RESPONSE OF PEA (*Pisum sativum* L. cv. Massey Gem) AND WHEAT (*Triticum aestivum* L. var.Machetti) SEEDLINGS TO SUPPLEMENTARY UV-B RADIATION

A thesis submitted by

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Summary

It is recognized that depletion of the atmospheric ozone layer or decline in the ozone column allows more UV-B radiation to reach the earth' s surface which will have detrimental effects on organisms. Terrestrial higher plants, in particular, would experience physical and physiological stresses if subjected to increased UV-B radiation. For example, they show significant decreases in their growth rate and development, as a result of changes in their morphological, physiological, and biochemical functions.

The aims of this study were to determine the effects of UV-B radiation on the morphological, anatomical and ultrastructural features of leaves as well as their photosynthetic and respiratory processes. In particular, the respiratory properties of leaf slices and the isolated leaf mitochondria were studied in detail.

Two species of terrestrial plants, *Pisum sativum* L. and *Triticum aestivum* L. were subjected to supplementary UV-B radiation. Pea and wheat seeds were planted in a mixture of potting soil and vermiculite in an unshaded glasshouse. On the 8th - 10th day after germination, UV-B radiation was applied. Four different treatments were used : Zero UV-B (in the glasshouse without UV-B, as a control), Natural UV-B (outside the glasshouse), Low UV-B (0.0896 Wm^{-2}), and High UV-B (0.164 Wm^{-2}). The 6 hour Low UV-B treatment was equivalent to the total UV-B sunlight radiation on a cloudless December day in 1993.

Pea leaves subjected to supplementary UV-B radiation showed waviness, bronzing, brown spots and chlorosis. Similar effects were not observed in wheat leaves. A reduction of the total above ground plant heights in both species was also evident. No significant decrease in plant fresh weight occurred either in pea or wheat species. Pea leaves experienced a reduction in both fresh and dry weights of their specific leaf pair. In peas, the reduction of plant height was due to a significant decrease in the length of the second internode.

In general, pea leaves exposed to UV-B responded by increasing their leaf thickness at the mid-rib area, but lowered thickness at non vascular bundle areas. This reduced leaf thickness was due to a reduction of thickness of the adaxial epidermis. On the other hand, significant increases in thickness of the epidermis were observed in wheat. Moreover, low and high UV-B appeared to damage the adaxial epidermal cells of pea leaves, as seen by a partial collapse of the epidermal cells. The damage was more pronounced under high UV-B

treatment. Wheat leaf epidermal and mesophyll exhibited no apparent damage in response to UV-B radiation.

Electron microscope studies showed that the general structure of cells from treated pea leaves was different from that of control leaves. The collapse of epidermal cells was extreme in some areas, leading to disappearance of the protoplasm with the cell walls becoming adpressed, leaving a small structure filled with granular material. The collapse of the epidermis was accompanied by some internal damage to palisade layers, where most of cells were disorganized, being empty and columnar. Damaged palisade sampled from low and high UV-B treated leaves contained altered chloroplasts, and cell contents which were disorganized in their orientation. The altered chloroplasts structure displayed a more open system of thylakoids and lamellar membranes. Their photosynthetic lamellae tended to be swollen, with a reduction in thylakoid number, smaller grana, an increase in intergrana spaces, more osmiophilic globuli, and poorly defined membranes. Mitochondria were sometimes smaller, with fewer cristae, and showed poor membrane structure. In contrast, the general ultrastructure of cells from treated wheat leaves was similar to that of the control leaves.

The respiration rates of leaf slices of pea and wheat seedlings decrease with age but those grown under supplementary UV-B radiation showed elevated levels of the uninhibited respiration. This decrease with age and increase with UV-B treatment was more pronounced in pea, than it was in wheat. Pea leaves, treated with UV-B showed a lower respiration sensitivity to KCN, indicating that the potential for CN-resistant oxidation was higher. In control wheat leaf slices, KCN had no effect on the O_2 uptake, but in UV-B treated leaf slices a stimulation by KCN was observed. No significant difference in the rate of residual respiration was found between treated and untreated leaves, in either species.

In pea leaf mitochondria, the uninhibited state 3 respiration was lower in UV-B treated pea leaves than in controls when glycine or glycine plus malate were used as substrates. However, the rate of uninhibited respiration in UV-B treated mitochondria was higher than in control, when NADH, succinate, succinate plus NADH, and malate plus glutamate were used as substrates. In wheat leaf mitochondria, no significant difference in the uninhibited respiration was observed between UV-B treated and untreated mitochondria with all substrates used.

In agreement with the KCN data, mitochondria isolated from UV-B treated pea leaves showed a higher resistance to myxothiazol inhibition than did control mitochondria. It is concluded that the lower inhibition by myxothiazol observed in mitochondria from UV-B treated plants was due to the higher presence of the alternative oxidase. The inhibition by myxothiazol in wheat mitochondria was the same for both UV-B and control.

The results of this study also showed that alternative oxidase respiration in leaf mitochondria from control and UV-B treated plants of both pea and wheat species was stimulated by pyruvate. In pea leaf mitochondria, the stimulation by pyruvate was more pronounced in UV-B treated leaves than in control. For wheat leaves, the greatest stimulation by pyruvate was found with NADH substrate.

SDS-PAGE gels showed that mitochondria of pea and wheat leaves contained many polypeptide bands. However, no differences were observed between mitochondria isolated from treated and untreated pea and wheat leaves. When immunoblots were used to identify the alternative oxidase proteins, it was found that this protein existed as an oxidized dimer (71-72 kDa) or a reduced monomer (28-29 kDa for peas, and 34-35 kDa for wheat) in all treatments. Although UV-B treatment induced greater activity of alternative pathway in peas, no significant increase in the amount of detectable alternative oxidase protein was observed in their mitochondria. In wheat leaf mitochondria, the bands were clearly evident. Again, treatment with UV-B radiation did not change the amount of the alternative oxidase protein either in low or high molecular weight range.

The rates of photosynthesis measured in leaf slices changed under UV-B treatment in both pea and wheat. When expressed on both leaf weight and chlorophyll basis, pea leaf slices from UV-B treated plants showed a significant decrease in the activity of CO_2-dependent O_2 evolution. Wheat leaves, also exhibited some reduction in photosynthesis rates, but it was not significant.

Rates of CO_2-dependent oxygen evolution in chloroplasts from UV-B treated pea leaves were lower than in controls. As UV-B has damaged the structure of chloroplasts, the reduction of photosynthetic rates could be related to the damage of thylakoid and envelope membrane. It was found that UV-B treatment also inhibited photosynthetic electron transport rates when artificial acceptors were used with broken chloroplasts.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan if it is accepted for award of the degree.

Maryani

January 1996

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Abbreviations

ADP	adenosine -5'-diphosphate
ATP	adenosine-5'-triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
CEMMSA	centre for Electron Microscopy & Microstructure Analysis
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethyl-ether)-N,N,N',N'-tetraacetic acid
FCCP	carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
g	gravity
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid
kDa	k-Dalton
MES	4-Morpholineethanesulfonic acid
Mn2+	manganase ions
MV	methylviologen
NADH	nicotinamide adenine dinucleotide, reduced
NADP	nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NAD(P)H	either NADH or NADPH
nPG	n-propylgallate
PAGE	polyacrilamide gel electrophoresis
PAL	phenylalaine ammonia lyase
PAR	photosyntetically active radiation
PS I	photosystem I
PS II	photosystem II
PVDF	polyvinylidene difluoride
PVP-40	polyvinyl-polypyrrolidone (average molecular weight= 40,000)
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SHAM	salicylhydroxamic acid

TES	2-(2'-Hydroxy-1,1-bis(hydroxymethyl)-ethylamino)ethanesulfonic acid
TRIS	Tris(hydroxymethyl)aminoethane
UQ	ubiquinone
UV	Ultraviolet

Chapter 1. Introduction and literature review

1.1. General Introduction

The initial discovery of a decline in ozone levels occurred in the mid 1970s, when an ozone hole was detected over Antarctica (Stolarski, 1988; Lothian, 1990). Through the 1980s, the decrease in ozone became progressively greater, and it was realised in 1985 that the ozone hole had opened (Farman, *et al.*, 1985; Wayne, 1991). An ozone hole has also been developing over Northern Europe and America (Brune *et al.*, 1991; Hofmann and Deshler, 1991). Satellite measurements carried out by NASA show that the ozone concentration over the North Pole decreased by ca 10% , compared with a 60% reduction over Antarctica (Levall and Bornman, 1993).

About 90 % of the ozone occurs in the stratosphere, an atmospheric layer between 20 and 50 km above the earth's surface, which although a very small proportion of the total stratosphere, it plays a major role in the chemistry of the earth's atmosphere. The primary function of the ozone layer is to act as a giant sun screen or filter in absorbing the sun's ultraviolet radiation which is potentially harmful to living things on earth, including plants (Hofmann & Salomon 1989). The ozone layer absorbs the shorter wavelengths of UV light, but not the longer wavelengths which are only weakly absorbed or unaffected. For example, the wavelengths 320 nm-380 nm (UV-A radiation) are not absorbed by ozone and pass unaffected through the stratospheric ozone layer, wavelengths 280-320 nm (UV-B radiation) are strongly absorbed, and wavelengths below 280 nm (UV-C radiation) are completely excluded.

The production of ozone occurs when radiation from the sun breaks down oxygen into oxygen atoms. By combining with oxygen molecules, these atoms form ozone. The greatest production of ozone occurs over the tropics and is redistributed towards the poles, around the globe and in the upper atmosphere (Lothian, 1990).

Since ozone acts as a filter by absorbing ultraviolet radiation, depletion of the ozone layer or a decline in the ozone column allows more UV-B to reach the earth's surface (Caldwell *et al.*, 1989; Frederick, 1993, NASA, 1988). For every one percent loss of ozone, there is a 1.5 to 2-fold increase in UV-B reaching the earth. Moreover, this dramatic ozone alteration in the upper atmosphere not only influences the energy balance on the earth, but also the nature of sunlight penetrating to the earth's surface.

Ultraviolet radiation is known to be quite important in biological photochemical reactions, and UV-B, particularly, is usually defined as the electromagnetic radiation between the wavelengths of 280 nm and 320 nm (Lothian, 1990). The level of UV-B reaching the earth's surface is influenced by many factors, such as geographical latitude, variations in stratospheric ozone, altitude, surface reflection, season, air pollution and clouds (WHO, 1992). The levels of UV-B are highest around noon when the angle of the sun's rays is smallest and lower in the morning and afternoon (Lothian, 1990). Energy spectra of UV-B irradiance during the night are two orders of magnitude lower than in the day and on clear days are about 5 times higher than during completely cloudy days (Henriksen *et al.*, 1991). Frederick and Snell (1990) also showed that scattering by clouds reduced the annually integrated surface UV-B irradiance on the ground to levels between 62 and 78% of the values prevailing under perpetually clear skies. The variability of cloudiness from year to year is the major factor accounting for interannual differences in the levels of UV irradiance (Frederick and Weatherhead, 1992).

The amount of UV-B radiation reaching the earth has not been stable through the years, making the detection of a long term change in radiation levels difficult (Kerr and McElroy, 1993). Canada's atmospheric Environment Service found that between 1988 and 1993 at a carefully monitored site in Toronto, wintertime levels of UV-B increased more than 5% every year (Appenzeller, 1993). Moreover, the UV-B levels in the mid-latitudes of the Northern and Southern Hemispheres have also been monitored lately (Roy *et al.*, 1990; Seckmeyer and McKenzie, 1992). The latter authors compared the UV-B levels at latitudes 45°S in New Zealand and 48°N in Germany during summer. They found that the level of UV-B radiation in New Zealand was about double that in Germany. Based on this information, it can be predicted that UV-B radiation reaching the earth's surface will increase in the future, but the extent of that increase cannot be determined. This is particularly so in the Southern Hemisphere, where the depletion of ozone is most severe and the atmosphere is relatively unpolluted.

The increase in UV-B reaching the earth's surface has generated interest in the effect that this will have on organisms, and particularly on plants. Solar radiation is essential for all plant life, with light not only the driving force for photosynthesis, but also triggering and regulating many morphogenic responses. Excessive photon flux density from UV-B radiation, however, is potentially harmful, if not lethal, for plants (Lothian, 1990). Tevini and Teramura (1989) have shown that enhanced UV-B caused significant stress in terrestrial plants, because it has

detrimental effects on plant morphology, physiology, and biochemical function. Some of the potential consequences of ozone decrease and corresponding increase in solar UV-B for higher plants are summarised in Fig.1.1.

Furthermore, as mentioned by Wayne (1991), if the ozone shield continues to be depleted and UV-B radiation increases, the most important biological processes, photosynthesis and respiration, may ultimately prove to be the most sensitive ones.

1.2. Literature review

1.2.1. UV-B absorbing compounds

It has been previously explained that UV-B exposure could enhance the synthesis of flavonoids and related phenolic compounds to protect plants from UV-B stress. These compounds which absorb strongly in the UV-B region, but transmit visible or photosyntheticaly active radiation (PAR) and appear responsible for epidermal attenuation (Day *et al.*, 1992), mostly accumulate in the vacuoles of epidermal cells (Clark and Lister, 1975), and, to a lesser extent, in the vacuoles of underlying mesophyll tissues (Cen and Bornman, 1993) or in chloroplasts (McClure, 1976). These pigments are water soluble, colourless flavonoids and include flavones, flavonols and isoflavonoids. Much of the UV-B attenuation can be removed by methanolic extraction of the epidermis, suggesting that such compounds as flavonoids, are important in the absorption of UV-B radiation (Teramura, 1983). In a study of the absorbance patterns of the flavonoid pigments in methanolic leaf extracts , an increase in UV-B absorbing compounds was found in most of the UV-B treated plants, but no changes were found in control plants (Murali and Teramura, 1986; Cen and Bornman, 1990, 1993; Teramura *et al* ., 1991; Adamse and Britz, 1992; He *et al.*, 1993, 1994). Schulze-Lefert *et al.* (1989) suggested a relationship between this increase and the stimulation of the synthesis of enzymes linked to the flavonoid pathway. Similarly, an increase in UV-B absorbing compounds was also found in soybean during both vegetative (Murali and Teramura, 1986; Reed *et al.*, 1992; Murali and Teramura,1984) and reproductive stages of development (Murali and Teramura, 1986), as well as in rice (Teramura *et al.*,1991, He *et al.*, 1993), *Brassica napus* L. (Cen and Bornman, 1993), peas (He *et al.*, 1993), bean (Cen and Bornman, 1990), and cucumber (Adamse and Britz, 1992).

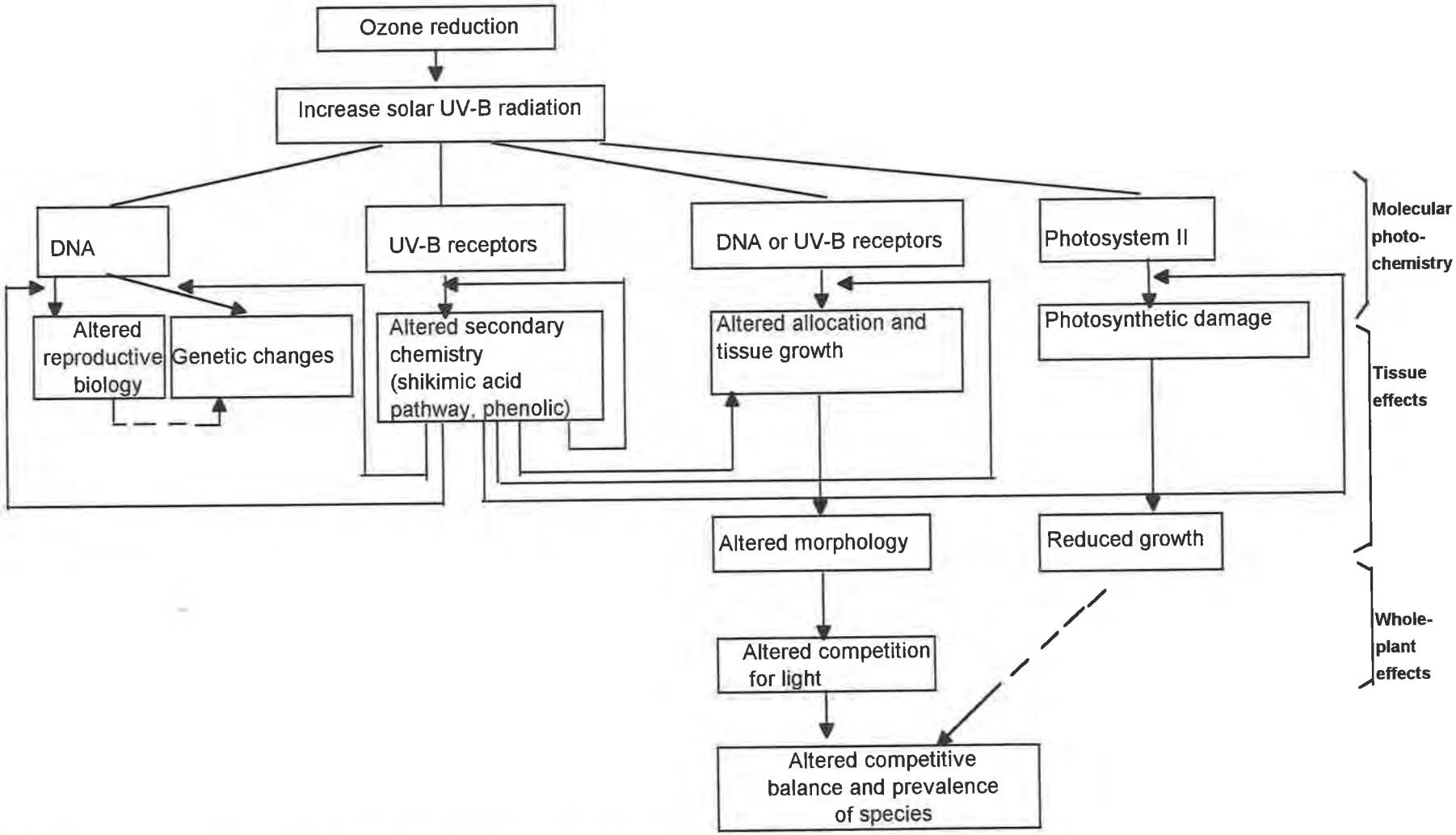

Fig.1.1. Potential consequences of ozone reduction and corresponding increase in solar UV-B radiation for higher plants at different levels of integration from molecular photochemistry to ecosystems. Solid lines indicate interactions for which there is experimental evidence. The dashed lines indicate interactions for which there is as yet no direct experimental evidence. Feedback loops indicate that the flavonoids and phenolics that may be induced by increased UV-B can serve as filtering agents, reducing the flux received by the photoreceptors and, thus, sometimes reducing the response to increased UV-B. (Caldwell *et al.*, 1989).

Different cultivars and species have different sensitivities to UV-B radiation which may be due to different levels of UV-B absorbing compounds in leaves in response to supplementary UV-B (Tevini *et al.*, 1991), and also to differences in other protective strategies (He *et al.*,1993). For instance, Murali *et al.* (1988) and Reed *et al.* (1992) reported that an increase in UV-B absorbing compounds in Williams soybean cultivar after exposure to UV-B radiation was greater than that in the Essex cultivar. This suggests that a relatively greater concentration of UV-B absorbing compounds was present in the epidermis of Williams after UV-B irradiation. Compared to these two soybean cultivars, pea plants have lower, while rice has much greater levels of UV-B absorbing compounds (He *et al.*, 1993)

Day *et al.* (1992) found differences in the depth of penetration and epidermal transmittance of UV-B in the foliage of different species. They determined that the leaf epidermis of herbaceous dicots was particularly ineffective at attenuating UV-B, having transmittance ranging from 18-41% and UV-B levels reaching 40-145 μm into the mesophyll tissue. However, all incident UV-B was attenuated by the epidermis of 1-year old conifer needles, with no radiation reaching the mesophyll. Between the leaves of herbaceous dicots and conifer needles, the leaves of woody dicots and grasses appeared to be intermediate with only 3 - 12% of the incident UV-B reaching the mesophyll. Recently, Cen and Bornman (1993) found that more of the UV-B screening pigments are present in the adaxial than in the abaxial epidermal layer of *Brassica napus* leaves. In their studies, UV-B screening pigments increased by 20% in UV-treated leaves relative to control leaves (Cen and Bornman, 1993) The accumulation of flavonoids in the epidermal layer (Vierstra *et al.*, 1982) may provide the major source of attenuation of UV-B radiation.

1.2.2. Growth and Morphological characteristics

The responses resulting from an increase in UV-B radiation clearly lead to a loss of crop productivity (Barnes *et al.*, 1988) through significant reduction in growth and development. It has been demonstrated that UV-B radiation can reduce growth characteristics such as plant height, dry matter production, and leaf area to various extents in sensitive plants (Teramura, 1983, Sulivan and Teramura, 1990; Reed *et al.*, 1992). However, alterations in morphology under UV-B irradiance were not always associated with these characters (Barnes et al., 1990).

A summary of changes in morphological characteristics induced by UV-B radiation is presented in Table 1.1.

1.2.2.1. Leaf features

Plant leaves are very sensitive to environmental stress because of their role in photosynthesis. Therefore, it is not surprising that leaf morphology is affected by UV-B radiation. In extensive growth chamber and glass house studies, it has been found that UV-B causes bronzing, stunting, scorching, glazing or chlorosis in many plants (Biggs *et al.*, 1981; Basiouny *et al.*, 1978; Strid *et al.*, 1990). There have been suggestions that these symptoms are often associated with the effectiveness and presence of UV-B absorbing pigments in the leaf epidermis. Moreover, sensitivity to UV-B depends on the level of photosynthetically active radiation (PAR) and varies from species to species (Teramura, 1980b). Robberecht and Caldwell (1978) reported that leaf bronzing did not appear in corn, but was apparent in tomato irradiated by UV-B in the greenhouse, and suggest this was partially due to differences in UV-B epidermal transmittance, because higher amounts of flavonoid compounds were found in corn than in tomato. Recently, Santos *et al.* (1993) found that under UV-B exposure, corn leaves although not bronzed could display dryness, with the margins of their distal fourth leaf somewhat involuted.

1.2.2.2. Dry weight and biomass allocation

Dry weight is a good parameter for determining total biomass accumulation, since it is an integration of all biochemical, physiological, morphological and growth parameters (Teramura, 1983). Even small supplementary UV-B radiation effects on physiological and biochemical processes will produce a noticeable result on accumulation of biomass. Most studies show that a number of species decrease their total dry biomass under increased UV-B (see in Table 1.1). In pea plants, Brandle *et al.* (1977) reported that dry weight per plant and biomass allocation decreased by 44% after 2 weeks exposure to UV-B. In addition, Murali *et al.* (1988) showed that soybean Essex cultivar produced less dry matter than its control, while a significant change was not found in Williams cultivar. A differential sensitivity to UV-B exposure has been found in rice cultivars (Ziska and Teramura, 1992; Day *et al.*, 1994). They reported that not all cultivars showed a significant decrease in leaf weight, but in three particularly sensitive

Table 1.1. Summary of some recently reported direct effects of UV-B radiation on growth of crops

Species	UV-B doses	Length of exposure	Effects locations	Experiments	Distance above canopy	References
A. Plant height						
Collards		2 weeks (continously)	- 25.7%	growth chamber		Basiouny *et al*, 1978
Oats		2 weeks (continously)	- 34.9%	growth chamber		Basiouny *et al*, 1978
Peanuts		2 weeks (continously)	- 8.2%	growth chamber		Basiouny *et al*, 1978
Soybean (Bragg cultivar)		2 weeks (continously)	- 7.7%	growth chamber		Basiouny *et al*, 1978
Sorghum		2 weeks (continously)	- 25.3%	growth chamber		Basiouny *et al*, 1978
Corn		2 weeks (continously)	- 2.4%	growth chamber		Basiouny *et al*, 1978
Wheat	17.5 mWm^{-2}	6 weeks (55% shade)	- 11.3%	glasshouse		Teramura, 1980
		6 weeks (88% shade)	+ 42.9%	glasshouse		Teramura, 1980
Soybean						
(Essex cultivar)	5.1 kJm^{-2}					
	(well watered)	8 weeks (6 hours day^{-1})	- 4.42%	field	75 cm	Sullivan & Teramura, 1990
	(drought-stressed)	8 weeks (6 hours day^{-1})	- 14.4%	field	75 cm	Sullivan & Teramura, 1990
(Glycine max)	13.6 kJm^{-2}	10 weeks (8 hours day^{-1})	- 18.45%	unshaded glasshouse		Reed *et al*, 1992
(Glycine soja)	13.6 kJm^{-2}	10 weeks (8 hours day^{-1})	- 17.22 %	unshaded glasshouse		Reed *et al*, 1992
Rice						
(IR-36)	15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 4.8%	unshaded glasshouse	40 cm	Teramura *et al*., 1991
(Carreon)	15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 9.3%	unshaded glasshouse		Teramura *et al*., 1991
(N22)	15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 14.8%	unshaded glasshouse		Teramura *et al*., 1991
Cassava	5.5 kJm^{-2}	13 weeks (8 hours day^{-1})	- 15.0%	field	70 cm	Ziska *et al*., 1993

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Soybean (Bragg cultivar)		2 weeks (continously)	- 7.7%	growth chamber		Basiouny *et al*, 1978
Sorghum		2 weeks (continously)	- 25.3%	growth chamber		Basiouny *et al*, 1978
Corn		2 weeks (continously)	- 2.4%	growth chamber		Basiouny *et al*, 1978
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(N22)	15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 14.8%	unshaded glasshouse		Teramura *et al.*, 1991
Cassava	5.5 kJm^{-2}	13 weeks (8 hours day^{-1})	- 15.0%	field	70 cm	Ziska *et al.*, 1993

B. Total dry weight

Peas	2 weeks	- 44.0%	glasshouse		Brandle *et al.*, 1977
Collards	2 weeks (continously)	- 31.7%	growth chamber		Basiouny *et al,* 1978
Oats	2 weeks (continously)	- 17.0%	growth chamber		Basiouny *et al*, 1978
Peanuts	2 weeks (continously)	- 0.4%	growth chamber		Basiouny *et al*, 1978
Soybean (Bragg cultivar)	2 weeks (continously)	- 17.9%	growth chamber		Basiouny *et al*, 1978
Sorghum	2 weeks (continously)	- 6.5%	growth chamber		Basiouny *et al*, 1978
Corn	2 weeks (continously)	- 4.8%	growth chamber		Basiouny *et al*, 1978
Soybean					
(Essex cultivar)					
5.1 kJm^{-2} (well watered)	8 weeks (6 hours day^{-1})	- 34.5%	field	75 cm	Sullivan & Teramura, 1990
5.1 kJm^{-2} (drought-stressed)	8 weeks (6 hours day^{-1})	- 55.5%	field	75 cm	Sullivan & Teramura, 1990
11.5 kJm^{-2}	5 weeks (6 hours day^{-1})	- 16.8%	unshaded glasshouse		Murali *et al.*, 1988
(Williams) 11.5 kJm^{-2}	5 weeks (6 hours day^{-1})	- 5.7%	unshaded glasshouse		Murali *et al.*, 1988
(Glycine max) 13.6 kJm^{-2}	10 weeks (8 hours day^{-1})	- 16.7%	unshaded glasshouse		Reed *et al*, 1992
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15.7 kJm^{-2}	5 weeks (6 hours day^{-1})	- 5.0%	unshaded glasshouse	35 cm	Teramura *et al.*, 1990
Rice					
(IR-36) 15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 41.1%	unshaded glasshouse	40 cm	Teramura *et al.*, 1991
(IR-36) 13.8 kJm^{-2}					
(+ 360 µbars CO_2)	16 weeks (8 hours day^{-1})	- 13.0%	unshaded glasshouse	50 cm	Ziska & Teramura, 1992
(+ 660 µbars CO_2)	16 weeks (8 hours day^{-1})	+ 4.3%	unshaded glasshouse	50 cm	Ziska & Teramura, 1992
(Carreon) 15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 37.9%	unshaded glasshouse	40 cm	Teramura *et al.*, 1991
(N22) 15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 39.3%	unshaded glasshous	40 cm	Teramura *et al.*, 1991
(Fujiyama-5) 13.8 kJm^{-2}					
(+ 360 µbars CO_2)	16 weeks (8 hours day^{-1})	- 11.0%	unshaded glasshouse	50 cm	Ziska & Teramura, 1992
(+ 660 µbars CO_2)	16 weeks (8 hours day^{-1})	+ 27.8%	unshaded glasshouse	50 cm	Ziska & Teramura, 1992
15.7 kJm^{-2}	5 weeks (6 hours day^{-1})	+ 1.0%%	unshaded glasshouse	35 cm	Teramura *et al.*, 1990

Rice cultivars	13.0 $kJm^{-2}day^{-2}$	3 weeks (6 hours day^{-1})	(-) 30 - (-) 24%	glasshouse	40 cm	Dai *et al.*, 1994
			(+) 8 - (+) 32%	glasshouse	40 cm	Dai *et al.*, 1994
Cassava	5.5 kJm^{-2}	13 weeks 6 hours day^{-1})	- 8.0%	field	70 cm	Ziska *et al.*, 1993
Bean	57.6 $mMm^{-2}s^{-2}$ (High PAR)	4 weeks 8 hours day^{-1})	32.0%	growth chamber		Deckmyen *et al.*, 1994
	21.2 $mMm^{-2}s^{-2}$ (Int. PAR)	4 weeks (8 hours day^{-1})	11.3%	growth chamber		Deckmyen *et al.*, 1994
	8.6 $mMm^{-2}s^{-2}$ (Low PAR)	4 weeks (8 hours day^{-1})	13.9%	growth chamber		Deckmyen *et al.*, 1994
Cucumber	18.2 $kJm^{-2}day^{-1}$	2 weeks (6 hours day^{-1})	decrease	growth chamber	55 cm	Krizek *et al.*, 1994

C. Total fresh weight

Peas		2 weeks	- 35.0%	glasshouse	Brandle *et al.*, 1977
Collards		2 weeks (continously)	- 5.3%	growth chamber	Basiouny *et al,* 1978
Oats		2 weeks (continously)	- 20.2%	growth chamber	Basiouny *et al*, 1978
Peanuts		2 weeks (continously)	- 22.5%	growth chamber	Basiouny *et al*, 1978
Soybean (Bragg cultivar)		2 weeks (continously)	- 26.6%	growth chamber	Basiouny *et al*, 1978
Sorghum		2 weeks 9continously)	- 51.8%	growth chamber	Basiouny *et al*, 1978
Corn		2 weeks (continously)	- 2.9 %	growth chamber	Basiouny *et al*, 1978
Bean	57.6 $mMm^{-2}s^{-2}$ (High PAR)	4 weeks (8 hours day^{-1})	25.2%	growth chamber	Deckmyen *et al.*, 1994
	21.2 $mMm^{-2}s^{-2}$ (Int. PAR)	4 weeks (8 hours day^{-1})	12.3%	growth chamber	Deckmyen *et al.*, 1994
	8.6 $mMm^{-2}s^{-2}$ (Low PAR)	4 weeks (8 hours day^{-1})	22.4%	growth chamber	Deckmyen *et al.*, 1994

D. Leaf fresh weight

Bean	57.6 $mMm^{-2}s^{-2}$ (High PAR)	4 weeks (8 hours day^{-1})	33.7%	growth chamber	Deckmyen *et al.*, 1994
	21.2 $mMm^{-2}s^{-2}$ (Int. PAR)	4 weeks (8 hours day^{-1})	14.1%	growth chamber	Deckmyen *et al.*, 1994
	8.6 $mMm^{-2}s^{-2}$ (Low PAR)	4 weeks (8 hours day^{-1})	4.2%	growth chamber	Deckmyen *et al.*, 1994

E. Length of petiole

Soybean (the third trifoliate) 9.5 $kJm^{-2}day^{-1}$ (with different levels and ratios of UV-A & PFD)					
	4 weeks	+ 16.8%	field		Caldwell *et al.*, 1994
	4 weeks	- 23.0%	field		Caldwell *et al.*, 1994
	4 weeks	- 7.0%	field		Caldwell *et al.*, 1994
	4 weeks	- 4.2%	field		Caldwell *et al.*, 1994
Soybean (the first trifoliate) 9.5 $kJm^{-2}day^{-1}$ (with different levels and ratios of UV-A & PFD)					
9.5 $kJm^{-2}day^{-1}$	4 weeks	+ 6.4%	field		Caldwell *et al.*, 1994
	4 weeks	- 9.6%	field		Caldwell *et al.*, 1994
	4 weeks	- 0.3%	field		Caldwell *et al.*, 1994
	4 weeks	- 2.3%	field		Caldwell *et al.*, 1994

F. Leaf area

Soybean						
(Essex cultivar)	5.1 kJm^{-2}					
	(well watered)	8 weeks (6 hours day^{-1})	- 34.7%	field	75 cm	Sullivan & Teramura, 1990
	(drought-stressed)	8 weeks (6 hours day^{-1})	- 57.1%	field	75 cm	Sullivan & Teramura, 1990
	11.5 kJm^{-2}	5 weeks (6 hours day^{-1})	- 19.4%	unshaded glasshouse		Murali *et al.*, 1988
(Williams)	11.5 kJm^{-2}	5 weeks (6 hours day^{-1})	- 11.4%	unshaded glasshouse		Murali *et al.*, 1988
(Glycine max)	13.6 kJm^{-2}	10 weeks (8 hours day^{-1})	- 19.4%	unshaded glasshouse		Reed *et al*, 1992
(Glycine soja)	13.6 kJm^{-2}	10 weeks (8 hours day^{-1})	- 20.8%	unshaded glasshouse		Reed *et al*, 1992
	15.7 kJm^{-2}	5 weeks (6 hours day^{-1})	- 5.0%	unshaded glasshouse	35 cm	Teramura *et al.*, 1990
Rice						
(IR-36)	15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 32.9	unshaded glasshouse	40 cm	Teramura *et al.*, 1991
(IR-36)	13.8 kJm^{-2}					
	(+ 360 μbars CO_2)	16 weeks (8 hours day^{-1})	- 20.8%	unshaded glasshouse	50 cm	Ziska & Teramura, 1992
	(+ 660 μbars CO_2)	16 weeks (8 hours day^{-1})	- 49.5%	unshaded glasshouse	50 cm	Ziska & Teramura, 1992
(Carreon)	15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 44.7%	unshaded glasshouse	40 cm	Teramura *et al.*, 1991
(N22)	15.7 kJm^{-2}	12 weeks (8 hours day^{-1})	- 48.7%	unshaded glasshouse	40 cm	Teramura *et al.*, 1991

(Fujiyama-5)	13.8 kJm^{-2}					
	(+ 360 μbars CO_2)	16 weeks (8 hours day^{-1})	+ 14.4%	unshaded glasshouse	50 cm	Ziska & Teramura, 1992
	(+ 660 μbars CO_2)	16 weeks (8 hours day^{-1})	+ 5.4%	unshaded glasshouse	50 cm	Ziska & Teramura, 1992
Rice cultivars	13.0 $kJm^{-2}day^{-2}$	3 weeks (6 hours day^{-1})	(-) 34 - (-) 23%	glasshouse	40 cm	Dai *et al.*, 1994
			(+) 9 - (+) 30%	glasshouse	40 cm	Dai *et al.*, 1994
Cassava	5.5 kJm^{-2}	13 weeks (6 hours day^{-1})	increase	field	70 cm	Ziska *et al.*, 1993
Bean	57.6 $mMm^{-2}s^{-2}$ (High PAR)	4 weeks (8 hours day^{-1})	34.0%	growth chamber		Deckmyen *et al.*, 1994
	21.2 $mMm^{-2}s^{-2}$ (Int. PAR)	4 weeks (8 hours day^{-1})	11.1%	growth chamber		Deckmyen *et al.*, 1994
	8.6 $mMm^{-2}s^{-2}$ (Low PAR)	4 weeks (8 hours day^{-1})	12.8%	growth chamber		Deckmyen *et al.*, 1994
Cucumber	18. kJm^{-2} (450 μmol mol^{-1})	4 days (10 hours day^{-1}	- 27%	growth chamber		Adamse & Britz, 1992
	18.2 kJm^{-2}	2 weeks (6 hours day^{-1})	increase	growth chamber	55 cm	Krizek *et al.*, 1994
Wheat	**17.5 mMm^{-2}**					
	0% shade (PAR)	6 weeks	- 11.6%	glasshouse		Teramura, 1990
	33% shade (PAR)	6 weeks	+ 52.3%	glasshouse		Teramura, 1990
	55% shade (PAR)	6 weeks	- 18.9%	glasshouse		Teramura, 1990
	88% shade (PAR)	6 weeks	+90.9%	glasshouse		Teramura, 1990
Wheat	**35.0 mMm^{-2}**					
	0% shade (PAR)	6 weeks	- 21.9%	glasshouse		Teramura, 1990
	33% shade (PAR)	6 weeks	- 50.3%	glasshouse		Teramura, 1990
	55% shade (PAR)	6 weeks	- 10.7%	glasshouse		Teramura, 1990
	88% shade (PAR)	6 weeks	- 109.1%	glasshouse		Teramura, 1990

G. Leaf dry weight

Soybean						
(Essex cultivar)	5.1 kJm^{-2}					
	(well watered)	8 weeks (6 hours day^{-1})	+ 2.0%	field	75 cm	Sullivan & Teramura, 1990
	(drought-stressed)	8 weeks (6 hours day^{-1})	- 1.2%	field	75 cm	Sullivan & Teramura, 1990
	11.5 kJm^{-2}	5 weeks (6 hours day^{-1})	+ 1.3%	unshaded glasshouse		Murali *et al.*, 1988

cultivars (Careon, IR36 and PTB13) there was a significant decline in stem weight. Although species or cultivar differences may determine the response to UV-B, decreases in total dry weight are often accompanied by changes in the partitioning of biomass between organs (Ziska and Teramura, 1992). In species such as soybean and cucumber, a greater proportion of biomass was allocated to leaves than to other parts, such as roots, sheaths or stems (Sulivan and Teramura, 1990; Krizek *et al.*, 1994). In contrast to the reduction of dry biomass weight, it has also been observed for *Phaseolus vulgaris* L., enhanced UV-B radiation affected a stimulation of dry matter accumulation (Deckmyen *et al.*, 1994).

The effect of UV-B radiation on plant growth depends on the availability of carbon dioxide. By combining levels of UV-B radiation and carbon dioxide, Ziska and Teramura (1992) showed that increased UV-B at ambient CO_2 reduced total plant biomass in rice, but increased total biomass significantly when combined with increasing CO_2. In addition, in cucumber cultivars, Adamze and Britz (1992) found a decrease in leaf dry weight after 4 days exposure to UV-B with low concentrations of CO_2. Instead of depending only on CO_2, the effect of UV-B radiation is also dependent upon the level of PAR incident during growth. As the incident levels of PAR decreased, UV-B irradiances were more effective in reducing soybean biomass (Teramura, 1980a). This indicates that UV-B affected reductions in total biomass are magnified under low PAR regimes (Teramura, 1983). This result is in contrast to a finding by Krizek *et al.* (1994) who determined an increase in dry matter production under UV-B with either low or high PAR.

Apparently, not all UV-B effects expressed under controlled greenhouse and growth chambers conditions occur in field studies. Hart *et al.* (1975) found no effect on dry biomass in corn, sorghum, soybean or peanut due to UV-B exposure in the field. In contrast, Biggs and Kossuth (1978) reported total dry weight reductions in field grown corn, pea, tomato and mustard (*Brassica juncea* cv. cripifolia) after irradiation with UV-B. In another field experiment, Dumpert and Knacker (1985) observed that with relatively large UV-B irradiances (corresponding to a 25% ozone depletion), dry weight was increased in kohlrabi, was unchanged in lettuce and savoy.

1.2.2.3. Leaf area and specific leaf weight

Plant leaf area is usually reduced by temperature, water, mineral, salt stress and other environmental stresses. It is reasonable to expect therefore, that leaf area would also be reduced by UV-B radiation. For example, under controlled

greenhouse conditions, screening over 21 economically important cultivars of 7 species, Dumpert and Knacker (1985) reported that 5 cultivars of 3 species produced less leaf area during exposure to UV-B radiation. Previously, in an extensive study, Biggs and Kossuth (1978) found that leaf area was significantly decreased in over 60 % of cases. In contrast to Biggs and Kossuth (1978), Murali *et al.* (1988) reported no marked change in leaf area in either Essex or Williams soybean cultivars exposed to enhanced UV-B radiation, though they did find an increase in specific leaf weight (SLW) in the Williams cultivar. Some recently reported direct effects of UV-B radiation on leaf area and specific leaf weight are presented in Table 1.1.

1.2.3. Anatomical changes

The penetration and distribution of UV-B within leaves varies among species and is strongly affected by leaf anatomy. Leaves are composed of several layers of tissues, with a few layers of mesophyll between the upper epidermis towards the adaxial side and the lower epidermis towards the abaxial side. The epidermal layers are responsible for making a protective barrier to prevent light entering and causing stress. The epidermis, which forms the first screen against radiation, is particularly effective in reducing the penetration of UV-B, while transmitting a large proportion of the photosynthetically active radiation (Day *et al.*, 1992). Epidermal transmittance at 300 nm is much less in most evergreen species than in most deciduous species. It has been well documented that UV-B penetrates deepest into leaves of herbaceous dicotyledons and least into conifer needles, with penetration into leaves of woody dicotyledons and grasses being intermediate (Day *et al.*, 1992). This difference results in different sensitivities in each of these groups when exposed to enhanced UV-B radiation.

Furthermore, plants exposed to supplementary UV-B in the greenhouse, growth chamber, or in the field, develop degenerative changes in their cell structure (Cen and Bornman, 1993; Santos *et al.*, 1993; He *et al.*, 1994). The effects of UV-B on anatomical and ultrastructural events occurring in leaves are summarized in Table 1.2. Anatomical changes caused by UV-B treatment include an increase of leaf thickness (Bornman and Vogelmann, 1991) due to thickening of the palisade and spongy layers of mesophyll tissues (Cen and Bornman, 1993). The total leaf thickness (those of adaxial and abaxial epidermis, palisade, and spongy layers) *of Brassica napus* increased after exposure to biologically effective UV-B at 8.9 $KJm^{-2}day^{-1}$ (Cen and Bornman, 1993).

Table 1 2. Summary of some recently reported effects of UV-B radiation on ultrastructural events occurring in leaves

Component affected	Damage/Symptom	References
Leaf	total leaf thickness	Murali *et al.* (1988) Cen & Bornman (1993)
	increased in epidermis adaxial thickness	Cen & Bornman (1993)
	increased in palisade thickness	Cen & Bornman (1993)
	increased in spongy thickness	Cen & Bornman (1993)
	increased in epidermis abaxial thickness	Cen & Bornman (1993)
	collapse of the adaxial epidermis	Cen & Bornman (1993) Santos *et al.* (1993)
Nucleus	slight dilation of nuclear membrane	Brandle *et al.* (1977)
Chloroplats	Thylakoid dilation	Brandle *et al.* (1977) He *et al.* (1994) Santos *et al.* (1993)
	outer membrane broken	Brandle *et al.* (1977) He *et al.* (1994)
	lost the pattern of grana and stroma thylakoid membrane	He *et al.* (1994)
	vesiculation in stroma	Brandle *et al.* (1977)
	swollen grana and intergrana lamellae	He *et al.* (1994)
	lost its structural integrity	He *et al.* (1994)
	large starch grains	He *et al.* (1994)
Mitochondria	fewer cristae	Brandle *et al.* (1977) Santos *et al.* (1993)
Endoplasmic reticulum	swollen citernae	Brandle *et al.* (1977)
	vesiculation	Brandle *et al.* (1977) Santos *et al.* (1993)
Plasmalemma	vesiculation	Brandle *et al.* (1977)
	disrupted	Brandle *et al.* (1977)

Looking at the upper layer of leaves, UV-B combined with low visible light apparently caused a partial collapse of the adaxial epidermis of soybean (Cen and Bornman, 1993) and corn leaf (Santos *et al.*, 1993). The degree of epidermal collapse was extreme in some cases and included the disappearance of protoplasm and the collapse of cell walls (Santos *et al.*, 1993). The epidermis can be an effective barrier to UV-B, not only by increasing its concentration of the UV-B screening pigments, but by the reflectance afforded by the increased amount of epicuticular wax. The existence of leaf hairs on the adaxial side has also been reported to provide a shield against UV-B radiation (Karabourniotis *et al.*,1993)

After exposure to UV-B irradiation, mesophyll chloroplasts in pea and red beet showed a conspicuous alteration (Brandle *et al.*, 1977). The effects of UV-B on chloroplasts structure included the reduction of their fractional volume observed by morphometric analysis (chloroplasts/grid) (Santos *et al.*, 1993). After 16 days of treatment under UV-B irradiation, this organelle appeared to be damaged to a great extent. The damage to chloroplasts could be seen as dilation or alteration of the thylakoid membrane, and the disruption of chloroplast envelope membranes which was related to destruction of membrane proteins and lipids (Brandle *et al.*, 1977; Bornman *et al.* in Worrest and Caldwell, 1986). Crystalline structures and vesicles also appeared in the stroma under UV-B treatment. A slight disorientation of grana, aggregation of plastoglobuli and misshaped chloroplasts (Anonym, 1975), and a higher content of starch in the chloroplasts (Santos *et al.*, 1993) have been reported in many species. The structural damage to the chloroplast membranes and thylakoids would probably tend to disrupt the electron transport system, thus significantly depressing photosynthetic rates (Brandle *et al.*, 1977; Cen and Bornman, 1990). Prolonged exposure to UV-B may cause detrimental effects to the cell surfaces (Wayne, 1991), and alter the distribution of photosynthetically active radiation (PAR, 400-700 nm) with depth in leaf tissue (Bornman and Vogelmann, 1991). Such changes in the microenvironment affect processes such as photosynthesis.

