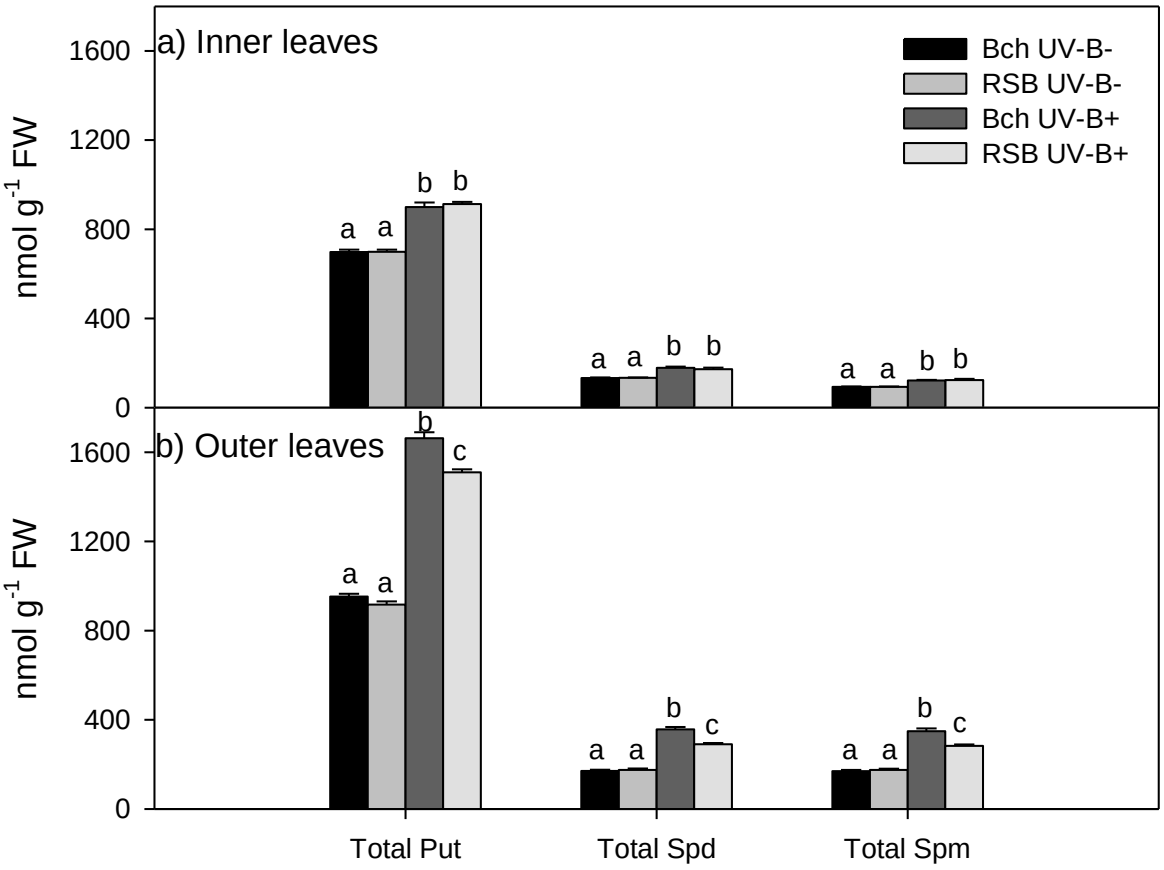

Figure 3.1 Mean total putrescine, spermidine and spermine in the a) inner and b) outer leaves of plants in the end-point experiment. Significantly different samples within polyamine types are indicated by different letters (Tukey's HSD, n = 12, α = 0.05). Error bars are one standard error.

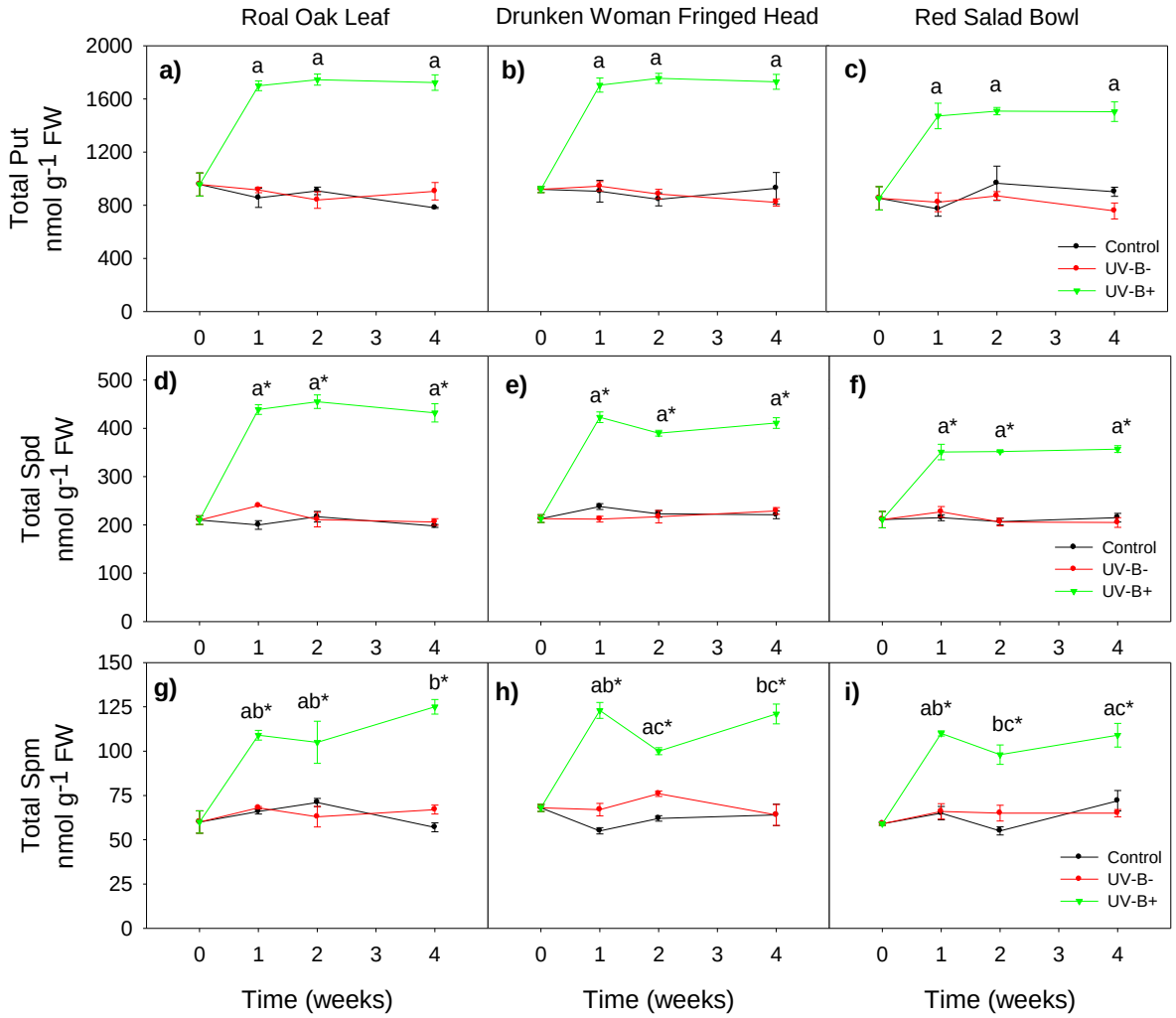

Figure 3.2 Total putrescine (a, b, c), spermidine (d, e, f) and spermine (g, h, i) by cultivar in the time-course experiment (a, d and g, Royal Oak Leaf; b, e, and h, Drunken Woman Fringed Head; c, f and i, Red Salad Bowl). Samples significantly different from untreated controls are indicated by different letters (Tukey's HSD, n = 3, α = 0.05). Error bars are one standard error.

3.3.2 Putrescine

In the end point experiment, MANOVA performed on the data for different forms of putrescine revealed significant effects of UV-B treatment ($p < 0.001$) and cultivar ($p < 0.001$). Univariate ANOVAs showed that UV-B+ treatment elevated the levels of all titers of putrescine in both inner and outer leaves ($p < 0.001$ in all cases, Fig. 3.1a, b). The effect of cultivar was non-significant except for in the case of TCA-soluble (free) and TCA-soluble-conjugated putrescine in outer leaves (Fig. 3.1b). An interaction between treatment and cultivar caused the extent of putrescine accumulation to differ between the cultivars, free and soluble-bound Put being higher in the cultivar 'Buttercrunch' than in 'Red Salad Bowl' after UV-B+ treatment ($p = 0.029$ and 0.025 for TCA-soluble and TCA-soluble-bound Put respectively; Fig 3.3a, b).

In the time course experiment, Putrescine levels in time = 0 samples did not differ significantly from controls, but MANOVA revealed significant differences in putrescine accumulation between treatments ($p < 0.001$) and cultivars ($p = 0.031$). Univariate ANOVAs indicated that treatment had a significant effect for all putrescine titers ($p < 0.001$ in all cases), UV-B+ treated plants accumulating higher levels of all titers of putrescine than controls (Dunnett's *t* test, $p < 0.001$ in all cases, Figs. 3.4a-f). Cultivar had significant effects on levels of free putrescine ($p = 0.013$) which appeared to be lower in the variety 'Red Salad Bowl' than in other varieties (Tukey's HSD test), especially in UV-B+ treated plants (Fig. 3.4c).

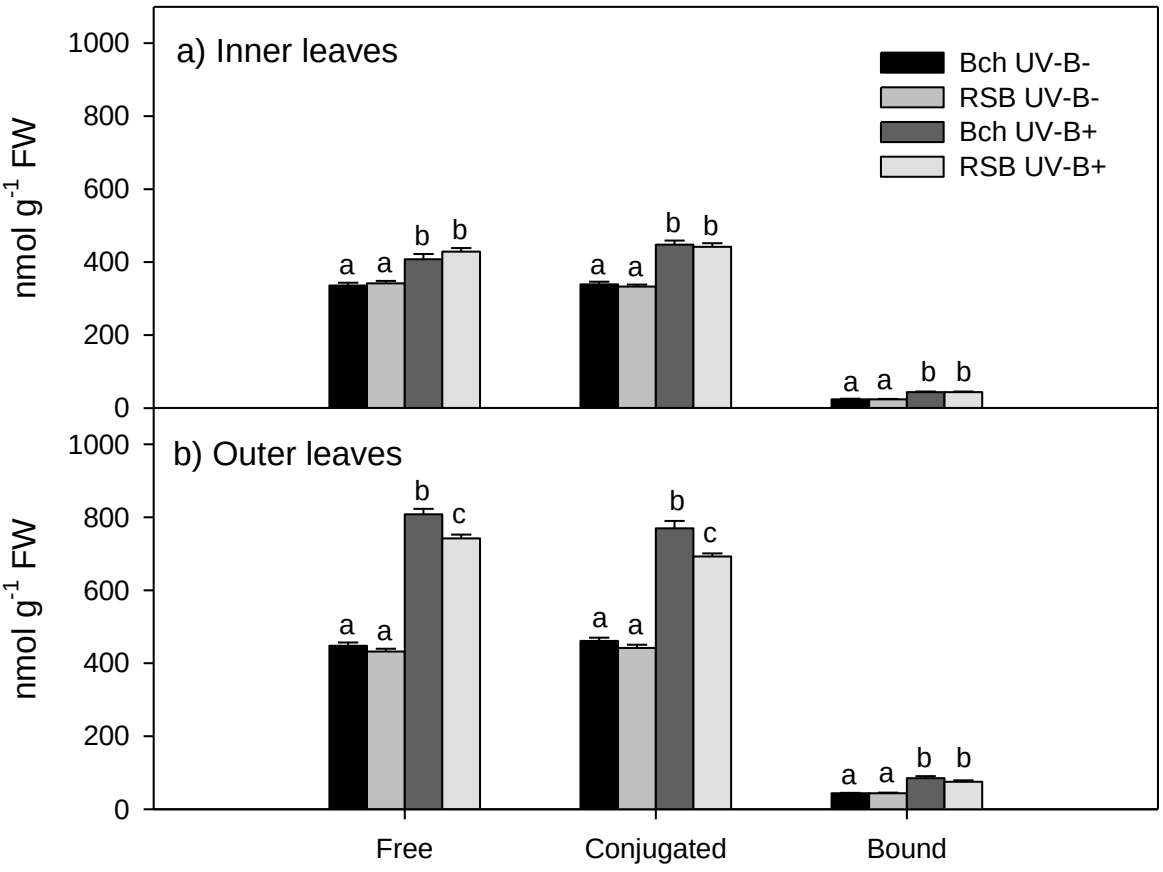

Figure 3.3 Mean free, soluble-conjugated and insoluble-bound putrescine in a) inner and b) outer leaves of lettuce plants. Significantly different samples within polyamine types are indicated by different letters (Tukey's HSD, n = 12, α = 0.05). Error bars are one standard error.

