The Effect of Ultraviolet-B Radiation on Growth, Photosynthesis and DNA Damage in Wheat (*Triticum aestivum* L. cv. Maris Huntsman and cv. Yecora Rojo).

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Abstract

The effect of UVB on two cultivars of *Triticum aestivum* L. (cv. Maris Huntsman and Yecora Rojo) was studied to determine if a cultivar bred for growth in Saudi Arabia (Yecora Rojo) showed a greater resistance to UVB than a cultivar from Britain (Maris Huntsman). Seedlings were grown in a growth cabinet with and without supplementary UVB (9.3 kJ m^{-2} day^{-1} biologically effective UVB; 16 hour photoperiod: 300-400 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF; λ 400-700nm)). UVB caused a decrease in leaf height, leaf weight and leaf area in both cultivars, although the British cultivar, Maris Huntsman was affected to a greater extent. Cell division and cell elongation were reduced in both cultivars with UVB, and this would account for the observed reduction in leaf growth. Plants were grown with and without supplementary UVB and then exposed to a short UVB irradiation. The amount of DNA damage, both cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6,4) pyrimidone dimers (6-4PPs) was measured using an ELISA. The results showed that when the plants were grown either with or without supplementary UVB Maris Huntsman accumulated more CPDs and 6-4PPs than Yecora Rojo. The difference in CPD and 6-4PP accumulation was in part due to Yecora Rojo being able to repair the damage at a faster rate. When grown without supplementary UVB, Yecora Rojo was found to contain more UVB-absorbing compounds in its leaves than Maris Huntsman. In contrast, when grown with supplementary UVB the concentration of UVB-absorbing compounds was found to be the same in both cultivars. Three flavonols were detected in the cultivars: quercetin, kaempferol and isorhamnetin. The concentration of these flavonols increased in the leaves of Maris Huntsman after 6 days growth under supplementary UVB but not in Yecora Rojo. Analysis of the penetration of light (wavelengths, 310nm and 430nm) into the leaf tissue, showed that the amount and the depth of penetration was greater in Yecora Rojo than in Maris Huntsman. When the cultivars were grown with supplementary UVB, Yecora Rojo selectively decreased the amount of 310nm radiation penetration into the leaf, while the amount of 430nm radiation increased. Therefore, the results from this study show that the Saudi Arabian cultivar, Yecora Rojo was affected to a lesser extent by UVB than the British cultivar, Maris Huntsman. The reasons for this difference will be discussed in this thesis.

Declaration

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

Pamela Gray
January 2000

Statement

I was admitted as a research student in October 1996 and as a candidate for the degree of Doctor of Philosophy in October 1996; the higher study for which this is a record was carried out in the University of St Andrews between 1996 and 2000.

Pamela Gray
January 2000

Certificate

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

Dr AK Tobin
January 2000

Copyright

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Abbreviations

A	absorbance
ANOVA	analysis of variance
cdt	cell doubling time
CFCs	chlorofluorocarbons
Chl	chlorophyll
Chl a	chlorophyll a
Chl b	chlorophyll b
cp	chloroplast
CPD	cyclobutane pyrimidine dimer
dwt	dry weight
ELISA	enzyme-linked immunosorbant assay
fwt	fresh weight
g	relative centrifugal force
HPLC	high pressure liquid chromatography
i	specific leaf segment under investigation
J	jolues
LER	leaf elongation rate
LM	light microscopy
MI	mitotic index
mV	millivolts
PAR	photosynthetically active radiation (400-700nm)
PBS	phosphate buffered saline
6-4 PP	pyrimidine (6,4) pyrimidone dimer
PPF	photosynthetic photon flux
PSI	photosystem I
PSII	photosystem II
RAF	radiation amplification factor
rbc S	mRNA encoding small subunit of Rubisco
rbc L	mRNA encoding large subunit of Rubisco
Rubisco	ribulose-1,5-bisphosphate
SER	segmental elongation rate
SLA	specific leaf area (mm^2 mg^{-1} dry weight)
UV	ultraviolet
UVA	ultraviolet-A
UVB	ultraviolet-B
UVC	ultraviolet-C

V_D	vertical displacement rate
v/v	volume/volume
W	watts
w/v	weight/volume

Chapter 1

Introduction

Introduction

1.1 Solar Radiation

The most important source of natural radiant energy is the sun, which emits a continuous spectrum of light (Hart, 1988). Over half of the wavelengths emitted by the Sun do not reach the Earth due to atmospheric effects, and so only wavelengths of light between 290 and 1500nm reach the Earth's surface (Attridge, 1990).

1.2 Ultraviolet Radiation

The ultraviolet (UV) region of the solar spectrum is between 40 and 400nm and although UV radiation contributes little energy to the solar spectrum, it is important because it is biologically active (Ambach *et al.*, 1991). UV radiation can be divided into four classes, Vacuum UV (40-200nm), UVC (200-280nm), UVB (280-320nm) and UVA (320-400nm) [see Fig. 1.1 and Table 1.1]. Vacuum UV and UVC radiation are not present at the Earth's surface because they are absorbed by oxygen, ozone and other gases in the stratosphere and troposhere (Tevini, 1993). Both UVB and UVA radiation reach the Earth's surface because ozone does not completely absorb these (Madronich, 1998). Exposure to shorter wavelengths are much more damaging to many biological processes than exposure to longer wavelengths, and even small increases in UVB can lead to considerable biological effects (Madronich, 1998). The amount of UVB at the Earth's surface can vary due to a number of factors such as altitude, latitude, time (day-length and season), amount of aerosols, cloud cover and amount of stratospheric ozone (Madronich, 1993).

1.3 Natural UVB Gradients

1.3.1 Latitude and Altitude

The angular position of the sun in the sky (solar zenith angle) varies with latitude, thus affecting the amount of UVB reaching the Earth's surface (Madronich, 1993). The highest UVB values are at the tropics and mid latitudes, with the lowest values occurring at the polar regions (Madronich, 1993). The altitude effect on UVB levels is highest at lower solar elevations and at shorter wavelengths since the altitude effect depends on the amount of irradiated air mass and on wavelength (Blumthaler, 1993). With increasing elevation above sea level, UVB

steep latitudinal gradient of solar UVB radiation exists along the Arctic/alpine life zone. This steep UVB gradient was the result of a number of factors, such as the natural latitudinal gradient in total atmospheric ozone column thickness, and the prevailing solar angles at different latitudes. The study of a comparison of UV radiation levels between Arctic and Alpine sites at high altitude stations in Switzerland and in Alaska (Ambach *et al.*, 1991), found that the UV flux in all seasons was greater for Switzerland than for Alaska. The differences in this study were more severe in winter because in the summer the lower solar elevation was compensated for by longer day length. Although natural UVB levels are higher at lower latitudes than higher latitudes, the UVB readings in the Antarctic (64°S) during the springtime ozone depletion, can exceed that in San Diego, USA (32°N) (Madronich, 1995).

1.3.2 Cloud Cover

Clouds can also change the amount of UVB reaching the Earth's surface (Caldwell *et al.*, 1980). Surface UVB irradiances are generally reduced with clouds, although the change in UVB levels with cloud cover is variable depending on cloud cell morphology, cloud amount and coverage, particle size and the position of the clouds relative to the sun (Madronich, 1993; Madronich *et al.*, 1998).

1.3.3 Time

There is a large natural variation in daily and seasonal effective UVB radiation reaching the Earth's surface. The UVB level depends on the daily and seasonal variation of the angular position of the sun relative to the Earth, and on the ozone concentration at that time of year (Madronich, 1993). Therefore, UVB levels vary with time of day and time of year (Blumthaler, 1993).

1.3.4 Natural Ozone Variations

Ozone occurs in trace amounts throughout the vertical extent of the atmosphere, although it is most important and abundant in the stratosphere (between an altitude of 15 and 40 km) (Drake, 1995). The distribution of ozone around the globe is not uniform and the ozone layer is thickest at the poles and thinnest at equatorial latitudes (Ott, 1993). At the equator, ozone varies little with season; however, at all other latitudes there is marked seasonal variation in ozone, with the maximum ozone concentration in spring and the minimum in autumn (Ott, 1993). As well as the natural variations in ozone concentration around the Earth, there is a decrease in stratospheric ozone concentration due to the release of ozone-depleting substances arising from human activity.

1.4 Stratospheric Ozone Depletion

Stratospheric ozone reduction results in an increase in ambient levels of UVB radiation reaching the Earth. For every 1% decrease in ozone there is approximately a 2% increase in UVB reaching the Earth's surface (Blumthaler & Ambach, 1990). Chlorofluorocarbons (CFCs) and other halocarbons, such as carbon tetrachloride and those containing bromine produced by human activity, are responsible for the thinning of the ozone layer (MacKenzie, 1987). CFCs are long-lived gases that accumulate in the atmosphere and break down only in the stratosphere and release chlorine there. Chain reactions initiated by chlorine atoms and involving ClO then act to deplete the ozone layer. A chlorine atom can be released into the atmosphere by stratospheric photolysis of CF_2Cl_2:-

$$CF_2Cl_2 + hv \text{ --->} Cl + CF_2Cl$$

Thus, the atomic chlorine atom produced can enter the catalytic cycle that efficiently destroys ozone between 35- 45 km altitude:-

$$Cl + O_3 \text{ ---> } ClO + O_2$$
$$ClO + O \text{ ---> } Cl + O_2$$

During the 1987 Antarctic Atmospheric Ozone Experiment when the polar ozone layer was sampled, it was confirmed that chlorine and bromine catalysed reactions are responsible for more than 50% loss of ozone over the South Pole each spring (Anderson *et al.*, 1989). In order to decrease the atmospheric abundance of chlorine before the year 2100 (the minimum necessary for recovery of the Antarctic ozone hole) almost all emissions of halocarbons need to be phased out (Prather & Watson, 1990).

As a result of ozone depletion there is a change in the amount of UVB at various latitudes. For example, a 16% ozone reduction would result in a 47% increase in daily biologically effective radiation at 40°N latitude which is equivalent to moving 20° further south in latitude without ozone reduction (Caldwell *et al.*, 1989).

1.5 Biological Weighting Functions

When assessing the biological consequences of ozone reduction, spectral responses must be determined. A reduction in ozone results in an increase of solar UVB between 290 and 320nm. The quality of this additional radiation resulting from ozone depletion is small compared with the background of total solar UV (Flint & Caldwell, 1996). Therefore, the additional radiation is only important if the biological responses are more sensitive to the shorter wavelength than the longer wavelength radiation. To calculate the biological

importance of UVB, a biological weighting function is incorporated into an equation along with the spectral irradiance at each wavelength. The biological weighting functions used may come from action spectra (Fig. 2). There are several action spectra associated with damage to plant tissue (Caldwell *et al.*, 1995) which all have a common feature: the shorter UVB wavelengths are the most important since they are more damaging to biological processes. However, the rate of decline (in relative quantum effectiveness) with wavelength varies considerably between action spectra. This decline is known as the radiation amplification factor (RAF). Small RAF values are the result of action spectra which do not decrease sharply with wavelength. Therefore, when calculating the biological importance of UVB it is critical to choose the correct action spectrum (see Fig. 1.2)

Action spectra have usually not been developed for the purpose of evaluating biological effects of UVB on plants. Photobiologists are able to use action spectra to predict how a pigment or molecule will absorb radiation and mediate the effect within an organism (Madronich *et al., 1995*). Thus, action spectra are developed usually by exposing the biological material to one wavelength at a time and measuring the effect. However, when studying the effects of UVB on biological systems it is more important to know how a molecule is affected by radiation in its normal state within an organism and how its effect may be altered by the activity of other absorbing molecules. For example, UVB will have a different effect on naked DNA (i.e. DNA *in vitro*), than it will have on DNA within a plant (i.e. DNA *in vivo*). So, a DNA action spectrum developed from *in vitro* DNA would differ from a DNA action spectrum evaluating the response of DNA *in vivo*. However, it is difficult to develop action spectra and as a result most are created using *in vitro* material. Therefore, when using biological weighting functions based on action spectra, these limitations must be remembered when considering the significance of biologically effective radiation.

1.6 Effects of Enhanced UVB Radiation on Biological Processes

UVB has an effect on various biological processes. It is absorbed by proteins and the nucleic acids, DNA and RNA, and this could have important biological consequences (Stapleton, 1992). UVB can alter the structure and function of nucleic acids in plants and cause damage to DNA, for example UVB inhibits replication and transcription and as a result interferes with cell growth, division and other activities (Rupert, 1986; Pang and Hayes, 1991). Trophic level interactions can be affected by UVB (Bothwell *et al.*, 1994), and the production and decomposition of plant matter can be modified by UVB which

may result in changes in the uptake and release of atmospherically-important trace gases (Zepp *et al.*, 1995).

1.7 Effects of Enhanced UVB on Plants

The amount of solar radiation which an object intercepts is dependent upon the orientation of the object with respect to the Sun. Because plants are sedentary they are constantly exposed to solar radiation and since their leaves are positioned to intercept large amounts of photosynthetically active radiation, an increase in UVB is likely to affect their growth and development.

1.7.1 Plant Growth

Many studies examining the effects of UVB on plants have focused on how UVB affected growth and morphology. A reduction in plant height is a common finding in UVB studies, found for example in soybean (*Glycine max*) (Teramura, 1980), rice (*Oryza sativa*) (Teramura *et al.*, 1991), maize (*Zea mays*) (Santos *et al.*, 1993) and wheat (*Triticum aestivum*) (Barnes *et al.*,1988). Leaf area is reduced by a number of stresses such as, water, temperature and salt stresses and therefore it is foreseeable that leaf area is reduced by UVB radiation (Teramura, 1983). Leaf area reduction with UVB has been reported in *Glycine max* (Teramura & Caldwell, 1981; Sullivan & Teramura, 1990), *Oryza sativa* (Teramura *et al.*, 1991) and *Zea mays* (Mark *et al.*, 1996). The extent to which growth is reduced by UVB is dependent on species and variety. For example, when the effect of increased UVB on the growth of European cultivars of *Zea mays* was examined, the growth reduction in the central European cultivars was much more pronounced than that of the other cultivars (Mark *et al.*, 1996).

With UVB, reductions in fresh and dry weight have also been observed (Mark *et al.*, 1996) as well as increases in leaf thickness (Cen & Bornman, 1993). An increase in leaf thickness with UVB is thought to be a protective mechanism resulting in a decrease in the amount of UVB penetrating through the epidermis to the photosynthetic tissue (Cen & Bornman, 1993).

Changes in plant growth with UVB could be caused by a number of factors, such as changes in cell elongation and/or cell division (Dickson & Caldwell, 1978) , changes in leaf expansion (González *et al.*, 1998) and changes in plant growth regulators (Ros & Tevini, 1995). These will be discussed in further detail in Chapter 3.

1.7.2 Photosynthetic Targets

As photosynthesis is one of the most critical processes affecting plant productivity, the effects of UVB on photosynthesis have been studied

extensively. The carbon balance of plants can be influenced by UVB radiation in four different ways, with effects on 1) the primary photochemical events and electron transport reactions, 2) the Calvin cycle, 3) dark respiration, and 4) stomatal resistance (Teramura, 1983).

Most evidence suggests that Photosystem II (PSII) is the main target for UVB-induced damage (He *et al.*, 1994; Chow *et al.*, 1992; Caldwell *et al.*, 1982; Wilson & Greenberg, 1993). However, more recent studies have shown that with UVB, a reduction in photosynthesis may occur without any damage to PSII (Middleton & Teramura, 1993; Allen *et al.*, 1997). It has also been shown that Ribulose 1,5-bisphosphate carboxylase (Rubisco) activity is inhibited by UVB exposure (Jordan *et al.,* 1992).

Chloroplast structure may be directly altered by UVB radiation (He *et al.*, 1994), and this can lead to inhibition of photosynthetic function. *Pisum sativum* seedlings were studied to determine whether physiological damage was paralleled by changes in ultrastructure. In plants treated with UVB, the chloroplasts lost their structural integrity, manifested by dilation of the thylakoid membranes and disintegration of the double membrane envelope surrounding the organelle. Ultrastructural damage to chloroplasts by UVB was also reported in sugar beet (*Beta vulgaris*) leaves where disruption of the chloroplastic envelope was observed as well as dilated thylakoids and membrane degradation (Bornman *et al.*, 1983). The effects of UVB on photosynthesis will be discussed in further detail in Chapter 3.

1.7.3 DNA Damage and Repair

When irradiated with UVB, DNA absorbs photons which cause several types of damage: single-strand breaks, DNA-protein cross-links and pyrimidine dimers. Most of the UVB-induced DNA damage is in the form of pyrimidine dimers (cyclobutane pyrimidine dimers (CPD) and pyrimidine (6,4) pyrimidone dimers (6-4PP)) (Grossweiner & Smith, 1989). Dimers have been detected in the nucleus, chloroplast and mitochondrion (Cannon *et al.*, 1995; Chen *et al.*, 1996; Hada *et al.*, 1998).

As plants are constantly exposed to UV it is conceivable that they have several mechanisms for either tolerance or elimination of UV-induced DNA damage. 'Tolerance' mechanisms exist that permit DNA replication even in the presence of DNA damage products. Hence, the cell manages to get through replication even though the dimer is unrepaired.

Two DNA repair mechanisms have been identified in plants; photoreactivation and excision repair (Sancar, 1996; Quaite *et al.,* 1994; Taylor *et al.*, 1996). Photoreactivation is the most common method used by plants and

in this process a class of photoactivated enzymes, photolyases, use energy from photons between 300 and 500nm to catalyse the repair of *cis* , *syn* -cyclobutane dipyrimidines (Sancar, 1996). Thus, longer wavelengths of light (UVA and visible wavebands) are integral to the repair of UVB-induced lesions. The other method of repair, excision repair, is a more complex mechanism but can take place in the dark (Stapleton, 1992). Both photoreactivation and excision repair have been observed in a number of plant species such as alfalfa seedlings (*Medicago sativa*) (Quaite *et al* ., 1994), mustard (*Sinapis alba*) (Bucholz *et al.*, 1995) and *Triticum aestivum* (Taylor *et al.*, 1996). DNA damage and repair will be discussed in more detail in Chapter 4.

1.8 PROTECTIVE MECHANISMS

1.8.1 Pigments

The synthesis of protective pigments is a well studied response of plants to UVB radiation (Flint *et al.*, 1985; Lui *et al.*, 1995; Karabourniotis *et al.*, 1992; Santos *et al.*, 1993). These pigments, mainly flavonoids, are phenolic compounds which absorb in the UVB waveband. Flavonoids are a class of secondary metabolites which are synthesised in vascular plants in response to environmental stimuli, and appear to act as 'sunscreens' for vulnerable plant tissues (Kootstra, 1994). UVB-absorbing pigments absorb light over a wide range of the light spectrum, however they do transmit photosynthetically active radiation. These metabolites are unique to plants and are essential for successful survival of plants as sedentary organisms living in diverse and constantly fluctuating environments (Shirley, 1996). An increase in UVB-absorbing pigments, with UVB, has been found in *Oryza sativa* (Sato & Kumagai, 1993), *Brassica napus* (Olsson *et al.*, 1998), *Pisum sativum* (Day *et al.*, 1996) and bean (*Vicia faba*) (Flint *et al.*, 1985) among others.

Most previous studies have measured the concentration of flavonoids and other phenolic compounds in whole leaf extracts. However, it may be misleading to only look at the concentration of flavonoids in whole leaf extracts. The most effective UVB filter would be phenolics such as flavonoids, located in the surfaces exposed to solar radiation for example in the epidermis. Epidermal leaf strips from the shaded and unshaded leaves of *Urginea maritima* were tested for their UVB-absorbing capacities and leaf optical properties (Grammatikopoulos *et al.*, 1999). It was found that exposed leaves had much higher UVB-absorbing capacities compared to shaded plants.

1.8.2 Epidermal Shielding

The epidermis provides the first line of defence in preventing UVB from penetrating the tissue. Leaf reflectance, in the vast majority of plants, is low and the dominant UVB screening mechanism appears to be epidermal attenuation (Robberecht & Caldwell, 1978). Waxes may also be of direct significance in attenuating the level of UV radiation reaching the sensitive targets in the leaf mesophyll.

UVB has a marked effect on the production and chemical composition of epicuticular wax in a range of crop plants (Steinmüller & Tevini, 1985; Gonzalez *et al.*, 1996). These studies showed that elevated levels of UVB stimulate wax production, but the extent of this effect seems to be determined by the biological effective dose of UVB, plant species and leaf orientation.

1.9 Growth Conditions Used To Study Effects of Enhanced UVB on Plants

Many experiments have been carried out on plants both in the field and in controlled-environment growth cabinets. Most experiments have shown UVB to have an effect on plant growth, although experiments under field conditions generally indicate that UVB has a smaller effect when compared with those carried out in growth cabinets (Caldwell *et al.*, 1989; Tevini, 1993 and Caldwell & Flint, 1993). Teramura and Murali (1986) conducted a long term study of soybean (*Glycine max*) varieties both in growth cabinets and under field conditions. They found large differences between the varieties and both positive and negative growth responses to UVB. For vegetative characters, the rank order of variety sensitivity was similar in growth cabinet and field conditions. However, the rank order for the seed yield of the varieties was different in growth cabinet and field conditions. Perhaps the absence of detectable effects in many field studies is partly due to the great environmental variability that plants experience in the field, and therefore small differences due to UVB treatments are more difficult to detect (Caldwell *et al.*, 1989).

When plants are grown in growth cabinets it is possible to change one variable while keeping all others constant. However, in the field most plants experience multiple stresses. In field experiments environmental factors fluctuate daily, seasonally and annually. This makes the interpretation of results difficult and there is a need for the experiment to be carried out over a number of years in order to rule out inconsistencies in plant responses.

The radiation conditions in growth chambers are very different from those in the field. Webb (1991) measured solar UVB over a period of four months and found that the changes are greatest at the short wavelengths on both a daily and annual basis. For the entire UVB waveband, the changes are much

less dramatic, and this indicates how the signal from the most biologically active end of the spectrum (i.e. at the shorter UVB wavelengths) can be subdued by the greater intensity of radiation at longer wavelengths in broadband measurements. Thus, it is necessary to know the exact UVB output of lights within a growth cabinet i.e. take a reading of each wavelength within the UVB range in order to assess the biological effective UVB dose.

It is important that a realistic balance is maintained between different spectral regions when using growth cabinets to study the effects of UVB. Both UVA (320-400nm) and visible (400-700nm) radiation can affect the plant's response to UVB (Caldwell *et al.*, 1994). If UVA and PAR are low, then the effects of UVB on plants will be much more severe. UVB effectiveness is often exaggerated under growth chamber and greenhouse conditions due to reduced photosynthetic photon flux density (PPFD between 400-700nm) levels (Mirecki & Teramura, 1984). This may be due to a reduction in photoprotection and photoreactivation mechanisms. However, it is often necessary to carry out experiments in a growth chamber as a first step to define the plant response (Caldwell *et al.*, 1995).

1.10 Variation Between Species & Cultivars

When plants are grown under UVB, a number of changes to the plant may occur such as reductions in growth and photosynthesis and increases in UVB-absorbing compounds, as already discussed. However, the effects of UVB on plants are extremely variable depending greatly on species and particular growth conditions (Caldwell & Flint, 1993) and some plant species do not seem to be affected by UVB at all. Some evidence is available which suggests species and ecotypes native to low latitudes are inherently more resistant to UVB irradiation than other plants (Caldwell *et al.*, 1982). Therefore, the ability of a plant to adapt to UVB might depend on where it originates, that is the altitude and latitude of its native habitat. It may be possible to identify specific characteristics or physiological traits associated with maintaince of productivity under increasing UVB irradiance if plant populations originating from contrasting UVB environments are studied. This may then lead to a greater understanding of the potential shifts in species composition arising from an increase in UVB. A number of studies have compared how species from different areas are affected by UVB.

The amount of UVB reaching the photosynthetic apparatus and nucleic acids in the mesophyll of Rocky Mountain plants was examined (Day *et al.*, 1992). Some life forms appeared to be more effective at screening out UVB radiation than others. Herbaceous dicots were particularly ineffective at

attenuating UVB, whereas in conifers virtually none of the UVB reached the mesophyll. The leaves of woody dicots and grasses were shown to be intermediate between these two extremes.

Seeds from plant species growing over a 3,000m gradient in Hawaii were collected (Teramura and Sullivan, 1991). Under the conditions used, only a small percentage of the species, collected from sea level to 500m, were tolerant to UVB. However, tolerance increased in species collected from higher elevations; 75% in species from 500- 1,000m, and 100% at higher elevations. Therefore, all of the species at an elevation greater than 2000m had adapted to high levels of UVB.

Most studies have concentrated on examining the effects of UVB radiation on the growth of temperate crop species with much less work focusing on the impact of UVB on tropical crops. Since there is a large natural variation in UVB, it has been suggested that adaptation to UVB may be more developed in species which occur in high UVB irradiance environments for example, low latitude, high elevation locations (Ziska *et al.*, 1992).

Carbon uptake, pigment content and maintenance of growth and development were examined in plant material grown (in growth cabinets) from seeds collected at contrasting elevations in Hawaii (Ziska *et al.*, 1992). The plants were chosen based on their differential sensitivity to UVB. Plants from higher elevations, where natural UVB radiation is high, were not affected when grown under UVB. Contrastingly, under UVB there was a significant decline in biomass production and photosynthesis in plants from the lower elevations. UVB-absorbing compounds increased in the plants from lower elevations but not in plants from higher elevations, although UVB-absorbing compounds were produced in larger amounts by the plants from higher elevations. Therefore, it seems that plants from higher elevations have adapted to maintain productivity in a high UVB environment. Perhaps ecotype differentiation in response to increased UVB over an elevated gradient may have occurred. Community processes and dynamics could be determined by the extent of species adaptability to UVB.

1.11 The Cultivars Under Investigation

The two *Triticum aestivum* cultivars examined in this study were Maris Huntsman and Yecora Rojo. Maris Huntsman was bred for optimum growth in the UK climate, for example Oxford; and Yecora Rojo bred for optimum growth in a high light intensity/high humidity environment such as Riyadh, Saudi Arabia. Climate information for Oxford and Riyadh is given in Table 1.2. The cultivars were not bred for their adaptation to UVB radiation but since there is a

difference in the amount of UVB in Oxford and Riyadh then it was hypothesised that Yecora Rojo would show a greater resistant to UVB than the other.

1.12 Aims of the thesis

The aim of the thesis was to investigate whether the two cultivars of wheat which have been bred for growth in different climates differed in their response to UVB radiation. The growth of the two cultivars under UVB was characterised and the differences between them examined. The amount of DNA damage, UVB-absorbing compounds and amount of light penetrating the leaves of both cultivars was compared to analyse why one cultivar was affected by UVB more than the other.

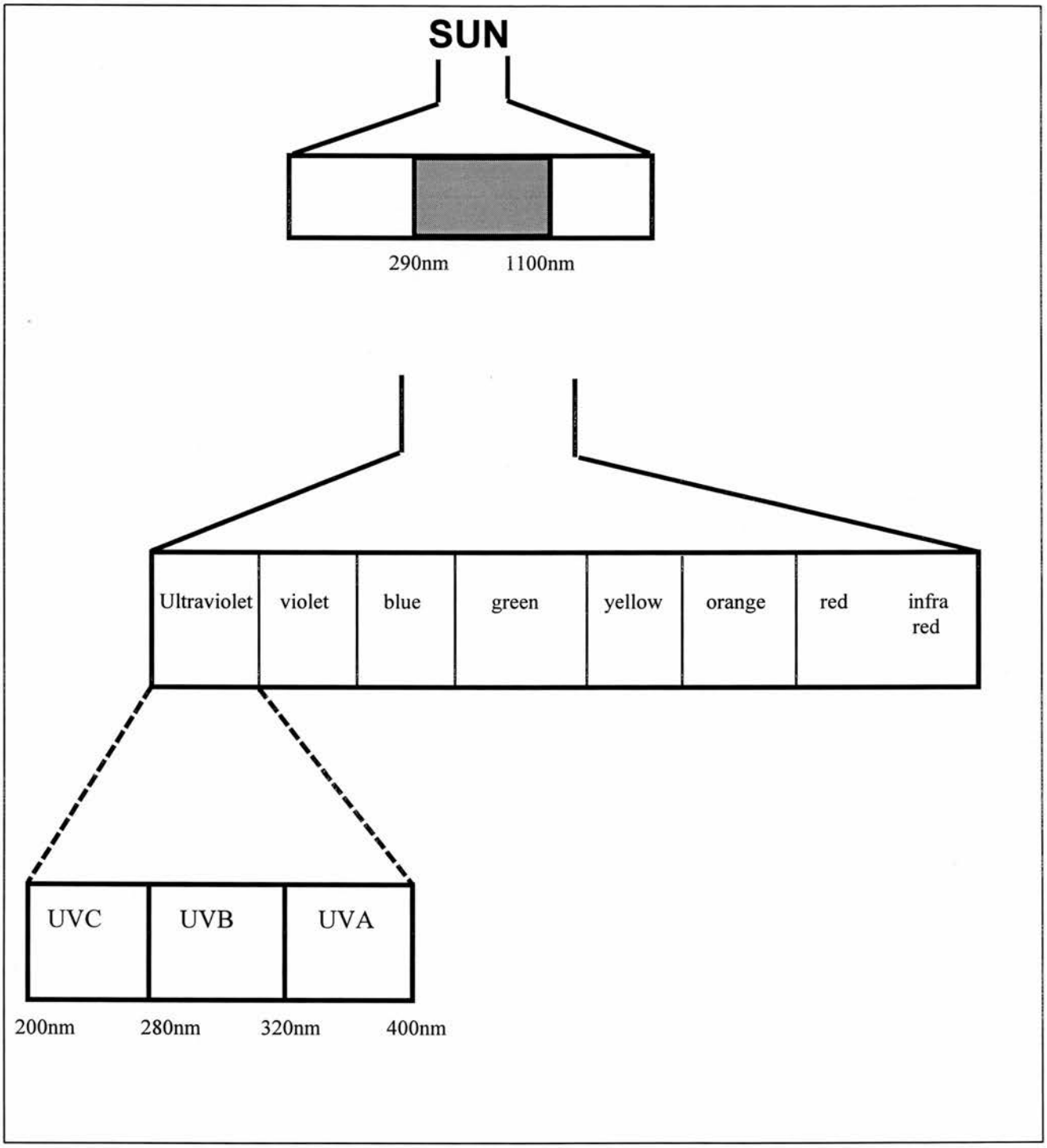


Figure 1.1 The Solar Spectrum (Hart, 1988).

Wavelength (nm)	% energy	Wm^{-2}
Below 200	0.1	1.36
200-300	3.0	40.8
300-400	8.9	121
400-700	36	490
700-1000	24	326
above 1000	28	381

Table 1.1 Spectral distribution of sunlight incident on the atmosphere (Hart, 1988).

	Oxford	Riyadh
Temperature (°C) High Low	35 -15	45 -7
Average Rainfall (yearly, mm)	674	81
Hours of Sunshine (Winter) (Summer)	1-3 5-7	6-8 12-13
Amount of UVB in the month of March ($KJm^{-2}d^{-1}$)*	1.70	7.90

Table 1.2 Climate information for Oxford, UK and Riyadh, Saudi Arabia (pers com. G. Loudon (Meterological Office), 1999).

* Biologically effective UVB radiation, calculated using the Caldwell (1971) generalised plant action spectrum.

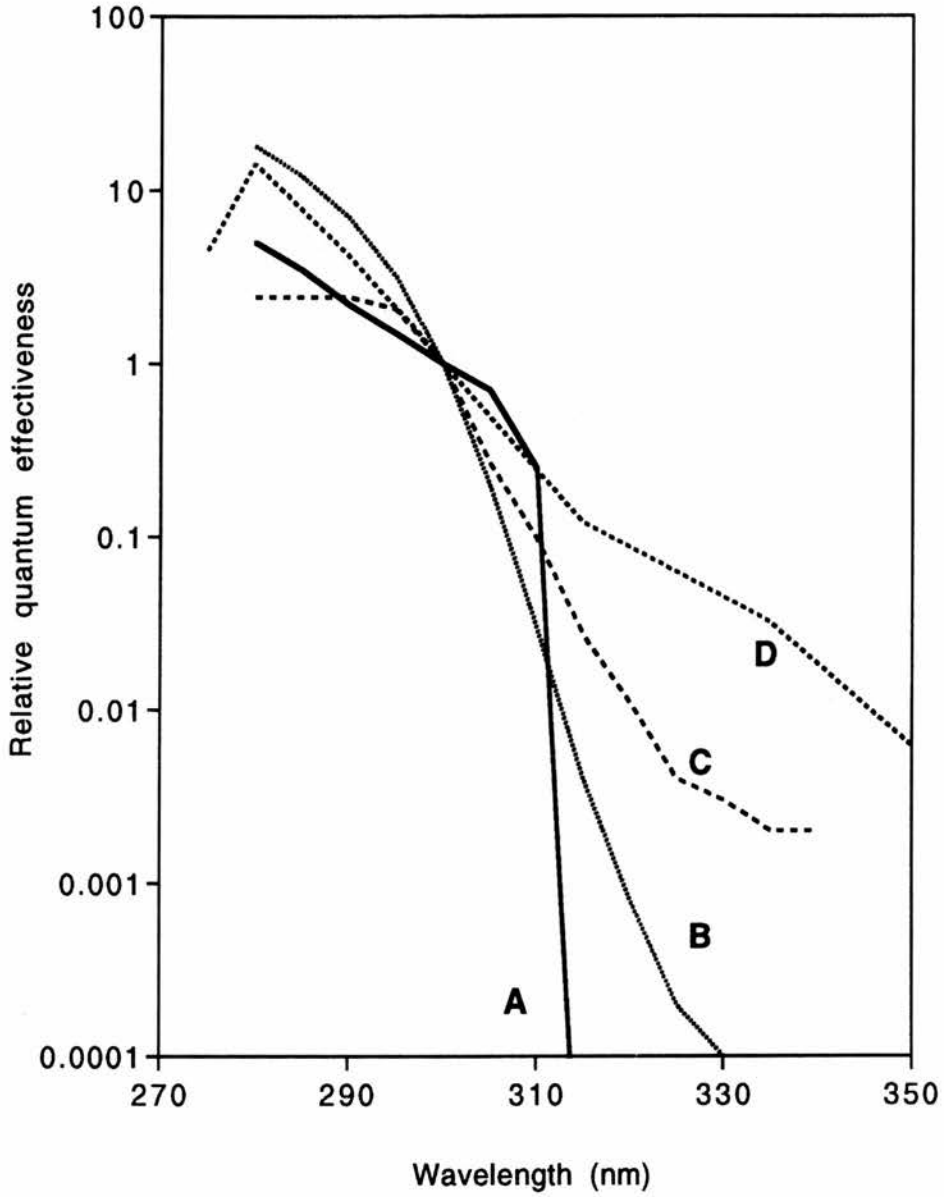


Figure 1.2 Various UV action spectra (Holmes, 1997). Generalised plant damage (A) (Caldwell, 1971); DNA damage (B) (Setlow, 1974); erythema (C) (McKinlay-Diffey, 1987); dimer production in alfalfa (D) (Quaite *et al.*, 1992).

Chapter 2

Materials and Methods

2.1 Materials

All chemicals were obtained from Sigma Chemical Company Ltd., Poole, U.K., and were of Analytical Grade, unless otherwise stated.

2.2 Plant Material and Growth Environments

Wheat (*Triticum aestivum* L.) cv. Maris Huntsman (Plant Breeding International, Cambridge, U.K.) and cv. Yecora Rojo (Arizona Crop Improvement Association, Arizona, USA) seeds were used in all experiments. Seeds were soaked in aerated tap water for 16 h at 20°C, sown at a density of 5g (pre-imbibition weight) of seed per seed tray (16 cm x 10 cm) in M2 medium nutrient potting compost (Levington Horticulture Ltd, Ipswich, U.K.), and covered with 1cm of fine grade vermiculite (Dupre, Hertford, U.K.). Seedlings were grown in a Fi-totron PG1400 environmental growth chamber (Sanyo-Gallenkamp, Loughborough, U.K.) under controlled conditions of 70% constant humidity and photoperiod of 16 h. Temperature was maintained at 20°C during the day and 10°C at night. Within the growth cabinet, visible light was supplied by a combination of high intensity discharge lamps (6 tubes HQI-TS 250W/NDL) and 8 X 60 W pearl tungsten bulbs (Sanyo-Gallenkamp, Loughborough, U.K.). The Quantum flux density was measured using a light meter (Macam Photometrics Ltd, Livingstone, U.K.), and ranged between 300-400 $\mu molm^{-2}s^{-1}$ photosynthetic photon flux (PPF, 400-700nm).

The UV-B treatment was supplied by 4 X 115 cm UV-B lights (Philips TL40/12: Starna Ltd, Romford, U.K.). The amount of UV-B supplied by the lamps was equivalent to 9.3 kJ m^{-2} day^{-1} biologically effective UVB based on Caldwell's (1971) generalized plant damage action spectrum normalised to 300 nm. The plants received this over 14 h. Caldwell's generalized plant damage action specrum was used because this was calculated from a number of parameters using various plant species. Compared to the DNA damage action spectrum which used DNA *in vivo*. Cellulose acetate, 0.13 mm thick, (Warne & Co. Ltd. London, U.K.) was used to cover the UV-B tubes and eliminate penetration of UV radiation below 292 nm. This was changed daily to avoid degeneration of the cellulose acetate.

2.3 Analysis of Leaf Growth

The plants were measured from day 4 to day 7 (days after planting). The seed, roots, coleoptile and secondary leaf were removed from the plants before subsequent experiments were carried out, unless otherwise stated.

2.3.1 Total Plant Height, Primary Leaf Height and Coleoptile Height

Four days post-imbibition, 30 plants from each cultivar were randomly selected and

labelled. Seedling height, primary leaf length and coleoptile height was measured in millimetres, to two decimal places, daily for each plant, using a ruler. Primary leaf length was taken as the distance between the basal meristem and the leaf tip.

2.3.2 Fresh and Dry Weight

The fresh and dry weight of ten primary leaves and coleoptiles from each cultivar was measured daily. The fresh weights were recorded and the leaves placed in paper bags and dried at 60°C for 96 hours. The dried leaves were allowed to cool to room temperature and the weight recorded. The fresh and dry weight of coleoptiles of each cultivar was also recorded as above.

2.3.3 Leaf Area

The area of 10 primary leaves was measured each day from 4 to 7 days post-imbibition. Leaves were mounted onto white paper using adhesive tape. Images of the leaves were captured onto an AnalySIS™ image-analyser using a monochrome CCD camera (Norfolk Analytical Ltd, Hilgay, U.K.). The area of each leaf was measured using the AnalySIS computer package (Software Imaging System, Münster, Germany)

2.3.4 Leaf Elongation

The segmental elongation rate (SER) and velocity of displacement (V_D) were measured in the primary leaves 5 days post-imbibition using a modified method of Schnyder and Nelson (1988). Fifteen primary leaves from each cultivar were randomly chosen, labelled and their height noted. Small holes were pierced 10 times from the leaf base vertically up the leaf, 2 mm apart, using fine wire (diameter 140 μm). The plants were then returned to the growth cabinet and measured again 24 hours later. The leaves were harvested, the coleoptile and secondary leaf removed, and the primary leaf was mounted onto white paper using adhesive tape. The distance between the holes was measured and the SER (i.e. rate of elongation of a leaf segment relative to the rate of elongation of the whole leaf blade) and V_D (i.e. the velocity at which a segment is moving up the leaf blade) were determined for each cultivar using the following equations:-

1) Relative segmental elongation (RSE_i)for the i th segment was calculated as

$$\mathbf{RSE_i = 2\,(D_{i,t_n} - D_{i,t_o})\ X\ (D_{i,t_n} + D_{i,t_o})^{-1}}$$

where **i**= particular leaf segment under analysis

$\mathbf{D_{i,t_o}}$ = the initial length of segment i.e. 2mm at t_o

$\mathbf{D_{i,t_n}}$ = the length of leaf segment after time (n) after piercing. In this case 24 h.

2) The segmental elongation rate ($\mathbf{SER_i}$ [mm.mm leaf length^{-1}. h^{-1}) was obtained by calculating

$$\mathbf{SER_i = LER\ X\ RSEi\ X\ (RSE_1 + RSE_2 + ...RSE_n)^{-1}\ X\ (L^{-1})}$$

where **i** = particular leaf segment under analysis

LER = the normal rate of growth of unpierced plants between day 5 and 6 (mm. h^{-1}).

$\mathbf{RSE_1 + RSE_2 + ...RSE_n}$ = The sum of the RSE of all segments

L = the length of the segment (i.e. 2 mm)

3) Velocity of displacement ($\mathbf{V_D}$[mm.h^{-1}])

$$\mathbf{V_{D_i} = 2\ (\ SERi_1 + ...SERi_{n-1}) + SERi_n}$$

where i = particular segment under analysis, for n segments per leaf

2.3.5 Mitotic Index

A modified method of Ougham *et al.*, (1987) was used to determine the mitotic index. Transverse sections of 1mm were taken from five primary leaves at 0-4mm above the basal meristem. The sections were fixed in 3:1 (v/v) ethanol to acetic acid and kept at 4°C for 24 h. The samples were transferred to 1.0M HCl and kept at 60°C for 8 min and then stained with Feulgens reagent (BDH, Poole, U.K.) for 30 min at 4°C. The leaf sections were removed from the Feulgens reagent, placed on a slide and squashed under a cover slip in 45% (v/v) acetic acid and viewed under a Nikon light microscope (Nikon, Kingston-Upon-Thames, U.K.). Eight random counts of 200 cells were taken for each section. By counting the number of cells in metaphase, anaphase and telophase the mitotic index was calculated as a percentage of total cells.

2.3.6 Cell Doubling Time

A modified method of Evans *et al.*, (1957) was used to calculate cell doubling time. While the plants were still in their trays a razor blade was used to remove a vertical section of the coleoptile, without damaging the primary leaf, on intact plants on day 6. A syringe was used to deliver 0.2 ml colchicine (1% (w/v) in distilled water) to the leaf base (where the coleoptile had been removed), of 14 plants and the plants returned to the growth chamber under the conditions described in Section 2.2. The solutions were re-applied every 2h to the rest of the plants over the time scale. Water (0.2 ml) was applied in a similar manner to 14 other plants as

a control. Every 2h two plants were harvested from 0-12 h after the start of the experiment from both the colchicine and control treatments.

The basal meristem was removed from the harvested plants, the tissue fixed by the method used for mitotic index count (see Section 2.3.5). Colchicine is used to determine the rate of cells arrested in metaphase. The number of cells in metaphase was counted in eight random counts of 200 cells for each time point for each cultivar in each treatment. Over the 12h period the accumulation of colchicine-arrested metaphase cells were determined for each cultivar in each plant growth treatment. The cell-doubling time (cdt) was determined using the formula of Clowes (1976):-

$$cdt\ (h) = \ln 2 \times 100/c$$

where c = the regression coefficient.

2.4 Photosynthesis Measurements

2.4.1 Leaf Disc Electrode

Photosynthesis was measured in the primary leaf of each cultivar 6 days post-planting using a leaf-disc electrode (Hansatech, Kings Lynn, U.K.). The electrode measures the O_2 concentration in an air filled chamber linked via a chart recorder. By circulating water through the surrounding water jacket from a temperature controlled water bath (Grant Cambridge Ltd, Hertfordshire, U.K.) a constant temperature of 20°C was maintained in the leaf-disc chamber.