Mitochondrial damage in the mesophyll cells of UV-B treated corn plants included fewer cristae, a less electron dense matrix, alteration and swelling in the endoplasmic reticulum and dictyosome (Santos *et al.*,1993). A reduction in cristae number within mitochondria was also observed in peas (Brandle *et al.*, 1977), although the researchers did not determine whether there was any damage to the mitochondrial structure.

1.2.4. Respiration

The carbon balance of crops can be altered by changes in respiration and mitochondrial respiration is one of the aspects of plant growth that may be affected by UV-B radiation. There have been few studies which have examined the effects of UV-B radiation on dark respiration, on either leaf slices or mitochondria and what is available is conflicting. In peas, the respiration rate of UV-B treated leaf slices was significantly higher than that of the control leaves (Brandle et al., 1977). A similar result was found in *Rumex patienta* (Sisson and Caldwell, 1976) and in tobacco (Owen, 1957). However, no significant change was observed in soybean plants (Teramura, 1980b), or in cassava (Ziska *et al.*, 1993) when treated with 4 different levels of UV-B radiation (0. 17.5, 35, and 70 mWm^{-2}). There is still insufficient information to ascertain whether respiration is one of the targets on which UV-B radiation impacts. With the paucity of information, it can not be concluded that dark respiration in sensitive species is generally affected by UV-B radiation. Furthermore, no conclusive data exist on the effects of UV-B radiation on oxygen uptake (or the activity of electron transport pathways) in crop plants.

1.2.5. UV-B radiation effects on photosynthesis

The physiological and biochemical processes of plant cells are sometimes vulnerable to environmental stresses. These changes cannot always be seen as morphological changes such as leaf colour and plant height. Photosynthesis, obviously, is closely related to light absorption, and any changes in light quality, such as an increase in UV-B, could directly or indirectly affect this process. There are at least three different aspects of photosynthesis which could be affected by UV-B and which would in turn, affect the carbon balance of crops. These are :

1. photosynthetic electron transport reactions and primary photochemical events,
2. dark reactions fixing carbon into reduced compounds,
3. stomatal resistance (Teramura, 1983).

It has been demonstrated that UV-B radiation inhibits multiple sites in the partial reactions of photosynthesis. This inhibition is closely associated with the chloroplasts, the site of photosynthesis. UV-B induced changes within the chloroplast include: impairment of electron transport, damage to the photosynthetic reaction centres, inhibition of photophosphorylation, loss of enzyme activity and

changes in the composition of chloroplast pigments (Bornman, 1989; Strid *et al.*, 1990).

Rates of net photosynthesis, measured as the rate of CO_2 assimilation in whole leaves in response to UV-B light under glasshouse and field conditions, have produced variable results. A reduction in photosynthesis has been noted but under certain conditions no effect or apparent increases in photosynthesis have been documented (Basiony *et al.*, 1978; Tevini and Teramura, 1989; Ziska *et al.*, 1993). These contrasting findings are thought to be due to inherent plant adaptation, the level of UV-B radiance, modification of UV effectiveness by interaction with natural climatic changes with season, differences in experimental conditions, different protocols (Teramura, 1987; Kramer *et al.*, 1992), differences in the sensitivity of individual species (Ziska *et al.*, 1992), and the effects of leaf age (Naidu *et al.*, 1993) which has been correlated with UV-B penetration of the leaf mesophyll (DeLucia *et al.*, 1992).

Net photosynthesis is also limited by the rate of diffusion of carbon dioxide through the stomata. Strid *et al.* (1990) demonstrated a decrease in net O_2 evolution as well as in maximum capacity for photosynthesis at light and CO_2 saturation in UV-B treated pea plants after 5 days treatment. Similarly, He *et al.* (1993) found a decrease in the quantum yield of photosynthesis, in O_2 exchange and maximum photosynthetic rates per unit area in pea and rice treated plants, but not in control plants. However, the decrease was much slower in rice than in peas over 8 days of treatment. In general, the rate of decrease in the quantum yield was somewhat slower than in that of maximum photosynthetic capacity.

1.2.5.1. Photosynthetic pigments

The stability of chlorophylls to light has also received considerable attention because the light dependent synthesis of chlorophyll is a powerful controlling factor in the overall process of chloroplast differentiation. The presence of chlorophyll is a prerequisite for the assembly of thylakoid membranes, in which the light reactions of photosynthesis occur (Hoober, 1984).

In many studies, the changes in chlorophyll in response to UV-B radiation have been assessed qualitatively and quantitatively (Deckmyn *et al.*, 1994; Strid *et al.*, 1990). The total chlorophyll content per unit leaf area decreased in UV-B treated oats, soybean (Teramura, 1983) and pea leaves (Strid *et al.*, 1990; He *et al.*, 1993, Jordan *et al.*, 1994). From 15-55% of pea leaf total chlorophyll was lost after 8 days of UV-B irradiance, due to a decrease in chlorophyll *a* (Chl.*a*) . In other experiments, the total chlorophyll declined slightly over the same period in

leaves of two different rice cultivars (He *et al.*, 1993), and did not change in cassava (Ziska *et al.*, 1993).

Chl. *a* is usually lost to a greater extent than is chlorophyll *b* (Chl.*b*). The content of Chl. *b* decreased by approximately 35%, whereas Chl.*a* dropped by 65% on the 8th day after treatment in pea leaves (Strid *et al.*, 1990). The lowered reduction of Chl.*b* under UV-B irrradiance may be due to conversion from Chl.*a* or to the greater stability of Chl.*b* (Brown *et al.*, 1991). Moreover,it was found that there was a slight increase in Chl.*b* after 7 days of UV-B treatment in pea species, even though Chl.*a* declined by 20% compared to control plants (Jordan *et al.*1994).

Expressed as a Chl.*a*/Chl.*b* ratio, a drastic reduction occurred in UV-B treated pea leaves (25-45%), while control plants changed from 3.45 to 3.12 (by less than 10%) (Strid *et al.*, 1990, He *et al.*, 1993, Jordan *et al.*, 1994). These results are in agreement with Vu *et al* (1981) who previously documented a decrease in the Chl.*a*/Chl.*b* ratio with increasing UV-B irradiation in soybean. The ratio of Chl.*a*/Chl.*b* was not found to change in cassava (Ziska *et al.*, 1993), and was only little altered (He *et al.*, 1993). The differences in results between studies on the effect of UV-B radiation on Chl.*a*/Chl.*b* ratios could reflect different sensitivities between one species or cultivar and another. Earlier studies on soybean suggested that William cultivar was more resistant to UV-B treatment than Essex cultivar (Murali *et al.*, 1988).

Generally, these studies suggested that the degree of chlorophyll destruction was a reflection of the level of UV-B irradiance, and that although less affected, carotenoids responded similarly (Strid *et al.*, 1990). On a unit leaf area basis, carotenoids decreased by approximately 50% after 8 days exposure to UV-B irradiation. However, this decrease was not significantly different to that of loss in chlorophyll content.

The reduction of chlorophyll and carotenoids could be a result of an inhibition of pigment biosynthesis, or due to the degradation of these pigments or their precursors. To answer this question, Strid and Porra (1992) found that the biosynthesic apparatus for chlorophyll was relatively stable under UV-B exposure and any decrease in chlorophyll was a result of increased degradation. The increase in chlorophyll degradation occurs when gene expression for the chlorophyll *a/b* binding protein is also severely inhibited by UV-B radiation (Jordan *et al.*, 1991). Although only a limited number of genes have been analysed, it was found that mRNAs for nuclear cab genes, encoding the chlorophyll a/b-binding proteins of the light harvesting antenna of PS II, are reduced much more rapidly than the mRNA for the plastid-localised psb A gene encoding the D1 protein of PS

II (Jordan *et al.*, 1991). This suggests that the nuclear DNA is more sensitive than the organelle DNA. The deleterious effects of UV-B radiation on the level of mRNA transcripts in mature pea plants has also been demonstrated by Jordan *et al.* (1994). They found that there was a great alteration in the developmental regulation of gene expression during UV-B treatment, which decreased the amounts of cab mRNA transcripts when mature green pea tissue is exposed to UV-B at low or even undetectable levels. Several factors could cause the decline in gene transcription during supplementary UV-B radiation, including the direct photodamage to DNA, indirect oxidative damage leading to changes in signal transduction, or involvement of UV-B receptors (Strid *et al.*, 1994).

1.2.5.2. Effects on protein

Most of the components involved in photosynthetic reactions are protein. It has been suggested that the UV-B radiation damage to plant cells was caused primarily by changes to protein and nuclear DNA, the two main chromatophores for UV absorption, with other pigments and lipids having a minor role (Caldwell, 1981). A decrease in pea soluble-leaf protein under UV-B exposure has been observed (Vu *et al.*, 1984), but in another experiment, soybean total leaf soluble protein was unchanged after 34 days exposure to UV-B in Essex (a sensitive cultivar) and William (a resistant cultivar) (Murali *et al.*, 1988). In contrast to these findings, Tevini *et al.* (1981) reported an increase in soluble protein in barley (*Hordeum vulgare* L.), radish (*Raphanus sativus* L.), bean (*Phaseolus vulgaris* L.), and in corn (*Zea mays* L., Santos *et al.*, 1993) leaves grown under UV-B. Teramura (1983) suggested an increase in soluble protein might be a reflection of the increased synthesis of aromatic amino acids.

Since ribulose 1,5-bisphosphate carboxylase (Rubisco), the primary enzyme of CO_2 fixation in C3 plants, often represents more than 50% of the total chloroplast protein (Albert *et al.*, 1989), and is a major resource of carbon and nitrogen within the plant, it is likely that a decline in leaf soluble protein may be a reflection of a decrease in the amount of Rubisco enzyme. A reduction in Rubisco activity or a reduction in Rubisco protein due to UV-B irradiance has been demonstrated (Vu *et al.*, 1982; He *et al.*, 1993, 1994) and this reduction is likely to be associated with protein degradation and or inactivation of the enzyme. Partial degradation of Rubisco enzyme could reduce the rate of carbon assimilation. Rubisco extracted from treated plants declined in activity and content with increasing UV-B exposure of both pea and soybean leaves (Vu *et al.*, 1982).

Recent studies show that based upon both chlorophyll content and leaf area, the functional activity of Rubisco in UV-B-treated pea plants was decreased by 38% after 1 day and by 71% after 3 days (Jordan *et al.*, 1992; Strid *et al.*,1990). The rice cultivar (IR 36) showed a similar trend, but no significant decrease was apparent in the 5 Fujiyama rice cultivar (Ziska and Teramura, 1992). Similarly, Mirecki and Teramura (1984) found no significant differences in Rubisco activity in soybean leaves either on a leaf area or a leaf fresh weight basis after 10 days. In contrast, tomato leaves produced an increase in their soluble protein content under UV-B irradiance (Vu *et al.*, 1982). This indicates that a tight correlation cannot be made between a UV-B induced decline in soluble protein and Rubisco content. Furthermore, Jordan *et al.* (1992) by examining the decline in maximum Rubisco activity after a given period of UV-B treatment, found it was greater than the decrease in either total soluble protein or Rubisco protein. The activation of Rubisco *in vivo* (measured as the ratio of the initial rate of carbon assimilation to the maximum rate after incubation with Mg^{2+} and bicarbonate for 7 min) was increased as the maximum rate decreased during the 3 days of UV-B exposure (Jordan *et al.* 1992). This decline included a clear reduction in the amount of both large (LSU) and small subunits (SSU) of Rubisco in isolated chloroplasts (Jordan *et al.*, 1992, Nedunchezhian and Kulandaivelu, 1991a). The smaller the remaining carboxylase activity during the experimental period, the more the enzyme could be activated *in vivo* (Strid *et.al.*, 1990). The reduction of Rubisco in plants exposed to UV-B irradiation, possibly results from low RNA transcription levels for both Rubisco subunits (LSU and SSU) (Jordan *et. al.*, 1992).

1.2.5.3. Effects on photosystems and electron transport reactions

The reduction of photosynthesis activity (Table 1.3) in whole leaves or in chloroplasts after UV-B radiation is mostly associated with the inactivation of photosystem II (PS II). This has been reported in peas (Brandle *et al.*, 1977, Strid *et al.*, 1990, He *et al.*, 1993), rice (Ziska and Teramura, 1992), and in soybean (Basiouny *et al.*, 1978, Vu *et al.*, 1981). There have been occasional reports of effects on photosystem I (PS I) (Brandle *et al.*, 1977).

PS II (Fig.1.2), which functions as a light harvesting system contains chlorophylls *a* and *b*, pheophytin, a reaction centre (P_{680}), quinones, carotene, one or two nonheme iron proteins, and at least 23 polypeptides including a core complex of six integral (intrinsic) polypeptides, two of which are D1(33 kDa) and D2

Table 1.3. Summary of some recently reported direct and indirect effects of UV-B radiation on photosynthesis (Teramura and Sullivan, 1994).

Component affected	Damage/Symptom	References
Primary direct effects		
Photosystem I	few direct effects reported	
Photosystem II	reduce oxidative capacity	Renger et al.(1989)
	D1 polypeptide turnover	Greenburg et al. (1989)
	photoreduction of PQ	Melis et al. (1992)
Rubisco	reduced mRNA transcripts	Jordan et al. (1992)
	reduced enzyme activity	Strid et al. (1990)
		Jordan et al. (1992)
Secondary direct effects		
CO_2 assimilation	reduced O_2 uptake	Sulivan and Teramura (1990)
RuBP regeneration	lower regeneration activity	Strid et al. (1990)
quantum yield	reduced F_v/F_m ratio	Sulivan and Teramura (1993)
		Ziska et al. (1993)
oxygen evolution	lower A_{max}	Ziska et al. (1992)
Indirect effects		
Light penetration	epicuticular waxes	Tevini and Steinmuller (1987)
	leaf thickness	Bornman and Vogelmann (1991)
Pigments	reduction in chlorophyll	Strid et al. (1990)
Stomatal function	reduced conductance	Negash and Bjorn (1986)
	loss of control	Teramura et al. (1983)
Canopy morphology	altered light interception	Barnes et al. (1990)
		Ryel et al. (1990)

F_v/F_m: ratio of variable to total chlorophyll fluorescence yield
A_{max} : light and CO_2 saturated rate of oxygen evolution

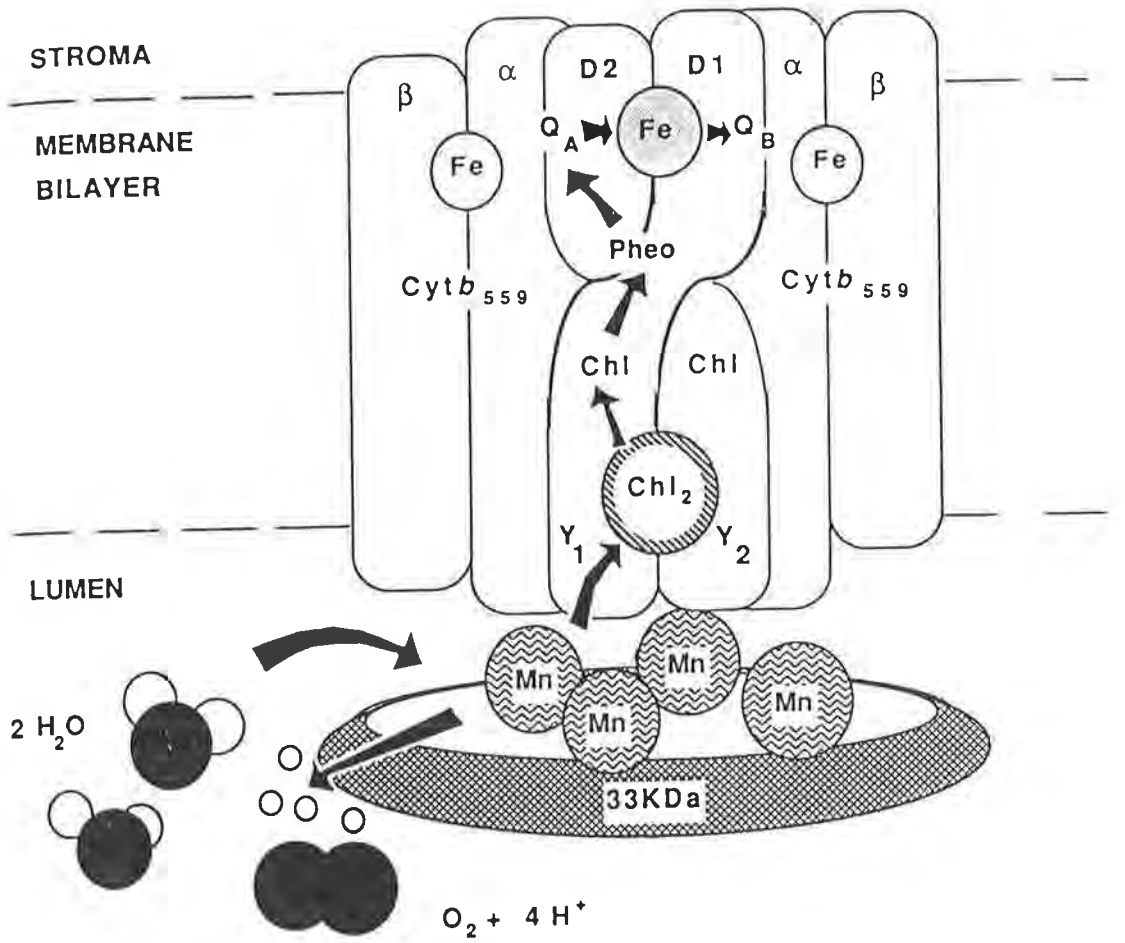


Fig.1.2. Representation of the photosystem II reaction centre with the extrinsic 33-kDa polypeptide. Cytochrome *b*-559 is depicted as two symetrically placed copies of an αβ dimer. D1-D2 contains the binding sites for the P680 Chl, pheophytin, and Q_A-Fe-Q_B. Abbreviations used : (Pheo), pheophytin; (Q), plastoquinone; (Cyt*b*), cytochrome. (Scheller *et al.*, 1989 as cited by Cramer *et al.*, 1991 in *Current Topic in Bioenergitics*, Vol.**16**, 1991).

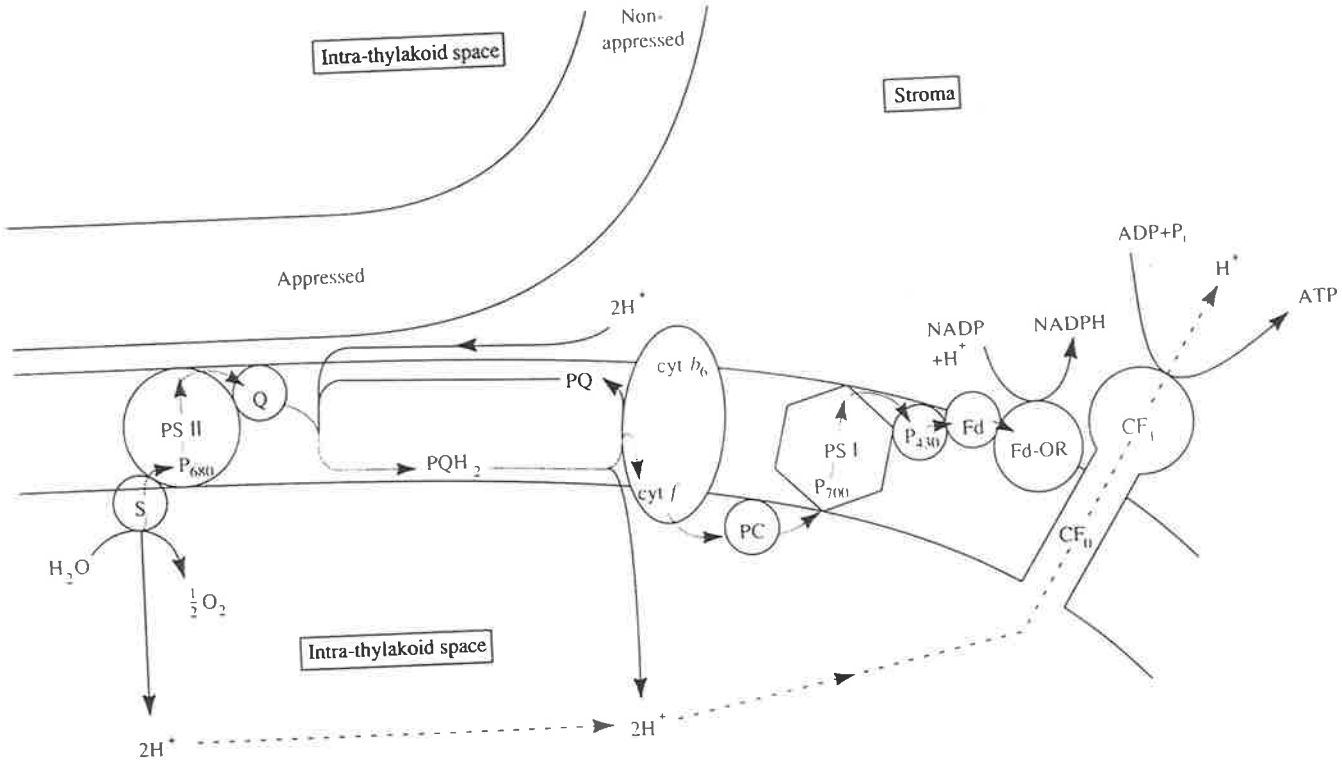


Fig.1.3. A stylized model of four structural units of the thylakoid membrane, which catalyze the light harvesting, electron transport, and energy coupling of photosynthesis. Abbreviations used : (S) water oxidizing Mn-protein; (PQ/QH_2), oxidized and reduced plastoquinone; (PC) plastocyanin; (Fd) ferredoxin; (Fd-OR) ferredoxin:NADP oxidoreductase. (Anderson and Beardall, 1991, p: 157).

(31 kDa), and three are peripheral (extrinsic) (Barber, 1987, Anderson and Beardall, 1990). The two reaction centre polypeptides (D1 and D2) directly bind P_{680} and quinones, which are involved in the oxidation of water and electron transport (Figure 1.3). It has been found that PS II was sensitive to UV-B radiation (Brandle *et al.*, 1977; Nooruden and Kulandaivelu, 1982). For instance, the function of PSA II reaction centres declined by 23% during 8 h of UV-B irradiation. Damage to the PS II reaction centres is partially responsible for the loss of quantum efficiency of photosynthetic O_2 evolution. UV-B radiation inactivates the reaction centre of PS II and degrades primarily the functioning of water oxidation (Renger *et al.*, 1989). Based on kinetic analyses, Renger *et al.* (1989) have shown that UV-B inhibits the PS II activity by destroying the D1. This is also supported by the research by Greenburg *et al.*(1989), who showed that UV-B-induced alterations on the reducing side of PS II were due to the continued degradation and but slower synthesis of the D1 polypeptide. To support this finding, Nedunchezhian and Kulandaivelu (1991b) also found that UV-B treated chloroplasts isolated from *Vigna sinensis* L.cv. Walp had a large decrease in the amount of D1 polypeptide present. This polypeptide is involved in transfer of electrons to plastoquinone (PQ). This indicates that the site of UV-B action must be prior to PQ in the electron transport chain (Kulandaivelu *et al.*, 1991). The fact that UV-B radiation lowered the overall levels of photoreduction of plastoquinone in isolated chloroplasts (Melis *et al.*,1992) is additional evidence that quinones and D1 are involved in that process. In this case, the D1 and D2 polypeptides act as apoproteins for the electron transport intermediates plastoquinone QA and QB respectively. Since quinone, semiquinone, and quinol absorb in the UV-B waveband, they are all potential targets for UV-B inhibiton (Bornman and Teramura, 1993). The D2 polypeptide on the receptor or oxidizing side was also found to be modified by UV-B radiation, so that the number and the activity of quinone binding sites were altered (Renger *et al.*, 1989). Furthermore, the reorganisation of the thylakoid components to form an internal protective system for the reaction centre complex, and the development of a 29 kDa light harvesting chlorophyll protein complex (LHCP) were also found to be consequences of UV-B radiation (Nedunchezhian and Kulandaivelu, 1991b).

Concerning the action of UV-B radiation on photosynthetic activity, the Hill reaction is also inhibited in chloroplasts and whole leaves after UV-B irradiation (Brandle *et al.*, 1977; Vu *et al.*, 1981, Bornman, 1989). Using intact leaves, Basiouny *et al.* (1978) found the Hill reaction activity of the chloroplasts of collards, oats, soybean was significantly reduced by prior exposure to UV-B, but in sorghum and corn. However, isolated *Amaranthus* chloroplasts treated with UV-B produced

an approximately linear decline of Hill activity over the first 60 min irrespective of the oxidant added, with as much as 85% of the activity being lost (Nooruden and Kulandaivelu, 1982). In addition, the rate of whole chain electron transport observed for $H_2O \rightarrow$ methylviologen (MV) was lower in UV-B treated chloroplasts (Brandle *et al.*, 1977; Kulandaivelu *et al.*, 1991). A total of 72 and 70% loss, with reference to the initial rates was noticed after 60 min UV-B in *Phaseolus* and *Triticum* chloroplasts, respectively, while in *Amaranthus*, *Zea* and *Pennisetum*, 65, 60 and 54% loss of activity was observed, respectively. Previously, Nooruden and Kulandaivelu (1982) found that a 30 min UV-B treatment of *Vigna sinensis* L. chloroplasts diminished the electron translocation between $H_2O \rightarrow$ FeCy (PS II + PS I) by 45%, whereas the $DCPIPH_2 \rightarrow$ MV (PS I) reduction remained unaffected (Fig.1.4).

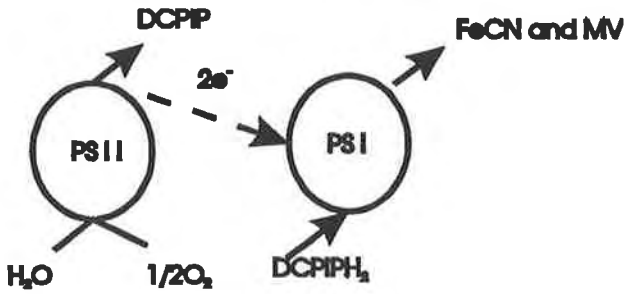


Figure 1.4. The role of DCPIP as an electron acceptor in form PS II and $DCPIPH_2$ as an electron donor for PS I.

1.3. The present study

A species of pea which had been selected to be grown in a growth room under 150 μmol photons m^{-2} s^{-1} PAR with 50 and 220 mW m^{-2} nm^{-1} at 273 nm and 313 nm from filtered UV-B lamps, demonstrated an increased sensitivity to UV-B radiation with increased time of radiation (Strid *et al.*, 1990). Peas, the second most important legume crop in Australia, have been found to be more sensitive to supplementary UV-radiation than other crops (Strid *et al.*, 1990). On the other hand, wheat was least affected by UV-B irradiances (Teramura, 1980a; Strid *et al.*, 1990).

In many experimental analyses, mature plants (or chloroplasts isolated from mature plants) were exposed to supplementary UV-B radiation. However, seldom have seedlings been treated with UV-B radiation, that would occur in their natural

environment. Whilst studies on the effects of UV-B on photosynthesis using both intact leaves and isolated chloroplasts have been well documented (He *et al.*1993, 1994; Teramura *et al.*, 1990; Nedunchezhian and Kulandaivelu, 1991b; Strid *et al.*, 1990), such has not been the case for the effects on respiration. Therefore, it was decided to examine the effects of UV-B radiation on both photosynthesis and respiration using leaf slices, and isolated chloroplasts and mitochondria.

In the present work a broad survey was carried out . The aims of this work were to determine the effects of UV-B radiation on:

1. the morphological features: plant height, specific leaf area, fresh and dry weight, specific leaf weight and petiole length.
2. leaf pigmentation including chlorophyll concentration and the accumulation of UV-B absorbing compounds.
3. the anatomical and ultrastructural features of leaves, chloroplasts, and mitochondria with a view to correlating the biochemical results to actual sites of change in response to UV-B radiation.
4. respiration of leaf slices and isolated leaf mitochondria,
5. photosynthetic oxygen evolution, using both leaf slices and properties of isolated chloroplasts,
6. and to relate the changes in morphologial and anatomical characteristics to the respiratory or photosynthetic processes.

For this purpose, two quite different species *Pisum sativum* L cv Massey Gem, a sensitive herbaceous dicotyledon, and *Triticum aestivum* L. (var Machetti), a tolerant monocotyledon, were studied.

Chapter 2: Material and Methods

2.1. Plant material and cultural conditions

Pea seeds (*Pisum sativum* L. cv. Massey Gem) and wheat seeds (*Triticum aestivum* L. var machetti) were planted 3 cm apart in a mixture of potting soil and vermiculite (3:1) in rectangular plastic basins in an unshaded glasshouse. The plants were watered every 2 days with tap water for a period of 8-10 days for peas and 5-6 days for wheat and with Hoagland's solution containing 5 mM KNO_3, 5 mM $Ca(NO_3)_3$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mM Fe-EDTA (ethylene-diaminetra-acetic acid), 4.63 mM H_3BO_3, 9.15 mM $MnCl_2$, 0.765 mM $ZnSO_2$, 0.32 mM $CuSO_4$, and 1.03 mM Na_2MoO_4 thereafter. The general environmental conditions prevailing in the glasshouse were as follows: 12 h photoperiod, average day/night temperatures of 27/20°C, and ambient CO_2.

2.2. UV-B treatment

In the greenhouse or the field, where larger-scale experiments have been performed, cellulose acetate and Mylar plastic films have been used (Barnes *et al.*, 1988; Dai *et al.*, 1992; Balakumar *et al.*, 1993; Caldwell, 1994; Sulivan and Teramura, 1994). The former was used to shape the spectral distribution of radiation eliminating harmful UV-C radiation, whilst the latter was used as a control (No UV-B), as the UV-B component is entirely absorbed by Mylar plastic films. Due to the absorption characteristics of glass, therefore, in most greenhouse studies, control plants did not receive natural UV-B radiation at all, which is unrealistic and unlike plants grown outdoors under a natural solar spectrum. For this study, the technique of using cellulose acetate and Mylar plastic films for UV-B lamps was not applied.

It is considered that all UV-B lamps emit a small amount of undesired radiation, either UV-A or UV-C. Using an IL 1700 Research Radiometer (International Light, New Buryport, MA, USA) generously provided by the South Australia Health Commission, it was found that only a small percentage of UV-A (15%) and UV-C (5%) (Fig. 2.1) was emanating from our UV-B lamps (NIS F40 T10, UV-B).

In this study, UV-B treatments were applied when the first pea leaf pair had fully expanded or when the first wheat leaf had appeared. Approximately a quarter

of the plants were kept in the unshaded glasshouse without the addition of supplementary UV-B radiation (zero UV-B or control). For the natural UV-B treatment, a quarter of the plants were kept outside, but adjacent to the glasshouse and exposed to direct sunlight (natural UV-B). For supplementary UV-B treatments, a special 4 x 2 m compartment with a bench (3 x 1.5 m) was built in the unshaded glasshouse and fitted out with 4 UV-B lamps at one end and with 2 UV-B lamps at the other (Fig.2.2). The lamps, 25 cm apart and suspended 110 cm above the plant canopy (Fig. 2.3), were equipped with a timer switch to allow the UV-B irradiance to be controlled precisely. For safety reasons, this switch was placed on the outside wall of the booth (Fig.2.4). As a standard protocol, lamps were aged 100 h prior to use. Plants were irradiated for 6 h per day in the middle of the photoperiod. The arrangement of the plants was rotated daily to minimize the effects of position under the lamps and unequal irradiation. Radiation levels were 0.0896 Wm^{-2} (low UV-B) and 0.164 Wm^{-2} (high UV-B) under the two treatments, as determined with an IL 1700 Research Radiometer. The integrated level of radiation under the low UV-B treatment was about the same as that measured outside glasshouse during the summer of 1993-1994, but concentrated into a 6 hour period. The high UV-B treatment gave double the amount of UV-B radiation.

2.3. Harvest

For tissue experiments, leaves of the third trifolium for peas or the second leaf for wheat were excised from stem tissue during the light period. When mitochondria and chloroplasts were to be isolated, all leaves were harvested.

2.4. Measurement of growth parameters

The growth of at least 10 plants was analyzed at a plant age of 4, 8, and 12 days of treatment. Plant height (above ground height, above cotyledon), leaf area (for peas), fresh plant weight (above ground fresh weight, above cotyledon), leaf fresh weight, and internode length (for peas) were recorded.

Fig.2.1. The spectrum of radiation from 6 UV-B lamps at 20 cm as measured using IL Research Radiometer connected to GM 200 double Monochromator (Kratos Analytical Instruments). The UV-B lamps were aged for 30 min before measurement.

Fig.2.2. The UV-B growth compartment used for experiment in unshaded glasshouse.

(a). Timer
(b). Main Switch
(c). UV-B lamps
(d). Temporary door
(e). Sign bulb (giving a sign when either lamps were on or off)

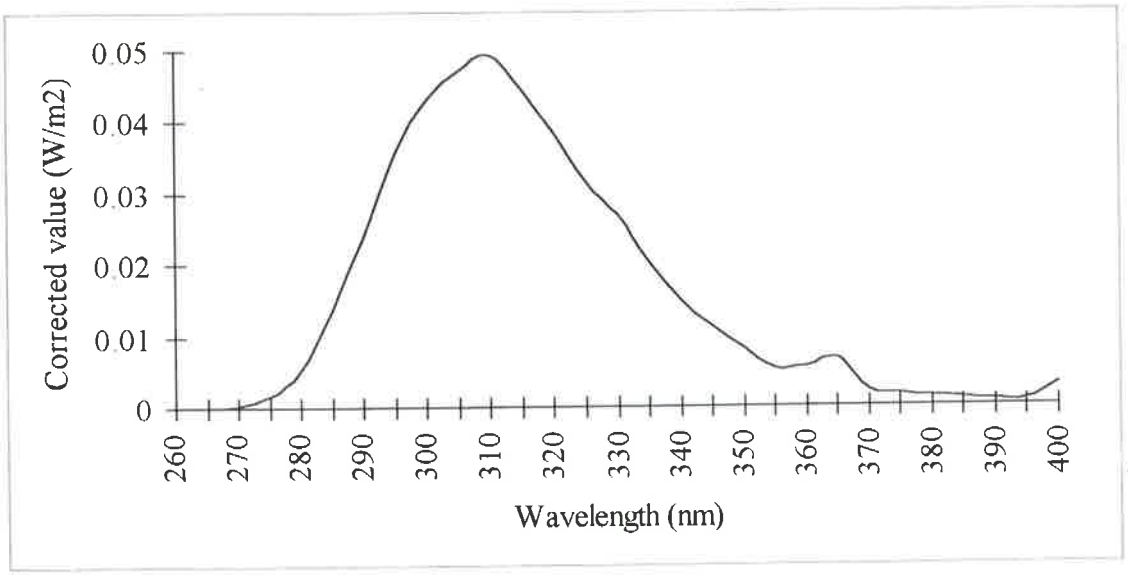

Corrected value (W/m2)
0.05
0.04
0.03
0.02
0.01
0
260
270
280
290
300
310
320
330
340
350
360
370
380
390
400
Wavelength (nm)

Fig.2.1


c
e
d
b
a
DANGER

Fig.2.2

Fig. 2.3. The automatic switch timer placed outside on the UV-B growth compartment

Fig. 2.4. The environment inside the UV-B growth compartment

(a). Bench
(b). UV-B lamps
(c). Door
(d). Sign bulb
(e). Thermohygrograph

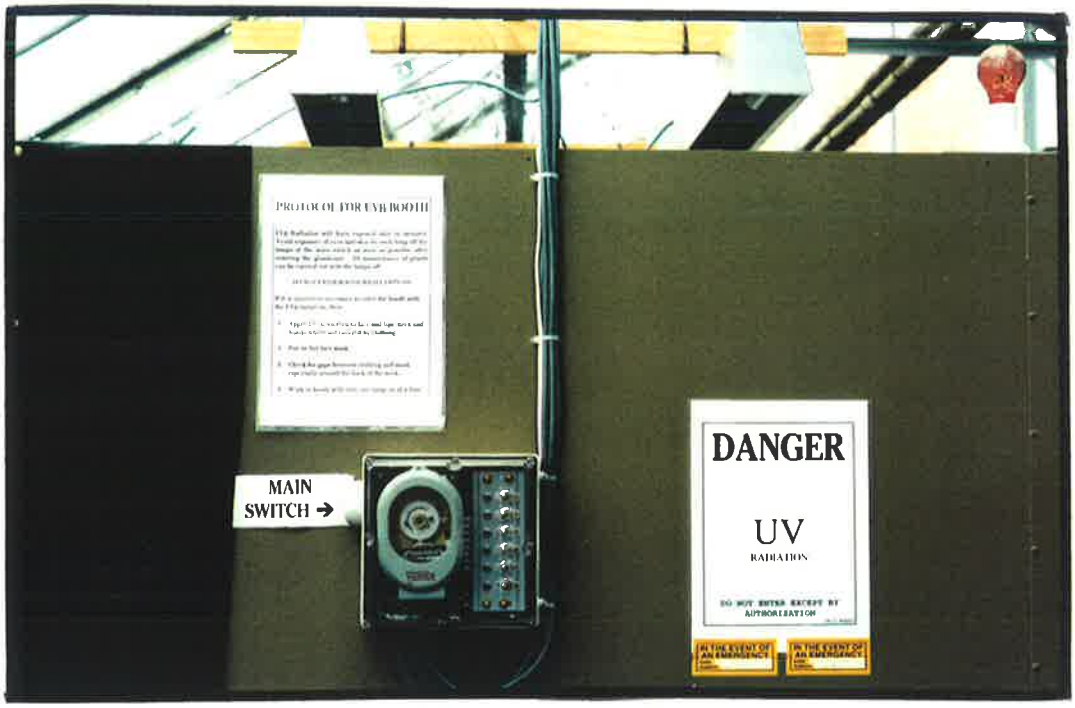

PROTOCOL FOR UVB BOOTH
MAIN
SWITCH
DANGER
UV
RADIATION
DO NOT ENTER EXCEPT BY
AUTHORIZATION
IN THE EVENT OF
AN EMERGENCY
IN THE EVENT OF
AN EMERGENCY

Fig.2.3


b
e

Fig.2.4

2.5. Anatomical study of pea and wheat leaves

2.5.1. Distribution of stomata

A piece, 0.5-1 cm^2, was cut from the edge of the leaf and placed in a test tube with 5 ml of 40% [w/v] hydrogen peroxide and 5 ml of 70-90% ethanol and heated until the colour disappeared and the cuticles began to peel back like a book opening (2 hours-2 days). The leaf segment was rinsed in water and cleaned of cellular debris from the cuticular envelope with two fine brushes. Ideally, the upper and lower cuticles remained attached via the leaf edge. Cuticles were rinsed briefly in 0.5% ammonia and stained with a solution of a drop of 0.1% crystal violet stain in 5 ml of water until a uniform light purple (30-120 second). Rinsed cuticles were mounted in phenol-glycerin-jelly (the outer surface of the cuticle toward the cover-slip). Stomata per unit area were counted at x 400 magnification.

2.5.2. Preparation of specimens for light and transmission electron microscopy

Leaf pieces, approximately 1 x 1 mm, for transmission electron microscopy (TEM) and leaf structure studies were excised randomly from the third pea leaf pair and the second wheat leaf harvested 8 days after treatment in the middle of the photoperiod for all treatments.

2.5.2.1. Fixation, dehydration, and embedding

Leaf pieces were fixed in 3% glutaraldehyde in 25 mM PIPES buffer, pH 7.2, overnight at 4°C and thoroughly rinsed in 25 mM PIPES buffer for 2 hours. After several buffer washes, the tissue pieces were postfixed in OsO_4 (1% OsO_4 in 25mM PIPES buffer, pH 7.2) at room temperature for 2-3 hours. The tissues were washed in distilled water and dehydrated in a graded acetone series (70, 80, 95, 100%), with 30 min in each solution. Samples were then infiltrated with a series of Spurr's resin:ethanol mixtures (1:1, 2:1, 3:1) followed by 100% resin, each step taking 12 hours on a rotary agitator (Spurs, 1969, with modifications by the Centre for Electron Microscopy & Microstructure Analysis (CEMMSA), Adelaide University, South Australia). The composition of Spurr's resin is: 10g vinyl cyclohexene dioxide (ERL), 5g diglicidyl ether of polypropylene glycol (DER), 26g nonenyl succinic anhydride (NSA) and 0.2ml 2-dimethylaminoethanol (DMAE), and was supplied by Probing & Structure, Queensland, Australia. The specimen was finally

embedded in fresh resin, polymerised at 70°C and applied overnight under vacuum.

2.5.2.2. Sectioning and staining

The resin block (containing the tissue) was trimmed to size and positioned to ensure that the tissue was correctly oriented for sectioning. Embedded tissues were cut with a glass knife on a Reichert-Jung Ultracut microtome .

For TEM, sections 50-70 nm thick were collected on the dull side of the grid by lowering the grid onto the sections. The grids containing sections were dried on filter paper and were kept in small petri dish.

Sections on grids were stained with 5% alcoholic uranyl acetate and lead citrate. The staining procedure was as follows. A drop of uranyl acetate was put onto a wax base in a petri dish. The grids with the tissue sections were placed face down on top of the drop and left for 20 min in the dark. The grids were quickly removed from the drop of stain, immersed in 50% ethanol and rinsed three times in distilled water before drying on filter paper. The grids were then stained again with lead citrate as above. To avoid trapping CO_2 in the lead matrix, some crystals of NaOH were placed around the drop of lead citrate. After 20 minutes, the grids were rinsed four times in distilled water and completely dried on filter paper.

For light microscopy, the microtome and glass knife were used to cut sections approximately 1mm long and 0.5 μm thick. These were transferred to a drop of water on a glass slide, dried on a hotplate, and then were stained with 0.5% of toluidine blue by dropping the stain on the tissue sections. After 20 second, the tissues were washed with distilled water and dried on the hotplate. The dried sections were mounted with Canada balsam and cover slips.

2.5.3. Microscopes

A Philips EM 100 transmission electron microscopy (TEM, in CEMMSA) was used to observe the cellular and chloroplasts structures. The images were recorded photographically.

An Olympus compound microscope (model CHT) was used for observation of leaf anatomy, leaf thickness and stomata number which were recorded on Kodak ASA 100 film in an Olympus SC 35 camera attached to the microscope. Photographic montages of each section were prepared at known magnifications.

2.6. Measurement of O_2 uptake in leaf slices

Pea and wheat leaf slices were prepared as described by Azcón-Bieto *et al.* (1983). The third pea leaf pair and the second wheat leaf were washed, their midribs removed, and the leaf transversely cut into 1 mm slices with a sharp razor blade under a solution of 0.2 mM $CaCl_2$ (pH 6.5). The leaves were positioned in such a way that the maximum number of veins were intercepted by the blade. The leaf slices were washed for a minimum of 30 minutes after slicing in the same solution, which was renewed several times. The rate of oxygen uptake was measured at 25°C in 3 ml of an air saturated solution containing 10 mM HEPES, 10 mM MES buffer (pH 6.5) and 0.2 mM $CaCl_2$ with a Rank O_2 Electrode (Rank Bros, Cambridge, England) connected to a Rikadenki chart recorder. Salicylhydroxamic acid (SHAM, 3 mM) was added to inhibit the alternative pathway, while KCN (1 mM) was used to inhibit the cytochrome pathway. The oxygen uptake rates were allowed to reach a steady rate before SHAM and/or KCN were added into the chamber. The rate of O_2 consumption was expressed on a fresh leaf weight basis (nmol O_2 uptake g^{-1}fr.wt sec^{-1}).

2.6.1. The determination of the alternative and the cytochrome pathways in leaf slices

The operation of the alternative and cytochrome pathways to O_2 uptake were measured in pea and wheat leaf slices using KCN to inhibit cytochrome pathway and SHAM to inhibit alternative oxidase. The final concentrations of inhibitors used were 3 mM for SHAM and 1 mM for KCN. FCCP was added at final concentration of 1 µM.

Total respiration (uninhibited oxygen uptake rates): represent the rates of respiration in the absence of inhibitor.

V_{alt} : is used to represent the rate of oxygen uptake sensitive to SHAM (the activity of the alternative oxidase). This value is determined as the rate of uninhibited oxygen uptake minus the rate of oxygen uptake in the presence of SHAM.