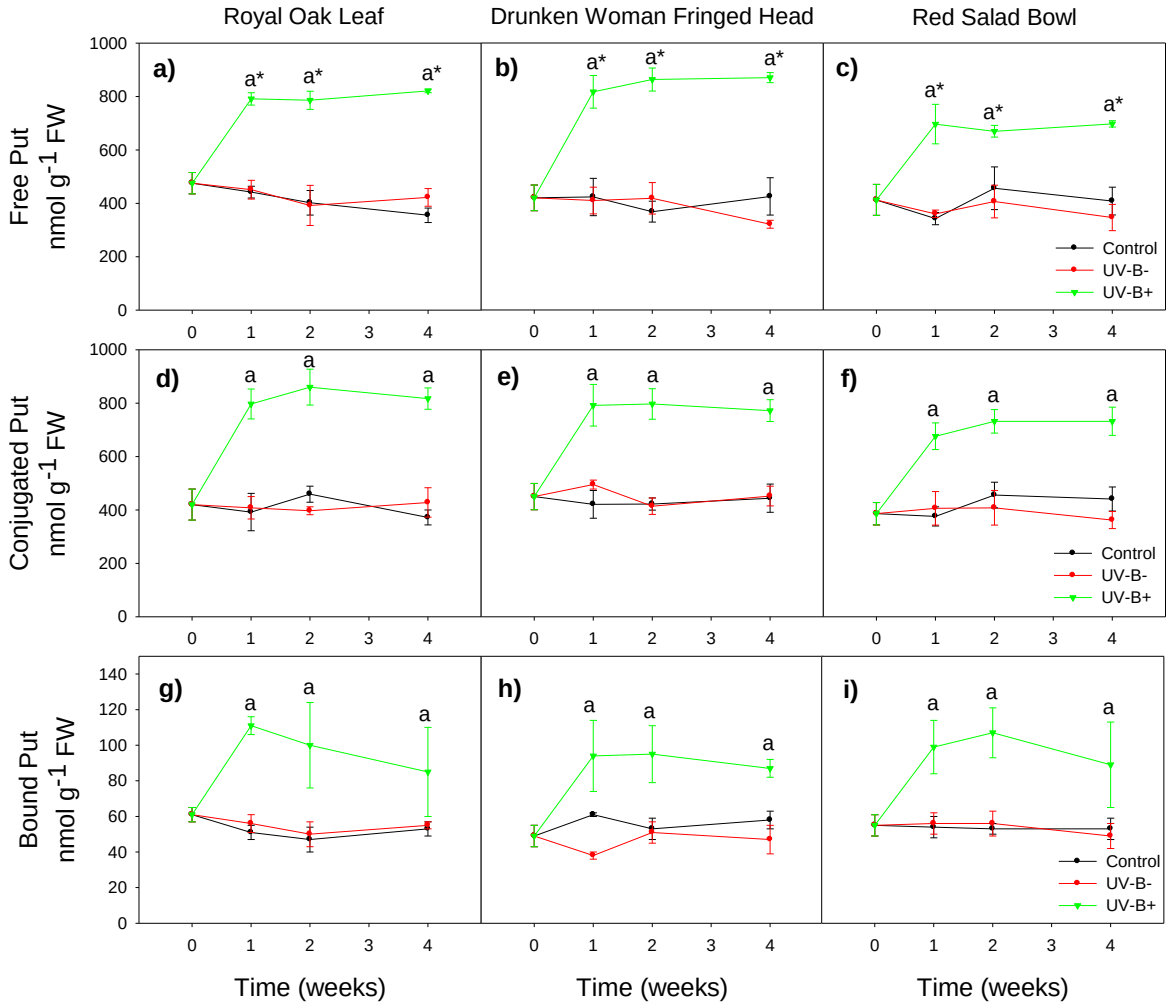

Figure 3.4 Mean levels of free (a, b, c), soluble-conjugated (d, e, f) and insoluble-bound putrescine (g, h, i) by cultivar in the time-course experiment (a, d and g, Royal Oak Leaf; b, e, and h, Drunken Woman Fringed Head; c, f and i, Red Salad Bowl). Samples significantly different from untreated controls at each time point are indicated with letters, an '*' also indicates a difference between varieties (Tukey's HSD, n = 3, α = 0.05). Error bars are one standard error.

3.3.3 Spermidine

MANOVA performed on the data for different forms of spermidine revealed a significant interaction between the effects of UV-B treatment and cultivar ($p = 0.002$) in the end point experiment. Univariate ANOVAs showed that UV-B+ treatment significantly elevated the levels of all titers of spermidine in both inner and outer leaves ($p < 0.001$ in all cases, Figs. 3.1a, b). The effect of cultivar was non-significant except for the case of soluble-conjugated and insoluble-bound Spd in outer leaves where an interaction between treatment and cultivar led the extent of UV-B induced Spd accumulation to differ between cultivars, soluble-conjugated and insoluble-bound Spd in outer leaves being greater in the cultivar 'Buttercrunch' than in 'Red Salad Bowl' after UV-B+ treatment ($p < 0.001$ for outer leaf soluble-bound Spd and $p = 0.043$ for outer leaf bound Spd; Fig. 3.5b).

Levels of spermidine at time = 0 in the time course experiment did not differ significantly from controls. MANOVA revealed significant interactions between treatment and cultivar ($p < 0.001$) and cultivar and time ($p = 0.009$). Univariate ANOVAs showed the level of all titers of spermidine to be dependent on an interaction between treatment and variety ($p < 0.001$ for free spermidine, $p = 0.001$ for soluble-conjugated spermidine and $p < 0.001$ for insoluble-bound spermidine). UV-B+ treatment significantly increased spermidine levels above that of the controls (Dunnett's *t* test, $p < 0.001$ in all cases), although the increase appeared less in the variety 'Red Salad Bowl' which had lower levels of all spermidine titers than other varieties (Tukey's HSD test, Fig. 3.6a-i). The interactive effect of variety and time was significant for soluble-conjugated ($p = 0.015$) and insoluble-bound spermidine ($p = 0.014$). The level of soluble-conjugated spermidine showed a slight decrease two weeks after the start of UV-B treatment in the cultivar 'Drunken Woman Fringed Head' (Fig. 3.6d), but not in 'Royal Oak Leaf' or 'Red Salad Bowl', both of which displayed a slight increase in conjugated spermidine at this time (Fig. 3.6e, f). The level of insoluble-bound spermidine in 'Royal Oak Leaf' was stable after two weeks in the cultivar 'Royal Oak Leaf' (Fig. 3.6g), but decreased slightly by the end of the experiment in cultivars 'Drunken Woman Fringed Head' and 'Red Salad Bowl' (Fig. 3.6h, i).

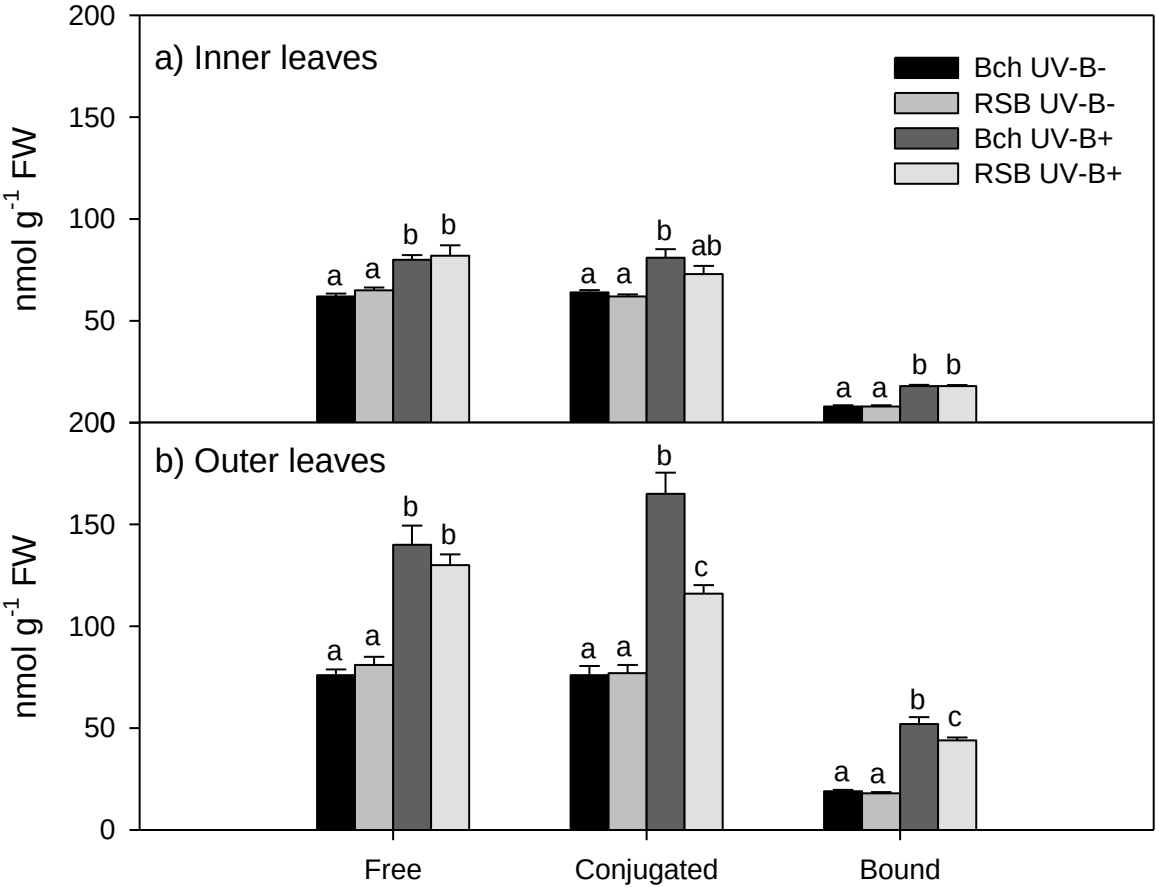

Figure 3.5 Soluble, soluble-conjugated and insoluble-bound spermidine in a) inner and b) outer leaves of plants in the end-point experiment. Significantly different samples within polyamine types are indicated by different letters (Tukey's HSD, n = 12, α = 0.05). Error bars are one standard error.