2.4.1.1 Calibration

Before each set of measurements the leaf-disc electrode was calibrated. A syringe filled with 1ml of air was attached to the leaf-disc chamber, the voltage output on the control box was noted (=R1). The 1 ml of air was passed into the chamber and the new voltage (=R2) recorded. The difference in the two voltages, i.e. R2-R1, was used to calculate the relationship between mV and O_2 concentration as follows. At 20°C and Standard Pressure 1 ml of air contains 8.73 μmoles O_2 (Lowry & Cavell, 1968). The amount of oxygen (x, in μmoles) that would generate 1mV was calculated by:-

$$x = 8.73/(R2-R1)$$

2.4.1.2 Photosynthesis Measurements

Sections (2 cm transverse) from the tip of approximately five primary leaves were used in each measurement. The fresh weight of the sections was recorded before placing the tissue into the leaf disc chamber. Capillary matting with 500 μl of 1.0 M sodium bicarbonate was added to the chamber to generate a

CO_2 concentration of 5% (v/v) in the chamber. The leaf chamber was sealed and after 2 minutes the light was turned on. Light and CO_2-dependent oxygen evolution was measured for 20 minutes at 20°C and a light intensity of 1,600 μmoles. m^{-2} .s^{-1} (PPF).

2.5 Chlorophyll Measurements

Sections (0.1 g) from the tip of five 6-day old primary leaves were ground in 1.5 ml of 80% (v/v) acetone in a mortar and pestle. This mixture was centrifuged at 10,000*g* for 2 min in a MSE Micro-centaur centrifuge (Scotlab, Coatbridge, U.K.). The supernatant was made up to 3 ml with 80% (v/v) acetone. The absorbance of the supernatant was measured against an 80% (v/v) acetone blank at 663 and 645nm in a Unicam Helios spectrophotometer (Unicam UV-Visible Heλios Spectrometery, Cambridge, U.K.). The chlorophyll content of the sample was then determined using the following equations (Wellburn, 1994):-

(i) Chl a (mg/ml) = $(12.25 \times A_{663}) - (2.79 \times A_{645})$

(ii) Chl b (mg/ml) = $(21.5 \times A_{645}) - (5.1 \times A_{663})$

2.6 UVB Absorbing Compounds

2.6.1 Measurement of UVB Absorbing Compounds

Ten primary leaves were divided into sections: coleoptile tissue, leaf tissue above the coleoptile and tissue from under the coleoptile. The tissue from each section was weighed and ground in 1ml methanol containing 1% HCl with a mortar and pestle. The supernatant was retrieved after 10 min centrifugation at 10,000*g* at room temperature in an MSE Micro-centaur centrifuge (Scotlab, Coatbridge, U.K.), 30 μl of supernatant was added to 1 ml acidified methanol. The absorbance of the supernatant was measured over 280-320 nm using a Unicam Helios spectrophotometer (Unicam UV-Visible Spectrometery, Cambridge, U.K.) against a blank of 1 ml acidified methanol. The area under the peak (280-320 nm) was calculated using the Vision software (Unicam UV-Visible Spectrometry, Cambridge, U.K.).

2.6.2 Identification of Flavonoids

2.6.2.1 Extraction and Hydrolysis Conditions

Five primary leaves from 6-day old plants were freeze dried, ground to a powder, and 20 mg of this tissue was mixed with 2 ml of hydrolysis buffer (60% (v/v) methanol, 20mM Sodium diethyldithiocarbonate, 1.6 M HCl). The mixture was hydrolysed at 90°C for 2 hrs in a Reacti-Therm Heating/Stirring Module (Pierce, Rockford, IL, USA). Extract aliquots of 100 μl, taken after the acid

hydrolysis, were made up to 250 µl with distilled water adjusted to pH 2.5 with trifluoroacetic acid and filtered through a 0.2 µm Anopore filter (Whatman, Maidstone, Kent, U.K.), prior to the analysis of 100 µl volumes by gradient elution reversed phase HPLC. All samples were analysed in triplicate.

2.6.2.2 High Performance Liquid Chromatography and Post-Column Derivatization

Samples were analysed using a Shimadzu (Kyoto, Japan) LC-10A series automated liquid chromatograph comprising a SCL-10A system controller, two LC-10A pumps, a SIL-10A autoinjector with a sample cooler, a CTO-10A column oven, and a SPD-10A UV-vis detector linked to a Reeve Analytical 2700 data handling system (Reeve Analytical, Glasgow, U.K.). Reversed phase separations were carried out at 40°C using a 150 x 3.0 mm internal diameter, 4 µm Genesis C18 cartridge column in an integrated holder (Jones Chromatography, Mid-Glamorgan, U.K.). The mobile phase was a 20 min, 20-40% gradient of acetonitrile in distilled water adjusted to pH 2.5 with trifluoroacetic acid, eluted at a flow rate of 0.5 ml/min. First the column eluent was directed to the SPD-10A absorbance monitor operating at 365 nm, after which post-column derivation was achieved by the addition of methanolic aluminium nitrate containing 7.5% glacial acetic acid and pumped at a flow rate of 0.5 ml/min by a pulse-free Model 9802 precision mixer/splitter (Reeve Analytical, Glasgow, U.K.). The mixture was directed to a RF-10A fluorimeter and fluorescent flavonol complexes detected by excitation at a wavelength of 425 nm and emission at a wavelength of 480 nm. The limit of detection was >5 ng at A 365 and linear 5-250 ng calibration curves were obtained for morin, rutin, quercetin, kaempferol and isorhamnetin. The fluorescent intensity of the individual flavonoid derivatives varied, however 0.1-100 ng linear calibration curves were obtained for myrcetin, morin, quercetin, kaempferol and isorhamnetin. Peak identities were verified using a Waters 996 photodiode array (Milford, USA), scanning from 210-440 nm. HPLC traces and spectra obtained were analysed using Millennium 32 chromatography software (Waters, Milford, USA).

2.6.3 Anthocyanin Content

The supernatant was extracted as in section 2.6.1 and was made up to 1ml with acidified methanol . The absorbance was measured at 535 nm on a Unicam Heλios spectrophotometer (Unicam UV-Visible Spectrometery, Cambridge, U.K.). The anthocyanin content was calculated as follows:

Concentration (µg $100ml^{-1}$) = absorbance/extinction coefficient

Anthocyanin (mg g^{-1} fwt tissue) = $(A_{535}/35{,}000) \times (10^6/100)/\text{fwt (g)}$

2.7 DNA Damage Analysis

2.7.1 Conditions Used to Induce DNA Damage

To induce DNA damage UVB radiation was supplied using four X 115 cm UV-emitting tubes (Philips TL40/12, Starna Ltd, Romford, U.K.) to day 6 plants post-imbibition which were lying on their side. Cellulose acetate (0.13 mm thick, Starna Ltd, U.K.) was wrapped round the UVB tubes to eliminate penetration of UV radiation below 292 nm. The cellulose acetate was changed at the beginning of each experiment. The level of UVB supplied was equivalent to 0.93 $kJm^{-2}h^{-1}$ (biologically effective radiation using the Caldwell (1971) generalised action spectrum) Post-irradiation the wheat leaves were harvested above or underneath the coleoptile, frozen in liquid nitrogen and stored at -80°C in the dark. The leaf tissue above and underneath the coleoptile were analysed separated since the tissue underneath the coleoptile would have less damage because coleoptile protection. When analysing repair, the wheat leaves were placed in the dark or light for differing lengths of time after the initial UVB dose. Amount of repair was calculated as a percentage of initial damage i.e. damage after 3 hours UVB radiation was equal to 100%.

2.7.2 Extraction of DNA

The method was adapted from that of Junghans and Metzlaff (1990). The primary leaf of wheat was harvested above the coleoptile then frozen and ground in liquid nitrogen to a fine powder with a mortar and pestle. The powder was suspended in 1.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 50 mM EDTA, 0.5% (v/v) sodium dodecyl sulphate, 10 mM β-mercaptoethanol) and incubated at room temperature for 15 minutes. The sample was divided into two equal volumes of 0.8 ml each, transferred into Eppendorf tubes and 300 µl of 100% liquified phenol buffered with Tris (Fisher Scientific, Loughborough, U.K.) was added to each sample. This was mixed and left at room temperature for two minutes. Chloroform (300 µl) was added and after centrifugation (10,000g for 3 minutes, MSE Microcentaur centrifuge, Scotlab, Coatbridge, U.K.) at room temperature, the supernatant was treated once more with 300 µl of 100% phenol and 300 µl chloroform. Phenol traces were removed by adding 300 µl of chloroform, mixing and then centrifuging (10,000g in a MSE Micro-centaur centrifuge, Scotlab, Coatbridge, U.K.) the sample. One volume of isopropanol was added to the supernatant and after 30 minutes incubation at -20°C the DNA was precipitated. The DNA was pelleted by centrifugation (10,000g) for 12 minutes, then the pellet was washed with 70% (v/v) ethanol and resuspended in 400 µl TNE buffer, pH 8.0 (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA). To remove RNA, 50 µg/ml RNase was added then the sample was incubated at 37°C for 30 minutes.

The RNase was removed by a phenol-Tris/chloroform (1:1 (v/v)) extraction followed by one chloroform (300 µl) extraction. By adding 1ml of ethanol (96% (v/v)) and incubating at -20°C for 30 minutes the DNA was precipitated again. After 12 minutes centrifugation (10,000g) the DNA was pelleted, the pellet washed with 1ml ethanol (70% (v/v))and resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA).

2.7.2.1 Quantification of DNA

The amount of DNA in a sample was determined by measuring the absorbance of 20 µl of the sample (from section 2.3) in 0.98 ml water, in a Unicam Heλios spectrophotometer (Unicam UV-Visible Spectrometery, Cambridge, U.K.) at 260 and 280 nm.

The amount of DNA was calculated by:-

A_{260} X 2500 = ng of DNA per ml of sample

The purity (P) of the DNA was determined by the ratio of the values at 260 to 280 nm :-

$A_{260} / A_{280} = P$

The highest value of P being 1.8 for pure DNA (Sambrook *et al.*, 1989).

2.7.3 DNA Damage Analysis

The amount of DNA damage was determined using an enzyme-linked immunosorbant assay (ELISA). Antibodies were used to detect the cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone dimers ([6,4] photoproducts). The monoclonal antibody TDM-1 is specific for the CPDs (Mizuno *et al.* 1991) and the 64M-2 antibody is specific for the (6-4) photoproduct (Mizuno *et al.* 1991).

The DNA extracts (300 ng) (Section 2.7.2) were plated into microtitre well plates at a concentration of 10ng/ml, and allowed to dry at 37°C for 48 hours. The plates were washed four times in phosphate- buffered saline (PBS)/0.1% Tween (v/v) (PBS-T) to remove any non-bound DNA. Blocking buffer (5% (w/v) Cadburys Marvel™ in PBS-T) was added to each well (100 µl) for 2 hours at Room Temperature. Excess blocking buffer was removed by four washes with PBS-T and the first antibody was added (50 µl). TDM-1 was used at a concentration of 1:250 and 64M-2 at a concentration of 1:1000, both diluted with PBS-T. The plates were incubated at room temperature for 90 minutes in the dark. The plates were washed five times with PBS-T, and the second antibody (biotinylated goat-anti-mouse antibody (Cambridge Biosciences, Cambridge, U.K.) diluted 1:8000 (v/v) in PBS-T) was added. After 90 minutes incubation in the dark the plates were washed again five times with PBS-T. The third antibody (streptavidin-linked horseradish peroxidase (Cambridge Biosciences, Cambridge, U.K.) diluted 1:4000 in PBS-T)

was added, and after incubation in the dark for 90 minutes the plates were washed four times in PBS-T. The plates were then given a single wash with sodium citrate buffer (0.2 M citric acid, 0.1 M sodium dihydrogen orthophosphate pH 5.0). A developer solution containing *o* -phenylenediamine (0.04% (w/v) 1,2-benzenediamine in sodium citrate buffer, then mixed with hydrogen peroxide (final concentration 0.04% v/v) was added to produce the colour. After 30 minutes incubation in the dark, colour formation was stopped by the addition of sulphuric acid solution (25 ml of 2.5N H_2SO_4).

To give a quantative evaluation of DNA damage levels the absorbance of samples was measured at 492 nm using a microtitre plate reader (EAR 400FW, SLT Labinstruments, Tecan, Reading, U.K.). In each assay a calf thymus standard (irradiated as in Section 2.6.4) was used to assess the level of colour development therefore giving standardisation between replicate assays.

2.7.4 Preparation of Standards

Calf thymus DNA was diluted to a concentration of 10ng/ml in PBS. This solution was irradiated in a box fitted with 2 X 58 cm UV tubes (Philips TL 20/12, Starna Ltd, Romford, U.K.) covered with 0.13mm thick cellulose acetate (Warne & Co. Ltd. London, U.K.). Doses ranging from 0 to 1000 $J.s^{-1}.m^{-2}$ of UV were applied to the DNA solution which was divided into 1ml aliquots and stored at 4°C.

2.8 Measurement of the Penetration of 310nm and 430nm Radiation

A quartz fibre optic microprobe (Polymicro Technologies, Inc, Phoenix, USA) with a tapered end of 8.8 μm diameter was used to measure the penetration of 310 and 430 nm radiation through the leaves of both cultivars. The 50% acceptance angle of the microprobe was 31°. Individual primary leaf sections from 6 day old plants were mounted in a leaf holder with the adaxial surface facing a xenon arc light source (xenon arc lamp, Hanovia 901C-1, 150W, Oriel, Stratford, USA) and a quartz lens (Oriel, Stratford, USA) was used to focus the monochromatic beam (Model 66011, Oriel, Stratford, USA) onto the leaf sample. The fibre tip was positioned so that it was touching the leaf section, between two veins, and a stepper motor (Model 18016, Oriel, Stratford, USA) was used to drive the fibre through the leaf at a speed of 2 μm s^{-1}. The other end of the fibre was attached to a spectroradiometer (Model 742, Optronic Laboratories, FL 32809, USA) interfaced with a computer which recorded the internal light level (relative steric energy flux) every 2μm through the leaf. The internal light level was measured at 310 nm and 430nm and was repeated 12 times for each cultivar grown under control and UVB conditions. Averaged curves were normalised against a standard curve taken when the probe faced the xenon lamp directly without the leaf sample.

2.8.1 Leaf Thickness

Handcut sections (0.30 mm thick) were examined under a light microscope (Nikon, LBi Instruments, Lund, Sweden) to measure the thickness of leaves of each cultivar grown under control and UVB conditions. The measurement was repeated 15 times for three independent growth studies and a mean value calculated.

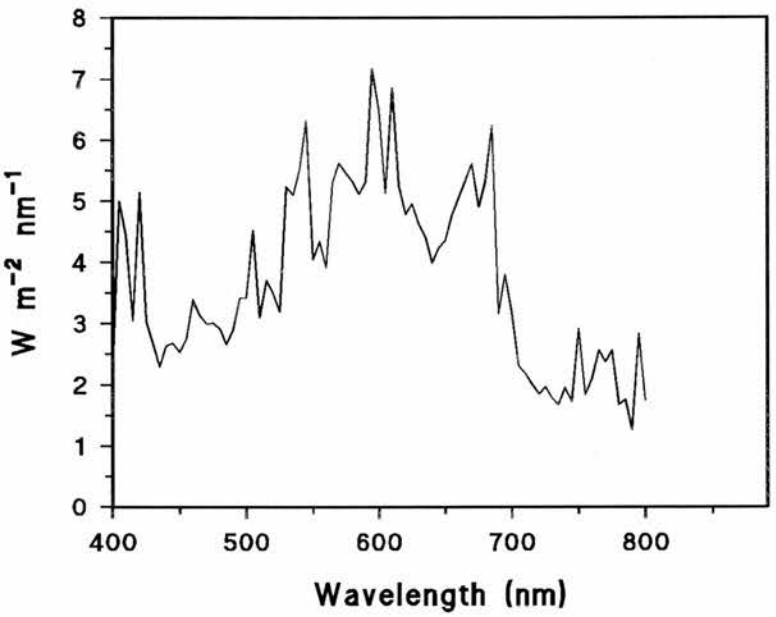


Figure 2.1 The relative spectral intensity of the output of the white light sources used in all experiments.

Chapter 3

The Effects of UVB on Growth, Morphology and Photosynthesis of Maris Huntsman and Yecora Rojo

Introduction

The purpose of the work described in this Chapter is to characterise the growth response of the two wheat cultivars to elevated UVB. Previous work has shown that responses to UVB differ between cultivars (Teramura *et al.*, 1990; Takayanagi *et al.*, 1994) and therefore it is necessary to determine the growth response of both cultivars under investigation.

3.1 Plant Growth and Development

Growth can be defined as a permanent increase in size (Taiz and Zeiger, 1991). Growth at a cellular level consists of a progression through different stages from division through to expansion and cell maturation. Morphogenesis and differentiation accompany growth. Morphogenesis is the development of the form of cells and organs and depends on cell division and expansion. The process by which cells undergo biochemical and structural changes to perform specialised functions is known as differentiation. As wheat is the species used in this study the growth and development of graminaceae will be described.

An intercalary meristem is established at an early stage in primordial development in the graminaceous leaf (Dale, 1988). It is from this meristem that all cells in the leaf originate. Cell division, like leaf growth, is predominantly unidirectional. The meristematic tissue produces parallel files of cells. As a result of production and longitudinal growth of younger cells, a cell within a file is displaced away from the site of division. The majority (85%) of epidermal cell expansion of grass leaves is along the longitudinal axis of the leaf blade (Schnyder & Nelson, 1987). As well as being displaced from the base, each cell expands and differentiates. Thus, the distance between a cell and its origin is a function of both age and developmental stage (Schnyder & Nelson, 1987). The region of cell division and cell elongation within the primary leaf is restricted to a region that is enclosed within a tissue sheath called the coleoptile, and is therefore not directly exposed to light. Thus, the coleoptile may protect the region of primary leaf growth from environmental stress.

3.1.1 Coleoptile Growth

During upward growth through the soil, the coleoptile acts as a protective sheath around the shoot. The coleoptile increases in size as a result of cell expansion (Wright, 1961). All the cells of the coleoptile are formed during embryogenesis, therefore cell division does not contribute to post-germination coleoptile growth. The coleoptile grows much more than the

enclosed shoot axis in darkness, and thus the deeper a grass seed is buried the longer the coleoptile. The relative rates of coleoptile and shoot growth change when the seedling is exposed to light. Under daylight conditions, the true primary leaves of the shoot emerge through the coleoptile tip (Hart, 1988).

3.1.2 What Controls Leaf Growth?

Plant growth is controlled by a number of factors including genetic, environmental and biochemical factors. For example, the length of the elongation zone of the primary leaf of *Festuca arundinacea* is increased with nitrogen fertilisation (Volenec & Nelson, 1983).

As the primary function of the leaf is photosynthesis (Dale, 1988) an important environmental factor influencing plant growth is light. Both light quantity and light quality play a role in influencing growth and the balance of irradiance at different wavelengths of the solar spectrum can influence how plants respond to UVB (Caldwell *et al.*, 1994).

3.2 Plant Cell Division and Elongation

Alterations in plant growth can be the result of changes in cell division and/or cell elongation. As external stresses usually affect plants by reducing plant growth (Francis, 1992), cell division and/or cell elongation will be affected. A number of factors can alter cell division in plant species, for example, increased temperature and CO_2 (Kinsman *et al.*, 1996); nitrogen applications (Volenec & Nelson, 1983); and UVB (Stáxen *et al.*, 1993). Specific aspects of the cell cycle can be affected by environmental stress, and these could be linked to the cellular mechanisms for tolerating stress and therefore may offer clues as to how plants adapt to UVB at a cellular level. In this chapter the effect of UVB on cell division and elongation was examined in order to investigate fully the effects of UVB on plant growth in the two wheat cultivars.

3.2.1 Cell Division

Within the basal meristem, actively dividing cells go through the cell cycle which consists of 4 phases: G1, S, G2 & Mi (Fig. 3.1). Cell cycle progression is regulated by a series of biochemical events, and division results from the interaction of both intrinsic and extrinsic signals and controls. The controls involved in the regulation of the plant cell cycle are varied and they may function at several phases in the cycle (Dunham & Bryant, 1985). For example, numerous proteins are required for DNA replication during the S

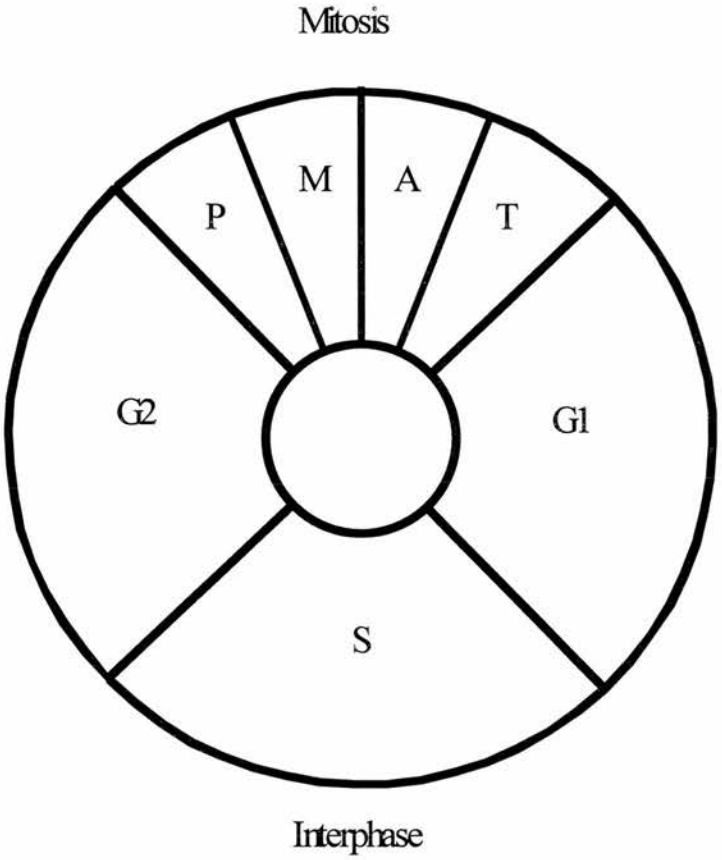


Figure 3.1 Diagram of the Cell Cycle

The cell cycle consists of four phases: pre-DNA synthetic interphase (G1); DNA synthetic interphase (S); post-DNA synthetic interphase (G2); and mitosis (Fosket, 1994). Mitosis involves the production of two daughter cells, each with half of the DNA complement of the interphase cell. Prophase (P): the first stage of mitosis, the nuclear membrane disappears and joined pairs of chromatids appear in the nucleus. Metaphase (M): the spindle fibres form and the chromosomes migrate to the equatorial plane where they attach to the spindle fibres. Anaphase (A): the paired chromosomes divide to give rise to two daughter chromosomes and these migrate to opposite poles of the cell. Telophase (T): the final stage, produces two daughter nuclei (Anderson & Beardall, 1991).

phase of the cell cycle and so the synthesis and/or activity of some of these proteins may be points of control of the plant cell cycle or function in regulating DNA replication. S phase duration may be affected by the total amount of DNA to be replicated (Francis *et al.*, 1985).

The cell cycle duration is determined primarily by the cell growth rate (Cavalier-Smith, 1985). Cell growth depends on nutrient transport, molecular

biosynthesis and organelle assembly (Lyndon, 1990), and therefore regulating any of these processes might alter the rate of cell division.

3.2.2 Cell Elongation

In monocotyledon leaves cell expansion accompanies cell division, with both processes partly overlapping in space and time (Brett & Waldron, 1996). Both plastic and elastic deformation can cause plant cell enlargement (Brett & Waldron, 1996). On a cellular level plastic deformation extends the wall permanently, whereas elastic wall deformation is reversible. Elastic wall deformation can produce an increase in cell size in nongrowing cells (Proseus *et al.*, 1999). Cell enlargement occurs when water is taken into the cell. Growth of cells is dependent on the supply of water, organic and inorganic solutes. As water enters expandable cells, growth occurs along with the dilution of cellular structures and solutes (Schnyder & Nelson, 1989).

Leaf elongation rate is influenced by the rate of cell production and the rate and duration of cell expansion (Nelson & MacAdam, 1989). Individual cell expansion is finite and thus cell division is necessary for continued growth of the leaf blade. The elongation zone is an active sink for carbohydrates, the majority of which are metabolised (Schnyder & Nelson, 1987). The greatest priority for use of sucrose in the basal region of the elongating leaf blade is for the synthesis of structural material (Nelson & MacAdam, 1989). The remaining carbohydrate is removed from the cytosol of the elongation zone and used for fructan synthesis.

Cell elongation can be influenced by growth substances, either produced by the tissue itself or transported into the tissue from other parts of the plant (Brett & Waldron, 1996). These include auxins, gibberellins and ethylene. Many studies have examined how auxins affect growth rate (Cleland, 1959; Kutschera & Schopfer, 1986; Hoson *et al.*, 1992), since they can stimulate the synthesis of wall polysaccharides and thus have a long-term effect on growth.

3.3 Leaf Metabolism

Many studies have looked at the effects of UVB on metabolism in plants, especially the effects on photosynthesis. (Reviews: Bornman, 1989; Teramura & Sullivan, 1994; and Allen *et al.*, 1998). UVB can affect photosynthesis in many plant species. Photosynthesis is affected directly when UVB impairs the performance of the component processes, for example, damage to PSII (see section 1.7.2), where changes in the reaction centre and function of the oxygen evolving complex have been reported (Renger *et al.*,

1989). Indirect effects to photosynthesis occur due to changes in plant growth and morphology which can alter plant competition and the microclimatic factors within the leaf itself (Barnes *et al.*, 1988).

3.3.1 Chloroplast Development

Along the leaf there is a progression of mesophyll development and changes in the structure and size of organelles. The plastids at the base of the leaf are all small and relatively undifferentiated (Boffey *et al.*, 1979; Tobin & Rogers, 1992). As the leaf develops, an increasing proportion of the mesophyll cell becomes occupied by chloroplasts due to an increase in chloroplast size and number (Ellis & Leech, 1985).

Plastids can be defined as self-replicating organelles surrounded by an envelope comprised of two membranes, and are only found in cells of photosynthetic eukaryotes (Newcomb, 1997). Chloroplasts are green plastids that contain chlorophyll, and are the site of photosynthesis. The internal membrane system contains stacks of flattened disk-shaped membranous sacs called grana. Thylakoids are membranous channels which transverse the stroma and interconnect the grana. During chloroplast development the thylakoid membranes become organised into granal stacks. The degree of stacking depends on the requirements of the cell, for example, when plants are grown under shade conditions the grana are well developed (Leech and Baker, 1983). In the thylakoids the concentration of photosystems I and II differ from that of the stroma and grana (Anderson & Beardall, 1991).

3.3.2 Photosynthetic Capacity

Along the wheat leaf there is a gradation of leaf cell metabolism from heterotrophic, nonphotosynthetic cells at the base through to autotrophic fully photosynthetic cells at the leaf tip (Tobin & Rogers, 1992). This transition has been observed in a number of studies. In the primary leaf of wheat, for example, photosynthetic oxygen evolution and photosynthetic enzyme activity increases with distance from the leaf base (Tobin *et al* ., 1988).

A number of factors contribute to the change in photosynthetic capacity along the leaf. These include increases in size, number and development of chloroplasts (Boffey *et al* ., 1980; Baker & Leech, 1977; Babani & Lichtenthaler, 1996), and synthesis of photosynthetic proteins, for example, of Ribulose-1,5-bisphosphate carboxylase (Rubisco) (Dean & Leech, 1982). This is further supported by the findings of Topping and Leaver (1990) who observed a steady increase in transcripts along the wheat leaf of

chloroplastic (*rbc L*) and nuclear (*rbc S*) genes encoding the large and small subunits of Rubisco.

3.4 Chapter Aims

UVB has been shown to affect cultivars of the same plant species in different ways. In this Chapter the growth, morphology and photosynthetic capacity of two cultivars of wheat will be compared under control and UVB conditions. The two cultivars have been bred for growth in very different climates, and the aim of the chapter is to characterise the growth of both cultivars under each of the experimental conditions and to investigate whether one cultivar is affected by UVB to a greater extent than the other.

RESULTS

N.B. Unless otherwise stated, 'significance' refers to 95% confidence limit (i.e. $p<0.05$). A one-way ANOVA was the statistical test used in each of the experiments to determine if there was a difference with UVB radiation.

3.5 The Effect of UVB on the Growth of Maris Huntsman and Yecora Rojo

3.5.1 Total Plant Height

Fig. 3.2 shows the effect of UVB on plant height for wheat seedlings from day 4 to day 7 after planting. For both cultivars, with and without supplementary UVB there was an increase in height from day 4 to day 7 after planting. On day 7 the height of Maris Huntsman (119cm) was significantly greater than the height of Yecora Rojo (115cm). When plants were grown with supplementary UVB the height of both cultivars was significantly reduced. The total height of Yecora Rojo was reduced by *c* . 19% under UVB whereas Maris Huntsman was reduced by *c*. 22%.

3.5.2 Primary Leaf Growth

Primary leaf length was significantly reduced when grown under supplementary UVB in both cultivars (Fig. 3.3). The leaf growth rate (Fig. 3.4) was reduced in both cultivars when grown under supplementary UVB, thus accounting for the reduction in leaf length. The primary leaf length of Maris Huntsman, after 7 days, was reduced by *c*. 25% with supplementary UVB, while Yecora Rojo was reduced to a lesser extent (*c*. 19%).

3.5.3 Primary Leaf Area

Between days 4 and 5 UVB had little effect on the area of the primary leaf of Maris Huntsman, whereas it was significantly reduced from day 6 onwards (Fig. 3.5a). The leaf area of the primary leaf Yecora Rojo increased at a linear rate under control conditions (Fig. 3.5b). UVB had little effect on leaf area until day 7 when it was significantly reduced in both Maris Huntsman (by 20%) and Yecora Rojo (by 15%).

3.5.4 Primary Leaf Fresh and Dry Weight

UVB had very little effect on the fresh weight of primary leaves of either cultivar until day 7 (Fig. 3.6), when the fresh weight of both cultivars was significantly reduced. The fresh weight of Maris Huntsman was reduced by *c* .

18% and Yecora Rojo by *c.* 10%. The dry weight of the primary leaf was maintained at *c.* 11% of the fresh weight, and this did not change with UVB. The dry weight of the primary leaf of both cultivars was not affected by UVB until day 7 when it was reduced by 14% in Maris Huntsman and 32% in Yecora Rojo (Fig. 3.7).

3.5.5 Specific Leaf Area

The specific leaf area (ratio of leaf area to leaf dry weight) of the primary leaf of control grown plants of both cultivars increased from day 4 to day 6, and then decreased by day 7 (Fig. 3.8). With supplementary UVB the specific leaf area of Maris Huntsman was significantly decreased with UVB on days 4, 5 and 6. However, when grown under supplementary UVB the specific leaf area of Yecora Rojo increased steadily from day 4 to day 7.

3.5.6 Coleoptile Height

The average height of the coleoptile in control grown plants of both cultivars increased to a maximum on day 6 (Fig. 3.9). When grown under supplementary UVB the coleoptile in both cultivars was inhibited significantly. The coleoptile height of Maris Huntsman was greatest on day 5 when grown with supplementary UVB, however in Yecora Rojo the maximum height was reached on day 6. With UVB the coleoptile height of Maris Huntsman was reduced by *c.* 27% and Yecora Rojo was reduced by *c.* 16.6%.

3.5.7 Coleoptile Fresh and Dry Weight

Under control conditions the fresh weight of the coleoptile of Maris Huntsman increased until it reached a maximum on day 7 (Fig. 3.10a). The maximum fresh weight of the Yecora Rojo coleoptile was reached on day 6 when grown with and without supplementary UVB (Fig. 3.10b). When grown under supplementary UVB the fresh weight of the coleoptile in Maris Huntsman was significantly reduced, however there was no change in the fresh weight of the coleoptile of Yecora Rojo when grown under supplementary UVB.

The dry weight of the coleoptile was maintained at *c.* 8.5% of the fresh weight in both cultivars under each treatment (Fig. 3.11). The coleoptile dry weight of Maris Huntsman was significantly reduced when grown with supplementary UVB, however there was no significant reduction in the coleoptile dry weights of Yecora Rojo.

3.6 The Effect of UVB on Cell Elongation

3.6.1 Spatial Distribution of Segmental Elongation Rate (SER) within the Cell Elongation Zone

The spatial distribution of SER (i.e. the rate of expansion of each segment relative to itself) within the basal meristem of 6-day old primary leaves of both Maris Huntsman and Yecora Rojo is shown in Fig. 3.12. In control grown plants of both cultivars the SER reached a maximum at 4mm above the leaf base, the SER then decreased until the cessation of the elongation zone at 12mm above the leaf base. However, in UVB grown plants the SER maximum was reached at 2mm above the leaf base and steadily decreased until the end of the elongation zone at 10mm from the leaf base. In addition, when grown under supplementary UVB the maximum SER was decreased in both cultivars. The size of the elongation zone was reduced in both cultivars when grown under supplementary UVB (from 12mm in control plants to 10mm in UVB grown plants).

3.6.2 Spatial Distribution of Vertical Displacement Velocity (V_D) within the Cell Elongation Zone

Fig. 3.13 shows the velocity of displacement of tissue within a 6-day old primary leaf of Maris Huntsman and Yecora Rojo. The V_D (i.e. the velocity of movement of a given section of the leaf with respect to the basal meristem) increased with distance from the leaf base until the end of the elongation zone where it reached a maximum and became constant. With UVB the V_D of Maris Huntsman was decreased in all regions of the elongation zone. Above 6mm from the leaf base UVB significantly reduced the V_D , that is, in UVB grown plants the tissue was being displaced more slowly through the elongation zone. The V_D of Yecora Rojo under UVB was greater than the control grown plants until 6mm from the leaf base where the V_D of the UVB grown plants levelled off and was significantly less than that of the control plants.

The final constant V_D in control grown Maris Huntsman was 0.923 $mm.hr^{-1}$ compared to 0.688 $mm.hr^{-1}$ in UVB grown plants i.e. a reduction of *c.* 25%. This was comparable to the mean growth rate of plants from day 5 to day 6 shown in Fig. 3.4a, *c.* 0.85 $mm.hr^{-1}$ for control grown plants and 0.685 $mm.hr^{-1}$ for plants grown under UVB. In Yecora Rojo the final constant V_D was 0.88 $mm.hr^{-1}$ in control plants, whereas in UVB grown plants it was 0.708

$mm.hr^{-1}$, a reduction of *c.* 20%. The mean growth rate of day 5 to day 6 Yecora Rojo plants shown in Fig. 3.4b is slightly less (*c.* 0.66 $mm.hr^{-1}$) compared to the V_D in Fig. 3.13.

3.7 The Effect of UVB on Cell Division in the Primary Leaf Basal Meristem

3.7.1 Mitotic Index

The mitotic index (MI) is defined as the percentage of cells undergoing mitosis, i.e. cells undergoing metaphase, anaphase and telophase. Using Feulgens reagent to stain the cells it is possible to identify the number of cells going through the stages of mitosis (Fig. 3.14). In 6 day old seedlings the zone of cell division was located within the basal 4mm of the primary leaf of both cultivars. When grown under supplementary UVB the mitotic index of Maris Huntsman was significantly reduced at 3mm from the leaf base (Fig. 3.15a). The MI in all regions of the cell division zone were significantly reduced in Yecora Rojo grown with supplementary UVB (Fig. 3.15b). In plants grown with and without UVB, the maximum percentage of mitotic cells was found within the first mm of the basal meristem. The proportion of mitotically active cells decreased to the end of the cell division zones in both cultivars for both treatments.

The proportion of cells in metaphase, anaphase and telophase was determined for leaf tissue of 6-day old seedlings (Figs. 3.16 to 3.18). The percentage of cells in each stage of mitosis within the cell division zone varies for the two cultivars under each treatment, although there is a general trend. That is, the maximum proportion of cells in each stage of mitosis was contained in the first mm of the basal meristem, after which there was a steady decline until the end of the cell division zone. The one exception was in control grown Maris Huntsman, where the maximum proportion of cells was reached in the second mm from the leaf base. The decline in the MI under UVB could be a result of a decline in any one of the stages. It appeared that UVB caused a reduction in the proportion of cells in all stages of mitotis.

3.7.2 Cell Doubling Time (cdt)

The cell doubling time (cdt) was determined by treating the primary leaf with colchicine (Section 2.3.6). Colchicine inhibits spindle formation and, as a result, actively dividing cells arrest in metaphase. Fig. 3.19 shows the accumulation of cells at metaphase for each cultivar over a 12 hour period.

There was an initial lag period (0-2 hours) before the colchicine started to act on the tissue, after which there was a linear increase before the rate of division was reduced. After prolonged exposure to colchicine, cells become less distinct as a result of spindle and chromosome collapse, known as 'ball-metaphase' (Utrilla *et al.*, 1989). This could be the reason for the reduction in number of cells at metaphase at the end of the treatment period (10-12 hours). From linear regression analysis (Fig. 3.20) of the curves the regression co-efficients were obtained and the cdt was calculated (Section 2.3.6). When grown under supplementary UVB there was no difference in the cdt of each cultivar. Control grown plants of Maris Huntsman had a cell doubling time of 41.32 $\pm$ 3.18 hours whereas plants grown with supplementary UVB had a cdt of 52.19 $\pm$ 4.94 hours. Yecora Rojo plants grown under control conditions had a cdt of 55.10 $\pm$ 5.34 hours compared to plants grown under supplementary UVB that had a cdt of 68.76 $\pm$ 4.23 hours.

3.8 The Effect of UVB on the Photosynthetic Rate of the Primary Leaf

Fig. 3.21 shows the rate of photosynthesis (CO_2 - dependent O_2 evolution per mm^2 leaf area) in the base and tip of both cultivars under control and UVB conditions. The rate of photosynthesis per unit leaf area increased between the base and the tip in both cultivars. With UVB the rate of photosynthesis at the tip of Maris Huntsman leaves decreased significantly, however at the base of the leaf there was no difference in photosynthetic rate. In Yecora Rojo, there was no significant difference in the rate of photosynthesis when grown under supplementary UVB at either the base or the tip of the leaf.

3.9 The Effect of UVB on the Chlorophyll Content of the Primary Leaf

The amount of chl a (Fig. 3.22) and chl b (Fig. 3.23) increased between the base and the tip of the leaf in both cultivars. When grown with supplementary UVB there was a significant increase in chl a and chl b at the tip of the leaf in both Maris Huntsman and Yecora Rojo.

There are no significant changes in the chl a:b ratio with UVB (Fig. 3.24) apart from in the base of the Maris Huntsman leaf where the chl a:b ratio is reduced. The total chl content (Fig. 3.25) at the tip of the leaf in both cultivars was increased significantly when grown under UVB, no significant difference was seen at the base of the leaf.

3.10 The DNA Content of the Primary Leaf

The DNA content of both cultivars was calculated per unit leaf area (Fig 3.26a), leaf fresh weight (Fig. 3.26b) and leaf dry weight (Fig. 3.26c). This was calculated to determine if there was a difference in the amount of DNA in the cultivars. No significant difference was found between cultivars irrespective of how the data are expressed.

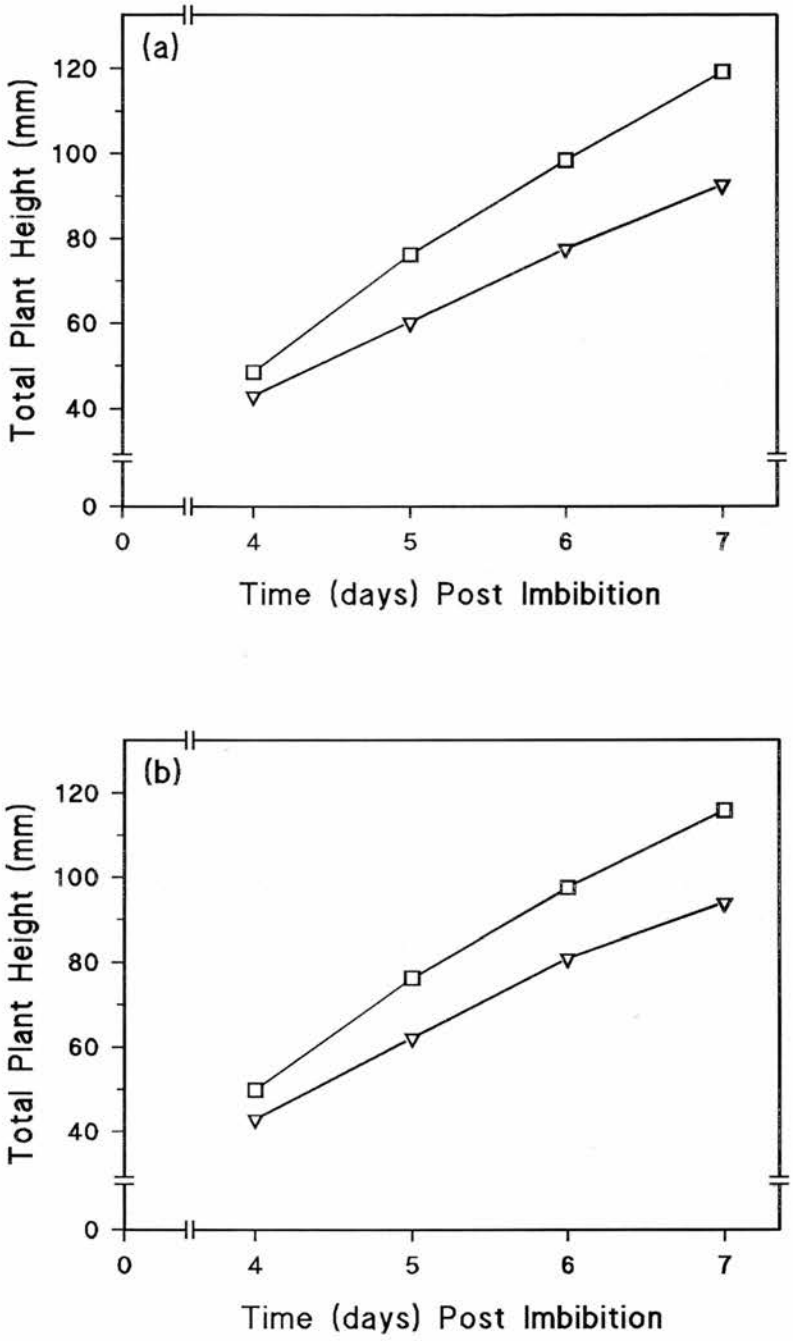


Figure 3.2 Total plant heights of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). Plant height was measured as described in Section 2.3.1. The data points represent the mean of four independent growth studies sampling 30 seedlings each. Standard errors are not shown for reasons of clarity. In all cases they were less than 1.5% of the mean.