V_{cyt} : represent the activity of the cytochrome pathway (the rate of oxygen uptake in the presence of SHAM minus residual respiration, the rate of oxygen uptake in the presence both SHAM and KCN).

2.7. Isolation of mitochondria

2.7.1. Pea leaves

Isolated mitochondria were prepared as described by Day *et al.* (1985) with modifications. Pea leaves (60~80 g) were harvested after 6, 12, and 16 days treatment and cooled to approximately 2°C before being homogenized for 2-3 seconds using a Polytron (Kinematica, GmbH, Model K, Krie-Luzein, Switzerland) with a probe PTA-35/2 (setting #6-6.5) in 400 ml of ice-cold extraction buffer containing 0.3M sucrose, 50 mM 2-(2'-hydroxy-1,1-bis(hydroxymethyl)-ethylamino)ethanesulfonic acid (TES) buffer, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM glycine, 1% polyvinyl-polypyrrolidone (PVP-40) [w/v], 0.4% bovin serum albumin (BSA) [w/v], and 10 mM isoascorbic acid, adjusted to pH 7.5 with KOH. The homogenate was filtered through two layers of "Finnweb" and centrifuged at 1,600 g_{av} for 10 min. The supernatant was centrifuged at 12,000 g_{av} for 10 min and the pellet resuspended in a few drops of wash medium containing 0.3 M sucrose, 1 mM glycine, 10 mM TES buffer (pH 7.5), and 0.1% BSA [w/v]. The crude mitochondrial suspension was loaded onto a 35 ml gradient solution consisting of 28% [v/v] Percoll, with a linear gradient of 0 to 2.2% PVP-40 [w/v] in wash medium and centrifuged at 30,000 g_{av} for 30-35 min. The mitochondria were found in a tight pale buff band near the bottom of the tube, whereas the thylakoids remained near the top of the tube. The purified mitochondria were removed from the gradient with a Pasteur pipette, washed in resuspension medium, and centrifuged at 10,000 g_{av} for 15 min. This was repeated once or twice to obtain a firm mitochondrial pellet. The pellet was resuspended in 300-500 µl of resuspension medium and kept on ice.

2.7.2. Wheat leaves

All the procedures were similar to those used for pea leaves, except that wheat leaves (30-40 g) were chopped into 1 cm lengths before homogenisation.

2.8. Measurement of O_2 uptake in mitochondria

Respiration was measured as O_2 uptake using a Rank-oxygen electrode (Rank Bros, Cambridge, England) in 3 ml of reaction medium containing 300mM sucrose, 10 mM TES buffer (pH 7.5), 5 mM KH_2PO_4, 2 mM $MgCl_2$ and 0.1% BSA

[w/v] at 25ºC and 200~500 μg of mitochondrial protein. The rate of respiration was expressed in nmol O_2 uptake mg^{-1}protein min^{-1}.

2.8.1. The determination of the activity of alternative oxidase in mitochondria

To asses the operation of the alternative oxidase, myxothiazol was used to a final concentration of 5 μM.

5.8.2. Stimulation by pyruvate

After the addition of myxothiazol, pyruvate (5 mM) was used to stimulate the alternative oxidase.

2.9. Gel electrophoresis

2.9.1. SDS-polycrylamide gel electrophoresis

Mitochondria protein (30~60 μg) was solubilised with 5 μl of non-reducing buffer (62 mM tris(hydroxymethyl)aminoethane (TRIS) [pH 6.8], 2% [w/v] sodium dodecyl sulfate (SDS), 10% glycerol, and 0.1% [w/v] bromophenol blue (BPB)) or reducing buffer (as above with the addition of 100 mM dithiothreitol (DTT)). Samples were denatured by heating at 85°C for 3~5 minutes.

Electrophoresis was performed with a Novex apparatus (Xcell II Mini-Cell) using purchased Novex Tris-Glycine gels (4-20% acrylamide and 2.6% bis-acrylamide, pH 8.6) filled with a TRIS-glycine running buffer (25 mM TRIS, 192 mM glycine, and 0.1% SDS, unadjusted pH 8.3). The gels were run at 125 V (constant) and 40 mA (initial) for 1-2 hours. The run was stopped when the BPB dye front reached the bottom of the gel.

2.9.2. Protein stain

The gels, were stained for 1 h in Coomassie brilliant blue R-250 stain (0.15% Coomassie, 50% [w/v] methanol and 10% acetic acid), destained for 1-2 hours, washed in a solution of 5% [w/v] methanol and 7% [w/v] acetic acid overnight and then dried down onto Whatman 3MM paper using a heated, vacuum pad.

2.9.3. Immunoblotting (Western blotting)

Proteins were transferred from gels to a PVDF membrane (Trans-Blot Transfer Medium, Bio-Rad) using a Novex transfer apparatus. The PVDF membrane was cut to size, washed in methanol and deionised water and then equilibrated with the gel for 15 minutes in the transfer buffer containing 12 mM TRIS, 96 mM glycine and 20% methanol (unadjusted pH ~8.3). Three fibre, blotting pads and two Whatman filter papers (cut to size) were saturated in transfer buffer, and stacked on the negative electrode in the order: blotting pad, filter paper, gel (face up) with a small amount of transfer buffer, the membrane, filter paper and two blotting pads. The positive electrode was sandwiched on top and the whole assembly fitted into the electrophoresis tank. The transfer chamber and tank were filled with transfer buffer and deionised water respectively. The system was run for 1 h at 30 V (constant) and 80 mA.

After transfer, the membrane was rinsed in deionised water, washed for 15 min in TBS (20mM TRIS and 500 mM NaCl pH 7.5) and incubated for 45 min in a blocking solution (as above with the addition of 3% [w/v] of BSA), to remove any sites of non-specific binding of antibody. The membrane was washed of blocking agent with TTBS (TBS, 0.25% Tween 80) (3 x 5 minutes) and incubated with primary (1°) antibody (kindly provided by Dr. L. McIntosh, Michigan State, University of Michigan, USA), in a solution of TTBS, 1%[w/v] BSA and 0.002% azide for 1-3 hours. Excess antibody was removed by washing 3 x 5 min in TTBS and the membrane was then incubated with the secondary antibody (rabbit anti-mouse, Sigma Immuno Chemicals) conjugated with alkaline phosphatase for 1-2 hours. The membrane was again washed in 3 x 5 min in TBS. The protein(s) recognised by anti-alternative oxidase primary antibody was visualised by incubation with alkaline phosphatase substrates 0.0065% [v/v] nitroblue tetrazolium (NBT) and 0.00325% [w/v] 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in buffer (100 mM TRIS pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$)(Promega). The reaction was stopped when a colour change occurred (2-10 min) by washing the membrane with deionised water.

2.10. Measurement of leaf pigments

2.10.1. UV-B absorbing compounds

Flavonoids and related phenolic compounds were extracted by soaking 1 cm^2 of leaf tissue for 10 min in 5 ml of an ethanol:acetic acid mixture (99:1[v/v]). Absorbance of the extract was determined with a Philips PU 8800 UV/VIS double beam spectrophotometer at 300 nm (Flint *et al.*, 1985).

2.10.2. Anthocyanin assay

Anthocyanins were measured as described by Lange *et al.* (1971). Approximately 1 g tissue, harvested from the first , second and third leaf pairs, was extracted in 10 ml of a mixture containing propanol, HCl, and H_2O (18:1:81, vol%), and the extraction vials were immersed in boiling water for 1.5 min. To ensure extraction, the leaves remained 24 h in the extraction medium at 25°C in the dark with occasional shaking. Extracts were centrifuged at 5000 g_{av} and the absorbance measured at 535 and 650 nm. The absorbance values at 535 nm were corrected according to the following equation:

$$\text{corrected } A_{525} = A_{535} - 2.2\ A_{650}$$

2.10.3. Chlorophyll determination

The chlorophyll estimation was essentially that described by Porra *et al.* (1989). Leaf pieces approximately 400 mm^2 (cut with razor blade) were extracted by grinding them in 2 ml buffered aqueous acetone in a mortar and pestle. The homogenate, combined with a further three washings of the pestle and mortar with the same solvent (each 1.5 ml), was centrifuged at 1500 g_{av} in an MSE bench centrifuge for 10 min. The pellet was extracted with a further 1 ml of solvent in a Potter-Elvejhem homogenizer and the pooled supernatants adjusted to a final volume of 8 ml. The spectrophotometer was set to zero absorbance at 750 nm. The absorbance was read at 663.6 and 646.6 nm and Chl. *a*, *b*, and *a* + *b* concentrations were calculated using the equations:

$$\text{Chl. } a \text{ (µg/ml)} = 12.25\ A_{663.6} - 2.55\ A_{646.6}$$

$$\text{Chl. } b \text{ (µg/ml)} = 20.31\ A_{663.6} - 4.91\ A_{646.6}$$

$$\text{Chl. } a + b \text{ (µg/ml)} = 17.76\ A_{663.6} + 7.34\ A_{646.6}$$

2.11. Determination of O_2 evolution in leaf slices

The third leaf pair and the second wheat leaf were washed with deionised water and their midribs removed. The halves of the leaflets were laid on top of each other in a Petri dish containing 0.5 mM $CaSO_4$ and transversely sliced into 1 mm wide strips with a brand-new razor blade. The rate of O_2 evolution was measured polarographically at 25°C in 4 ml of 10 mM phosphate buffer solution (pH 6.5). Before adding the slices, the buffer solution was bubbled with N_2 in order to reduce the O_2 concentration to 20% saturation. $NaHCO_3$ (5 mM) was added to the reaction medium and followed by the leaf slices. The leaf slices were illuminated using a 150 W projector lamp (Rondette, 1500 RF, Hanimex, Ireland). The intensity of light reaching the reaction vessel was about 200 $\mu\varepsilon m^{-2}s^{-1}$ PAR as measured with a light meter (Li-COR, Li-1000, Q 11648). O_2 evolution was monitored until the O_2 concentration reached approximately 200 µM. The rate of O_2 evolution was expressed both on a fresh leaf weight basis (nmol O_2 evolved g^{-1}fr.wt sec^{-1}) and on chlorophyll content (nmol O_2 evolved mg^{-1} chl. sec^{-1}).

2.12. Preparation of chloroplasts from pea leaves

The procedure was similar to that described by Robinson and Wiskich (1977). Pea leaves (60-80 g) were ground in a Polytron blendor (Kinematica, GmbH, Model K, Krie-Luzein, Switzerland) with a probe PTA-35/2 (setting # 3-4) for 2 to 3 sec in 200 ml of ice-cold medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM NaCL, 0.5 mM NaH_2PO_4, 2 mM isoascorbate, 50 mM MES, and 0.4% BSA adjusted to pH 6.2. The brei was squeezed through a double layer of miracloth containing a layer of cotton wool, and the filtrate was centrifuged at 2000 g_{av} for 30 sec in an M.S.E. Super Minor centrifuge. The crude chloroplast pellet was resuspended in 6-8 ml of wash medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM NaCl, 0.5 mM NaH_2PO_4, 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and 0.4% BSA adjusted to pH 6.4.

For intact chloroplasts, this resuspended pellet was loaded onto a 4 ml resuspension medium consisting of 40% Percoll and recentrifuged at 2500 g_{av} for 1 min. The pellet was suspended in 1 ml of the wash medium using a smooth brush. All procedures were carried out at 2°C using chilled solutions and apparatus.

For grana preparation, the pellet of intact chloroplasts above was resuspended in 50 ml of a medium containing 100 mM sorbitol and 5 mM $MgCl_2$ and centrifuged at 4000 g_{av} for 5 min. The final pellet was resuspended in 2-3 ml resuspending medium consisting of 100 mM sorbitol, 5 mM $MgCl_2$, and 0.5% BSA [w/v].

2.13. Measurements of photosynthesis

2.13.1 CO_2-dependent O_2 evolution measurement

CO_2-dependent O_2 evolution was measured polarographically at 25°C in a Rank O_2 electrode illuminated as described in Section 2.11. A volume of intact chloroplast suspension to give 100~200 μg chlorophyll was added to 3 ml reaction medium consisting of 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 mM HEPES adjusted at pH 7.6. Before adding the chloroplasts and 4 mM $NaHCO_3$, the assay medium was bubbled with nitrogen to reduce the O_2 concentration to about 20% saturation.

2.13.2. Electron flow

2.13.2.1. Non-cyclic electron flow

Non-cyclic electron flow was measured in an O_2 electrode. The vessel was illuminated with a 150 W light projector giving a light intensity of 200 $\mu\varepsilon\ m^{-2}\ s^{-1}$ at the centre of the vessel. The vessel was maintained at 25°C. A volume of grana to give 30~50 μg chlorophyll was added to 3 ml reaction medium consisting of 100 mM sorbitol, 10mM HEPES, 5 mM $MgCl_2$, 10 mM NaH_2PO_4, 1.3 mM FeCN all at pH 7.6. Before adding the grana, the assay medium was bubbled with nitrogen to reduce the O_2 concentration to about 20% saturation. The effects of 5 mM NH_4Cl and 1.7 mM nigericin were studied. The rate of electron flow was expressed in units of nmol O_2 evolved $mg^{-1}chl.min^{-1}$.

2.13.2.2. Pseudocyclic electron flow

Pseudocyclic electron flow was measured in an electrode maintained at 25°C and illuminated with a 150 W light projector giving a light intensity of 200 με m^{-2} s^{-1} at the centre of the vessel. A volume grana to give 30~50 μg chlorophyll was added to 3 ml reaction medium consisting of 100 mM sorbitol, 10 mM HEPES, 5 mM $MgCl_2$, 10 mM NaH_2PO_4, 0.3 mM MV, 3.3 mM azide at pH 7.6. The effects of 5 mM NH_4Cl was studied. The rate of electron flow was expressed in units of nmol O_2 uptake mg^{-1} chl.min^{-1}.

Chapter 3. UV-B absorbing compounds and morphological characteristics of pea and wheat plants irradiated with UV-B

3.1. Introduction

Plants may be particularly predisposed to damage by enhanced UV-B because their leaves are positioned to intercept large amounts of photosynthetically-active solar radiation. When exposed to UV-B radiation, they display a variety of changes in their physiology and morphology, some of which are protective mechanisms.

The attenuation of UV-B radiation by outer layers of leaf tissue is one possibility for reducing the UV-B flux received at potentially sensitive sites. Leaf surface reflectance would provide a first line of defence in preventing UV-B from reaching internal chromophores, but in the vast majority of plants UV reflectance is low (<10 %) and epidermal attenuation appears to be the dominant UV-B screening mechanism (Robberecht *et al.,* 1980). By increasing the amount of UV-B absorbing compounds, such as flavonoids and anthocyanin, plants can alleviate the harmful effects of UV-B light (Caldwell *et al.,* 1983; Beggs *et al.,* 1986; Murali *et al.*, 1988; Tevini *et al.,* 1991; Reddy *et al.,* 1994). These compounds, which absorb strongly in the UV-B but transmit visible or photosynthetically active radiation are thought to act as protective pigments in shoots and leaves (Day *et al.,* 1992).

The UV-B absorbing compounds are a group of phenylalanine-derived aromatic secondary products (Li *et al.,* 1993) which generally accumulate in the vacuoles of the upper or sub-epidermal cells (Clark and Listen, 1975; Cen and Bornman, 1993), or even in chloroplasts (McClure, 1976). In general, species relatively resistant to UV-B radiation contain high concentrations of flavonoids while sensitive species contain very little.

The morphological features of plants also can be altered by a change in their environment. The adaptation of herbs and grasses to different physical environments (temperature, water supply and light intensity) is due to differences in morphological, anatomical and biochemical characteristics (Björkman and Berry, 1973; Black, 1973). An increase in UV-B may either inhibit or stimulate plant growth (Dai, *et al*, 1994; Krizek *et al.,* 1994). Growth characteristics such as plant height, leaf area, dry matter production and biomass accumulation were reduced to

various extents with increased UV-B radiation (Teramura, 1983; Tevini and Teramura, 1989; Tevini *et al*, 1989; Teramura *et al.*, 1990; Dai *et al.*, 1994; Krizek *et al.,* 1994). This also has been observed in the agronomic species of wheat, barley, soybean, tomato, cucumber, lettuce and rice (Krupa and Kickert, 1989; Dai *et al.,* 1994). This reduction is because UV-B radiation is mostly absorbed by nucleic acids, proteins and some pigments in the plant system altering their functions. However, alterations in morphology are not always associated with reduced dry matter production or plant height for some species (Barnes *et al.*, 1988, 1990). Overall, the effectiveness of UV-B and the degree of UV-B induced change varies among species, maturity or their sensitivity to UV-B radiation (Tevini and Teramura, 1989). For example, it has been suggested that plants may be particularly sensitive to radiation before full leaf expansion, when great metabolic activity and growth are still occurring (Teramura and Caldwell, 1981).

In the present study, morphological changes in leaves (colour, fresh weight, area), the above ground height, fresh weight and dry weight of plants that might serve as suitable criteria for identifying sensitive and less-sensitive species were examined. The UV absorption of pea and wheat leaf extracts at 280, 300, and 320 nm was also examined.

3.2. Results

3.2.1. Absorbance at 280, 300 and 320 nm

The absorbance of UV-B absorbing compounds were measured at three single wavelengths (280, 300 and 320 nm), as a lowest, middle and highest peak of UV-B radiation wavelengths. Treatment of pea plants with UV-B radiation resulted in higher flavonoids levels in their leaves (Fig.3.1). This trend was evident in both January and March plantings but the overall level of UV-B absorbing compounds was much higher in the summer crop (January). The high UV-B treatment consistently induced the highest level of UV-B absorbing compounds over 12 days of treatment (Fig.3.1B). Measurements were made at a single wavelength of 300 nm because there is a relationship between 300 nm absorbing compound concentrations and the penetration of 300 nm in foliage (Day *et al.*, 1994). The increase in the accumulation of UV-B absorbing compounds with increasing time of radiation was observed in both pea and wheat (Fig.3.2).

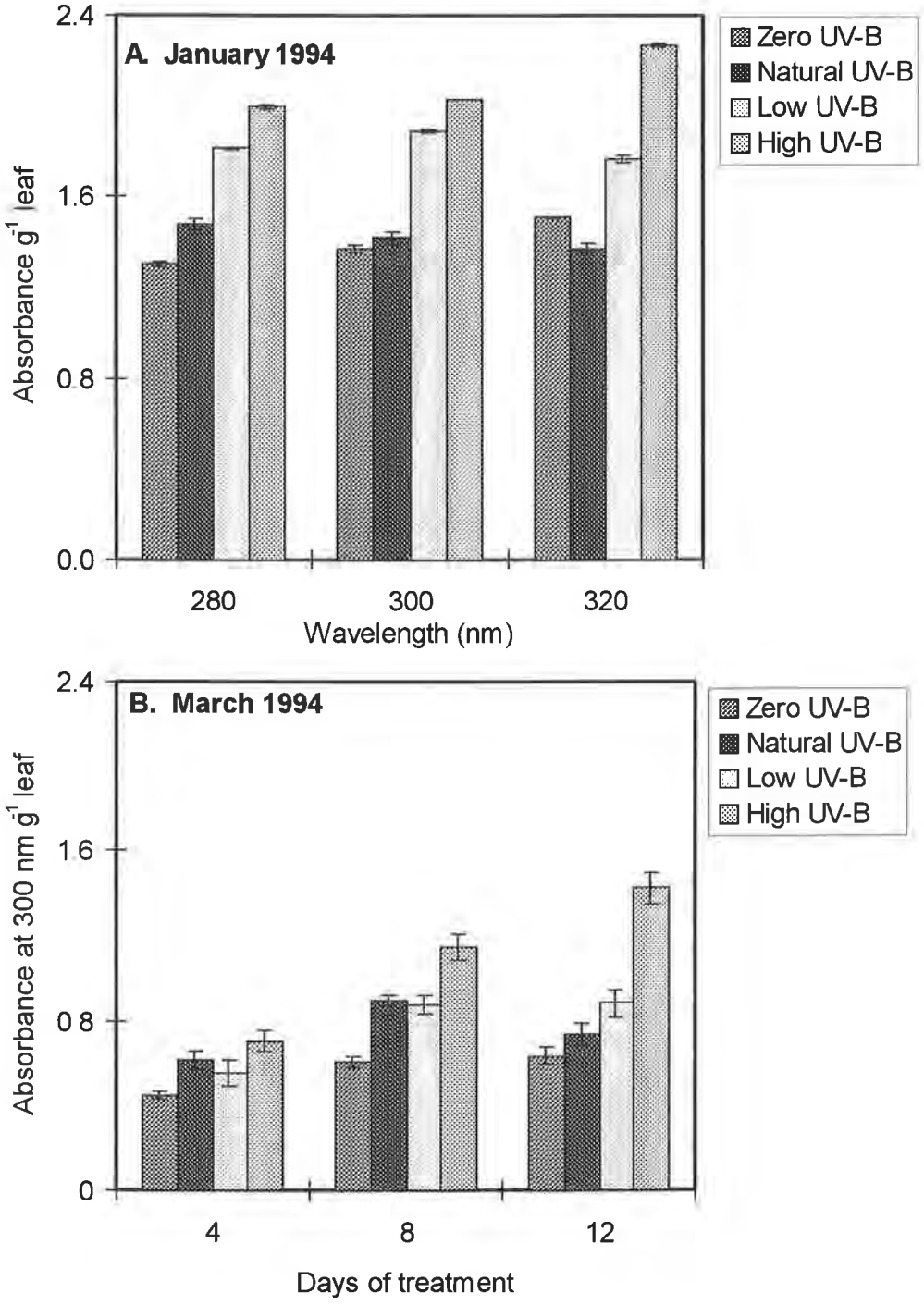


Fig. 3.1. UV-B-absorbing compounds in ethanolic extract from the third pair of pea leaf determined in January 1994 (after 12 days treatment, A) and March 1994 (B). Values are means of 6 replicates from at least two independent experiments.

For the first leaves, the absorbance in wheat were much more enhanced after 12 days treatment than that in peas, even though the absorbance in peas after 4 days exposure was higher than in wheat (Fig.3.2). A similar trend was obtained for absorbance in the second leaf (Fig.3.2C,D). However, the third leaves of both species had similar absorbances at 8 and 12 days of supplementary UV-B irradiation (Fig.3.2E,F). In general, the rate of increase in UV-B absorbing compounds was much greater in low and high UV-B treated plants than in control plants.

3.2.2. Anthocyanin content

Anthocyanins were estimated as the absorbance at 535 nm of acidic propanol extracts (Fig.3.3). In this experiment, wheat synthesized more anthocyanins than peas in all leaves assayed. In the first pair of pea leaves, there was no increase in anthocyanin content up to 8 days treatment (Fig.3.3A). However, after 12 days, differences among the treatments in the accumulation of this pigment become apparent. This trend was also found in the second leaf pair (Fig.3.3C). UV-B radiation appeared to have no effect on the antocyanins content within pea leaves, as well as observed in the third leaf pair (Fig.3.3E).

In the first wheat leaf, anthocyanins were remarkably constant after 8 and 12 days treatment (Fig.3.3B). Anthocyanins in the second leaf was variable but highest after 12 days exposure under supplementary UV-B radiation (Fig.3.3F).

Overall, there was no increase in some extent to the accumulation of anthocyanins under UV-B treatment in relation to the control, there appeared to be no significant relationship between the accumulation of anthocyanins and UV-B treatment.

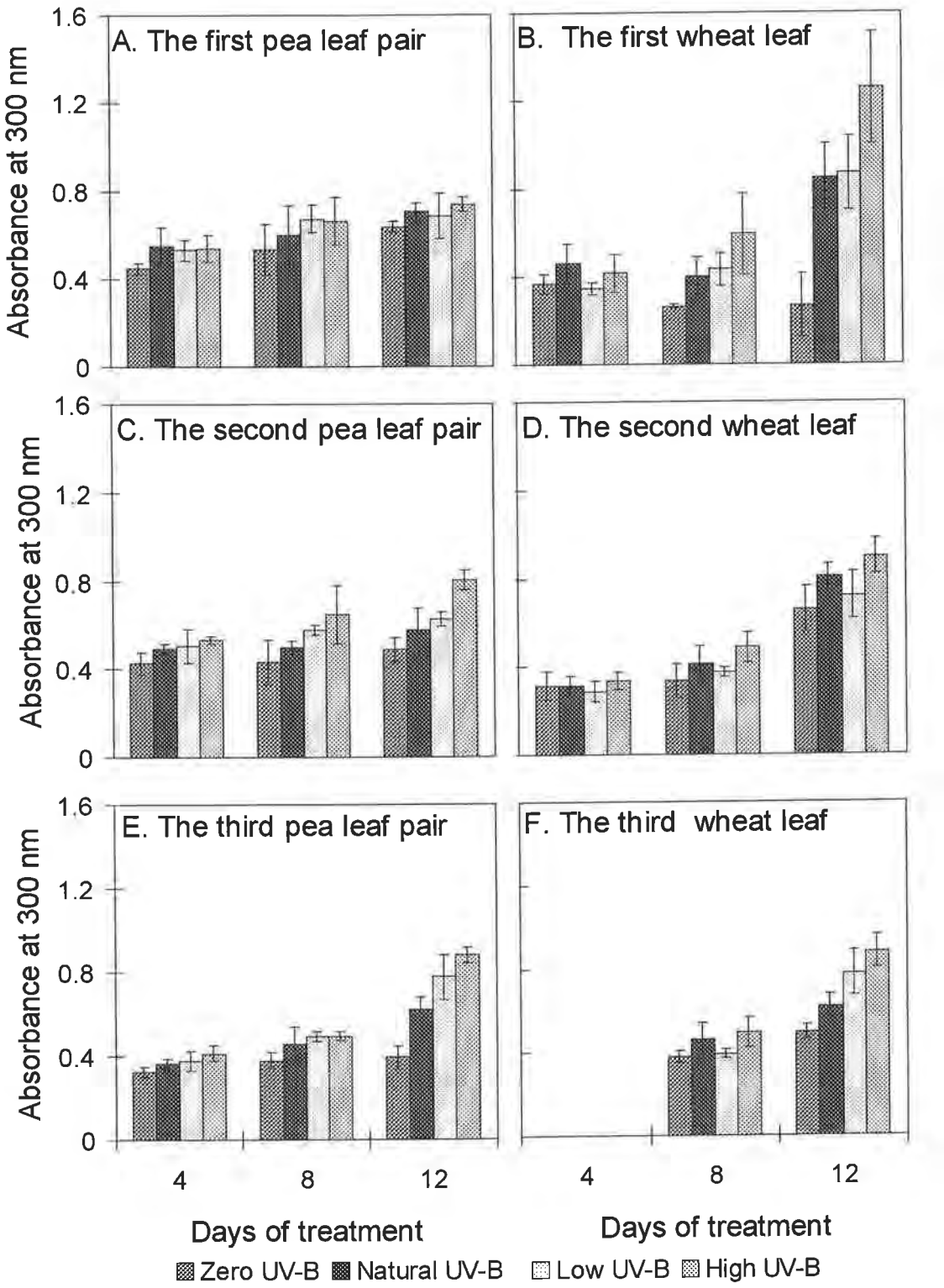


Fig.3.2. UV-B-absorbing compounds determined as absorbance at 300 nm in ethanol: acetic acid (99:1) extracts on a leaf area basis from leaves of pea (A, B, and E) and wheat (B. D, and F) after 4, 8 and 12 days treatment. Values are means of 6 replicates from at least two independent experiments.

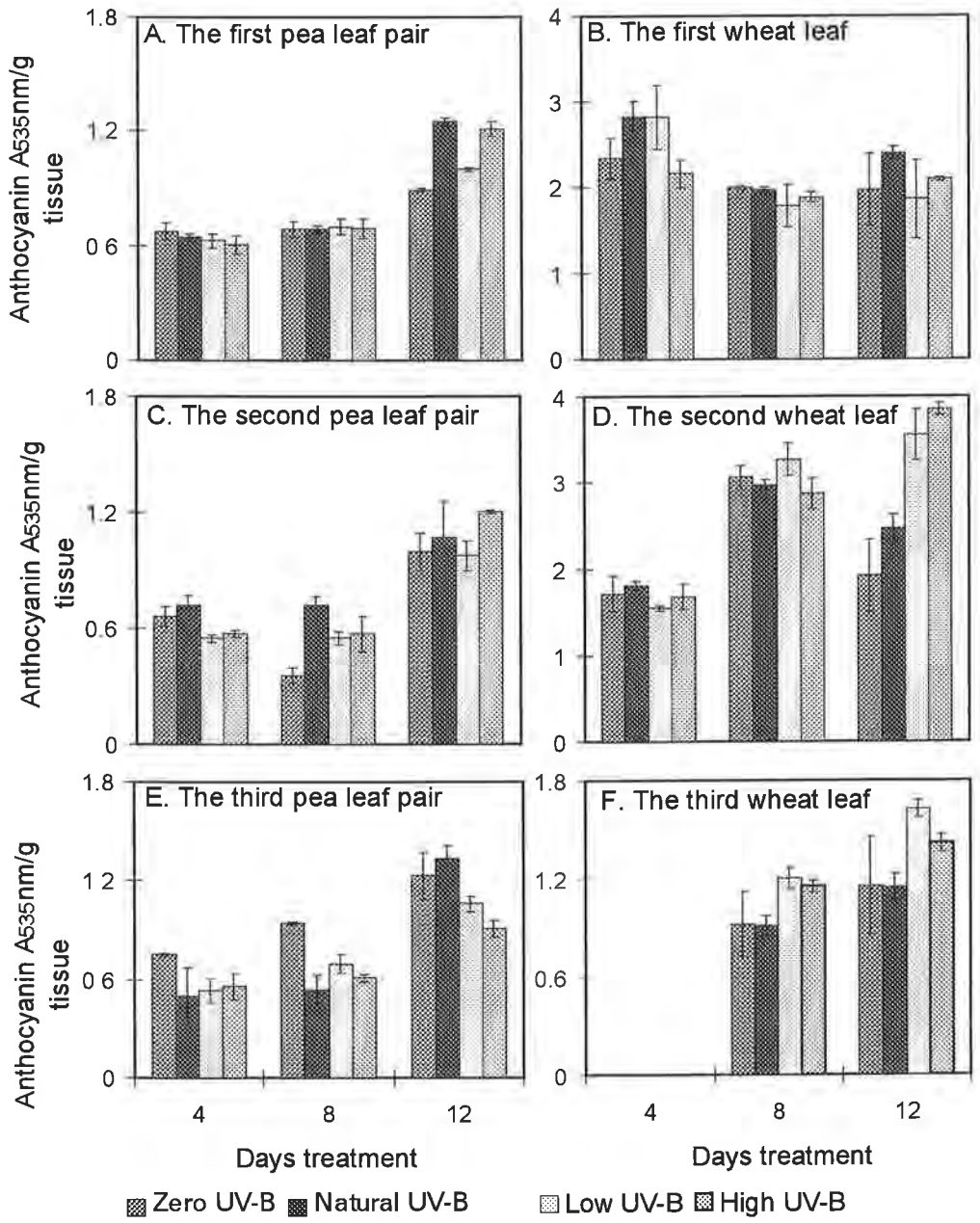


Fig.3.3. Anthocyanin contents determined as the absorbance at 535 nm of an extract of pea (A, C, and E) and wheat (B, D, and F) leaves of as a function of days of UV-B treatment. Values are means of 6 replicates from at least two independent experiments.

3.2.3. Leaf morphology

3.2.3.1. Peas

The leaves of control plants developed normally with fully expanded, flat laminae of a fresh green colour (Fig.3.4 and 3.5). Fresh and green stems were also evident. Natural UV-B plants were not as green as control plants, they showed more white spots on the adaxial leaf surface, and looked paler and tougher than leaves of the control plants (Fig.3.5). In contrast, plants subjected to low and high supplementary UV-B showed visible changes in their leaf morphology. After 4 days treatment, the third leaf pair was wavy, rather than flat, and was partially bronzed with brown and black spots on both the adaxial and abaxial leaf surfaces. The waviness started at the leaf tip, and spread down both margins which became brown and dry. The leaf margin became involuted. Furthermore, with continued irradiation the brown and black spots continued to enlarge on the adaxial leaf surfaces. Bronzing was observed much more on the adaxial than the abaxial leaf surface and was particularly evident in the high UV-B plants . In this treatment, bronzing appeared as brown to dark shiny yellow colours at the lamina margin, which extended inwards and by day 12 had developed parallel to the midrib. These effects were less apparent in the low UV-B plants and absent in both control plants and plants grown under natural UV-B (Fig.3.5).

3.2.3.2. Wheat

UV-B radiation had no effect on wheat leaf morphology after 8 days of treatment (Fig.3.6). Leaf laminae were still flat in shape and mostly green in colour, though a little yellowing at their tips and some drying could be noticed.

Fig.3.4. Pea plants after 8 days treatment (top). Close-up of the same plants (bottom).

(a). Control (Zero UV-B)

(b). Natural UV-B

(c). Low UV-B

(d). High UV-b

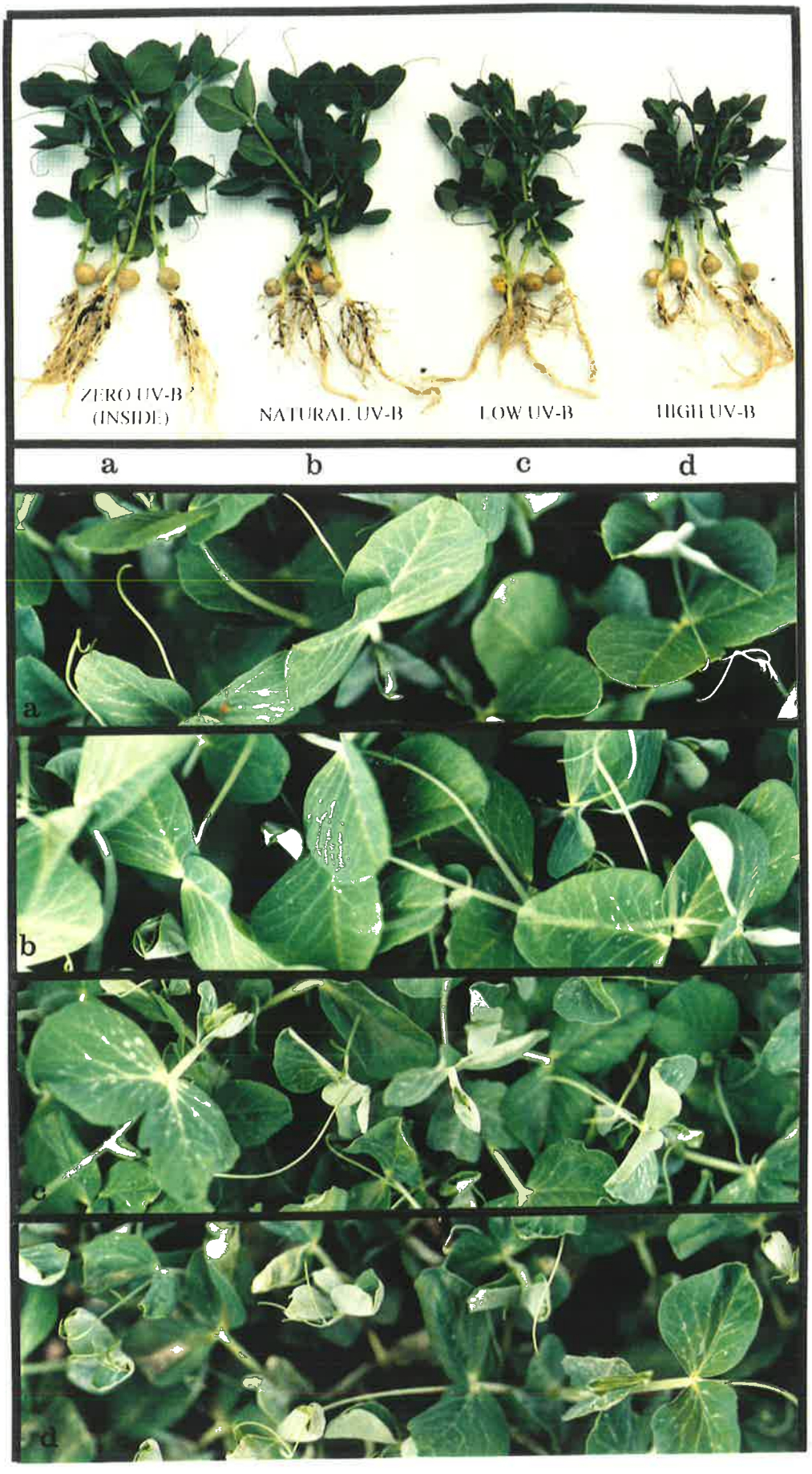

ZERO UV-B
(INSIDE)
NATURAL UV-B
LOW UV-B
HIGH UV-B
a
b
c
d
a
b
c
d

Fig.3.5. The third pair of pea leaves after 8 days treatment.

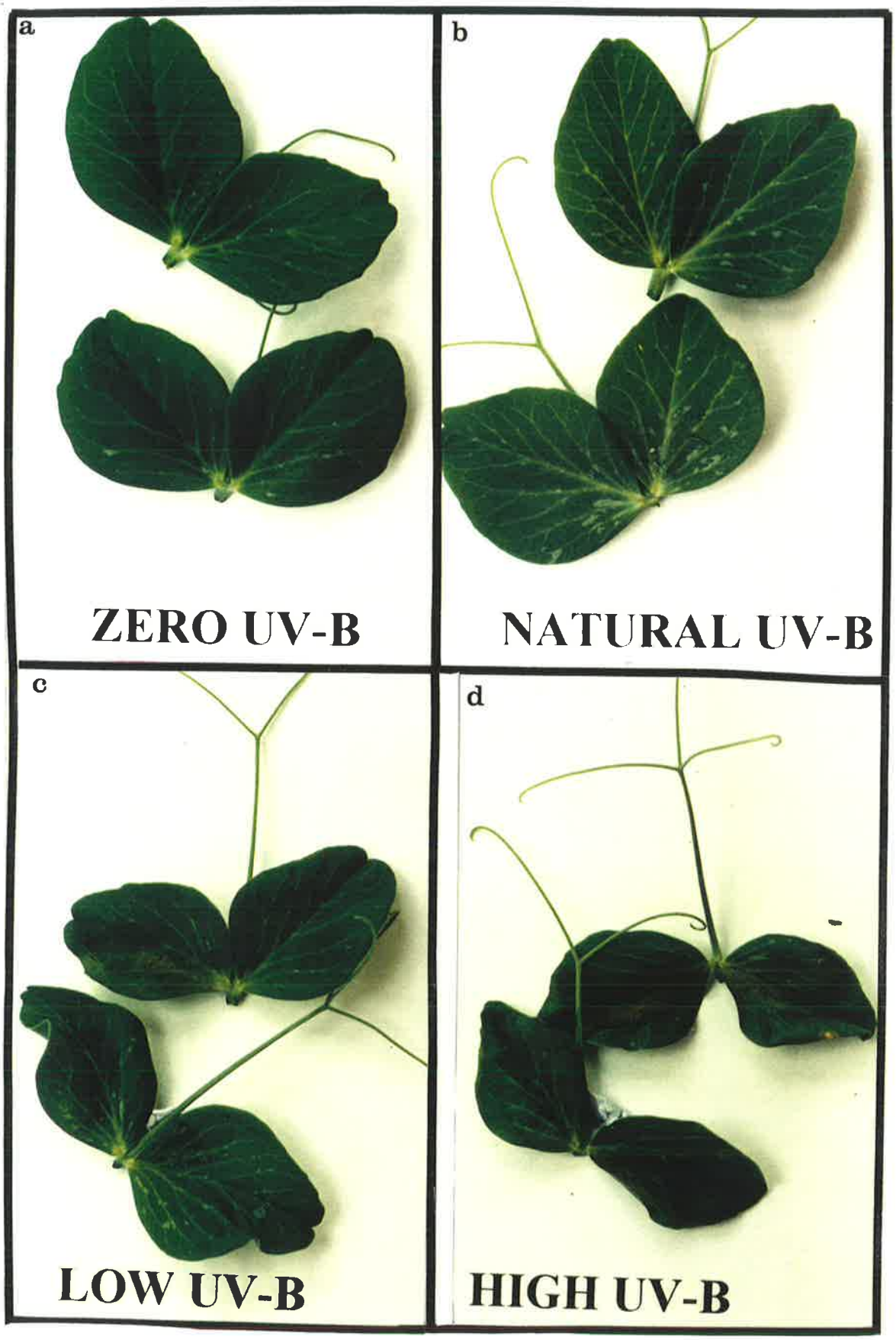
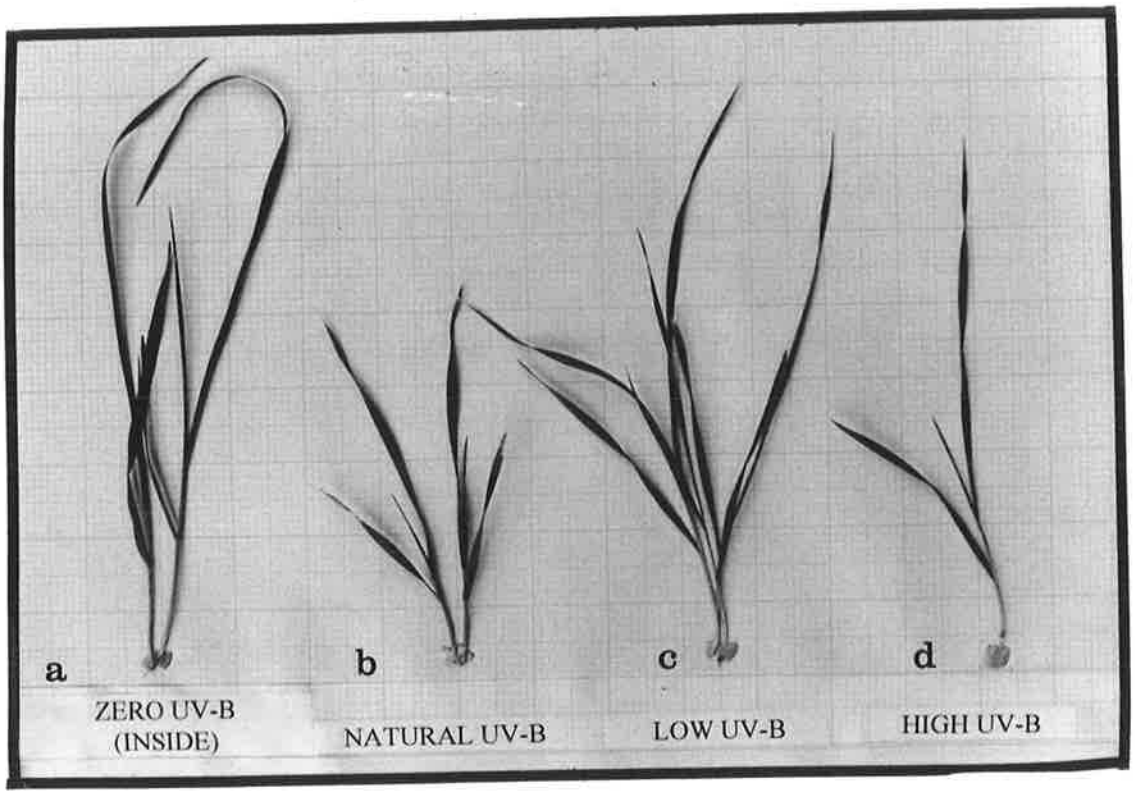

a
ZERO UV-B
b
NATURAL UV-B
c
LOW UV-B
d
HIGH UV-B

Fig.3.6. Wheat plants after 8 days treatment.


a
ZERO UV-B
(INSIDE)
b
NATURAL UV-B
c
LOW UV-B
d
HIGH UV-B

3.2.4. Plant height

The pattern of above ground plant height during development differed between peas and wheat (Fig.3.7). For peas, after 4 and 8 days treatment, the above ground plant heights were not significantly different (Fig.3.7A), even though after 8 days control plants appeared to be taller than treated plants. With continued treatment, plants grown under natural and low UV-B grew much less and those under high UV-B radiation actually showed a reduction in height. This reduction was more pronounced and significant in low and high UV-B plants, but not in natural UV-B plants.

For wheat, the control plants were always taller than treated plants at all ages. The reduced growth of treated plants was evident after 4, 8 and 12 days treatment. Although there was no significant difference among the various UV-B treatments (Fig. 3.7B), they were significantly shorter than the control.

3.2.5. Plant fresh weight

In general, above ground fresh weight of pea plants increased with age for all treatments. Wheat also showed an increase in fresh weight after 8 days. However, UV-B radiation had no significant effect on either pea or wheat above ground fresh weights at any age (Fig. 3.8), except high UV-B reduced the fresh weight gain after 12 days.