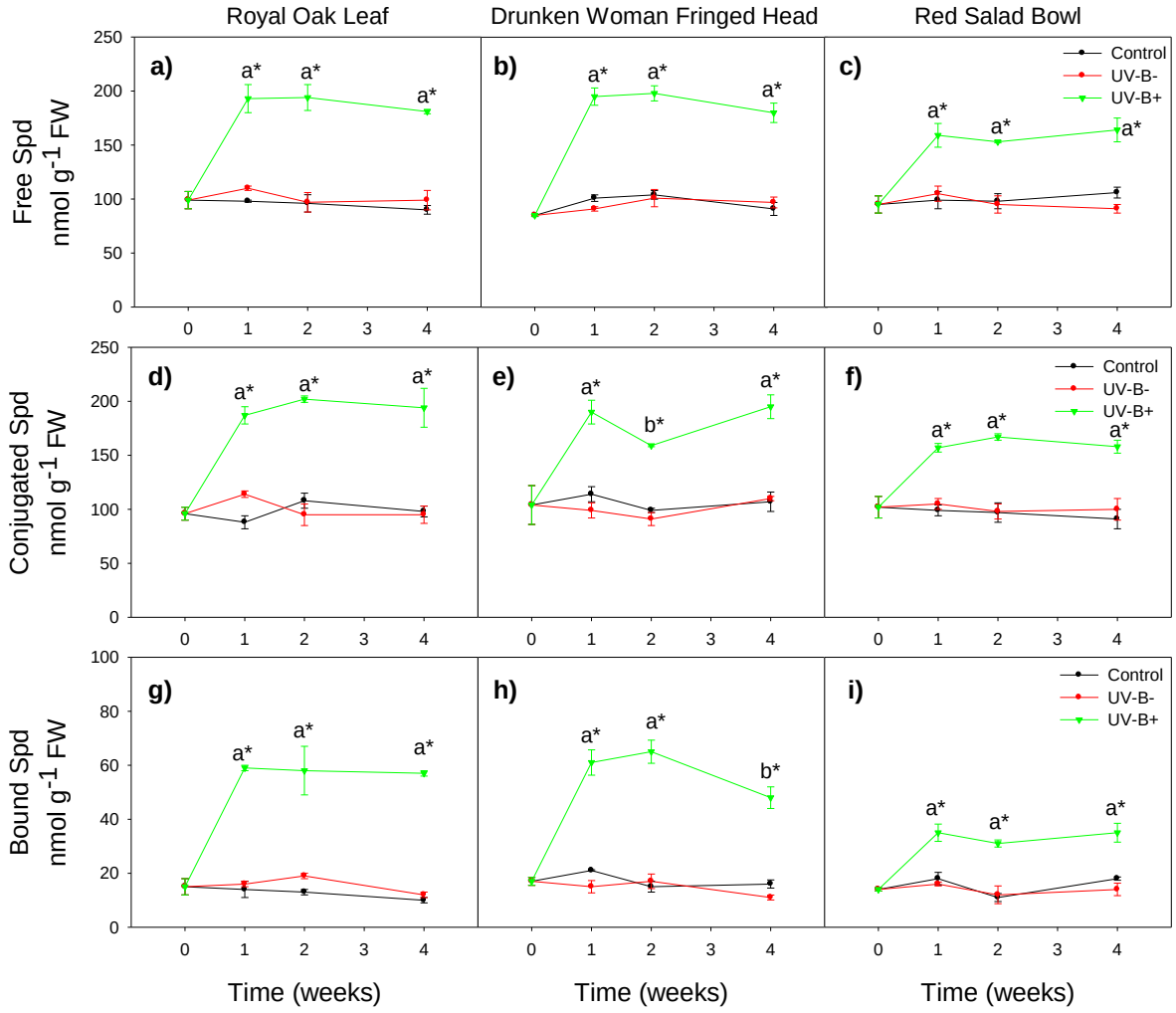

Figure 3.6 Soluble (a, b, c), soluble-conjugated (d, e, f) and insoluble-bound spermidine (g, h , i) by cultivar in the time-course experiment (a, d and g, Royal Oak Leaf; b, e, and h, Drunken Woman Fringed Head; c, f and i, Red Salad Bowl). Samples significantly different from untreated controls at each time point are indicated with letters, an '*' also indicates a difference between varieties (Tukey's HSD, n = 3, α = 0.05). Error bars are one standard error.

3.3.4 Spermine

MANOVA conducted on data for spermine levels showed a significant interaction between the effects of UV-B treatment and cultivar ($p < 0.001$) in the end point experiment. Univariate ANOVAs showed a significant increase in insoluble-bound spermine in both inner and outer leaves of UV-B+ treated plants ($p < 0.001$ in both cases), and an interaction between treatment and cultivar resulted in differing accumulation of free and soluble-conjugated spermine between cultivars, accumulation of both free ($p = 0.006$) and soluble-conjugated spermine ($p < 0.001$) after UV-B+ treatment being greater in the cultivar 'Buttercrunch' than in 'Red Salad Bowl' (Fig 3.7a, b).

Like other polyamines, the levels spermine in samples from time = 0 in the time course experiment did not differ significantly from controls. MANOVA revealed a significant interaction between the effects of treatment and time ($p = 0.020$). Univariate ANOVAs showed the interaction between treatment and time was non-significant for free spermine ($p = 0.811$), although as with all other spermine titers there was a significant effect of treatment ($p < 0.001$ in all cases). UV-B+ treated plants displayed higher levels of free spermine than controls (Dunnett's *t*, $p < 0.001$ in all cases; Figs. 3.8a-c). The interaction between treatment and time however, was significant for soluble-conjugated and insoluble-bound forms of spermine ($p = 0.048$ and 0.010 respectively) although Tukey's HSD test gave only poor resolution of differences between samples. The level of soluble-conjugated spermine in UV-B+ treatments appeared to decrease at two weeks after the start of UV-B treatment and remained steady thereafter (Figs. 3.8d-f). The level of insoluble-bound spermine in these plants remained steady between one and two weeks after the start of UV-B treatment and then increased dramatically in the cultivar 'Royal Oak Leaf' (Fig. 3.8h), and was increased one week after the start of treatment in 'Drunken Woman Fringed Head' before decreasing at two weeks and then increasing again before harvest (Fig. 3.8i). In the cultivar 'Red Salad Bowl', the level of bound spermine appeared to increase slightly but steadily throughout the experimental period (Fig. 3.8h).

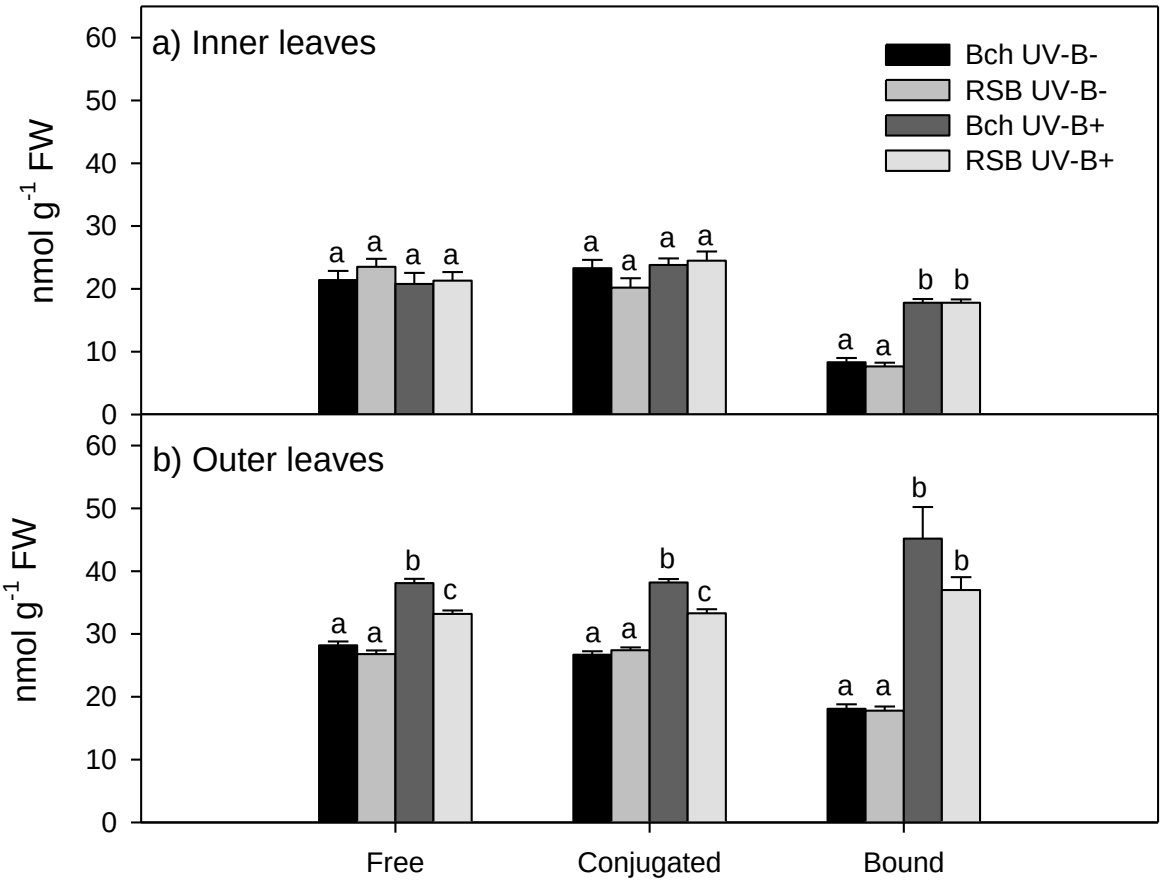

Figure 3.7 Soluble, soluble-conjugated and insoluble-bound spermine in a) inner leaves and b) outer leaves in the end-point experiment. Significantly different samples within polyamine types are indicated by different letters (Tukey's HSD, n = 12, α = 0.05). Error bars are one standard error.

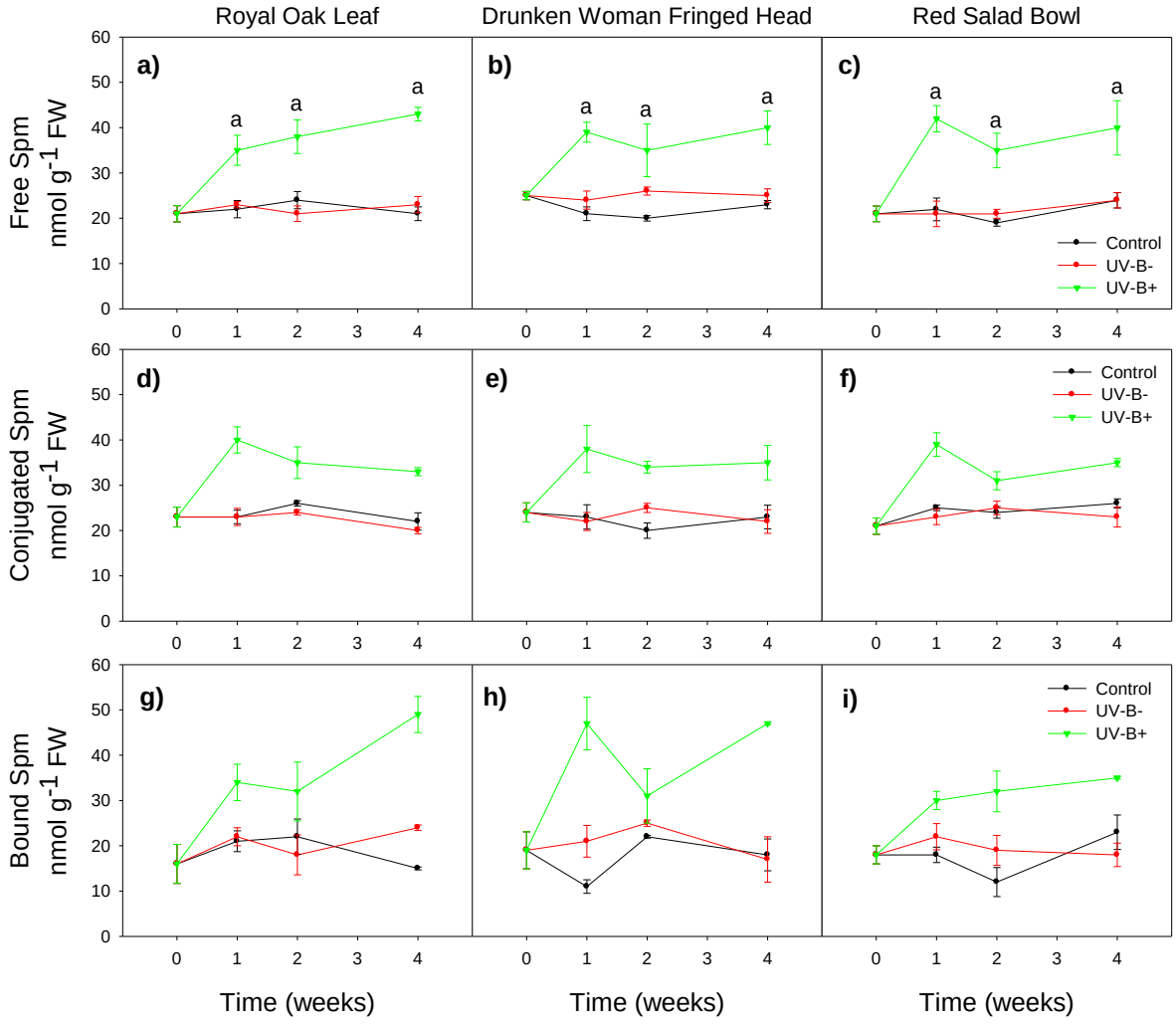

Figure 3.8 Soluble (a, b, c), soluble-conjugated (d, e, f) and insoluble-bound spermine (g, h i) by cultivar in the time-course experiment (a, d and g, Royal Oak Leaf; b, e, and h, Drunken Woman Fringed Head; c, f and i, Red Salad Bowl). Samples significantly different from untreated controls are indicated with letters (Tukey's HSD, n = 3, α = 0.05) although for conjugated and bound Spd Tukey's HSD poorly resolved differences and are not shown. Error bars are one standard error.

3.3.5 Enzymes involved in PA biosynthesis

In the end-point experiment, activities of ADC and ODC were measured in outer leaves of the variety 'Red Salad Bowl' only. The activity of PA biosynthesis enzymes, especially ADC, was elevated in UV-B+ treated plants, although the effect of treatment was non-significant in both cases (Fig. 3.9).