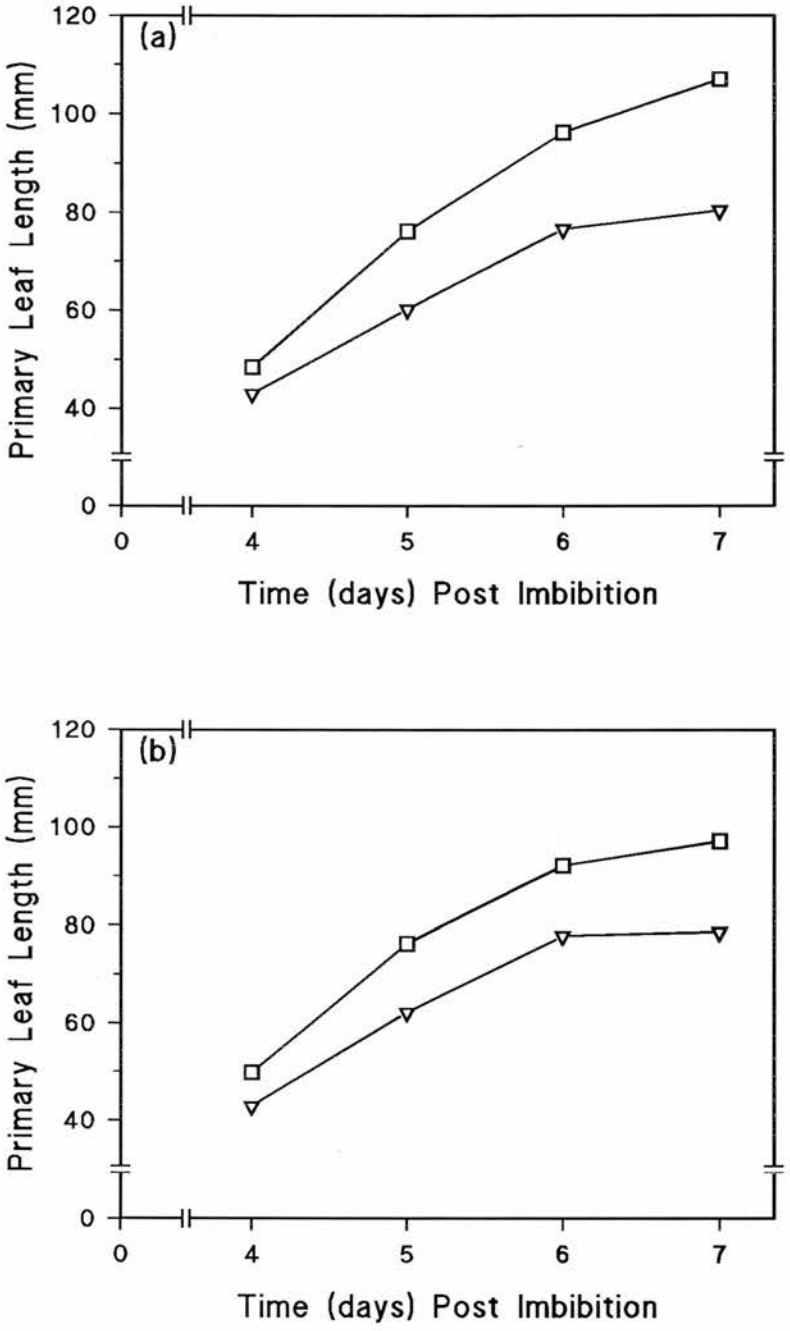


Figure 3.3 Primary leaf length of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). Primary leaf length was measured as described in Section 2.3.1. The data points represent the mean of four independent growth studies sampling 30 seedlings each. Standard errors are not shown for reasons of clarity. In all cases they were less than 2% of the mean.

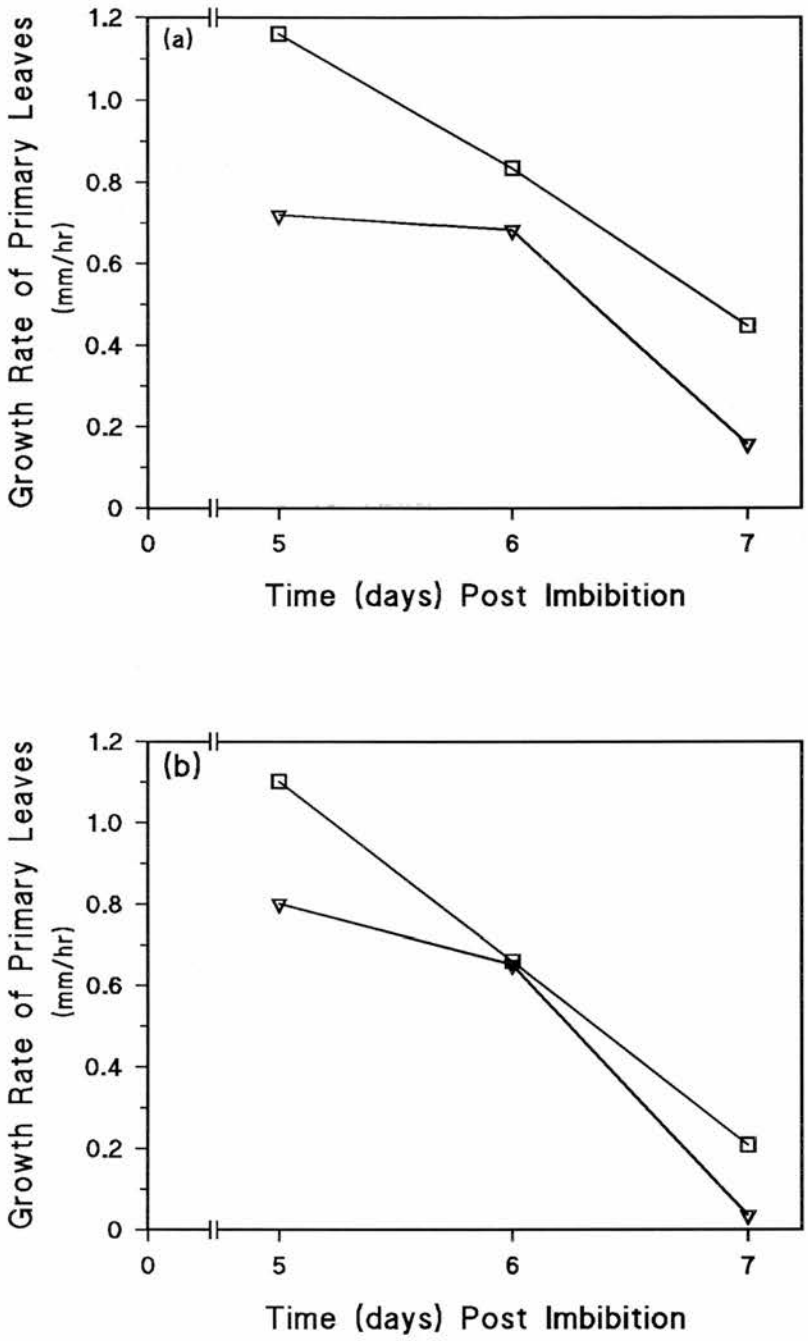


Figure 3.4 Growth rate of the primary leaf of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). Plant growth rate was calculated as described in Section 2.3.1. The data points represent the mean of four independent growth studies sampling 30 seedlings. Standard errors are not shown for reasons of clarity. In all cases they were less than 2% of the mean.

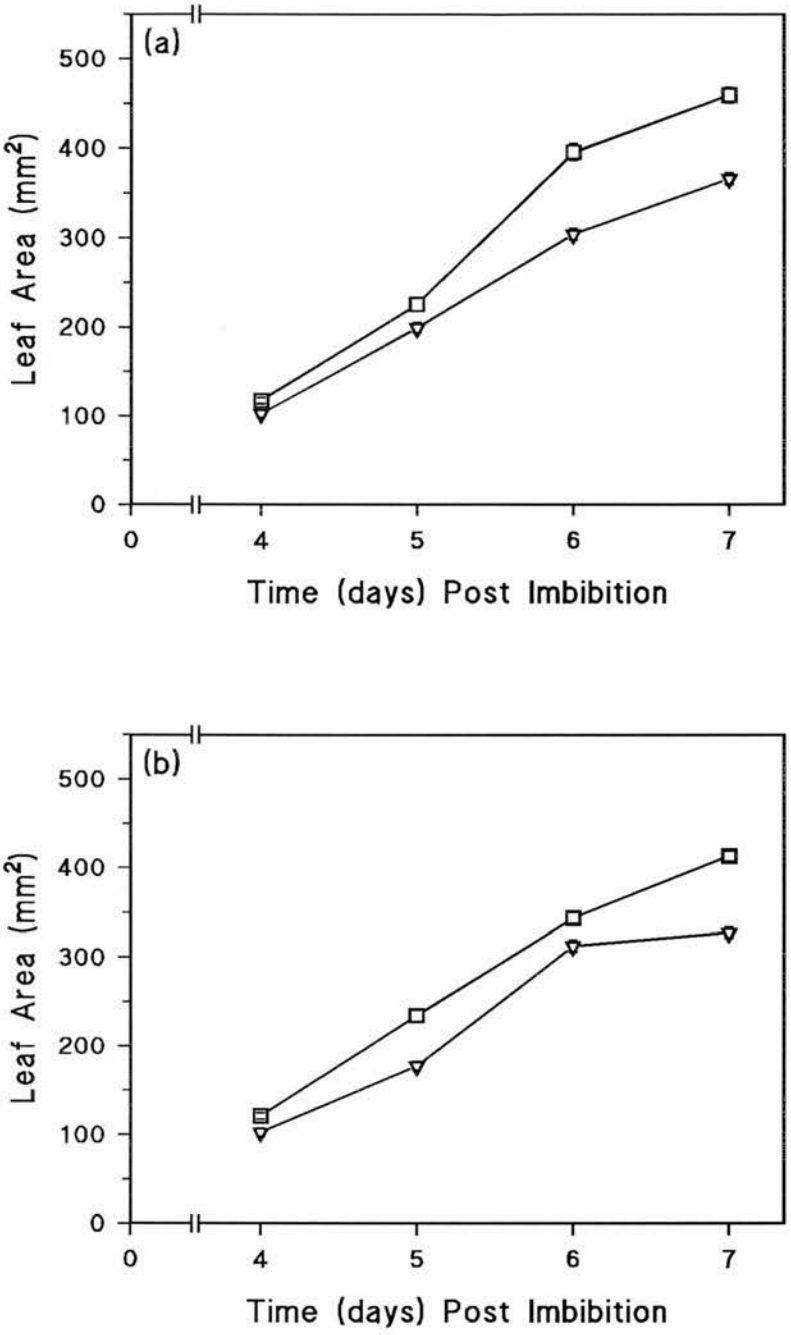


Figure 3.5 Area of the primary leaf of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). Leaf area was measured as described in Section 2.3.3. The data points represent the mean of four independent growth studies sampling 30 seedlings each, with error bars showing ± one standard error from the arithmetic mean.

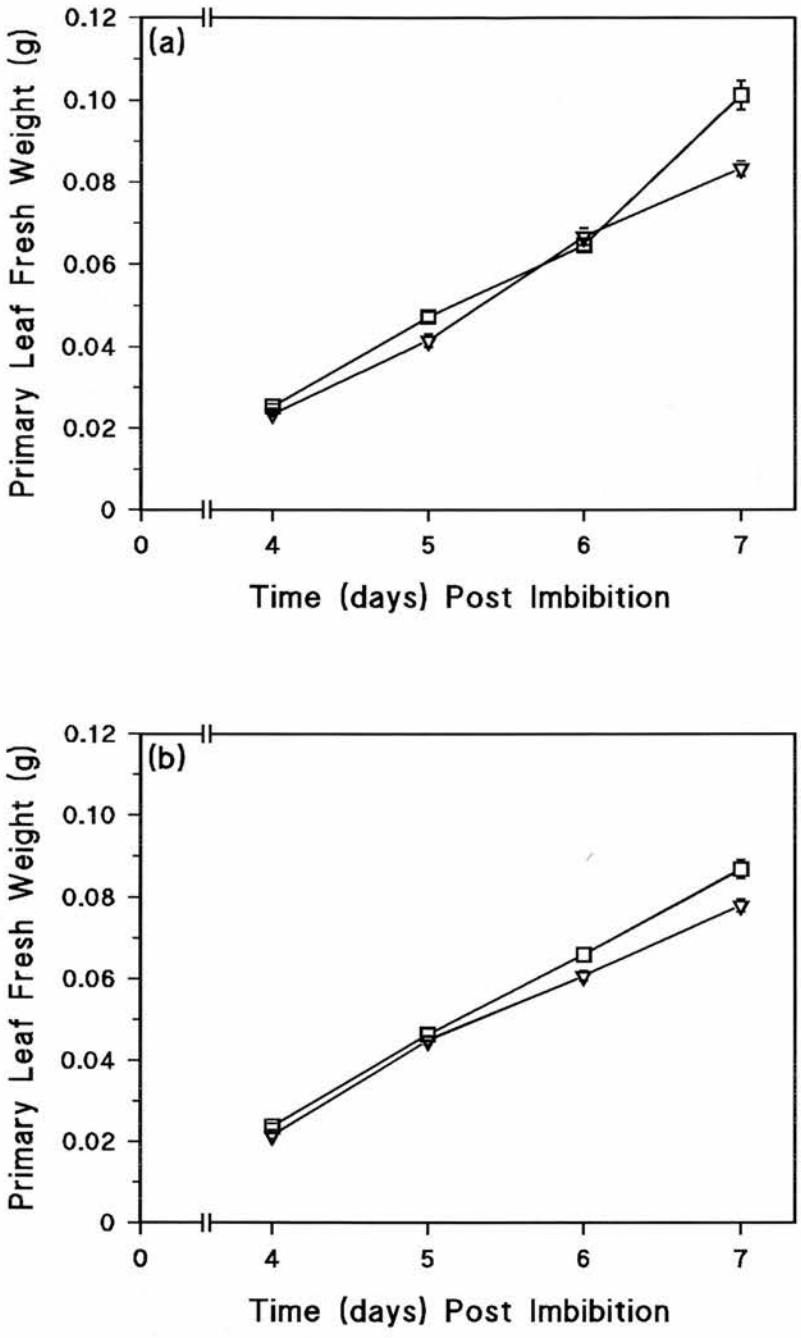


Figure 3.6 Fresh weight of primary leaves of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). Leaf fresh weight was measured as described in Section 2.3.2. The data points represent the mean of four independent growth studies sampling 12 seedlings each, with error bars showing ± one standard error from the arithmetic mean.

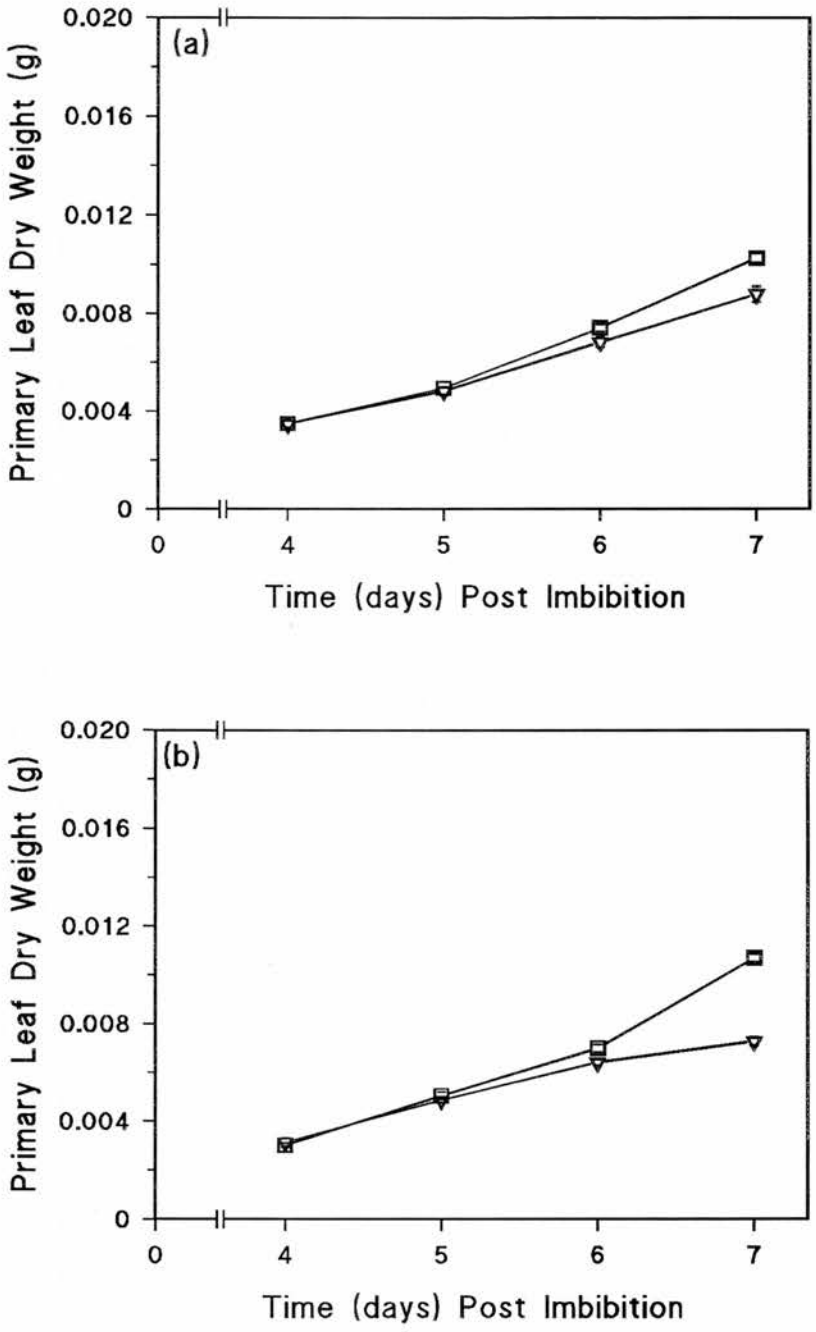


Figure 3.7 Dry weight of primary leaves of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). Leaf dry weight was measured as described in Section 2.3.2. The data points represent the mean of four independent growth studies sampling 12 seedlings each, with error bars showing ± one standard error from the arithmetic mean.

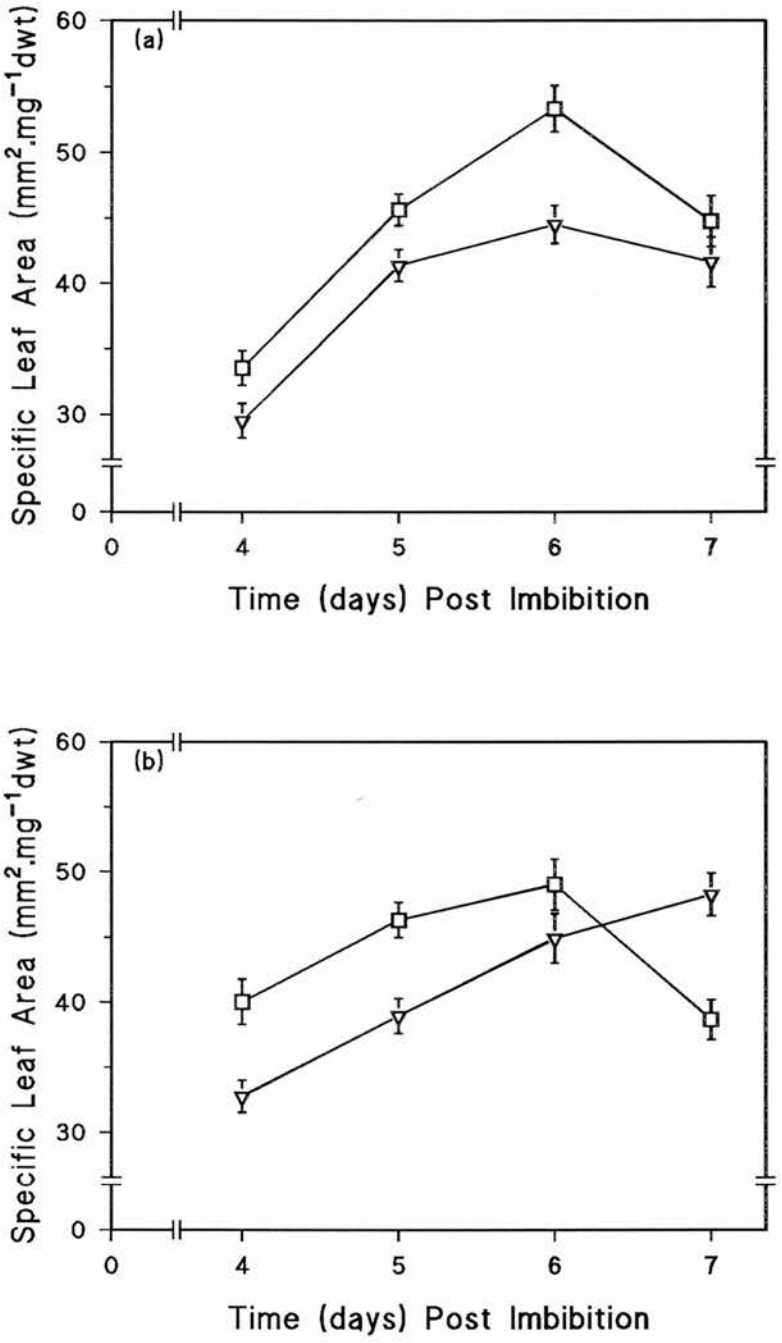


Figure 3.8 Specific leaf area of the primary leaves of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). Specific leaf area was calculated by dividing the leaf area by the dry weight. The data points represent the mean of four independent growth studies sampling 12 seedlings each, with error bars showing ± one standard error from the arithmetic mean.

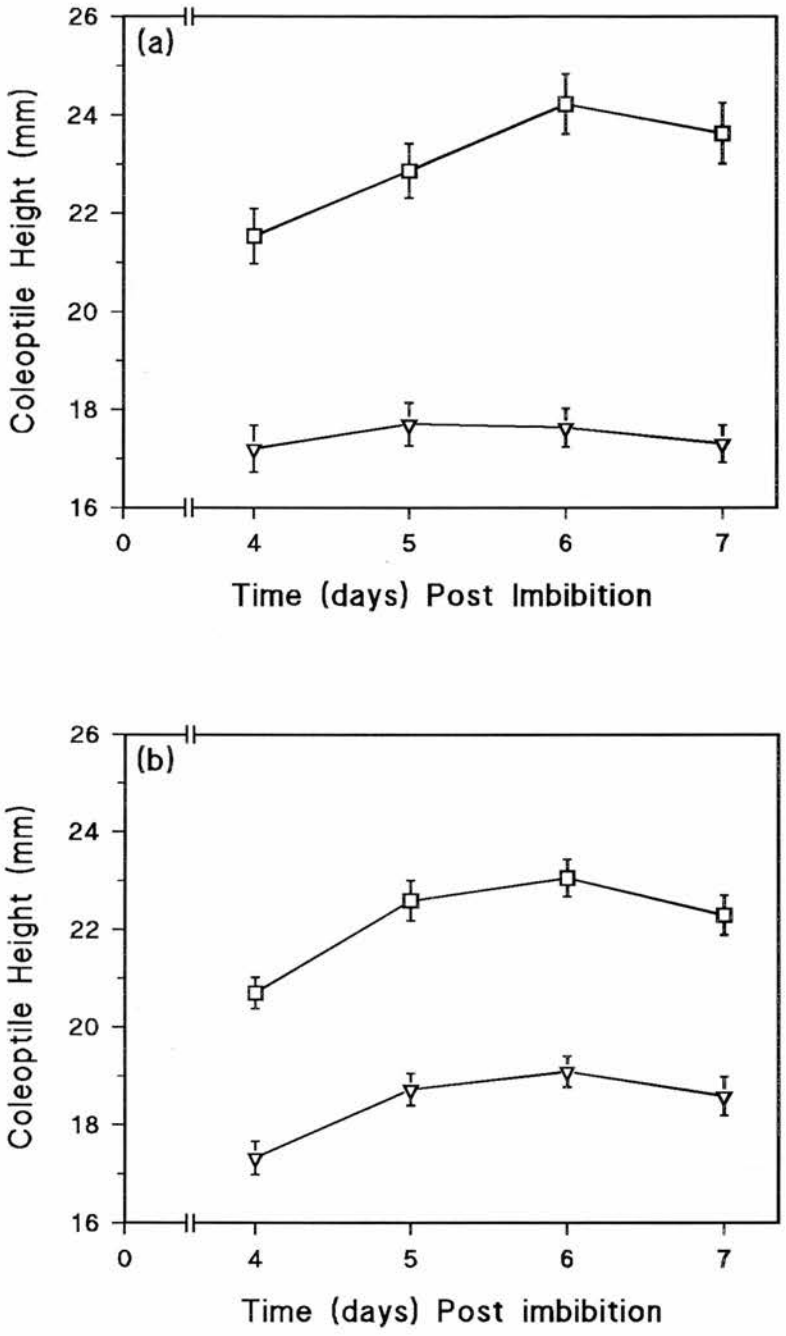


Figure 3.9 Coleoptile height of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). Coleoptile height was measured as described in Section 2.3.1. The data points represent the mean of four independent growth studies sampling 12 seedlings each, with error bars showing ± one standard error from the arithmetic mean.

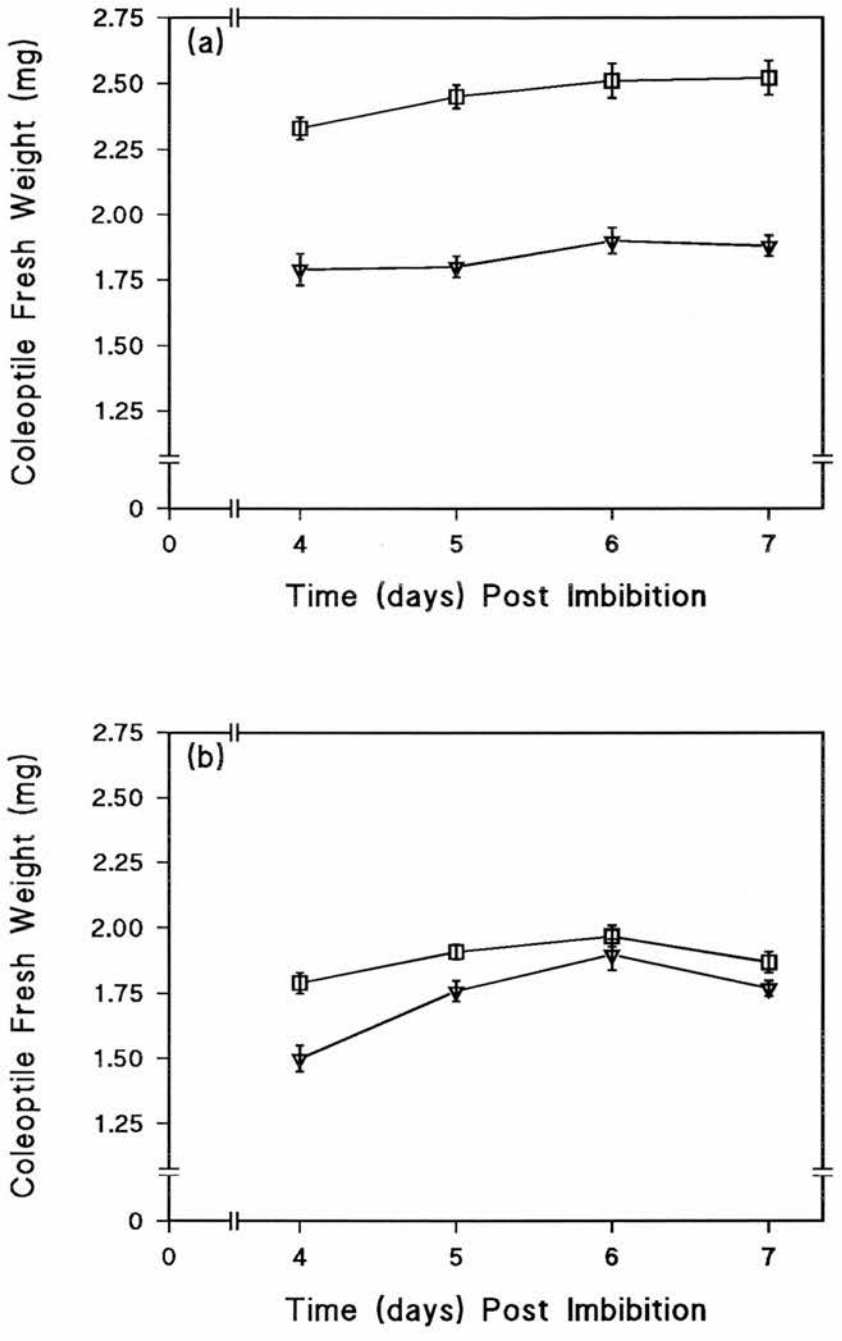


Figure 3.10 Coleoptile fresh weight of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). Coleoptile weight was measured as described in Section 2.3.2. The data points represent the mean of four independent growth studies sampling 12 seedlings each, with error bars showing ± one standard error from the arithmetic mean.

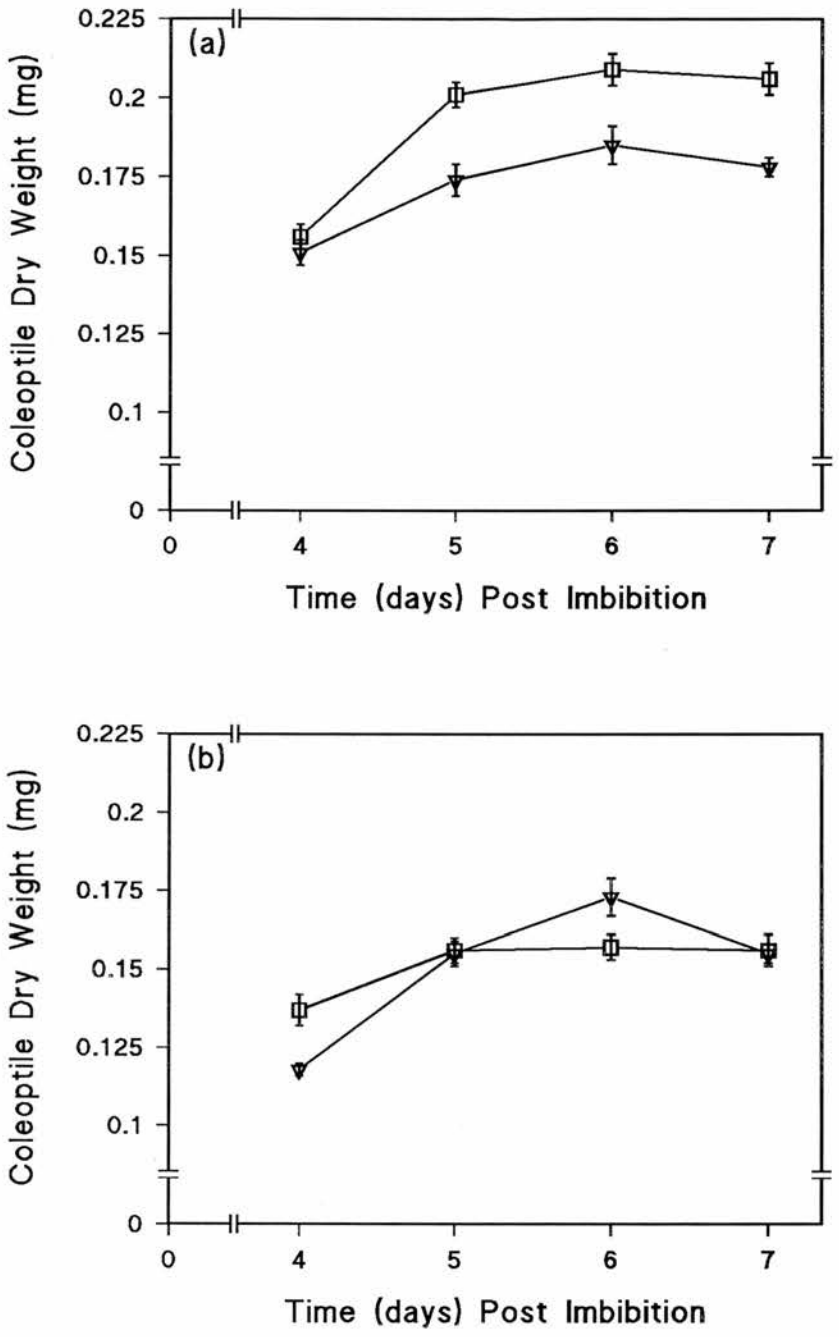


Figure 3.11 Coleoptile dry weight of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (∇) and without supplementary UVB (□) (Section 2.2). Coleoptile weight was measured as described in Section 2.3.2. The data points represent the mean of four independent growth studies sampling 12 seedlings each, with error bars showing ± one standard error from the arithmetic mean.

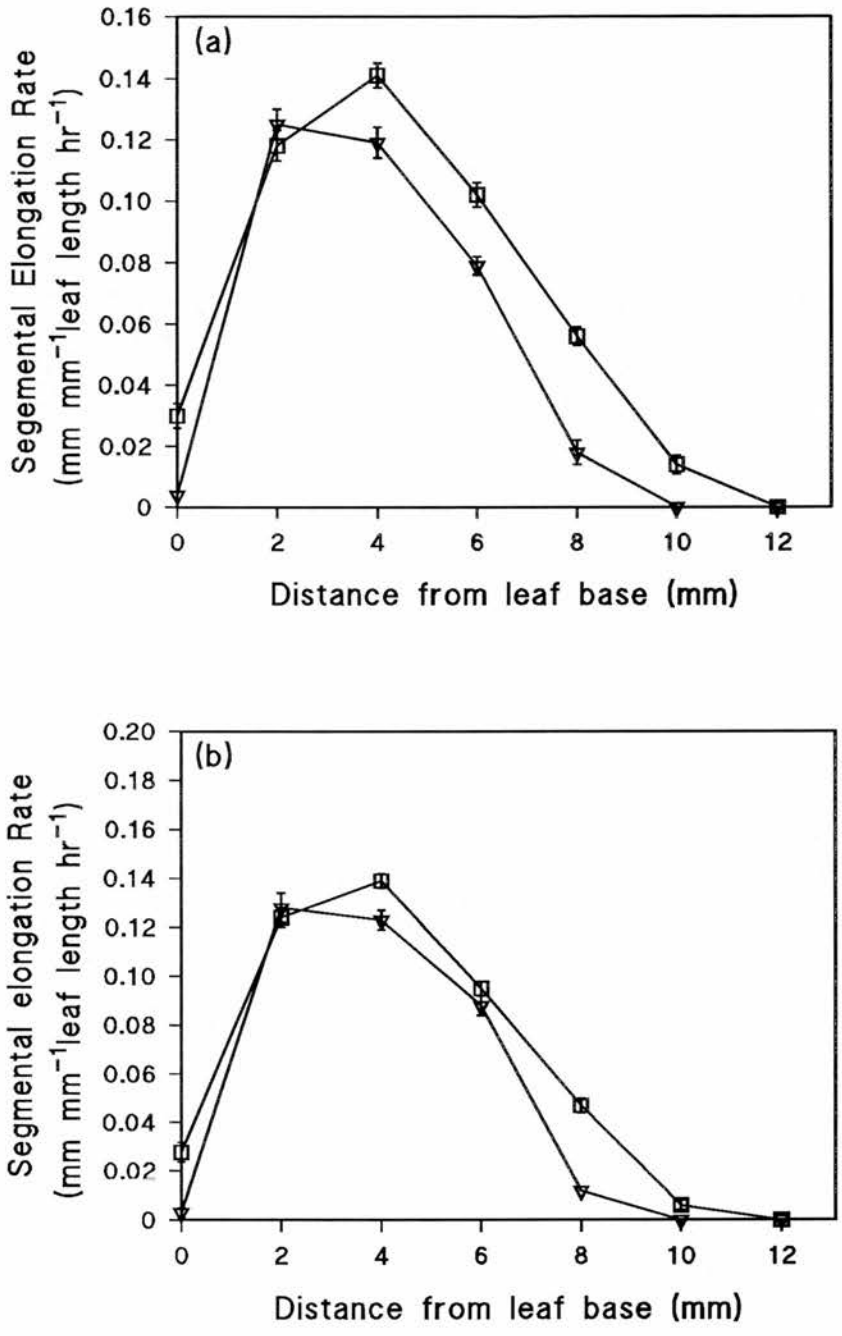


Figure 3.12 Segmental elongation rates of different regions within the primary leaf of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▽) and without supplementary UVB (□) (Section 2.2). The segmental elongation rate was measured in five-day old seedlings as described in Section 2.3.4. The data points represent the mean of four independent growth studies sampling 30 seedlings each, with error bars showing ± one standard error from the arithmetic mean.

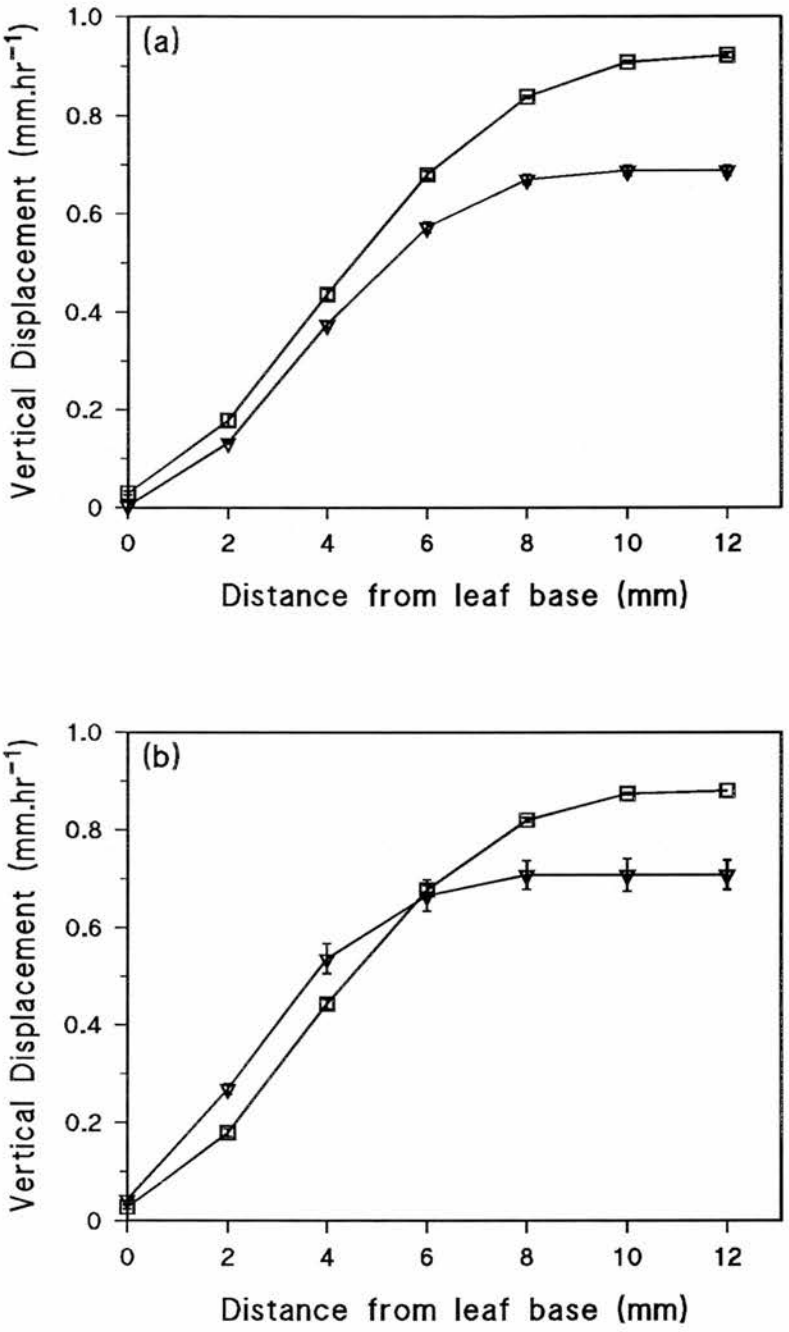


Figure 3.13 The velocity of displacement of cells along the primary leaf of 5 day old seedlings of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (∇) and without supplementary UVB (□) (Section 2.2). The vertical displacement velocity was measured in five-day old seedlings as described in Section 2.3.4. The data points represent the mean of four independent growth studies sampling 30 seedlings each, with error bars showing ± one standard error from the arithmetic mean.

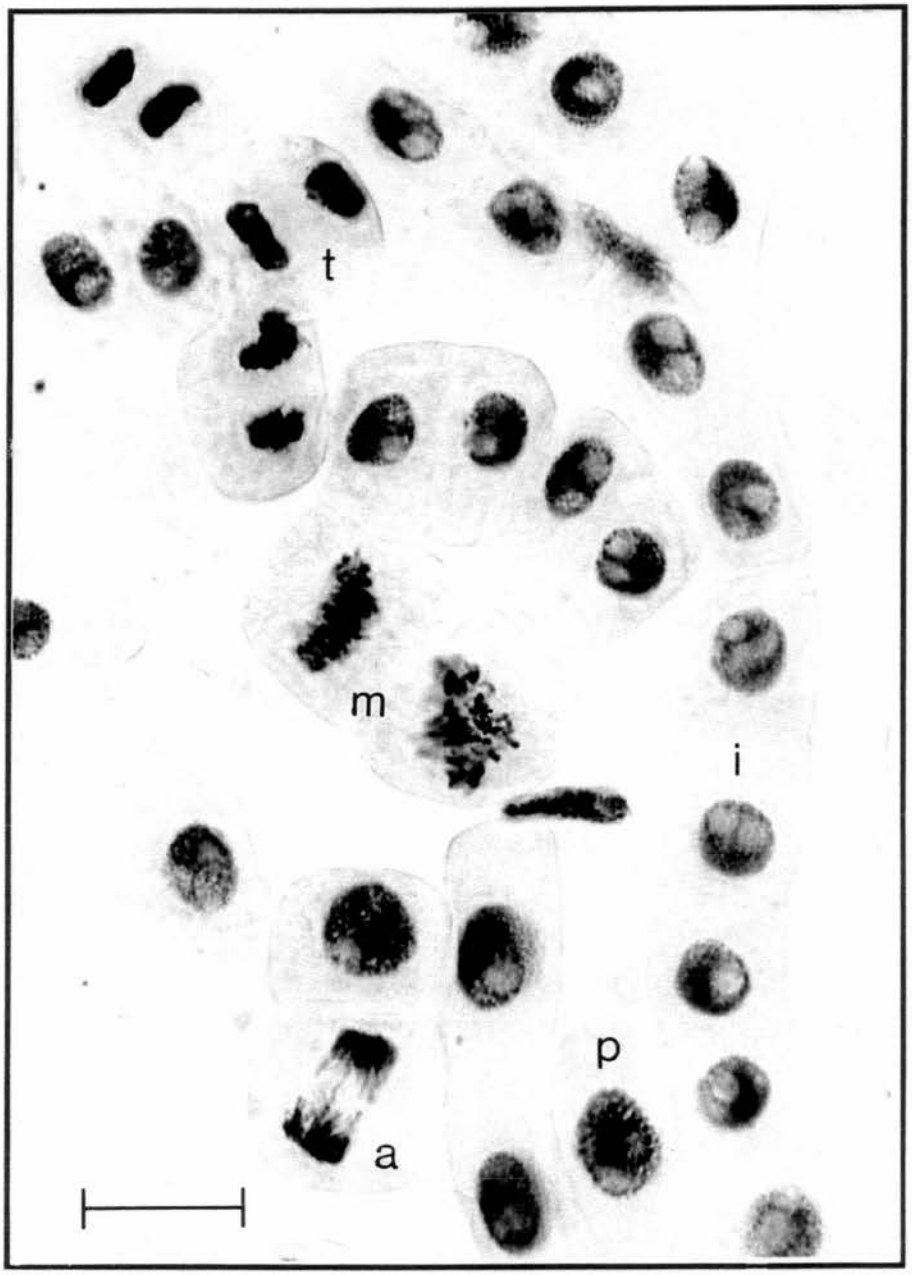


Figure 3.14 A Feulgen-stained preparation of cells within the primary leaf of wheat.

Tissue was prepared as described in Section 2.3.6. Photograph courtesy of M.A. Bond (MA Bond, 1997).