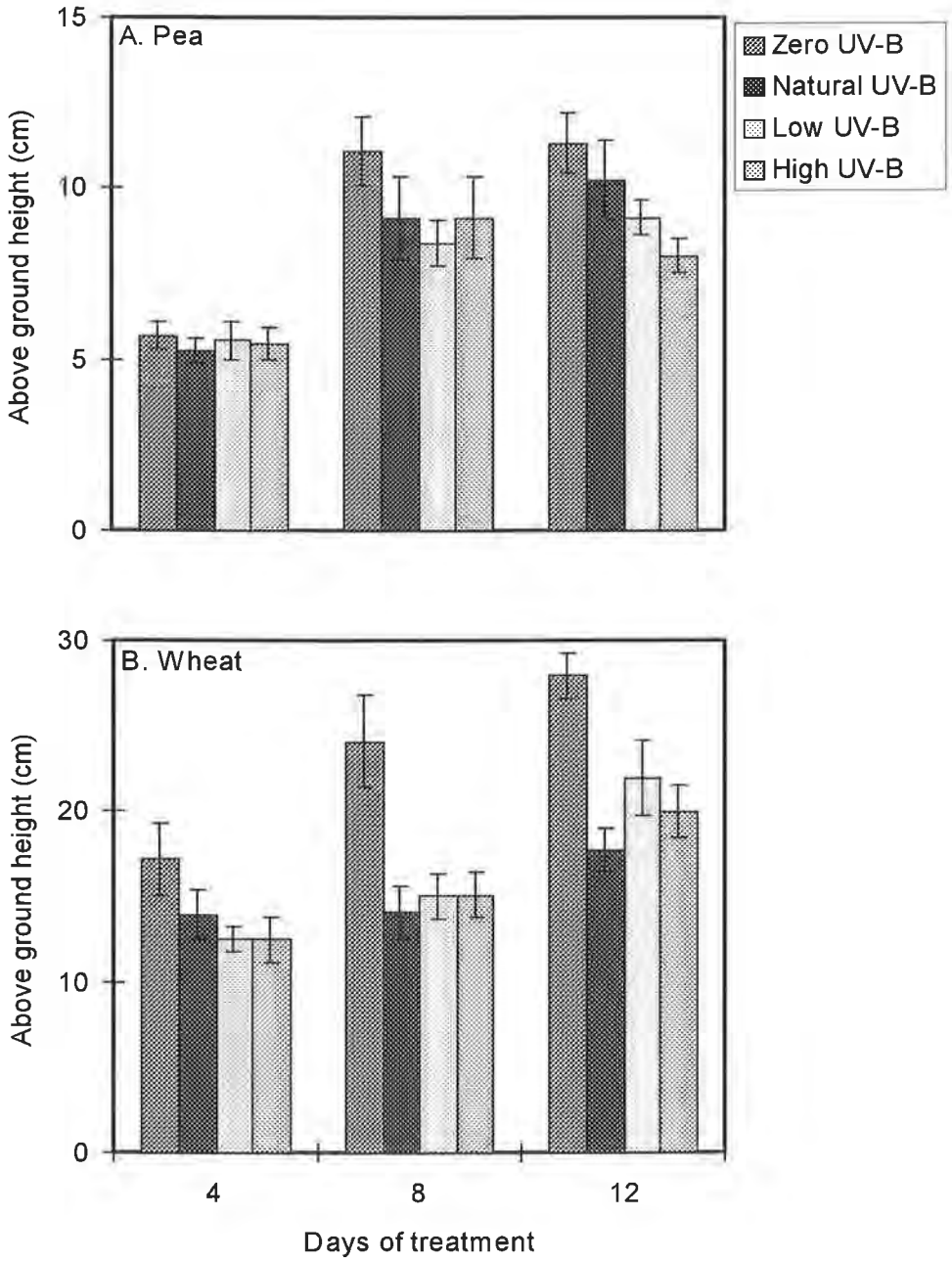


Fig. 3.7. Above ground height of peas (A) and wheat (B) after 4, 8, and 12 days treatment. Each value is the mean of 10 replications.

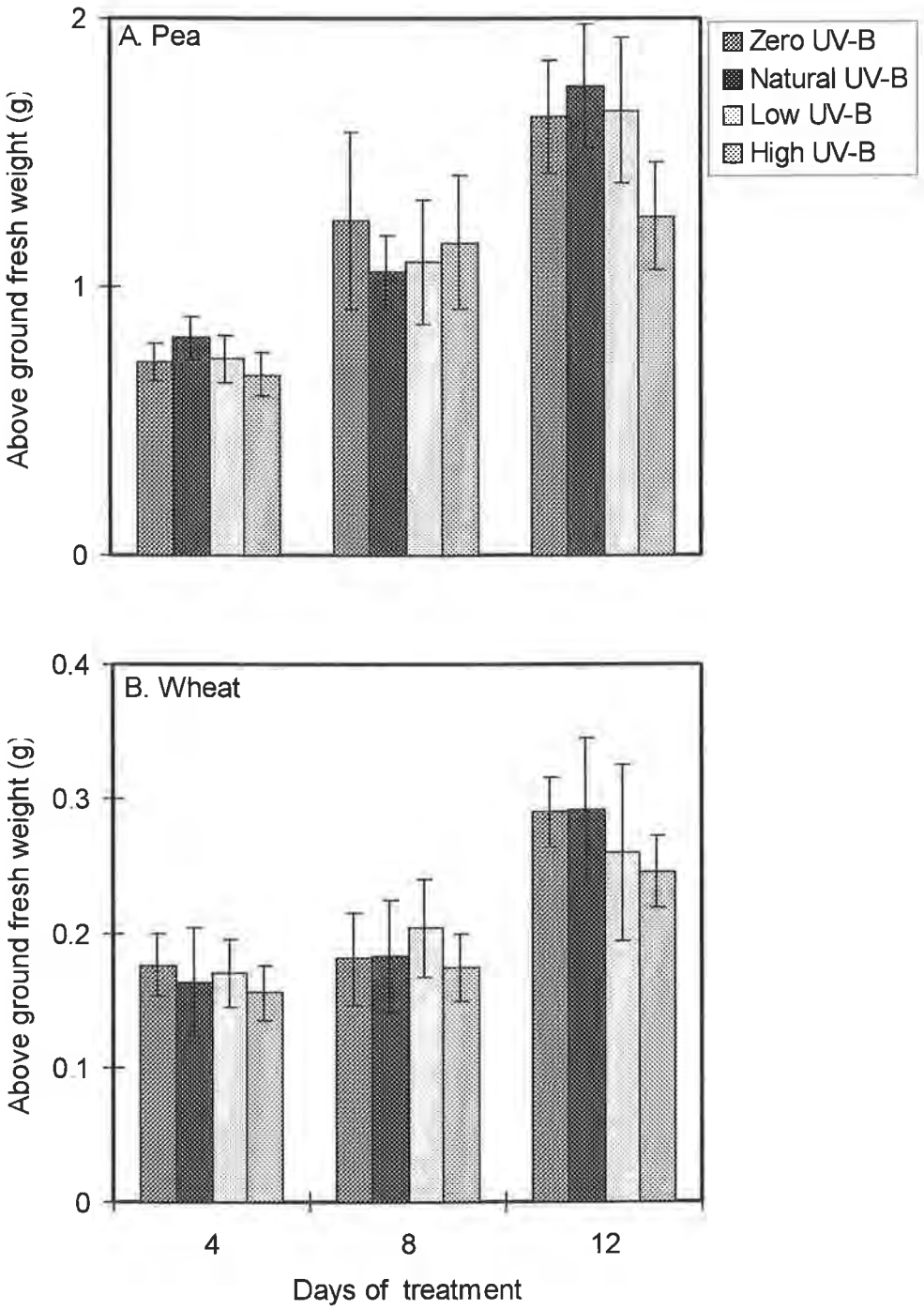


Fig. 3.8. Above ground fresh weight of peas (A) and wheat (B) after 4, 8, and 12 days treatment. Each value is the mean of 10 replications.

3.2.6 Length of the second internode

The second internode length of pea plants increased with plant age (Fig.3.9), but the increase was more apparent in the control than in the UV-B treated plants. UV-B radiation tended to reduce the elongation of the second internode. In comparison with control plants, significant reductions were observed after 8 days and 12 days for all UV-B treatments.

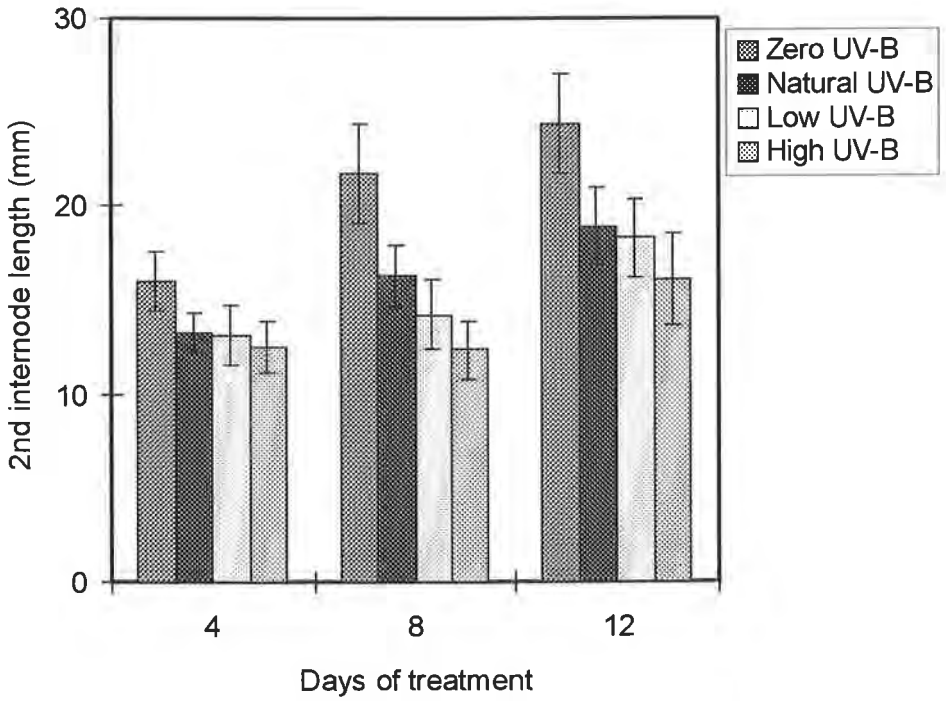


Fig. 3.9. The 2nd internode length of pea plants after 4, 8, and 12 days treatment. Each value is the mean of 10 replications

3.2.7. Plant dry weight

In pea plants, the above ground dry weight tended to increase with plant age, especially after 8 days treatment (Fig.3.10A). The dry weights of natural UV-B plants were always greater than those of the control, but it was not significant. After 12 days, the dry weight of the low UV-B plants was similar to that of the natural UV-B plants, while the dry weight of the high UV-B plants was about the same as that of the control. Wheat plants showed no significant changes over 8 days treatment. After 12 days exposure (Fig. 3.10B), an increase in plant dry weight was only observed under natural UV-B treatment.

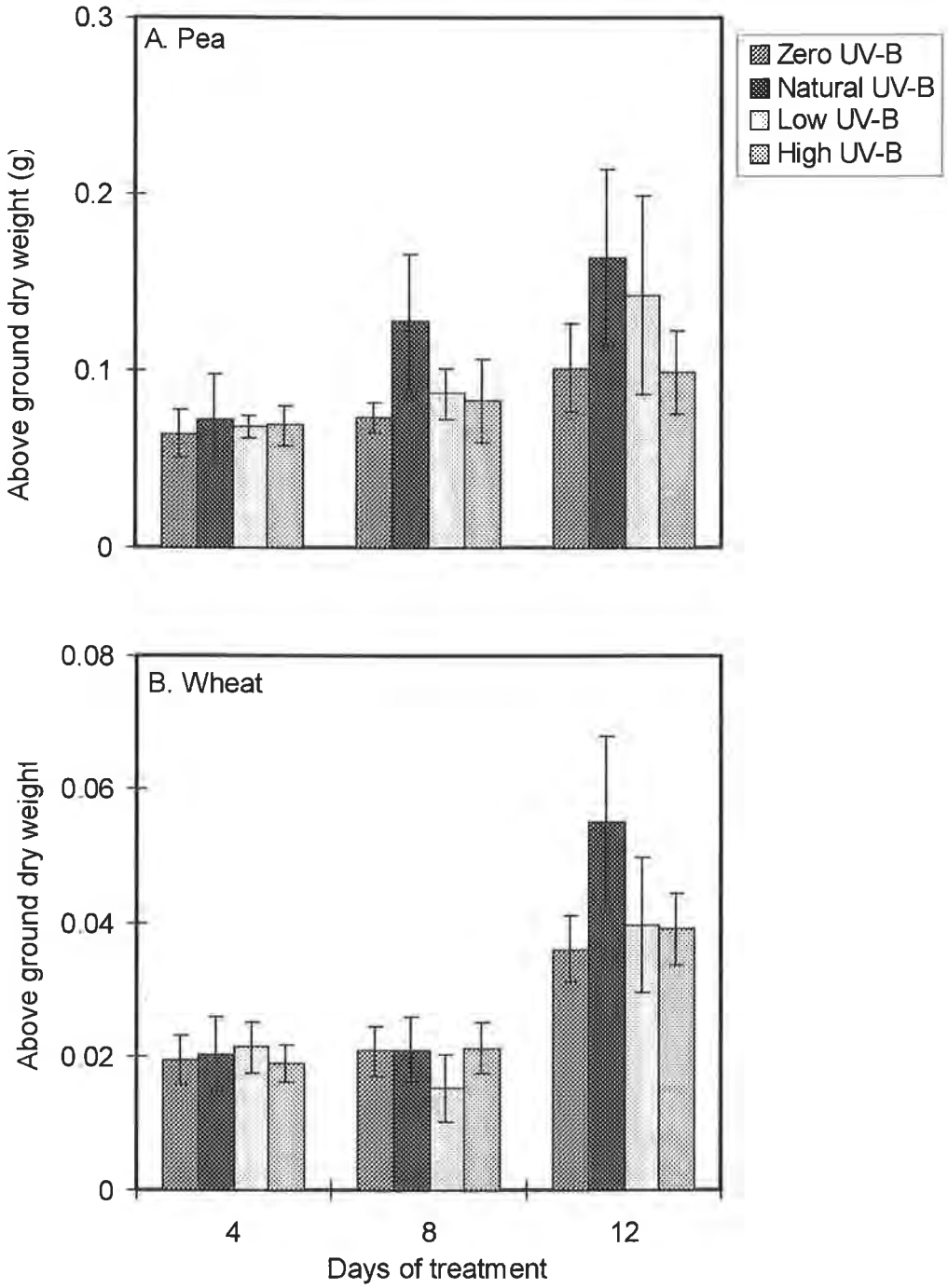


Fig. 3.10. Above ground dry weight of peas (A) and wheat (B) after 4, 8 and 12 days treatment. Each value is the mean of 10 replications.

3.2.8. Leaf fresh weight and leaf area

3.2.8.1. Peas

The measurements of fresh weight and area of the third leaf pair in peas are shown in Fig.3.11. In general, there both parameters increased with age but increased less with UV-B treatment. Relative to control, both leaf fresh weight (Fig.3.11A) and area (Fig.3.11B) were less under UV-B treatment after 8 and 12 days treatment. However, only high UV-B radiation was significantly less throughout the treatment.

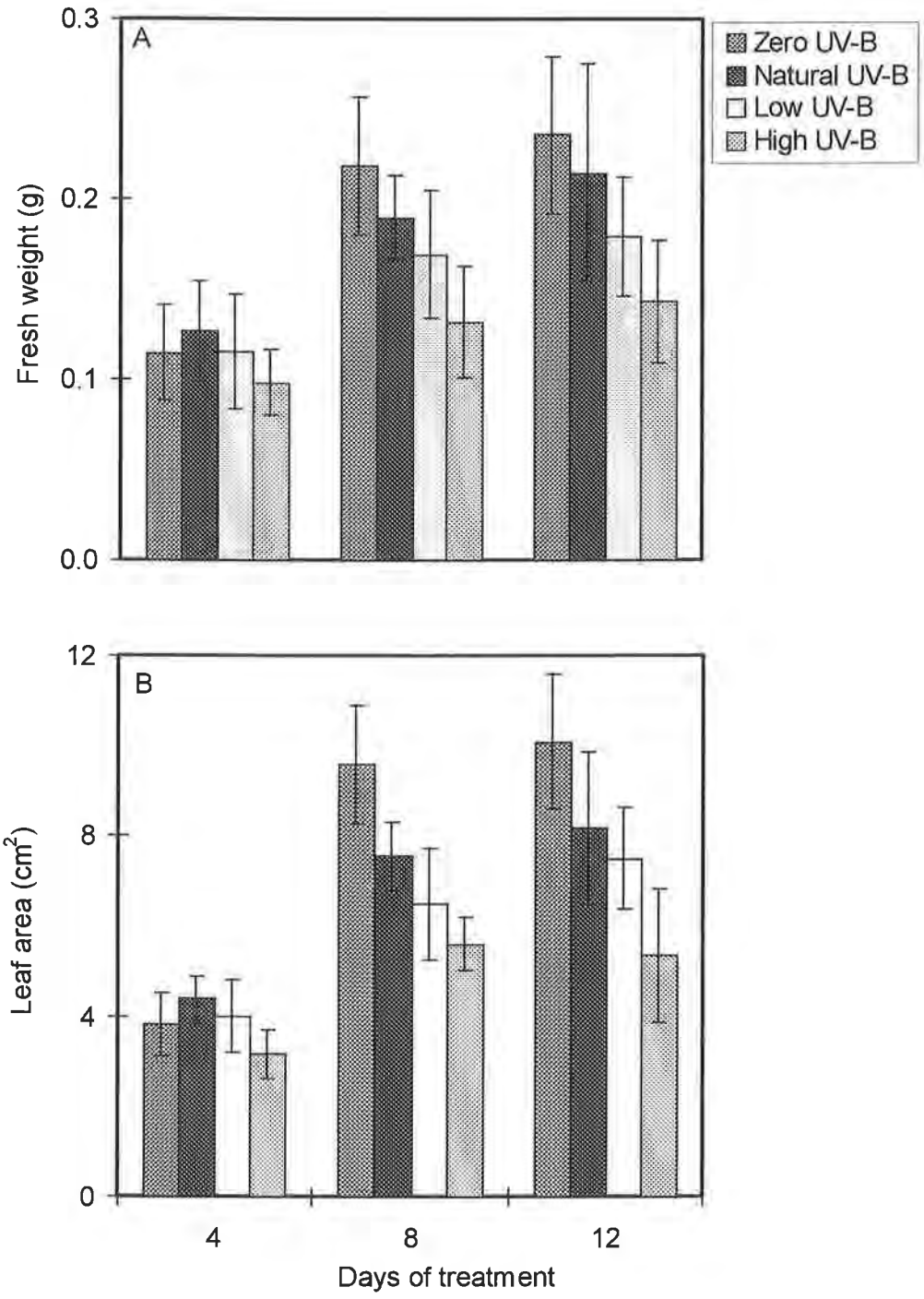


Fig. 3.11. The 3rd leaf pair fresh weight (A) and area (B) of peas after 4, 8, and 12 days treatment. Each value is the mean of 10 replications.

3.2.8.2. Wheat

In wheat, the second leaf fresh weight increased with plant age but only slightly under UV-B treatment (Fig.3.12). Even though UV-B treated leaves always weighed less than the controls, only under high UV-B was fresh weight significantly less.

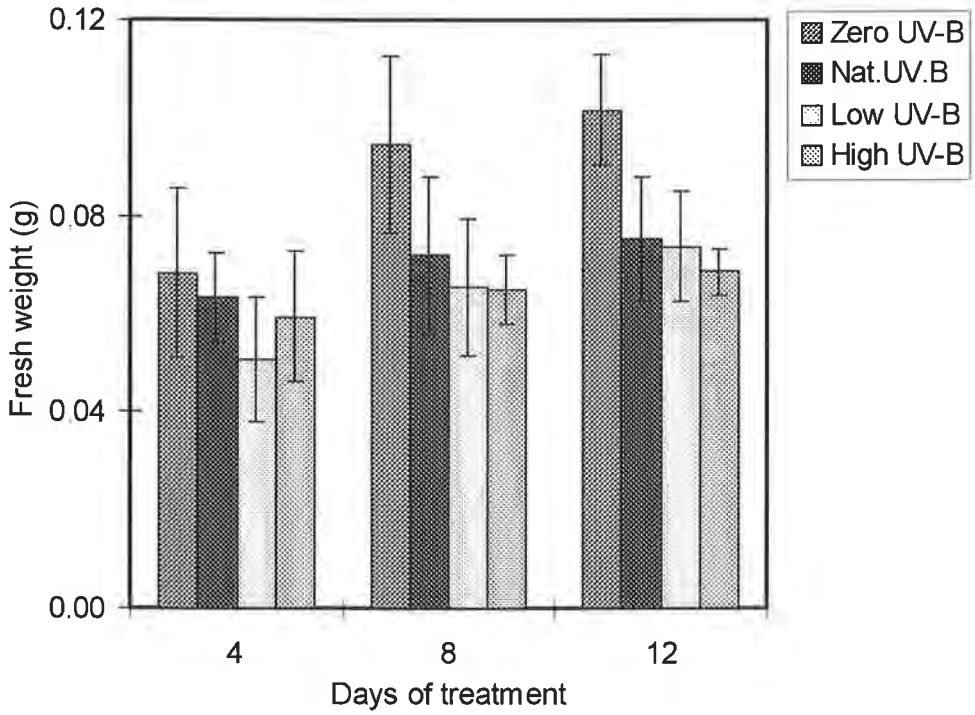


Fig. 3.12. The 2nd leaf fresh weights of wheat after 4, 8, and 12 days treatment. Each value is the mean of 10 replications. .

3.3. Discussion

3.3.1. UV-B absorbing compounds

Plants have different mechanisms for responding to UV-B radiation. It has been suggested that one mechanism which accounts for the different responses of different species to UV-B radiation is the accumulation of UV-B absorbing compounds in their leaves (Robberecht and Caldwell, 1978; Tevini *et al.*, 1991). These pigments which mainly absorb light between 230 and 380 nm, are a group of aromatic secondary products (phenylpropanoids) derived from phenylalanine. They are water soluble and colourless flavonoids including flavones, flavonols and isoflavonoids (Hahlbrock and Scheel, 1989; Strid and Porra, 1992). In the present study, the production of UV-B absorbing compounds was stimulated by UV-B

radiation in peas and wheat. This finding is in agreement with previous results for peas (He *et al.,* 1993 and 1994; Jordan *et al.*, 1994) and wheat (Teramura *et al.*, 1990). Furthermore, the accumulation of UV-B absorbing compounds on January was higher than on March. This may because a longer period of daylight occurs in January than in March, so it will allow more light to penetrate into the foliage and stimulate the production of flavonoids.

The increase in UV-B absorbing compounds under UV-B radiation may indicate a similar UV-B induction stimulus for their biosynthesis. The synthesis of UV-B absorbing compounds requires the presence of phenylalanine ammonia lyase (PAL); an enzyme regulating the first reaction of phenylpropanoid synthesis (Hahlbrock and Scheel, 1989). Krizek *et al.* (1993) found an increase in PAL in cucumber seedlings after 8 and 15 days of UV-B irradiation. A similar result was also found by Stafford (1990) in soybean cell suspension cultures, where flavonoid accumulation was linked to UV-B induced activation of chalcone synthase and the flavonoid synthesis pathway. The pathway of flavonoid synthesis is summarized in Fig. 3.13. Induction of flavonoids is mediated by the induction of genes of the phenylpropanoid pathway which induce the synthesis of the enzymes (Hahlbrock and Scheel, 1989). Both protein and *chs* mRNA have been found to increase within epidermal cells as flavonoids accumulate (Schmelzer *et al.*, 1988)

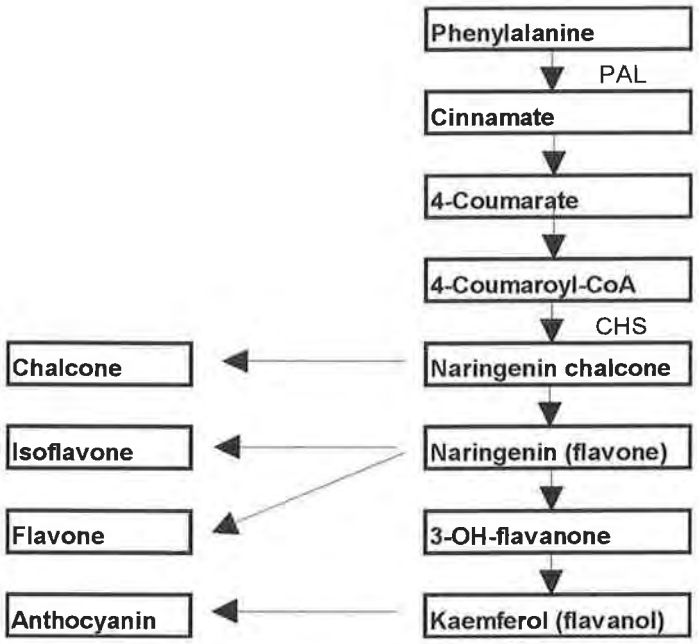


Fig.3.13. A simplified pathway for the synthesis of flavonoids derived from phenylalanine in plants (after Harbone, 1980). PAL (phenylalanine ammonia lyase), CHS (chalcone synthase).

Furthermore, anthocyanins which also belong to the flavonoids group may also accumulated under UV-B radiation, although they absorb maximally around 530 nm. Therefore, anthocyanins can provide UV-B protection. A high UV-B irradiance has been reported to increase anthocyanin production in sorghum (Drumm-Herrel and Mohr, 1981), apple skin (Arakawa *et al.,* 1985) and maize (Beggs and Wellman, 1985; Khare and Guruprasad, 1993). In the present study, there was no difference in the anthocyanin content between control and treated leaves either in peas or wheat. This finding is in contrast to a slight increase in anthocyanin production under supplementary UV-B radiation reported for sorghum (Yatsuhashi *et al.*, 1982) and maize (Khare & Guruprasad, 1993).

3.3.2. Morphological parameters

Peas grown under UV-B treatments showed bronzing, interveinal waviness and yellowing. These effects are consistent with findings previously reported for peas (Strid *et al.*, 1990), collards (Basiouny *et al.*, 1978) and soybean (Teramura, 1980). The changes in colour demonstrated threshold effects and the higher presence of UV-B-absorbing compounds. As they developed, UV-B treated leaves produced more UV-B absorbing pigment than the control leaves, even though the precise nature of these responses might be quite complex. Bronzing may also be followed by tissue degradation (Cen and Bornman, 1990). Interveinal waviness and yellowing were observed only if leaves were exposed to UV-B irradiance from early leaf expansion to maturity. Fully expanded leaves exposed to high UV-B irradiance did not develop these features. This suggests that the waviness is probably a result of UV-B effects on cell division or on cell expansion early in leaf development. Also, yellowing was manifested only in leaves which had expanded under UV-B radiation and not in fully expanded mature leaves. Hence UV-B radiation is probably interfering with normal proplastid differentiation rather than accelerating mature leaf senescence (Teramura *et al.*, 1980b).

Grasses were found to have a better ability to screen UV-B radiation from reaching the mesophyll than herbaceous dicots (Day *et al.*, 1992). In this study, wheat leaves were not affected by UV-B radiation. Higher percentage of the UV-B screening ability, higher amount of UV-B absorbing compounds and leaf positions may all contribute to the resistance of wheat plants to UV-B radiation. The higher the epidermal screening effectiveness, the more UV-B radiation will be attenuated. Flavonoids and related phenolic compounds, which absorb strongly in the UV-B region, appear responsible for epidermal attenuation and underlying tissue

region, appear responsible for epidermal attenuation and underlying tissue protection (Tevini *et al.*, 1991; Reedy *et al.*, 1994). The position of wheat leaves which are nearly vertical would allow less UV-B radiation to penetrate into leaves or a greater UV-B radiation being reflected from leaves.

The mean values of plant height of pea and wheat plants under low and high UV-B treatments were significantly lower than those of control plants after 12 days treatment. The reduction in plant height was also observed in collards and sorghum (Basiouny *et al.*, 1978), wheat (Teramura, 1980b, Barnes *et al.*, 1988, 1990), soybean (Sullivan and Teramura, 1990; Reed *et al.*, 1992), rice (Teramura *et al.*, 1991; Dai *et al.*, 1994) and cassava (Ziska *et al.*, 1993). A decrease in plant height is a characteristic commonly observed in sensitive species after UV-B exposure (Biggs and Kossuth, 1978). In pea, a sensitive species, the slight reduction in plant height under UV-B treatments was reflected in a reduction in internode length and not in node number. This suggested that UV-B radiation did not simply delay the rate of plant development but involved some intrinsic growth characteristic.

In this study, the taller control plants had higher fresh weight, but similar dry weight to high UV-B treated plants, indicating that taller plants probably resulted from differences in cell elongation, and were not due to dry matter production. In addition, the taller plants may consist of more water relative to dry matter. Therefore, the reduction in plant height under UV-B may be due to decreased cell expansion and elongation within the stem. As well, the greater height and cell elongation in control plants may be associated with lower intensity and narrower range of wavelengths received during development, as they grew in the glasshouse without any UV. By day 12, the control plants looked weaker than natural, low and high UV-B treated plants, suggesting that they probably lacked mechanical tissue. Light is very important element for mechanical tissue development (Salisbury and Ross, 1992) . Since light is also crucial in regulating cell expansion and elongation, height should not be used alone as an indicator of UV-B stress.

Above ground fresh weight was slightly lower in treated plants after 12 days of treatment, but not significant by so. This may be due to a reduction in leaf thickness, leaf area, or may be merely due to the reduction in plant height. The reduction in above ground fresh weight supported results in the growth cabinet experiments previously found for peas (Brandle *et al.*, 1977), peanuts and soybean (Basiouny *et al.,* 1978). The quantitative differences found by various workers are

probably due to differences in growth conditions during treatment (See also Table 1.1).

UV-B has been shown to decrease leaf area in some sensitive plants, such as soybean cultivars (Sullivan and Teramura, 1990; Reed *et al.*, 1992), IR36 and Careon rice cultivars (Teramura *et al.*, 1991) and cucumber (Adamze and Britz, 1992). In contrast, the stimulation of leaf area was also observed in Fujiyama-5 rice cultivar (Ziska and Teramura, 1992), cassava (Ziska *et al.*, 1993) and cucumber (Krizek *et al.*, 1994).. In this study, there was a significant reduction in area of the third leaf pair of peas. The reduction of leaf area under UV-B treatment might be associated with cell division and expansion, as these leaves were exposed to UV-B during their active development since they were still closed.

Peas also respond to a supplementary UV-B environment by reducing their specific leaf weight, as do the second leaves of wheat. This agrees with pervious studies using wheat (Teramura, 1990) and rice cultivars (Dai *et al.* 1992, 1994).

The reduction of leaf area or weight indicates that these two parameters would be better parameters for the selection of UV-B tolerance than plant height. This conclusion was reached by Dai *et al.* (1994) who found that although rice cultivars showed a more significant reduction in plant height than in leaf area and plant dry weight under enhanced UV-B radiation, the sensitivity of plant height of rice to elevated UV-B radiation was much lower than that of leaf area and dry weight. The relation of the third leaf area and fresh weight with above ground fresh weight suggest that this character may also play a role in sensitivity of peas to UV-B radiation. However, this change may not correspond with UV-B radiation resistance (Big and Kossuth, 1978).

Dry biomass represents a long term integration of all biochemical, physiological and growth parameters. It was previously found that UV-B reduced the dry matter production in peas (Brandle *et al.,* 1977), soybean (Murali *et al.*, 1988; Sullivan and Teramura, 1990; Reed *et al.*, 1992). However, dry matter production was stimulated by UV-B radiation in some rice cultivars (Ziska and Teramura, 1992) and bean (Deckmen *et al.*, 1994). In this study, natural UV-B pea and wheat plants showed a slight increase in dry matter production, whilst high UV-B had no effect. This indicate that UV-B radiation can stimulate the synthesis of dry biomass at low levels. Usually, a greater reduction in total biomass is often found in leaves (for dicotyledons). This trend is not nearly as clear in monocotyledons, where a multitude of species specific responses are found. The lack of any change in pea and wheat of dry biomass under UV-B is in agreement with data previously found in corn, sorghum, soybean and peanut under unfiltered

lamps in the glasshouse (Hart *et al.*, 1975). On the other hand, pea, tomato and mustard radiated with filtered lamps in the field had reductions in their total dry weight (Big and Kossuth, 1978). From this information, it could be argued that the protocol of each experiment is a reasonable cause for the different responses to UV-B radiation, even though the trends found in field studies can be remarkably similar to those found in growth chambers. It should be noted that the experiments reported here were conducted in the glasshouse for control, low and high UV-B treatments, while natural UV-B plants were grown outside. Different environment conditions between the outside and the inside of the glasshouse would interfere with the analysis of effects of UV-B on these plants. In addition to the genetic differences of species, variation between experiments could also be due to differences in growth conditions, lengths of UV-B irradiation, stage of growth and the ratio of UV-B radiation to total incident radiation, all of which have been demonstrated to greatly modify UV-B response (Teramura *et al*, 1991).

3.4. Conclusion

In conclusion, results obtained during this study showed that supplementary UV-B radiation increased the accumulation of UV-B absorbing compounds in pea plants, but had less effect in wheat plants. The absorbance at 280, 300 and 320 nm generally increased with UV-B treatment, while anthocyanin content was not affected by UV-B radiation in either species.

UV-B radiation treatments also induced leaf bronzing, waviness and yellowing in peas, but not in wheat. Reduction in plant height under high UV-B was observed in both species, even though the pattern of their growth are different. In peas, the stunted plants were associated with a reduction in their internode lengths. Fresh and dry matter production was not correlated with stunted growth in either wheat or peas since no change was observed. However, the weight and area of the third pair of pea leaves were less under UV-B radiation, as well as of the second leaf in wheat.

Chapter 4: Anatomical changes in pea and wheat leaves exposed to UV-B radiation

4.1. Introduction

To be effective in light absorption, leaves consist of a band of chlorenchyma which is not thicker than the depth of light penetration, otherwise the lower cells would be completely shaded by the upper ones (Mauseth, 1988). The shape of leaves helps in the harvesting of light as well as providing a balance between the need to harvest light energy for photosynthesis and the need to preserve water and prevent over-heating. A varied spectrum of light penetrates into leaf tissue causing different responses within leaves, and even though all autotrophic plants need visible light for photosynthesis, in some cases the light can be too intense (Mauseth, 1988). For example, penetration of UV-B into leaf tissues could cause damage to leaf cells (Brandle *et al.*, 1977; Cen and Bornman, 1990; Santos *et al.*, 1993; Staxén and Bornman, 1994).

Changes in leaf anatomy due to UV-B radiation have been documented (Brandle *et al.*, 1977; Cen and Bornman, 1990; Cen and Bornman, 1993; Staxén and Bornman, 1994). Changes in leaf thickness in response to UV-B, for example, is one protection mechanism which can decrease UV-B radiation to inner leaf tissues (Tevini *et al.*, 1991). The changes in leaf thickness such as its reduction as measured in leaf cross section was observed in *Zea mays* (Santos *et al.*, 1993) and *Petunia hybrida* (Staxén and Bornman, 1994), while an increase was found in *Phaseolus vulgaris* (Cen and Bornman, 1990) and *Brassica napus* (Cen and Bornman, 1993). The protective mechanisms against excessive solar radiation which involve leaf thickness are also associated with the adaxial epidermal layer (Robberect and Caldwell, 1978), which has an epicuticular wax. By selectively transmitting longer wavelengths, the epidermis plays a major role in mitigating against potential ultraviolet damage in the mesophyll. Reflectance of UV-B from a leaf surface is generally low, approximately 10% of incident irradiation (Clark and Lister, 1975) and most of the UV-B is absorbed within the epidermis. The dense arrangement of epicuticular wax on the adaxial leaf surface of *Brassica napus* under UV-B treatment may decrease penetration of UV-B radiance by reflectance (Cen and Bornman, 1993). Accordingly, the elasticity of the epidermal layer determines the direction of growth of underlying tissue and thereby acts as a

mediator of UV-B effects by absorbing detrimental UV-B before it reaches the photosynthetically active region of the mesophyll.

Another possible mechanism of changing leaf anatomy is by affecting cell division in the leaf meristem, altering cell size and shape (Staxén and Bornman, 1994). The changes of cell size and shape would in turn affect leaf thickness. Several meristems are found in dicotyledonous and monocotyledonous species, some of which are active until late in the development of the leaf (Mauseth, 1988). Divisions that occur at the time of leaf expansion, as is the case for divisions of the guard-cell mother cells in the epidermis (Esau, 1977) would be more susceptible to inhibition by UV-B because the developing leaf becomes more exposed to the radiation.

Alteration in the number of stomata differentiating under increased levels of UV-B has been observed (Staxén and Bornman, 1994). Fewer stomata (on a leaf area basis) on both epidermal layers were found in cucumber cotyledons *Petunia hybrida* Vilm (Staxén and Bornman, 1994). By contrast, Stewart and Hoddinott (1993) observed an increase in stomatal density in Jack pine. When the division leading to the formation of stomatal complexes took place in expanding leaves at a time when they were subjected to UV-B radiation, a direct effect on their cell division would result in an alteration in the number of stomata in the fully differentiated leaf (Staxén and Bornman, 1994).

In the case of a direct action of UV-B, other factors could also explain the changes in leaf anatomy, for instance a collapse of adaxial epidermis, disruption of the first layer of palisade mesophyll tissue, changes in cellular expansion or elongation and the disruption of chloroplasts (see Table 1.3). Changes in these tissue properties would tend to inhibit plant growth or to interfere with the physiological and biochemical activities of cells. As a result, the photosynthetic process would be one that was altered. Since photosynthesis has been found to be affected by UV-B radiation (Brandle *et al.*, 1977: He *et al.*, 1993, 1994), the changes in the machinery of photosynthetic, the chloroplasts needs to be observed. Therefore, information concerning the UV-B radiation effects on plant tissues is important for an understanding of plant responses to UV-B radiation at the cell and tissue levels.

This chapter examines the internal changes in leaf tissues and attemps to relate this information on ultrastructural changes to the physiological results with actual sites responding to UV-B radiation.

4.2. Results

4.2.1. Anatomy of pea leaves

The anatomical structure of a pea leaf in transverse section (Fig.4.1) consists of epidermal layers, mesophyll tissues and vascular bundles. Epidermal cells of both surfaces tend to be tubular and have extremely thin cell walls. The adaxial epidermis characteristically has a thicker cuticle and shorter cells than the abaxial. Stomata are found in about the same density on both the adaxial and abaxial epidermal layers. The mesophyll consists of two different tissue layers. Firstly, immediately below the adaxial epidermis is one row of palisade parenchyma, a layer of columnar chlorenchyma cells, rich in chloroplasts pressed firmly against the plasmalemma and cell wall by a large central vacuole. Secondly, between the palisade parenchyma and the abaxial epidermis there are 5-6 layers of spongy mesophyll, a region in which cells are very widely separated from each other, rounded in shape and with fewer chloroplasts inside. Vascular bundles are collateral and opened, with cambium between xylem and phloem.

4.2.2. Anatomy of wheat leaves

The anatomical structure of a wheat leaf in cross section is different to that of peas. Differences are found in (i) its mesophyll which can not be distinguished into two different tissues and (ii) its vascular bundle type (Fig.4.2). The mesophyll consists of 4-5 layers of spongy parenchyma cells which are all the same shape, tending to be round. The vascular bundles, consisting of xylem toward the adaxial side and phloem toward the abaxial side, are surrounded by a sheath of sclerenchyma cells, the bundle sheath. The vascular bundles are collateral and closed, and oval shaped. Stomata and trichomes are located on both epidermal layers.

4.2.3. Leaf thickness

The effects of UV-B radiation on leaf epidermis, palisade thickness, the number of stomata and chloroplasts (in the first layer of mesophyll tissue) are summarized in Table 4.1. Pea and wheat leaves exposed to UV-B responded with increased leaf thickness (measured from the adaxial to abaxial epidermis). In pea

Table 4.1. Anatomical response of pea and wheat leaves to supplementary UV-B radiation after 8 days of exposure. Values are the average of 25 replications, from 5 different leaves for each treatments. * indicates the significant different (P=0.05) to the control. † indicates a field view at 400 X magnification using light microscope.

Treatment	Leaf thickness at the mid-rib (μm)	Leaf thickness at non vascular region (μm)	The thickness of adaxial epidermis (μm)	The thickness of abaxial epidermis (μm)	Number of chloroplasts in the first layer of mesophyll tissue (per cell)	The thickness of palisade layer (μm)	Number of stomata (adaxial epidermis)†	Number of stomata (abaxial epidermis)†
Peas								
Control (Zero UV-B)	211.5 ± 8	184.7 ± 10	20.4 ± 2	16.8 ± 2	38.4 ± 6	55.2 ± 5	49.2 ± 5	45.6 ± 6
Natural UV-B	206.9 ± 8	184.9 ± 4	19.5 ± 3*	14.0 ± 2*	32.9 ± 5	51.6 ± 4	39.5 ± 4	32.5 ± 7*
Low UV-B	212.9 ± 13	166.6 ± 8*	4.4 ± 1*	19.6 ± 4*	29.2 ± 4*	43.4 ± 6*	47.0 ± 6	41.4 ± 6
High UV-B	236 8 ± 12*	172.0 ± 12*	0.1 ± 0*	18.1 ± 2*	23.3 ± 3*	28.2 ± 3*	56.4 ± 7*	32.3 ± 3*
Wheat								
Control (Zero UV-B)	165.6 ± 23	102.9 ± 10	8.7 ± 1	13.1 ± 2	25.8 ± 3		7.3 ± 1	4.2 ± 1
Natural UV-B	275.3 ± 12*	172.5 ± 10*	23.8 ± 4*	24.9 ± 4*	26.9 ± 5		7.4 ± 1	3.5 ± 1
Low UV-B	259.4 ± 9*	178.6 ± 13*	27.7 ± 6*	25.8 ± 3*	30.4 ± 6*		7.3 ± 1	3.6 ± 1
High UV-B	259.2 ± 3*	194.7 ± 13*	24.4 ± 4*	28.5 ± 5*	36.7 ± 7*		7.7 ± 1	4.0 ± 1

leaves, high UV-B resulted in a significant increase of total leaf thickness at the mid-rib, while there was a thinning of the non-vascular regions. In wheat leaves, significant increases in thickness were obtained under all UV-B treatments, at both mid-rib and non-vascular sites. Leaf thickness at the mid-rib in wheat showed no significant differences between low and high UV-B treated leaves, but was about 57% thicker compared to the control. The increases in thickness of wheat leaves were even greater at non-vascular sites.

In peas, the thickness of the adaxial epidermis was reduced under both low and high UV-B radiation. Wheat epidermal cells on the other hand, significantly increased in thickness under all UV-B treatments.

4.2.4. Number of stomata and of chloroplasts

In peas, the density of stomata on the adaxial and abaxial epidermis were about the same (Table 4.1) and about 8 times higher than the density of wheat leaf' stomata. Under high UV-B treatment, pea leaves produced an increase in stomatal density on the adaxial epidermis, and a decrease on the abaxial epidermis. On the other hand, no differences in stomatal density were observed on either epidermis of wheat leaves.

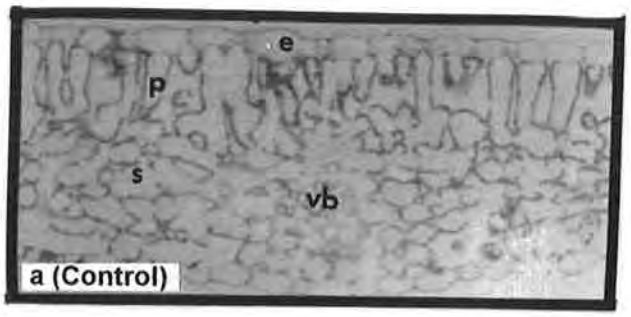
In peas, a reduction in palisade cell elongation and a reduction in chloroplast number of 24% for low UV-B and 39% for high UV-B was observed. In contrast, wheat leaves showed marked increases in the number of chloroplasts, 20% for low UV-B and 44% for high UV-B.