In the time course experiment while time = 0 samples did not differ from controls, MANOVA did reveal a significant three-way interaction between the effects of cultivar, treatment and time ($p = 0.014$). Subsequent univariate analysis showed this interaction to be effective in the case of ADC only ($p = 0.001$). UV-B+ treatment elevated the activities of ADC in all varieties above that of controls (Dunnett's *t* test, $p < 0.001$), although Tukey's HSD provided

only poor resolution of differences between samples. ADC activity in UV-B treated plants of the cultivars 'Royal Oak Leaf' and 'Drunken Woman Fringed Head' appeared to increase substantially one week after treatment began, before decreasing at two weeks and then increasing again prior to harvest (Figs. 3.10a, b). In contrast, ADC activity in UV-B treated plants of the cultivar 'Red Salad Bowl' peaked two weeks after the start of treatment (Fig. 3.10c). ODC levels were not altered by experimental treatment (Figs. 3.10d-f). Univariate tests also showed SAMDC activities to be significantly affected by UV-B treatment ($p < 0.001$), being elevated above the level of controls in UV-B+ treated plants (Dunnett's *t* test, $p < 0.001$; Fig. 3.10g-i).

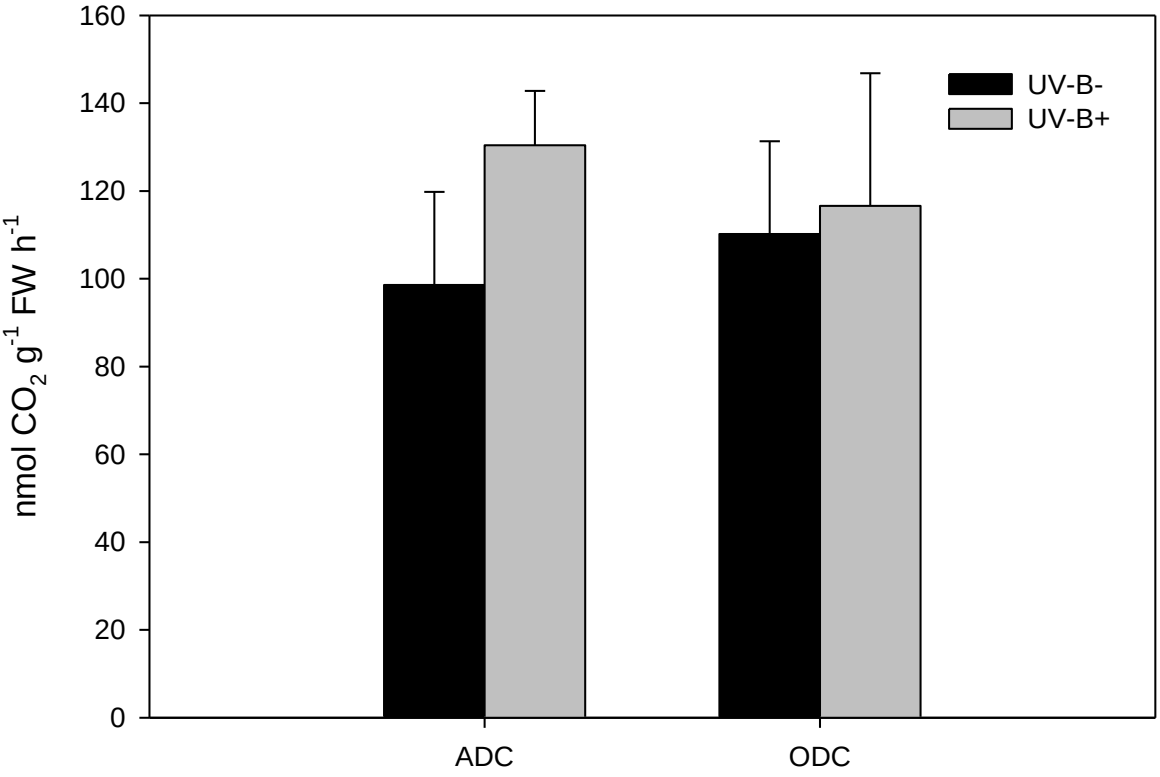

Figure 3.9 Activities of ADC and ODC in the outer leaves of the lettuce variety "Red Salad Bowl". Samples are not significantly different, error bars are one standard error.

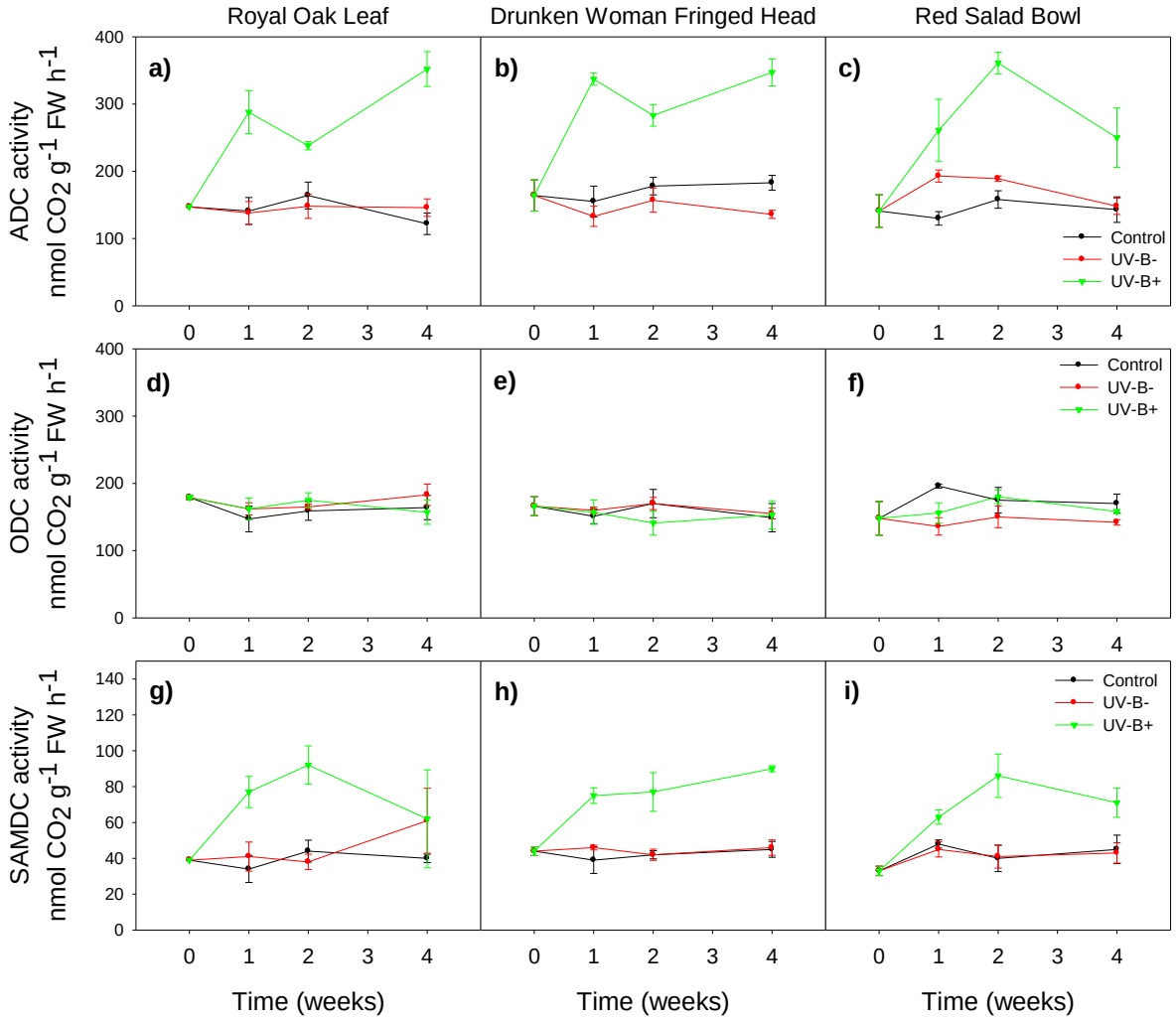

Figure 3.10 ADC (a, b c), ODC (d, e, f) and SAMDC (g, h, i) activities by cultivar (a, d and g, Royal Oak Leaf; b, e, and h, Drunken Woman Fringed Head; c, f and i, Red Salad Bowl). While ADC and SAMDC activities of UV-B+ treated plants were significantly different from controls, Tukey's HSD provided only poor resolution of differences between samples. Error bars are one standard error.

3.4 Discussion

In the current experiments, polyamines were rapidly accumulated in the leaves of lettuce plants in response to UV-B exposure in a cultivar dependent manner. This is comparable to the results of researchers such as Radyukina *et al.* (2011) and Rakitin *et al.* (2009), who recorded the accumulation of Put and Spd in sage plants and *Arabidopsis* exposed to UV-B. The polycationic nature of polyamines enables them to bind to cellular membranes, which may aid in preventing lipid peroxidation (Roussos and Pontikis, 2007). Spermine has a greater positive charge the diamine putrescine and is proposed to lay parallel to membranes, interacting with negative charged particles in the membrane and aiding membrane stabilization (Roy *et al.*, 2005). The accumulation of spermine under UV-B stress has been considered to be an important factor in UV-B tolerance. While putrescine and spermidine

were the most common polyamines in the current experiment, spermine was also accumulated in response to UV-B exposure, a greater portion of the spermine accumulated being found in bound and conjugated form than was the case for putrescine and spermidine. The concentrations of both total spermine and bound spermine also increased as UV-B treatment progressed, suggesting a role for bound-spermine in UV-B acclimation.

Gomez-Jimenez *et al.* (2010) reported that higher spermidine contents in olive fruits corresponded with increased cell division, while increased putrescine contents relative to spermidine and spermine was possibly related to developmental acquisition of cell size. In the current experiment, putrescine was the most abundant polyamine in both UV-B exposed and UV-B unexposed lettuce leaves suggesting that cell enlargement was of primary importance for leaf expansion under both UV-B regimes. Polyamines are important in plant responses to UV-B stress because they assist in the protection of the photosynthetic apparatus, relieve oxidative stress and are able to protect DNA from UV-B induced damage. PSII is particularly sensitive to UV-B radiation as high energy photons may cause damage to the water-splitting Mn clusters of the D1 protein and over-excitation of plastiquinone electron acceptors, causing photoinhibition and a consequent decrease in oxygen evolution (Besford *et al.*, 1993; Sfichi-Duke *et al.*, 2008). Polyamines, especially spermine, are able to induce non-phytochemical quenching which absorbs excess energy in the photosystem before it can cause damage to PSII and disrupt the electron transport chains required for carbon fixation and oxygen evolution (Ioannidis and Kotzabasis, 2007; Sfichi-Duke *et al.*, 2008). The secondary structure of PSII may be altered by positively charged Spd and Spm, resulting in greater efficiency of the PSII antenna (Ioannidis and Kotzabasis, 2007). Apoproteins in the chlorophyll a and b antenna, and the large subunit of RUBISCO are known to be substrates of transglutiminases that function in the conjugation of polyamines to proteins (Delduca *et al.*, 1995; Lutz *et al.*, 2005). Bound and conjugated forms dominate the polyamine pools of chloroplasts, suggesting that polyamines conjugated to PSII apoproteins play a key role in the dissipation of high-energy photons under UV-B stress (Lutz *et al.*, 2005). Put is also known to activate ATP synthesis which may be helpful under stress conditions by directing excess energy through non-damaging pathways (Ioannidis and Kotzabasis, 2007). Lettuce plants in the current experiment showed little if any signs of photoinhibition (see chapter 2), and increased content of Put, Spd and Spm. A particularly high portion of Spm was found in conjugated and bound forms after the initiation of UV-B irradiation, suggesting that polyamine synthesis under UV-B stress may have been localized to the chloroplasts in the

manner described by (Lutz *et al.*, 2005), where it would have been available for aiding in the protection of the photosynthetic apparatus.

In addition to providing protection to the photosynthetic apparatus through the enhancement of non-phytochemical quenching, polyamines may provide protection from stress by acting as antioxidants. UV-B radiation results in the formation of ROS (detailed in chapter 1.1.2) and polyamines have been shown to have direct radical scavenging activity *in vitro* that arises from their ability to interact with negatively charged particles (Groppa and Benavides, 2008; Rider *et al.*, 2007). *In vivo*, Spd and Spm depletion of mouse fibroblasts has been shown to result in increased oxidative damage to DNA (Rider *et al.*, 2007). The inhibition of key enzymes in polyamine biosynthesis in the aquatic liverwort *Riccia fluitans* also resulted in increased levels of oxidative damage after exposure to phenathrene, a toxic hydrocarbon (Burritt, 2008). However, other authors report that the radical-scavenging activity of PAs is low, radical scavenging in phenolic-acid conjugated PAs investigated by (Bors *et al.*, 1989) being little increased on that of unconjugated phenolic acids. The production of H_2O_2 in the catabolism of PAs may also give them a pro-oxidant action that could be important in signaling during the antioxidant defense response (Groppa and Benavides, 2008). Thus while PAs increased dramatically in the current experiment during the period of most intense oxidative stress, further research is needed before it is possible to say whether they exhibit a direct antioxidant function or whether they function in signaling pathways to promote the up-regulation of other antioxidant defenses.