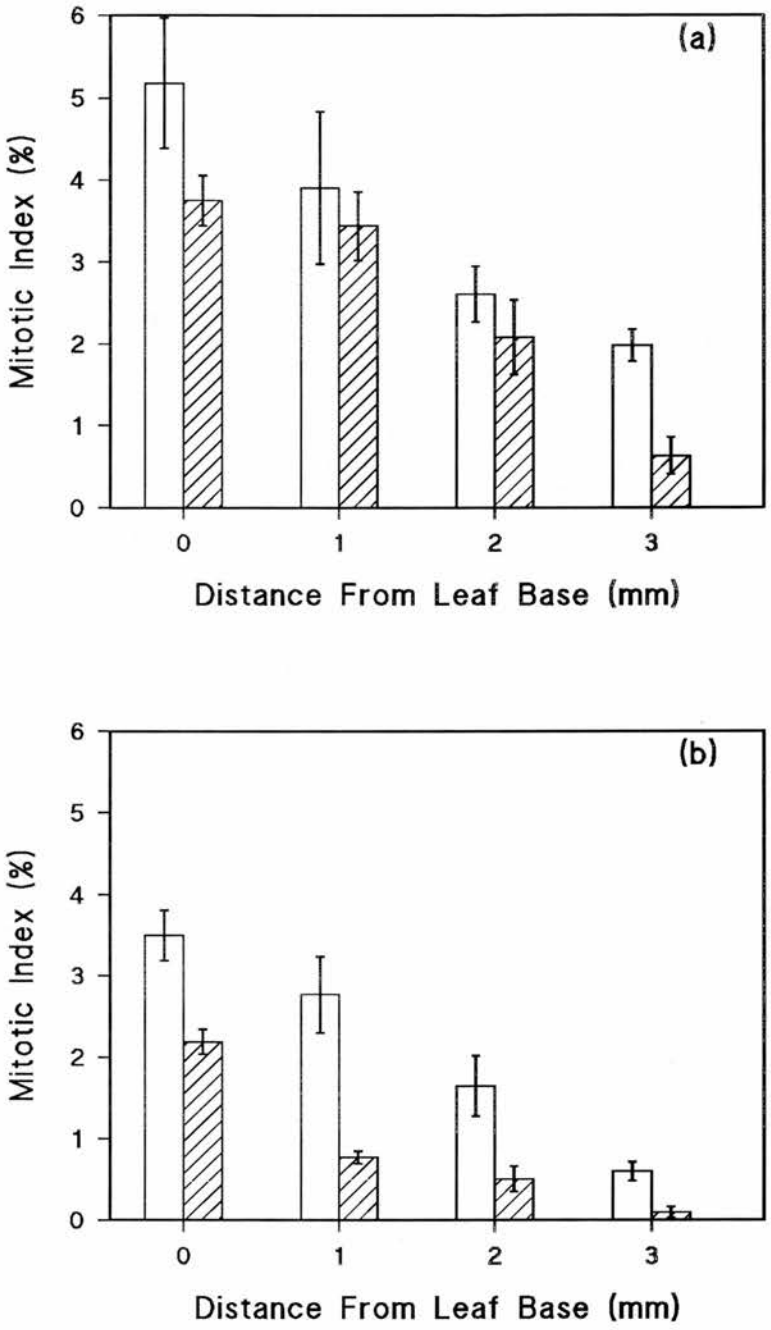


Figure 3.15 The mitotic index of cells at the primary leaf base of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The mitotic index was determined for six-day old seedlings as described in Section 2.3.5. Each point represents the mean value of 8 random counts of 200 cells, from 5 replicate treatments, with error bars showing ± one standard error from the arithmetic mean.

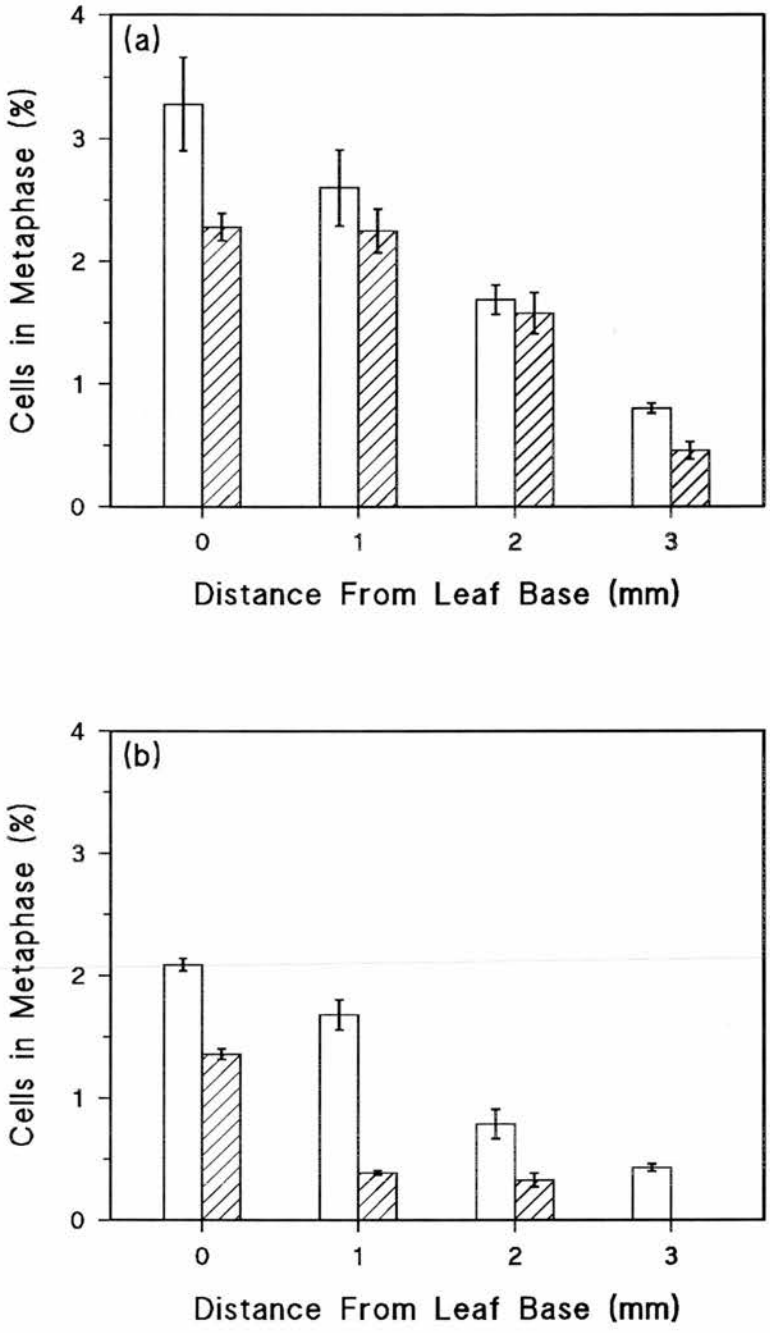


Figure 3.16 The proportion of cells in metaphase within the primary leaf base of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The proportion of cells in metaphase was calculated as described in Section 2.3.5. Each point represents the mean value of 8 random counts of 200 cells, from 5 replicate treatments, with error bars showing ± one standard error from the arithmetic mean.

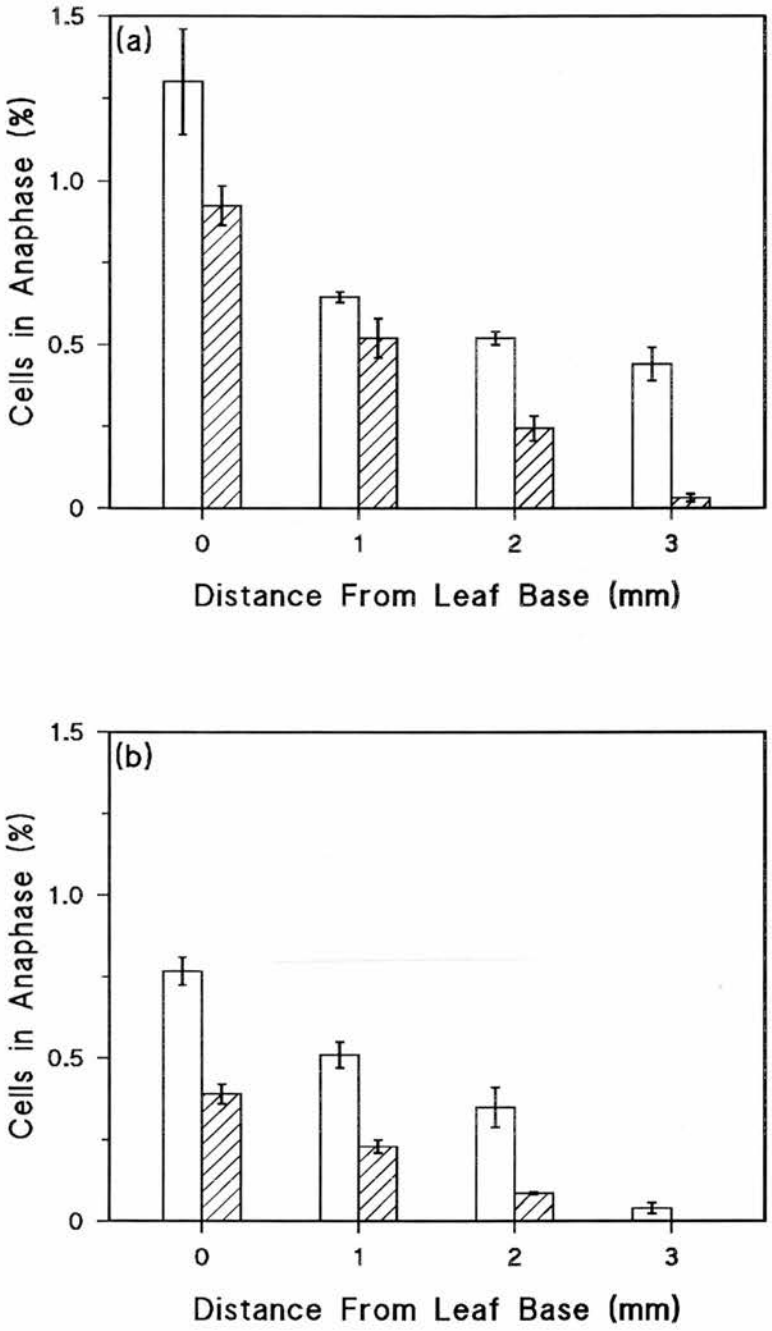


Figure 3.17 The proportion of cells in anaphase within the primary leaf base of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The proportion of cells in anaphase was calculated as described in Section 2.3.5. Each point represents the mean value of 8 random counts of 200 cells, from 5 replicate treatments, with error bars showing ± one standard error from the arithmetic mean.

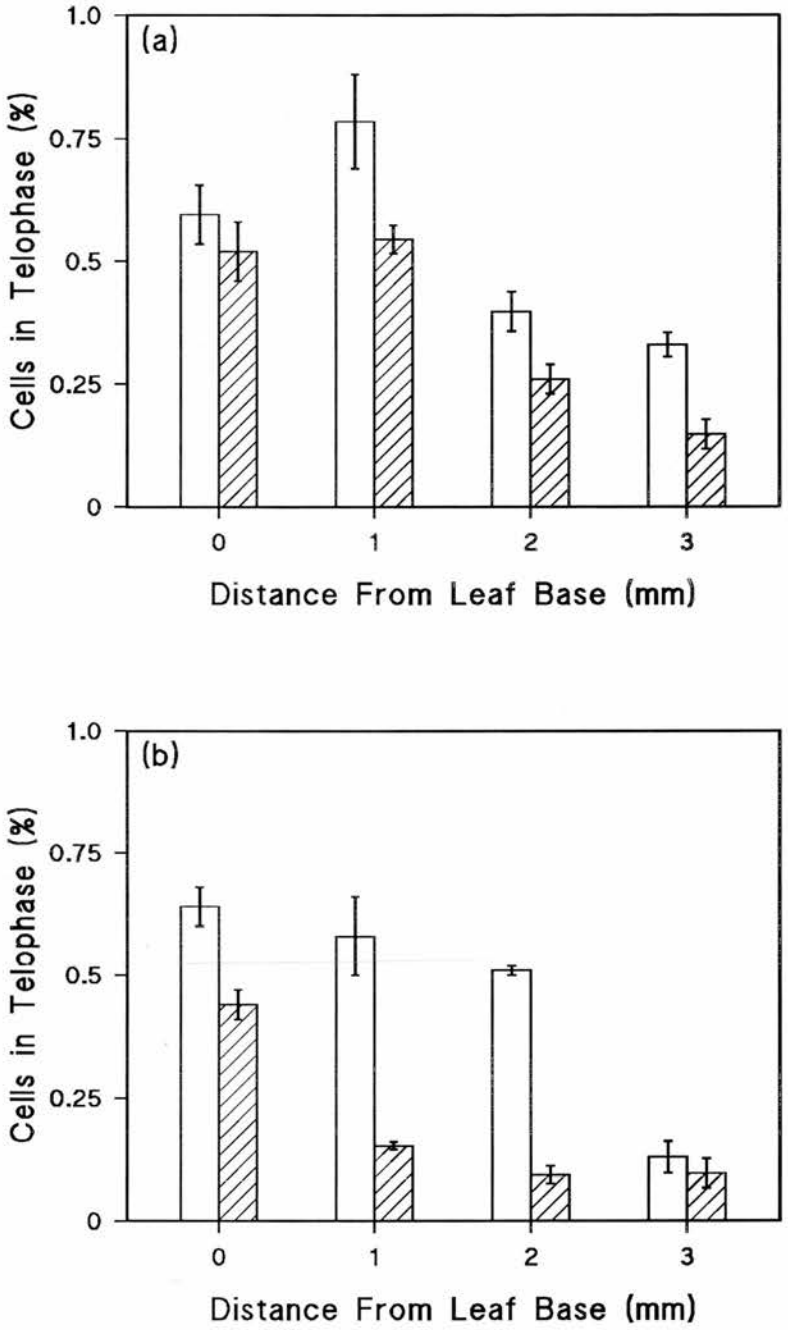


Figure 3.18 The proportion of cells in telophase within the primary leaf base of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The proportion of cells in telophase was calculated as described in Section 2.3.5. Each point represents the mean value of 8 random counts of 200 cells, from 5 replicate treatments, with error bars showing ± one standard error from the arithmetic mean.

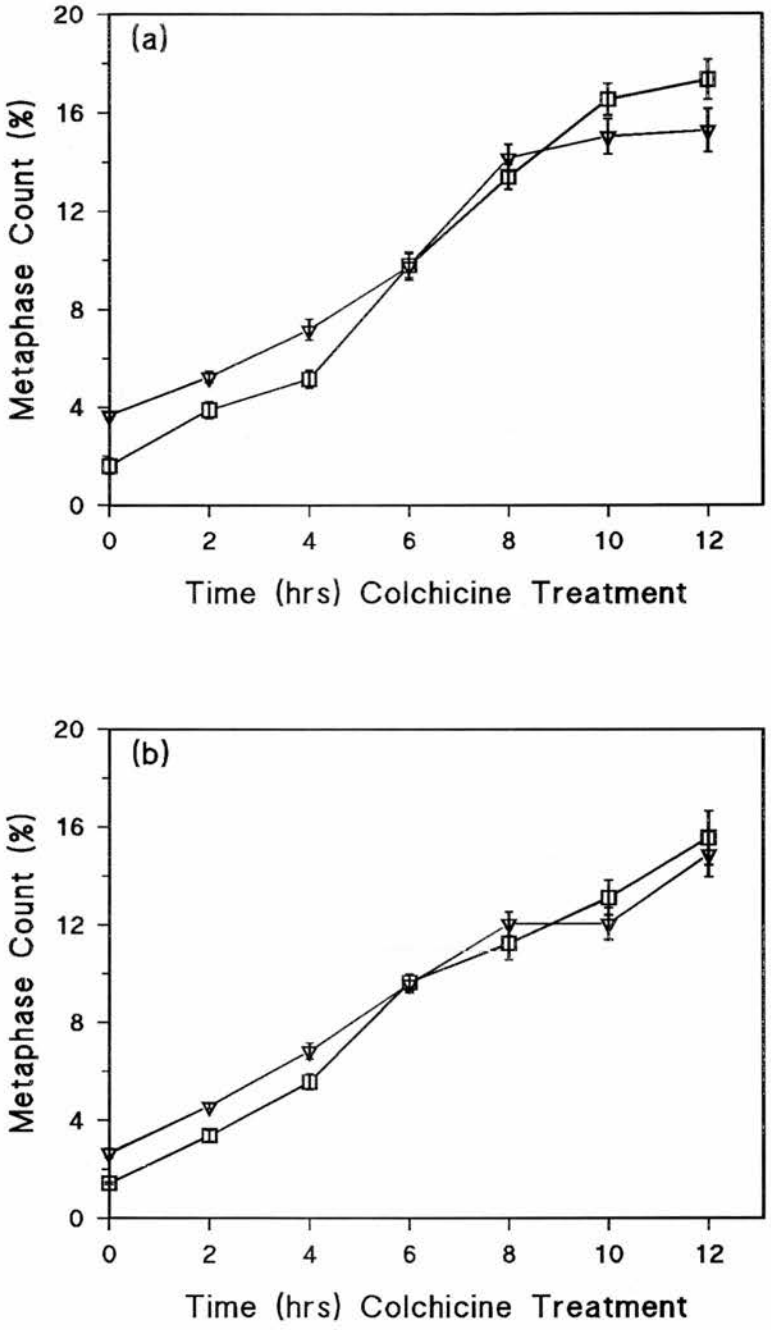


Figure 3.19 Determination of cell division rates within the basal intercalary meristem of Maris Huntsman (a) and Yecora Rojo (b).

Plants were grown with (∇) and without supplementary UVB (□) (Section 2.2). The cell division rates were determined for six day old seedlings as described in Section 2.3.6. The accumulation of metaphase-arrested cells is shown as a proportion of the total cell population. Each point represents the mean value of 8 random counts each of 400 cells from 3 replicate treatments with errors bars showing ± one standard error from the arithmetic mean.

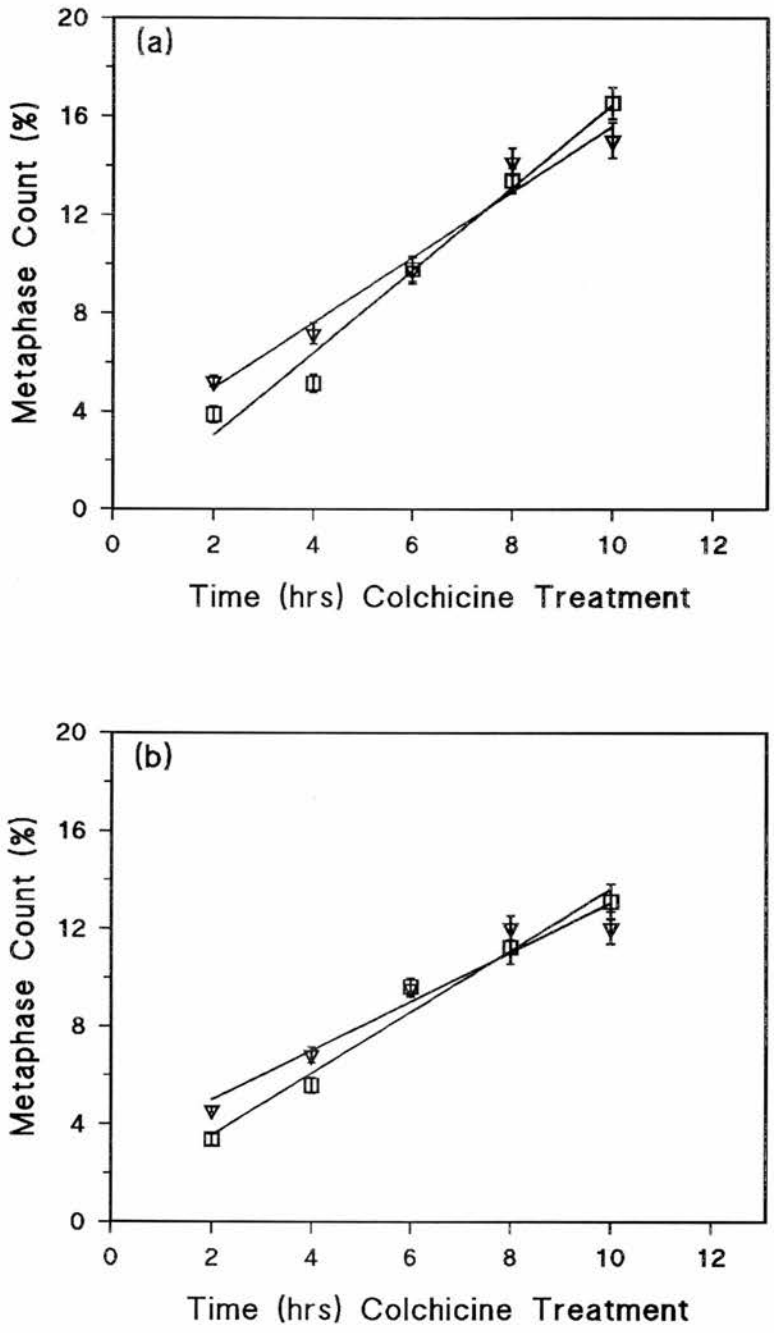


Figure 3.20 The linear regression of the cell division rates within the basal meristem of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (∇) and without supplementary UVB (□) (Section 2.2). The cell division rates were determined for six-day old seedlings as described in Section 2.3.6. The accumulation of metaphase-arrested cells is shown as a proportion of the total cell population. Each point represents the mean value of 8 random counts each of 400 cells from 3 replicate treatments with errors bars showing ± one standard error from the arithmetic mean.

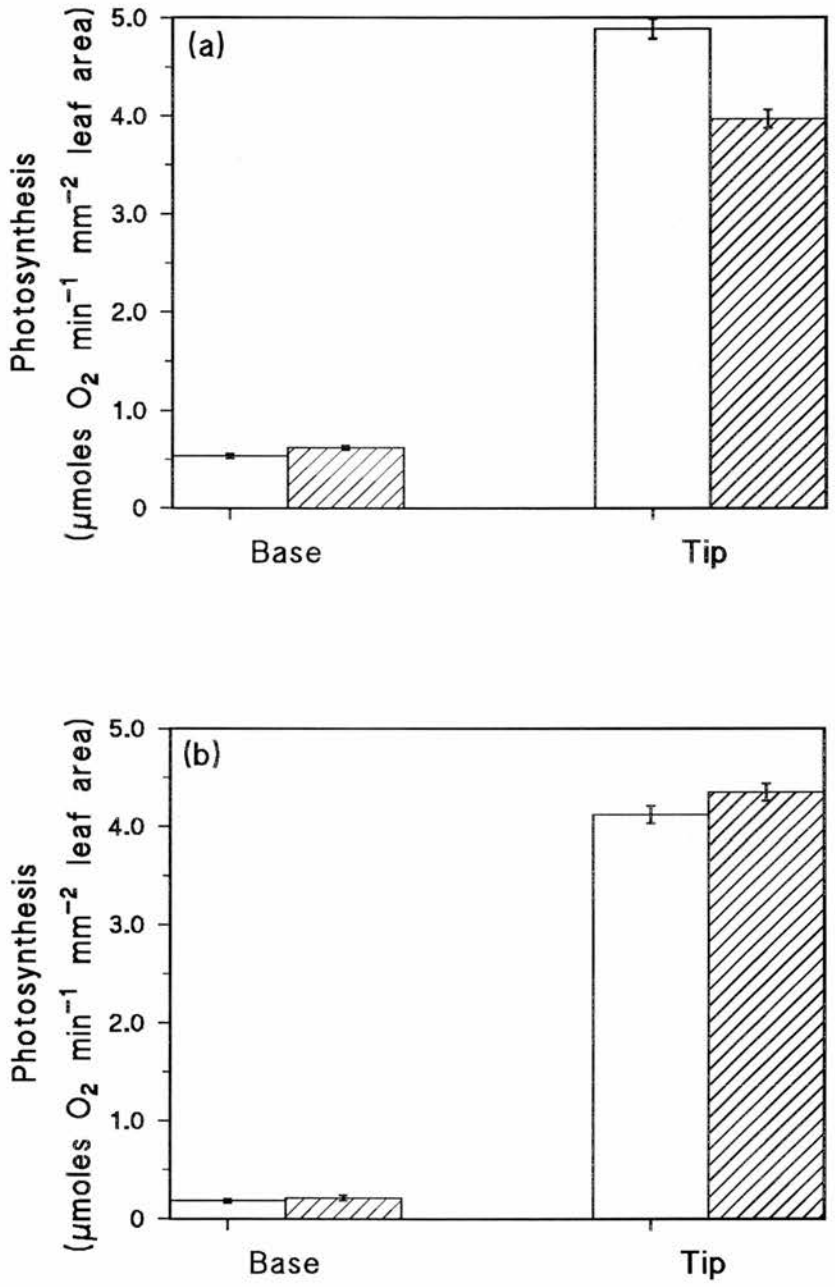


Figure 3.21 Changes in the rate of photosynthesis in the base and tip of the primary leaf of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The photosynthetic rate was determined for six-day old seedlings as described in Section 2.4. Each point represents the mean of 5 replicates from each treatment, with error bars showing ± one standard error from the arithmetic mean.

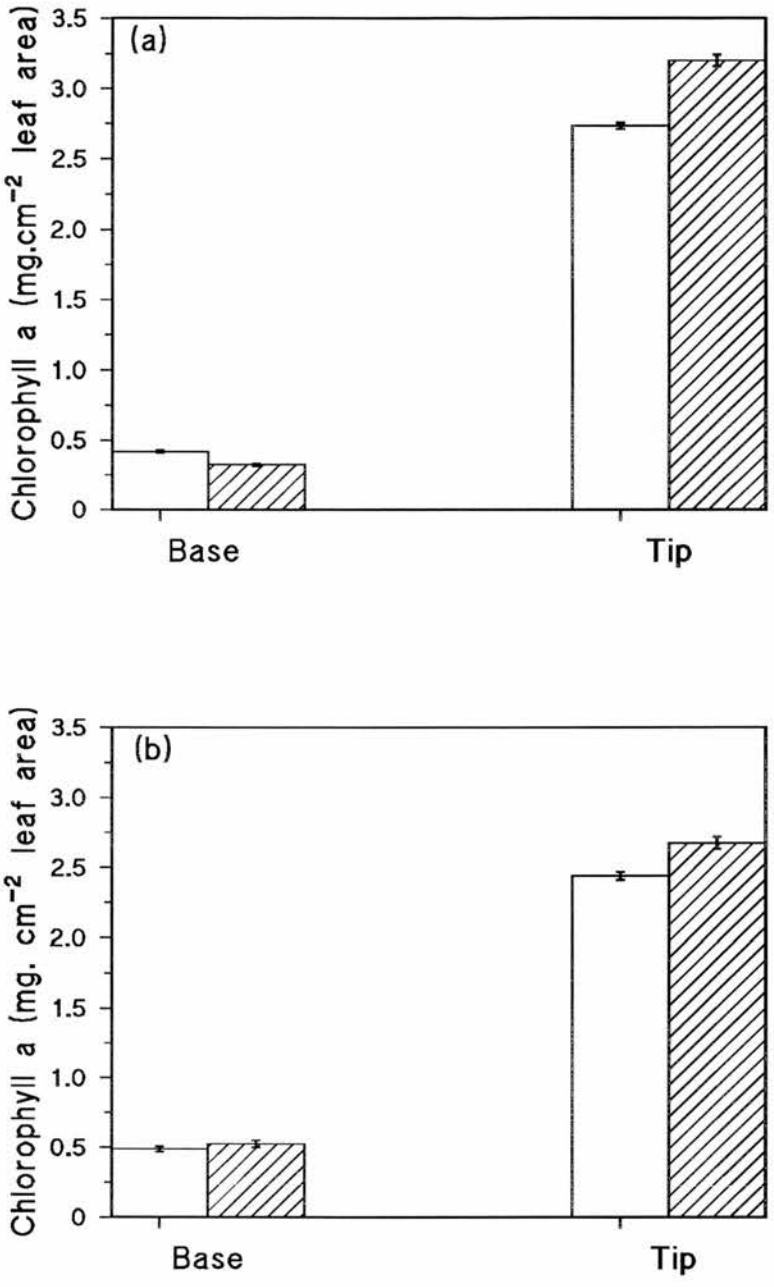


Figure 3.22 Changes in the Chlorophyll a content in the base and tip of the primary leaf of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The chla content was determined for six-day old seedlings as described in Section 2.5. Each point represents the mean of 5 replicates from each treatment, with error bars showing ± one standard error from the arithmetic mean.

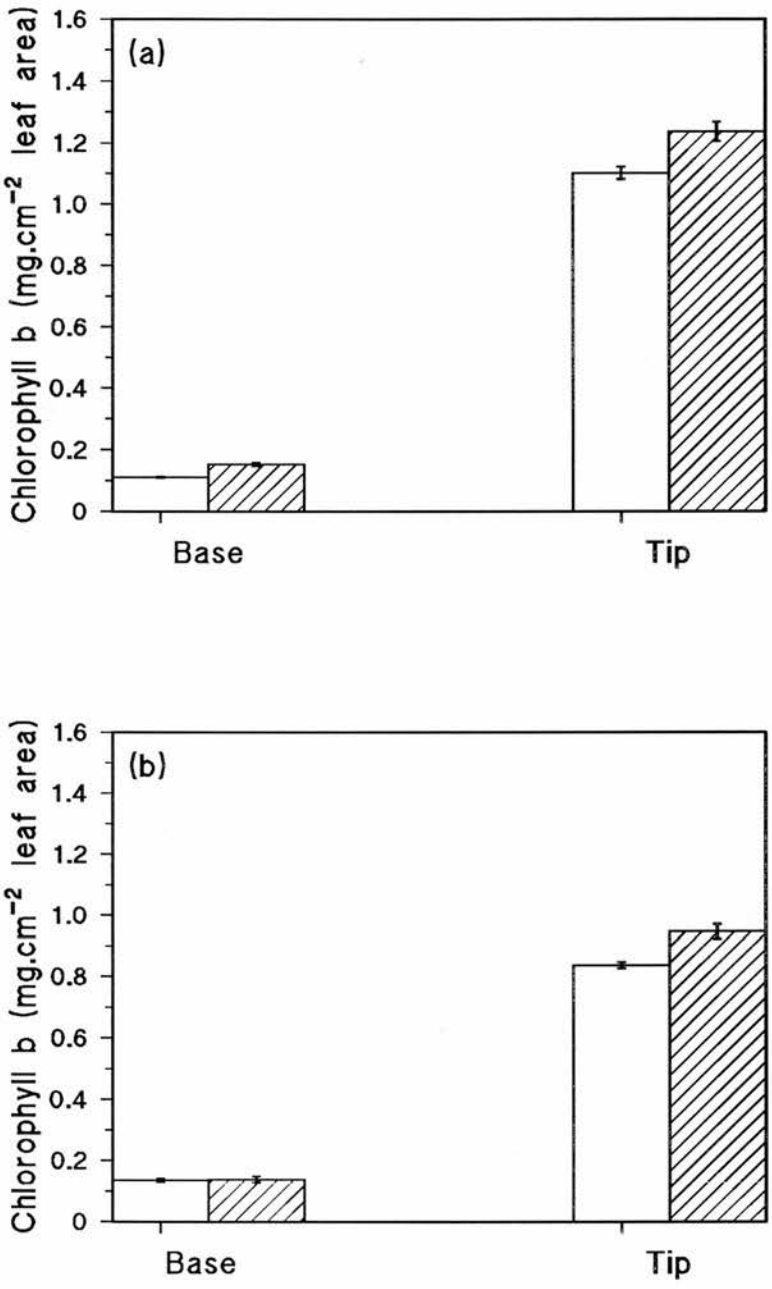


Figure 3.23 Changes in the Chlorophyll b content in the base and tip of the primary leaf of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The chlb content was determined for six-day old seedlings as described in Section 2.5. Each point represents the mean of 5 replicates from each treatment, with error bars showing ± one standard error from the arithmetic mean.

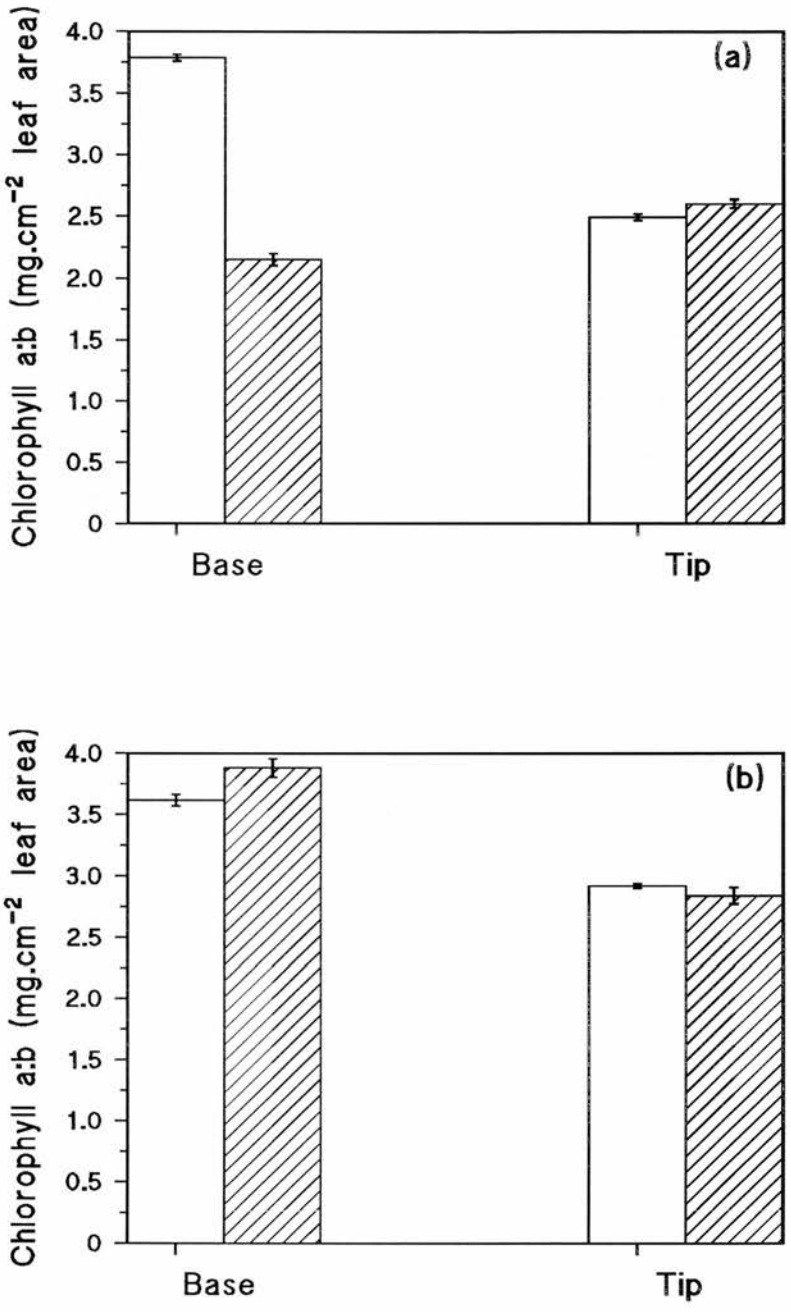


Figure 3.24 Changes in the Chlorophyll a:b ratio in the base and tip of the primary leaf of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The chla:b ratio was determined for six-day old seedlings as described in Section 2.5. Each point represents the mean of 5 replicates from each treatment, with error bars showing ± one standard error from the arithmetic mean.

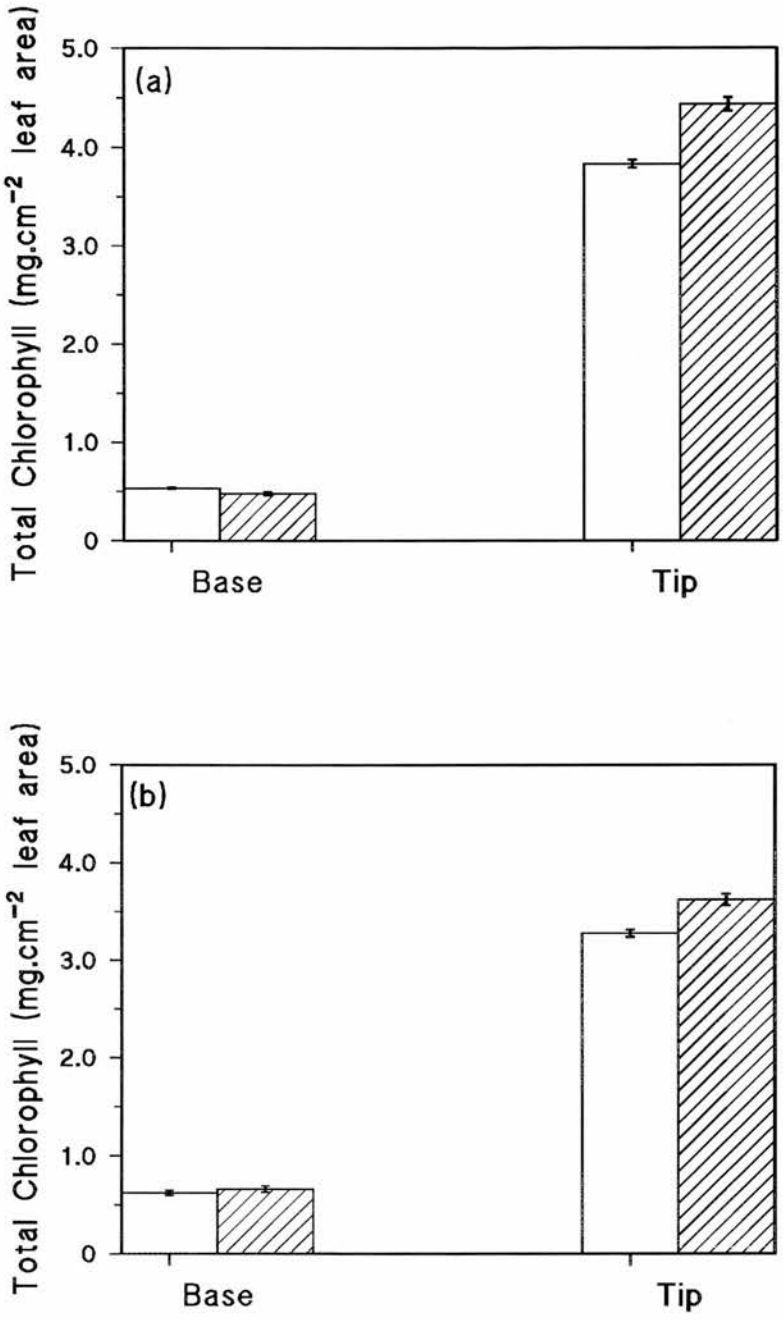


Figure 3.25 Changes in the total Chlorophyll content in the base and tip of the primary leaf of Maris Huntsman (a) and Yecora Rojo (b).

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The total chl content was determined for six-day old seedlings as described in Section 2.5. Each point represents the mean of 5 replicates from each treatment, with error bars showing ± one standard error from the arithmetic mean.

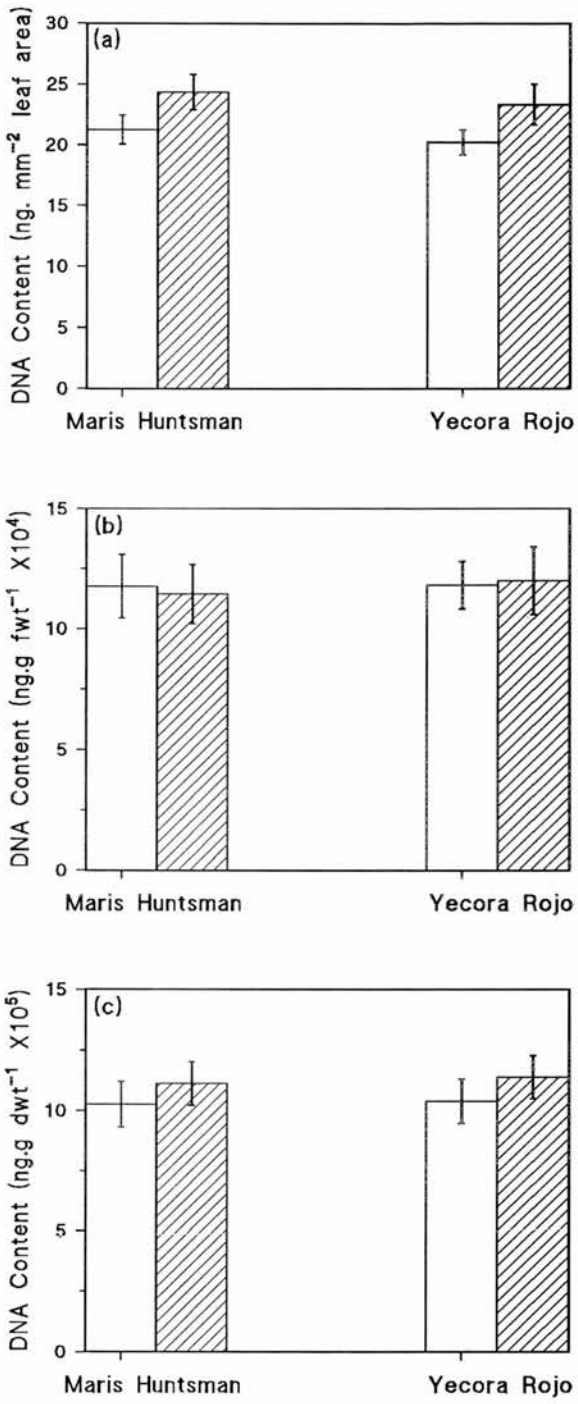


Figure 3.26 The DNA content of the primary leaf of Maris Huntsman and Yecora Rojo grown with and without supplementary UVB.

The growth conditions are described in section 2.2. DNA was extracted from the leaves as described in section 2.7.2. The DNA content of the leaves was calculated per unit leaf area (a), per g leaf fresh weight (b) and per g leaf dry weight (c) for plants grown with (▨) and without UVB (□). Each point represents the mean value of 200 samples from each treatment, with the error bars showing ± standard error from the arithmetic mean.

Discussion

3.11 The Effect of UVB on Plant Growth

The total plant height, primary leaf length and primary leaf area of Maris Huntsman and Yecora Rojo decreased significantly when grown under supplementary UVB (Figs. 3.2, 3.3 and 3.5). The reduction in total plant height under UVB was greater on day 7 than day 4 in both cultivars. One explanation for this could be that on day 4 almost half of the leaf is covered by the coleoptile which acts as a protective barrier against UVB (Haussuhl *et al.*, 1996). The primary leaf length of Maris Huntsman, the British cultivar, was reduced to a greater extent when grown with supplementary UVB, than the Saudi Arabian cultivar Yecora Rojo (25% and 19% respectively), and the same pattern can be seen for total plant height. Larson *et al.* (1990) found a similar result when examining the effects of UVB on two species of columbines, *Aquilegia caerulea* (an alpine species) and *A. canadensis* (a lower elevation species). Although both species showed a significant reduction in height when grown with supplementary UVB, the degree of shortening was greater in the non-alpine species. A reduction in growth under UVB may be caused by changes in cell elongation and/or cell division, both of which will be discussed later in this chapter.

A number of plant species have shown a reduction in height when grown under UVB, such as *Rumex patientia* (Sisson & Caldwell, 1976), *Zea mays* (Mark *et al.*, 1996), *Hordeum vulgare* (Lui *et al.*, 1995) and *Triticum aestivum* (Becwar *et al.*, 1982; Barnes *et al.*, 1990). Teramura (1980) concluded that when plants are grown in a greenhouse the magnitude of response of wheat to UVB depends upon the PAR level during growth, since UVB radiation is much more effective in producing large shifts in biomass allocation at reduced PAR levels. However, when Becwar *et al.* (1982) examined the effects of UVB on pea (*Pisum sativum*), potato (*Solanum tuberosum*), radish (*Raphanus sativus*) and wheat (*Triticum aestivum*) at a 3000m elevation field site, wheat was the only plant to show a significant response. With an increase in UVB, the height of wheat was significantly reduced even though the plants were receiving high levels of PAR.