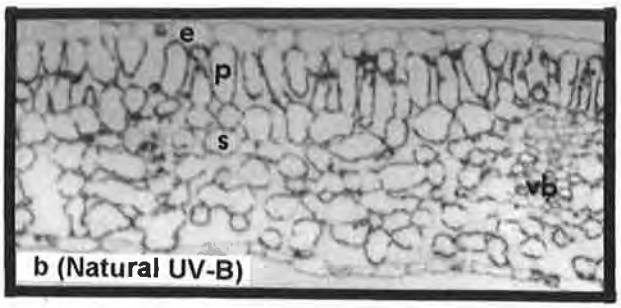
4.2.5. Anatomical responses to UV-B radiation

4.2.5.1. Pea leaf

UV-B treatment did affect the anatomical structure of pea leaves, and particularly that tissue layer which received direct UV-B (Fig.4.1 and 4.3). Since UV-B penetrates only a few μm into the leaf (Day, 1992), its effects were restricted to the adaxial epidermis and the upper layers of mesophyll cells. From transverse sections of leaves, low and high UV-B appeared to cause partial collapse of the adaxial epidermis and some internal damage to palisade layers (Fig.4.3c,d). High UV-B caused more damage than did low UV-B radiation. Under high UV-B, the

Fig.4.1. Light micrograph of cross sections of pea (*Pisum sativum* L.) leaves after 8 days treatment. Sections from the control and natural UV-B plants showed a very well-organized leaf structure, while those from low and high UV-B treaments show most of the adaxial epidermal cells were damaged. Shown are adaxial epidermis (e), palisade parenchyma (p), spongy parenchyma (s), vascular bundle (vb) and collapsed epidermal cells (ce). 100x.


e
p
s
vb
a (Control)


e
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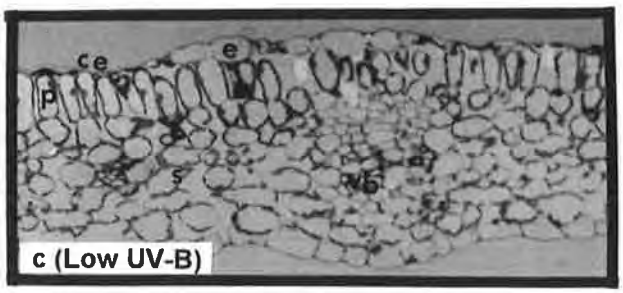
s
vb
b (Natural UV-B)


ce
e
p
s
vb
c (Low UV-B)

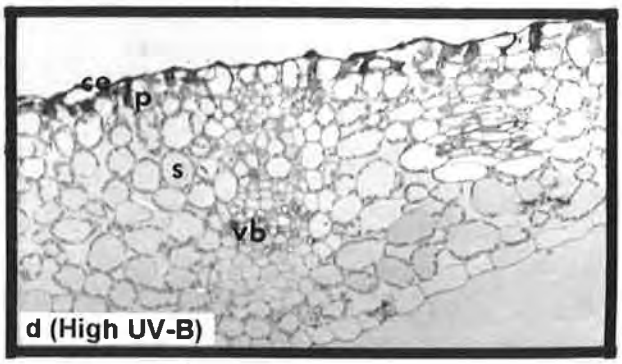
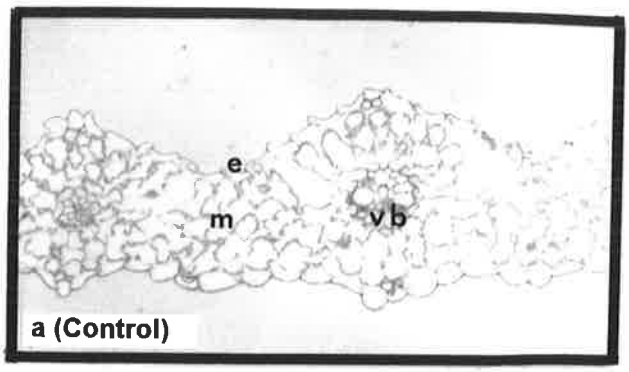
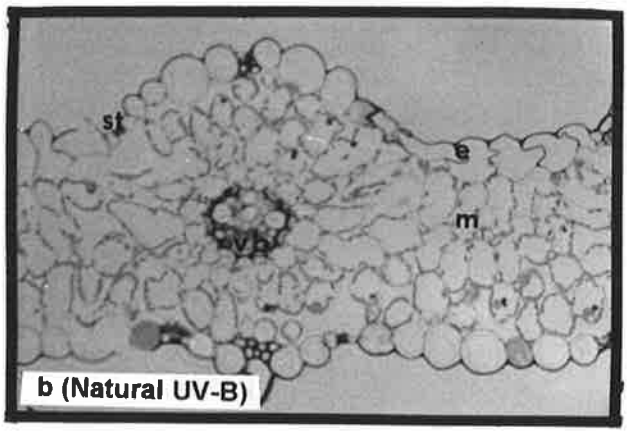
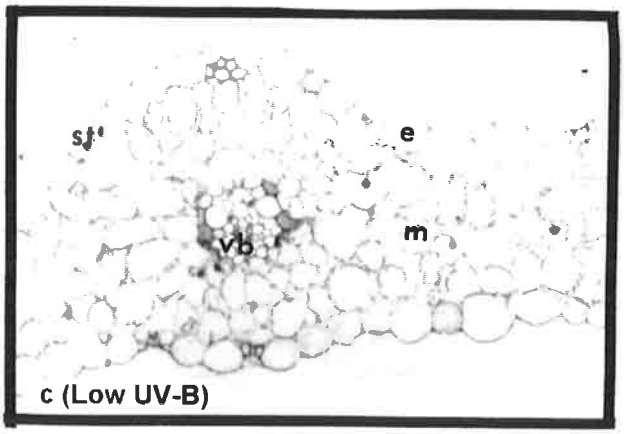

ce
p
s
vb
d (High UV-B)

Fig.4.2. Light micrograph of the cross sections of wheat (*Triticum aestivum* L.) leaves after 8 days treated with supplementary UV-B radiation. Shown are adaxial epidermis (e), mesophyll (m), vascular bundle (vb) and stomata (st). 100x.


e
m
vb
a (Control)


st
e
m
vb
b (Natural UV-B)


st
e
vb
m
c (Low UV-B)

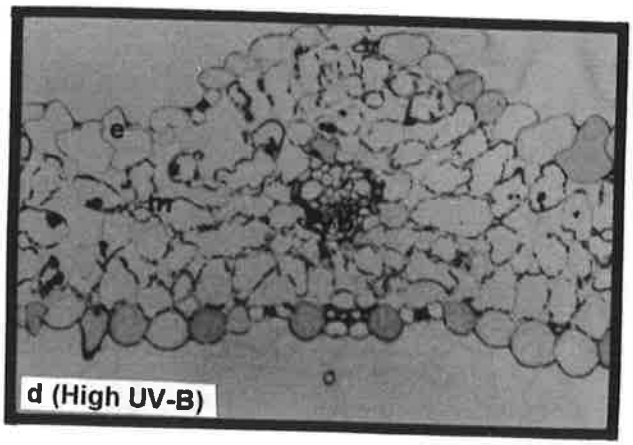
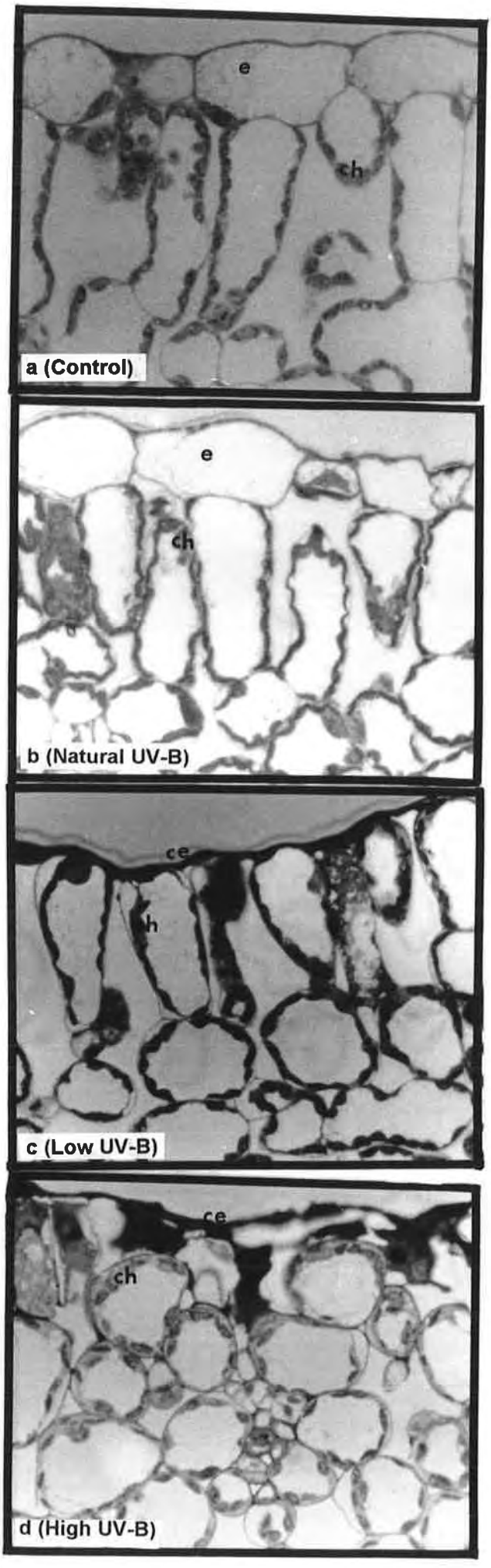

e
m
d (High UV-B)

Fig.4.3. The high magnification (400x) of pea (*Pisum sativum* L.) leaves cross sections after 8 days of UV-B treatment. The collapsed or damaged epidermis cells were clearly apparent. Shown are adaxial epidermis (e), collapsed epidermis (ce) and chloroplast (ch). See Fig.4.1. for further detail of complete cross section.


e
ch
a (Control)
e
ch
b (Natural UV-B)
ce
h
c (Low UV-B)
ce
ch
d (High UV-B)

adaxial epidermis looked like a horizontal line on top of the palisade tissue. A cellular structure of epidermal cells was no longer recognizable, and palisade cells were no longer columnar and some of them were empty.

4.2.5.2. Wheat leaf

Wheat leaves displayed a different response to UV-B radiation. They did not appear to suffer any damage at the cellular level, even at the adaxial epidermis, but the epidermii were thicker as a consequence of the UV-B treatment (Fig.4.4).

4.2.6. Ultrastructure responses

4.2.6.1. Pea leaf

The ultrastructure of the cells from treated leaves was different to that of the control leaves, particularly on the adaxial side. The adaxial epidermal cells of control and natural UV-B leaves developed normally (Fig.4.5a,b). They displayed highly vacuolated cells with a peripheral layer of cytoplasm containing organelles and their structure was typical of epidermal cells. However, under both low and high UV-B radiation, the adaxial epidermis was damaged and the palisade cells were partly altered (Fig.4.5c,d). The epidermal cells showed an irregular cell wall outline which became adpressed and collapsed and adhered to the palisade cells. This collapse was extreme in some areas, with total disappearance of the protoplast. Almost the entire cell contents of the epidermal cells disappeared, and only a small structure filled with granular material remained All the organelles within damaged epidermal cells were disrupted and could no longer be identified.

Palisade cells from control and natural UV-B leaves showed the normal columnar morphology with a large vacuole at the centre (Fig.4.5a,b). The cytoplasm, bound by the plasmalemma and tonoplast, contained the usual organelles, including a single layer of chloroplasts at the cell boundary. In contrast, palisade cells from low and high UV-B treated leaves were collapsed, especially those located adjacent to the damaged epidermis (Fig.4.5c,d). They contained altered chloroplasts, nuclei, and other organelles with small vacuoles.

Fig.4.4. The high magnification (400x) of wheat (*Triticum aestivum* L.) leaves cross sections after 8d of UV-B treatment. Shown are adaxial epidermis (e), stomata (st) and mesophyll (m).

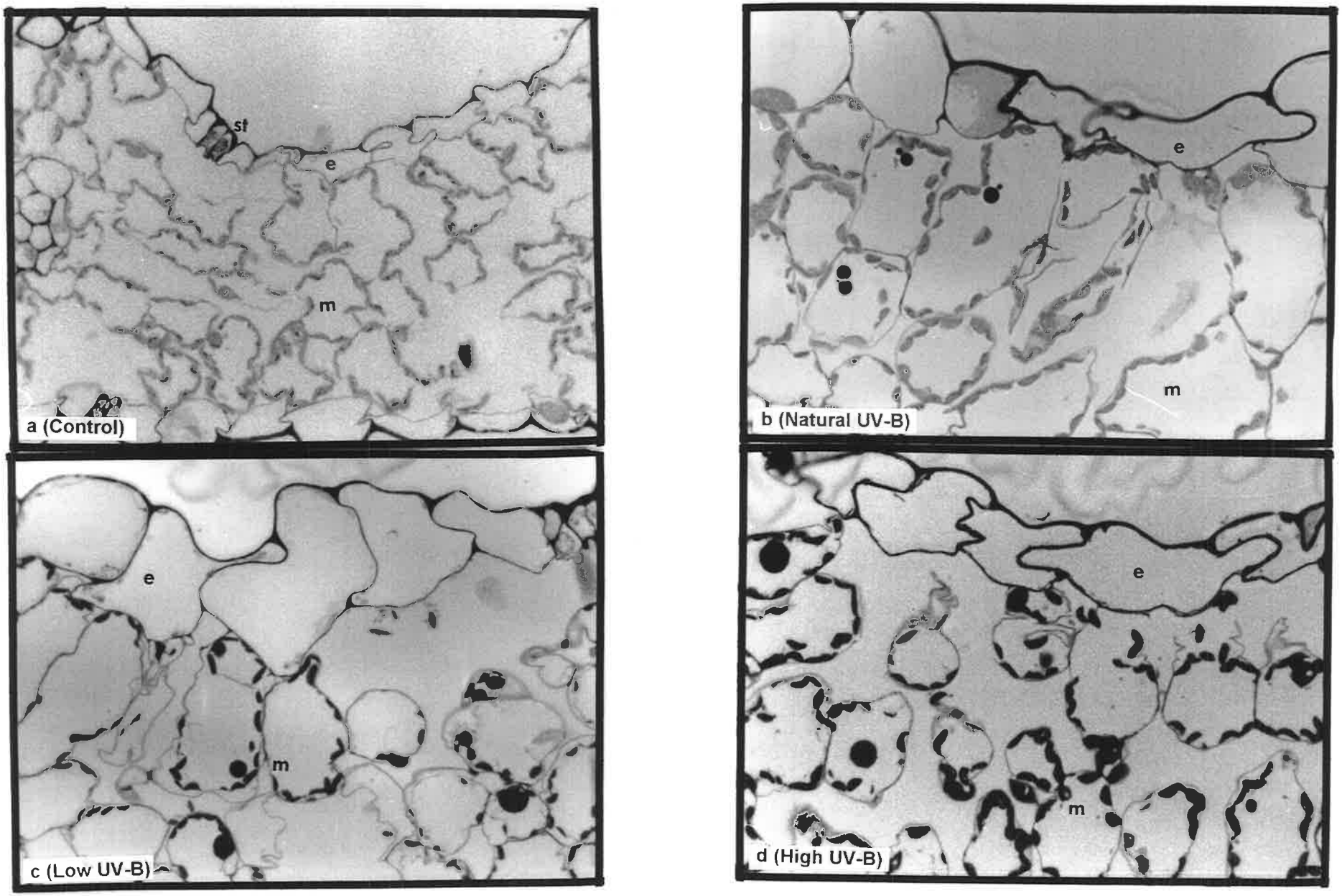
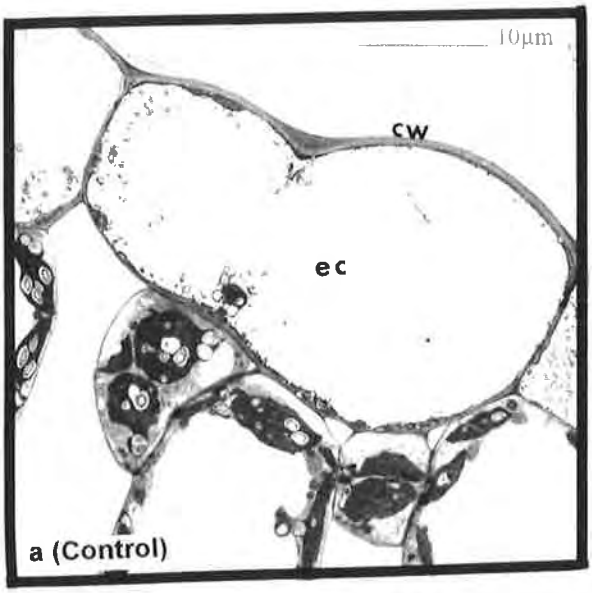
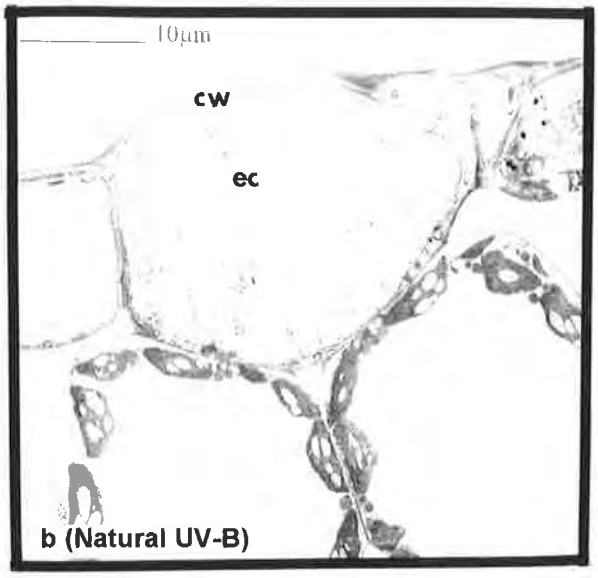
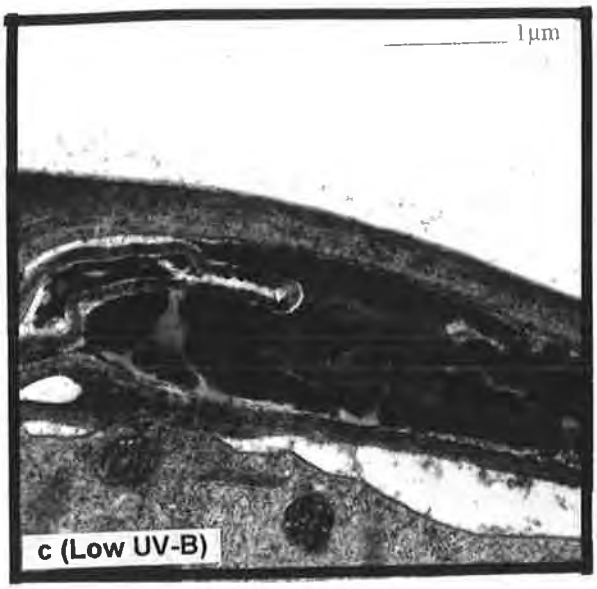
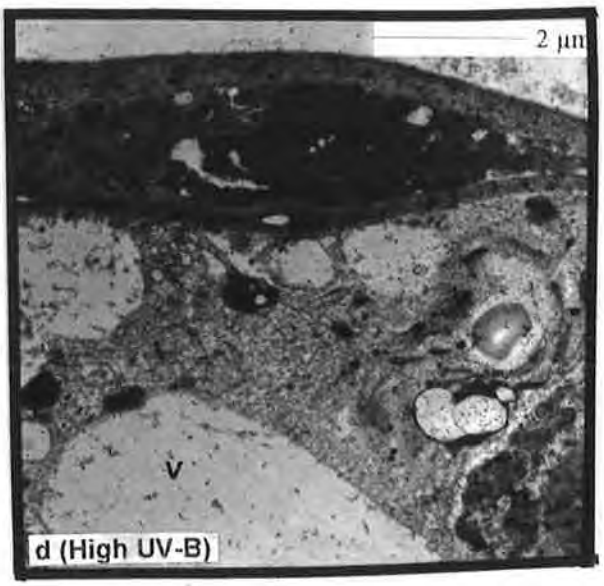

st
e
m
a (Control)
e
m
b (Natural UV-B)
e
m
c (Low UV-B)
e
m
d (High UV-B)

Fig.4.5. Transmission electron micrograph of epidermal cells from pea (*Pisum sativum* L.) leaves after 8 days of UV-B treatment. Epidermal cells from the control and the natural UV-B leaves contained a large vacuole and a normally organized protoplast. The epidermal and palisade cells of the low and the high UV-B treated leaves displayed an irregular cell wall. Shown are cell wall (cw), epidermis cell (ec), nucleus (n) and vacuole (v).


10µm
cw
ec
a (Control)


10µm
cw
ec
b (Natural UV-B)


1µm
c (Low UV-B)


2 µm
v
d (High UV-B)

Chloroplasts taken from control and natural UV-B treated leaves appeared normal (Fig.4.6a,b). They were roughly elliptical to elongate in shape, with dense, uniform and dark stroma, they had a complex and well developed internal membrane system with densely piled thylakoids. The chloroplast envelope remained intact. Stroma and grana lamellae were generally oriented parallel to the long axis of the chloroplast. The homogenous compact stroma contained one or more starch granules and osmophilic globuli.

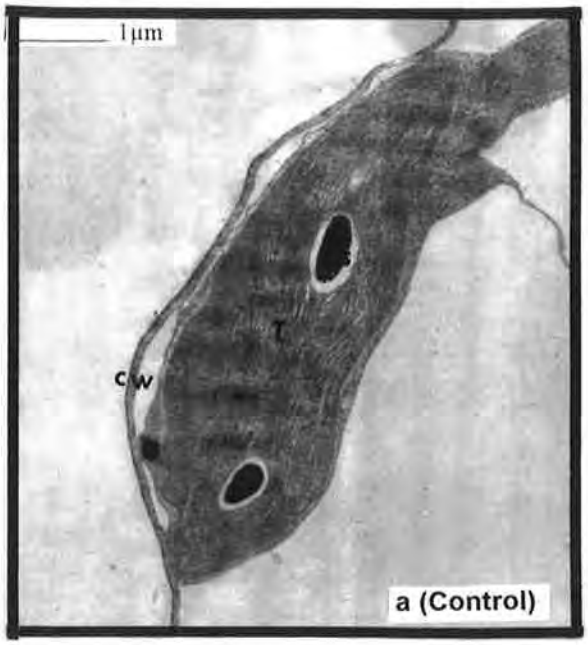
The chloroplasts observed in the damaged palisade cells from low and high UV-B treated plants were characterized by a more open system of thylakoid and lamellar membranes (Fig.4.6c,d). The chloroplasts tended to become rounded or spherical and their photosynthetic lamellae exhibited various degrees of swelling, with a reduction in thylakoid number and smaller grana with poorly defined membranes. The most severe damage was represented by rounded chloroplasts with swollen thylakoids, increased intergrana spaces, and more osmiophilic globuli which were compact and loosely granular. Swelling of the lumen spaces within thylakoids and the formation of electron-transparent gaps in stroma were also observed. The lamellar system was disoriented and distended. The increase of intergrana space relative to controls was more pronounced in high UV-B treatments. Furthermore, alteration of the thylakoids was not apparent in all chloroplasts from UV-B treated leaves. It occurred only in chloroplasts within palisade cells located beneath the damaged epidermal cells (Fig.4.6c,d). Normal chloroplasts (similar to control) were observed in palisade cells in areas where the epidermis was not damaged, and in spongy parenchyma from low and high UV-B treated leaves (Fig.4.6e,f).

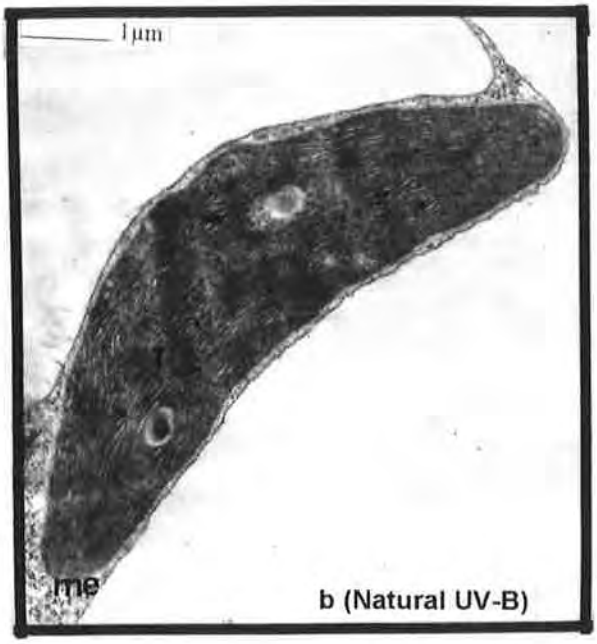
In sections of cells in which the chloroplasts were mostly severely altered, the mitochondrial membrane was also sometimes poorly defined. The mitochondria in the high UV-B cells appeared to be smaller, with fewer cristae than those in control cells (Fig.4.7). However, the degree of these differences was not uniform in all mesophyll cells. The most obvious damage caused by low and high UV-B radiation was to the chloroplasts which tended to lose their integrity.

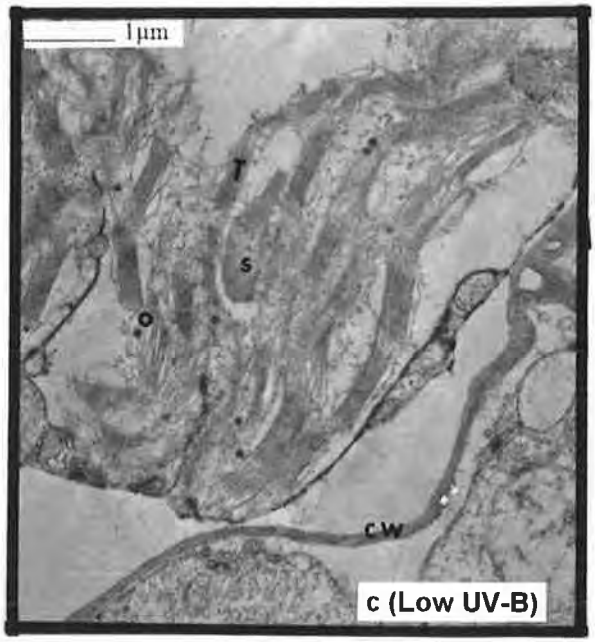
4.2.6.2. Wheat leaf

The ultrastructure of the cells from treated wheat leaves was similar to that of the control leaves. The adaxial epidermis and the mesophyll cells photographed from all treatments developed normally, and no damage to the adaxial epidermis under UV-B treatment was found. In cells of 8 day treated leaves wheat, chloroplasts and other organelles occupied the thin band of cytoplasm surrounding the large central vacuoles. Chloroplasts from all treatments seemed to be similar in shape and in their thylakoid arrangement. They were roughly elliptical and had a complex lamellar system (Fig.4.8). Stroma and grana lamellae were generally oriented parallel to the long axis of plastid. The compact stroma contained ribosomes and small osmiophilic globuli. Seemingly intact mitochondria could be observed in leaves from all treatments.

Fig.4.6. Transmission electron micrograph of chloroplasts in palisade cells of pea (*Pisum sativum* L.) leaves after 8 days of UV-B treatment. The control and the natural UV-B chloroplasts contained a very well-organized thylakoid system. The ultrastructural changes during treatment can be seen in Fig.4.6c,d. The chloroplasts taken from normal palisade cells of the low and the high UV-B treated leaves are shown in Fig.4.6e and Fig.4.6f respectively. Shown are cell wall (cw), thylakoid (T), osmiophilic globuli (o), membrane envelopes (me) and starch (s).


1µm
T
cw
a (Control)


1µm
T
me
b (Natural UV-B)


1µm
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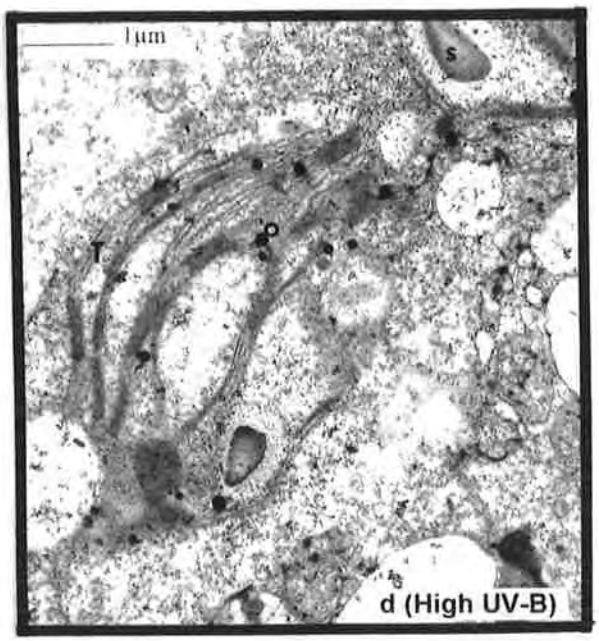
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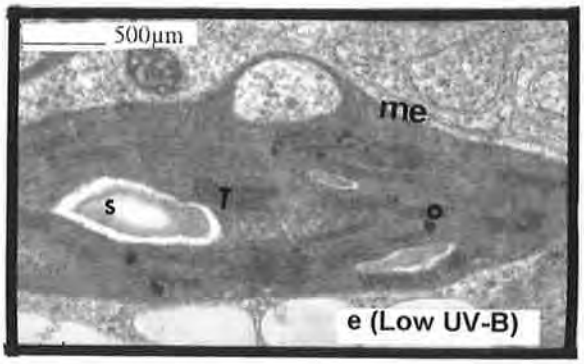
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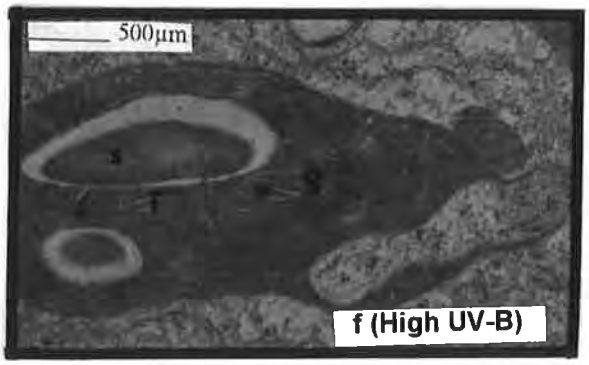
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Fig.4.7. Transmission electron micrograph of mitochondria in palisade cells of pea (*Pisum sativum* L.) leaves after 8 days of UV-B treatment. Shown are mitochondria (m), the outer membrane (om) and cristae (c). 19 000 X.

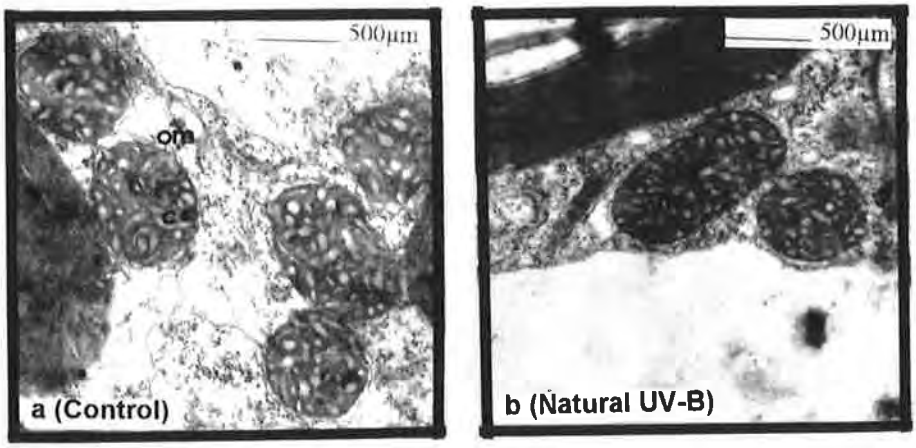
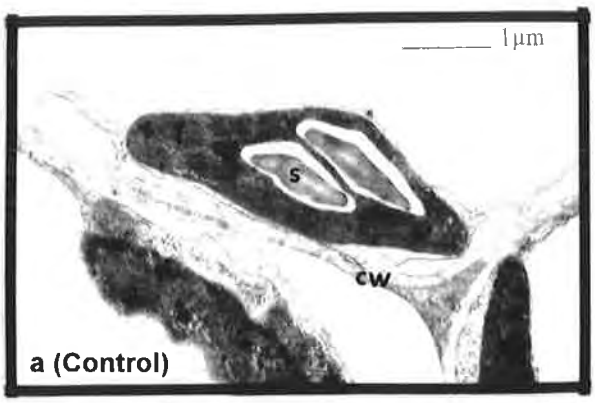
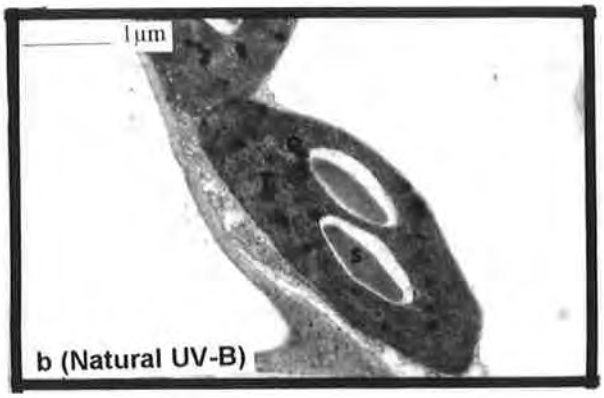
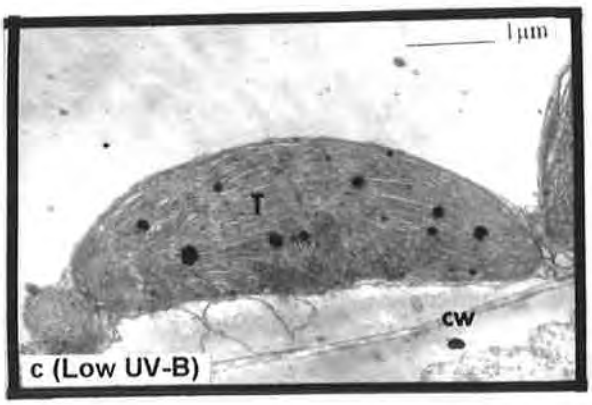

500µm
om
a (Control)
500µm
b (Natural UV-B)
500µm
c (Low UV-B)
500µm
d (High UV-B)

Fig.4.8. Transmission electron micrograph of chloroplasts in the first layer of mesophyll tissue of wheat (*Triticum aestivum* L.) leaves after 8 days of UV-B treatment. The control and the natural UV-B chloroplasts contained a very well-organized thylakoid system. The ultrastructural changes during treatment can be seen in Fig.4.8c,d. Shown are cell wall (cw), thylakoid (T), osmiophilic globuli (o) and starch (s).


1 µm
S
CW
a (Control)


1 µm
S
S
b (Natural UV-B)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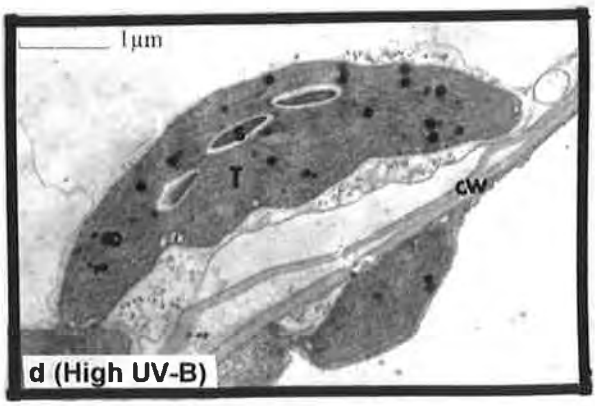
T
CW
c (Low UV-B)


1 µm
T
CW
d (High UV-B)

4.3. Discussion

Changes in leaf thickness under low and high supplementary UV-B radiation were different at different parts of pea leaves, but were uniform in wheat across leaves. In peas, increased leaf thickness at the mid-rib only, while it decreased in non-vascular areas. This decrease was related to the reduced thickness of both the adaxial and abaxial epidermal cells and palisade cell thickness. Apart from a dramatic decrease in leaf thickness in found *Petunia hydbrida* (Staxén and Bornman, 1994) the increase of leaf thickness observed here agrees with other results observed with *Brassica sp* (Bornman and Vogelmann, 1991), *Phaseolus vulgaris* (Cen and Bornman, 1990) and *Brassica napus* (Cen and Bornman, 1993). The increase in leaf thickness in peas appeared to be caused by an increase in number of vascular bundle cells, while in wheat it is due to an increase in the number of spongy parenchyma cells and an increase in the thickness of adaxial and abaxial epidermal cells, similar to that found by Cen and Bornman (1993). Bornman and Vogelmen (1991) explained that the increase in leaf thickness in *Brassica carinata* and *Medicago sativa* was due to an increase in the number of spongy parenchyma cells, while in *Brassica campestris* it was caused by an increase in the number of layers of palisade cells. Thicker leaves could act as a protection against UV-B radiation and would conceivably attenuate radiation to deeper lying tissues (Cen and Bornman, 1993). However, the increase in thickness more likely represents a photomorphogenic response to UV-B, with the protective aspect being secondary when compared to the effect of the epidermis in filtering UV-B (Cen and Bornman, 1993; Day *et al.,* 1993). The latter authors showed that only a small amount of short wavelength radiation penetrated to lower regions of the leaf.

In the present study, I found an increase in the number of stomata per unit area on the adaxial epidermis and a reduction on the abaxial epidermis of pea leaves, while there was no significant changes in wheat leaves. The increase in stomata number on the adaxial epidermis of UV-B treated pea plants was in contrast to that found in *Petunia hybrida* (Staxén and Bornman, 1994) where a decrease in the number of stomata per unit area on both the adaxial and the abaxial epidermal surfaces was reported. These authors stated that a small decrease in the ratio of stomata to epidermal cells of leaves exposed to UV-B was concomitant with a small increase in the number of epidermal cells per unit area. Furthermore, they determined that the UV-B exposed *Petunia* plants had higher transpiration rates and therefore a lower water use efficiency. These higher

transpiration rates could explain why the cells in the UV-B-exposed plants were smaller.

UV-B radiation decreased the chloroplast number in pea palisade cells (Table 4.1). There was no significant change in chloroplast number in the first mesophyll layer of wheat leaves. The decrease in chloroplast number in pea indicates that there was tissue damage, which could result from an effect either on chloroplast division or chloroplast development. The development of chloroplasts is coupled to the formation of the leaf meristem and the production of mesophyll cells. During development, plastid volume per cell increases dramatically and plastid composition changes in parallel with the acquisition of photosynthetic competence. Plastid composition also varies in response to environmental conditions or to meet specialized needs of the plant (Mullet, 1988). The number of chloroplasts per cell increases proportionately with increases in cell size, and chloroplasts divide and expand to occupy a constant proportion of the mesophyll cell surface (Robertson and Laetsch, 1974). In this study, mesophyll cells from UV-B treated pea plants were damaged and their size was smaller than in control material. Therefore, any reduction in palisade cell size was concomitant with a reduction in the chloroplast number per cell. Inhibition of leaf development appears to lead to a concomitant inhibition of chloroplast development and plastid transcriptional activity. This suggests that the development of chloroplasts in pea and wheat, as vascular plants, is regulated in part by internal signals that control differentiation of the leaf (Mullet, 1988).

Since UV-B does not penetrate deeply into leaf tissue (Day *et al.,* 1993), one can expect that the radiation will affect the most superficial layers of leaves. Damage to the adaxial epidermis of pea leaves under low and high UV-B radiation was evident in this present study. The epidermis is considered as UV-B radiation filter and usually attenuates transmission to lower lying leaf tissue (Roberrecht and Caldwell, 1978). Although flavonoids will provide some protection, harmful effects of UV-B are still likely to occur given the strong absorption in the UV-B region of the spectrum by many important biomolecules. Cen and Bornman (1990) suggested that flavonoid synthesis was enhanced by UV-B radiation. This may have been partly due to the damaged epidermal layer of leaves. Furthermore, Cen and Bornman (1993) observed that 310 nm radiation was strongly attenuated by the epidermis and underlying mesophyll tissue in *Brassica napus* and only very small amounts of scattered UV-B were found to penetrate deeply into the leaf tissue. However, these small amounts may cause damage. Palisade cells from pea

leaves were also considerably disrupted under low and high UV-B , indicating that UV-B must indeed have been able to penetrate well below the epidermal tissue.

The changes in epidermal and palisade cells under UV-B radiation seemed in turn, to change their shape. This could be related to the pattern of microfibrils deposition. Miicrotubules, which have been implicated in the deposition of cell wall through the orientation of the cellulose synthetase complexes (Emons *et al.*, 1992) and in the morphogenesis of mesophyll cells (Panteris *et al.*, 1993), have been shown to be depolymerized in protoplasts when irradiated by UV-B (Staxén *et al.*, 1993). However, this change in microtubule organization was not found in the epidermal cells of *Petunia hybrida* leaves of whole plants grown under UV-B radiation (Staxén and Bornman, 1994).

Chloroplasts from supplementary UV-B treated plants showed marked changes from those of the control and natural UV-B plants. These included the dilation of thylakoid membranes and the disruption of envelope membranes. He *et al.* (1994) suggested that there is a relationship between the dilation of thylakoids and the transmembrane ion flux across the chloroplasts membrane under UV-B radiation.

The disruption of chloroplasts membranes by UV-B radiation has previously been found in pea (Brandle *et al.,* 1977; He *et al.,* 1994). In their studies, the ultrastructural changes in chloroplast membranes became more pronounced with increasing level of UV-B radiation.

In contrast to pea leaves, the anatomical structure of wheat leaves was less affected by UV-B radiation. Wheat leaves are hairy. Apart from a possible role as a defence mechanism against biotic attack (Johnson, 1975), leaf hairs may also function as fine modifiers of the leaf micro-environment in some adverse physical situations. It has been suggested that leaf hairs may constitute a shield and protect the underlying tissues against UV-B radiation damage (Karabourniotis *et al.*, 1992, 1993) as they also contain flavonoid pigments and the attenuation of UV-B radiation tended to be more effective the greater the number of leaf hairs (Karabourniotis *et al.*, 1992).

4.4. Conclusion

This study has shown that the morphological and cytological structures of plants subjected to supplementary UV-B radiation were changed. UV-B radiation increased the leaf thickness in wheat, but partly in peas. This response may help prevent damage to deeper lying tissue. Damage of the adaxial epidermis and of the palisade cells was observed in pea plants grown under supplementary UV-B radiation, in parallel with changes in chloroplast ultrastructure, including the swelling or dilation of thylakoids. In leaves from UV-B treated wheat plants, neither collapsed adaxial epidermii nor the damaged of mesophyll tissue was found and the ultrastructure of wheat chloroplasts was not affected.

Chapter 5: The effects of supplementary UV-B radiation on respiration

5.1. Introduction

5.1.1 Respiration

Respiration is the process whereby carbon compounds (usually sugars) are oxidized to release energy, and is an important parameter in the carbon economy of plants, because about half the carbon fixed in photosynthesis is lost via respiratory processes (Amthor, 1989). Plants with lower rates of mature leaf respiration will consistently produce higher dry matter yields, and respiratory rates provide a measure of metabolic activity (Wilson, 1975). Thus respiration rates have been used to screen for high yields in pasture plants.

Respiration occurs in two stages. The first is the breakdown of carbohydrate molecules to pyruvate, the second is the oxidation of pyruvate to carbon dioxide and water with the uptake of oxygen and the release of energy. The dominant pathway for the first stage is glycolysis, although other secondary pathways such as the oxidative pentose phospahte pathway can provide intermediates for the glycolytic process. This all takes place in the cytosol. The second stage takes place in the mitochondria via the tricarbocxylic acid (TCA) cycle and electron transport chain (ETC) of the inner mitochondrial membrane.

Respiration is an important process occuring in all plant cells, in the light and in the dark. It supplies the cell with usable energy (as ATP) and essential metabolic intermediates. Thus it was felt important to investigate the effects of UV-B on plant respiration and especially on the mitochondrial aspects of plant respiration.

5.1.2. Glycolysis

Glycolysis is the process through which glucose or other carbohydrates are converted in the cytosol into two molecules of pyruvate (Mierynk, 1990). This is the dominant pathway of carbohydrate oxidation (Kruger, 1990) and can be summarized:

1 glucose + 2 ATP + 2 Pi + 4 ADP + 2 NAD $\rightarrow$ 2 pyruvate + 2 ADP + 4 ATP + 2 NADH

Glycolysis can be separated into two different stages as outlined in Fig. 5.1. Firstly, the transformation of glucose to form fructose 1,6-biphosphate, and secondly, hydrolysis of fructose biphosphate to produce triose phosphate which is oxidized to generate ATP. With the metabolism of phosphoenol pyruvate via irreversible steps to form pyruvate, the second step of glycolysis is complete.

Pyruvate and malate, as final products of glycolysis may enter mitochondria to be further metabolized via the TCA cycle. It is also possible for intermediates from other pathways to enter the glycolytic pathway, as well as for glycolytic intermediates to enter other pathways.

5.1.3. Oxidative pentose phosphate pathway

Similarly to glycolysis, the oxidative pentose pathway occurs in the cytosol. The reactions in the oxidative pentose phosphate pathway are reversible, and the intermediates summarized in Fig. 5.2. Glucose 6-phosphate is degraded through a series of reactions to form ribulose 5-phosphate. In this case, two molecules of NADPH and one molecule of CO_2 are generated. There is an interactive and an associative process between glycolysis and the oxidative pentose pathway as there are enzymes and several intermediates common to both (Mierynk, 1990).