Polyamines are also involved in promoting the condensation of DNA, which may function to reduce stress induced DNA damage by limiting target sites for oxidative attack (Nayvelt *et al.*, 2010; Vijayanathan *et al.*, 2002). Interaction between positively charged polyamines and the negatively charged phosphate groups of DNA promotes the packaging of DNA into nanoparticles. Features of polyamine structure especially the distribution and density of positively charged amino and imino groups impact the size of the nanoparticles formed in this manner (Vijayanathan *et al.*, 2002) and so may have important implications for stress resistance. However, nuclear-localized polyamines tend to be synthesized via the enzyme ODC, which is not commonly up-regulated in plant stress responses, and further research is needed before the role of nuclear polyamines during stress can be fully described.

The balance of polyamine titers is also controlled by the varying activities of enzymes involved in polyamine biosynthesis. Predominance of synthesis via ODC is frequently recorded along with low levels of SAMDC activity and is thought to favour Put accumulation.

ADC activity on the other hand, increases under stress and frequently co-occurs with an increase in SAMDC activity, a rate-limiting factor in Spd and Spm synthesis (Gomez-Jimenez *et al.*, 2010). Increased ADC and SAMDC activity in stress conditions is also frequently reported to coincide with an increase in the portion of bound and conjugated polyamines (Roy *et al.*, 2005), so it may be that shifting PA biosynthesis to the ADC-SAMDC pathway reflects a greater investment in protective metabolites. As ADC is localized in the chloroplasts, especially the thylakoid membranes (Bortolotti *et al.*, 2004), the increased activity of ADC and increased production of bound and conjugated forms is possibly indicative of the functioning of polyamines in the photosynthetic apparatus. Previous studies show stress-induced increases in AD activity in *Arabidopsis* to be accompanied by increases in transcript levels of the gene *ADC2*. Other genes encoding for SAMDC (*SAMDC2*) and spermine synthase (*SPMS*) are also up-regulated under stress (Alcazar *et al.*, 2006; Urano *et al.*, 2003). The phytohormone abscisic acid has been implicated in increased PA biosynthesis through regulation of the ADC-SAMDC pathway. The stress-inducible genes *ADC2*, *SAMDC2* and *SPMS* all include ABA-inducible elements in their promoters, also pointing to a role for ABA in the stress response (Alcazar *et al.*, 2006). Interestingly, ABA signaling is involved in the down-regulation of stress-induced ethylene evolution, which competes with the biosynthesis of polyamines through shared precursors of Spd and Spm (Rakitin *et al.*, 2008a). The role of polyamines in other physiological events during plant development may also be mediated by ABA in a similar manner to the stress response. (Urano *et al.*, 2003) found high levels of the stress-inducible *ADC2* transcript**s** in the reproductive organs of *Arabidopsis*, and conjugated forms of polyamines have been recorded in the flowers of many plant species. In the current experiment increased ADC and SAMDC activities in UV-B+ treated plants were reflected in the increased accumulation of spermidine and spermine, a greater portion of which were accumulated in the bound and conjugated forms than was the case for putrescine. Putrescine on the other hand was accumulated mostly as free Put. Putrescine is a necessary precursor for the synthesis of spermidine (and thus spermine), and the accumulation of free putrescine may provide a pool for the synthesis of the larger polyamines (Liu *et al.*, 2006). This could be established by further research into the effects of stressors on plants over-expressing genes for DAO, an enzyme involved in putrescine degradation.

In both the end-point and time-course experiments, polyamine accumulation was less in the outer leaves of the variety 'Red Salad Bowl' when exposed to UV-B radiation than it was in other varieties. It has sometimes been suggested that reduced ability to accumulate polyamines is a marker of UV-B sensitive cultivars (Krishnamurthy and Bhagwat, 1989; Lutz

et al., 2005). 'Red Salad Bowl', however, was significantly less sensitive to UV-B stress than other varieties, experiencing less oxidative damage and recovering from damage more fully (see section 2.3.2.2), so the differential induction of polyamine accumulation cannot be considered a marker of stress sensitivity in this case. The lowered accumulation of polyamines in the cultivar 'Red Salad Bowl' is more likely the result of reduced severity of UV-B stress due to higher levels of UV-B screening metabolites such as anthocyanins in red-coloured lettuces like 'Red Salad Bowl (Tomas-Barberan and Espin, 2001). The constitutive ability of the variety 'Red Salad Bowl' to accumulate polyamines was no less than other cultivars, as was displayed by accumulation of polyamines in inner leaves which did not display the same degree of red colouration as outer leaves.

An additional unexpected result was that in inner leaves of both 'Red Salad Bowl' and 'Buttercrunch' polyamine accumulation was less than in outer leaves on the same plant. Inner leaves are generally younger and not fully expanded, and most reports show polyamine concentrations to be higher in tissues that are growing rapidly (Fujihara and Yoneyama, 2001; Peeters *et al.*, 1993; Szalai *et al.*, 1997). It is possible however, that inner leaves of lettuce heads are not the most rapidly growing part of the organism. Leaf lengths in the second experiment increased most between weeks two and three, increasing little in the first week (see section 2.3.2.1). A similar lag phase between leaf formation and the period of most rapid expansion may exist in the interior of lettuce heads.

As molecules that are involved in a wide range of cellular processes, polyamines are vital for the good health of all organisms including humans. To fulfill this requirement humans may synthesize polyamines themselves, or absorb polyamines from gut microbiota or dietary sources (Hunter and Burritt, 2011). A polyamine-rich diet may be of particular importance to the elderly as the level of polyamines in many organs is known to decrease with age, due to the decreasing activity of enzymes involved in polyamine biosynthesis (Eisenberg *et al.*, 2009; Majumdar, 2003; Nishimura *et al.*, 2006). A diet rich in polyamines may help the body maintain adequate intracellular polyamine pools. High-polyamine diets have been recorded as promoting increased lifespan in nematodes, flies and rats as well as human cells (Eisenberg *et al.*, 2009; Soda *et al.*, 2009). Polyamine supplementation also enhances the stress tolerance of cells grown *in vitro* and reduces markers of oxidative stress (Eisenberg *et al.*, 2009). Oxidative stress is thought to be involved in the pathogenesis of many age-related diseases, and the reduction of oxidative stress may help to maintain health into old age. Additionally, PAs are thought to be necessary for the maintenance of gut mucosa throughout life (Majumdar, 2003; Zou *et al.*, 2008), and play a key role in suppressing cellular adhesion and

maintaining an appropriate inflammatory status within the body (Soda *et al.*, 2005). As a significant portion of polyamines in the gut may be absorbed and retained in the body (Bardocz *et al.*), environmental manipulations that produce crops with higher polyamine content could produce a product with a higher nutritional value.

Some authors however, have cautioned the consumption of polyamine-rich foodstuffs due to their role in the promotion of tumour growth (Larque *et al.*, 2007; Saunders and Wallace, 2007). While PA supplementation does accelerate tumour growth and decrease lifespan in animals with tumours (Soda *et al.*, 2009), PA intake has not been found to correlate with increased risk of tumour occurrence (Nishimura *et al.*, 2006). In human intestinal epithelial cells, polyamines have been found to suppress tumour formation by promoting the expression of genes involved in tumour suppression such as the nuclear phosphoprotein p53 (Seiler and Raul, 2005). So while it may still be necessary for patients with tumours to avoid polyamine-rich foods it is likely that a high polyamine diet is generally beneficial.

In conclusion, the level of all polyamines in lettuce leaves was increased in response to UV-B exposure. The increased activity of the ADC biosynthethic pathway accompanied by increased synthesis of spermidine and spermine may indicate the localization of PA biosynthesis in the chloroplast in plants undergoing UV-B stress. Polyamines of higher molecular weight have a greater positive charge and may be able to better protect membranes from oxidative damage, and are accumulated in more in conjugated and bound forms that may in the protection of PSII in plants exposed to UV-B. Outer leaves of the red lettuce 'Red Salad Bowl' did not display the same level of polyamine accumulation as green varieties, perhaps because of the presence of UV-B screening metabolites. Unusually, younger leaves from the inner whorl had lower concentrations of polyamines than more fully expanded leaves in the outer whorl, possibly because of the continuous growth and rapid senescence patterns peculiar to lettuce leaves.

4 Ability of lettuce leaf material to protect human cells from oxidative damage *in vitro*

4.1 Introduction

Oxidative stress in human metabolism has been implicated in the causation of diseases commonly associated with ageing, such as cardiovascular disease, cancer and neurodegenerative diseases (Evans *et al.*, 2004; Jansen *et al.*, 2008; Khan *et al.*, 2010). In cancer especially, the spontaneous mutations in genetic material that can lead to increased cell proliferation and the deactivation of tumour suppressor genes are a crucial step in carcinogenesis and it is well established that such mutations can be induced by ROS-induced damage to DNA. Oxidative damage to DNA has also been implicated as a factor leading to sub-optimal inflammatory status and in the process of aging (Evans *et al.*, 2004; Marcil *et al.*, 2011; Moller *et al.*, 2007). Evidence suggests that a diet high in antioxidants may be able to aid in the prevention of certain cancers (Khan *et al.*, 2010), so it is important to investigate the effects of antioxidant containing foodstuffs on DNA oxidation in human cells.

ROS interact with DNA in a variety of ways that may lead to single- and double-strand breaks, sister chromatid exchange, the formation of cross-links between DNA molecules and between DNA and proteins, and the modification of purine and pyrimidine bases (Garg and Manchanda, 2009). Strand breakage is the result of oxidative modification of sugars in the sugar-phosphate backbone of DNA, and cross-linking is a result of oxidative modification to DNA bases leading to the formation of covalent bonds between the modified DNA bases, or DNA and proteins (Evans *et al.*, 2004). Oxidative damage to DNA bases is the result of attack by OH· or $^1O_2$ (Moller *et al.*, 2007). OH· may add to the double bonds of heterocyclic DNA bases, forming cytosine- and thyrosine-adduct radicals, or may abstract a proton to form a methyl group resulting in an allyl radical. OH· may also be added to purines at the C4, C5 and C8 position (in guanine only), resulting in adduct radicals (Evans *et al.*, 2004). Depending on a range of factors, including the redox environment, available reaction partners and the presence of O_2, these radicals may undergo further decomposition to form a wide variety of potentially mutagenic products (Evans *et al.*, 2004). OH· addition at the C8 position results in formation of a C8-OH-guanisine radical, further oxidation of which forms the lesion hydroxy-2-deoxyguanosine (8-oxodGuo), which is one of the most common products of oxidative insult to DNA and a commonly used biomarker of oxidative damage (Garg and Manchanda, 2009). 8-oxodGuo is a potentially mutagenic lesion as it pairs with adenine and cytosine at almost the same efficiency, promoting mutation through errors in

replication. 8-oxodGuo may also promote misreading of adjacent bases as well (Evans *et al.*, 2004; Yoshimura *et al.*, 2007).

In the human body, the intestine is a key interface through which the body interacts with its environment. The intestinal epithelia cells (IECs) form a barrier through which foodstuffs and potential toxins must pass before they are able to be incorporated into metabolism and exert a beneficial or detrimental effect (Langerholc *et al.*, 2011). As such, IECs may be exposed to concentrations of both oxidants and antioxidants that are not found elsewhere in the body, a factor that helps explain why colorectal cancer is one of the most common types of cancer (Duthie and Dobson, 1999; Sun *et al.*, 2008). Caco-2 is a line of human adenocarcinoma cells that display many of the characteristics of IECs. Caco-2 cells undergo structural and functional differentiation patterns similar to those of mature enterocytes, developing microvilli on the apical surface and tight junctions between cells, making caco-2 a plausible model of the small intestine in drug-uptake and nutritional studies (Langerholc *et al.*, 2011; Robichova and Slamenova, 2001; Sun *et al.*, 2008).

Previous studies have shown some plant extracts and compounds with antioxidant action to be effective in protecting caco-2 cells from oxidatively-induced DNA damage *in vitro* (Aherne and O'Brien, 2000; Aherne and O'Brien, 1999; Duthie and Dobson, 1999; Ramos *et al.*, 2010). The current studies have shown the activity of antioxidant enzymes, as well as levels ascorbate, glutathione and polyamines to be increased in lettuce plants grown under UV-B transmitting plastic films (see sections 2.3 and 3.3), and it seems reasonable to hypothesize that material from these plants might also be able to protect caco-2 cells from oxidative damage. The aim of the current experiment is to test this hypothesis by comparing the levels of 8-oxodGuo lesions in caco-2 cells pretreated with digested plant material and challenged with H_2O_2.

4.2 Methods

4.2.1 Plant material

Lettuce seeds were germinated, grown and harvested as detailed in section 2.2.2. To save resources, only material harvested from the cultivars 'Drunken Woman Fringed Head' and 'Red Salad Bowl' after four weeks UV-B+ or UV-B- treatment was used in this assay.