A reduction in leaf length and leaf area was found for 5 monocot species, including wheat, when comparing the effects of UVB on crop and weedy species (Barnes *et al* ., 1990). The results showed that monocots were more morphologically responsive to UVB than dicots and there seemed to be no difference in the morphological response of crop and weedy species. However, when Tevini *et al.*, (1981) examined the effects of UVB on barley (*Hordeum*

vulgare), corn (*Zea mays*), bean (*Phaseolus vulgaris*) and radish (*Raphanus sativus*), the most sensitive plant was bean with the monocots, barley and corn, showing much smaller reductions in growth.

The results from this chapter show that primary leaf growth rate decreased significantly when grown with supplementary UVB for both cultivars from day 5 to day 7. The reduction in the growth rate under UVB can, in part, account for the decline in growth of both cultivars. The decline in growth rate of Maris Huntsman and Yecora Rojo under UVB can perhaps be explained by early senescence of the leaf. It seems that the primary leaf reaches its maximum height more rapidly when grown with supplementary UVB than when grown under control conditions. Early leaf maturation was suggested as a reason to explain UVB effects on soybean (*Glycine max*; Teramura & Caldwell, 1981) as UVB seemed to accelerate normal leaf development.

When studying the inhibition of growth by UVB in the *Pisum sativum* cultivar, Guildo, González *et al.* (1998) concluded that the reduction in growth was a result of an irreversible reduction in leaf expansion and the reversible inhibition of development. The response of a plant to UVB appears to depend on the developmental stage at which the stress occurs. This has been reported by Teramura *et al.* (1984) who showed that soybean plants were more sensitive to elevated UVB during the vegetative phase of growth rather than later on in development. Since the epidermis plays a limiting role in leaf expansion (Kutschera, 1992), the effect of UVB on epidermal cell number and cell enlargement may be the reason for reduced growth under UVB. This will be discussed further in Section 3.13 and 3.14.

Another reason for the reduction in growth could be that photooxidative destruction of the plant growth regulator indole acetic acid (IAA) is taking place followed by lower cell wall extensiblity. This was examined in *Helianthus annuus* by Ros & Tevini (1995) who observed *in vivo* concentrations of IAA in UVB irradiated seedlings were reduced by more than 50% compared to controls. Therefore, IAA destruction could be a mechanism for the inhibition of elongation of UVB irradiated seedlings.

In this study the fresh and dry weights of Maris Huntsman and the fresh weights of Yecora Rojo were also reduced when grown with supplementary UVB. Plant biomass has been found to decrease in some species but not others when grown under UVB. For example, dry weight and leaf area were both reduced when *Glycine max* was grown under UVB (Sullivan and Teramura, 1990); one cultivar of bean (*Phaseolus vulgaris*) showed a reduction in leaf area and dry matter under UVB but the other cultivar under investigation showed no significant difference (Pinto *et al.*, 1999); UVB had no

effect on plant biomass in *Pisum sativum* (Day *et al.*, 1996); and UVB caused a reduction in height but no reduction in dry weight in monocots (Barnes *et al.*, 1988; Barnes *et al.*, 1990).

In this study, supplementary UVB decreased the leaf area of both cultivars, although the dry weight was not reduced to the same extent. The specific leaf area (ratio of leaf area to dry weight) was reduced with UVB in both cultivars (Fig. 3.7). Specific leaf area takes into account both the density and thickness of the leaf. Therefore, when grown under UVB the leaves of both cultivars increase in thickness and density. An increase in leaf thickness is a common observation when plants are grown under UVB and it has been suggested that this is a protective response (Cen & Bornman, 1990; Bornman & Vogelmann, 1991; Cen & Bornman, 1993).

It is important to look at a number of factors when analysing the effects of UVB on plants since alterations in morphology involve both the inhibition and stimulation of various parts of the plant, and therefore morphology may be a more sensitive indicator of UVB exposure than leaf photosynthesis or total biomass production (Barnes *et al.*, 1990). Morphology alterations caused by UVB are important since they can change the amount of light a plant intercepts and may therefore alter the competitive balance of the plant.

3.12 Effects of UVB on the Coleoptile

UVB significantly reduced the height, and the fresh and dry weights of the coleoptile of Maris Huntsman. In Yecora Rojo fresh and dry weight was unaffected and only the height of the coleoptile was significantly reduced when grown under supplementary UVB. The coleoptile acts as a protective sheath to the developing leaf during upward growth through the soil (Hart, 1988) and it has been suggested that in addition to this role the coleoptile may also protect the seedling from harmful radiation (Haussuhl *et al.*, 1996).

Although coleoptile growth is inhibited by short wavelengths of white light (Baroncelli *et al.*, 1984), it is unlikely that this is the reason for the reduction of coleoptile height under UVB since, both control and UVB conditions contained the same amount of blue light (420-450nm). Coleoptile growth is entirely due to cell elongation, and therefore UVB must be affecting cell elongation in order to cause a reduction in coleoptile growth. Studies have shown that growth in coleoptiles is promoted by auxins, IAA (Wright, 1961; Rose & Crossman, 1982), and therefore the growth of coleoptiles may be inhibited due to the photooxidative destruction of IAA, as discussed above for leaf tissue (Ros & Tevini, 1995).

Studies have shown that coleoptiles are photosynthetically active (Bette & Kutschera, 1996) and that they provide carbohydrates for the developing seedling (Fröhlich & Kutschera, 1995). Therefore, the changes observed in the coleoptile of UVB grown plants could affect the carbohydrate metabolism within the developing leaf system.

3.13 The Effect of UVB on Cell Elongation

In grasses, leaf elongation occurs in a region at the base of the leaf that is enclosed by sheaths of older tissue. Therefore, experiments examining the elongation zone can usually only be assessed using destructive techniques. The technique used in this project was the hole-punching method (Schnyder & Nelson, 1988) and this has been used in a number of studies (Ben-Haj-Salah & Tardieu, 1995; Ferris *et al.*, 1996). This method involves piercing the base of the leaf, and fine wire was used in order to minimise growth reductions. Leaf elongation is determined by the length of the segmental elongation rate (SER) and the length of the elongation zone (Schnyder *et al.*, 1990; Skinner & Nelson, 1994), both of which were measured in this study.

The results in this chapter show that when Maris Huntsman and Yecora Rojo were grown with supplementary UVB, the length of the elongation zone and SER decreased. Leaf elongation has been shown to change under various growth conditions, such as high temperatures (Ben-Haj-Salah & Tardieu, 1995; Durand *et al.*, 1999); drought (Durand *et al.*, 1995); CO_2 (Ferris *et al.*, 1996); nitrogen supply (Nelson & MacAdam, 1989; Roggatz *et al.*, 1999) and UVB (Ballaré *et al.*, 1995; Nogues *et al.*, 1998).

Cell elongation is caused by changes in turgor pressure and cell wall extensiblity, and therefore factors which alter these cell properties will in turn alter cell elongation. With increased CO_2 the SER in ryegrass (*Lolium perenne*) increased (Ferris *et al.*, 1996), and it was concluded that the increase was due to changes in cell wall loosening enzymes and cell wall plasticity. Fricke & Flowers (1998) suggested that leaf expansion in barley relies on high rates of water and solute supply which are not available during periods of limiting N, and this is why cell elongation is altered with changes in N concentration.

There are a number of possible explanations for the decrease in the elongation zone and the maximum elongation rate with UVB. The amount of assimilates imported into the elongation zone depends upon irradiance (i.e. PPFD the plants are receiving; Schnyder & Nelson, 1989). Carbohydrates are translocated mainly in the form of sucrose and if the levels of imported sucrose

are altered this may change the turgor pressure within the cell. Auxins such as IAA, and phenols such as ferulic acid, can change cell wall extensibility (Brett & Waldron, 1996; Fry, 1986). With UVB, alterations in the amounts of IAA (Ros & Tevini, 1995) and ferulic acid (Lui *et al.*, 1995) have been observed and this may explain why cell elongation was decreased in this study.

Ballaré *et al.* (1995) constructed an activity spectrum to determine the cause of UVB inhibited elongation in tomato (*Lycopersicon esculentum*) seedlings. Since the maximum effectiveness was at 297nm they suggest that a flavin chromophore is likely to be involved in this elongation response.

3.14 The Effect of UVB on Cell Division

Changes in cell division, like cell elongation, can occur under different growth conditions, for example, changes in CO_2 (Kinsman *et al.*, 1996; 1997); temperature (Harrison *et al.*, 1998); and UVB (Nogues *et al.*, 1998; Dickson & Caldwell, 1978). The changes in cell division may result from variations in cell division rates (i.e. mitotic index) and/or cell doubling time (cdt). Both parameters were measured to study the effect of UVB on cell division. In the primary leaf of wheat mitotically active cells are located within the basal 4mm and make up around 5 to 8% of the total proportion of cells (Ellis *et al.*, 1983).

The percentage of cells undergoing mitotis recorded in this chapter are similar to that in other studies (Casero *et al.*, 1989). When grown under supplementary UVB, the amount of mitotically active cells in Yecora Rojo decreased. The cdt for each cultivar increased significantly when grown with supplementary UVB; therefore since the time taken for cell division was increased, growth of the plants will be reduced. A reduced number of cell division cycles was also observed in *Rumex patientia* under UVB (Dickson & Caldwell, 1978); however it appeared that the duration of the cell cycle was not affected.

Cell elongation and division can be influenced by growth substances, such as auxins (Hoson *et al.*, 1992; Wernicke & Milkovits, 1987). In the absence of auxins, such as IAA, cells of *Triticum timopheevi* ceased to divide (Wernicke & Milkovits, 1987) and perhaps the photooxidation of auxins under UVB may affect cell division.

The expression of genes involved in cell division was examined in *Petroselinum crispum* under UV radiation and genes involved in cell cycle regulation were found to be repressed, and as a result an increase in cdt was observed (Logemann *et al.*, 1995). A reduction in cell division may be an adaptive rather than an injurious response. Isolated protoplasts from *Petunia*

hybrida were examined to assess whether UVB has an effect on cell division (Staxén *et al.*, 1993). The results showed an arrest of cells in G1, S and G2 phases by UVB. The maximum absorbance of DNA is at 280nm (Quaite *et al.*, 1992) which is in the UVB range and studies have shown that DNA damage accumulates in plant tissue following UVB irradiation (Taylor *et al.*, 1996). Perhaps the DNA damage must be repaired before the cell can progress through the cell cycle thus the increasing cdt. The cell would be likely to spend a longer time in G1 and S phase repairing DNA damage and synthesising new DNA for progression through the rest of the cell cycle. DNA damage will be discussed in greater detail in chapter 4.

The amount of mitotically active cells in Maris Huntsman is unaffected by UVB, therefore, the reduction in growth of this cultivar is caused by an increase in cell cycle duration and a decrease in cell elongation.

3.15 The Effect of UVB on Photosynthesis

Along the leaf there is an increase in the rate of photosynthesis from the base to the tip (see Section 3.3). Both cultivars show an increase in photosynthesis from base to tip under control and UVB conditions. When Maris Huntsman is grown with UVB there is a decrease in photosynthesis at the tip of the leaf. However there is no significant difference in the photosynthesis of UVB grown Yecora Rojo compared to control conditions. A reduction in photosynthesis in UVB grown plants has been observed in a number of studies (Bornman & Vogelmann, 1991; Sullivan & Teramura, 1989; Day & Vogelmann, 1995). When grown under supplementary UVB, the amount of chl increases in both cultivars thus, the decrease in photosynthesis in Maris Huntsman is not due to a decrease in chlorophyll.

UVB can directly affect photosynthesis by damaging PSII, changing the chloroplast ultrastructure or reducing Rubisco activity. In order to have any direct or developmental effects on photosynthetic productivity, UVB must penetrate through the leaf and be absorbed (Allen *et al.*, 1998). Various studies have shown PSII to be the most sensitive component on exposure to UVB. As a result, PSII damage has often been implicated as the major potential limitation to photosynthesis in UVB irradiated plants (Bornman, 1989; Stapleton, 1992; Teramura & Sullivan, 1994). Chlorophyll fluorescence studies have shown that PSII is damaged by UVB (Bornman & Vogelmann, 1991; Middleton & Teramura, 1993; Ziska *et al.*, 1993). It was suggested that UVB radiation damages PSII by changing the oxidative capacity of the reaction center (Renger *et al.*, 1989). UVB can stimulate lipid peroxidation which can cause membrane

alteration and damage, and if chloroplast membrane stability is affected this could be reflected by a change in photosynthesis.

The activity of Rubisco, the primary enzyme of carbon dioxide fixation, may decline under UVB due to either deactivation or loss of the enzyme. The UVB-induced decline of Rubisco in *Brassica napus* was due to a reduction in the amount of Rubisco present rather than deactivation of the enzyme (Allen *et al.*, 1997). A reduction in the Rubisco content under UVB was also observed in *Glycine max*, *Pisum sativum* and *Lycopersicon esculentum* (Vu *et al.*, 1982). Within hours of UVB exposure the mRNA levels coding for both the large and small subunits of Rubisco declined before any effect at the protein level was evident (Jordan *et al.*, 1992), indicating that the UVB radiation photoreceptor may not be Rubisco itself. However, the UVB effect on Rubisco appears to be reversible under high PAR since the mRNA levels were much higher under high PAR and it was suggested that this may be a protective mechanism at the DNA level (Strid *et al.*, 1996).

Photosynthesis can also be indirectly affected by UVB. For example, numerous experiments have shown a reduction in stomatal conductance in response to UVB irradiation (Teramura *et al.*, 1983; Middleton & Teramura, 1993). Changes in leaf thickness may affect the amount of light penetrating the leaf and thus change photosynthesis. Changes in primary photosynthetic pigments with UVB such as chlorophyll, have been found in a number of species. Under UVB the chlorophyll in some plants increased (Dai *et al.*, 1992), in others there was a decrease (Bornman & Vogelmann, 1991; Hidema *et al.*, 1996; Jordan *et al.*, 1994) and other studies showed that chlorophyll content was not affected (Day *et al.*, 1999; Day *et al.*, 1996; Gwynn-Jones & Johanson, 1996). Not only have studies observed changes in chlorophyll concentration with UVB, but a change in distribution of chlorophyll has also been noted. Following UVB treatment there was a shift in the distribution of chlorophyll to deeper layers of the mesophyll in *Pisum sativa* plants (Day & Vogelmann, 1995).

The ratio of chl a:b changes during leaf development because of the location of chl a and chl b in the chloroplast. PSI contains chl a and a small amount of chl b, and is located on the unstacked stromal thylakoids, whereas PSII contains both chl a and b and is located on the stacked granal thylakoids (Anderson & Beardall, 1991). Therefore, the chl a:b ratio is highest at the base of the leaf and decreases towards the tip. In this chapter the results show that when the two cultivars were grown with supplementary UVB there was a decrease in both chlorophyll a and b. Since chl a and b both decreased at the tip of the leaf in the two cultivars there was no change in the chl a:b ratio, and

therefore there was presumably no change in the PSI:PSII ratio with UVB, although this was not measured.

Structural changes in chloroplasts have been observed in plants grown under UVB, such as disruption of the envelope and dilation of thylakoids (Bornman *et al.*, 1983; Brandle *et al.*, 1977). The PSII complex is embedded in the thylakoid membrane so structural disturbances can have an effect on the photosynthetic capacity. Studies have shown that under UVB gene expression for chloroplastic proteins is repressed (Jordan *et al.*, 1991). However, this response appears to be dependent on the developmental stage of the plant. Other studies have found that chloroplastic DNA is damaged by UVB (Cannon *et al.*, 1995; Chen *et al.*, 1996; Hada *et al.*, 1998) and it is possible that this could lead to a decrease in photosynthetic capacity of the plant. When grown under supplementary UVB, the leaves of Maris Huntsman appear to be showing early senescence and this could be a reason to account for the decrease in photosynthesis in this cultivar with UVB. Photosynthesis is an integrated physiological process which requires the integrity of membrane systems and the co-ordinated response of enzymatic and photochemical processes. Therefore, it is a useful indicator of plant response to stress and it is not surprising that Maris Huntsman shows a reduction in photosynthesis with UVB.

Various studies have examined how plants from different geographical regions respond to UVB and whether plants which naturally grow in areas where UVB is higher are less damaged. When examining photosynthesis of plants grown under UVB, arctic species appeared to be more inherently sensitive than alpine species (Caldwell *et al.*, 1982); plants collected from equatorial, alpine sites had no UVB-induced damage to photosynthesis, however, plants from higher latitudes were inhibited (Barnes *et al.*, 1987); and UVB did not affect photosynthesis in an Ethiopian cultivar of *Brassica carinata* even though the Swedish cultivar of *Brassica campestris* was affected (Bornman & Vogelmann, 1991). It has been suggested that plants may evolve some physiological adaptations to UVB due to their geographical origin (Bornman & Vogelmann, 1991). In this study, the British cultivar, Maris Huntsman, had a reduced photosynthetic capacity under UVB; but photosynthesis in the Saudi Arabian cultivar, Yecora Rojo was unaffected.

3.16 DNA Content of the Leaf

The DNA content of the two cultivars was measured in order to examine whether it changed under UVB and if the amounts in each cultivar were comparable. The two cultivars contain similar amounts of DNA and under UVB there was no significant difference in DNA content. This is important for

the experiments in the next Chapter which look at DNA damage in the two cultivars.

3.17 Conclusions

The aim of this Chapter was to characterise the growth, morphology and photosynthetic capacity of the two cultivars under control and UVB conditions. When grown with supplementary UVB, both cultivars have shorter, thicker primary leaves with shorter coleoptiles. The British cultivar is affected to a greater extent by UVB than the Saudi Arabian cultivar, and this is especially reflected in the photosynthetic capacities of each. This difference may be the result of UVB-induced DNA damage thus affecting various processes in the leaf, and the following Chapter will examine the amount of DNA damage which accumulates in the leaf following UVB exposure.

Chapter 4

The Effects of UVB on DNA Damage Accumulation and Repair in Maris Huntsman and Yecora Rojo

Introduction

4.1 DNA Damage

DNA is functionally more stable than the other two cellular macromolecules, RNA and protein. This stability is due to a number of properties such as the double helix structure which carries the information in duplicate, and the fact that all that is needed for the transfer of genetic information is the primary structure. Various repair mechanisms are also essential for the functional stability of DNA (see Section 4.3) (Sancar & Sancar, 1988; Buchholz *et al.* , 1995).

When irradiated with UV-B, DNA absorbs photons which cause several types of damage: single-strand breaks, DNA-protein cross-links and production of pyrimidine dimers. Pyrimidine dimers make up about 98% of UVB-induced DNA damage (Mitchell & Karentz, 1993) and the two pyrimidine dimers occurring are cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone dimers (or 6-4PP) (Grossweiner & Smith, 1989). The CPD and 6-4PP make up approximately 75% and 25% of the UV-induced DNA damage products respectively (Mitchell & Nairn, 1989). Dimers (both CPDs and 6-4PPs) are most efficiently induced by radiation at approximately 260nm (Britt, 1996).

CPDs are formed between the 5,6 bonds of any two adjacent pyrimidines, whereas 6-4PPs are characterised by a stable bond between positions 6 and 4 of two adjacent pyrimidines. Extensive rotation of the neighbouring pyrimidines, from their usual B-form DNA alignments, is required for their formation. CPDs cause small but significant deformations of the DNA double helix, while 6-4PPs may produce more significant deformations. An important requirement for UV damage induction is the ability of the DNA to unwind and bend, as sequences that can easily do this are favourable sites for damage formation (Tornaletti & Pfeifer, 1996).

A single pyrimidine dimer (either CPD or 6-4PP) in the absence of repair, is sufficient to completely eliminate expression of a transcriptional unit (Britt, 1996; Sancar & Sancar, 1993). Only a fraction of pyrimidine dimers result in a mutation while every dimer acts as a block to transcription and replication. Therefore, in plant tissue the inhibitory effects of UVB on transcription and replication are likely to have more significant biological effects than its mutagenic effects. Both CPDs and 6-4PPs have been shown to inhibit the progress of DNA polymerases. During replication when one of these lesions is reached by the advancing polymerases, the enzyme will attempt to install a nucleotide opposite the lesion only to recognise this as a

mismatched base. Then, the newly installed base is excised by the 3' to 5' mismatch repair exonuclease function of the polymerase. Pyrimidine dimers cannot base-pair with other nucleotides effectively, and therefore, they are not directly mutagenic. Instead, they act as blocks to DNA replication (Britt, 1995).

The action spectrum for DNA damage in plants depends on the DNA absorption spectrum and on the attenuation by shielding layers and pigments (Quaite *et al.* 1992). When the wavelength dependence of DNA damage formation in intact *Medicago sativa* seedlings was measured, the action spectrum had a maximum near 280nm (Quaite *et al.* 1992), which differs from that for unshielded DNA (i.e. DNA *in vitro*) where the maximum occurs at 260nm (Davies, 1995). This means that increases in UVB radiation are likely to cause DNA damage *in vivo*. Accumulation of CPDs and 6-4PP have similar action spectra below 313nm with a maximum peak at 260nm. Above 313nm, the action spectra of the two photoproducts are different since the 6-4PPs are converted to Dewar isomers (Matsunaga *et al.*, 1991).

4.2 Damage to Organelle DNA

Three distinct genomes are contained in the plant cell, within the mitochondrion, the plastid and the nucleus . They contain 1, 15 and 84% of the total cellular DNA in the plant respectively (Mantell *et al.*, 1985). UV light can damage all three genomes and the damaged bases can act as blocks to DNA replication and transcription (Chen *et al.*, 1996). It has been known for some time that the chloroplasts of eukaryotic algae and higher plants contain genetically active DNA. The chloroplast (cp) and mitochondrial genomes have been fully sequenced in a number of species (Dennis *et al.*, 1997). The genome of a chloroplast exists as a covalently closed circular molecule of double-stranded DNA ranging in size from 80 to 200kbp. It contains a number of genes which code for components of the plastid's own protein synthesising apparatus and for important proteins for photosynthesis (Cannon *et al.* , 1995). It is in young leaf cells that most cp DNA synthesis takes place, and after amassing high copy numbers of the plastome per organelle, plastid division during cell development results in redistribution of the existing cp DNA copies among daughter organelles (Tymms *et al.*, 1983). Therefore, chloroplasts must have an efficient means to remove DNA damage otherwise its use as a transcriptional template would be greatly reduced as the leaf matures. Damage to cp DNA could affect the

photosynthetic capacity of a plant, thus sufficient repair mechanisms must exist to prevent a reduction in photosynthesis.

The chloroplast and mitochondrial genomes are not known to encode any repair proteins, and the three plant genomes may contain different DNA damage repair and tolerance pathways. When the rate of repair of CPDs in mitochondrial, plastid and nuclear sequences were compared, the rate in nuclear sequences corresponded to that obtained for total cellular extracts (Chen *et al.*, 1996). No significant repair was observed in either mitochondrial or plastid genomes in the presence or absence of visible light. Damage tolerance mechanisms must exist in the organelles in order for the genomes to replicate. It is possible that dimer bypass or recombinational repair (see Section 4.3.2) may take place. Perhaps it is due to this lack of repair that organellar genomes are present in 5 to 100-fold excess of the nuclear genome.

Few experiments have examined organellar DNA damage and repair directly since high nuclear DNA background is present in *in vivo* experiments and this complicates evaluation of organellar contributions. A photoautotrophic *Glycine max* cell line containing high cpDNA levels was exposed to UV (254nm) in order to study the repair of damage in chloroplast DNA (Cannon *et al.*, 1995). Cp DNA was found to accumulate half as many lesions as the nuclear DNA. This may be due to shielding of the plastome or perhaps the nuclear DNA is a more accessible target. These results suggest that chloroplasts do not posses a light-independent repair system and under white light DNA damage in the choloplasts was repaired at a much slower rate than that of nuclear damage.

When spinach (*Spinacia oleracea*) leaves were irradiated with UVB, cp DNA was found to contain 30% less CPDs than nuclear DNA but the amount of 6-4PPs was similar in both (Hada *et al.*, 1998). After exposure to blue/UVA light both CPDs and 6-4PP decreased in nuclear DNA but no repair was observed in cp DNA. In this experiment when Hada *et al.* (1998) looked at repair of DNA damage, the cp DNA contained less CPDs than nuclear DNA and therefore it is not valid to compare the amount of repair as the initial damage is different. When assessing DNA repair it is important to have the same amount of initial damage, as a certain level of damage may be required before repair takes place, also different repair mechanisms operate at different damage levels, as suggested by Quaite *et al.* (1994).

4.3 DNA Repair & Damage Tolerance Mechanisms in Plants

Beggs *et al.* (1985) classified plants into three groups: 1) Plants which prevent UVB from reaching sensitive targets e.g. via increased epidermal absorbance; 2) Plants which minimise damage by delaying growth; and 3) Plants which repair damage caused by UVB.

4.3.1 Avoidance

Plants can avoid DNA damage by accumulating pigments or waxes which absorb or reflect UVB thus minimising penetration into the leaf. These pigments, mainly flavonoids, are phenolic compounds synthesised in plants to act as 'sunscreens' for vulnerable plant tissues (Kootstra, 1994). This will be discussed in further detail in Chapter 5.

4.3.2 DNA Damage Tolerance Mechanisms

'Tolerance' pathways are known to exist which allow cells to go through replication even though DNA damage has occurred. Two 'tolerance' pathways have been identified; dimer bypass and recombinational repair (Britt, 1996). Dimer Bypass occurs when some organisms are able to produce a modified DNA polymerase, capable of performing translesion synthesis on non-informational DNA lesions. Generally, adenine residues are installed by the altered polymerase opposite the non-informational DNA damage products, thus DNA replication is permitted but it is at the expense of accuracy. This type of repair has not been identified in plants (Britt, 1996).

Recombinational Repair occurs when a pre-existing complementary strand is transferred from a homologous region of DNA to the site opposite the damage. Although the lesion is left unrepaired the cell manages to progress through replication. This mechanism has not been found in plants, although UVB irradiation have been found to induce chromosomal rearrangements (Britt, 1995).

In both of the 'tolerance' mechanisms, described above, the lesion is left unrepaired and so the gene sequence may be changed.

4.3.3 Light Dependent Repair of DNA Damage

For a variety of organisms it has been demonstrated that the damaging effects of UVB can be diminished or abolished by irradiation with long-wavelength UV or blue light (Batschauer, 1993). This process is called photoreactivation and uses enzymes, termed photolyases, to repair both CPDs and 6-4PPs. The photolyase enzyme binds specifically to CPDs and 6-4PPs to directly reverse damage (error-free) upon absorption of a photon of the

appropriate wavelength (350-450nm) (Britt, 1995). Photoreactivation is a rapid and efficient pathway for the excision of UV-induced dimers and probably provides the bulk of protection against UVB-induced DNA damage. A single protein molecule performs photoreactivation of a single dimer in DNA, with a slow (dark) step, during which the photoreactivating enzyme locates a dimer and forms a productive enzyme-substrate complex. The second step, dimer photolysis, is rapid in the presence of saturating light.

Numerous experiments have examined the repair of CPDs by photoreactivation (Pang & Hays, 1991; Quaite *et al.*, 1994; Buchholz *et al.*, 1995). Recently, light-dependent removal of 6-4PPs has been identified in *Drosophila melanogaster* (Todo *et al.*, 1993) and the 6-4PP photolyase gene was isolated from *Xenopus laevis* (Todo *et al.*, 1997). Light-dependent elimination of 6-4PPs was observed in an excision repair-deficient *A. thaliana* seedling (Chen *et al.*, 1994). The results showed that exposure to visible light prior to UVB was necessary for photoreactivation of CPDs but not for 6-4PPs. Therefore, the CPDs and 6-4PP must be eliminated by two different pathways.

Photolyases carry two cofactors, one chromophore absorbs the photoreactivating light and transfers the excitation energy to the other chromophore which serves as an electron donor (Britt, 1995). The specific binding of photolyase to the substrate is independent of light but catalysis requires light in the region of 300-500nm (Kim & Sancar, 1993).

CPD-photolyases from microbial organisms, referred to as class I photolyases, were the first photolyases to be studied and cloned (class I). They all contain reduced flavin adenine dinucleotide (FAD), and a second species-dependent chromophore is either 5,10-methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deaza-riboflavin (8-HDF) (Kleiner, 1999). This second chromophore transfers light energy to reduced FAD. An electron is transferred from the excited $FADH^-$ to the CPD leading to formation of an unstable CPD radical anion. After the carbon bonds to the cyclobutane ring and splits it, the electron is channelled back to the neutral flavin radical to begin the process again (Sancar, 1994). The absorption characteristics of the enzyme are therefore determined by the second chromophore.

In order to understand the molecular structure and expression of photolyases in plants, Batschauer (1993) attempted to clone a plant photolyase gene from mustard (*Sinapis alba*) and analyse its expression. This protein, however, was found to have no photoreactivating activity when it was over-expressed in *E. coli* (Malhorta *et al.* , 1995). The gene appears to code for a blue-light photoreceptor which is highly homologous

with the photoreactivating DNA repair enzymes. Recently, a second group of CPD-photolyases (class II) has been cloned (Taylor *et al.*, 1996; Ahmad *et al.*, 1997) and these are only distantly related to Class I photolyases. As little is known about the reaction mechanism of Class II photolyases, Kleiner *et al* (1999) purified, analysed and examined the enzymatic activity. The enzyme contained FAD, like Class I photolyases, but the second chromophore was not detectable. However, the photolyase was active, and the results suggested that the dimer-splitting mechanism of the two classes is the same.

Photolyase activity appears to be dependent on temperature and amount of initial damage. Photolyase formation was reduced at low temperatures in mustard, *Sinapis alba*, although the activity of the enzymes was high enough to compensate for CPD formation even at high UVB irradiances (Buchholz *et al.*, 1995).

With increasing initial damage in *Medicago sativa* seedlings the rate of photorepair increased (Quaite *et al.*, 1994). This could be the result of an increase in the rate of enzyme-dimer complex formation.

4.3.4 Excision Repair

Dark repair pathways do not directly reverse the DNA damage, instead the damaged DNA is replaced with new, undamaged nucleotides. There are two classes of dark repair: base excision repair (BER) and nucleotide excision repair (NER) (Britt, 1996). In BER, a system which has not yet been identified in plants, a single damaged base is removed and the DNA is restored to its original sequence by exonucleases.

NER has been deemed to be the second major DNA repair pathway and it is thought that it is needed for the repair of minor, non-dimer UV-induced photoproducts. In this process the damaged strand is removed and the undamaged strands are used as a template to fill the resulting gap. NER requires repairosome, a large protein coupler containing more than ten independent gene products. These recognise CPDs and 6-4PP and introduce incisions into the neighbourhood of the damage sites on DNA (Takao *et al.*, 1996). In contrast to BER, NER has been detected in a number plants such as *Wolffia microscopia*, *Chlamydomas reinhardtii*, and *Medicago sativa* (Degani *et al.*, 1980; Small & Greimann, 1977; Small, 1987; Quaite *et al.*, 1994). Although most experiments have shown that excision repair plays a small role in repair of UVB-induced DNA damage, this may be due to the initial damage levels as at high damage levels, excision repair contributed almost as much as photorepair in *Medicago sativa* plants (Quaite *et al.*, 1994).

4.4 Detection of DNA Damage

In this study an enzyme-linked immunosorbent assay (ELISA) was used to detect 6-4PPs and CPDs in wheat DNA using the 64M-2 and TDM-1 antibodies respectively. The monoclonal antibody, TDM-1, specifically recognises cyclobutane-type thymine dimers in DNA (Mizuno *et al.* 1991) and the 64M-2 antibody is specific for the 6-4PP (Mori *et al.*, 1991).

The binding of the TDM-1 monoclonal antibody was measured by an ELISA using 254nm UV-irradiated calf thymus DNA (Mizuno *et al.*, 1991). The absorbance of the ELISA was compared to the amount of CPDs determined by HPLC. The results showed that the binding of TDM-1 to DNA increased in a UV dose dependent manner corresponding to the induction rate of CPDs determined by HPLC, thus indicating that CPDs could be the UV lesion recognised by TDM-1 antibody. The 254nm UV-irradiated DNA was exposed to various doses of 313nm UV to clarify whether TDM-1 antibody can recognise 6-4PPs since 313nm radiation is known to photolyase the 6-4PP. With increasing doses of 313nm radiation the binding of 64M-2 to the DNA decreased, compared to the binding of TDM-1 to the DNA which did not change. This suggests that the 6-4PPs are not recognised by the TDM-1 antibody. A similar procedure was carried out for the 64M-2 antibody (Mori *et al.*, 1991). UV irradiated (254nm) DNA containing both 6-4PPs and CPDs was exposed to 313nm radiation, which converts 6-4PPs to Dewar Isomers. The binding of 64M-2 antibody decreased with increasing 313nm radiation indicating that the DNA damage targeted by 64M-2 antibody is the 6-4PP.

Normally it is desirable to test the specificity of antibodies before proceeding with experiments. However, in this study there was no suitable testing procedure available as the antibodies recognise DNA damage products rather than a protein, for example. It was necessary to establish that the antibodies detected DNA damage in plant extracts and that with increasing DNA damage the amount of absorbance on the ELISA increased. Therefore a number of experiments were carried out to test the linearity of the antibodies response.

4.5 Aims of Chapter

The aim of this Chapter is to examine the relative susceptibility of the two wheat cultivars to UVB-induced DNA damage and their capacity for repair. In the last Chapter, when the cultivars were grown under UVB, the British cultivar, Maris Huntsman was affected to a greater extent than the Saudi Arabian cultivar, Yecora Rojo. Perhaps one reason for this is that Yecora Rojo accumulates less UVB-induced DNA damage i.e. less CPDs and 6-4PPs than

Maris Huntsman. Can the amount of dimers accumulated account for the differences in growth and photosynthesis under supplementary UVB in the two cultivars?

Results

4.6 Calibration of the ELISA

To establish the appropriate antibody concentration and amount of DNA needed in the ELISA experiments, calibration curves were constructed (Figs. 4.1 to 4.3). Calf thymus DNA was irradiated with UVB as described in Section 2.6.4. Using the irradiated DNA, different antibody dilutions were used to determine which concentration was suitable for the ELISA experiments (Fig. 4.1). For the TDM-1 antibody, which detects CPDs, a 1:250 dilution was most suitable as the other concentrations (1:500 to 1:1000) showed a significant decrease in absorbance for the same UVB dose (Fig. 4.1a). There was no difference in the absorbance between the different 64M-2 (detects 6-4PPs) antibody dilution, and therefore the antibody could be used at a 1:1000 dilution (Fig. 4.1b). For the 1:250 concentration of TDM-1 and the 1:1000 dilution of 64M-2 the absorbance of the ELISA is proportional to the UVB dose i.e. with increasing UVB dose the absorbance increases. It should be noted that direct quantification of CPDs and 6-4PP was not possible with the ELISA method (see section 4.4).

A range of amounts of UVB-irradiated (Section 2.6.4) calf thymus DNA (0-1000 ng) was used in an ELISA to construct a standard curve (Fig. 4.2). Fig. 4.2a shows the curve for the TDM-1 antibody. An increase in absorbance i.e. increase in amount of CPDs, was detected with increasing amounts of DNA until a maximum was reached at 500ng. After 500ng there was a decrease in absorbance with increasing amounts of DNA. For the 64M-2 antibody there was an increase in absorbance with DNA, amount until 700ng, after which the absorbance stayed the same (Fig. 4.2b).

The same experiment was carried out with plant DNA to ensure that by increasing the amount of plant DNA the absorbance, i.e. amount of CPDs and 6-4PPs detected, increased. Fig. 4.3 shows that with increasing amounts of plant DNA, the absorbance increases until 600ng for TDM-1 and 700ng for 64M-2, after which it levels off. Therefore, the response of the assay is not completely linear. There are two reasons which might account for this non linear response: too much DNA or too high lesion content. If there is too much DNA in the assay then there may not be enough antibody to attach to the DNA damage sites, this is also true if too many lesions are present. The amount of DNA used in all further experiments was 300ng which falls into the linear part of the graph.

4.7 Accumulation of DNA Lesions in the Leaf Tissue Under the Coleoptile

Plants were grown with or without supplementary UVB and on day 6 after planting they were irradiated with UVB, in the absence of other light sources, for up to 3 hours to assess their susceptibility to DNA damage. This exposure to UVB will be termed 'UVB irradiation' to distinguish it from the longer term exposure to enhanced UVB (referred to as 'supplementary UVB') during growth studies. Under these conditions, both CPDs and 6-4PPs accumulated in the leaf tissue under the coleoptile (Figs. 4.4 & 4.5).

When plants were grown without supplementary UVB, Yecora Rojo accumulated significantly more CPDs than Maris Huntsman after 0.5, 1 and 2 hours of UVB irradiation. However, after 3 hours of UVB irradiation there was no difference in the amount of CPDs in the two cultivars (Fig. 4.4a). Accumulation of 6-4PPs also differed between the two cultivars under these conditions (Fig. 4.4b). Yecora Rojo accumulated 6-4PPs at an approximately linear rate, whereas, in Maris Huntsman 6-4PP accumulation levelled off after 1 hour. Therefore, after 3 hours UVB irradiation, Yecora Rojo had significantly more 6-4PPs than Maris Huntsman.

Plants grown under supplementary UVB accumulated lower levels of CPDs in response to UVB irradiation than those grown without supplementary UVB (Figs 4.4 & 4.5). As was the case for the plants grown without supplementary UVB, Yecora Rojo, accumulated significantly more CPDs than Maris Huntsman (Fig. 4.5a). However, after 3 hours of UVB irradiation Yecora Rojo had significantly more CPDs than Maris Huntsman, in contrast to when they were grown without supplementary UVB, where after 3 hours UVB irradiation both cultivars accumulated the same amount of CPDs. Yecora Rojo accumulated significantly less 6-4PPs in response to UVB irradiation when grown with supplementary UVB, than when grown under white light only. The effect of growth under supplementary UVB did not change the 6-4PP accumulation in Maris Huntsman i.e. there was no difference in the amount of 6-4PPs accumulated in Maris Huntsman when grown with or without supplementary UVB. When the cultivars were grown with supplementary UVB, Yecora Rojo accumulated significantly less 6-4PPs than Maris Huntsman after 0.5, 1 and 2 hours UVB irradiation (Fig. 4.5b).

4.8 Accumulation of DNA Lesions in the Primary Leaf Tissue

Plants were irradiated with UVB in the absence of other light sources, and the leaf tissue from above the coleoptile to the leaf tip was assayed to assess

the amount of CPDs and 6-4PPs accumulating in the leaf tissue. Under these conditions both CPDs and 6-4PPs accumulated in the leaf tissue (Figs. 4.6 & 4.7) and the amount of both increased with increasing UVB irradiation time.

Fig. 4.6 shows the amount of CPDs and 6-4PPs accumulated in primary leaf tissue of plants grown without supplementary UVB. Under these conditions, Maris Huntsman accumulated significantly more CPDs and 6-4PPs than Yecora Rojo after 0.5 hours UVB irradiation.

The accumulation of CPDs and 6-4PPs in primary leaf tissue of plants grown with supplementary UVB is shown in Fig. 4.7. Plants grown under supplementary UVB accumulated lower levels of CPDs in response to UVB irradiation than those grown without supplementary UVB. After 3 hours of UVB irradiation, Maris Huntsman accumulated significantly more CPDs than Yecora Rojo (Fig. 4.7a). The 6-4PP accumulation in Maris Huntsman and Yecora Rojo was significantly greater for plants grown under supplementary UVB, than when grown under white light only. At each UVB irradiation time point Maris Huntsman accumulated more 6-4PPs than Yecora Rojo (Fig. 4.7b).

4.9 Removal of DNA Damage

In order to determine the capacity for DNA repair, plants were irradiated with UVB in the absence of other light sources, the UVB source was removed and the tissue was allowed to repair the damage for different lengths of time either in the dark or with white light.

Dark repair of CPDs and 6-4PPs was analysed to determine if the reason for the difference in accumulation of CPDs and 6-4PPs between Maris Huntsman and Yecora Rojo (Figs. 4.6 & 4.7) was that one cultivar repaired the damage at a faster rate than the other. Since the plants were irradiated with UVB in the absence of other light sources, the only repair that could take place was dark repair. When studying the accumulation of CPDs and 6-4PPs, the plants were irradiated with UVB for up to 3 hours. When analysing dark repair between the two cultivars, only the proportion of CPDs and 6-4PPs after 3 hours in the dark was determined.

Fig. 4.8 shows the proportion (as a percentage) of the initial DNA damage remaining when plants were given a three hour period in the dark, after 3 hours UVB irradiation had been given in the absence of other light sources. Only a small amount of dark repair of 6-4PPs and CPDs was detected when the plants were grown without supplementary UVB. Under these conditions, there was no significant difference in the amount of repair of CPDs between Maris Huntsman and Yecora Rojo (Fig. 4.8a). However, Yecora Rojo repaired significantly more 6-4PPs than Maris Huntsman when grown without

supplementary UVB (86.86% left compared to 97.15%, after three hours in the dark).

When grown with supplementary UVB, prior to UVB irradiation, both cultivars repaired significantly more CPDs and 6-4PPs in the dark, than when grown under white light only. Both cultivars repaired the CPDs and 6-4PPs at the same rate when grown with supplementary UVB (Fig. 4.8b).

When assessing light repair in the two cultivars it was important that the amount of initial damage was the same in both cultivars in order to compare repair. Therefore, the cultivars were irradiated with UVB for different lengths of time in order to generate the same amount of initial damage. From Figs. 4.6 and 4.7, a UVB irradiation time was calculated to generate the same amount of DNA damage in each cultivar.

Figs. 4.9 and 4.10 show the proportion of initial DNA damage remaining in the primary leaf of plants grown without and with supplementary UVB. A significantly greater proportion of CPDs and 6-4PPs are repaired in the light than in the dark in both cultivars (compare Figs. 4.8 to 4.9 & 4.10).

Fig. 4.9 shows the proportion of initial damage left in the primary leaf of plants, grown without supplementary UVB, after different lengths of time in the light (400-700nm). There was no significant difference in the proportion of CPDs repaired between Maris Huntsman and Yecora Rojo (Fig. 4.9a). After 30 minutes and 1 hour Yecora Rojo had removed a significantly greater proportion of 6-4PPs than Maris Huntsman; however, after 3 hours light repair there was no difference in the proportion of 6-4PPs repaired between the two cultivars (Fig. 4.9b).

Fig. 4.10 shows the proportion of initial damage left in the primary leaf of plants, grown with supplementary UVB, after different lengths of time in the light. When the plants were grown with supplementary UVB the proportion of light repair of CPDs and 6-4PPs was significantly less ($p<0.05$) than when grown without supplementary UVB. There was no difference in the amount of CPDs removed from the leaf tissue in Maris Huntsman and Yecora Rojo (Fig. 4.10a). Also, there was no difference in the amount of 6-4PPs removed in the two cultivars (Fig. 4.10b).