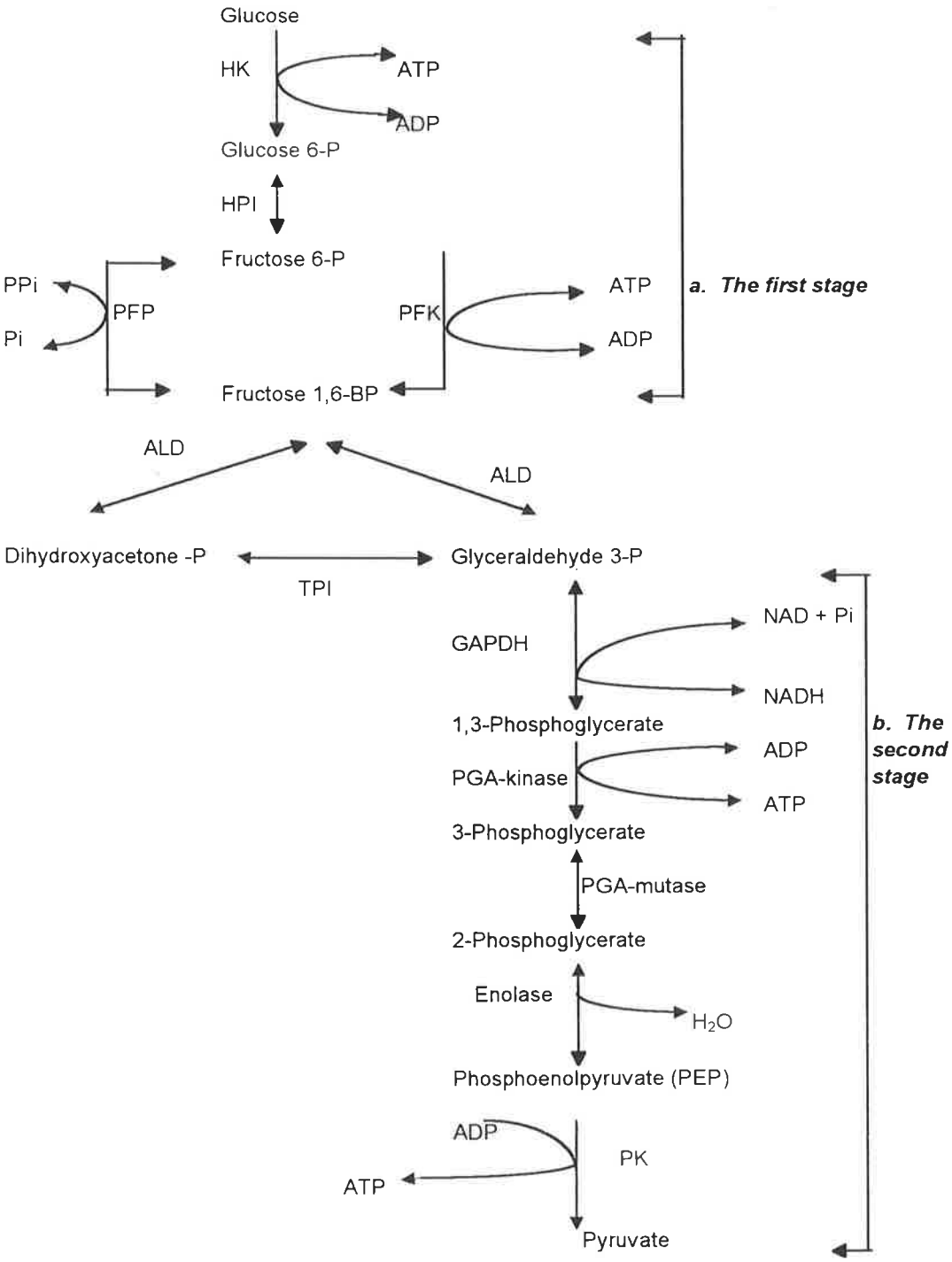


Fig.5.1. The glycolysis process within plant cytosol. Abbreviations used: HK, hexokinase; HPI, hexose phosphate isomerase; PFP, phophofructophosphate; PFK, phosphofructokinase; ALD, fructose 1,6-biphosphate aldolase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PK, pyruvate kinase; PEPC, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase.

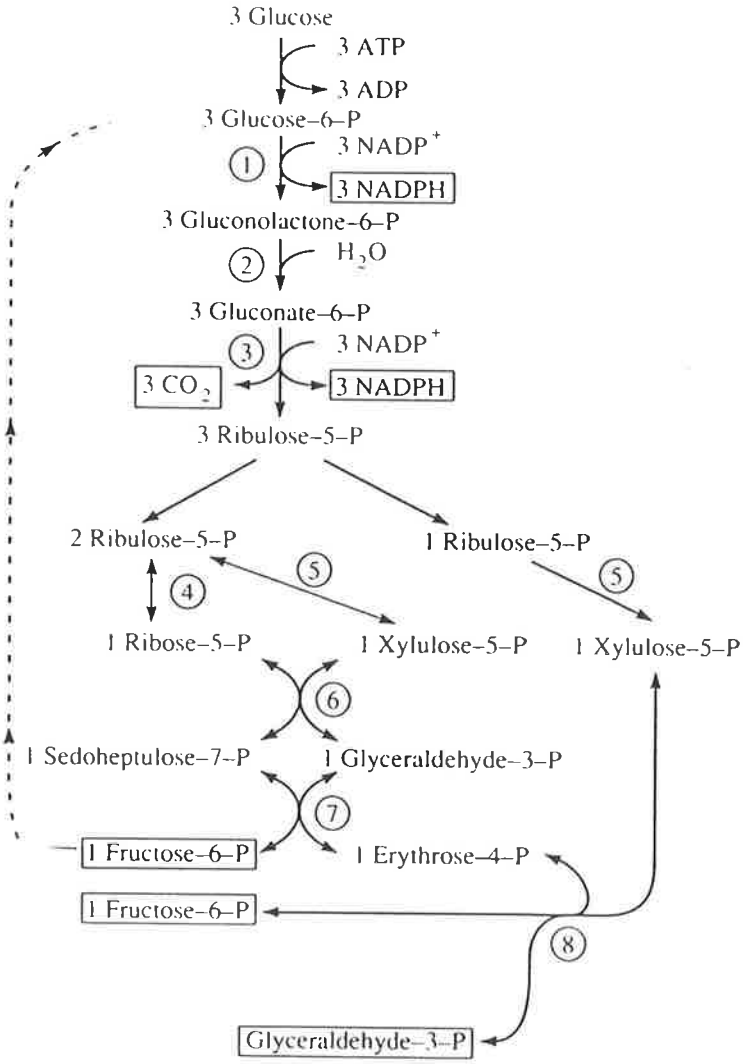


Fig.5.2. The oxidative pentose phosphate pathway. Abbreviations used: (1) glucose-6-P dehydrogenase, (2) gluconate-6-P lactonase, (3) gluconate-6-P dehydrogenase, (4) ribose-5-P isomerase, (5) ribulose-5-P 3-epimerase, (6) transketolase, (7) transaldolase, (8) transketolase. (Anderson and Beardall, 1991, p 128).

5.1.4. Structure of plant mitochondria

Plant mitochondria are approximately 1-2 μm x 0.5 μm in size (Fig.5.3). In general, they appear to be rod-shaped with hemispherically shaped ends, or to be cup or filament-shaped (Newcomb, 1990). Each mitochondrion is composed of two highly specialized membranes which are distinct in their appearance and function. These membranes together create two mitochondrial compartments, the internal matrix and the intermembrane space (Albert *et al.*, 1989).

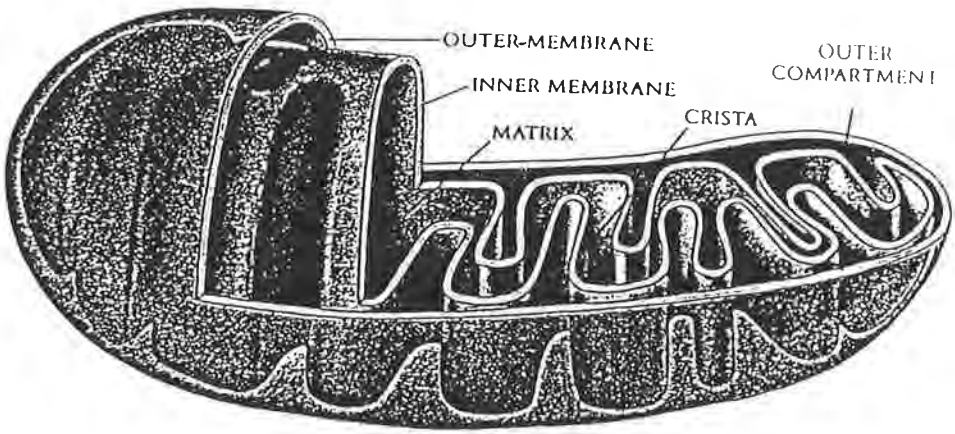


Fig.5.3. Longitudinal section of a mitochondrion (After Raven and Johnson, 1986)

The outer membrane of mitochondria occasionally appears to be connected to endoplasmic reticulum (ER). A large channel-forming protein embedded in the outer membrane provides permeability to all highly uncharged molecules of 10 kda or less (Newcomb, 1990).

The intermembrane space contains various enzymes used in nucleotide phosphorylation. They oxidize pyruvate to CO_2 with concomitant phosphorylation of ADP to ATP in processes involving the tricarboxyclic acid cycle (TCA cycle) and oxidative phosphorylation.

The inner membrane, which is highly invaginated to form cristae, consists of 70% protein and 30% lipid, and has particles on its inner surface which bounds the mitochondrial matrix. This membrane is impermeable to charged molecules and to large uncharged molecules, but is permeable to low molecular weight uncharged

molecules. Various transport proteins within the inner membrane make it selectively permeable, and allow the transport of metabolites into and out of mitochondria (Mitchell, 1966). Also it contains an electron tarnsport chain to transfer electrons from organic molecules to oxygen and embedded within it are the enzymes essential for reactions in ATP synthesis.

The internal space, the matrix contains the enzymes required for the oxidation of pyruvate and other organic acids in the TCA cycle, in which the dominant component of carbon metabolism takes place.

5.1.5. Tricarboxylic Acid Cycle (TCA)

This pathway is the dominant component of carbon metabolism occurring in the mitochondria. Pyruvate, malate and other carbon source intermediates are the main substrates for the TCA cycle. The reactions within the TCA cycle are summarized in Fig.5.4. The sum of one turn of the TCA cycle is the consumption of 1 pyruvate, 4 NAD, 1 ADP, 1 Pi, 1 FAD, and 3 H_2O to form 3 CO_2, 4 NADH, 1 ATP and 1 $FADH_2$. There are two turns of the TCA cycle for each glucose degraded , the yield from glucose is doubled. The NADH and $FADH_2$ produced by the TCA cycle enter the mitochondrial electron transport chain (ETC) and drive the formation of ATP from ADP and Pi during oxidative phosphorylation.

5.1.6. The ETC and Oxidative Phosphorylation

Within the inner membrane of mitochondria there is a mobile electron transport chain consisting of redox complexes, which transfer electrons from NADH and $FADH_2$ to oxygen. In plant mitochondria, there are four major complexes associated with electron transport and one with the production of ATP (Fig.5.5).

Complex I catalyzes the transfer of electrons from NADH produced in the matrix space to ubiquinone. This transfer occurs with the translocation of H^+ from the matrix to the intermembrane space, probably 2 H^+ per electron (Lambers, 1990).

Complex II is capable of transferring electrons from succinate to ubiquinone, via FAD during operation of the TCA cycle. This transfer does not move H^+ across the inner mitochondrial membrane.

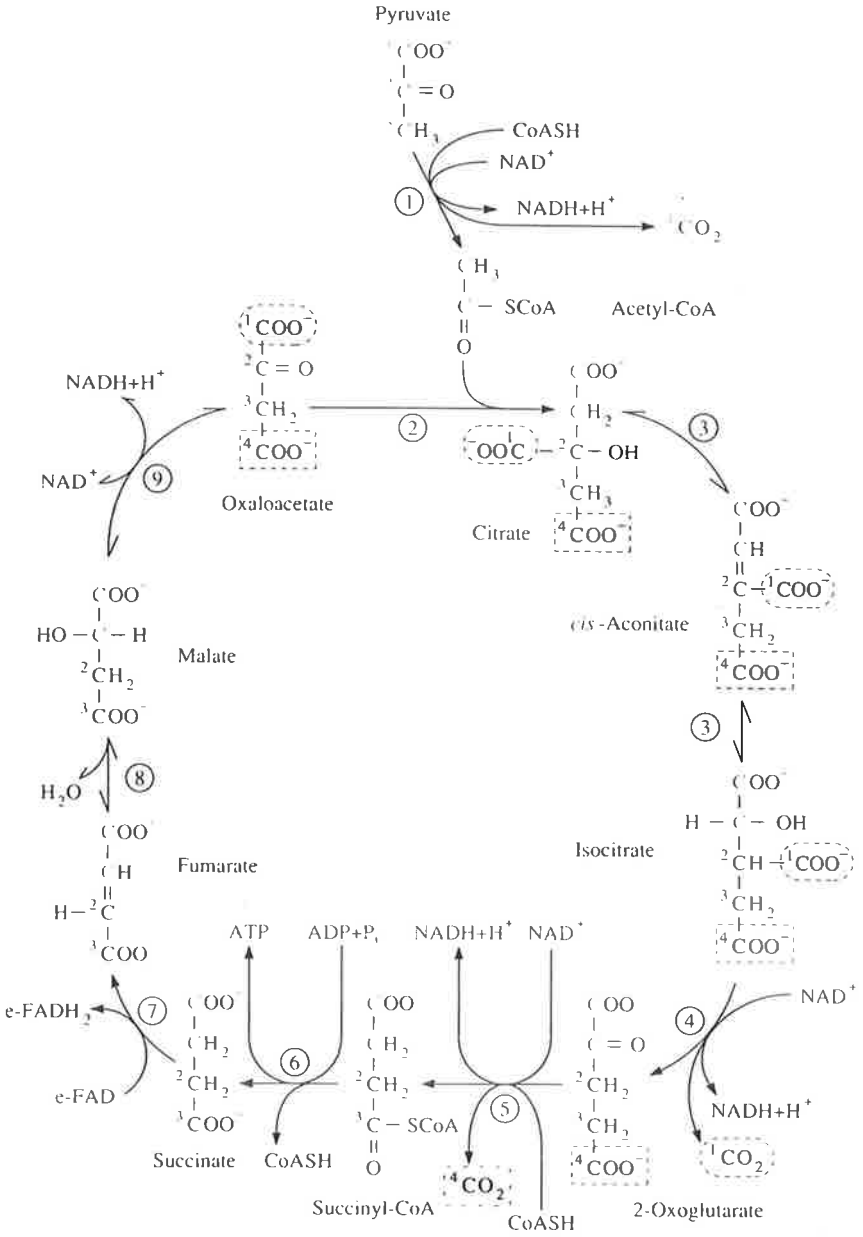
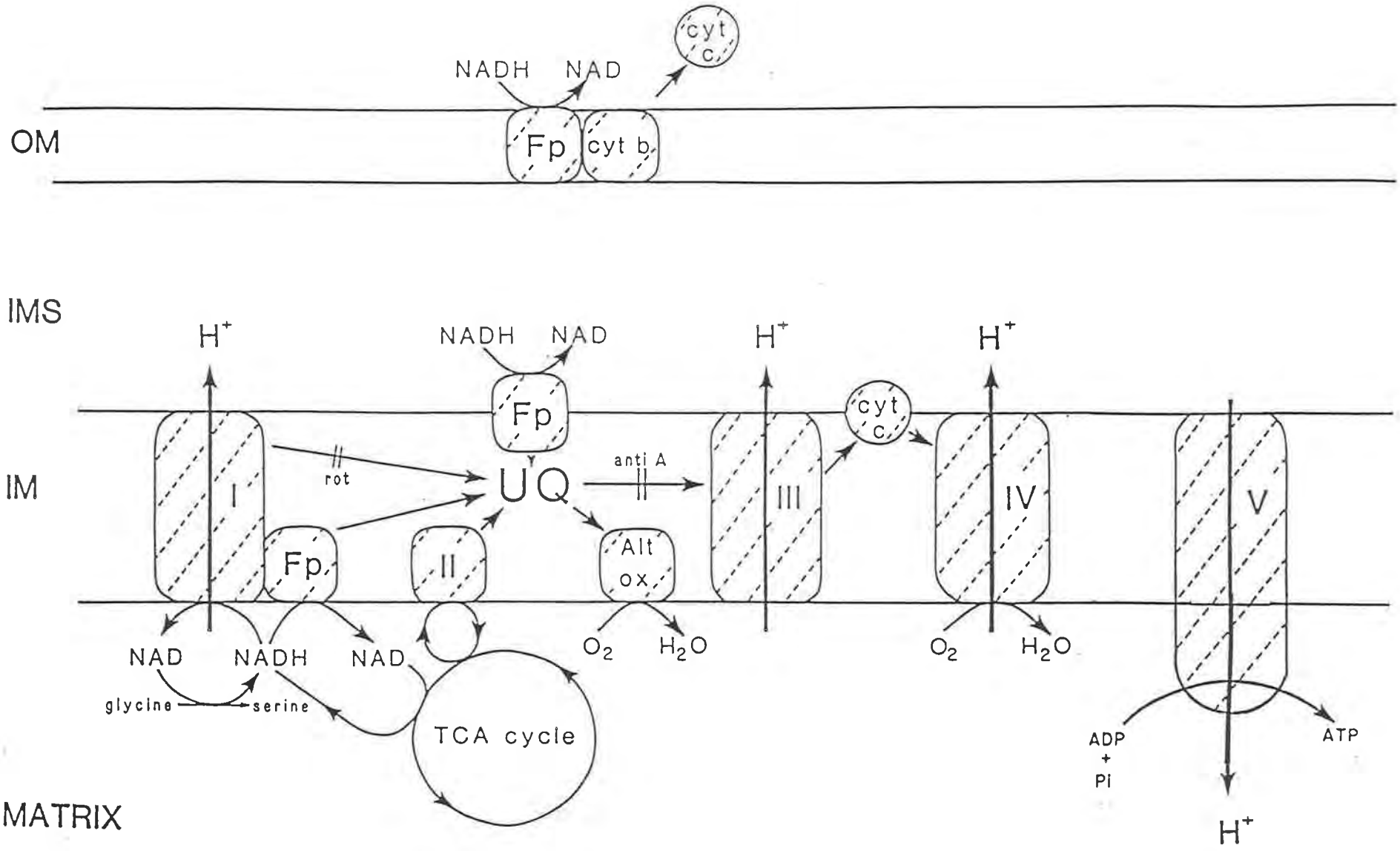


Fig.5.4. The TCA cycle. Abbreviations used: (1) pyruvate dehydrogenase; (2) citrate synthase; (3) aconitase; (4) isocitrate dehydrogenase; (5) 2-oxoglutarate dehydrogenase; (6) succinate thiokinase; (7) succinate dehydrogenase; (8) fumarase; (9) malate dehydrogenase. (Anderson and Beardall, 1991, p: 109).

Figure 5.5. The organization of electron transport chain in higher plant mitochondria. All components are located in the inner mitochondrial membrane. Numerals (I-IV) refer to the electron transporting complexes, and V refers to the ATP synthesis. Shown are outer membrane (OM), inter membrane space (IMS), inner membrane (IM), flapoprotein (Fp), ubiquinone (UQ), alternative oxidase (Alt ox), rotenone (rot), antimycin A (anti A), cytochrome b (cyt b), and cytochrome c (cyt c).


cyt c
NADH
NAD
OM
Fp
cyt b
IMS
H+
NADH
NAD
H+
H+
cyt c
IM
I
rot
UQ
anti A
III
IV
V
Fp
Fp
II
Alt ox
NAD
NADH
NAD
glycine
serine
TCA cycle
O2
H2O
O2
H2O
ADP + Pi
ATP
MATRIX
H+

Complex III is the second site of proton translocation. This complex transfers electrons from ubiquinone to cytochrome *c*. Two protons are transported into the intermembrane space for every electron, through the Q cycle (Mitchell, 1966) .

Complex IV is the terminal oxidase of the cytochrome pathway and consists of cytochrome *a* and cytochrome a_3. Cytochrome *a*, accepts electrons from cytochrome *c* and donates them to cytochrome a_3 which reacts with oxygen to form water. This complex extrudes 1 or 2 protons per electron (Lambers, 1990).

Besides these four main complexes, there are some additional components occurring in the plant mitochondrial ETC. Firstly, a NADH dehydrogenase located in the innermembrane, but with its binding site for NADH facing the intermembrane space, can oxidize cytosolic NADH. Secondly, a NADPH dehydrogenase, similarly located can oxidize cytosolic NADPH. Thirdly, alternative oxidase, located in the inner membrane, and facing the matrix also reacts with oxygen to form water. The last component is the rotenone-insensitive NADH dehydrogenase, which is distinct from complex I, but also faces the matrix and is not blocked by inhibitors of complex I. None of these additional components extrude H^+ during electron flow to ubiquinone or, in the case of the alternative oxidase, from ubiquinol.

5.1.7. Mitochondrial electron transport chain (mETC)

In plants, fungi and microorganisms, respiration by the mitochondrial electron transport chain can advance via two pathways, the phosphorylating cytochrome pathway and the nonphosphorylating alternative pathway (Fig.5.6). The branch point between these two pathways is the mobile ubiquinone pool.

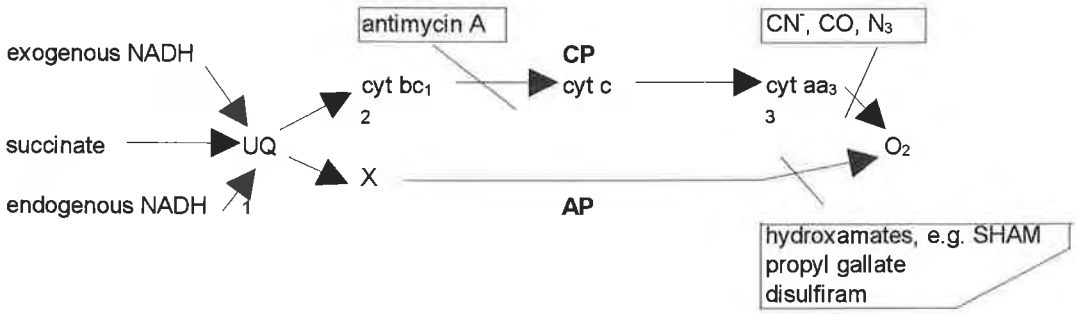


Fig. 5.6. A schematic drawing of the electron transport pathways in plant mitochondria. Coupling sites of electron flow to proton gradient formation are shown by numbers 1, 2, and 3. UQ, ubiquinone; CP, cytochrome pathway; AP, alternative pathway; X, alternative oxidase (Møller *et al.*, 1988).

5.1.7.1 Characteristics of the alternative (cyanide-resistant) pathway

The alternative pathway catalyzes the well known phenomenon of cyanide-resistant (CN-resistant) respiration. It generates no proton motive force, releases energy as heat, and is energetically inefficient. The CN-resistant pathway has been widely found among higher plants, fungi and microorganisms (Day *et al.*, 1980; Lance *et al.*., 1985; Douce and Neuburger, 1989) and plays an important role in plant survival, growth and development. The contribution of the non-phosphorylating cyanide-resistant pathway of mitochondrial electron transport to the respiration rate of intact tissue has been studied for years (Bingham and Farrar, 1987; de van Werf *et al.*, 1991). However, no study on the effect of UV-B radiation on the alternative pathway has been made.

The transport of electrons from endogenous NADH to O2 via the CN-resistant pathway is associated with the formation of one ATP molecule per NADH, whilst there are three ATP formed when transport occurs via the cytochrome pathway. Since the CN-resistant pathway is non-phosphorylating, this pathway offers a potential explanation for the low yield of ATP, thus requiring a high rate of respiration to give the same rate of ATP production (Møller *et al.*, 1988), even though it is different within species. For example, in male-sterile *Pisum sativum* varietes, Musgrave *et al.* (1986) found that higher yields were due to the absence of an CN-resistant pathway, while in *Lolium perenne,* faster respiration is not associated with a high activity of this pathway (Day *et al.*, 1985).

The CN-resistant respiration has several functions. It generates heat to volatilizes oils in order to attract insects required for cross pollination in thermogenic tissues of certain lilies (Meeuse, 1975). More importantly, it has three roles in plant carbon metabolism. Firstly, the CN-resistant pathway allows carbon flow through glycolysis and the TCA cycle to continue even when cytosolic energy status is high. This prevents the build-up of glycolytic intermediates during periods of high carbon input into glycolysis which may otherwise lead to fermentation. Secondly, it provides carbon skeletons for biosynthetic reactions, especially if it is coupled to the operation of phosphoenolpyruvate carboxylase in the cytosol and malic enzyme in the mitochondrion (Wiskich and Dry, 1985). This may have particular relevance to the production of secondary compounds such as happens in disease resistance. Thirdly, the CN-resistant pathway may play a protective role in the mitochondrion by preventing over-reduction of the respiratory chain and consequent production of reactive oxygen species which may cause organelle and cell damage (Pervis and Shewfelt, 1993).

5.1.7.2. Activity of CN-resistant respiration

Mitochondrial respiration has been studied for years in intact tissue (leaf and root) and isolated mitochondria. Respiration in intact tissues can be measured by following the O2 concentration in a solution in which the intact tissues or parts thereof are present and manipulated by the addition of inhibitors. The concentration of inhibitors of the cytochrome pathway and the CN-resistant pathways to be used varies between tissues. For KCN, the usual range is 0.1 - 1.0 mM and for SHAM 2-25 mM. However, side effects of SHAM may occur at both low and high concentrations (Møller *et al.*, 1988). The CN-resistant, SHAM sensitive respiration *in vivo* is generally reflected in the CN-resistant of the mitochondria isolated therefrom (Lambers *et al.,* 1983) and there is no evidence to indicate that other enzymes account for the phenomenon of CN-resistant, SHAM sensitive O2 uptake observed in intact tissues.

In intact tissues, the presence of the CN-resistant pathway in intact tissues is demonstrated by measuring SHAM-sensitive O2 uptake in the presence of KCN. An inhibition of tissue oxygen uptake by SHAM, in the absence of KCN, can be interpreted as being due to some alternative pathway activity. However, quantitative measurements of the activity of either the CN-resistant or cytochrome pathway can not be made using inhibitors. This is because inhibitors change the steady state activity of the CN-resistant pathway (by activation and /or reduction) and because switching of electron flow can occur between the two pathways (Hoefnagel *et al.*, 1995).

Myxothiazol and *n*-prophylgallate (nPG) are not suitable for use with intact tissues (Moore and Siedow, 1991). SHAM has been used extensively as a potent and seemingly selective inhibitor of the CN-resistant pathway in a variety of tissues and species. Low concentrations of SHAM are claimed to specifically inhibit the CN-resistant pathway (Lance *et al.*, 1985) in intact barley roots (Bingham and Farrar, 1987) and in green algae (Weger *et al.*, 1990). In intact tissue, there may be a small component of residual respiration which is not inhibited by either cyanide or SHAM (Møller *et* al., 1988). The concentration of SHAM (0.03 - 0.4 mM) most commonly used to inhibit the CN-resistant pathway almost completely (Schonbaum *et al.*, 1971) has been shown to stimulate O_2 uptake in several plant species (Bingham and Farrer, 1987; Weger *et al.*, 1990). However, at higher concentrations above 2 mM, SHAM may inhibit the cytochrome pathway (Lambers *et al.*, 1983; Bingham and Farrer, 1987). With intact roots, in the absence of cyanide, only high concentrations were inhibitory (>5mM), while in the presence of cyanide, low SHAM concentrations (2.5 - 5.0 mM) give maximum inhibition

(Bingham and Farrar, 1987). These results further illustrate the uncertainty involved when using inhibitors.

In isolated mitochondria, respiration can be measured by following the change in O_2 concentration in a solution with additional exogenous substrates. Antimycin A, myxothiazol and KCN are used as specific inhibitors of the cytochrome pathway, while the CN-resistant pathway is sensitive to the hydroxamic acids salicylhydroxamic acid (SHAM), benzhydroxamic acid (BHAM), and to nPG.

Use an inhibitor of one pathway to measure the capacity of the other pathway is straightforward, at least in isolated mitochondria. Assessing the contribution of either oxidase by inhibiting respiration leads to switching of electron flow to the other pathway, resulting in under estimation of the activity of the inhibited pathway prior to the addition of the inhibitor.

5.1.8. Substrates for electron transport chain

The major substrates for mitochondrial oxidations are pyruvate, malate, succinate, intermediates of the TCA cycle, NADH from glycolysis, NADPH from the oxidative pentose phosphate pathway, and glycine (in C3 leaves) produced via the photorespiratory pathway (Fig.5.7) (Lambers, 1990). Pyruvate and malate, as endproducts of glycolysis are two major substrates for the TCA cycle. Malate may be oxidized in mitochondria by either malate dehydrogenase producing oxaloacetate or malic enzyme producing pyruvate and CO_2. This pyruvate becomes available for subsequent oxidation via pyruvate dehydrogenase and the TCA cycle. Extra-mitochondrial NADH from glycolysis can be oxidized via the external NADH dehydrogenases, on the outer surface of the inner membrane. Endogenous NADH is oxidized via a dehydrogenase bound to the inner side of the inner membrane and the rotenone-sensitive.

Moreover, the expression and activity of the CN-resistant pathway within a plant is often tissue specific. In isolated mitochondria from non-thermogenic tissues, large variations have been seen in CN-resistant activity as substrates are varied (Lance *et al.*, 1985). For example, succinate and malate seemed to give the maximum rates (Lance *et al.*, 1985), whereas NAD-linked substrates often gave slower rates that correlate with a lower level of quinone (Q) reduction (Day *et al.*, 1991). Exogenous NADH may be poorly oxidized via the alternative oxidase even though it maintains QH_2 at levels seen with succinate, and exogenous quinols barely oxidized at all (Day *et al.*, 1991; Moore and Siedow, 1991). The activity with

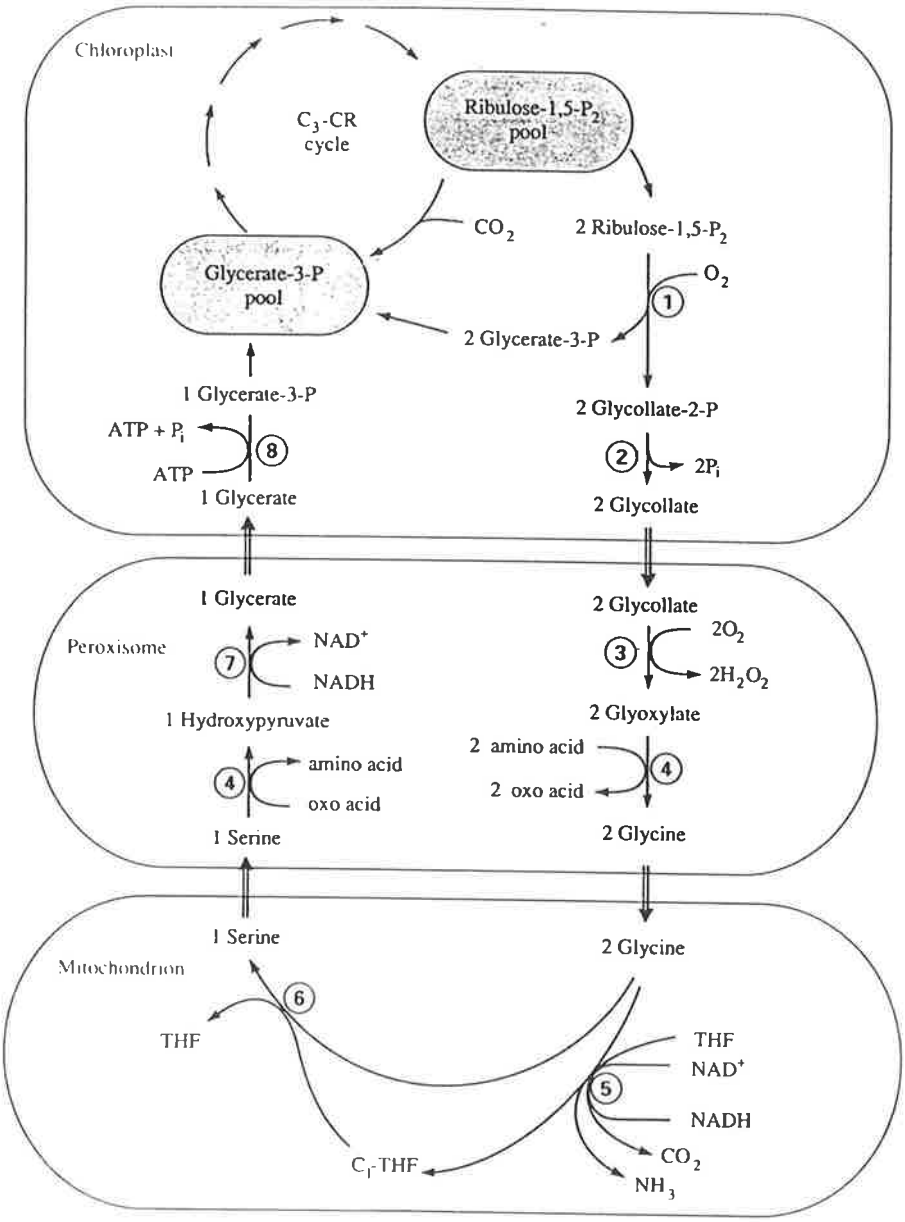


Fig.5.7. Pathways of photorespiration. Enzymes are : (1) ribulose-1,5-P_2 oxygenase; (2) glycollate-2-P phosphatase; (3) glycollate oxidase; (4) aminotransferase; (5) glycine dehydrogenase; (6) serine hydroxymethyltransferase; (7) glycerate dehydrogenase; (8) glycerate kinase. (Anderson and Beardall, 1991, p: 188).

succinate or malate as substrates may be substantially greater than with exogenous NADH (Lance *et al.*, 1985), even though NADH oxidation via the cytochrome pathway is rapid and reduces the Q pool greatly (Day *et al.*, 1991). Current findings have demonstrated that reaction conditions can influence CN-resistant activity greatly, with succinate able to stimulate activity with NADH and low temperature stimulating QH_2 oxidation (Lidén and Akerlund, 1993). More recently, it has been shown that these differences in preference of substrates oxidation can be abolished by the addition of certain organic acids; (e.g. pyruvate) which act as an allosteric activators of the oxidase in soybean and some other species (Millar *et al.,* 1993)

5.1.9. Effect of UV-B on respiration

The CN-resistant pathway is supposed to be part of a stress response in plants and fungi. In a variety of plants, especially non-thermogenic plants, plants cell cultures and yeasts, CN-resistant respiration can be induced by various stresses. In other words, an increased respiration rate is sometimes associated with higher activity of the alternative pathway of electron transport. Low temperature and chilling injury, for example, have been found to induce the CN-resistant respiration in corn seedlings (Stewart, 1990), wheat coleoptiles (McCaig and Hill, 1977), cucumber seedling (Sasson and Bramlage, 1981), soybean cotyledons (Leopold and Musgrave, 1979), and in suspension cells of tobacco (Vanterberghe and McIntosh, 1992). UV-B radiation, which is expected to increase simultaneously with other changes in the global climate, also induces a stress response in plants. The portion of UV-B not absorbed by the epidermis passes to the mesophyll, and could lead to additive, compensatory or other synergic effects on plant photosynthesis and respiration. The shorter wavelengths of UV-B have been reported to stimulate respiration in *Rumex patientia* L.(Sisson and Caldwell, 1976), in peas (Brandle *et al.*, 1977) and in cassava (Ziska *et al.,* 1993), but not in seagrass (Larkum and Wood, 1993). The mechanism by which UV-B stimulates respiration is unclear and no work has focused on the interactions and changes in the respiratory alternative pathway. Therefore, a study on the extent and pattern of leaf slice and isolated mitochondrial respiration after supplementary UV-B treatment is of interest.

In this present study, morphological and anatomical changes in leaves as well as a reduction in plant biomass were found under supplementary UV-B

radiation (Chapter 3 and 4). It was hypothesized that one of the reasons for this was the presence of, and high rates of respiration of, the alternative pathway. Therefore an attempt was made to investigate in some detail the effects of supplementary UV-B radiation on the respiration of both leaf slices and isolated of pea and wheat leaf mitochondria.

5.2. Results and discussion

5.2.1. Respiration in leaf tissue

5.2.1.1 Uninhibited O_2 uptake (total respiration)

The rates of uninhibited O_2 uptake by leaf slices in the presence and absence of carbonyl cyanide p-(trifluoromethoxy)phenyhydrazone (FCCP) as an uncoupler, were measured in pea and wheat slices after a given period of UV-B irradiation. In general, the uninhibited rates of O_2 uptake decreased with leaf age over all treatments, were always lower in pea (Fig 5.8) than in wheat (Fig 5.9) when expressed on fresh weight basis, and were greatest under high UV-B.

Respiration *in vivo* would depend upon the endogenous levels of substrates, as well as on the availability of ADP and these may change with age. It could be that in older leaves, the production of substrates was lower than that in young leaves, and this would be a reason that O_2 uptake decreased with leaf age.

Total respiration was lower in pea leaf tissue than in wheat. This may be due to pea leaves having fewer mitochondria per gram tissue than wheat, which would contribute to a lower respiration rate. The preparation of the tissue for experiments may also have caused these differences in respiration. Slicing the leaf into thin strips may induce a wounding response which briefly increases the O_2 uptake. The leaf thickness affects the permeability properties to O_2, and the uptake of respiratory inhibitors.

Supplementary UV-B significantly stimulated the rates of total O_2 uptake (Fig.5.8A). These stimulations were (13%, 51%, 20%) for low UV-B and (34%, 107%, 92%) for the high UV-B after 4, 8, 16 days of treatment respectively. Respiration in wheat leaf slices was also stimulated by UV-B (Fig 5.9A). It has been previously found that UV or UV-B treatment increased respiration in *Rumex patientia* L.(Sisson and Caldwell, 1976), in pea (Brandle *et al.*, 1977) and in cassava (Ziska *et al.*, 1993).

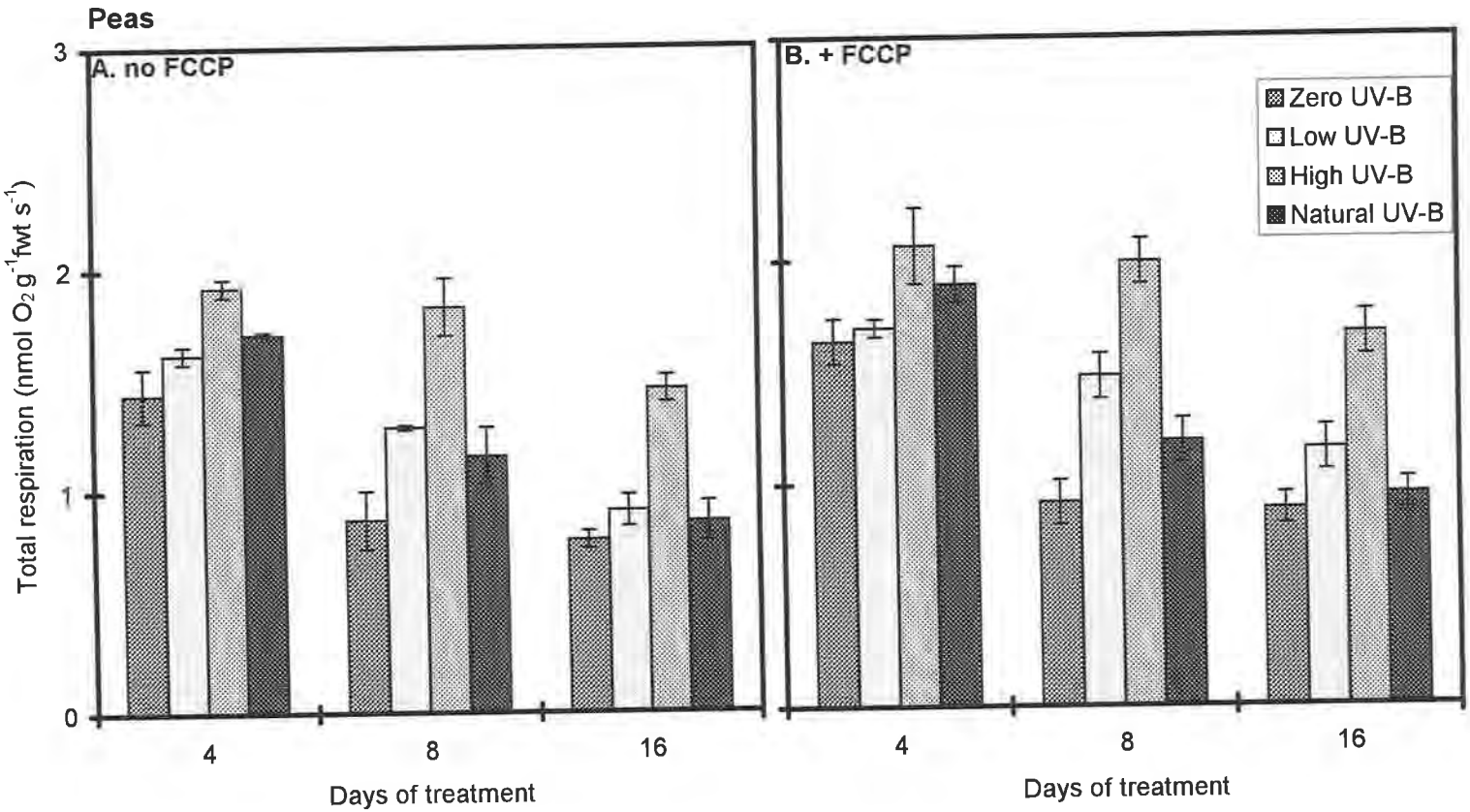


Fig.5.8. Total respiration rates of pea leaf slices, in the absence (A) and presence (B) of FCCP. Values are the means of of 4 replicates from at least two independent experiments.

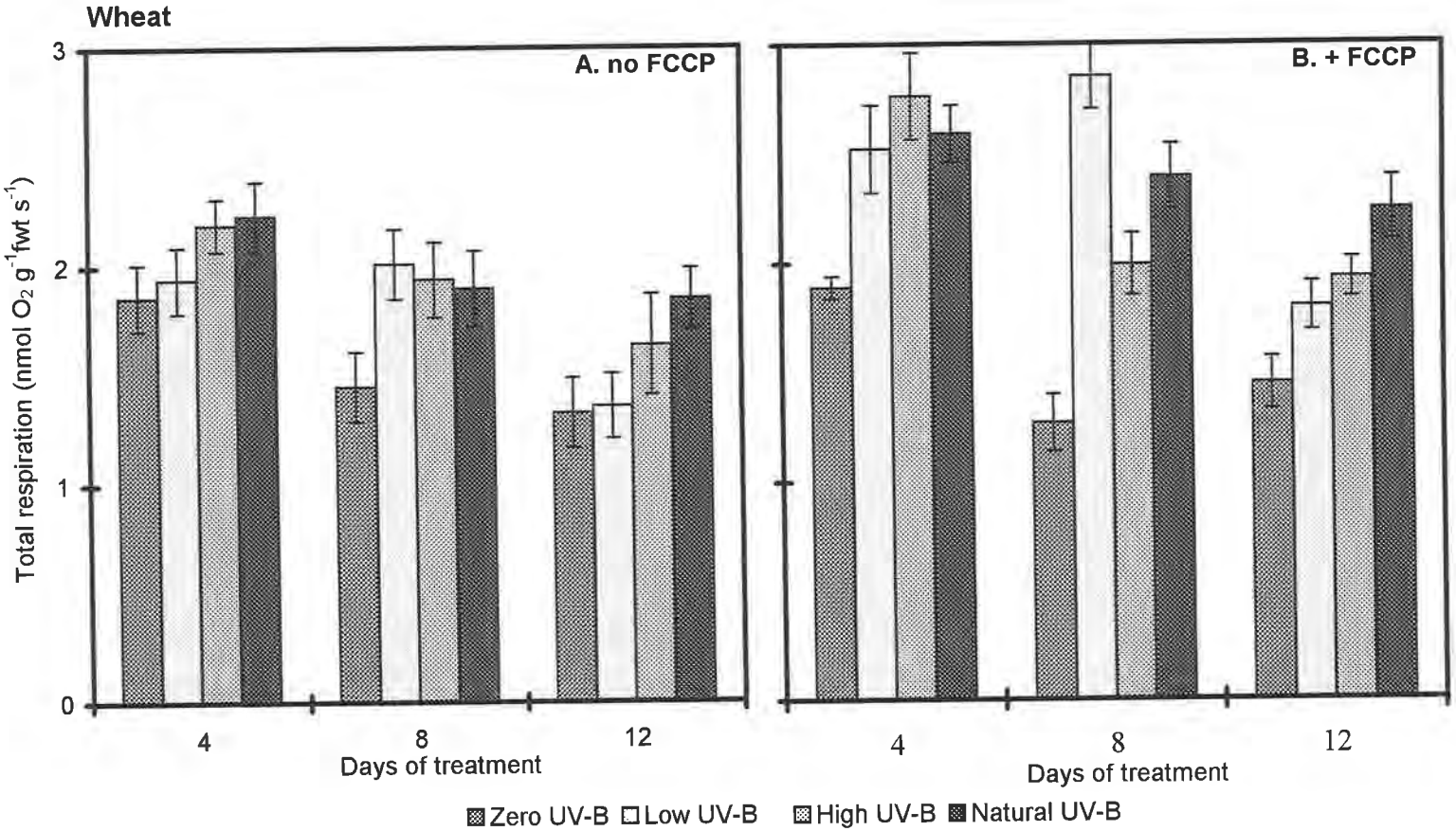


Fig.5.9. Total respiration rates of wheat leaf slices, in the absence (A) and presence (B) of FCCP. Values are the means of

In contrast, Whitecross *et al* (1994) found that there was no significant difference between untreated and treated plants when the respiration was measured with intact tissue. The increase in respiration rates under UV-B radiation is probably one response to environmental stress, and suggest that this increase must be is related to a useful process of maintenance and adaptation

Uncoupling respiration by the addition of FCCP generally increased the rate of total O_2 uptake. However, there appeared to be little or no effect on the high UV-B pea leaves (Fig.5.8) and on the zero UV-B wheat leaves (Fig.5.9). This would suggest that ATP turnover was not limiting the respiratory process in these treatments. This indicates that the initial rate of O_2 uptake was limited by the magnitude of the electrochemical gradient (Elthon and Stewart, 1983). In bean leaf slices also, respiratory stimulation by FCCP decreased with age (Azcön Bieto *et al.*, 1983). FCCP, by catalyzing a net unindirectional transport of protons abolishes the electrochemical gradient and allows maximum electron flux through the respiratory pathways. This uncoupler also increases the permeability of membranes to protons. This prevents the synthesis of ATP, resulting in a decrease in the cellular level of ATP and an increase in the concentration of ADP. Hence, the rate of glycolysis increases due to increase of availability of ADP.