4.2.2 Cell culture

In vitro peptic and intestinal digestion was performed according to the method of Glahn *et al.*, (1999), with modifications. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in 75 cm^2 flasks (Corning Inc., Corning,

NY, USA) at 37 °C in a humidified atmosphere of air/carbon dioxide (95:5 v/v), seeded at a density of 10^4, with a seven day passage frequency. The growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mmol glucose and 4 mmol glutamine and supplemented with 0.1% (v/v) non essential amino acids (Invitrogen, Carlsbad, CA, USA) and 10% (v/) heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), and was changed every 48 or 72 hours.

Porcupine pepsin (Sigma-Aldrich, St. Louis, MO, USA), pancreatin (Sigma-Aldrich) and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts, Sigma-Aldrich) were used for *in vitro* digestion. Pepsin, pancreatin, and bile extracts were further prepared to remove any contamination and to disperse the enzymes in solutions at physiological pH. Shortly before use, 0.2 g pepsin was dissolved in 5 ml 0.1 mol/L HCl, added to 2.5 g of Chelex-100 (Bio-Rad Laboratories, Hercules, CA, USA) and shaken gently on a platform with tabletop shaker for 30 minutes. To filter out the Chelex, the pepsin solution containing Chelex was eluted through a filtration column 1.6 cm in diameter. 5 ml of 0.1 mol/L HCl was added to the column and the filtrate collected in to the pepsin solution, the final volume of the eluted pepsin solution being 8 ml.

For intestinal digestion, 0.05 g of pancreatin and 0.3 g of bile extract were dissolved in 25 ml of 0.1 mol/L $NaHCO_3$, and 12.5 g of Chelex-100 added. The resulting mixture was shaken for 30 minutes on a tabletop shaker, before being poured into a 1.6 cm diameter filtration column to remove the Chelex. An additional 10 ml of 0.1 mol/L $NaHCO_3$ was added to the column and the filtrate collected into the pancreatin/bile solution, giving a total volume of 27 ml. Treatment of the pepsin and pancreatin-bile solutions by the methods described above did not affect the activity of the enzymes.

Peptic and intestinal digestions were carried out on a laboratory shaker in an incubator at 37 °C with an atmosphere of 5 % CO_2 and 95 % air, maintained at constant humidity. The intestinal digestion was carried out in the upper chamber of a two-chamber system in 6-well plates (Fig. 4.1). The cell monolayer was attached to the bottom surface of the lower chamber, chambers being separated by 15,000 molecular weight cut-off dialysis membranes fitted to appropriately-sized Transwell® insert rings (Costar®, Corning Inc., Corning, NY, USA). The membranes were soaked in deionized water before use and held in place with a silicone ring (Web Seal Inc., Rochester, NY, USA). After the dialysis membrane was fastened to the insert ring, the entire unit was sterilized in 70 % ethanol and kept in sterile water until use.

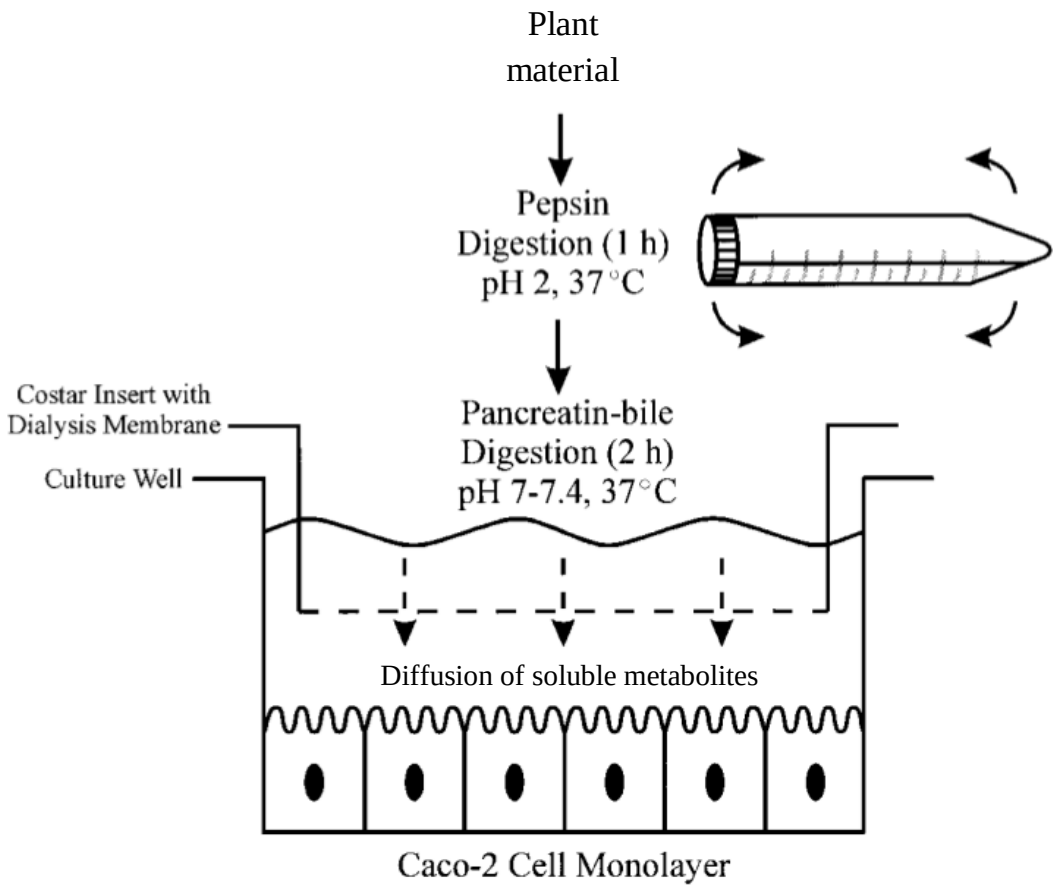

Figure 4.1 Diagram of *in vitro* digestion/Caco-2 cell culture model (figure and caption modified from (Glahn *et al.*, 1999).

For peptic digestion, 0.1 g FW of ground plant tissue and 1 mL of 140 mmol/L NaCl, 5 mmol/L KCl solution was placed in a 5 mL screw cap culture tube, and the pH of the mixture adjusted to 2.0 with 5.0 mol/L HCl. Then, 0.05 mL of pepsin solution was added. The tube was capped, placed horizontally and incubated for 60 minutes on a lab shaker at 10 oscillations/minute. For intestinal digestion, the pH of the sample (also referred to as 'digest') was raised to 6.0 by adding 1 mol/L $NaHCO_3$ dropwise, and 0.25 ml of the pancreatin-bile extract mixture was added. The mixture was then adjusted to pH 7.0 with 1 M NaOH and the volume brought to 2 ml with 120 mmol/L NaCl, 5 mmol/L KCl.

4.2.2.1 Preparation of 6-well culture plates with cell monolayers

Immediately before intestinal digestion, the growth medium was removed from the culture wells and each cell layer was washed twice with 37 °C Hanks' Balanced Salt Solution (containing 5.6 mmol/L glucose and buffered to pH 7.4 with 25 mM/L HEPES). A 1.0 mL aliquot of fresh Hank's Balanced Salt Solution (HBSS) covered the cells during the

experiment. The two-chamber system was then created by inserting the insert ring and dialysis membrane into the well, and a 1.5 ml aliquot of intestinal digest was pipetted into the upper chamber. The plate was covered and incubated on a laboratory shaker for 120 minutes at approximately 10 oscillations/min.

4.2.3 Cytotoxicity assay

After the intestinal digestion period, the digest and salt solutions were removed. Cells were washed twice with 2.5 ml of FBS-free growth medium and then incubated for a period of 60 minutes in FBS-free growth medium. The FBS-free growth medium was then removed and replaced with FBS-free growth medium supplemented with either deionized water or 50 μmol H_2O_2. Cells were then incubated for a further 60 minutes, before being harvested and DNA extracted as detailed below.

4.2.4 DNA extraction and measurement of DNA oxidation

DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), using the cultured cells protocol as per the manufacturer's instructions, with the following modifications: the AL buffer was supplemented with 5 mM deferoxamine and 20 mM EDTA, and the DNA purification columns were pre-treated with 250 μl of a solution containing 5 mM deferoxamine, 20 mM EDTA, 5 mM o-phenathroline, 20 mM tris-HCL (pH 8.0) and then washed with 500 μl of sterile deionized water before use. The quantity of DNA in each sample was determined by measuring the absorbance at 260 nm and DNA purity was determined by the ration of absorbance at 260 nm to absorbance at 280 nm (A_{260}/A_{280} ratio), all values being in the range 1.7 – 1.9 (Sambrook *et al.*, 1989). All extraction procedures were carried out under minimal lighting at between 0 and 4 °C and all solutions were deoxygenated by bubbling with nitrogen or argon gas.

A sample of extracted DNA was precipitated by the addition of 0.1 ml of 4 mol NaCl and 2.5 ml of cold ethanol, and digested as per (Shigenaga *et al.*, 1994), with modifications. Briefly, the precipitated DNA was re-dissolved in 200 μl of DNA hydrolysis buffer containing 1 mmol deferoxamine, 20 mmol sodium acetate, pH 5.0. 4 μl of 3.3 mg ml^{-1} nuclease P1 was then added and samples were incubated at 65 °C for 15 minutes. Alkaline phosphatase (4 U in 1 M tris-HCl [pH 8]) was added and the samples were incubated at 37 °C for a further 60 minutes. 20 μl 3 mol sodium acetate was added to each sample, followed by 20 μl of chelating solution (containing 50 nM EDTA, 10 mM deferoxamine). The solutions were transferred to microcentrifuge filters with a 30 kDa cut-off, before being centrifuged at 5,000 x *g* for 15 minutes. The filtered solutions, containing the nucleotides, were then collected for 8-hydroxy-2-deoxyguanosine (8-oxodGuo) analysis.

Digested DNA was analyzed using high-performance liquid chromatography (HPLC) followed by UV detection of deoxyguanosine (dGuo) and electrochemical detection (coulometric) of 8-oxodGuo. The procedure was essentially the same as that described by Dany *et al.* (1999), using a 4.6 x 250 mm, 5 μm particle size, C_{18} reverse-phase column (JASCO, Ishikawa-cho, Hachioji-shi, Toyko, Japan), a Perkin-Elmer HPLC system (Boston, MA, USA) and an ESA model 5100 electrochemical detector (ESA, Chelmsford, MA, USA). Oxidation potentials of the analytical cell of the electrochemical detector were 150 mV for electrode 1, and 350 mV for electrode two, the guard cell potential being set to 400 mV. Unmodified nucleosides were detected by measuring absorbance at 260 nm. Separation of 50 μl of digested DNA was achieved using an isocratic mobile phase consisting of 50 mmol potassium phosphate (pH 5.5) and 10 % methanol, with a flow rate of 1 ml min^{-1}, the column maintained at 30 °C. Absorbance peak data were collected and analyzed using a DataCenter 4000 general-purpose laboratory data interface, and Delta chromatography data acquisition and analysis software (DataworkX, Brisbane, QLD, Australia). The retention time for dGuo was 12 minutes, and 17 minutes for 8-oxodGuo. Solutions of dGuo and 8-oxydGuo (Sigma Chemical Co., St Louis, MO USA), prepared in HPLC-grade water (Merck, Darmstadt, Germany) and sterilized by passage through 0.2 mm filters (Millipore, Bedford, MA, USA) were used as standards. The signal for dGuo was used to estimate the amount of DNA injected into the column, and 8-oxodGuo was quantified by comparison to external standards.

4.2.5 Statistical methods

Statistical analysis was performed using SPSS 19 for Windows XP. A univariate ANOVA was performed to detect any significant effects of UV-B treatment and cultivar on the level of 8-oxodGuo, and significant differences between sample means distinguished using Tukey's HSD tests.

4.3 Results

Oxidative damage to the DNA of caco-2 cells challenged with H_2O_2 varied significantly depending on the plant material in the digest. The protective effect of the digest was affected both by the cultivar (p = 0.006) and pre-harvest UV-B treatment (p = 0.004) of plant material used in the digest. Levels of 8-oxodGuo decreased caco-2 cells treated with digest containing plant material from lettuces grown under the UV-B transparent cellulose diacetate film, and were lower digests containing material from the cultivar 'Red Salad Bowl' than in those containing that of the cultivar 'Drunken Woman Fringed Head' (Tukey's HSD, Fig. 4.2).