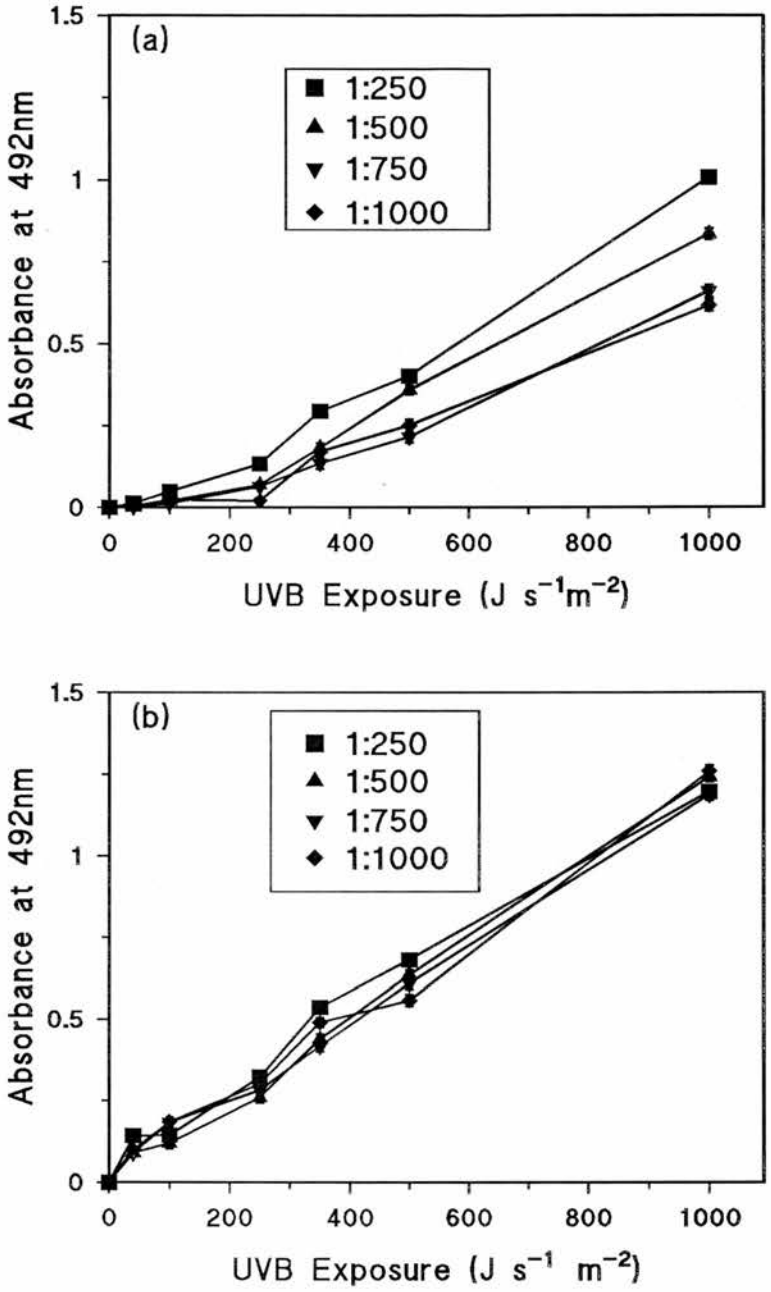


Figure 4.1 The detection of CPDs (a) and 6-4PP (b) in calf thymus DNA standards by different concentrations of TDM-1 and 64M-2 antibodies respectively.

Calf thymus (300ng) DNA was irradiated as described in Section 2.7.4 and DNA damage was detected using an ELISA method (Section 2.7.3). Different concentrations of antibody were used to determine the most suitable for the ELISA method. Each data point represents the mean from 16 samples, with error bars showing $\pm$ one standard error from the arithmetic mean.

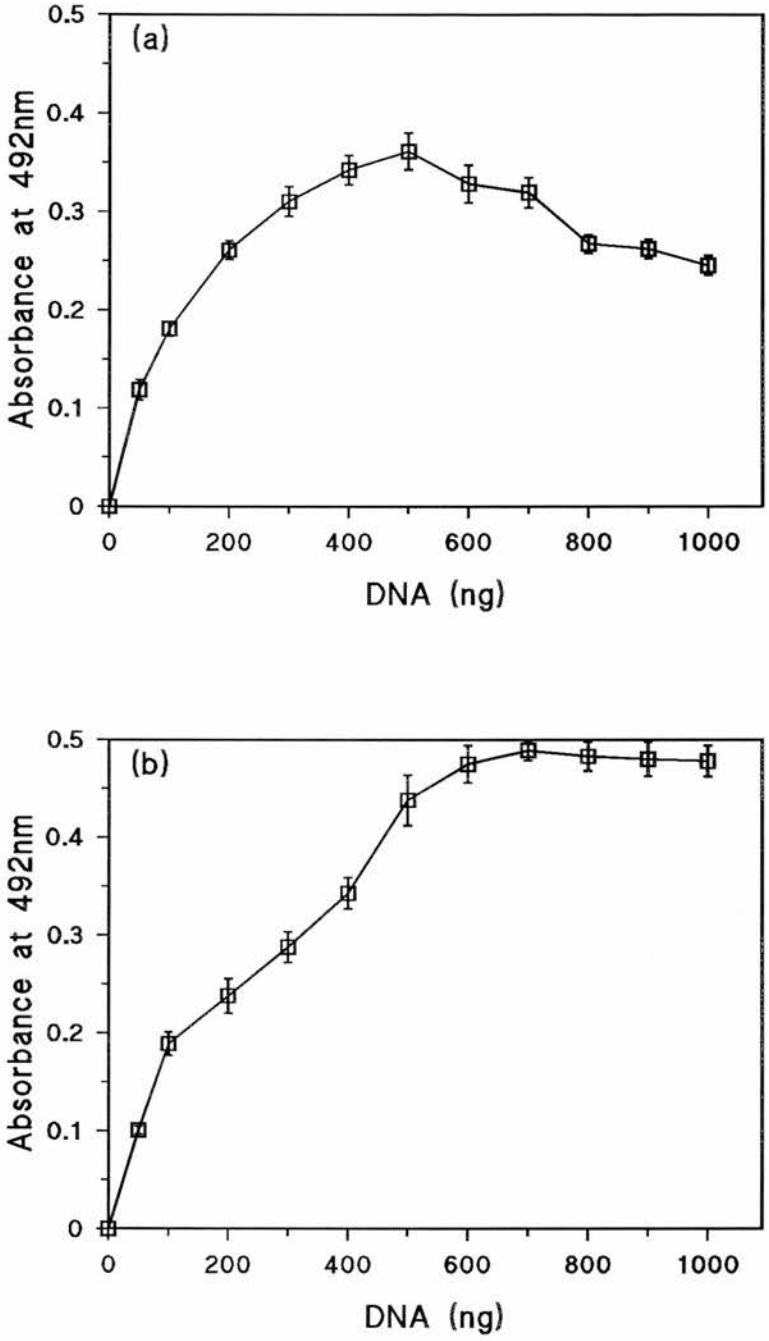


Figure 4.2 The detection of CPDs (a) and 6-4PP (b) in calf thymus DNA standards by TDM-1 and 64M-2 antibodies respectively.

Calf thymus DNA was irradiated as described in Section 2.7.4 and DNA damage was detected using an ELISA method (Section 2.7.3). Each data point represents the mean from 16 samples, with error bars showing $\pm$ one standard error from the arithmetic mean. Each value was read against a blank containing only buffer.

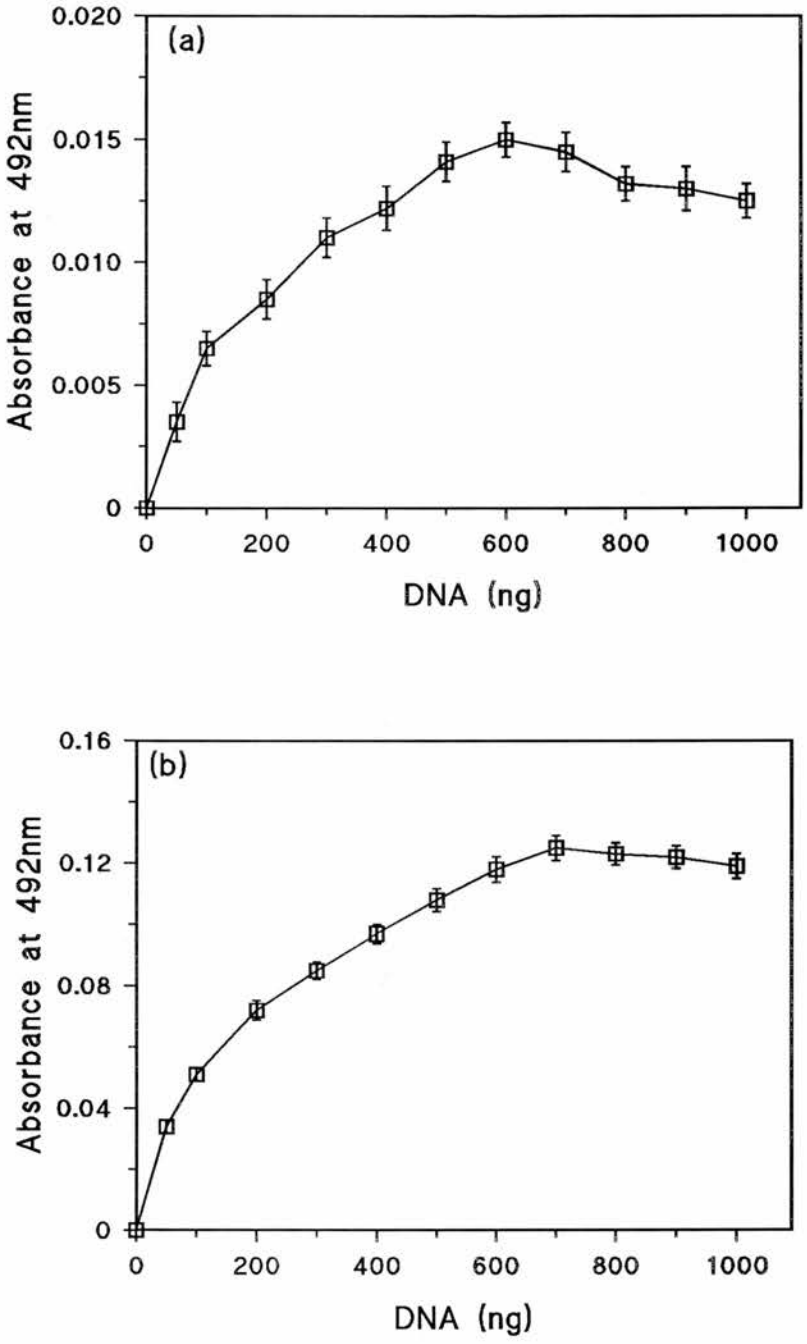


Figure 4.3 The detection of CPDs (a) and 6-4PP (b) in plant DNA standards by TDM-1 and 64M-2 antibodies respectively.

Plant DNA was irradiated *in vivo* as described in Section 2.7.4 and DNA damage was detected using an ELISA method (Section 2.7.3). Each data point represents the mean from 16 samples, with error bars showing $\pm$ one standard error from the arithmetic mean. Each value was read against a blank containing only buffer.

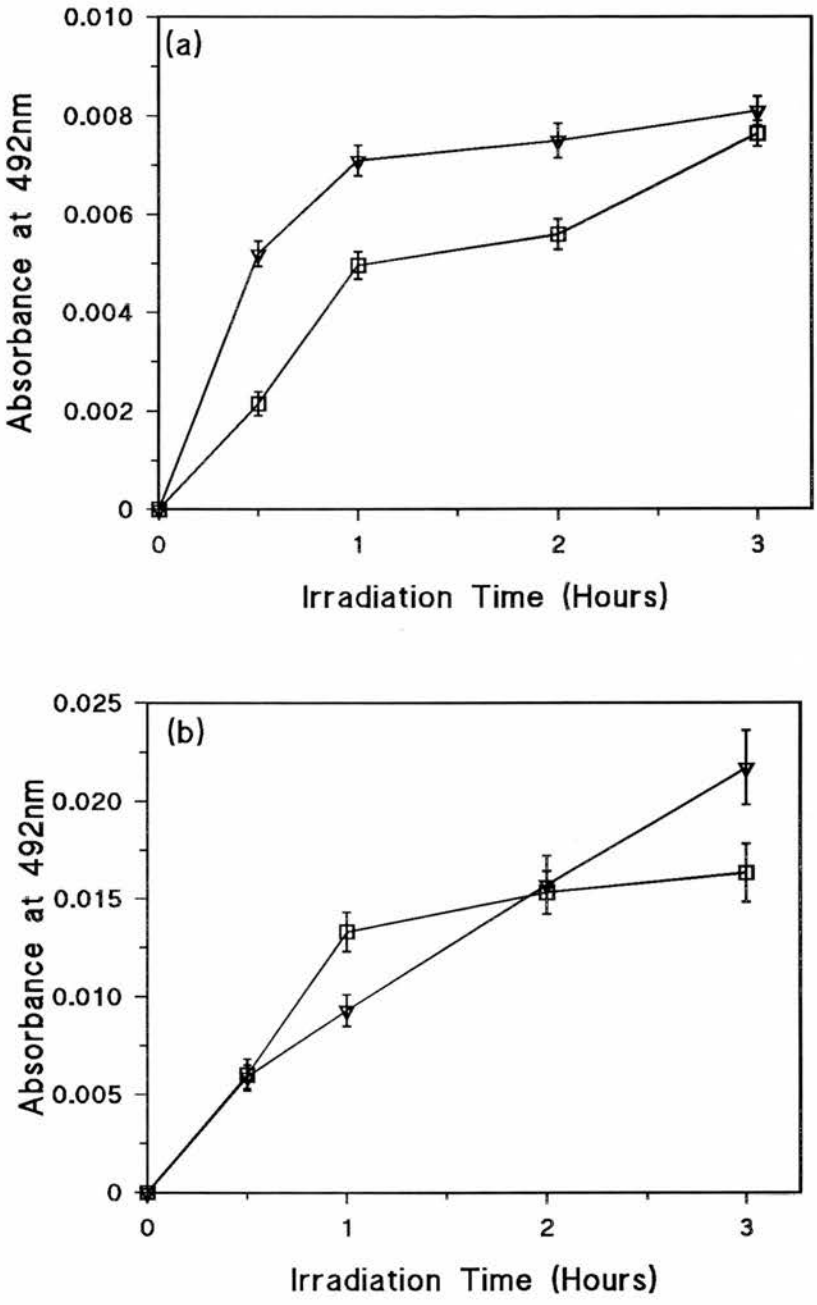


Figure 4.4 Accumulation of CPDs (a) and 6-4PPs (b) in primary leaf tissue under the coleoptile of 6-day old wheat seedlings of Maris Huntsman (□) and Yecora Rojo (▽) grown without supplementary UVB and irradiated with UVB in the dark.

Plants were grown without supplementary UVB (Section 2.2). Seedlings were irradiated with UVB, in the absence of other light sources, for different lengths of time, the DNA extracted from the leaf tissue covered by the coleoptile, and an ELISA performed to determine the lesion content (see Section 2.7). The data points represent the mean of four independent growth studies sampling 18 seedlings each, with error bars showing ±one standard error from the arithmetic mean.

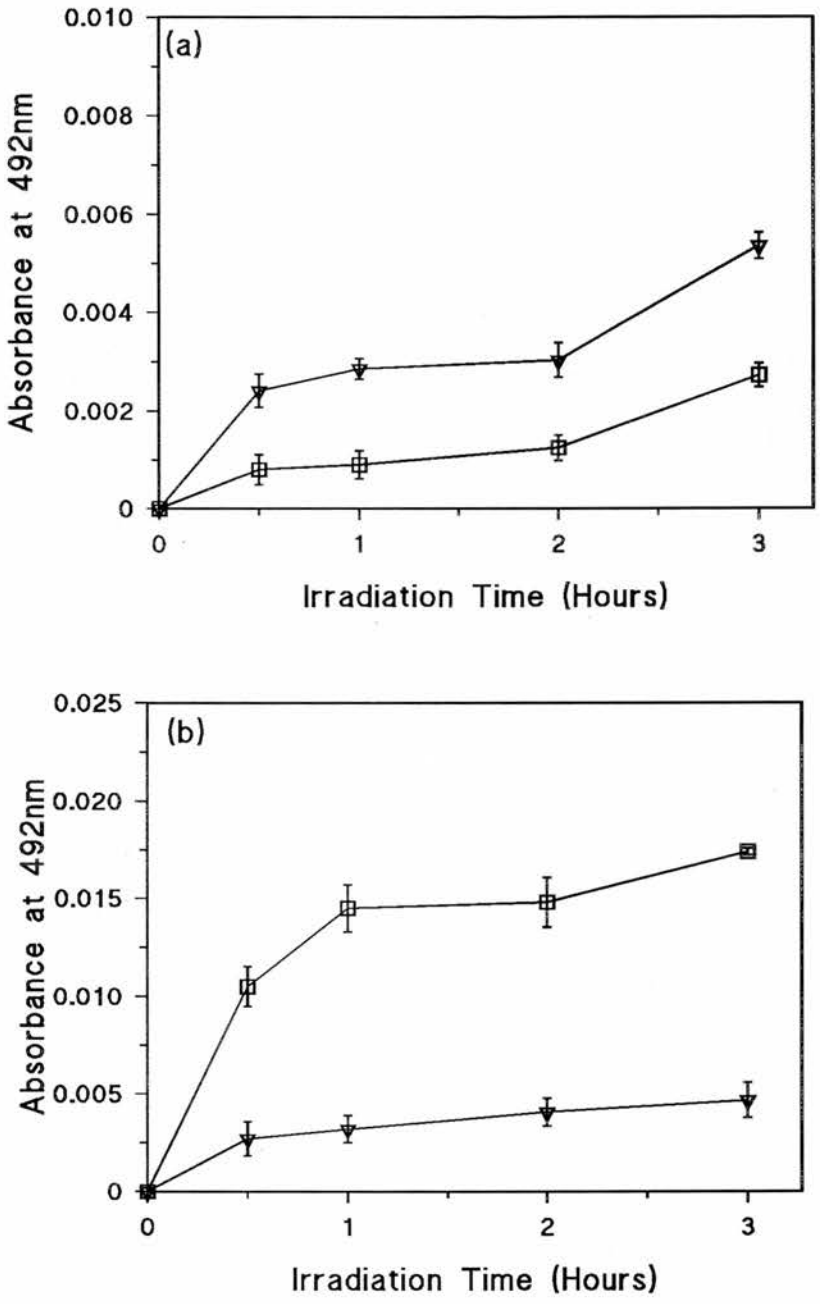


Figure 4.5 Accumulation of CPDs (a) and 6-4PPs (b) in primary leaf tissue under the coleoptile of 6-day old wheat seedlings of Maris Huntsman (□) and Yecora Rojo (▽) grown with supplementary UVB and irradiated with UVB in the dark. Plants were grown with supplementary UVB (Section 2.2). Seedlings were irradiated with UVB, in the absence of other light sources, for different lengths of time, the DNA extracted from the leaf tissue covered by the coleoptile, and an ELISA performed to determine the lesion content (see Section 2.7). The data points represent the mean of four independent growth studies sampling 18 seedlings each, with error bars showing ±one standard error from the arithmetic mean.

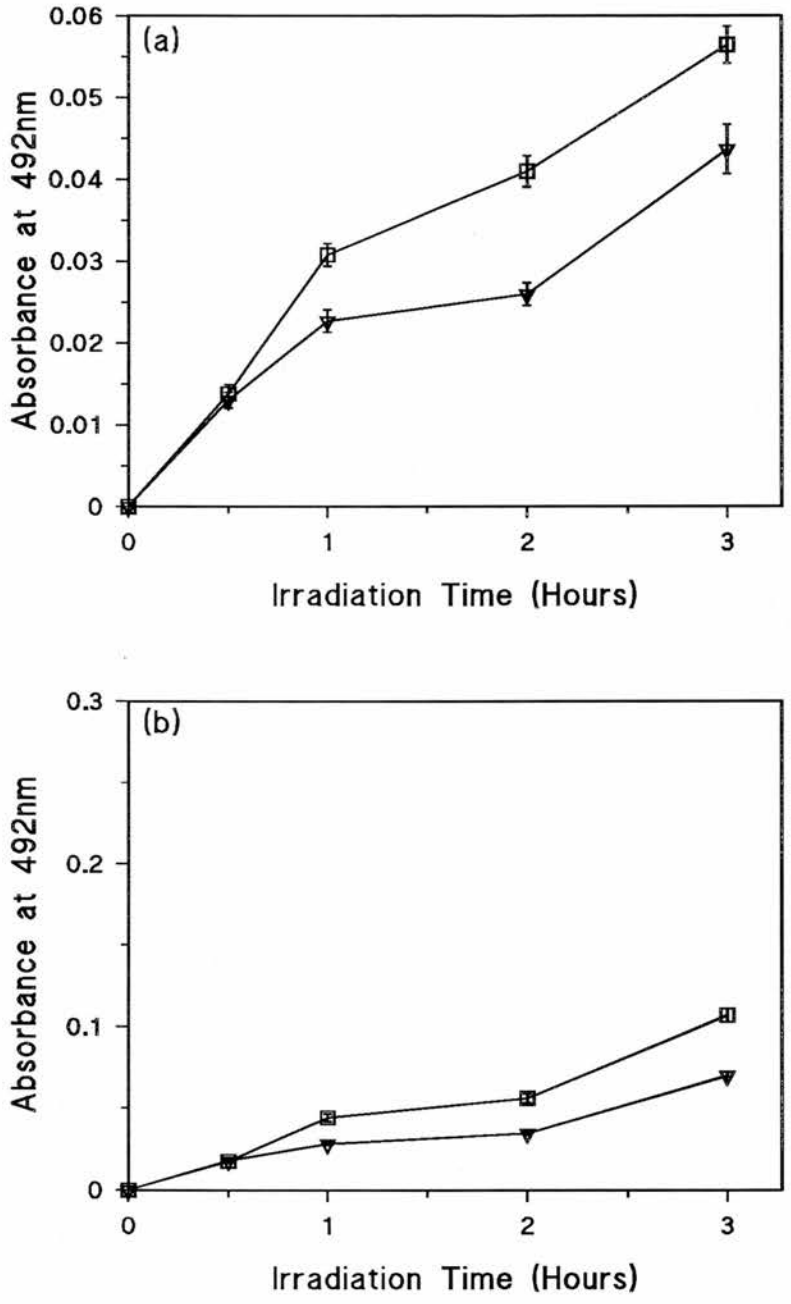


Figure 4.6 Accumulation of CPDs (a) and 6-4PPs (b) in the primary leaf of 6-day old wheat seedlings of Maris Huntsman (□) and Yecora Rojo (▽) grown without supplementary UVB and irradiated with UVB in the dark.

Plants were grown without supplementary UVB (Section 2.2). Seedlings were irradiated with UVB, in the absence of other light sources, for different lengths of time, the DNA extracted and an ELISA performed to determine the lesion content (see Section 2.7). The data points represent the mean of four independent growth studies sampling 18 seedlings each, with error bars showing ±one standard error from the arithmetic mean.

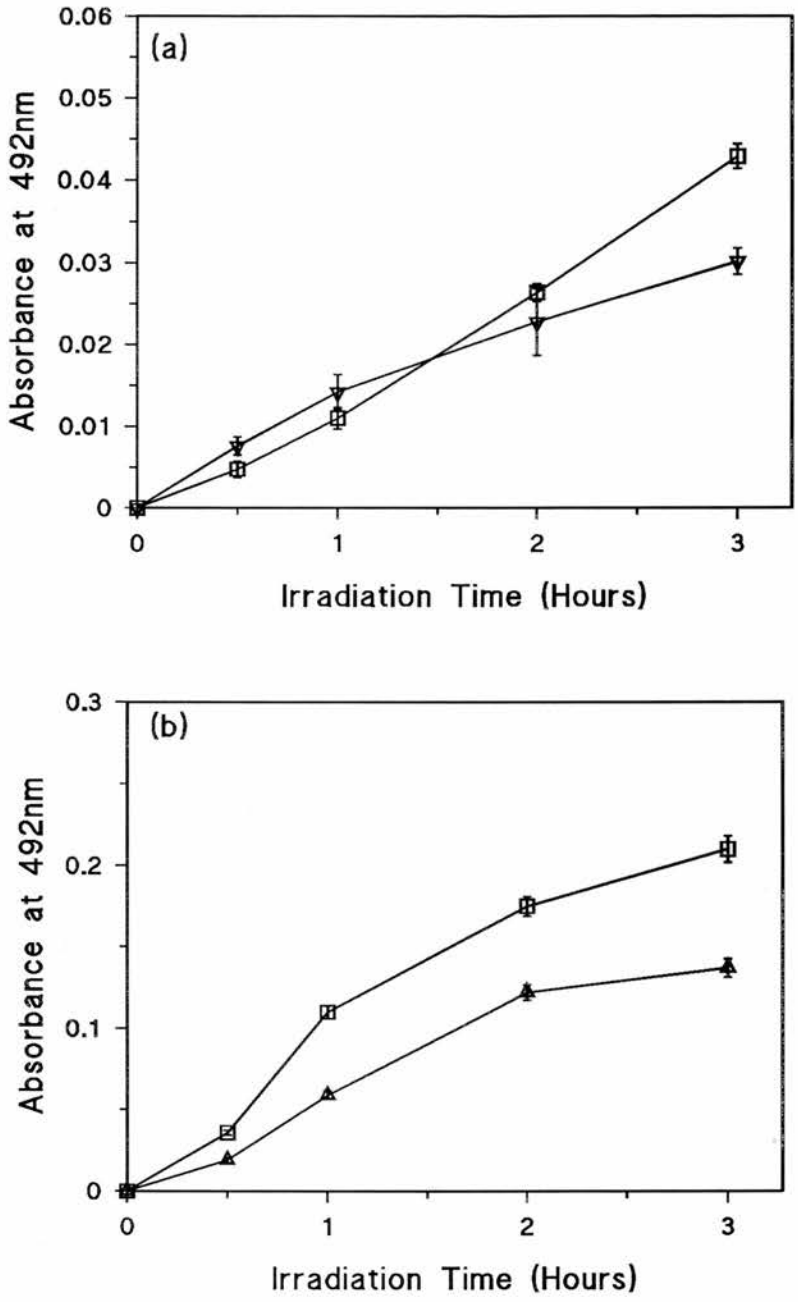


Figure 4.7 Accumulation of CPDs (a) and 6-4PPs (b) in the primary leaf of 6-day old wheat seedlings of Maris Huntsman (□) and Yecora Rojo (▽) grown with supplementary UVB and irradiated with UVB in the dark.

Plants were grown with supplementary UVB (Section 2.2). Seedlings were irradiated with UVB, in the absence of other light sources, for different lengths of time, the DNA extracted and an ELISA performed to determine the lesion content (see Section 2.7). The data points represent the mean of four independent growth studies sampling 18 seedlings each, with error bars showing ±one standard error from the arithmetic mean.

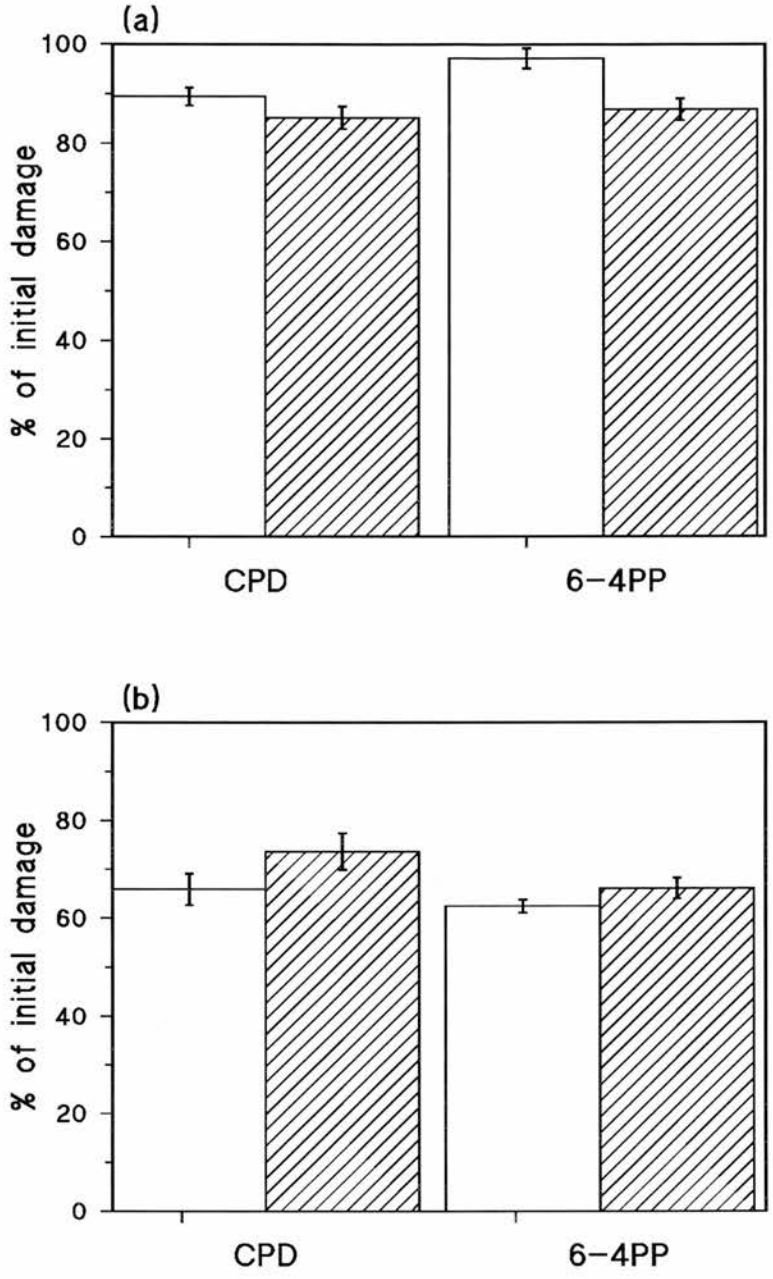


Figure 4.8 The removal of DNA lesions, in the dark, from the primary leaf in 6-day old wheat seedlings of Maris Huntsman (□) and Yecora Rojo (▨) grown with (b) and without (a) supplementary UVB.

Plants were grown without supplementary UVB (a) and with supplementary UVB (b) for 6 days prior to UVB irradiation (Section 2.2). Seedlings were irradiated with UVB (0.93 kJ m^{-2} h^{-1}, Caldwell weighting) for 3 hours, in the absence of other light sources, followed by a 3 hour period in the dark without the UVB source, the DNA extracted and an ELISA performed to determine the proportion (i.e. percentage of initial damage) of CPDs and 6-4PPs remaining (see Section 2.7). The data points represent the mean of four independent growth studies sampling 18 seedlings each, with error bars showing ±one standard error from the arithmetic mean.

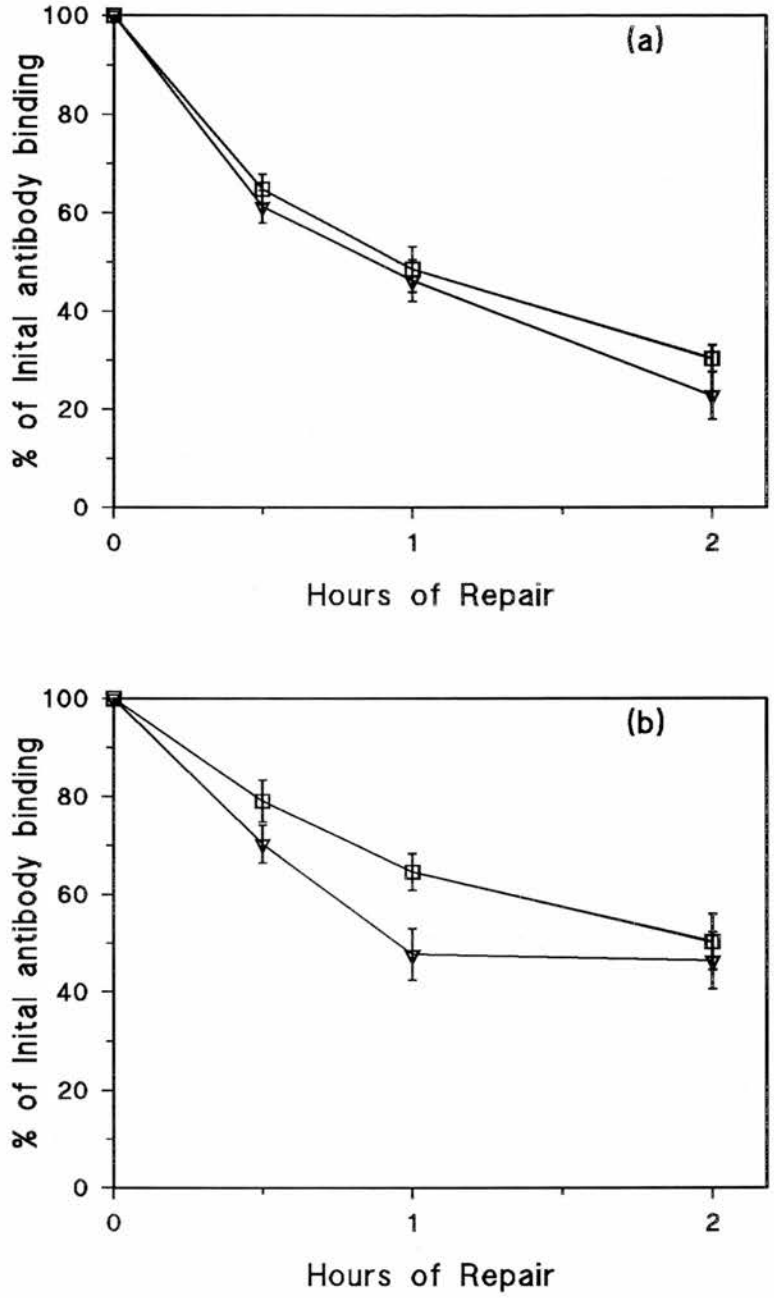


Figure 4.9 The removal of CPDs (a) and 6-4PPs (b), in the light, from the primary leaf in 6-day old wheat seedlings of Maris Huntsman (□) and Yecora Rojo (▽) grown without supplementary UVB.

Plants were grown without supplementary UVB (Section 2.2). Seedlings were irradiated with UVB (0.93KJ m^{-2} d^{-1}), in the absence of other light sources, followed by different lengths of time in the light (400-700nm) without the UVB source, the DNA extracted and an ELISA performed to determine the lesion content (see Section 2.7). The data points represent the mean of four independent growth studies sampling 18 seedlings each, with error bars showing ±one standard error from the arithmetic mean.

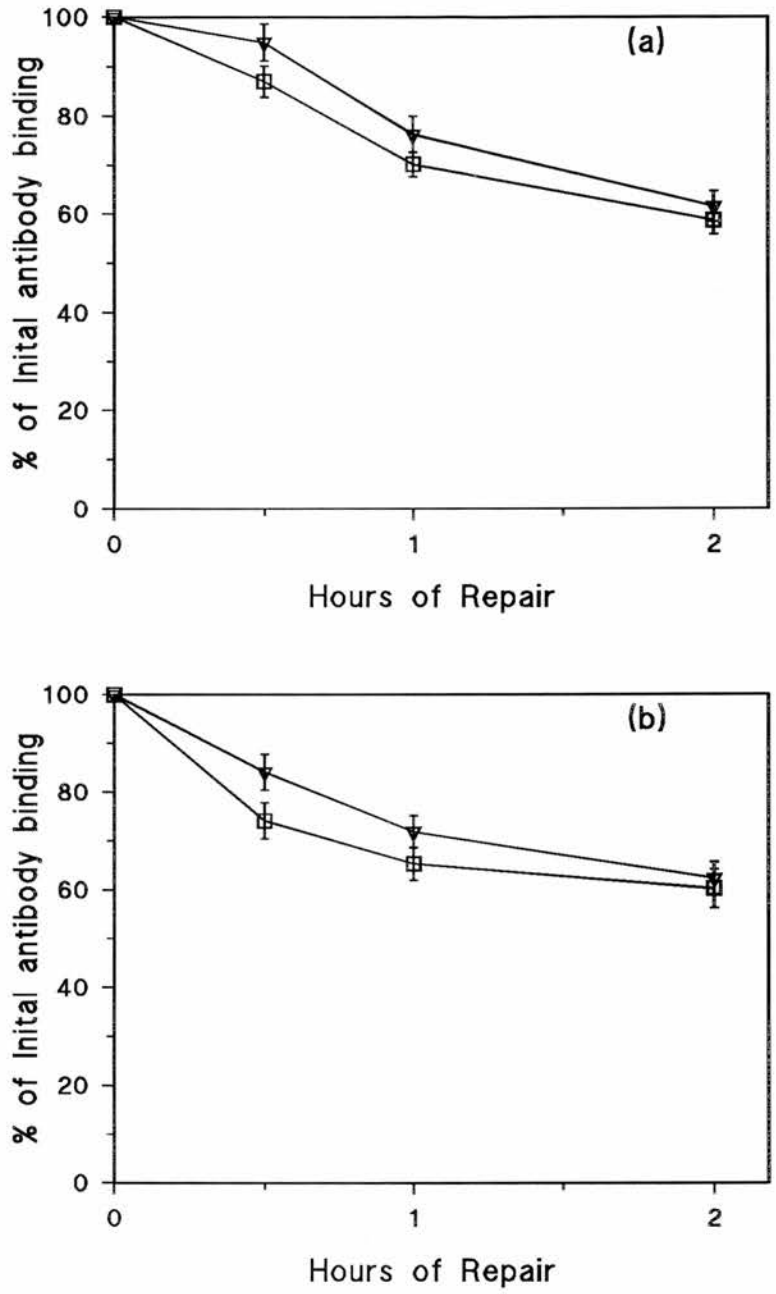


Figure 4.10 The removal of CPDs (a) and 6-4PPs (b), in the light, from the primary leaf in 6 day old wheat seedlings of Maris Huntsman (□) and Yecora Rojo (▽) grown with supplementary UVB.

Plants were grown with supplementary UVB (Section 2.2). Seedlings were irradiated with UVB (0.93KJ m^{-2} d^{-1}), in the absence of other light sources, followed by different lengths of time in the light (400-700nm) without the UVB source, the DNA extracted and an ELISA performed to determine the lesion content (see Section 2.7). The data points represent the mean of four independent growth studies sampling 18 seedlings each, with error bars showing ±one standard error from the arithmetic mean.

Discussion

4.10 Measurement of DNA Damage

The ELISA technique used in this chapter provides a method for the measurement of UVB-induced DNA lesions in a large variety of DNA samples. This method can be described as semi-quantitative because although the actual numbers of CPDs and 6-4PPs cannot be calculated from the assay, the absorbance between samples can be compared in order to obtain a relative estimate of the extent of damage present in the tissue. The TDM-1 and 64M-2 antibodies have been used in a number of laboratories to detect CPDs and 6-4PPs in a variety of different tissues using various techniques. Chadwick *et al.* (1995) used these antibodies to detect CPDs and 6-4PPs in human skin using an immunohistochemical method. The TDM-1 and 64M-2 antibodies were also used to detect CPDs and 6-4PPs in *Triticum aestivum* seedlings using the ELISA technique (Taylor *et al.*, 1996).

It was important to establish that the antibodies gave a quantitative response, that is, whether the absorbance of the ELISA increased with increasing DNA damage. The results (Fig. 4.1 to 4.3) show that the antibodies are specific as they give a linear response with increasing DNA damage. The results for the TDM-1 antibody (which detects CPDs) and the 64M-2 antibody (which detects 6-4PPs) cannot be compared directly since the absorbance reading is not a direct measure of the number of DNA lesions.

4.11 DNA Damage in Tissue Under the Coleoptile

When seedlings were grown with supplementary UVB and then irradiated, much less damage, both CPDs and 6-4PPs, accumulated in the tissue under the coleoptile as compared to seedlings grown without supplementary UVB. The amount of dimers accumulated by tissue under the coleoptile was much less than that accumulated in leaf tissue above the coleoptile. The tissue covered by the coleoptile would not receive as much UVB light as the other leaf tissue since the coleoptile has a UVB protective role (Haussühl *et al.*, 1996). Therefore, it is not surprising that the amount of CPDs and 6-4PPs accumulated is less than in the tissue above the coleoptile. Repair of damage in the tissue under the coleoptile was not examined since the amount of damage detected was very low, and therefore analysis of repair would be difficult.

In the previous Chapter, the growth of Maris Huntsman was reduced to a greater extent than that of the Saudi Arabian cultivar, Yecora Rojo. Perhaps the effect of UVB on growth of Maris Huntsman is due to the accumulation of UVB-induced DNA damage. When grown without

supplementary UVB, Yecora Rojo accumulates more CPDs and 6-4PPs than Maris Huntsman, although with supplementary UVB Maris Huntsman accumulates more 6-4PPs. Even though Yecora Rojo accumulates more CPDs than Maris Huntsman when grown with supplementary UVB the growth of this cultivar appears to be less affected. The tissue under the coleoptile is where cell division and cell elongation takes place and the amount of DNA damage accumulated in this tissue may have an affect on growth.

4.12 DNA Damage in the Leaf Tissue

When the leaf tissue above the coleoptile to the leaf tip was analysed for DNA damage, the results showed that Maris Huntsman, the British cultivar, accumulated more CPDs and 6-4PPs than the Saudi Arabian cultivar, Yecora Rojo.

There are two possibilities to account for the reason why Maris Huntsman is damaged to a greater extent than Yecora Rojo. Either Yecora Rojo is protected better and thus does not accumulate dimers to such an extent, or it repairs the damage more quickly. Protection of Maris Huntsman and Yecora Rojo against UVB radiation will be discussed in detail in Chapter 5, whereas DNA damage repair (i.e. repair of CPDs and 6-4PPs) will be examined in this Chapter. Since the seedlings were irradiated in the absence of other light sources any repair taking place would have been excision repair as this does not require light.

Maris Huntsman accumulates more CPDs and 6-4PPs when irradiated with UVB. Cp DNA makes up 15% of the total cellular DNA and so some of the CPDs and 6-4PPs measured will be from damage accumulated in the cp DNA. The results from the previous chapter showed that when plants were grown with UVB the photosynthesis of Maris Huntsman was reduced, but the same decrease did not occur in Yecora Rojo. It is likely that if photosynthesis is affected by UVB the chloroplasts could be accumulating CPDs and 6-4PPs rather than the photosynthetic apparatus being directly damaged by UVB. This may be one explanation why Maris Huntsman had more CPDs and 6-4PPs compared to Yecora Rojo.

When CPD repair was measured in two rice cultivars, one was found to have a reduced CPD repair capacity which could be correlated with the sensitivity of this cultivar to UVB (Hidema *et al.*, 1996). This suggests that DNA damage may account for the UVB-induced damage rather than UVB damage to the photosynthetic apparatus. In Norin 1, UVB caused a reduction in Rubisco content (encoded in the chloroplast) but not in the chl a/b protein which is encoded in the nucleus. This reduction of Rubisco may be due to a

deficient DNA repair capacity in the chloroplast since this has been found in other plants (Cannon *et al.*, 1995; Chen *et al.*, 1996; Hada *et al.*, 1998), whereas the nuclear DNA repair is not affected.

4.13 The Effect of Growth Conditions on DNA Damage

To analyse whether growth conditions were having an effect on the accumulation or removal of CPDs and 6-4PPs in the leaves of both cultivars, plants were grown with and without supplementary UVB before UVB irradiation. This was designed to test if repair or protection against CPD and 6-4PP accumulation was UVB inducible.

When plants were grown with supplementary UVB the amount of CPDs accumulating in the primary leaf, in response to UVB irradiation, was less than when plants were grown without supplementary UVB. However, the amount of 6-4PPs was almost double in plants grown with supplementary UVB. Although supplementary UVB-grown plants had a greater capacity for repair of CPDs (see Fig. 4.8), this cannot be the reason for the decrease in CPD accumulation compared to the increase in 6-4PP accumulation in plants grown with supplementary UVB. In plants grown with supplementary UVB, the removal of 6-4PPs increased along with the increased capacity for CPD repair compared to that for plants grown without supplementary UVB (see Section 4.13). Perhaps when the cultivars are grown with supplementary UVB the DNA is protected in some way which prevents CPDs from forming but 6-4PPs continue to accumulate.