5.2.1.2. Activities of respiratory pathways in the presence of inhibitors

The response to inhibitors of pea and wheat leaf slices was different. Pea leaf slices showed some sensitivity to both KCN and SHAM when inhibitors were applied individually (Fig.5.10 and 5.12). However, wheat leaf slices displayed an almost complete resistance to KCN, when it was added alone (Fig.5.11), but were sensitive to SHAM (Fig.5.13).

The effects of KCN in the absence of SHAM on the rates of O_2 uptake of pea leaf slices from UV-B treated and untreated seedlings, as a function of seedling age, are shown in Fig. 5.10. In all pea leaf measurements, the initial addition of KCN after approximately 6 min of uninhibited steady rate brought about a considerable decrease in O_2 uptake in either all UV-B treated leaves or with leaf age. The actual rate of O_2 uptake in the presence of KCN was measured for about 8-10 min and it was virtually constant from 8 to 16 days of treatment. Thus the percentage of cyanide resistance increased with leaf age. Although all treatments showed a decrease in respiration after the addition of KCN, low and high UV-B treatments still gave higher rates than control leaves at all ages, but it was only significantly higher after 8 days of treatment. This indicates that under

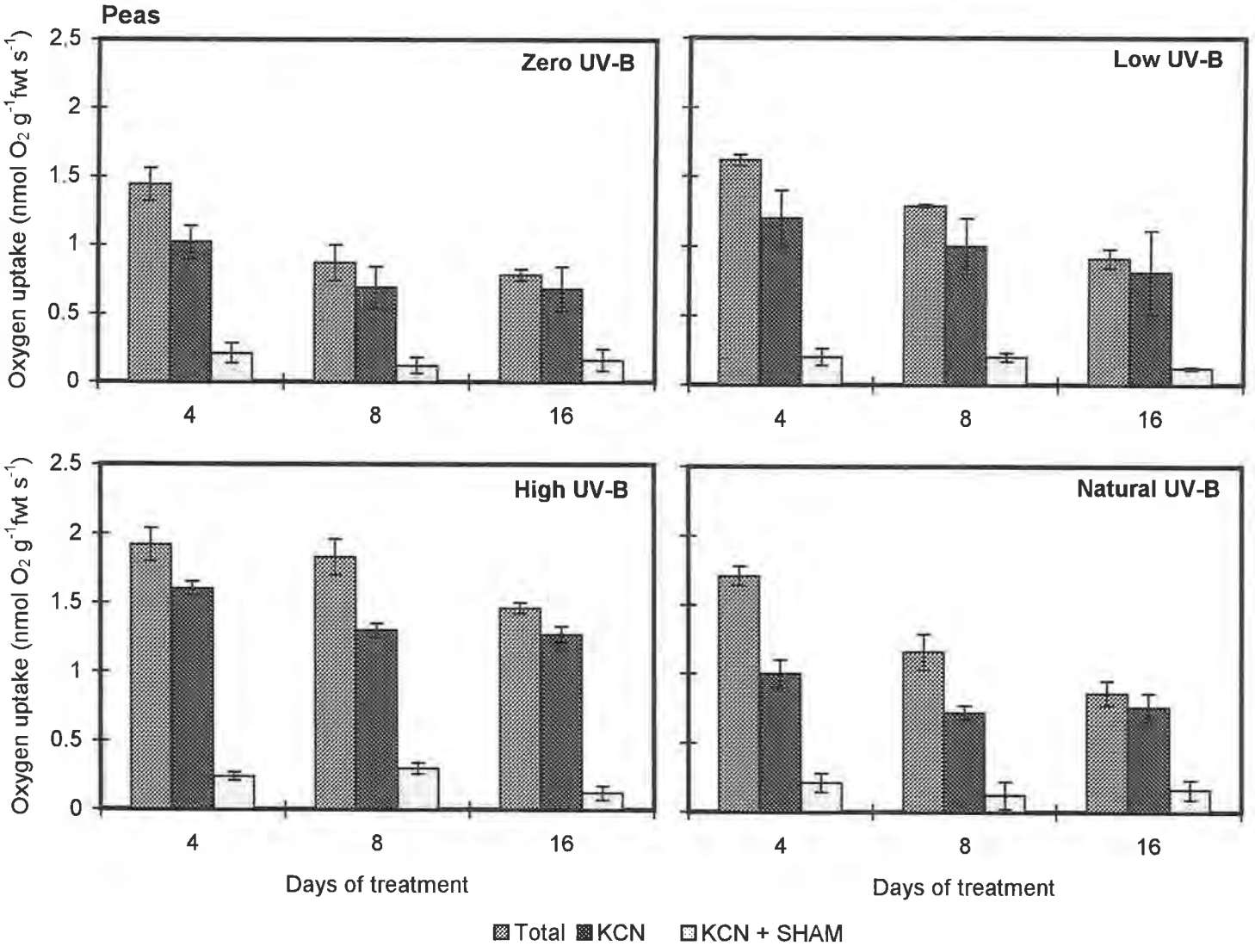


Fig. 5.10. Coupled respiratory rates of pea leaf slices. KCN and SHAM were added at 1.0mM and 3.0mM respectively. Values are the means of 4 replicates from at least 2 independent experiments.

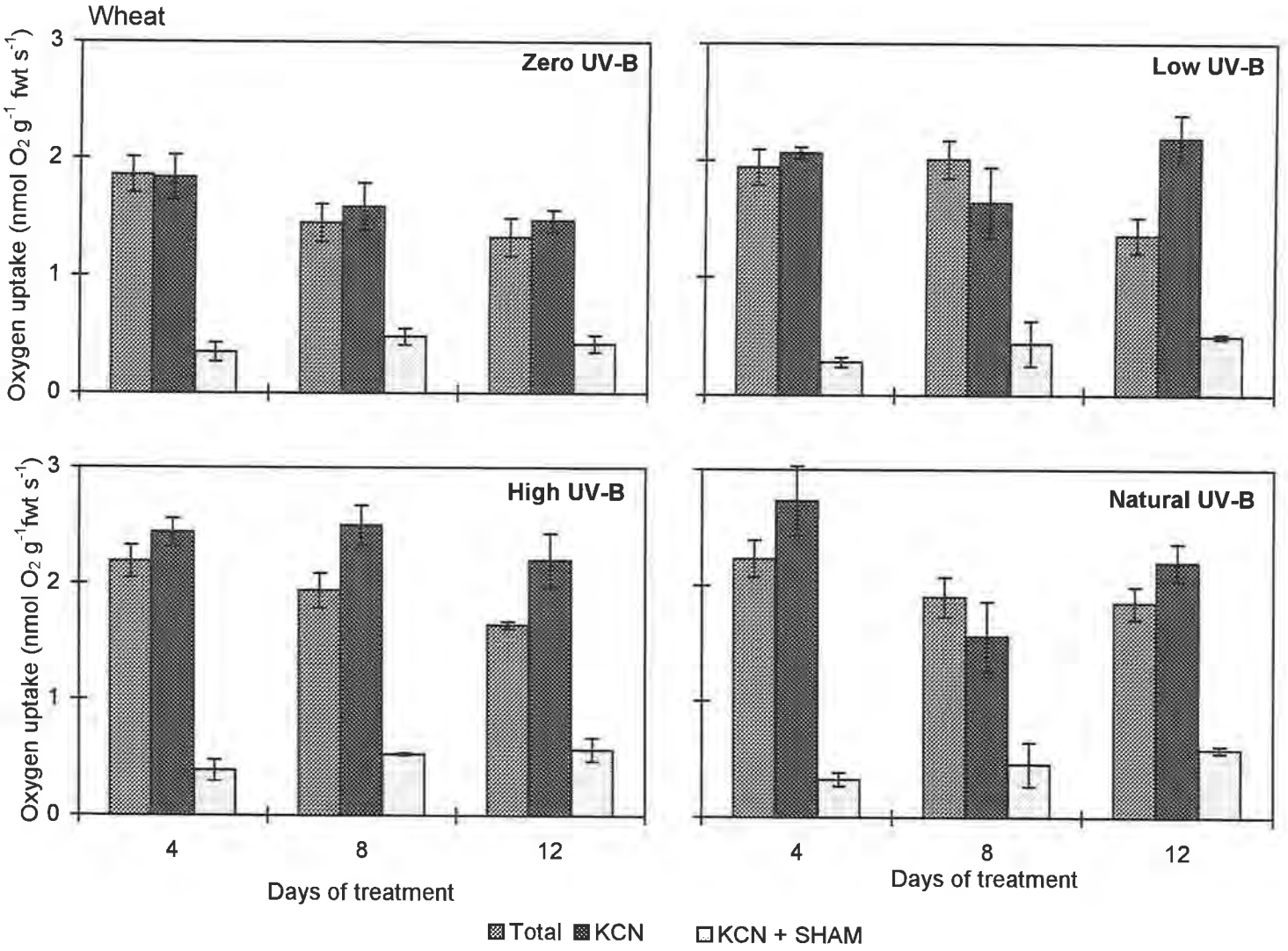


Fig.5.11. Coupled respiratory rates of wheat leaf slices. KCN and SHAM were added at 3.0mM and 1.0mM respectvely. Values are the means of 4 replicates from at least independent experiments.

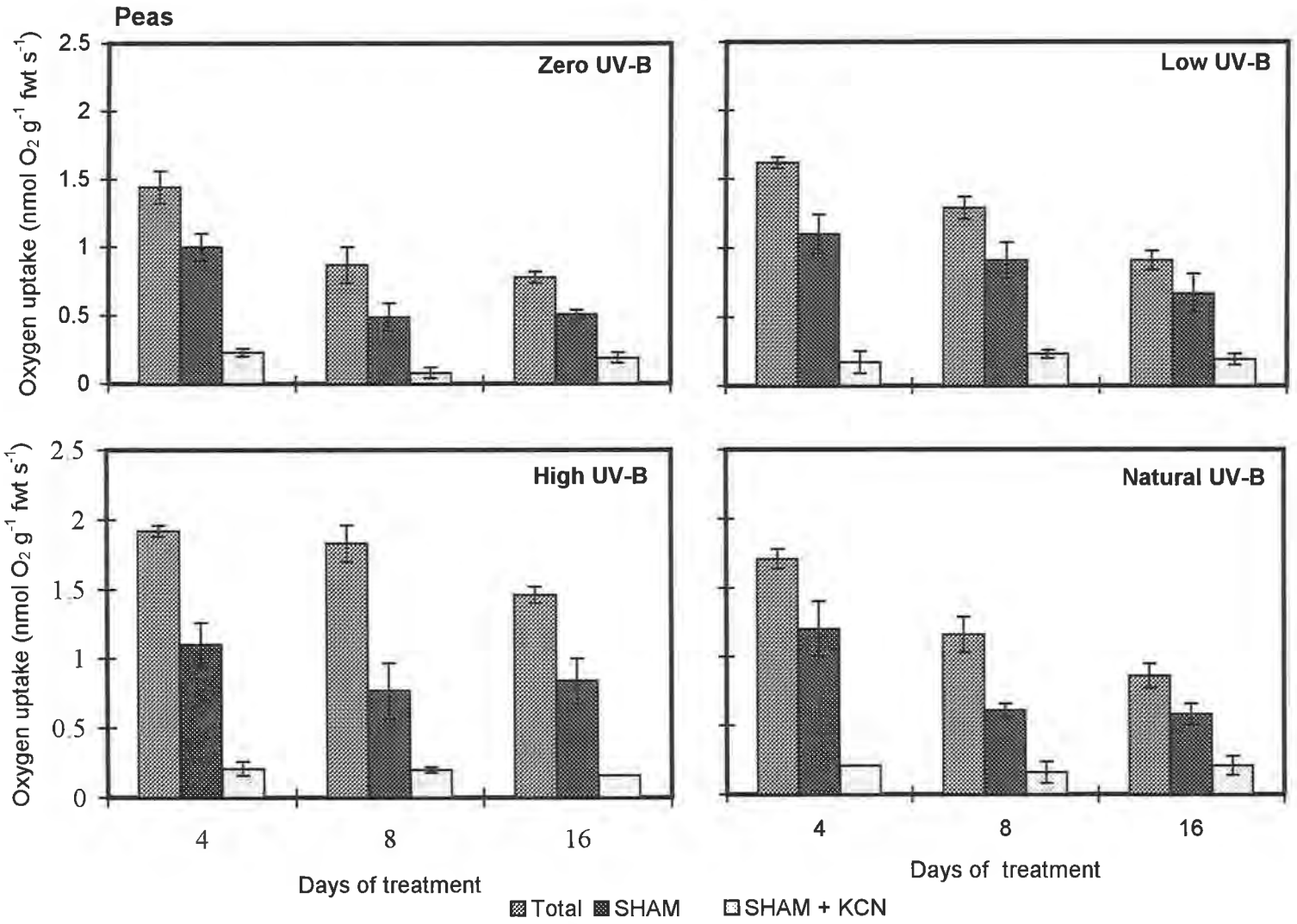


Fig. 5.12. Coupled respiratory rates of pea leaf slices. SHAM and KCN were added at 3.0mM and 1.0mM respectively. Values are the means of 4 replicates from at least 2 independent experiments.

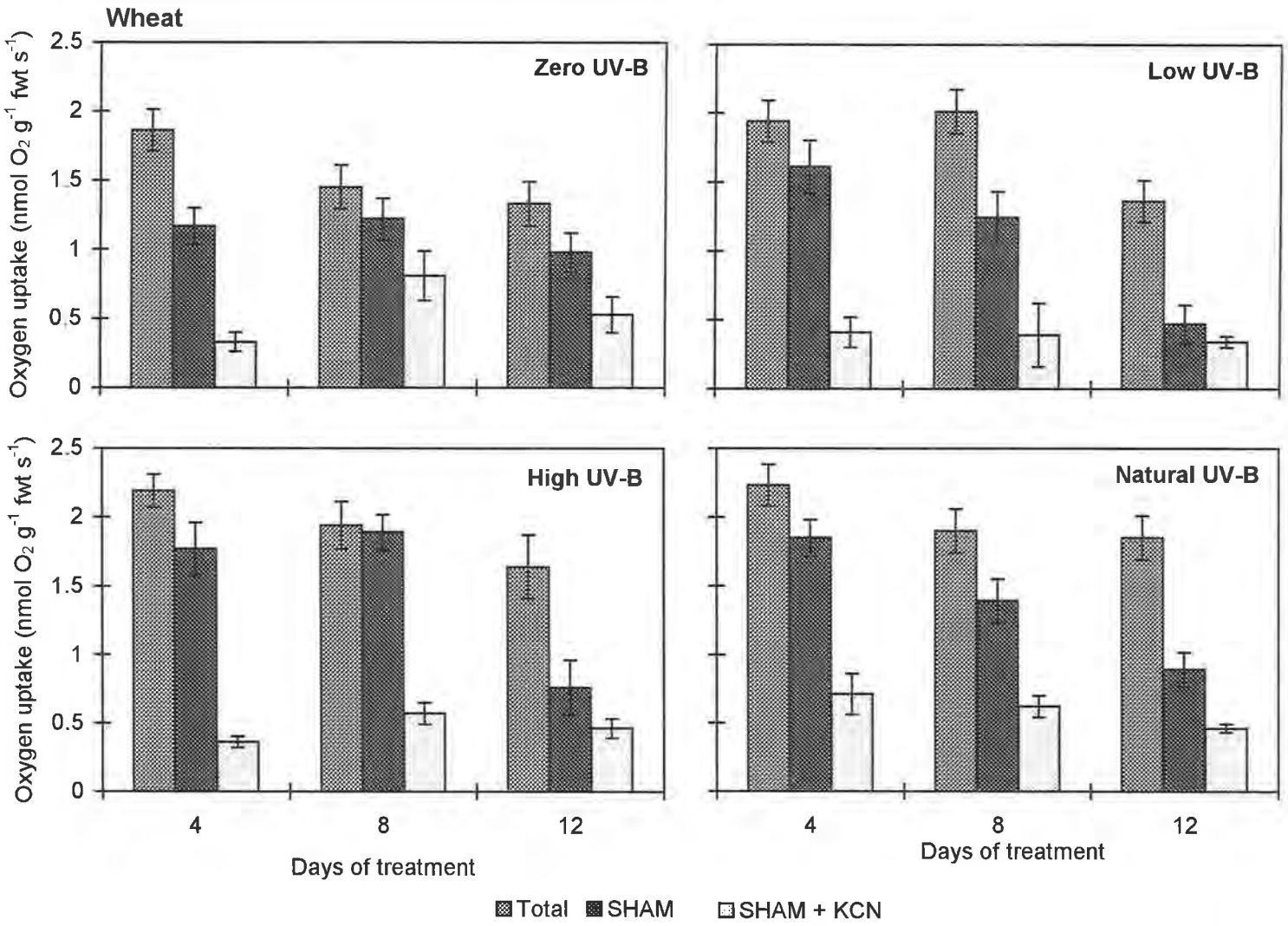


Fig.5.13. Coupled respiratory rates of wheat leaf slices. SHAM and KCN were added at 1.0mM and 3.0mM respectively. Values are the means of 4 replicates from at least independent experiments.

supplementary UV-B radiation, there may be an increased amount of the alternative oxidase protein or there was an activation of the cyanide-resistant activity.

The respiration of wheat leaves was entirely cyanide resistant (Fig.5.11), in fact, there was a KCN stimulation of respiration of high UV-B leaves. The absence of KCN inhibition suggests that electron flow was diverted to the alternative pathway when the cytochrome pathway was blocked, and implies that the activity of the alternative pathway is at least as great as the engagement of the cytochrome pathway in the absence of KCN. The stimulation of O_2 uptake by cyanide has been interpreted as a result of decreased ATP production which in turn stimulates carbohydrate catabolism. The electrons produced are transferred to O_2 via the CN-resistant (Lambers. 1985). If this is the correct interpretation, then the unused activity of CN-resistant must exceed the activity of the cytochrome pathway. Under high UV-B treatment, the O_2 uptake was higher than in the control, indicating that treated leaves were relatively more resistant than the control leaves.

In the presence of both KCN and SHAM in the reaction medium, the rates of O_2 uptake were not totally inhibited. Pea and wheat leaf tissue had residual respirations up to ±15% and ±20% respectively of the uninhibited O_2 uptake. In pea leaves, this residual respiration was low and did not change with age or UV-B treatment. In wheat leaves, the residual respiration increased slightly with age, but was not alterd by UV-B treatment.

The initial addition of SHAM to inhibit the CN-resistant pathway showed that the respiration was partially inhibited by SHAM at all ages and at all treatments (Fig.5.12 and 5.13) in both species. An increased participation of the cyanide-resistant pathway in young tissue may reflect the inability of the tissue to store those carbohydrates which are not being utilised in structural growth, energy production, or storage within the plant body, but are subsequently oxidized via the alternative pathway (Lambers, 1980). However, with age, the capacity to store carbohydrates increases. A decreased availability of substrates for metabolism may match the requirements for the cytochrome chain alone which will result in a negligible flow of electron through the alternative pathway and a decreased participation.

In pea leaves, SHAM alone obviously reduced the total rate of O_2 uptake to some extent (Fig.5.12). The rates of O_2 uptake in the presence of SHAM decreased concomitantly with age up to 8 days, and then remained relatively constant. The inhibition by SHAM of high UV-B leaves seemed to be greater (42

%, 55%, and 43% for 4, 8, and 16 days of treatment respectively) than that of either control (30%, 43%, and 31%) or low UV-B (30%, 30%, 25%), even though low UV-B produced higher rates. This indicated that UV-B radiation produced a relatively high degree of sensitivity to SHAM or a high cyanide resistance in pea.

The values of O_2 uptake in the presence of SHAM for wheat leaves are presented in Fig.5.13. In UV-B treated leaves, the rates were slightly higher than in control.

5.2.1.3. The alternative and the cytochrome pathways in leaf slices

The changing patterns of cyanide-resistant O_2 uptake by slices of pea leaf during the UV-B treatment and leaf development are presented in Fig.5.14. In the absence of FCCP (Fig.5.14A), from 4 to 16 days of treatment there was a gradual decrease in the activity of the cyanide-resistant respiration rate (v_{alt}, the total respiration rate minus the rate in the presence of SHAM). Treatment with low UV-B radiation brought about a slight increase (50%, 140%, 5% at day 4, 8 and 16 of treatment) in the activity of the cyanide-resistant pathway but a marked and significant increase was seen under high UV-B treatment (100%, 400%, 100% at 4, 8, and 16 days of treatment) respectively. Uncoupled tissue showed similar patterns of SHAM-sensitive respiration except for the 16-day treatment under high and low UV-B. In this case FCCP induced a marked increased in the amount of SHAM-sensitive respiration suggesting that substrate supply may have been limiting the respiration rate.

The effects of UV-B treatment on the activity of CN-resistant wheat leaf slices are presented in Fig.5.15. From day 4 to day 12 of treatment, the values of participation of the cyanide-resistant to total respiration O_2 uptake declined with age, both in the absence and the presence of FCCP. Throughout the study, no significant increases under UV-B treatments were observed. FCCP stimulated the SHAM-sensitive respiration of both low and high UV-B treated leaves.

No significant enhancing effect of UV-B radiation on the activity of cytochrome pathway (v_{cyt}, the rate in the presence of SHAM minus the residual respiration) was observed in either pea or wheat leaf slices. This occurred when this activity was measured either in the absence or presence of FCCP (Fig.5.16). However, the pattern whereby control leaves had lower activity and treated leaves showed considerably greater rates were still determined at all ages. The mean values of the activity of the cytochrome pathway continuously decreased

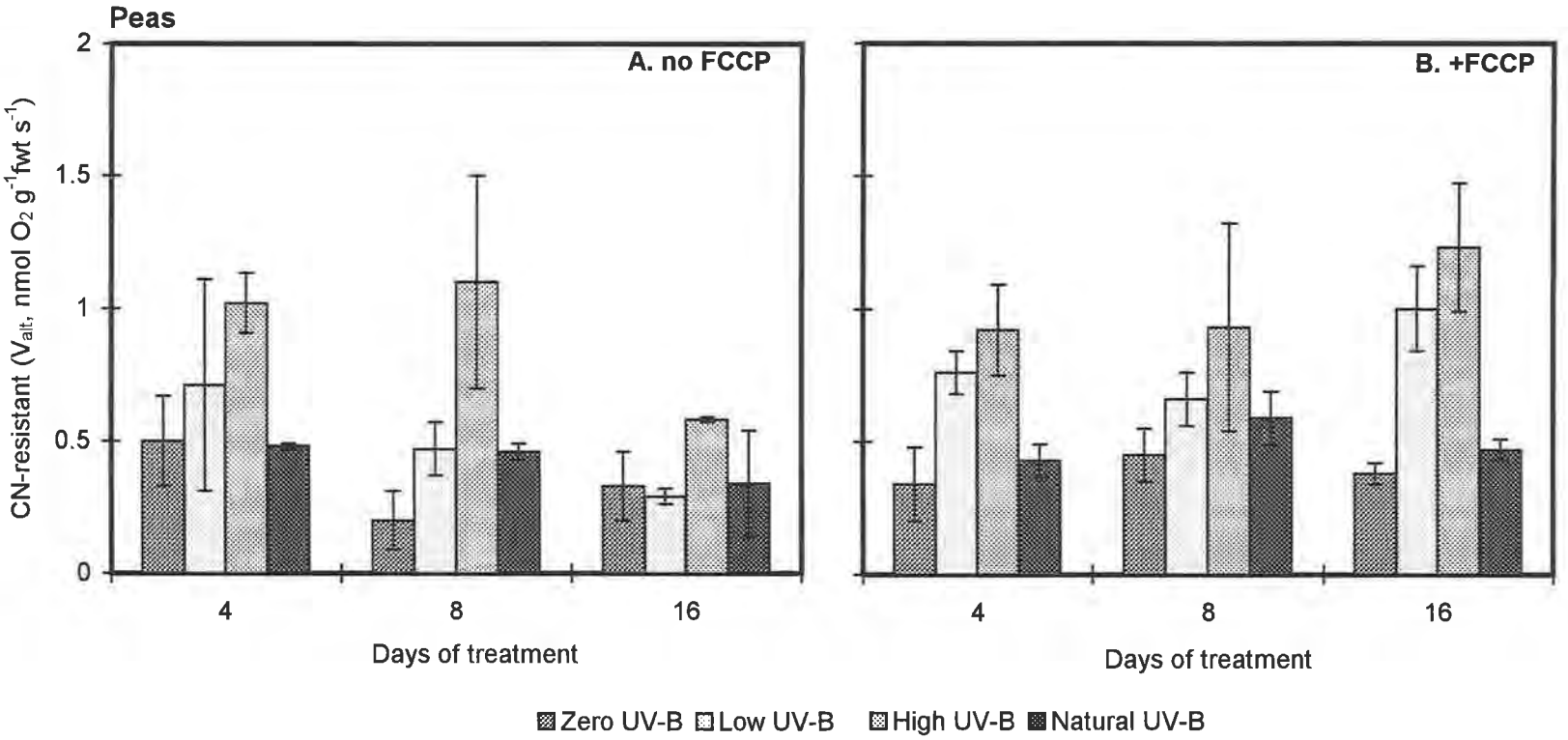


Fig.5.14. Respiration of the CN-resistant pathway in pea leaf slices in the absence (A) and presence(B) of FCCP, estimated by measuring the fraction of O_2 uptake sensitive to SHAM. SHAM and FCCP were added to final concentration of 3mM and 1mM respectively. Values shown are means of 4 replicates from at least 2 independent experiments.

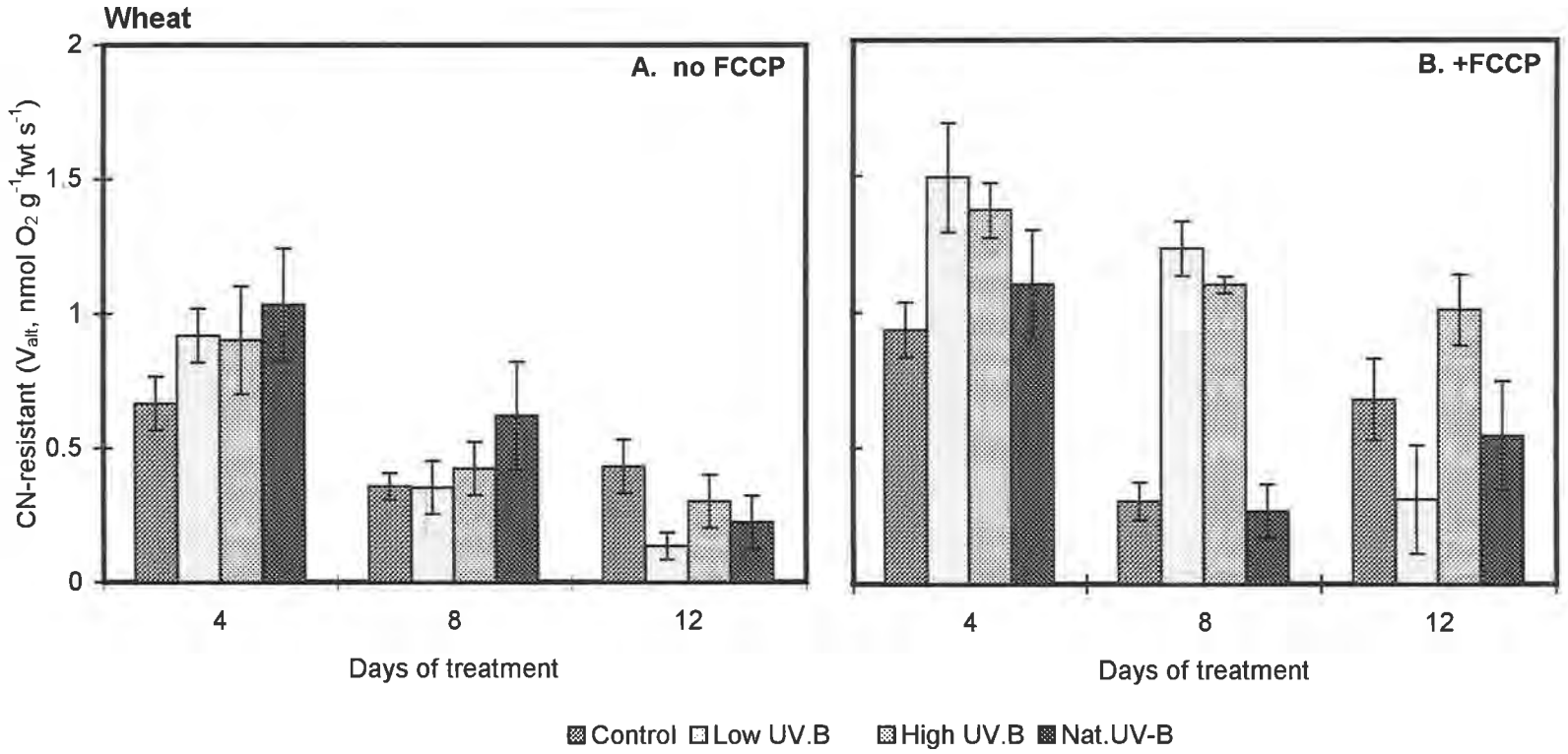


Fig.5.15. Respiration of the CN-resistant pathway in wheat leaf slices in the absence (A) and presence(B) of FCCP, estimated by measuring the fraction of O_2 uptake sensitive to SHAM. SHAM and FCCP were added to final concentration of 3mM and 1mM respectively. Values shown are means of 4 replicates from at least 2 independent . experiments.

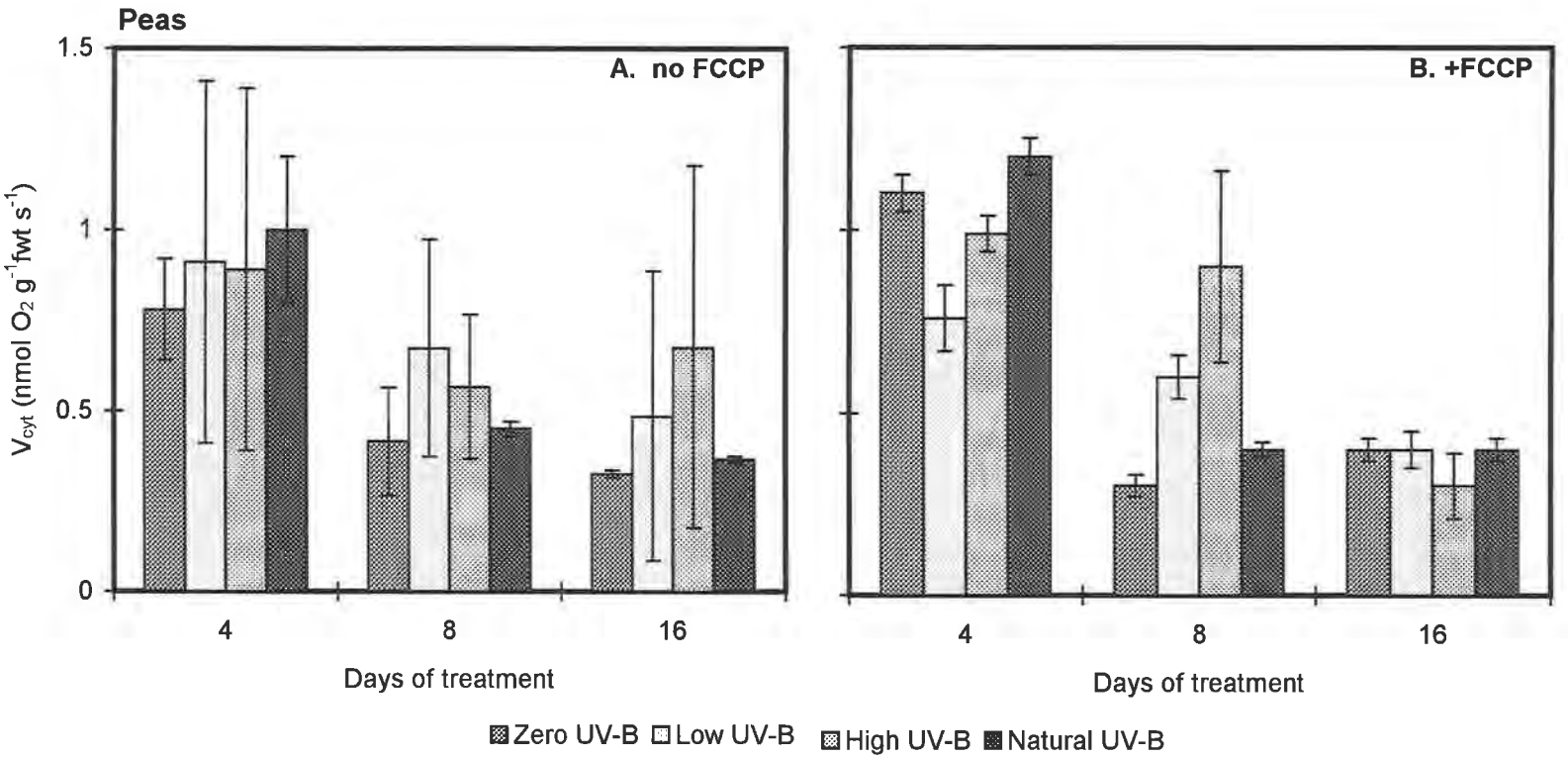


Fig.5.16. Respiration of the cytochrome pathway in pea leaf slices in the absence (A) and presence(B) of FCCP, estimated by measuring O_2 uptake in the presence of SHAM minus residual respiration. SHAM and FCCP were added to final concentration of 3mM and 1mM respectively. Values shown are means of 4 replicates from at least 2 independent experiments.

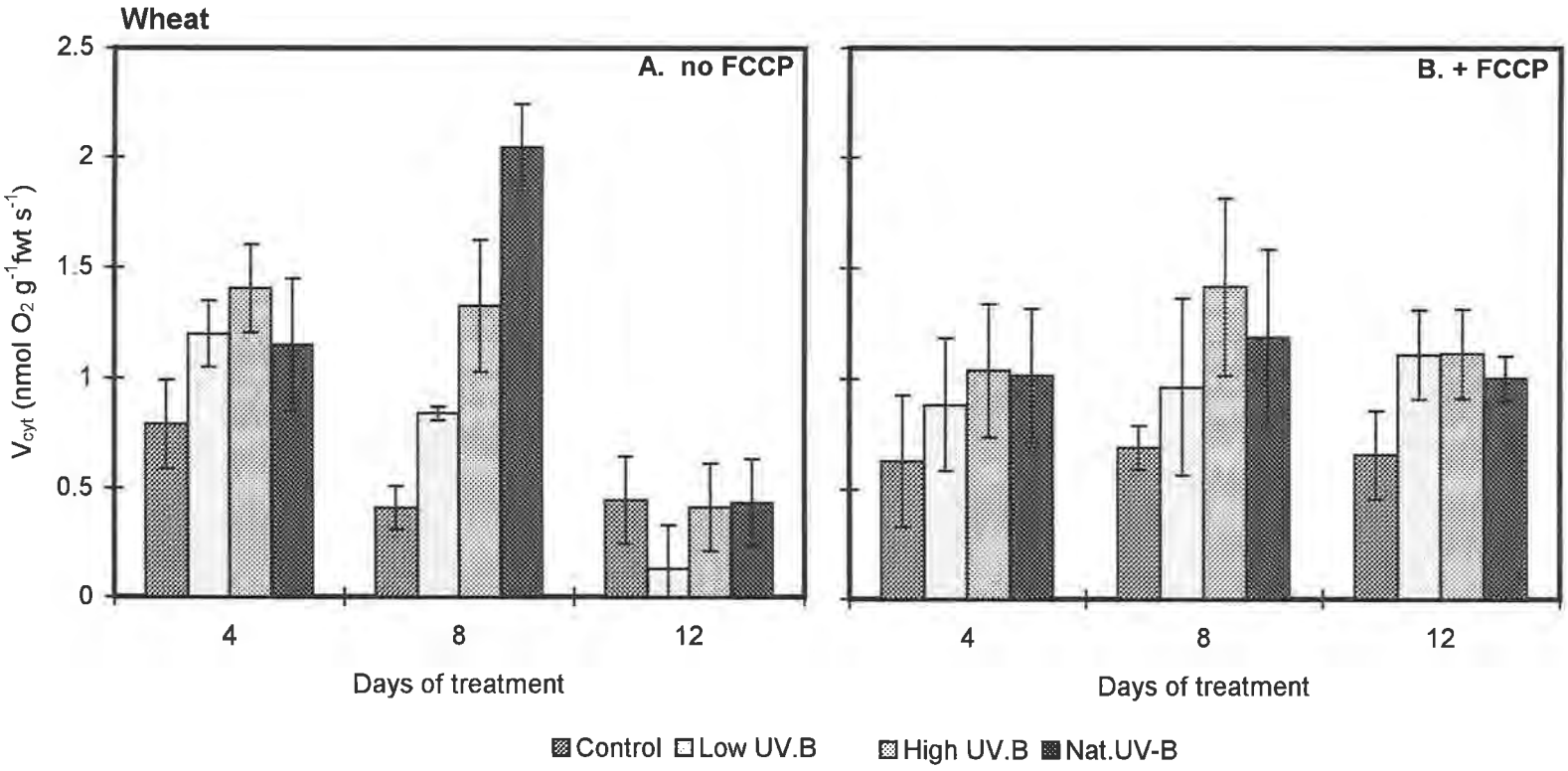


Fig.5.17. Respiration of the cytochrome pathway in wheat leaf slices in the absence (A) and presence(B) of FCCP, estimated by measuring O_2 uptake in the presence of SHAM minus residual respiration. SHAM and FCCP were added to final concentration of 3mM and 1mM respectively. Values shown are means of 4 replicates from at least 2 independent experiments.

with age. A similar pattern where the activity of the cytochrome pathway decreased with age was again observed in wheat leaf (Fig.5.17).

Because the level of the electrochemical gradient present can influence the measurable Cyt chain capacity and may also have an influence on the flow of electrons through the alternative pathway (Elthon and Stewart, 1983), the activities of both pathways were measured in the presence of FCCP. In general, FCCP did not stimulate the SHAM-sensitive respiration (except for the 16 day low and high UV-B pea leaves). Thus, rates were more sensitive to cyanide in the presence of SHAM. When the initial rates (uninhibited O_2 uptake) are measured in the absence of FCCP, there is electron flow through both pathways. In the presence of FCCP, the initial rate is stimulated because the electrochemical gradient has been abolished. Under these conditions, the sensitivity to cyanide in the presence of SHAM is greater because electron flow through the Cyt pathway was not restricted by the electrochemical gradient before cyanide was added. A slight increase in the activity of alternative oxidase in the presence of an uncoupler was due to the supply of substrates from glycolysis to the ETC.

5.2.2. Respiration in isolated mitochondria

UV-B treatment of pea and wheat seedlings increased respiration and the alternative oxidase activity in leaf slices, (Section 5.3.1). Therefore, the respiration was further studied in isolated mitochondria.

Isolated mitochondria do not consume oxygen rapidly even when additional substrates are added. A rapid O_2 uptake without any additional substrates is called "state1" (Fig. 5.18). "State 2" refers to O_2 consumption in the presence of substrate (without ADP). After addition of ADP, there is an increase in the rate of O_2 uptake, which is referred to as "state 3", while the state when the rate declines as ADP becomes limiting is termed "state 4".

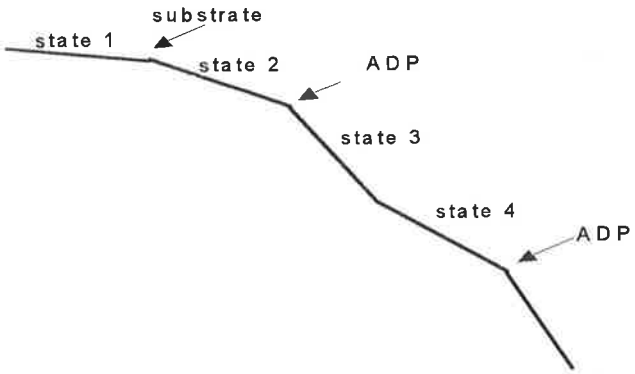


Fig.5.18. A schematic drawing of the "states" of isolated mitochondria during the oxidation of substrate.

5.2.2.1. Total respiration of isolated mitochondria

The effects of UV-B radiation on the total respiration of state 3 of pea and isolated wheat leaf mitochondria with treatment are presented in Fig.5.19 and 20. In general, pea leaf mitochondria showed a higher rate of oxygen uptake than did wheat leaf mitochondria. A greater amount of respiratory enzymes may exist in pea leaf mitochondria.

In pea leaf mitochondria, respiration with all substrates decreased with leaf maturity in mitochondria from both UV-B treated and untreated leaves (Fig.5.19). This change in pea leaf mitochondrial respiration suggests that it is part of a common development process reflecting the rates of synthesis and /or turnover of mitochondrial proteins. Compared to other treatments, mitochondria isolated from natural UV-B leaves showed a lower rate of oxygen uptake with TCA intermediates at 6 days of treatment. This difference tended to disappear as leaves became older and was absent after 16 days. A possible explanation is that the natural UV-B treatments were grown outside the glasshouse and were subject to different environmental conditions, such as temperature.

Moreover, the effect of UV-B on the rate of oxygen uptake in pea leaf mitochondria was not the same for all substrates. UV-B treatment resulted in a slight increase in the rate of oxidation with TCA intermediates, whereas rates of oxidation of the photorespiratory substrate, glycine tended to decreae. The decrease in glycine respiration under UV-B treatment was much more pronounced in mitochondria from younger leaves (Fig.5.19).

Mitochondria from control leaves showed a dramatic reduction in respiration with glycine with age than did UV-B treated leaf mitochondria. Zhang and Wiskich (1995) reported that glycine oxidation in isolated pea leaf mitochondria decreased with aging in the absence of glycine. This was due to the loss of glycine

decarboxylase activity, the enzyme important in maintaining glycine oxidation (Zhang and Wiskich, 1995). Glycine is produced in peroxisomes during photorespiration and transported into mitochondria to be decarboxylated. The large quantities of NADH produced during this decarboxylation are reoxidized via the respiratory chain or via malate dehydrogenase when OAA is supplied. This NADH will compete for the oxidation with that from TCA intermediates. The decrease in respiration rate with glycine under UV-B treatment may be due to a decrease in available substrate. As UV-B is known to directly affect the photosynthesis of pea leaves (Strid *et al.*, 1993, 1994, Chapter 6), a further impact would be to reduce photorespiration and the production of glycine. This would be related to Rubisco enzyme during UV-B treatment (Strid *et al.*, 1993) resulting in less production of glycine. UV-B radiation may also reduce both the synthesis and activity of glycine decarboxylase.