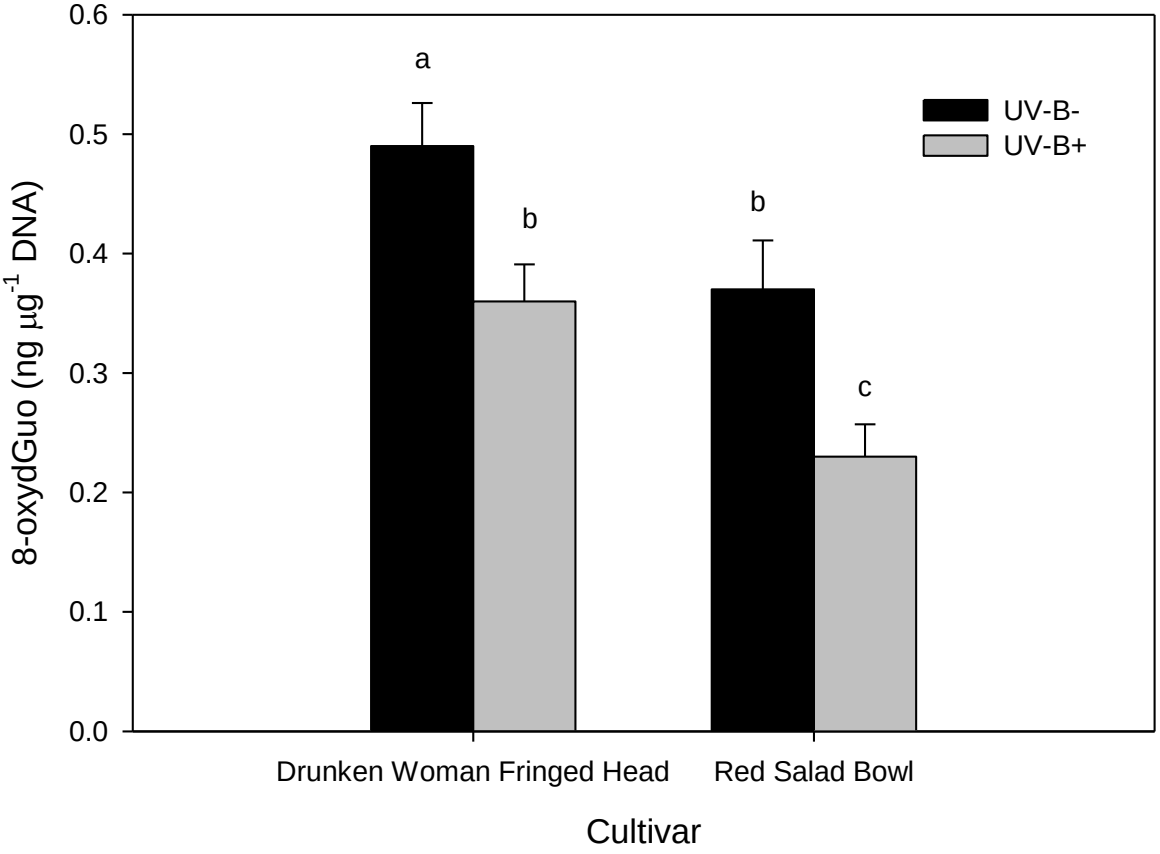

Figure 4.2 Mean levels of 8-oxydGuo in caco-2 cells fed digests containing lettuce tissue of different cultivars grown in UV-B- and UV-B+ conditions. Samples that are significantly different are labelled with different letters (Tukey's HSD, n = 3, α = 0.05) and error bars represent one standard error.

4.4 Discussion

As expected, material from plants growing under a UV-B transmitting film conferred greater protection on Caco-2 cells challenged with H_2O_2 than material from plants grown under a UV-B blocking film. Material from plants grown under the UV-B transmitting film cellulose diacetate contained a higher content of low-molecular weight antioxidants that that of plants grown under the UV-B blocking Mylar-D® film (see section 2.3), and it is possible that ROS-scavenging by antioxidants present in digests of UV-B+ treated plants contributed to the protective effect. GSH is one of the principal antioxidants in human cells, and was elevated in samples from which the UV-B+ digests were derived (section 2.3.2.10). Activities of enzymes that use GSH as a substrate have previously been recorded as increasing in Caco-2 cells exposed to oxidative stress and exogenous GSH was shown to be effective in protecting mouse fibroblasts from oxidative stress, scavenging ROS independently of other compounds that also exhibited protective effects (Rider *et al.*, 2007; Wijeratne *et al.*, 2005). However, the

action of other antioxidants might not be as straight-forward. Human milk is thought to be high in the antioxidant enzymes SOD and CAT but a study by (Shoji *et al.*, 2005) showed that while human IEC-6 cells pretreated with human milk showed reduced sensitivity to H_2O_2, there was no evidence of the absorption of antioxidant enzymes into IEC cells. The action of AsA is also ambiguous. While it is well-demonstrated to have a powerful antioxidant ability, in the presence of iron AsA may also be a source of highly reactive OH· radicals through the Fenton reaction (Aherne and O'Brien, 1999; Griffiths and Lunec, 2001; Halliwell, 2006; Premkumar *et al.*, 2007). This may be of particular importance in the current experimental conditions as H_2O_2 does not directly interact with DNA and must first be converted to OH· if damage to DNA is to occur. Plant material from UV-B+ treated lettuces of the cultivar 'Drunken Woman Fringed Head' contained a higher level of total ascorbate than that of 'Red Salad Bowl', and increased OH· generation in the digests obtained from 'Drunken Woman Fringed Head' might have reduced its effectiveness in protecting Caco-2 cells from oxidative damage. AsA is also suspected to have other effects on cells beyond its capacity to modulate levels of ROS. It may be able to directly protect DNA by binding to sights of oxidative attack (Robichova and Slamenova, 2001) and may provide indirect protection against ROS-mediated DNA damage by promoting the increased expression of genes involved in antioxidant defense and DNA repair (Bevan *et al.*, 2010). However, further research is needed before the protective effects of individual antioxidants can be established.

Polyamines, especially spermidine and spermine, have also been shown to enhance resistance to oxidative DNA damage in a variety of cellular systems including mouse fibroblasts, prostate carcinoma cells and human IECs (Nayvelt *et al.*, 2010; Rider *et al.*, 2007; Shoji *et al.*, 2005). Elevated levels of polyamines in the plant material from UV-B+ treated plants (see section 3.3) and could have contributed to the protective effect of digests containing material from these plants. Previous studies have proposed three mechanisms by which polyamines may protect nuclear material from oxidative attack: scavenging of ROS, protection of the structural integrity of DNA and the regulation of gene expression (Rider *et al.*, 2007; Tkachenko *et al.*, 2001). As discussed previously, polyamines are protonated at physiological pH, carrying a positive charge that enables them to scavenge OH· and $^1O_2$ radicals (Das and Misra). Other research however, has cast doubt on the ability of polyamines to act as radical scavengers. Polyamines have been shown to enhance the generation of OH· in the presence of Fe^{3+} and consequently may increase damage to DNA where there is an excess of transition metal ions (Mozdzan *et al.*, 2006). However, polyamines may protect DNA through mechanisms other than radical scavenging. By forming non-covalent bonds with DNA,

polyamines are able to limit sites of ROS attack by inducing conformational changes in the DNA molecule and physically blocking ROS (Muscari *et al.*, 1995; Nayvelt *et al.*, 2010; Rider *et al.*, 2007). Alterations to DNA topography may also have the further function in the regulation of genes involved in oxidative stress responses (Tkachenko *et al.*, 2001). However, further research is needed before the exact contribution and mode of action of polyamines in digested plant material can be ascertained.

The protective effect of digested plant material was greater in digests containing material from the cultivar 'Red Salad Bowl' than those containing 'Drunken Woman Fringed Head'. Cv. 'Drunken Woman Fringed Head' had higher contents of both low-molecular weight antioxidants and polyamines than 'Red Salad Bowl', so while it is likely that both low-molecular weight antioxidants and polyamines contributed to the protection of DNA these compounds are unlikely to be responsible for the full effect. Other compounds must be responsible for at least part of the effect. Phenolics are a class of compounds occur in red lettuce varieties at higher levels than in green lettuces, and have been shown to be able to protect DNA from oxidative damage (Duthie and Dobson, 1999; Ramos *et al.*, 2010). At least one class of phenolics, anthocyanins, was recorded in the present experiment as being present in material of the cultivar 'Red Salad Bowl' (see section 2.3.2.2), and flavonoids have been reported to vary between lettuce varieties in previous studies (Heimler *et al.*, 2007; Hohl *et al.*, 2001; Llorach *et al.*, 2008; Nicolle *et al.*, 2004). Flavonoids possess antioxidant capacity through their ability to donate H atoms to OH·, which may be an effective mechanism of protection against oxidative attack on DNA (Aherne and O'Brien, 2000). Flavonoids are also reported to possess metal chelating capacity and reduce oxidative damage to DNA through a reduction in the rate OH· production through the Fenton reaction, and may also promote the activity of the DNA repair enzymes (Aherne and O'Brien, 1999; Ramos *et al.*, 2010). Although phenolics are seldom recorded in human-tissues at biologically-meaningful levels, cells lining the gut come into much higher levels of phenolics and could receive a protective effect (Galleano *et al.*, 2010). Levels of phenolics were likely to have been higher in the red-leaved cultivar 'Red Salad Bowl' than in 'Drunken Woman Fringed Head', and might explain the increased protection against oxidative stress afforded by digests containing material from the former cultivar.

In conclusion, the results of the current experiment show material from plants grown in UV-B exposed conditions confers greater protection from oxidative damage to DNA in human cells than material from plants unexposed to UV-B. Additionally, material from red lettuce varieties may confer greater protective capacity than that of predominantly green varieties.

Further research is needed to establish the mechanism by which digested plant material may assist in defense against oxidative attack, but low-molecular weight antioxidants and polyamines, as well as phenolic compounds are likely candidates.

5 General Discussion

5.1 Overview

Exposure to UV-B in the current experiments increased the levels of antioxidant enzymes, ascorbate, glutathione and polyamines in all varieties of lettuce studied. UV-B exposed plants experienced oxidative stress as evidenced by the increased levels of protein carbonyls and lipid hydroperoxides, and by the increased oxidation of ascorbate and glutathione – all of which were most intense one week after the start of UV-B treatment. Antioxidant enzymes and the low molecular-weight antioxidants ascorbate and glutathione are important for the detoxification of excess ROS and the maintenance of an appropriate redox status within plant cells, although the induction of each antioxidant is dependent on its mode of action and localization (Mittler, 2002; Foyer and Noctor, 2009). SOD is involved in the detoxification of highly reactive $O_2\cdot^-$ which may be produced through the over-reduction of election transport chains in the thylakoid membranes of UV-B exposed plants (Noctor and Foyer, 1998). H_2O_2 is another ROS common in UV-B exposed plants and is detoxified through the action of CAT, peroxidases and low-molecular-weight antioxidants (Garg and Manchanda, 2009; Mittler, 2002). CAT which is localized mainly in peroxisomes reduces H_2O_2 to water and O_2 (Garg and Manchanda, 2009). APox and GPox, more efficient antioxidant enzymes with a higher affinity for H_2O_2 are located in the chloroplast, mitochondria and cytoplasm (Mittler, 2002; Moller *et al.*, 2007). Ascorbic acid is required as a substrate for APox, and reduced glutathione is required as a substrate for GPox. Both ascorbate and glutathione must therefore be regenerated enzymatically, by MDAHR and DHAR in the case of ascorbate and GR in the case of glutathione (Gill and Tuteja, 2010; Mittler, 2002). In their reduced forms, ascorbate and glutathione may also exhibit antioxidant action of their own (Noctor and Foyer, 1998). In situations of prolonged oxidative stress, the less complex detoxification systems centered on SOD and CAT are up-regulated more rapidly before additional ascorbate and glutathione can be synthesized (Dawar *et al.*, 1998), a pattern broadly reflected in the current experiments where SOD activity peaked two weeks after the start of UV-B exposure.

The role polyamines play in plant stress responses has been less well characterized. It is thought that they may play a role in stabilizing cellular membranes at risk of lipid peroxidation and by compacting DNA may reduce the risk of oxidative injury to nuclear material (Nayvelt *et al.*, 2010; Roy *et al.*, 2005; Vijayanathan *et al.*, 2002). They have also been demonstrated to possess antioxidant capacity and may participate in the scavenging of ROS (Das and Misra, 2004; Groppa and Benavides, 2008). Two pathways control the

synthesis of putrescine and larger polyamines in plants, one starting from ornithine and the other from arginine. Bound and conjugated titers of spermidine and spermine have also been posited to play a role in the protection of PSII through the promotion of non-phytochemical quenching (Ioannidis and Kotzabasis, 2007; Sfichi-Duke *et al.*, 2008). Enzymes catalyzing the biosynthesis of polyamines from arginine are localized to the chloroplasts (Bortolotti *et al.*, 2004). The activity of these enzymes (especially ADC) is up-regulated in response to oxidative stress. The increases in ADC activity and increases in levels of bound spermidine and spermine seen in the current experiment in plants exposed to UV-B, lend support to the hypothesis that stress-induced polyamine accumulations function to protect the photosynthetic apparatus.