In this Chapter, the amount of CPDs, formed in response to UVB irradiation, decreased when the seedlings were grown with supplementary UVB. However, the opposite was found in *Oryza sativa* when the effects of the light environment on CPD accumulation were studied (Kang *et al.*, 1998). The results showed that when seedlings are grown with supplementary UVB, the amount of CPDs accumulating in the tissue increased. One explanation as to why the amount of CPDs decreased in this study, could be that with UVB the amount of UVB absorbing compounds and leaf optical properties changed (see Chapter 5).

4.14 Dark Repair of DNA Damage

Two types of repair have been identified in plants: photoreactivation and excision repair. Photoreactivation requires light and therefore, when repair of DNA damage in the dark was studied only excision repair was taking place. Excision repair was examined in the two cultivars to see if the difference in CPD and 6-4PP accumulation between the cultivars

could be accounted for by the fact that one cultivar repaired the damage at a faster rate than the other cultivar. When excision repair was examined in plants grown without supplementary UVB, Yecora Rojo had a greater capacity to repair 6-4PPs than Maris Huntsman, although there was no difference in the rate of repair of CPDs between the cultivars. However, this higher capacity to repair 6-4PPs could not account for the large differences in accumulation in response to irradiation (see Fig. 4.6). After UVB irradiation, Maris Huntsman accumulated 29% more CPDs than Yecora Rojo (from Fig. 4.6) and the 4% higher excision repair capacity (from Fig. 4.8) would not account for this difference. Yecora Rojo had 53% less 6-4PPs than Maris Huntsman after UVB irradiation (Fig. 4.6) and the 10% higher excision repair capacity of Yecora Rojo (Fig. 4.8) could not compensate for this difference.

When plants were grown with supplementary UVB, Yecora Rojo accumulated 42% less CPDs than Maris Huntsman after UVB irradiation (Fig. 4.7), but since Maris Huntsman had a greater excision repair capacity (Fig. 4.8) that could not account for the difference in damage accumulation. After UVB irradiation, supplementary UVB-grown Maris Huntsman accumulated 53% more 6-4PPs than Yecora Rojo (Fig. 4.7), but the 3% higher excision repair capacity (Fig. 4.8) of Yecora Rojo could not compensate for the difference. Thus, the difference in the amount of damage accumulated by Maris Huntsman cannot be explained by Yecora Rojo's ability to repair the damage more quickly under the conditions used in this experiment.

Therefore, the reason for Maris Huntsman accumulating more CPDs and 6-4PPs is not totally due to the fact that Yecora Rojo repairs the damage at a faster rate than Maris Huntsman. It is likely that Yecora Rojo has a protective mechanism which reduces the effect to which CPDs and 6-4PPs accumulate.

In control-grown Maris Huntsman plants, CPDs were repaired at a faster rate than 6-4PPs, however under the other experimental conditions there was no difference in the rate of repair of CPDs or 6-4PPs. In *Arabidopsis thaliana* excision repair of 6-4PPs occurred much more rapidly than that of CPDs with nearly half of the lesions being repaired within 2 hours (Britt *et al.*, 1993).

4.14.1 The Effect of Growth Conditions on Dark Repair

Both cultivars had a higher excision repair capacity when grown with supplementary UVB, suggesting that excision repair might be UVB-inducible. The enzymes involved in excision repair may be UVB inducible and although no other studies have examined this, UVB treatment has

been found to significantly increase the levels of photolyase (the enzyme involved in light repair of CPD and 6-4PPs) in *A. thaliana* (Pang & Hays, 1991).

4.15 Light Repair of DNA Damage

The repair of CPDs and 6-4PPs in the primary leaf of wheat was much more rapid in the presence of white light than in the dark and there are two possible reasons to account for this. The first is that enzymes are involved in various stages of excision repair such as removing, resynthesising and replacing DNA (Britt, 1996), therefore excision repair will be a slower reaction than photoreactivation where only one enzyme reaction takes place. The second reason is that when plants were allowed to repair the damage in the light, both excision repair and photoreactivation would be taking place, whereas in the dark only excision repair could take place.

When grown without supplementary UVB, there is no difference in the amount of CPDs repaired by the two cultivars, and after 3 hours 75% of CPDs are removed from the primary leaf. Although Yecora Rojo repairs 6-4PPs at a faster initial rate than Maris Huntsman, after 3 hours light repair both cultivars repair 50% of 6-4PPs. Only 60% of CPDs and 6-4PPs were repaired after 3 hours light repair when plants were grown with supplementary UVB.

At 313-334nm 6-4PPs are converted to Dewar isomers, and therefore if 6-4PPs are exposed to this radiation their frequency would decrease. This has been used to explain the reduction of 6-4PPs in a number of studies, however in this chapter only repair of dimers in the dark and in the light (400-700nm) was examined and therefore the radiation required to convert the 6-4PPs to Dewar isomers would not be present. The presence of Dewar isomers in *Triticum aestivum* tissue after light repair was examined by Taylor *et al.* (1996) to test whether the decrease in 6-4PPs was due to Dewar isomer formation. Using a Dewar isomer-detecting antibody in an ELISA, no presence of the Dewar isomer was detected. Therefore, it is extremely unlikely that conversion of 6-4PPs to Dewar isomers is responsible for the decrease in 6-4PPs in this chapter.

The repair of CPDs especially, was much more rapid in the light than in the dark and this has been observed in other studies (Pang & Hays, 1991; Taylor *et al.*, 1996; Takeuchi *et al.*, 1996; Hidema *et al.*, 1997). The main pathway for removal of CPDs in a number of plants is photoreactivation as found in e.g. *Arabidopsis thaliana* (Pang & Hays, 1991); *Medicago sativa* seedlings (Quaite et al., 1994), soybean tissue culture cells (Cannon *et al.*,

1995), *Triticum aestivum* (Taylor *et al.*, 1996) and *Zea mays* (Stapleton *et al.*, 1997).

It is possible that the repair mechanism in the two cultivars in this study may have been underestimated. The Saudi Arabian cultivar, Yecora Rojo, has been bred for growth under high light intensity and may therefore require more light for photoreactivation. However, under growth cabinet conditions it is impossible to recreate the light levels which this cultivar would experience in Saudi Arabia. The effect of the light environment on repair was examined in seedlings of *Medicago sativa* , the results showed that there were lower rates of dimer repair under artificial light than under natural light (Takayanagi *et al.*, 1994).

Other studies have compared DNA damage and repair between cultivars with different sensitivities to UVB. The amount of CPDs accumulated in two rice cultivars, Norin 1 which is UV sensitive and Sasanishiki which is UV tolerant, was compared (Hidema *et al.*, 1997). Under the conditions used, both cultivars accumulated similar CPD levels, although repair of CPDs differed. Under white light Sasanishiki removed 75% of the CPDs in less than 5 minutes whereas, the rate of photoreactivation in Norin 1 was one fifth of that of Sasanishiki. Norin 1 excised CPDs at a rate of 20% that of Sasanishiki's excision rate. Therefore, the UV-sensitive cultivar was deficient in both excision and photoreactivation repair. Hidema & Kumagai (1998) suggest two possibilities for the deficiency in photorepair of Norin 1. First, there may be a structural mutation of photolyase which would affect the enzyme substrate complex, or the levels of photolyase could be decreased due to a regulatory mutation. The ability of Norin 1 and Sasanishiki to photorepair CPDs increased rapidly during development, and reached a maximum which remained constant after full expansion of the leaf (Hidema & Kumagai, 1998). This increase in ability to photorepair could be a result of an increase in the amount of photoreactivating enzyme.

4.15.1 The Effect of Growth Conditions on Light Repair

The plants were grown under supplementary UVB to test if this would affect the light repair of CPDs and 6-4PPs. When the plants were grown with supplementary UVB, the amount of light repair was less than when grown without UVB. This may be due to a change in the amount of photolyase present when the plants are grown with supplementary UVB. UVB treatment significantly increased the photolyase levels in *Arabidopsis thaliana* (Pang & Hays, 1991), and the expression of the yeast photolyase gene also increased with UV treatment (Sebastian, Kraus & Sancar, 1990). It is unclear why the

opposite effect, i.e. reduced photorepair, was observed in the present study. Future measurement of photolyase activity might help to clarify this. Growth under supplementary UVB seems to cause early senescence of the leaf so perhaps photorepair is decreased due to a decrease in photolyase with senescence.

4.15.2 Is Light Repair Inducible?

It has been found that the amount of repair depends on the initial damage level (Howland, 1975; Quaite *et al.*, 1994) i.e. perhaps a certain threshold of damage is needed before repair takes place. The photolyase may need a certain concentration of substrate before the enzyme becomes activated. When the plants are grown under supplementary UVB, the threshold level of damage needed before repair takes place may be different from that of plants grown without supplementary UVB. This might also depend on the amount of photolyase present in the tissue. Also, the different cultivars may have different damage thresholds. At low initial dimer frequencies *Medicago sativa* seedlings only used photoreactivation to repair dimers. However, at higher damage levels both excision and photorepair contributed to the repair process (Quaite *et al.*, 1994). Photoreactivation involves the enzyme photolyase forming a complex with a dimer, thus when the amount of substrate increases the time required for enzyme association may decrease, resulting in increase in photoreactivation.

Quaite *et al.* (1994) suggest that the reason for low excision repair in *Medicago sativa* may be due to the lower affinity of excision enzymes for CPDs relative to other lesions, or to lower accessibility of the lesions. At low damage levels photorepair of CPDs takes place simultaneously with the excision repair of non-CPD lesions. At higher damage levels the capacity of photorepair is saturated thus plants use excision repair.

4.15.3 Photolyase

Both 6-4PPs and CPDs were removed rapidly in the light, and therefore both cultivars in this study contain mechanisms for photorepair of both dimers. There are many published examples of photorepair for CPDs (e.g. Taylor *et al.*, 1996) but more recently other studies have shown photoreactivation of 6-4PPs. The first demonstration of a 6-4PP photolyase was in *Drosophilia melanogaster* (Todo *et al.*, 1993). Two types of photolyase were identified, one specific for CPDs and the other specific for 6-4PPs. Before the Todo *et al.* (1994) study, 6-4PPs were thought to be removed only by excision repair (Mitchell & Nairn, 1989). In *Arabidopsis thaliana* seedlings

6-4PPs were rapidly removed (Chen *et al.*, 1996), and since then the 6-4PP photolyase has been cloned to examine the properties of this enzyme.

The two cultivars in this study may have different temperature maxima for photorepair and repair may also change with leaf development. The plants used in this experiment were 6-day old primary leaves and therefore fully developed (as discussed in chapter 1). The rate of photoreactivation has been shown to change with temperature and plant development (Pang & Hays, 1991; Takeuchi *et al.*, 1996; Hidema & Kumagai, 1998). In cucumber (*Cucumis sativus*), high repair activity of CPDs and 6-4PPs was observed at 25-30°C and at lower or higher temperatures the photorepair activities were much lower (Takeuchi *et al.*, 1996). To test if temperature had an effect on repair, UVB irradiated plants would have to be incubated under white light at different temperatures and an ELISA carried out to analyse repair at each temperature.

4.16 Conclusions

The British cultivar, Maris Huntsman accumulates more CPDs and 6-4PPs than the Saudi Arabian cultivar, Yecora Rojo. This could, in part, account for the greater reduction in growth and photosynthesis of Maris Huntsman when grown under supplementary UVB. However, the reasons for Yecora Rojo accumulating less DNA damage are not clear. Although Yecora Rojo has a greater capacity to repair CPDs and 6-4PPs, the difference in repair is not enough to account for the huge difference in damage accumulation. Therefore, one possible explanation is that Yecora Rojo is better protected against UVB than Maris Huntsman.

Chapter 5

Protection of the leaves of Maris Huntsman and Yecora Rojo From UVB

Introduction

The previous two Chapters have shown that the tropical cultivar, Yecora Rojo, is less affected by UVB than the British cultivar, Maris Huntsman. One reason for this could be that Yecora Rojo is better protected against UVB than Maris Huntsman; either by having more UVB-absorbing compounds or by filtering out UVB in some other way. In this Chapter the amount of UVB-absorbing compounds and the amount of radiation penetrating the leaves of both cultivars will be compared.

5.1 Protection Against UVB

Plants, being sedentary organisms, are constantly exposed to solar radiation and because their leaves are positioned to intercept large amounts of photosynthetically active radiation, UVB is likely to affect their growth and development. Therefore, plants have evolved strategies such as shielding and repair to limit the amount of damage caused. Repair of DNA damage was discussed in the previous Chapter and this Chapter will concentrate on shielding mechanisms employed by the plant.

5.1.1 Epidermal Shielding

The leaf epidermis forms the first filter for radiation and is particularly effective in reducing the amount of UVB penetration whilst transmitting a large portion of PAR. Higher plant surfaces develop a layer of epicuticular wax on the outmost surface of the cuticle which forms the first line of defence against external influences. Although these waxes do not absorb strongly in the UVB region they may reflect and protect from high UV-irradiances and visible light (Bornman & Teramura, 1993).

UVB radiation may have a regulatory effect on wax biosynthesis which may result in increased wax cover. The physicochemical changes in the cuticle of *Nicotiana tobacum* when grown under UVB were examined and although UVB caused significant changes in the adaxial surface leaf wax composition, the total amount of wax did not change (Barnes *et al.*, 1996). The total cuticular wax of *Cucumis sativus*, *Phaseolus vulgaris* and *Hordeum vulgare* increased when grown under UVB. With UVB, the amounts of alkanes were different and this additional wax in the cuticle may reduce water loss from cuticular transpiration which could occur under enhanced UVB (Steinmüller & Tevini, 1985). However, the reflectance from the leaf surface is relatively low (<10%) in most plants that have been studied and the primary means of filtering out harmful UVB appears to be the presence of UVB-absorbing pigments (Caldwell *et al.*, 1983).

5.1.2 UVB Absorbing Pigments

Following exposure to UVB radiation most higher plants accumulate UVB-absorbing pigments (Sullivan & Teramura, 1989; Strid & Porra, 1992; Skaltsa *et al.*, 1994; Lui *et al.*, 1995) . These compounds are a class of water-soluble derivatives called flavonoids, including colourless pigments, which effectively absorb in the UV region of the spectrum with an absorbance peak at 295nm (Bornman & Teramura 1993). This group of secondary metabolic products includes flavones, flavonols and isoflavonoids. The accumulation of flavonoids in response to UV light is due to an increase in the rate of transcription of the chalcone synthase (*CHS*) gene (Chappell & Hahlbrock, 1984). CHS catalyses the first step in flavonoid biosynthesis (see Fig. 5.1) and depending on species and stage of development *CHS* gene expression is controlled by UV/blue photoreceptors and phytochrome (Jenkins, 1997).

The relationship between pigment accumulation and UVB levels has been examined using two main approaches. Either the induction and enzymatic regulation of UVB-absorbing compounds in plants grown under UVB can be studied or the response of flavonoid-deficient mutants can be examined. Flavonoid-deficient mutants of *Petunia, Hordeum vulgare* and *Arabidopsis thaliana* have been used to test the role of flavonoids in UVB protection (Reuber *et al.*, 1996; Jordan *et al.*, 1998; Ryan *et al*, 1998). In each of these experiments the flavonoid-deficient mutants were more susceptible to the effects of UVB than wild type as determined via reductions in growth and photosynthetic capacity. UVB radiation caused an increase in the UVB-absorbing pigments of *Brassica napus* (Ålenius *et al.*, 1995), *Pisum sativum* (Day & Vogelmann, 1995), *Aquilegia caerulea* (Larson *et al.*, 1990) and *Pinus taeda* (Sullivan & Teramura, 1989).

Flavonoids are a highly diverse group of plant secondary metabolites and it is likely that species-specific groups of flavonoids will be synthesised in response to UVB (Wilson *et al.*, 1998). A number of experiments have found that UVB changes the amounts of flavonoids, in particular those with additional hydroxyl groups on ring B of the flavonoid skeleton (Cen *et al.*, 1993; Lui *et al.*, 1995; Reuber *et al.*, 1996; Ryan *et al.*, 1998; Wilson *et al.*, 1998). When *Brassica napus* was grown under UVB there was a change in the relative amounts of the two major flavonoid compounds, kaempferol and quercetin glycosides (see Fig. 5.1). The proportion of quercetin glycosides to kaempferol glycosides increased when *Brassica napus* was grown under supplementary UVB (Olsson *et al.*, 1998). The ratio of quercetin to kaempferol glycosides was increased in *Petunia* spp. grown under supplementary UVB (Ryan *et al.*, 1998).

Quercetin and kaempferol, the two flavonols, differ from each other only in the degree of hydroxylation on the B ring of the flavonoid skeleton (Fig. 5.1). Quercetin is dihydroxylated and kaempferol monohydroxylated and it may be that the dihydroxylated flavonol is effective under conditions of UVB stress. Quercetin is a more effective free radical scavenger and therefore an increase in this flavonoid will provide the plant with UVB screening and antioxidant properties. Plants produce free radicals during normal metabolism (Mackerness *et al.*, 1998), although during stress conditions such as drought or exposure to UVB radiation the level of active oxygen metabolites may increase (Bornman & Teramura, 1993). Free radicals can react with bases in DNA causing mutations and denature proteins (Smirnoff, 1995). As UVB radiation generates free radicals, an increase in flavonoid antioxidant activity would be beneficial (Olsson *et al.*, 1998).

Another group of pigments that accumulates in response to UVB is the anthocyanins. Although they absorb mainly between 500-550nm they generally have another absorption maximum between 270-280nm (Waterman & Mole, 1994). The UVB absorption of anthocyanins is increased by acylation with aromatic organic acids and an additional absorption maximum in the 310-320 range is produced (Woodall & Stewart, 1998). When anthocyanins accumulated in cells of *Centaurea cyanus* under UVB irradiation the extent of pyrimidine dimer formation was reduced (Takahashi *et al.*, 1991). The UVB tolerance of 3 genotypes of *Brassica rapa*, that differ in their anthocyanin production, were analysed to determine whether anthocyanins function as UV-protective pigments. From the results of this experiment it was concluded that factors other than anthocyanin pigment accumulation must be involved in the UVB response (Klapper *et al.*, 1996). A similar conclusion was drawn when the amount of anthocyanins in juvenile leaves of *Syzygium* spp. were analysed to assess whether the high presence of anthocyanins was related to UV protection (Woodall & Stewart, 1998). The absence of anthocyanins with aromatic acyl groups and the abundance of other compounds which strongly absorb UV radiation both suggest that the UV protection hypothesis is not valid for the anthocyanins in developing leaves of *Syzygium* .

5.2 Penetration of Light Through the Leaf

UVB-absorbing pigments, changes in epidermal waxes and leaf reflectance all contribute to changing the quality and quantity of light penetrating the leaf of plants grown under UVB. By measuring the penetration of light through the leaves, direct comparisons can be made of the amount of UVB radiation and white light reaching the mesophyll cells. This is likely to

have important influences on the susceptibility to DNA damage and the photosynthetic activity of the leaf, respectively.

Using optical fibres of quartz, the light distribution within plant tissues can be measured directly. Optical fibre, when heated, stretched and sealed only allows light entry into the extreme tip. Thus, the fibre optic microprobe is small (*c.* 2 μm), durable and has high spatial resolution (Vogelmann *et al.*, 1991). This probe can be inserted into plant tissues to estimate the amount of light within different tissues and organs.

To determine whether the differences in the response to UVB reported in previous Chapters is due to the differences in leaf optical properties, the amount of 310nm (within the UVB range) and 430nm (within the PAR range) radiation penetrating the primary leaves of Maris Huntsman and Yecora Rojo was measured. These wavelengths were chosen so that one wavelength from the UVB range and one from the white light range was studied i.e. decreases in both suggest non-specific screening, decreases in UVB relative to PAR would indicate a specific UVB protection response

5.3 Aims of the Chapter

Results presented in Chapter 4 indicated that Yecora Rojo accumulated less DNA damage than Maris Huntsman and this was not due to Yecora Rojo being able to repair the damage at a faster rate. Therefore, this chapter will examine pigment composition and optical properties in the leaves of both cultivars to determine if there is a greater capacity to reduce UVB penetration in the more tolerant cultivar, Yecora Rojo.

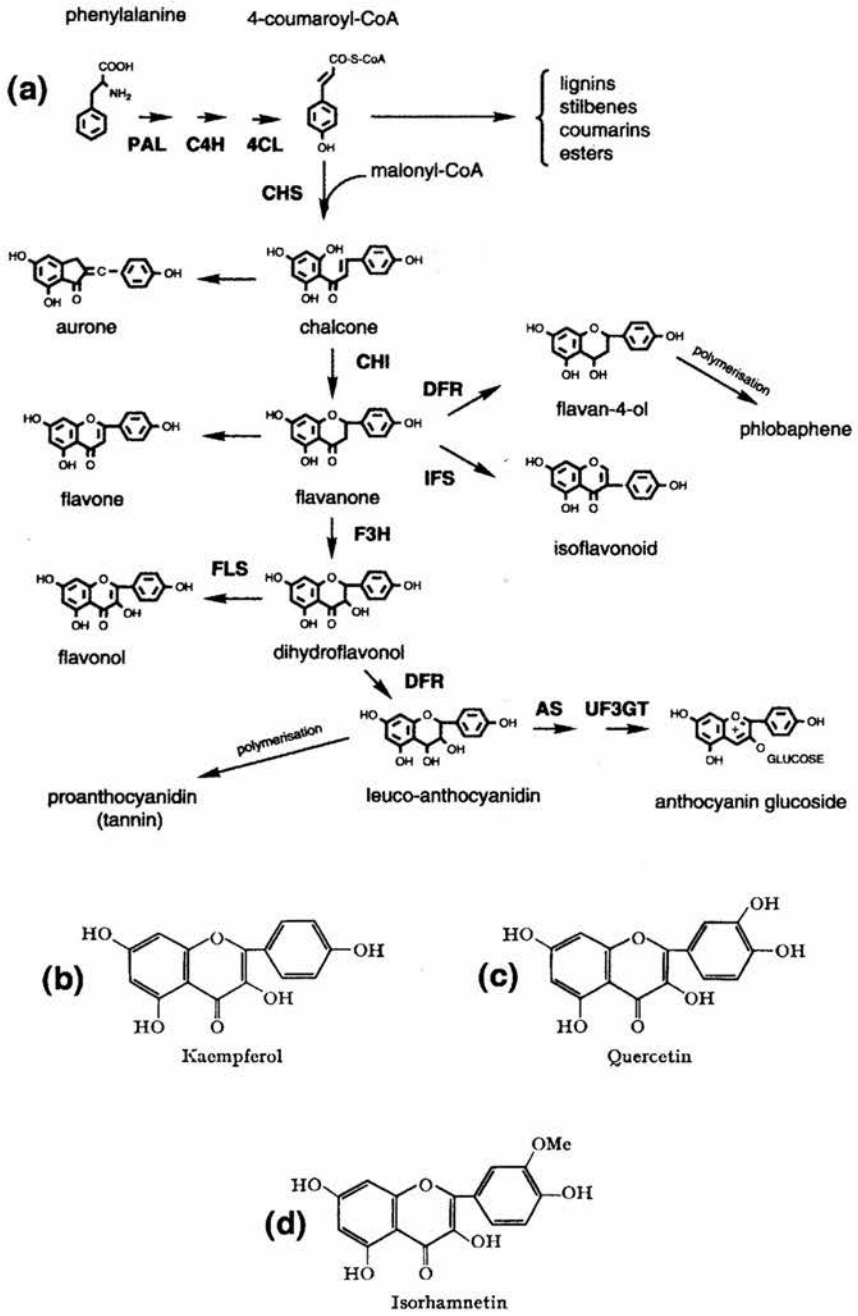


Figure 5.1 The general flavonoid pathway (a) (Koes *et al.*, 1994). The structures of the major classes of flavonoids are illustrated. Three flavonols are shown, kaempferol (b), quercetin (c) and isorhmnetin (d) (Markham & Geiger, 1993).

The enzymes catalysing the reactions are indicated by the following abbreviation: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-coenyzme A ligase; CHS, chalcone synthase; CHI, chalcone flavanone isomerase; F3H, flavanone 3β-hydroxylase; DFR, dihydroflavonol 4-reductase; FLS, flavonol synthase; AS, anthocyanin synthase; UF3GT, UDP-glucose: flavonoid 3-O-glucosyl-transferase.

Results

5.4 The Effect of UVB on Pigments

The amount of anthocyanins produced by Maris Huntsman and Yecora Rojo is shown in Fig. 5.2. There was no significant difference in the amount of anthocyanins produced in either the coleoptile or leaves of Maris Huntsman when grown with or without supplementary UVB. The amount of anthocyanins was calculated for unit area of fresh tissue and therefore takes into account the changes in leaf area with UVB. When Yecora Rojo was grown under supplementary UVB there was significantly more ($p<0.05$) anthocyanins produced in the coleoptile than when grown without supplementary UVB. However, in the leaf, there was no difference in the amount of anthocyanins produced when grown without supplementary UVB compared to UVB conditions in Yecora Rojo. When grown without supplementary UVB the coleoptiles of Maris Huntsman plants contained more anthocyanins than the coleoptiles of Yecora Rojo. With supplementary UVB this difference was not significant because of the increase in anthocyanin accumulation in Yecora Rojo. The leaves of both cultivars contained similar concentrations of anthocyanins under both growth conditions.

Fig. 5.3 shows the amount of UVB-absorbing compounds in the leaf and coleoptile of Maris Huntsman and Yecora Rojo per unit leaf area. UVB radiation resulted in a significant increase ($p<0.05$) in UVB-absorbing compounds in the leaf tissue and coleoptile of both Maris Huntsman and Yecora Rojo. When grown without supplementary UVB, Yecora Rojo had significantly more ($p<0.05$) UVB-absorbing compounds than Maris Huntsman. However, when grown under supplementary UVB there was no difference in the amount of UVB-absorbing compounds in the primary leaves of the two cultivars. The coleoptile of Yecora Rojo, plants grown without supplementary UVB, had significantly less ($p<0.05$) UVB-absorbing compounds per unit area than Maris Huntsman. With supplementary UVB, there was no difference in the amount of UVB-absorbing compounds in the coleoptile of the two cultivars. The leaf tissue of both cultivars contained more UVB absorbing compounds than the coleoptile under both experimental conditions. There was a significant increase in the amount of UVB-absorbing compounds per unit area when the cultivars were grown with supplementary UVB.

Fig 5.4 shows the absorption spectra from the spectrophotometer. From these graphs the area under the curve between 280 and 320 was calculated to measure the amount of UVB absorbing compounds present in the tissue samples of each cultivar.

The particular flavonols present in the leaf tissue of both cultivars were measured using HPLC and the results are shown in Fig. 5.5. The three flavonols identified in the leaf tissue were quercetin, kaempferol and isorhamnetin. When Maris Huntsman was grown under supplementary UVB there was a significant increase ($p<0.05$) in the amount of all three flavonols present, although the biggest difference was in the amount of quercetin, which increased by 200%. There was no significant difference in the amount of the three flavonols when Yecora Rojo was grown with supplementary UVB. With supplementary UVB, Maris Huntsman had significantly more ($p<0.05$) quercetin and kaempferol than Yecora Rojo, but there was no difference in the amount of isorhamnetin between the two cultivars. When the plants were grown under supplementary UVB the quercetin:kaempferol ratio increased significantly ($p<0.05$) in Maris Huntsman but not in Yecora Rojo.

5.5 Absorbance Spectra of Quercetin and Kaempferol

Fig 5.6 shows an absorption spectra of quercetin and kaempferol. The is no difference in the absorption of quercetin and kaempferol *in vitro*.

5.6 Differences in Leaf Optical Properties of Maris Huntsman and Yecora Rojo

The spectral data are presented as absolute (a) and normalised values (b) (Figs 5.7 to 5.9). The different spectral quality of the light within leaves which have different fluxes is illustrated when the data are normalised to 1 (i.e. the maximum value is equal to 1). When the data are normalised to 1 the differences in the shape of the curves are more apparent, thus they are more easily compared. The amount of light penetrating the leaves is measured as the relative steric energy flux and it is relative to the amount of light measured by the fibre optic probe when no leaf is present.

5.6.1 Penetration of 310nm Radiation into the Leaf Tissue

The amount of 310nm radiation penetrating the primary leaves of Maris Huntsman and Yecora Rojo grown with and without supplementary UVB is shown in Figs. 5.7 and 5.8. Radiation with a wavelength of 310nm was attenuated strongly by the epidermis and mesophyll tissues in both Maris Huntsman and Yecora Rojo. The measurements show that light penetrated further into the leaf of Yecora Rojo than Maris Huntsman. In plants grown without supplementary UVB Yecora Rojo had 47% greater steric energy flux at the adaxial epidermis than Maris Huntsman (Yecora Rojo 0.5; Maris Huntsman, 0.34). and *c.* 75% of the relative steric energy was absorbed by the adaxial

epidermis in Maris Huntsman compared to *c.* 50% in Yecora Rojo. However, when the plants were grown with supplementary UVB, Yecora Rojo and Maris Huntsman had the same relative steric energy at the adaxial epidermis, and the adaxial epidermis of Maris Huntsman absorbed *c.* 75% of the relative steric energy flux compared to *c.* 70% in Yecora Rojo. Growth under supplementary UVB resulted in more relative steric energy being absorbed by the adaxial epidermis of Yecora Rojo plants. When grown without supplementary UVB, Yecora Rojo had a greater relative steric energy flux throughout the entire depth of the leaf than Maris Huntsman. When grown under supplementary UVB the relative steric energy of light reaching of Maris Huntsman increased slightly and Yecora Rojo decreased slightly and thus the response of Maris Huntsman and Yecora Rojo are very similar. In summary, Yecora Rojo allowed more 310nm radiation to penetrate the leaves than did Maris Huntsman.

5.6.2 Penetration of 430nm Radiation into the Leaf Tissue

Figs. 5.9 and 5.10 show the amount of 430nm radiation penetrating the primary leaves of Maris Huntsman and Yecora Rojo when grown with and without supplementary UVB. Again, the light penetrated further into the leaf of Yecora Rojo than Maris Huntsman. When the plants were grown with supplementary UVB, Yecora Rojo had 26% greater steric energy at the adaxial epidermis than Maris Huntsman and *c.* 68% of the relative steric energy flux was absorbed by the adaxial epidermis of Maris Huntsman compared to *c* 50% in Yecora Rojo. When grown under supplementary UVB the relative steric energy was increased by 22% in Maris Huntsman and by 100% in Yecora Rojo. The adaxial epidermis of Maris Huntsman absorbed *c.* 68% of the steric energy compared to 80% in Yecora Rojo. When grown without supplementary UVB, both cultivars had a similar response, with Yecora Rojo having an increased steric energy throughout the depth of the leaf. However, when grown with supplementary UVB, the relative steric energy flux of Yecora Rojo was increased to a greater extent than in Maris Huntsman.

5.7 Ratio of 310 to 430nm Radiation Penetrating the Leaves

The amount of 310 to 430nm radiation penetrating the upper epidermis of each cultivar grown with and without UVB was calculated (Table 5.1). When grown without supplementary UVB, the ratio of 310 to 430nm radiation penetrating the upper epidermis of Maris Huntsman is significantly ($p<0.05$) less than that of Yecora Rojo. UVB results in a significant ($p<0.05$) increase in the ratio of 310 to 430nm radiation penetrating upper epidermis of Maris Huntsman. However, in Yecora Rojo UVB results in a significant ($p<0.05$)

decrease in the amount of 310 to 430nm radiation penetrating the upper epidermis.

5.8 Leaf Thickness

When the plants were grown with supplementary UVB, an increase in leaf thickness was observed in both cultivars (Fig. 5.11). The leaves of Yecora Rojo increased by 23% whereas Maris Huntsman increased by 9%. Under UVB the mesophyll of Maris Huntsman, and the upper epidermis and mesophyll of Yecora Rojo increased significantly ($p<0.05$).

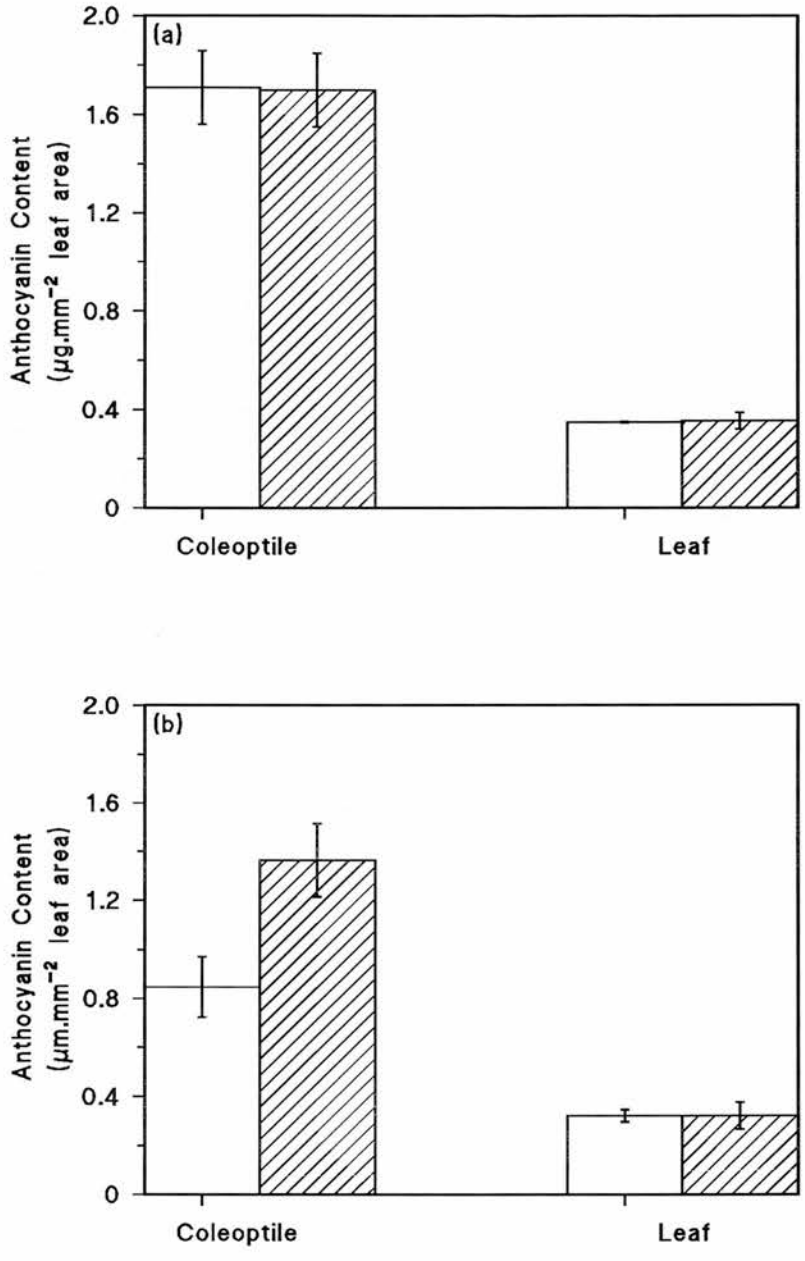


Figure 5.2 The amount of anthocyanins in the primary leaf of 6-day old seedlings of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB.

Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The amount of anthocyanins per unit leaf area was determined using the method in Section 2.6.3. The bars represent the mean from 24 samples from four independent growth studies with error bars showing ± one standard error from the arithmetic mean.

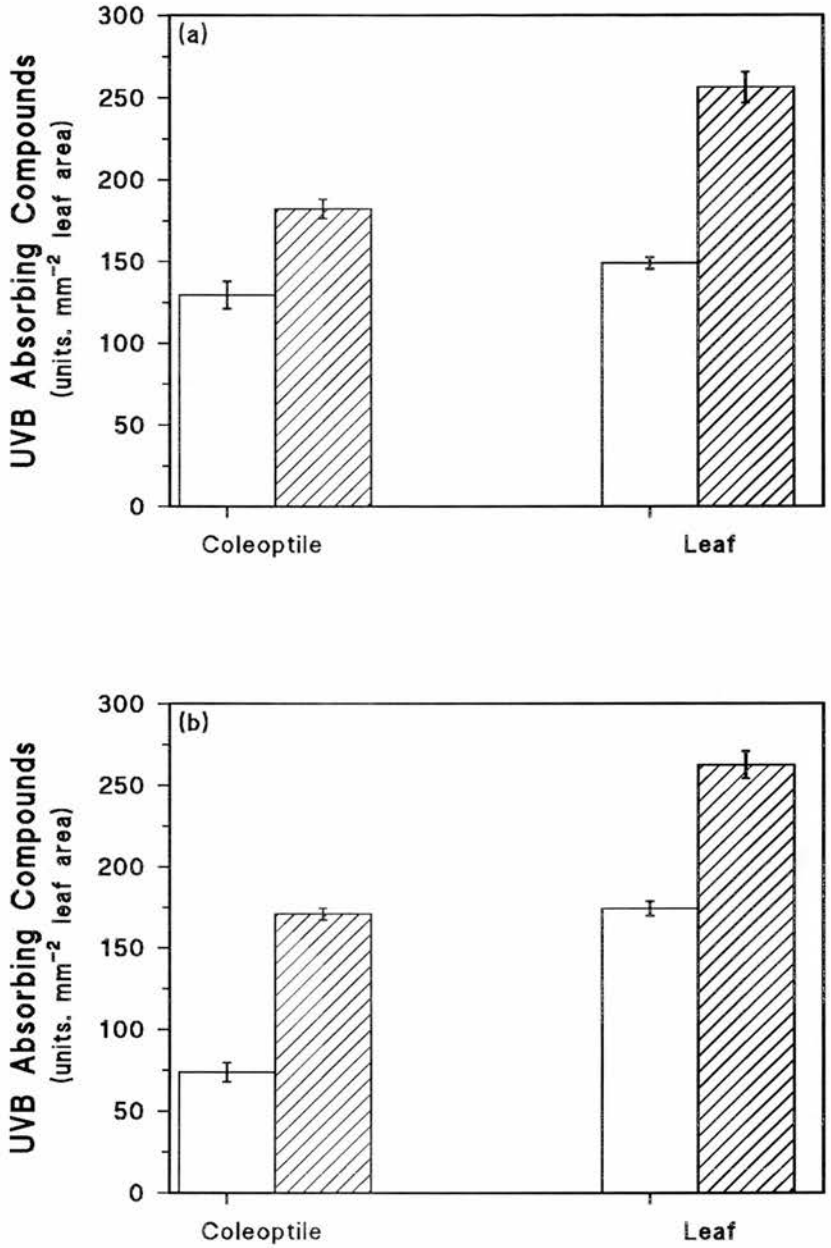
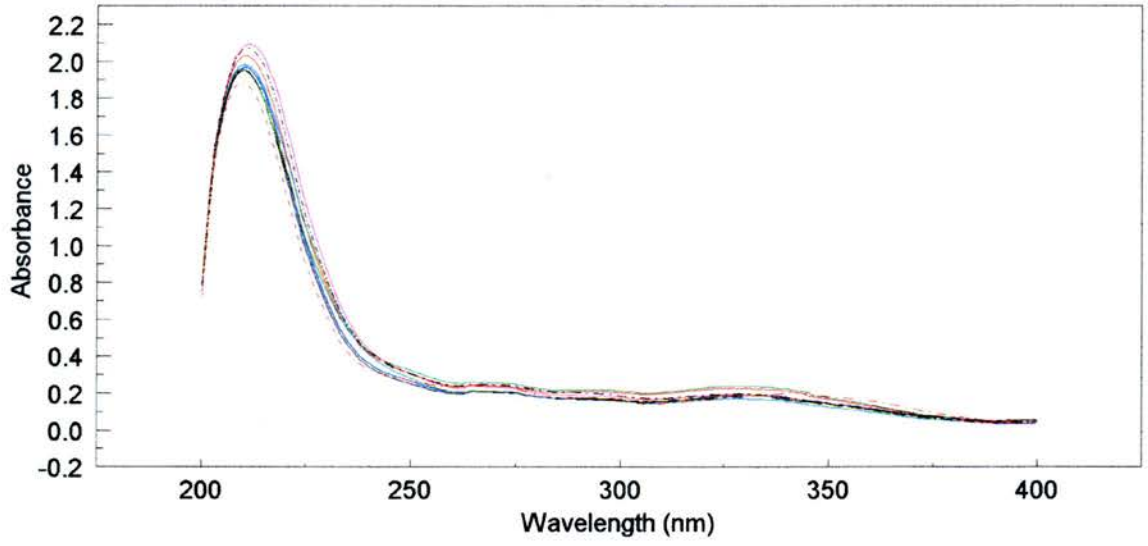


Figure 5.3 The amount of UVB-absorbing compounds in the primary wheat leaf and coleoptile of 6-day old seedlings of Maris Huntsman (a) and Yecora Rojo (b). Plants were grown with (▨) and without (□) supplementary UVB (Section 2.2). The relative amount of UVB-absorbing compounds was determined using the method in Section 2.6.1. The bars represent the mean from 24 samples from four independent growth studies with error bars showing ± one standard error from the arithmetic mean.



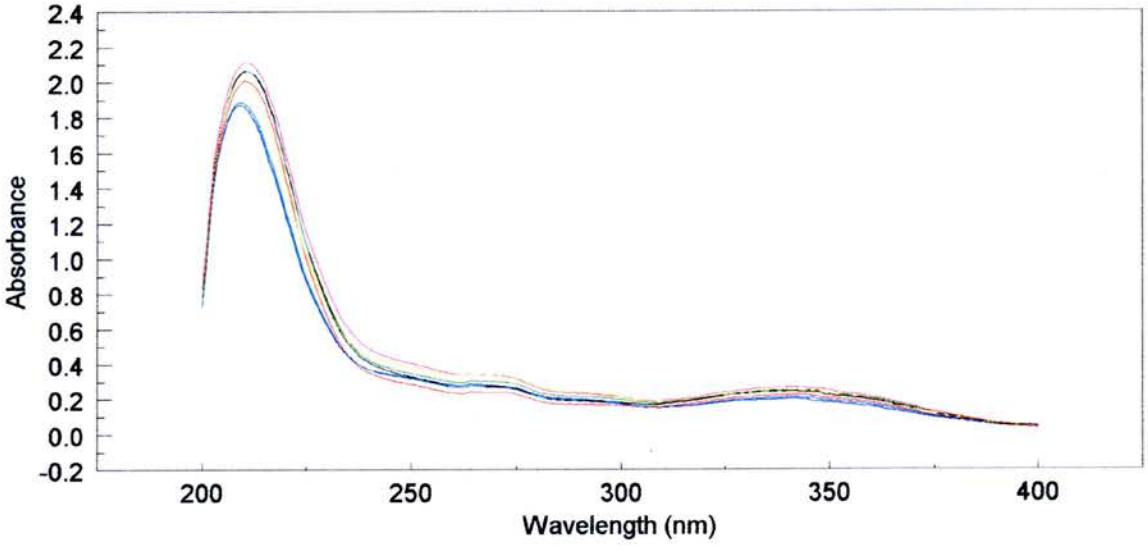


Figure 5.4 An example of an absorption spectra of primary leaf extracts of Maris Huntsman (a) and Yecora Rojo (b) grown without supplementary UVB.
Plants were grown without supplementary UVB (Section 2.2). The UVB absorption was measured using the method in Section 2.6.1. Each line represents one sample from one growth study. The area under the graph between 280 and 320nm was used to calculate the amount of UVB-absorbing compounds in each cultivar when grown with and without UVB.

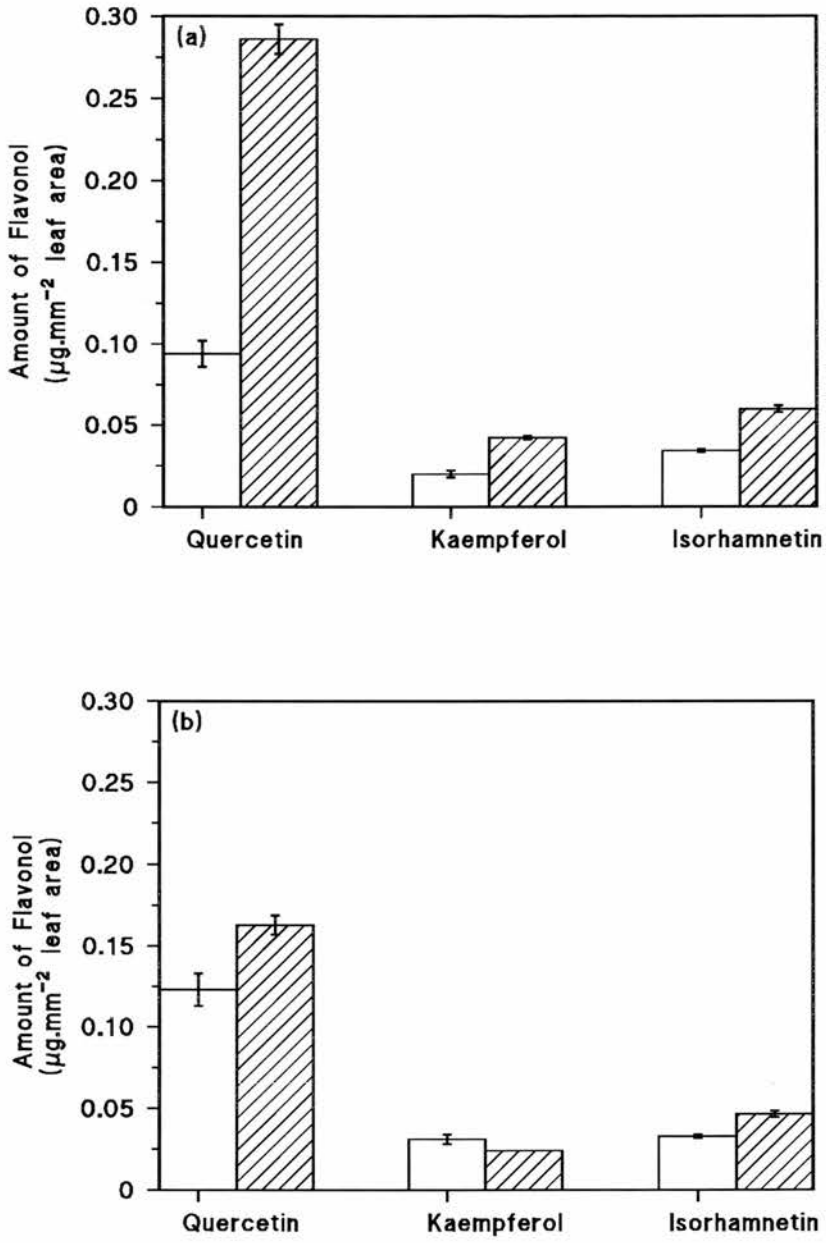


Figure 5.5 The Amount of flavonols in the primary wheat leaf of 6-day old seedlings of Maris Huntsman (a) and Yecora Rojo (b).

Plants were grown under with (▨) and without (□) UVB (Section 2.2). The amount of flavonols was determined using the HPLC method in Section 2.6.2. The bars represent the mean from 3 samples with error bars showing ± one standard error from the arithmetic mean.

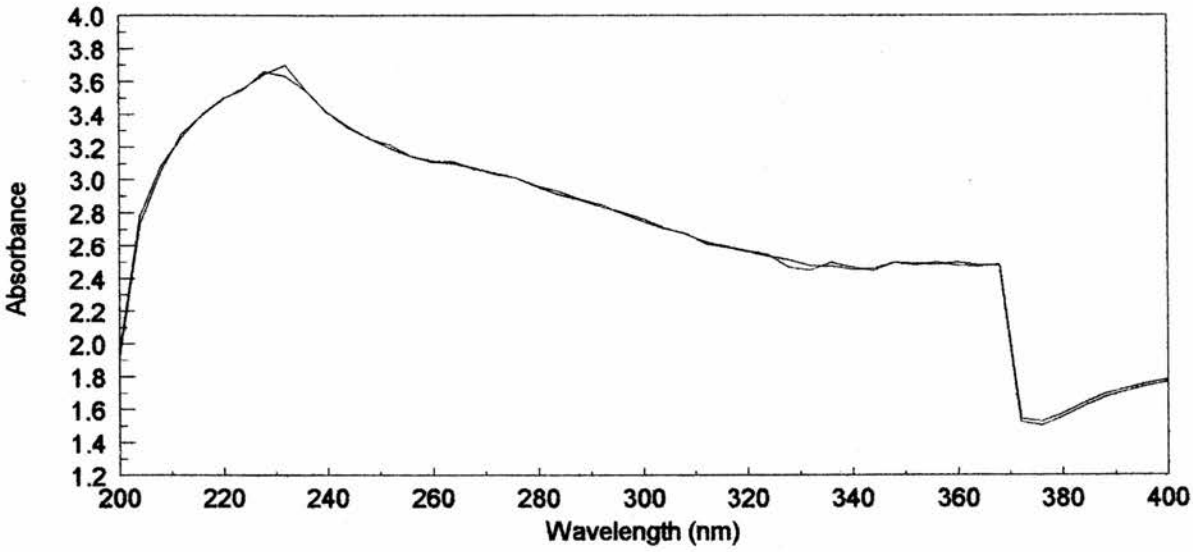


Figure 5.6 An example of the absorption spectra of the quercetin and kaempferol.

The UVB absorption was measured using the method in Section 2.6.1. A scan of each compound (5mM solutions in acidified methanol) was taken using a spectrophotometer to compare the absorption spectra within the UVB range (i.e. between 280 and 320nm). Each compound was scanned against a blank of acidified methanol.

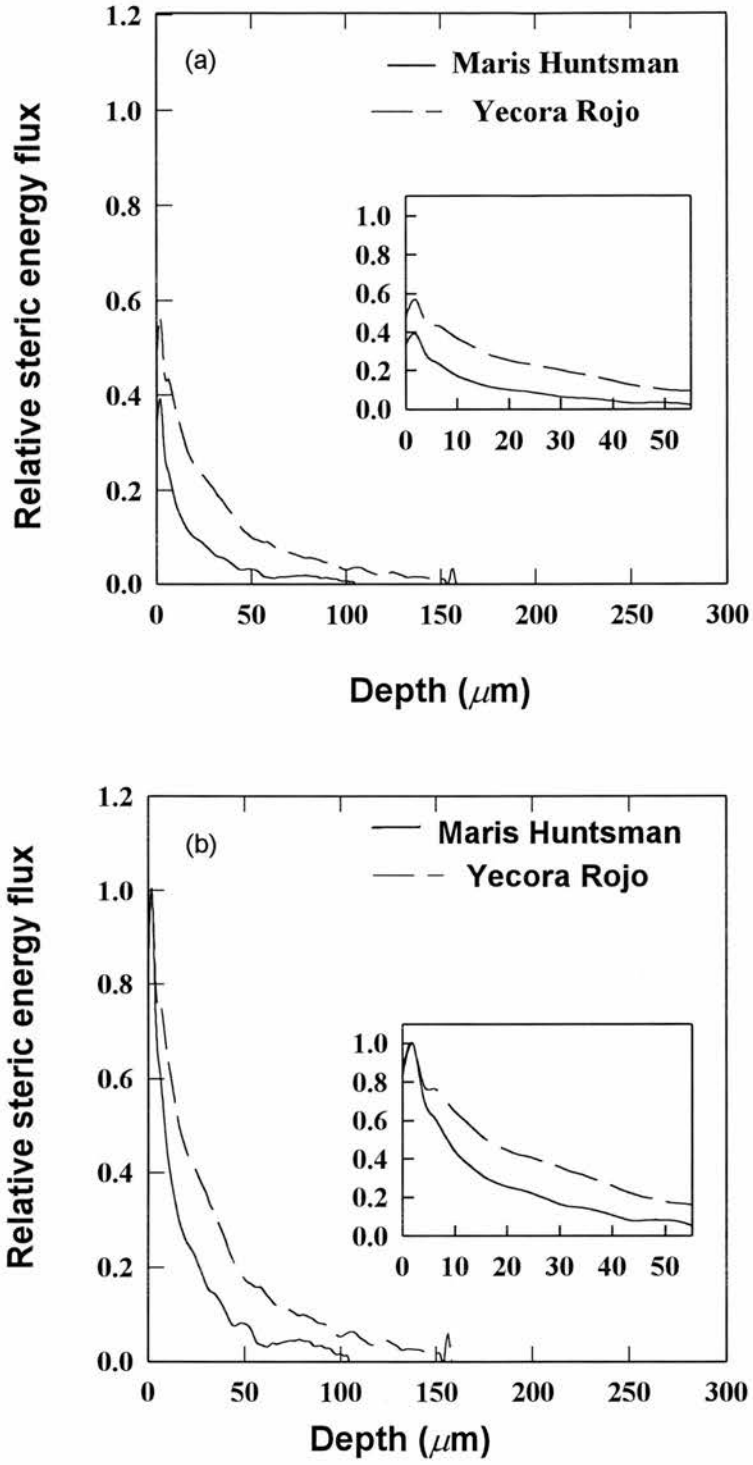


Figure 5.7 Penetration of 310nm radiation through the primary leaf of Maris Huntsman and Yecora Rojo grown without supplementary UVB.

Plants were grown without supplementary UVB (Section 2.2). The relative steric energy flux was measured using a fibre optic probe as described in Section 2.8. The line represents the mean from a total of 16 leaves from two independent growth studies. The insert shows the x-axis expanded. The actual values are shown in Fig a and the values at the leaf surface have been normalised to 1 in Fig b.

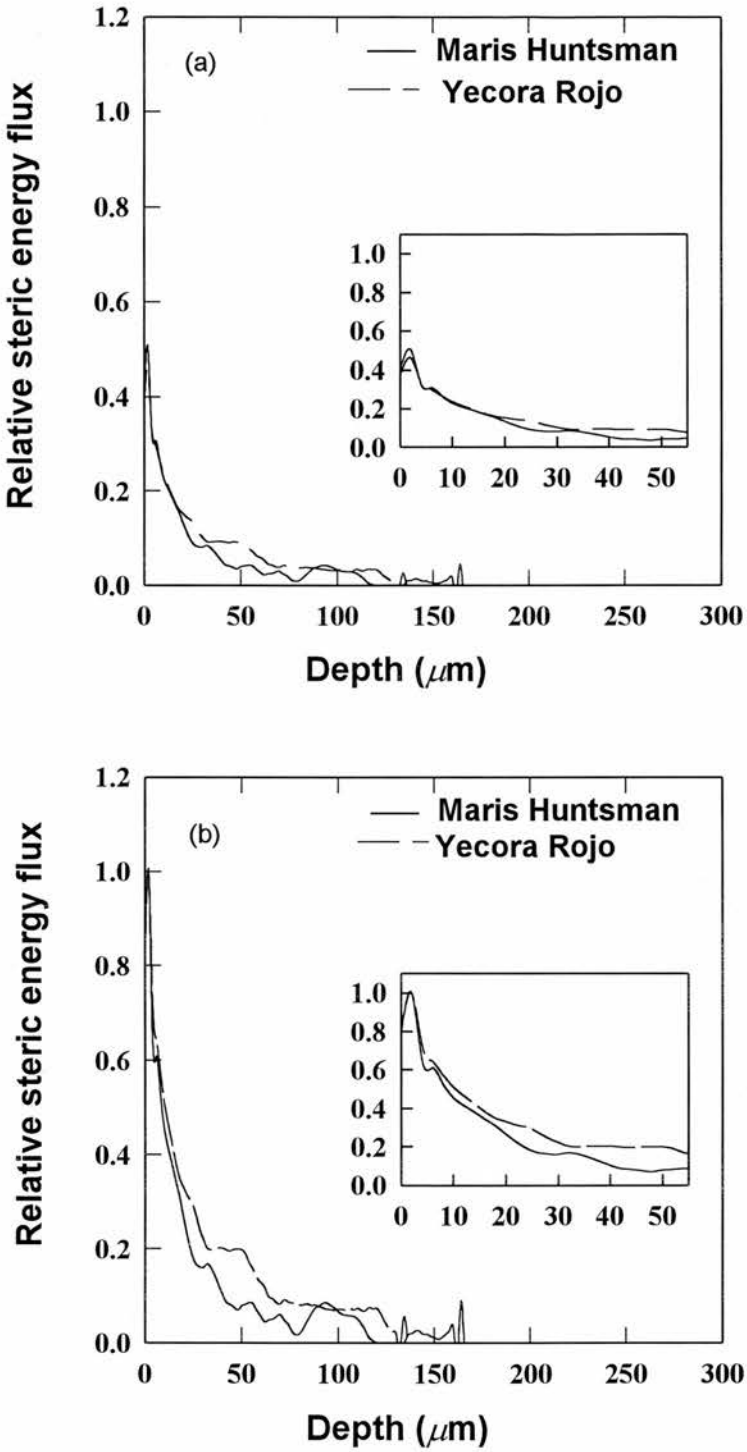


Figure 5.8 Penetration of 310nm radiation through the primary leaf of Maris Huntsman and Yecora Rojo grown with supplementary UVB.

Plants were grown with supplementary UVB (Section 2.2). The relative energy flux was measured using a fibre optic probe as described in Section 2.8. The line represents the mean from a total of 16 leaves from two independent growth studies. The insert shows the x-axis expanded. The actual values are shown in Fig a and the values at the leaf surface have been normalised to 1 in Fig b.

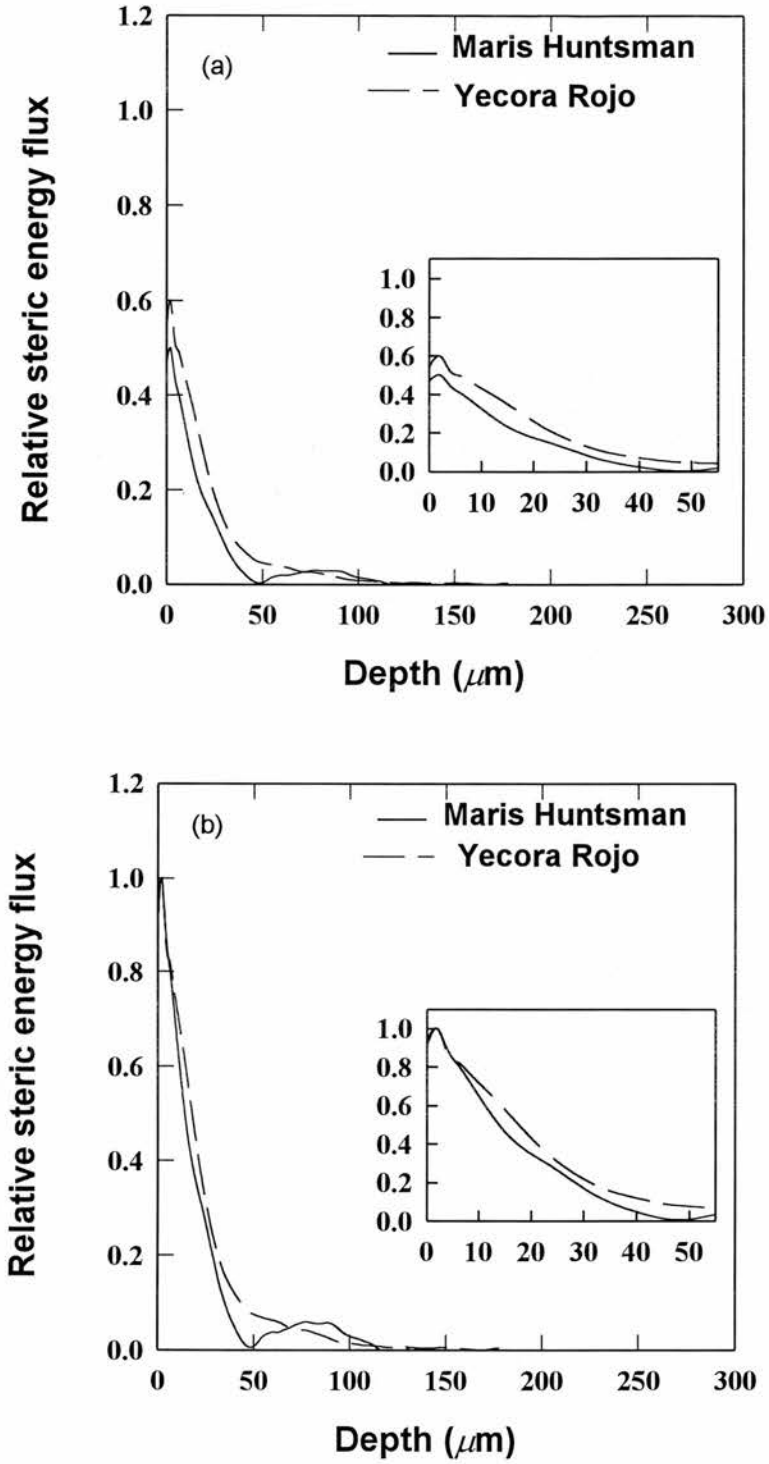


Figure 5.9 Penetration of 430nm radiation through the primary leaf of Maris Huntsman and Yecora Rojo grown without supplementary UVB.

Plants were grown without supplementary UVB (Section 2.2). The relative energy flux was measured using a fibre optic probe as described in Section 2.8. The line represents the mean from a total of 16 leaves from two independent growth studies. The insert shows the x-axis expanded. The actual values are shown in Fig a and the values at the leaf surface have been normalised to 1 in Fig b.

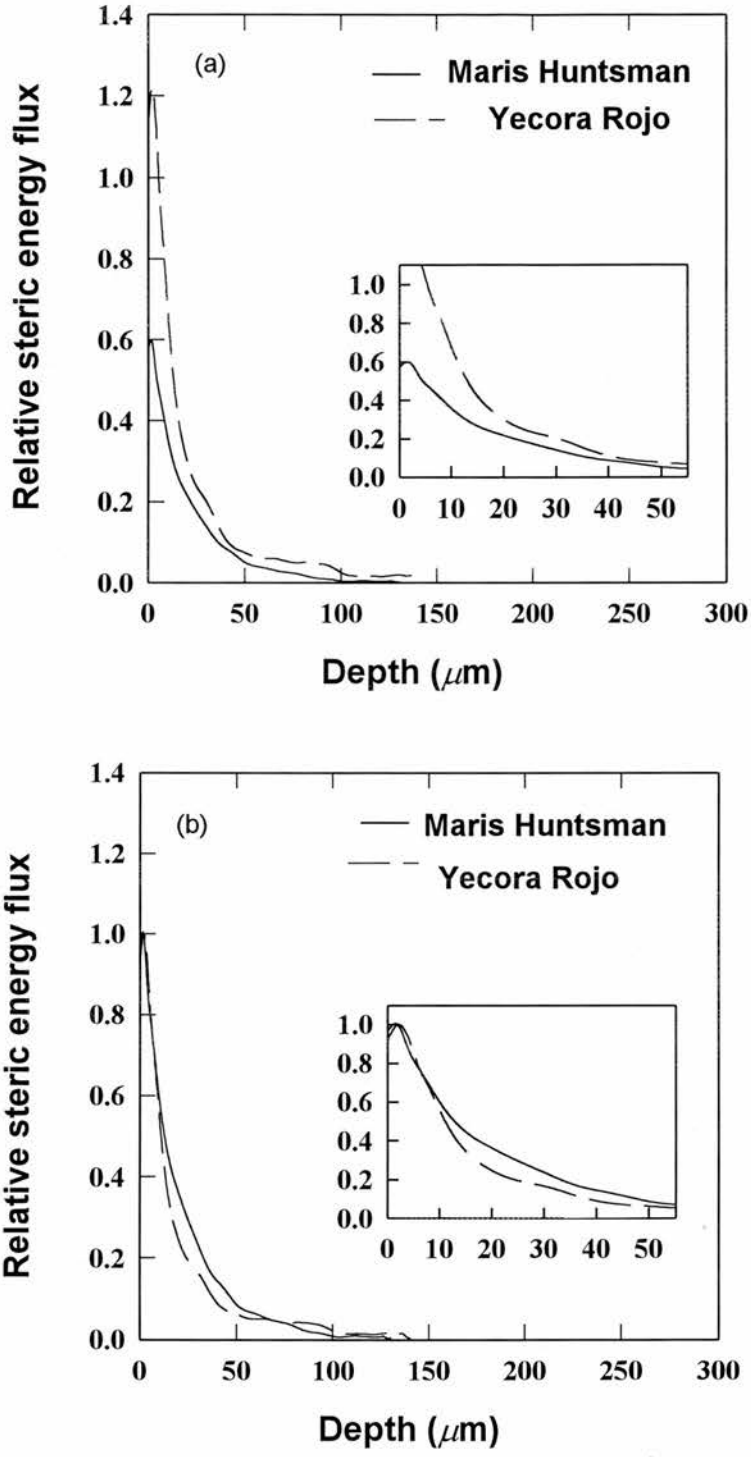


Figure 5.10 Penetration of 430nm radiation through the primary leaf of Maris Huntsman and Yecora Rojo grown with supplementary UVB.

Plants were grown with supplementary UVB (Section 2.2). The relative energy flux was measured using a fibre optic probe as described in Section 2.8. The line represents the mean from a total of 16 leaves from two independent growth studies. The insert shows the x-axis expanded. The actual values are shown in Fig a and the values at the leaf surface have been normalised to 1 in Fig b.

	Without UVB			UVB		
	310	430	ratio of 310:430	310	430	ratio of 310:430
Maris Huntsman	0.202	0.330	**0.612**	0.250	0.368	**0.679**
Yecora Rojo	0.402	0.462	**0.870**	0.242	0.654	**0.370**

Table 5.1 The relative steric energy values of 310 and 430nm radiation and the ratio of 310:430nm radiation in the upper epidermis of primary leaves of 6-day old Maris Huntsman and Yecora Rojo seedlings grown with and without supplementary UVB.

Plants were grown with and without supplementary UVB (Section 2.2). The relative energy flux was measured using a fibre optic probe as described in Section 2.8. The real value represents the mean from a total of 16 leaves from two independent growth studies. The ratio of the relative steric energy of 310 to 430nm radiation was calculated and is shown in bold. There are no units because relative values are being used.

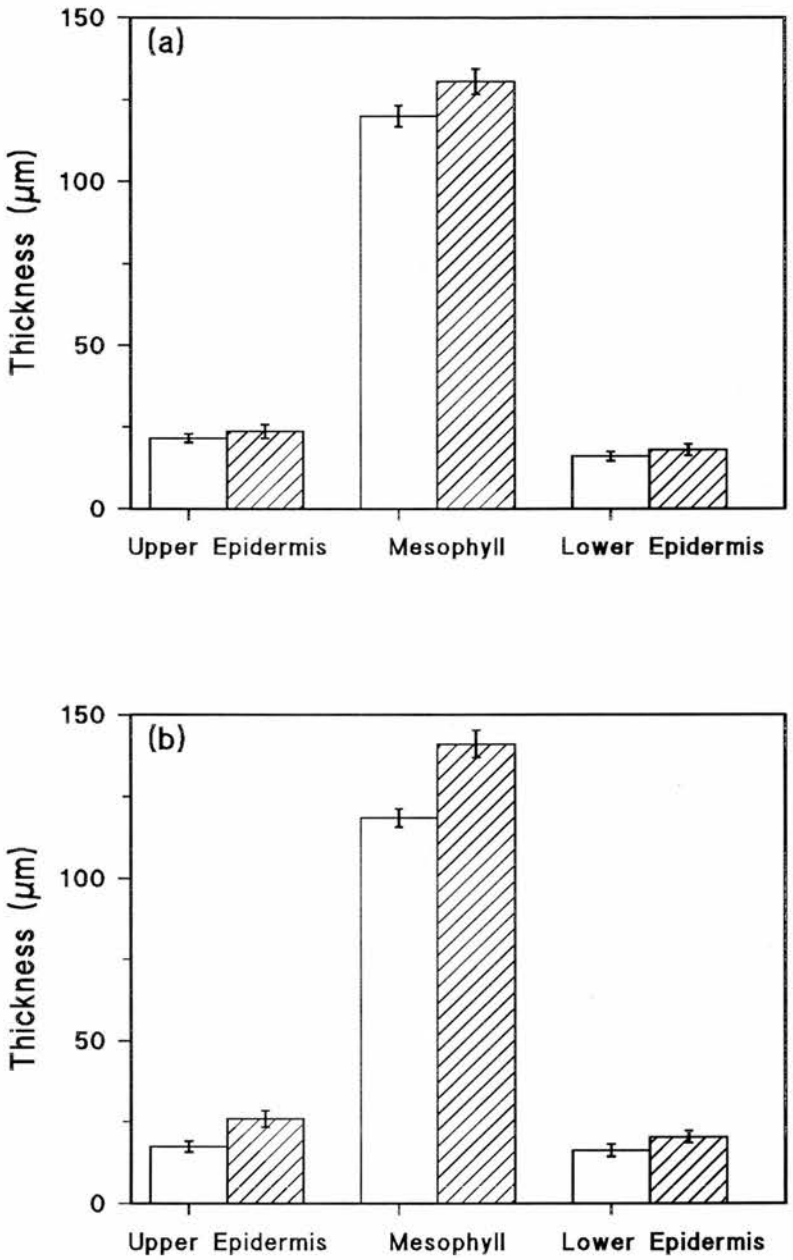


Figure 5.11 The thickness of each tissue type in the primary leaf of Maris Huntsman (a) and Yecora Rojo (b) grown with and without supplementary UVB. Plants were grown with (▨) and without supplementary UVB (□) (Section 2.2). The leaf thickness was calculated using hand-cut sections as described in Section 2.8.1. The bars represent the mean of 15 samples from three independent growth studies with error bars showing ± one standard error from the arithmetic mean.

Discussion

In this Chapter pigment composition and spectral differences in both cultivars will be discussed to assess the role of UVB protective pigments and the extent of radiation penetration into the leaf.

5.9 The Effect of UVB on Pigment Content in Maris Huntsman and Yecora Rojo

There was no difference in the amount of anthocyanins in the primary leaves of either cultivar when grown with supplementary UVB compared to those grown without UVB. Therefore, it would seem that anthocyanins are not related to the observed difference in accumulation of CPDs and 6-4PPs between the cultivars. However, the level of UVB protection the plant receives from anthocyanins depends on where the pigment is located in the leaf. For example, if anthocyanins are present in the abaxial epidermis then this will not offer much UVB protection, although if the pigments are present in the adaxial epidermis then the plant will be provided with a barrier against UVB. In order to assess the role of anthocyanins in UVB protection of these cultivars, it would be necessary to find out where exactly in the leaf the anthocyanins are accumulating.

The coleoptile of both cultivars contained more anthocyanins than the leaf tissue. The coleoptile protects the basal meristem of the plant from harmful radiation (Haussuhl *et al.*, 1996) and therefore anthocyanins may help with this function. When grown under supplementary UVB, the amount of anthocyanins in the coleoptile of both cultivars was the same and so this is unlikely to be the reason for the difference in the effect of UVB on growth (Chapter 3). Therefore, it seems that anthocyanins do not have a large role in UVB protection in Maris Huntsman and Yecora Rojo, although in order to test this fully the anthocyanins would have to be isolated and their UVB absorption range analysed.

When grown without supplementary UVB, the leaves of Yecora Rojo contained more UVB-absorbing compounds than Maris Huntsman. This method measured the total amount of UVB-absorbing compounds, which included flavonoids and any other compound which absorbed in the UVB range. The higher concentration of UVB-absorbing compounds in Yecora Rojo could be one reason why Maris Huntsman accumulates more CPDs and 6-4PPs than Yecora Rojo. When the plants were grown under supplementary UVB, the amount of UVB-absorbing compounds increased in both cultivars relative to those grown without but there was no difference in the amount between the two cultivars. With UVB, the amount of UVB-absorbing compounds also increased

in the coleoptile which will increase the coleoptile's protective role against UVB. This experiment simply measured the total amount of UV-absorbing compounds in the leaf tissue and therefore did not give any indication of where in the leaf the compounds are situated or what compounds are present. As already argued for anthocyanins, these factors are important in determining the protective role of these pigments.

HPLC analysis was used to identify which particular flavonoids were present in the leaves of each cultivar. The HPLC procedure did not detect all of the UVB-absorbing compounds, therefore, the flavonols detected (Fig. 5.5) cannot be correlated directly to the total amount of UV-absorbing compounds (Fig. 5.3). The three flavonols, quercetin, kaempferol and isorhamnetin were present in different amounts under each treatment. When grown under UVB, Maris Huntsman had more quercetin and kaempferol than Yecora Rojo and thus the quercetin to kaempferol ratio (Q:K) increased in Maris Huntsman with UVB. With UVB there was no change in the amount of isorhamnetin in either cultivar. Although both quercetin and kaempferol absorb in the UVB range, their absorbance maxima are in the UVA and UVC parts of the light spectrum. As quercetin is dihydroxylated, compared to kaempferol which is monohydroxylated, it may be that quercetin was selectively increased in Maris Huntsman because it is more effective under conditions of UVB stress. An increase in this flavonol, which is a more effective free radical scavenger, may be beneficial to the plant. An absorption spectra of quercetin and kaempferol showed that there is no difference in their absorption in the UVB range. Therefore, the increase in quercetin when grown with supplementary UVB cannot be due to the fact that this compound absorbs more in the UVB range than kaempferol.

Under supplementary UVB the primary leaves of the cereal, *Hordeum vulgare* showed increases in lutonarin and saponarin, the two main flavonols in barley leaves (Reuber *et al.*, 1996). It is likely that *Triticum aestivum* may have similar flavonols to those present in *Hordeum vulgare*. However, because these two flavonoids are mainly glycosylated on the A ring of the flavonol structure and the HPLC column used in this study was not optimised for such compounds, lutonarin and saponarin would not have been identified had they been present. Therefore, perhaps all the flavonols present in the two cultivars were not identified and there could be other differences which have not been examined in this study. Further work is needed to identify the other UVB-absorbing compounds present in the two cultivars under investigation.

The absorption of anthocyanins in the UVB range has led to the conclusion that they may protect leaves from UVB damage and a number of

studies have shown that anthocyanins accumulate with UVB. For example, anthocyanins were induced by UVB in eggplant seedlings (*Solanum melongena*; Toguri *et al.*, 1993). The UVB protective effects of anthocyanins were observed in mature leaves of red *Coleus* cultivars containing epidermal anthocyanins which showed less damage under UVB than the cultivars containing no anthocyanins (Burger & Edwards, 1996). Takahashi *et al.* (1991) demonstrated that the cells of *Centaurea cyanus* were protected from the harmful effects of UVB by anthocyanins since an increase in anthocyanins caused a reduction in the amount of UVB-induced pyrimidine dimers. However, when Woodall & Stewart (1998) assessed the role of anthocyanins in juvenile leaves of *Syzygium* species containing high concentrations of the pigments, the results showed that the UVB protective role of anthocyanins is not a valid hypothesis for this plant species.

UV-absorbing compounds have been shown to increase with UVB in a number of experiments e.g. UVB-absorbing compounds increased after UVB treatment in *Aquilegia caerulea* (Larson *et al.*, 1990); *Hordeum vulgare* (Liu *et al.*, 1995); *Pisum sativum* (Day *et al.*, 1996) and *Oryza sativa* (Hidema *et al.*, 1996). Like anthocyanins, the protective effect of UV-absorbing compounds depends on what compounds are present and where in the leaf they are situated. To show the correlation between UVB penetration and the internal distribution of UVB-screening pigments of *Brassica napus* (Ålenius *et al.*, 1995) used three-dimensional representation, plotting UV-absorbing compounds against wavelength and depth. There was a large increase in the UVB screening pigments relative to the controls at the adaxial tissue layer whereas at the abaxial surface there was no difference in the amount of pigments. Since the pigments were located in the top surface of the leaf this would help to prevent UVB radiation from reaching susceptible sites within the *Brassica napus* leaf. Flavonoid-deficient mutants of *Zea mays* were used to test if flavonoids offer protection from UVB-induced DNA damage *in vivo* (Stapleton & Walbot, 1994). The results showed that less CPDs accumulated in *Zea mays* plants that contained flavonoids compared to the plants which lacked the compounds.

In the present study, an increase in the quercetin:kaempferol ratio was observed when Maris Huntsman was grown with supplementary UVB. Such an increase was also found in *Brassica napus* (Olsson *et al.*, 1998) and mutants of *Petunia* (Ryan *et al.*, 1998). Quercetin and kaempferol only differ from each other in the degree of hydroxylation on the B-ring of the flavonoid skeleton, and it is likely that under conditions of UVB stress the dihydroxylated flavonol may be more effective since it is a more efficient free radical scavenger.

The level of free radicals may increase after exposure to UVB radiation (Hideg & Vass, 1996) and these free radicals can induce strand breaks and base modifications in DNA (Abdi & Ali, 1999). With UVB, CPDs and 6-4PPs are induced directly due to absorption of energy not by free radicals (Britt, 1996). Therefore, an increase in quercetin may be beneficial since it is an efficient free radical scavenger, however, the amount of quercetin cannot be correlated with the amount of CPDs or 6-4PPs produced by either cultivar.

5.10 The Effect of UVB on Leaf Optical Properties

Changes in pigment content of the cultivars grown with and without supplementary UVB, were accompanied by changes in the internal gradients of light at a wavelength of 310nm and 430nm within the leaves. The differences in penetration of 310nm and 430nm when grown with UVB, are probably due to changes in UVB-absorbing pigment content and structural changes in the leaf, such as an increase in the depth of the adaxial epidermis. With UVB, the adaxial epidermis of Yecora Rojo increased significantly unlike the thickness of the epidermis of Maris Huntsman which did not change when grown under UVB. The thickness of the epidermis may contribute to screening out UVB.

When grown with supplementary UVB, the total amount of UVB-absorbing compounds increased in primary leaves of both cultivars and this was accompanied by an increase in total leaf thickness. An explanation for the increase in leaf thickness in response to UVB radiation has been that some of the adverse effects are alleviated by reducing penetration of UVB radiation (Flint *et al.*, 1985; Cen & Bornman, 1993). Thus, an increase in leaf thickness and UVB-absorbing compounds will decrease the amount of UVB penetrating to the mesophyll tissues of the leaf, and at the same time possibly reduce the penetration of visible light. However, in this study the absolute amounts of 430nm radiation penetrating the leaf in both cultivars, when grown under supplementary UVB, was higher than that for the plants grown without UVB. UVB resulted in a 100% increase in relative steric energy of 430nm radiation in Yecora Rojo, which may explain why the photosynthesis of Yecora Rojo was not affected when grown with supplementary UVB.

Growth under supplementary UVB resulted in a increase in the ratio of 310 to 430nm radiation within the upper epidermis of Maris Huntsman primary leaves and an decrease in the ratio of 310 to 430nm radiation within the upper epidermis of leaves of Yecora Rojo (Section 5.7). Yecora Rojo selectively reduced the amount of UVB penetrating the leaf which does not affect the amount of white light entering the mesophyll layer of the leaf. This may be another reason why photosynthesis of Yecora Rojo does not decrease when the

plant is grown under UVB, since much more photosynthetically active radiation is penetrating the upper epidermis of this cultivar.

When the plants are grown without supplementary UVB, Yecora Rojo allows more 310nm radiation to penetrate through the leaf tissue than Maris Huntsman, yet still accumulates less CPDs and 6-4PPs. The amount of UVB that the plants were grown under was equivalent to ambient levels for the tropical cultivar, Yecora Rojo, but was greater than ambient for the British cultivar, Maris Huntsman. Therefore, because Yecora Rojo has been bred to grow in high light irradiance environments, perhaps it has some physiological adaptations to cope with higher levels of UVB radiation. Although a considerable amount of UVB penetrates the leaf of Yecora Rojo, photosynthesis is not affected even though UVB radiation is damaging to photosynthetic machinery (Bornman, 1989). An explanation for the lack of damage to the photosynthetic apparatus could be that it may be protected from UVB at the organelle level by UVB-absorbing compounds. Some plants have been shown to have flavonoids in the outer membrane of chloroplasts (Haupt & Scheuerlein, 1990) which provides a final line of defence against UVB radiation. It is possible that other antioxidant systems may play a role in protection of chloroplasts also.

The amount of light penetrating the leaves of spinach (*Spinacia olercea*) grown under sun and shade conditions was measured (Cui *et al.*, 1991). Even though the sun-grown leaves were thicker and had a chlorophyll content similar to that of shade-grown leaves, the light penetrated further into the leaf. The palisade cell shape and intercellular air spaces played an important role in determining the amount of light penetrating the leaf tissue. This is similar to the situation with Yecora Rojo, since its leaves are thicker and have a similar chlorophyll content to Maris Huntsman. Therefore, perhaps one of the most important factors in determining the light gradients is the anatomy of the palisade layer.

A range of epidermal transmittance has been found among species of different plant life forms (Day *et al.*, 1992). The difference seemed to be due to different optical characteristics of the epidermis rather than difference in thickness. The location of UVB-screening compounds was one of the optical properties which may explain epidermal screening effectiveness.

The epidermal UVB screening effectiveness is dependent upon the epidermal thickness and concentration of UVB-absorbing compounds, their specific absorbance spectra and their location within the epidermis (Day *et al.*, 1993). Optical techniques were used to determine whether the differences in UVB screening effectiveness in herbaceous species and conifers could be

explained by location of UVB absorbing compounds. UVB penetrated further into the foliage of the herbaceous species than the conifers (Day *et al.*, 1993). The results showed that spatial location and uniformity of the UVB-absorbing compounds was important. The epidermis of herbaceous species was a deficient UVB screen due to the poor attenuation of UVB of cell walls, whereas, conifers which contain large amounts of flavonoids within the cell walls as well as in the protoplasts, provided a much more effective UVB screen.

In UVB treated plants of *Brassica napus* the epidermis was a more effective barrier to UVB radiation than in control grown plants (Cen & Bornman, 1993). This increased effectiveness was due to an increase in the amount of UVB-screening pigments, increased reflectance and thicker leaves. An important factor in determining the distribution of light within the leaf palisade layer is the lens properties of the epidermis (Poulson & Vogelmann, 1990), and this could be altered after exposure to UVB radiation.

5.11 Conclusion

Anthocyanins do not seem to provide UVB protection for either of the cultivars. When grown without supplementary UVB, the tropical cultivar, Yecora Rojo has a greater concentration of UVB-absorbing compounds which will explain why it accumulates less CPDs and 6-4PPs. With UVB, the leaves of Yecora Rojo transmit much more 310 and 430nm radiation which explains why photosynthesis of this cultivar does not decrease when grown under supplementary UVB.

Chapter 6

Summary

Summary

The results from this study are summarised in Table 6.1

6.2 The Effects of UVB on the Primary Leaf of Maris Huntsman and Yecora Rojo

Maris Huntsman and Yecora Rojo were selectively bred for optimum growth in different climates (Chapter 1). Maris Huntsman was bred for optimum growth in the British climate whereas Yecora Rojo was bred for optimum growth in Saudi Arabia. The climate in Saudi Arabia is warmer and drier with more sunshine than Britain; therefore when the cultivars are grown in their respective environments, there is a difference in the amount of UVB they experience. The cultivars were not selected for their response to UVB radiation, but since the amount of UVB differs where they are normally grown, the plants may show different growth characteristics when grown under UVB.

The results from Chapter 3 showed that the Saudi Arabian cultivar Yecora Rojo, was less affected by UVB than the British cultivar, Maris Huntsman. The growth of the primary leaf of Yecora Rojo was not reduced by UVB to the same extent as the primary leaf of Maris Huntsman. The coleoptile height and fresh and dry weights of Maris Huntsman were reduced when grown with supplementary UVB. In contrast, only the coleoptile height of Yecora Rojo was reduced with UVB. UVB reduced the rate of photosynthesis in the primary leaf of Maris Huntsman but did not change the rate of photosynthesis in the primary leaf of Yecora Rojo.

The difference in reduction of growth and photosynthesis of the primary leaf of the two cultivars was, in part, due to the fact that Yecora Rojo accumulated less DNA damage (both CPDs and 6-4PPs) in the primary leaf than Maris Huntsman (Chapter 4). There was no difference between the cultivars in the amount of CPDs and 6-4PPs repaired, and so the difference in accumulation of CPDs and 6-4PPs was not due to one cultivar being able to repair the damage at a faster rate than the other. This suggested that perhaps the amount of UVB penetrating to the primary leaf of Maris Huntsman was greater than that of Yecora Rojo, and thus inducing more CPDs and 6-4PPs.

When grown without supplementary UVB, Yecora Rojo had a higher concentration of UVB-absorbing compounds in the primary leaf than that contained in the primary leaf of Maris Huntsman. Both cultivars accumulated more UVB-absorbing compounds when grown with supplementary UVB, but there was no difference in the amount of UVB-absorbing compounds between the cultivars. Three flavonols were detected in the leaves of Maris Huntsman

and Yecora Rojo: Quercetin, kaempferol and isorhamnetin. Under supplementary UVB, Maris Huntsman contained more quercetin and kaempferol than Yecora Rojo. When grown with supplementary UVB, the amount of UVB radiation (310nm radiation) penetrating the leaf of Yecora Rojo was selectively decreased, while allowing more white light (430nm) penetration (Chapter 5). In contrast, in Maris Huntsman, the amount of UVB penetrating the primary leaf increased but the amount of white light penetrating the primary leaf decreased under these conditions. This decrease in the amount of white light (430nm) penetrating the leaf of Maris Huntsman when grown with UVB, could be one reason for the observed decrease in rate of photosynthesis.

This study has shown that the cultivars do differ in their response to UVB, but since the cultivars have not evolved naturally it can not be concluded that it is an adaptive response. However, because the cultivars were selected for their performance (e.g. yield and quality) under field conditions that included high (in the case of Yecora Rojo) and lower (in the case of Maris Huntsman) ambient UVB levels, it is possible that the susceptibly of the primary leaf to UVB damage might have been an important component of this. This is consistent with my observation that young seedlings of Yecora Rojo at the primary leaf stage, are more tolerant of UVB than those of Maris Huntsman.

The aim of my work was to investigate whether two wheat cultivars, bred for growth in different climates, differed in their response to UVB. This study has proven that this is the case since Maris Huntsman was affected to a greater extent than Yecora Rojo. It would appear that the amount of DNA damage accumulated by each cultivar and how each cultivar has protected the primary leaf from the penetration of UVB radiation, both make an important contribution to this response.

6.1 Summary of Results

	Maris Huntsman	Yecora Rojo
Total Plant Height	-	-
Primary Leaf Length	-	-
Leaf Area	-	-
Fresh and Dry Weight	-	-
Coleoptile Height	-	-
Coleoptile Fresh and Dry Weight	-	**0**
SER	-	-
V_D	-	-
Cell Division	-	-
Cell Doubling Time	+	+
Photosynthesis	-	**0**
Chlorophyll Content	+	+
Accumulation of CPDs in Coleoptile	-	-
Accumulation of 6-4PPs in Coleoptile	**0**	-
Accumulation of CPDs	-	-
Accumulation of 6-4PPs	+	+
Dark Repair of CPDs	+	+
Dark Repair of 6-4PPs	+	+
Light Repair of CPDs	-	-
Light Repair of 6-4PPs	-	-
Anthocyanin Content	**0**	**0**
UVB-absorbing Compounds	+	+
Amount of Flavonols	+	**0**
Amount of 310nm Radiation Penetrating the Leaf	+	-
Amount of 430nm Radiation Penetrating the Leaf	-	+
Leaf Thickness	+	+

Table 6.1 Summary of results for the primary leaf of each cultivar when grown with supplementary UVB.
The symbols indicate an increase (+), decrease (-) or no effect (0) when the plants are grown with supplementary UVB.

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