5.2 Plant foods and human health

It has long been known that fruits and vegetables are an essential component of a healthy diet. They contain a large array of nutritionally valuable compounds, some of which cannot be readily obtained from any other source (Jansen *et al.*, 2008). Ascorbate is one such nutrient which is poorly available in meat products, deficiency in ascorbate resulting in abnormal collagen synthesis leading to the potentially fatal condition known as scurvy (Olmedo *et al.*, 2006). In addition to the role of plant-based foods as sources of essential nutrients, recent attention has focused on the function that diet plays in the prevention of chronic diseases. ROS play a role in the pathogenesis of several diseases such as cancer, heart disease, diabetes and neurodegenerative diseases that together are responsible for the majority of age-related deaths in developed countries and are increasingly prevalent in developing countries (Poiroux-Gonord *et al.*, 2010). Antioxidants found in plant foods may be able to help prevent chronic diseases not only by directly scavenging ROS but also by promoting increased activity of endogenous antioxidants and by promoting the activity of mechanisms to repair oxidative damage to cellular macromolecules. Ascorbate for example, has been reported to induce processes similar to nucleotide-excision repair independently of UV-light, reducing T-T adducts in oxidatively stressed mononuclear peripheral blood cells (Bevan *et al.*, 2010). Ascorbate has also been reported to play a key role in slowing the growth of malignant tumours (Kuiper *et al.*, 2010). Plant foods are a particularly good source of dietary antioxidants and epidemiological studies provide evidence that consumption of a diet rich in fruits and vegetables lowers the risk of cardiovascular disease, adult-onset diabetes and several forms of cancer (Gomez-Romero *et al.*, 2007; Psaltopoulou *et al.*, 2011).

Polyamines are also an important factor in human health. While studies of their role in tumour growth have generally warned against consuming a diet high in polyamines,

polyamines play a wider role beyond their classical function as promoters of cell proliferation and insufficient polyamine intake may itself be a factor contributing to the pathogenesis of chronic diseases (Hunter and Burritt, 2011). Decreased polyamine levels in mammalian cells are associated with increased expression of tumour suppressor genes. A reduction in tumour suppressor gene expression is an important step in carcinogenesis and maintaining adequate intracellular polyamine pools in healthy patients may aid in cancer prevention (Seiler and Raul, 2005). Polyamines also suppress the expression of adhesion molecules involved in inflammation, helping the body to maintain an appropriate inflammatory status and reducing risks associated with high blood pressure (Soda *et al.*, 2005; Hunter and Burritt, 2011). In addition to their other roles there is some evidence that polyamines may be able to protect against oxidative stress by directly scavenging free radicals, although *in vivo* evidence for this function is debated (Mozdzan *et al.*, 2006). They may also provide protection to DNA from ROS attack through their role in promoting compaction of DNA, and by functioning as metal chelators thereby limiting the production of OH· through the Fenton reaction (Mozdzan *et al.*, 2006; Muscari *et al.*, 1995). As the body's ability to synthesise endogenous polyamines decreases with age, a polyamine rich diet may be beneficial in reducing the incidence of chronic disease amongst the elderly (Majumdar, 2003; Nishimura *et al.*, 2006).

All foodstuffs are complex mixtures of chemicals that may alter the functioning of individual compounds. The combination of compounds in foodstuffs may have potentiating, antagonising or synergistic effects (Jansen *et al.*, 2008; Poiroux-Gonord *et al.*, 2010). Interactions between active components are particularly important when considering the action of foods thought to promote good health. Berberine, for example, is a compound with anti-cancer properties thought to be an active component of the medicinal herb *Coptidis rhizoma* however the anti-cancer effect of whole-plant extract is more effective than pure berbine, suggesting potentiating or synergistic interactions also exist within the extract (Raskin and Ripoll, 2004). In other mixtures individually ineffective compounds may exhibit potent effects when combined, as is the case of some flavonoids, saponins and volatile oils found in some plants with mild diuretic properties (Raskin and Ripoll, 2004). In the current experiments, material from lettuce leaves was shown be able to protect the DNA of Caco-2 cells from oxidative damage *in vitro*. The heightened ability of lettuce leaves from a red variety to protect against oxidative damage to DNA in Caco-2 cells suggests a synergistic effect of anthocyanins and other antioxidants. However, while synergistic interactions between anthocyanins and ascorbic acid have been recorded before (McCune *et al.*, 2011), further research is needed before the effect of each component can be verified.

5.3 Environmental manipulation and plant quality

While there is considerable evidence that a healthy diet including fruit and vegetables can reduce the risk of chronic disease, attempts at encouraging people to eat more fruit and vegetables such as "5+ a day" campaigns have met with only limited success (Poiroux-Gonord *et al.*, 2010). It may therefore be beneficial to increase the content of health-promoting phytochemicals in commonly consumed crop plants. Two approaches towards this goal are possible: genetic approaches targeted at increasing the yield of desirable phytochemicals and manipulation of the growth environment to achieve the same end. Genetic approaches have had success in increasing the levels of nutritionally beneficial phytochemicals such as ascorbate and β-carotene in several crop plants, 'Golden Rice' being a famous example. However, genetic approaches must necessarily involve the methods of traditional breeding which are time-consuming and not all consumers are willing to consume genetically-modified food (Poiroux-Gonord *et al.*, 2010; Sands *et al.*, 2009). As many nutritionally or pharmacologically valuable phytochemicals play a key role in plant stress responses, horticultural practices that moderately increase environmental stressors could improve the quality of existing crops without the need to develop new varieties.

Altering environmental conditions has been shown to have a positive effect on the antioxidant contents of fruits and vegetables. Broccoli (*Brassica oleracea* L.) heads have been shown to accumulate ascorbic acid when greenhouse temperature was lowered, an effect that was enhanced under increased light intensity (Schonhof *et al.*, 2007). Similar effects of low temperature and high light intensity have been observed in a variety of vegetables including tomatoes and spinach (Eskling and Akerlund, 1998; Poiroux-Gonord *et al.*, 2010). Exposure to moderate levels of UV-B during growth is another factor shown to enhance ascorbate levels lettuce and spinach (Jansen *et al.*, 2008), findings that the current experiments corroborate. Environmental manipulations have also been shown to affect the levels of phenolics, which may also exhibit antioxidant activity. The level of anthocyanins in a wide variety of food crops has been shown to increase with low doses of UV-B, including maize, rice, apple and cherries (Guo *et al.*, 2008; McCune *et al.*, 2011). However, while physiological studies have indicated scope for increasing the level of antioxidants in plant crops through environmental manipulations, the results of agronomic studies are mixed and effects often vary significantly between cultivars of the same species. In studies where crops have been exposed to differing UV-B irradiation through the use of horticultural plastics of increased UV-B transparency, leafy vegetables such as lettuce and pak choi (*Brassica rapa* L.) have shown the greatest increases in phenolics and antioxidant capacity, an effect

particularly marked in red-coloured cultivars (García-Macias *et al.*, 2007; Ordidge *et al.*, 2010; Tsormpatsidis *et al.*, 2008; Zhao *et al.*, 2007b). Fruits such as blueberries and strawberries on the other hand show little or no variation regardless of the UV-B environment, possibly because commercial varieties of these fruits are bred for consistency of color (Ordidge *et al.*, 2010). While there is considerable scope for the improvement of the antioxidant contents of crops through environmental manipulations, the genetic factors are obviously of importance in facilitating or limiting responses to environmental conditions.

Fewer studies have focused on the effect of horticultural factors on polyamine levels. While there is evidence that polyamine levels are increased by abiotic stress, most studies to date have been short-term and some authors suggest that polyamine levels are up-regulated only temporarily during the first phases of adaptation to environmental stressor (Jansen *et al.*, 2008; Sfichi-Duke *et al.*, 2008). Temporary accumulation of polyamines early in the stress response could limit the gain to be had from manipulating environmental conditions if levels return to normal prior to harvest. However, a recent study by Neelam *et al.* (2008) suggests that environmental variables can promote polyamine accumulation. Non-transgenic tomatoes grown in legumous hairy vetch (*Vacia villosa*) mulch displayed higher polyamine accumulation than those grown with black polythene instead of mulch. Gene expression of mulch-grown plants revealed altered carbon and nitrogen assimilation, partially mediated by polyamine biosynthesis and SAMDC (Neelam *et al.*, 2008). While nitrogen metabolism was not investigated in the current experiments, there was generally no decrease in polyamine accumulation over time similar to that suggested by other authors and it is tempting to suggest that high levels of polyamine accumulation could be maintained by a combination of abiotic stress and adequate nitrogen supply.

Although exposure to environmental stressors is able to promote the accumulation of several nationally and pharmacologically valuable phytochemicals in crop plants, such treatments frequently come at the cost of decreased yield (Tsormpatsidis *et al.*, 2010). This is often the case of plants exposed to UV-B radiation. While significant growth inhibition was not always found in the current experiments, previous studies have found lettuces in tunnels constructed from a covering to transparent to small amounts of UV-B yield 34 % less fresh weight than lettuces grown in tunnels that blocked UV-B, and 44 % less in tunnels made of covering that transmitted the whole UV-B spectrum (García-Macias *et al.*, 2007). Another study found above-ground biomass of lettuces to be up to 2.2 times less under UV-B transparent plastic films compared to standard horticultural film (Tsormpatsidis *et al.*, 2008). In light of these effects, a recent study suggested that to maximize both the levels of beneficial phytochemicals

and yield, plants should be transferred from UV-B free to ambient UV-B conditions a few days before harvest (Tsormpatsidis *et al.*, 2010). However, while plants treated in this manner did have comparable biomass to those grown in UV-B free conditions, such treatment may not be advisable. In the current experiment markers of oxidative stress and oxidation of the ascorbate and glutathione pools suggest significant levels of ROS remained seven days after the start of UV-B irradiation. Consuming food with high levels of ROS or lipid hydroperoxides is likely to place cells in the gut under oxidative stress (Wijeratne *et al.*, 2005). Allowing insufficient time between environmental manipulation and harvest may thus limit or even damage the health benefits to be gained from the product. The current experiments demonstrate the need to fully investigate the dynamics of antioxidant responses in plants before recommendations can be made for horticultural practice.

5.4 Conclusions

Considerable potential exists to improve the health-promoting qualities of lettuce crops through interventions aimed at increasing UV-B levels in the growth environment, such as growing lettuces under UV-B transparent coverings. Moderately increased exposure to UV-B results in oxidative stress and increased activity of the enzymes SOD, CAT, APox, GPox and GR, and in the accumulation of ascorbate and glutathione. These compounds function as important moderators of the redox status of cells. Accumulation of the polyamines Put, Spd and Spm is also increased by synthesis via the ADC and SAMDC pathways, polyamine pools tending towards the accumulation of longer-length and bound polyamines as UV-B adaptation progresses. The increased activity of ADC in UV-B exposed plants supports the hypothesis that Spd and Spm are involved in protection of the photosynthetic apparatus under stress as previous studies have indicated that this enzyme is primarily localized to chloroplasts. Lettuce varieties, however, differed in their accumulation of nationally valuable phytochemicals. Both antioxidants and polyamines accumulated less in the red cultivar 'Red Salad Bowl' when exposed to UV-B, possibly because UV-B absorbing pigments interfered with induction mechanisms. Finally, the potential of lettuce as a functional food was shown through its ability to protect against oxidative attack in Caco-2 cells. Red lettuce was more effective than green lettuce in this respect, probably because of the action of additional antioxidants that were not present in green lettuce, such as anthocyanins. However, recommendations for horticultural practice must take into account the kinetics of the antioxidant response as harvesting too soon after the application of a stressor could result in a product with high levels of ROS.

5.5 Future directions

The current study highlights several possible areas of future research that could enhance our understanding of stress responses in plants and their implications for human health. Investigating the localization and specific isoforms of antioxidative enzymes up-regulated in response to UV-B could provide valuable insights into the mechanisms behind UV-B stress and the wider processes of redox regulation in plant cells (Foyer and Noctor, 2009). Likewise, studying the localization of polyamines within the plant cell during stress responses would help build a more complete picture of their functioning. Finally, while the ability of lettuce material to protect human cells from oxidative stress was demonstrated, studies of whole foods are unable to determine how compounds present in the food are responsible for the effect (Duthie, 2007). Further research to quantify the contribution of both antioxidants (including phenolics) and polyamines to the protective effects is needed before more detailed recommendations can be made in respect to human nutrition and horticultural practices.

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