In the presence of both malate and glycine, the oxygen uptake rates of pea leaf mitochondria were higher than in the presence of glycine alone. The increase in the respiration rates could be due to the marked increase in NADH levels (Wiskich *et al.*, 1990). It has been found that the state 3 respiration rates of leaf mitochondria, at pH 7.2 in the presence glycine and a second NAD-linked substrate, such as malate are greater than the sum of the individual oxidation rates with these substrates (Dry *et al.*, 1983; Dry and Wiskich, 1985). This suggests that the respective dehydrogenases compete for access to the respiratory chain. Under this situation, glycine appears to be oxidized at the expense of the other TCA cycle substrates (Bergman and Ericson, 1983). The addition of malate stimulates glycine metabolism by producing oxaloacetate, and the malate dehydrogenase enzyme fraction remote from glycine decarboxylase will oxidize malate (Wiskich, *et al.*, 1990). Glycine decarboxylase (a photorespiratory enzyme) may not act to increase rates of electron flow via respiratory chain only, but may also support the operation of an OAA removal system. Mitochondria from UV-B treated pea leaves gave lower rates of oxygen uptake with glycine than did control mitochondria. This indicates that the rate of photorespiration was inhibited, whereas the rates of malate oxidation tended to increase with UV-B treatment.

When NADH, succinate, NADH plus succinate and malate plus glutamate are used as substrates, the rates of uninhibited state 3 oxygen uptake of mitochondria from UV-B treated pea leaves were higher than the controls. This agrees with the result found in soybean leaf mitochondria when succinate or NADH were used as substrate (Whitecross *et* al., 1994). These organic acids can be readily found within the mitochondria matrix.. The higher rate of oxygen uptake is

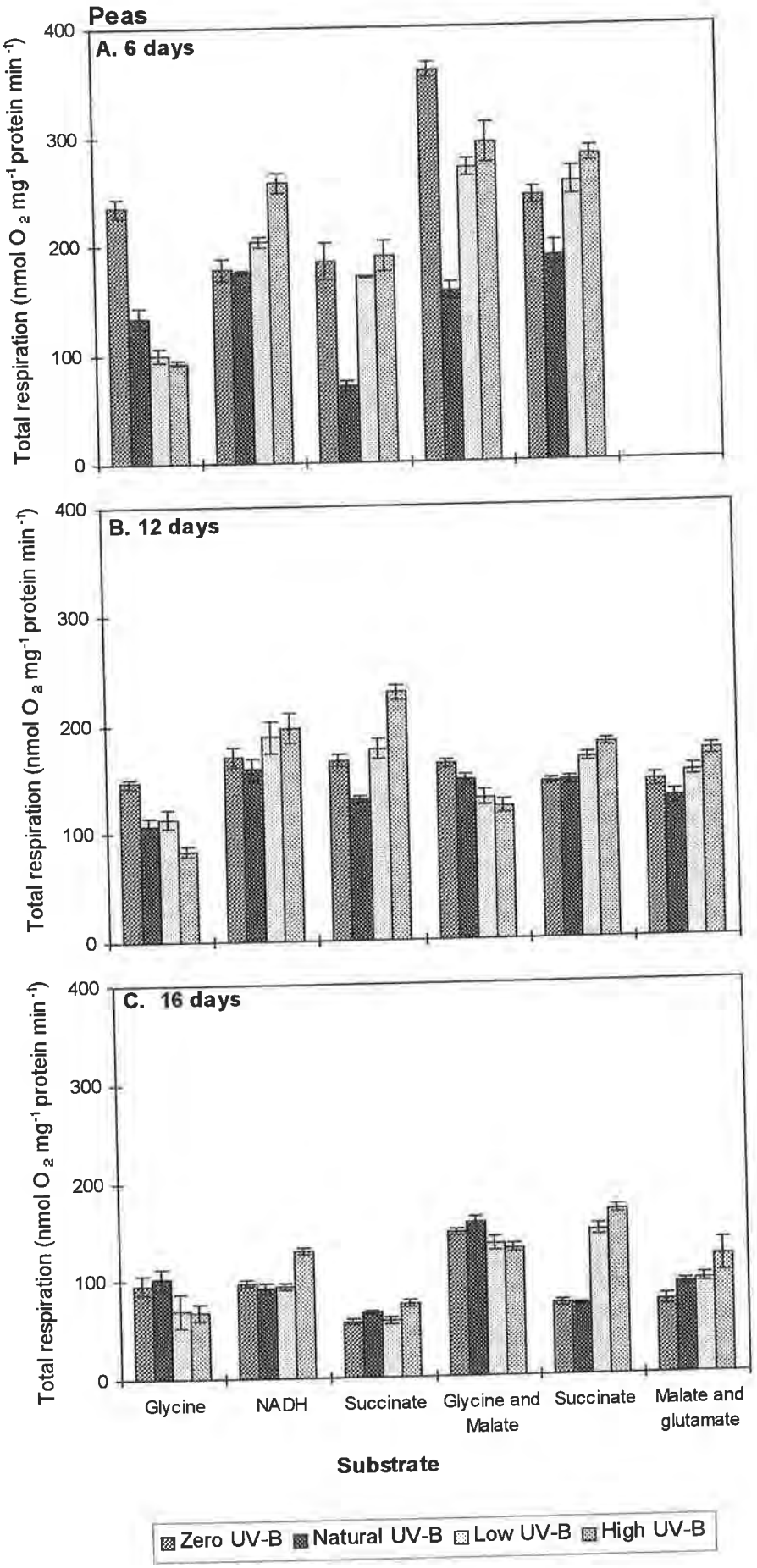


Fig.5.19. Total respiration (state 3) of isolated mitochondria from pea leaves after 6 (A), 12 (B) and 16 (C) days of UV-B treatment. Substrates were provided at final concentrations of 10 mM (glycine, malate, glutamate, succinate), 1 mM (NADH), and 0.5 mM (ADP). Values are means of 6 replicates from at least 2 independent experiments

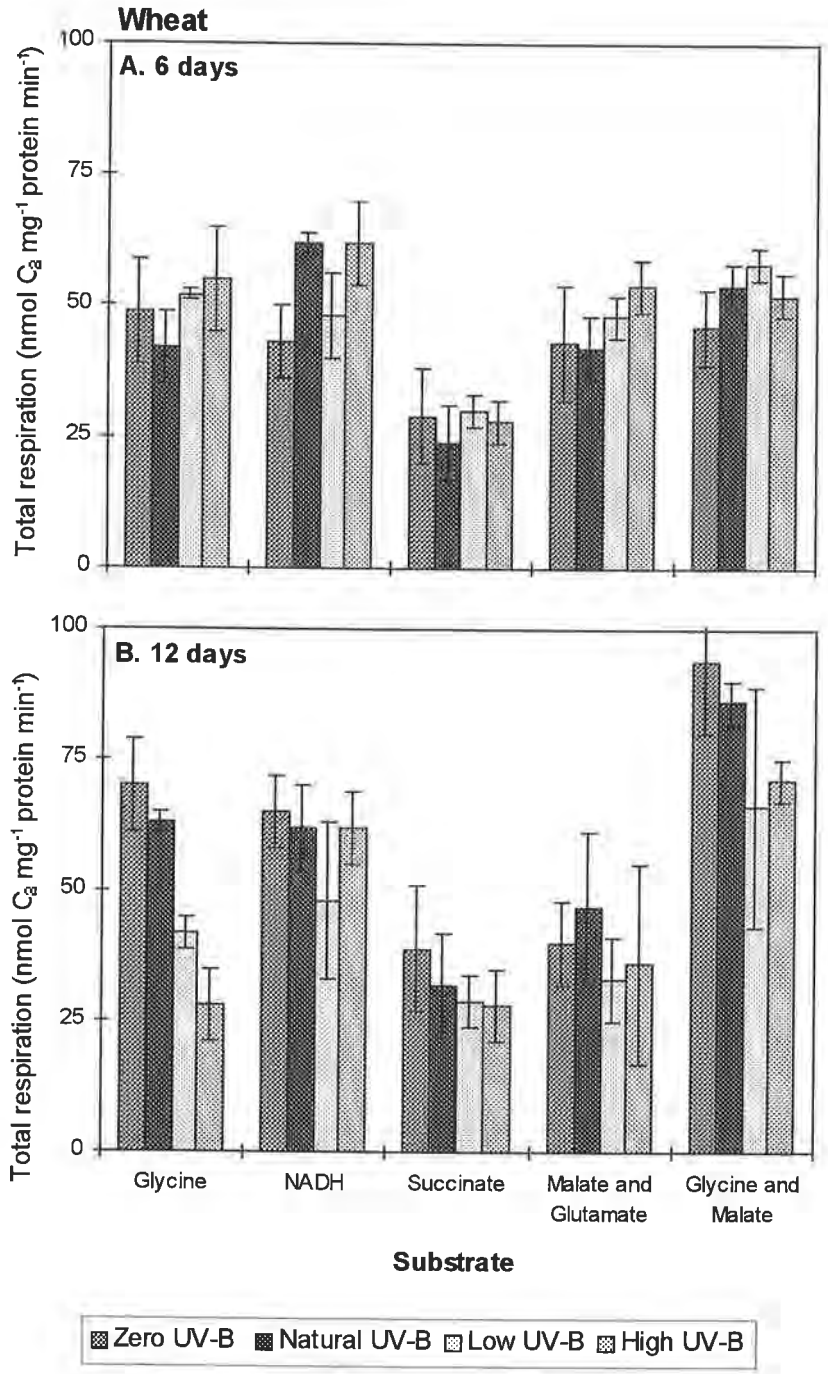


Fig.5.20. Total respiration (state 3) of isolated mitochondria from wheat leaves after 6 (A) and 12 (B) days of treatment. Substrates were provided at final concentrations of 10 mM (glycine, malate, glutamate, succinate), 1 mM (NADH), and 0.5 mM (ADP). Values are means of 6 replicates from at least two independent experiments.

probably used to maintain a continuity of UV-B protection for plants during treatment, suggesting that both glycolysis and TCA cycle become faster.

In wheat leaf mitochondria, no significant difference in the rates of oxygen uptake was found between treatments with any substrates after 6 days treatment, except NADH gave higher rates under UV-B treatment than the control (Fig.5.20A). This result suggests that photorespiration, TCA cycle and glycolysis operated normally in wheat leaves, and the rate of these processes are not strictly affected by UV-B radiation. UV-B radiation did not penetrate deeply into the tissue, and no biochemical processes were restricted. After 12 days of treatment, the state 3 oxygen uptake rates tended to show a decline with UV-B treatment (Fig.5.20B). This was especially marked with glycine oxidation, while the TCA substrates appeared to be little affected. Perhaps, longer exposure may have produced different results.

5.2.2.2. Myxothiazol resistance

To better estimate the activity of the alternative oxidase in leaf slices, mitochondria were isolated from treated plants and assayed with inhibitors. Myxothiazol was used to inhibit the cytochrome path recognizing that the presence of this inhibitor could divert electron flow to the alternative pathway. Fig.5.21 and 5.22 show the resistance of oxygen uptake to myxothiazol in pea and wheat leaf mitochondria. It was found that just as leaf slices from UV-B treated plants displayed a high resistance to cyanide, so the isolated mitochondria also displayed resistance to myxothiazol. Compared to wheat, pea leaf mitochondria were more sensitive to myxothiazol, thus indicating less alternative oxidase activity in pea leaf mitochondria.

In pea leaf mitochondria, the general pattern of the myxothiazol resistance increased with leaf age from 6 to 12 days, and from 12 to 16 days for all substrates, indicating that the alternative pathway activity was increasing. After 6 days of exposure to UV-B radiation there was a clear induction of myxothiazol resistance of oxygen uptake for all substrates, except succinate. However, this induction was not so clear after 16 days. Even though the % inhibition by myxothiazol was lower under UV-B treatments, the inhibited rates of O_2 uptake were still higher in mitochondria isolated from UV-B treated leaves (Table 5.1). In other words, contribution of alternative pathway to total respiration was greater under UV-B treatment. There are two possible reasons for this increase in alternative oxidase activity. Firstly, it may be caused by an increase of the amount of the alternative

Table 5.1. Oxygen uptake (state 3) in the presence of myxothiazol in isolated mitochondria from pea leaves. Substrates were provided at final concentrations of 10 mM (glycine, malate, glutamate, succinate), 1 mM (NADH), and 0.5 mM (ADP). Myxothiazol was added to give a final concentration of 5 μM. Values are means of 6 replicates from at least two independent experiments.

Substrate	Treatment	Oxygen uptake (nmol O_2 mg^{-1} protein min^{-1})		
		6 days of treatment	12 days of treatment	16 days of treatment
	Peas			
Glycine	Zero UV-B	27 ± 2.9	26 ± 1.9	18 ± 3.5
	Natural UV-B	31 ± 4.5	21 ± 2.5	23 ± 2.1
	Low UV-B	31 ± 2.2	35 ± 2.4	18 ± 3.8
	High UV-B	34 ± 2.1	33 ± 1.8	21 ± 2.6
NADH	Zero UV-B	16 ± 0.7	22 ± 3.8	10 ± 1.7
	Natural UV-B	15 ± 1.0	15 ± 0.8	9 ± 0.5
	Low UV-B	18 ± 1	43 ± 3.4	9 ± 0.5
	High UV-B	32 ± 3	46 ± 3.7	23 ± 1.7
Succinate	Zero UV-B	32 ± 3.1	35 ± 4.3	11 ± 0.7
	Natural UV-B	18 ± 1.5	28 ± 2.2	13 ± 0.5
	Low UV-B	29 ± 2.0	44 ± 2.6	17 ± 0.4
	High UV-B	39 ± 1.5	95 ± 9.8	28 ± 0.5
Glycine, Malate	Zero UV-B	12 ± 1.5	27 ± 0.9	32 ± 3.0
	Natural UV-B	11 ± 1.5	26 ± 0.9	39 ± 2.5
	Low UV-B	32 ± 2.5	26 ± 0.8	34 ± 2.0
	High UV-B	46 ± 1.5	23 ± 1.2	45 ± 2
Succinate, NADH	Zero UV-B	18 ± 2.0	24 ± 1.3	18 ± 1.7
	Natural UV-B	23 ± 2.0	22 ± 1.5	38 ± 1.5
	Low UV-B	44 ± 2.1	42 ± 1.8	50 ± 1.5
	High UV-B	51 ± 2.5	70 ± 1.3	52 ± 1.5
Malate, Glutamate	Zero UV-B		31 ± 4.5	24 ± 1.2
	Natural UV-B		31 ± 2.3	27 ± 2.6
	Low UV-B		47 ± 5.2	30 ± 1.5
	High UV-B		52 ± 12	29 ± 1.5

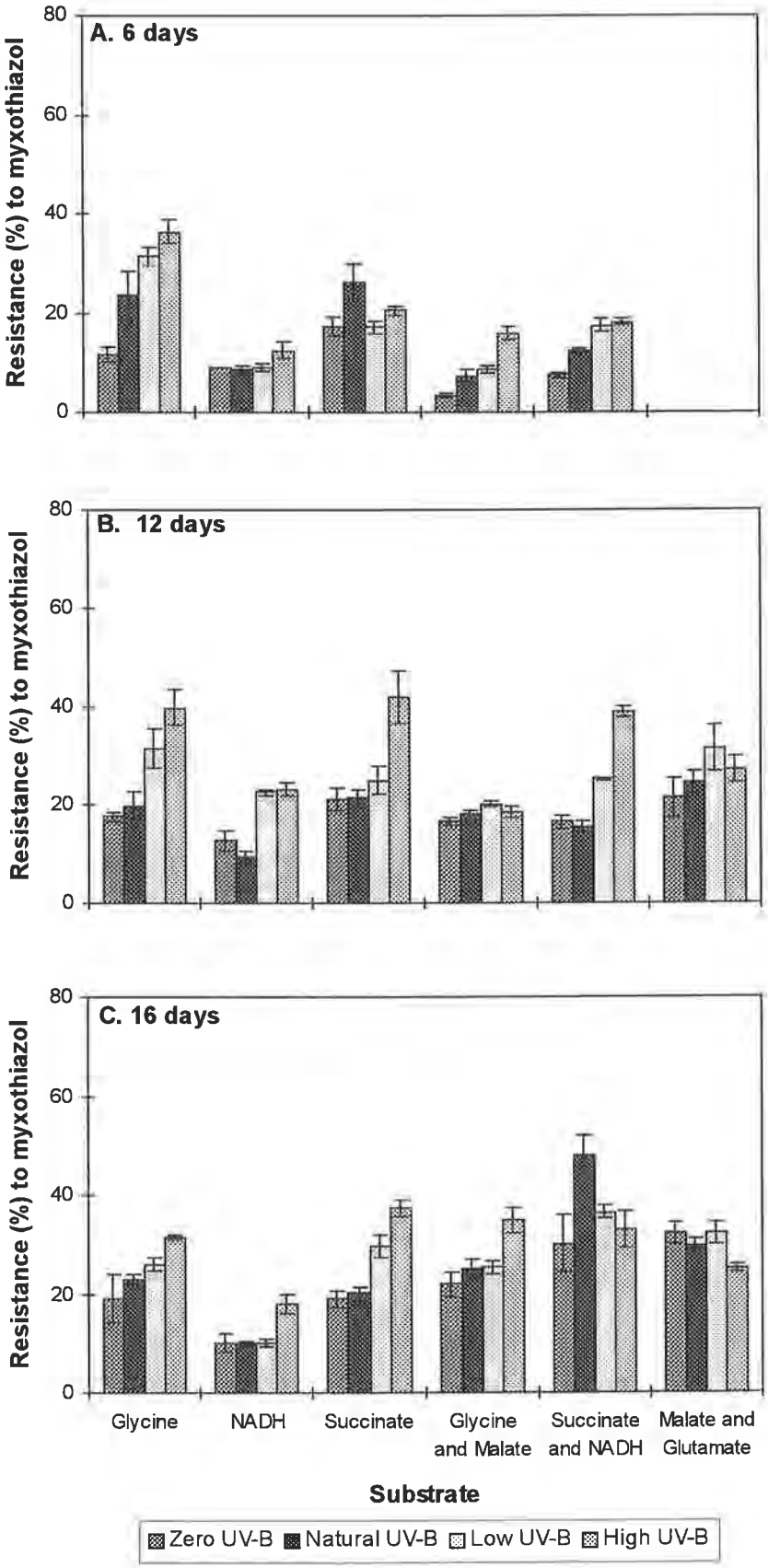


Fig.21. Activity of alternative pathway in isolated mitochondria from pea leaves expressed as percentage resistance of total respiration to myxothiazol after 6 (A), 12 (B) and 16 days of treatment. Substrates were provided at final concentrations of 10 mM (glycine, malate, glutamate, succinate), 1 mM (NADH), and 0.5 mM (ADP). Myxothiazol was added to give a final concentration of 5 μM. Values are means of 6 replicates from at least two independent experiments.

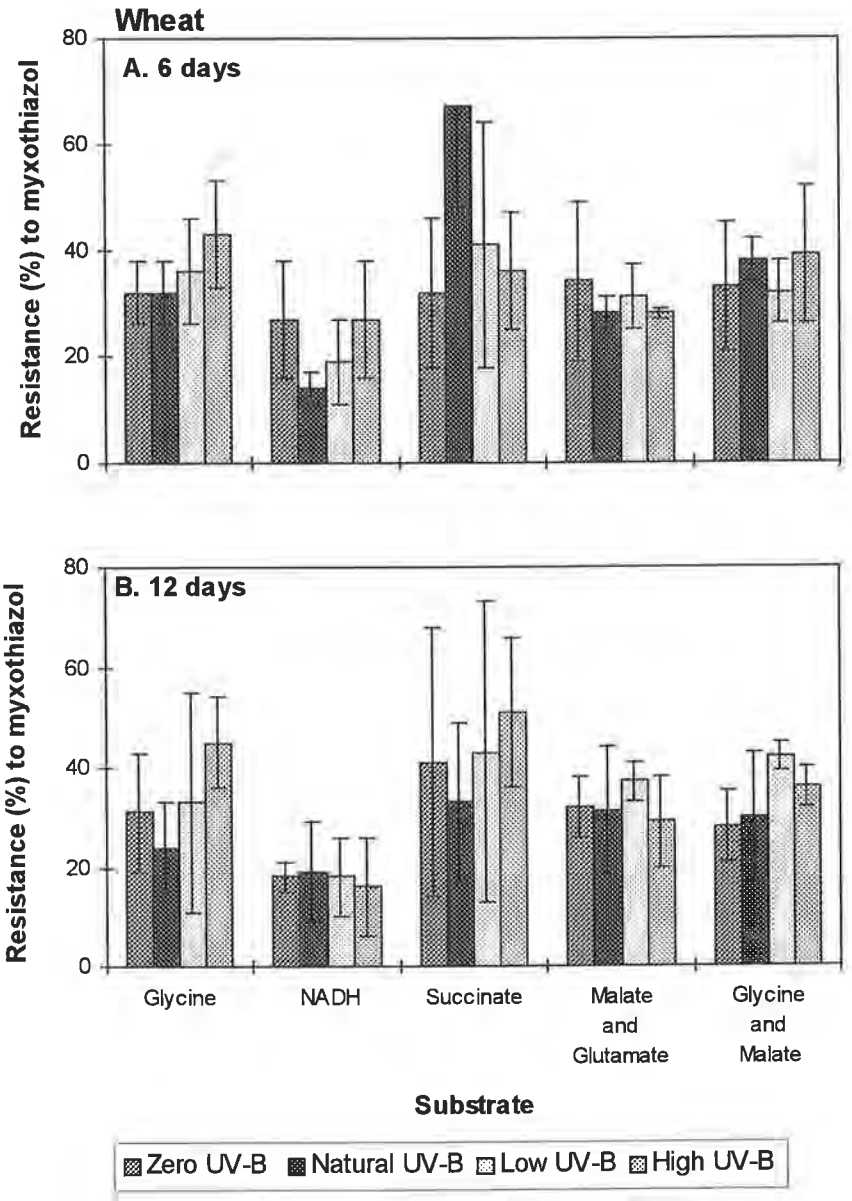


Fig.22. Activity of alternative pathway in isolated mitochondria from wheat leaves expressed as percentage resistance of total respiration to myxothiazol after 6 (A) and 12 (B) days of treatment. Substrates were provided at final concentrations of 10 mM (glycine, malate, glutamate, succinate), 1 mM (NADH), and 0.5 mM (ADP). Myxothiazol was added to give a final concentration of 5 μM. Values are means of 6 replicates from two independent experiments.

oxidase protein. Secondly, there may have been an increase in the activity of alternative oxidase. This allows the alternative oxidase proteins to compete for electrons with the cytochrome pathway and to contribute a greater rate of oxygen uptake to respiration when the cytochrome path is inhibited.

UV light has been found to stimulate the synthesis of salicylic acid in tobacco (Yalpani *et al.*, 1994), and salicylic acid has been involved in the induction of the alternative pathway (Elthon *et al.*1989a). These authors reported that salicylic acid dramatically increased both the alternative pathway and the levels of the 35, 36, and 37-kDa alternative oxidase protein in *Sauromatum gutattum.* Most recently Wen and Liang (1994) found an apparent increase in the operation of the alternative pathway in mitochondria isolated from dormancy-breaking potato slices treated with salicylic acid, but not in dormant potato slices. It is possible that the activation of the alternative oxidase in pea leaf mitochondria by UV-B treatment was mediated the de-novo synthesis of salicylic acid throughout the plants. The increase in salicylic acid may also lead to the development of strategies for protecting plants againsts environmental stresses.

In wheat leaf mitochondria, there was no obvious change in myxothiazol resistance with substrates, age, or UV-B treatments. The resistance was about the same with all substrates, with NADH giving a lowest resistance in the absence of added pyruvate. These results indicate that the amount and activity of the alternative oxidase within leaf mitochondria does not change with UV-B radiation.

Compared to the cyanide resistance in leaf slices, the myxothiazol resistance in isolated mitochondria was higher. In leaf slices, the percentage of resistance may depend on the extent of saturation of the respiration chain from the oxidation of endogenous substrates. However, in isolated mitochondria, the oxidation of exogenously provided substrates and ADP can completely saturate the respiratory chain. So differences in response may be due to substrate availability and not to activity of the electron transport chain.

5.2.2.3. The stimulation of alternative oxidase by pyruvate

Pyruvate, an organic acid known to stimulate the alternative oxidase has been postulated to act in a feed-forward control manner (Day *et al.*, 1995). The activation by pyruvate was assayed in the hope of determining the degree of activation under the various conditions. Figures 5.23 and 5.24 display the stimulation by pyruvate in mitochondria from pea and wheat leaves. After the inhibition by myxothiazol, pyruvate was added, and substrate oxidation increased

under state 3 conditions. In general, the percentage stimulation by pyruvate in pea leaf mitochondria was slightly greater than that in wheat leaf mitochondria.

In pea leaf mitochondria (Fig.5.23), no differences in pyruvate activation was found between zero and natural UV-B treatment during plants development. The results recorded here are derived from absolute respiration rates by alternative oxidase shown in Fig.5.25. The oxygen uptake measured in the presence of both myxothiazol and pyruvate is indicative of alternative oxidase activity, there being no residual respiration in isolated mitochondria when n-propylgallate (nPG, an inhibitor of the alternative pathway) was added. Mitochondria from control and natural UV-B leaves might retain large amounts of pyruvate, so that the addition of exogenous pyruvate would result in a lower percent stimulation. On the contrary, significant increases in pyruvate stimulation were noted in mitochondria isolated from low and high UV-B-treated leaves at all ages (Fig.5.23), and the pattern was the same in older leaves. Likewise, there may be differences in intramitochondrial pyruvate levels between mitochondria isolated from UV-B treated and untreated pea leaf, regarding electron partitioning between pathways. It was the low and high UV-B treatments which produced the greatest stimulation by pyruvate, and the fastest absolute rate. These data are shown in Fig. 5.23. Slower production of pyruvate during treatment may lead to de-activatation of alternative oxidase, changing the partitioning of electrons between the oxidases. This may be an important function of stress response to UV-B treatment. In stressed plants, there may be a change in the energy demands, resulting from a slight change in intramitochondrial pyruvate level which will modify the rate of electron flux through the alternative oxidase or the cytochrome pathway. If carbon intermediates are required for biosynthesis, increased flux via glycolysis will increase the pyruvate concentration, which in turn increases the activity of the alternative oxidase. UV-B treatment may lower the relative energy requirement and put more emphasis in the production of carbon intermediates.

Pyruvate is known as an activator of the alternative oxidase (Day *et al.,* 1994). In the presence of pyruvate the CN-resistant pathway will compete with the cytochrome pathway for electrons from ubiquinol (Hoefnagel *et al.*, 1995). With NADH as a substrate, the stimulation was higher, because unlike other substrates, NADH does not produce pyruvate (Day *et al.*, 1994). Mitochondria oxidizing substrates that produce pyruvate intramitochondrially may not be further stimulated as the addition of exogenous pyruvate, succinate, and malate can be converted to pyruvate.

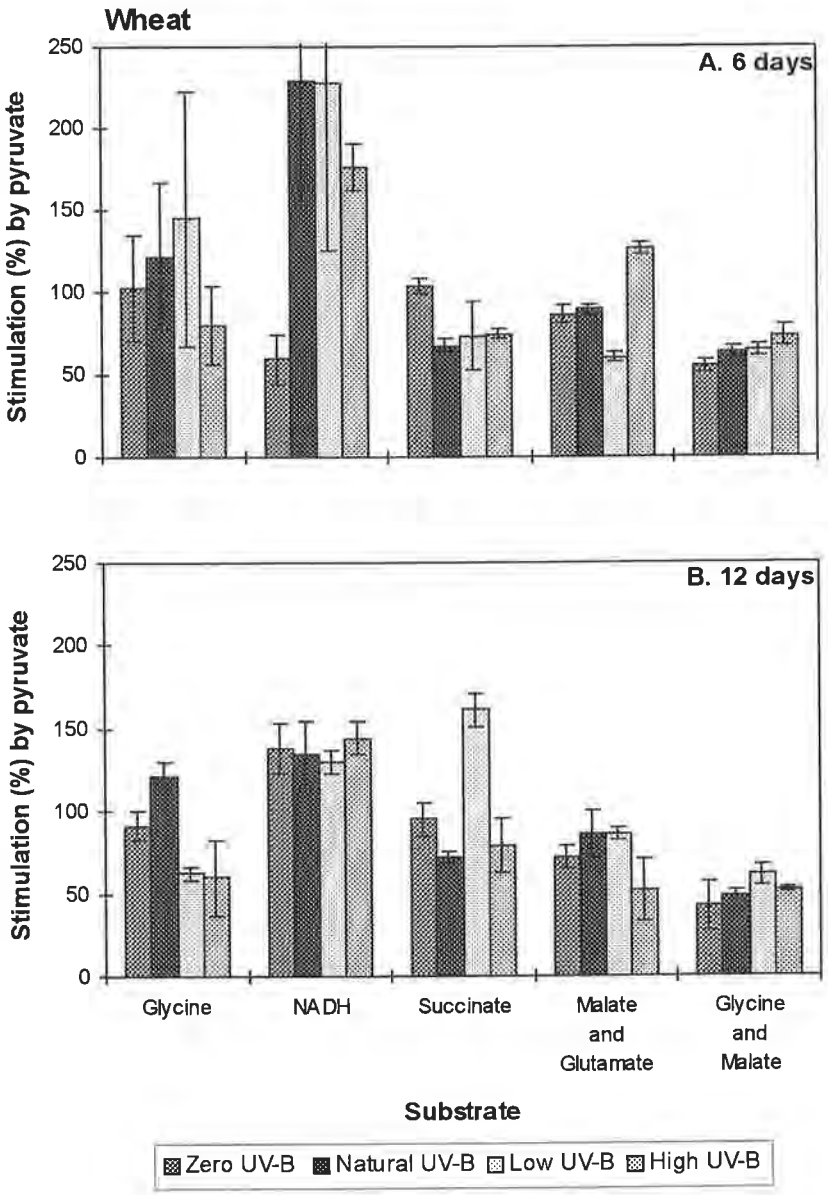


Fig.5.24. Pyruvate stimulation on alternative oxidase of mitochondria isolated from wheat leaves after 6 (A) and 12 (B) days of treatment. Substrates were provided at final concentrations of 10 mM (glycine, malate, glutamate, succinate), 1 mM (NADH), and 0.5 mM (ADP). Myxothiazol and pyruvate were added to give a final concentration of 5 μM and 5 mM respectively. The % of stimulation of the alternative oxidase was calculated as the (rate with myxothiazol in the presence of pyruvate minus the rate with myxothiazol / rate with myxothiazol) * 100. Values are means of 6 replicates from two independent experiments.

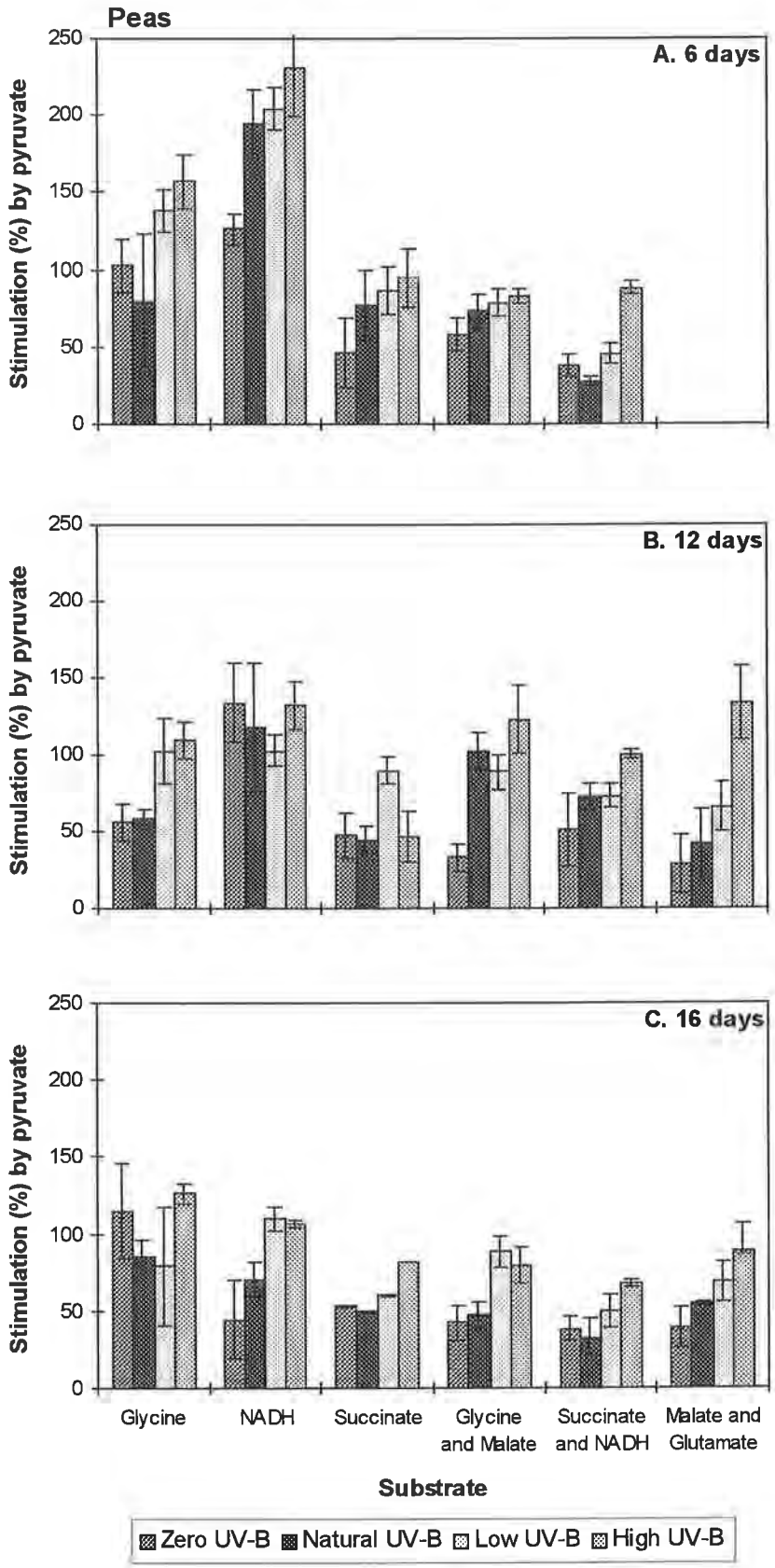


Fig.5.23. Pyruvate stimulation on alternative oxidase of mitochondria isolated from pea leaves after 6 (A), 12 (B) and 16 (C) days of treatment. Substrates were provided at final concentrations of 10 mM (glycine, malate, glutamate, succinate), 1 mM (NADH), and 0.5 mM (ADP). Myxothiazol and pyruvate were added to give a final concentration of 5 μM and 5 mM respectively. The % of stimulation of the alternative oxidase was calculated as the (rate with myxothiazol in the presence of pyruvate minus the rate with myxothiazol / rate with myxothiazol) * 100. Values are means of 6 replicates from two indipendent experiments.

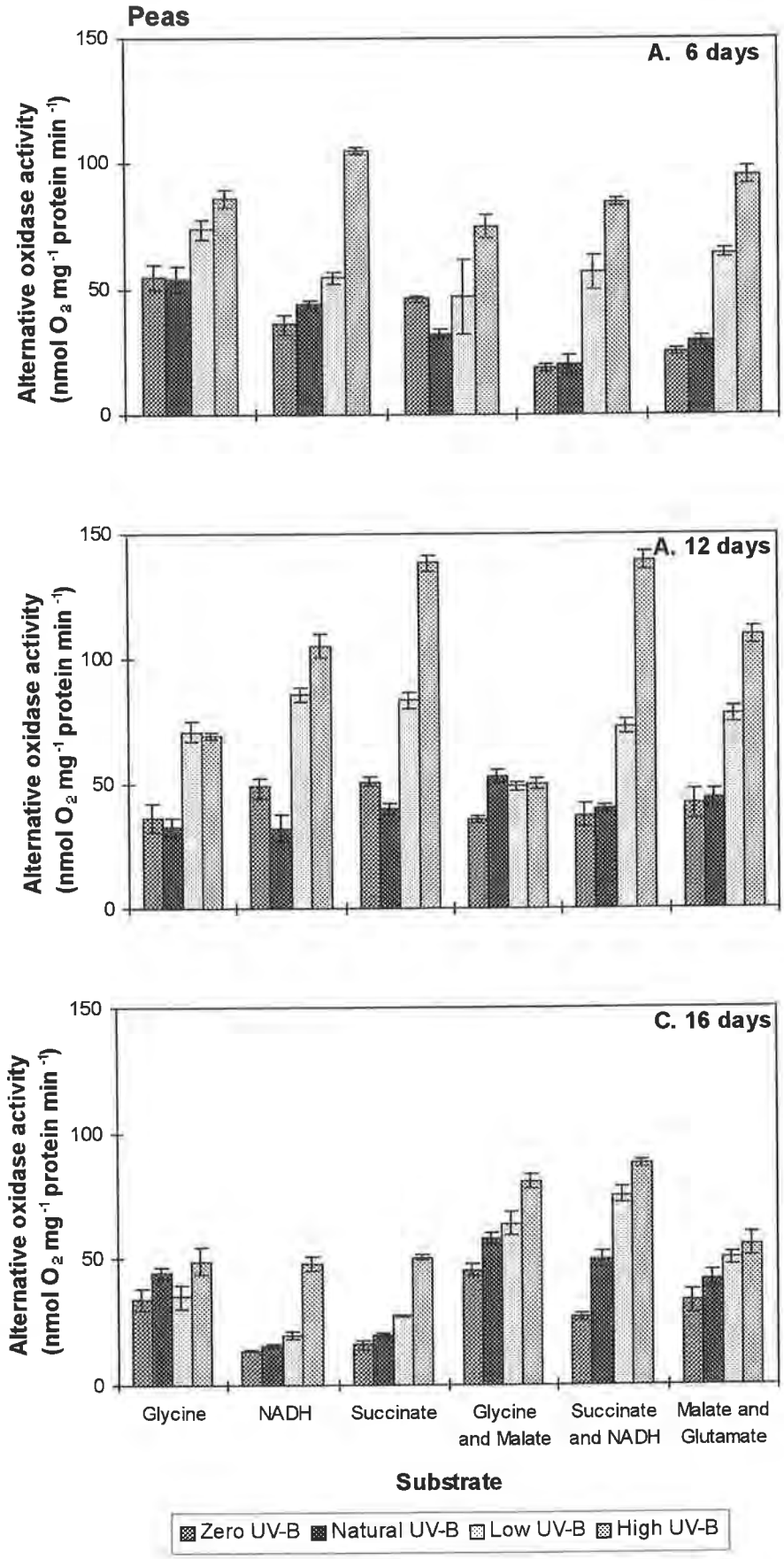


Fig.5.25. The activity of alternative oxidase of mitochondria isolated from pea leaves after 6 (A), 12 (B) and 16 days of treatment. Substrates were provided at final concentrations of 10 mM (glycine, malate, glutamate, succinate), 1 mM (NADH), and 0.5 mM (ADP). Myxothiazol and pyruvate were added to give a final concentration of 5 μM and 5 mM respectively. The % of stimulation of the alternative oxidase was calculated as the (rate with myxothiazol in the presence of pyruvate minus the rate with myxothiazol / rate with myxothiazol) * 100. Values are means of 6 replicates from at least two independent experiments.

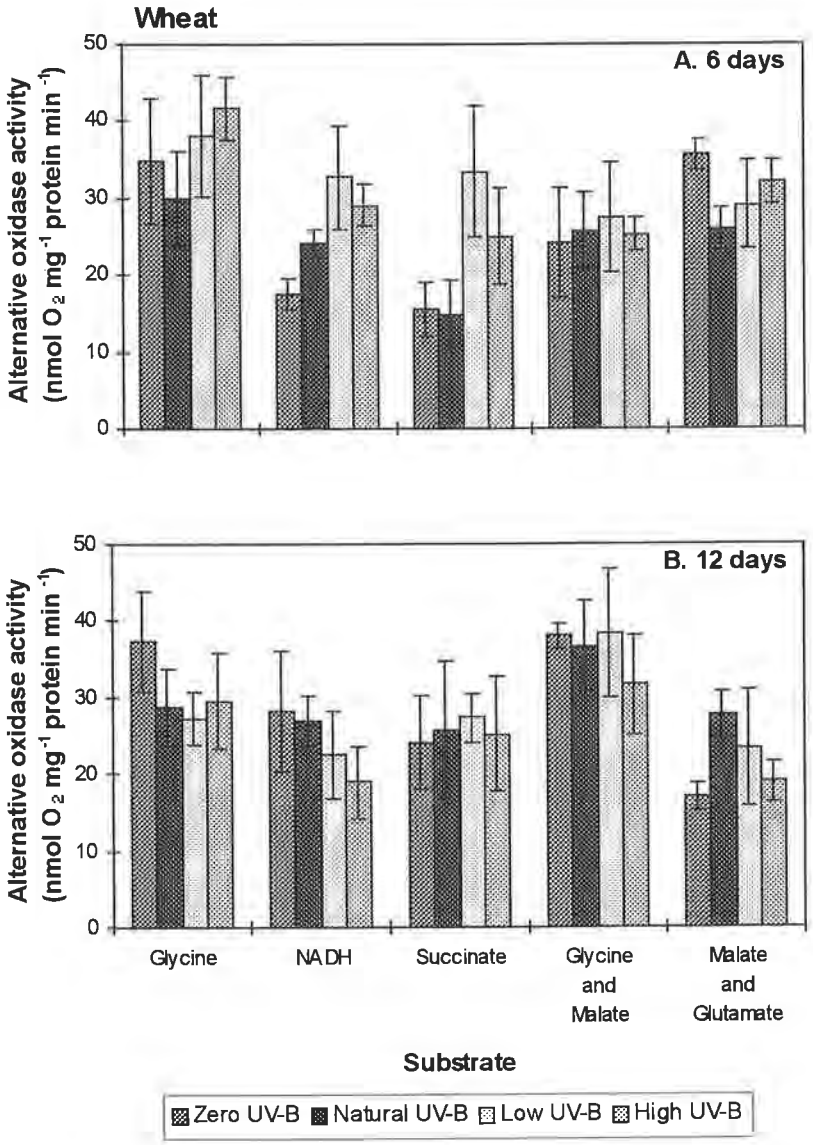


Fig.5.26. The activity of alternative oxidase of mitochondria isolated from wheat leaves after 6 (A) and 12 (B) days of treatment. Substrates were provided at final concentrations of 10 mM (glycine, malate, glutamate, succinate), 1 mM (NADH), and 0.5 mM (ADP). Myxothiazol and pyruvate were added to give a final concentration of 5 μM and 5 mM respectively. The % of stimulation of the alternative oxidase was calculated as the (rate with myxothiazol in the presence of pyruvate minus the rate with myxothiazol / rate with myxothiazol) * 100. Values are means of 6 replicates from at least two independent experiments.

In pea leaf mitochondria, the degree of stimulation was different between substrates. Pyruvate stimulated the myxothiazol-resistant rate to a greater extent with NADH than any other substrate at day 6 and 12. This stimulation decreased as the leaves became older.

The pattern of pyruvate stimulation in wheat leaf mitochondria was different to pea leaf mitochondria. Overall, the greatest stimulation was determined when NADH was used as substrate. At 6 days treatment, the stimulation of NADH, malate plus glutamate, and glycine plus malate oxidation was higher in the to UV-B treatments. However, this increase was not observed at 12 days treatment. When expressed as the absolute rate alternative oxidase oxygen uptake (Fig.5.26), UV-B treatment had no effect on pyruvate stimulation.

5.2.3. Alternative oxidase protein and its activity

The polypeptides of pea leaf mitochondrial protein as separated by SDS-PAGE gel are presented in Fig.5.27. The various bands seemed to increase with leaf age. This pattern occurred whether reductant DTT was either present (Fig.5.27A) or not (Fig.5.27B) in the SDS-PAGE sample buffer . UV-B treatment, moreover, did not influence the pattern of mitochondrial protein. In other words, no changes in protein expression were observed in mitochondria isolated from UV-B treated pea leaves. The polypeptides isolated from wheat leaf mitochondria are presented in Fig. 5.28. Compared to pea leaf mitochondria, the number of polypeptides appeared to be fewer, but also increased with leaf age.

To identify alternative oxidase protein, immunoblots were performed with an alternative oxidase antibody. From the same amount of mitochondrial protein (40µg), two polypeptides were detected for each plant species (Fig.5.29 and Fig.5.30). These had molecular weights of 71-72 kDa (oxidized dimer) and 28-29 kDa (reduced monomer) for peas, and 71-72 kDa and 34-35 kDa for wheat. These species were found in the presence and absence of reducing agent (DTT) in the sample buffer. Two different populations of enzymes, reduced and oxidized forms also exist in soybean mitochondria (Umbach and Siedow, 1993; Umbach *et al.*, 1994). However, the only active enzyme is the reduced form (Umbach and Siedow, 1993). In wheat leaves, the amount of the alternative oxidase protein appeared to be lower than in peas, but the sizes of polypeptides of proteins found are similar to those seen in pea leaf mitochondria. This result suggest that the alternative oxidase protein in pea and wheat leaves mitochondria may have a similar degree of homologous sequence.

Fig.5.27. SDS-PAGE gels of pea leaf mitochondrial protein in the absence (A) and presence (B) of DTT in the sample buffer at different days from pea plants grown under UV-B treatments. Proteins (40 μg per lane) were stained with Coomassie blue. Apparent molecular masses of the markers in kD are indicated on the left.

Legend

M	=	M.W. markers
Z	=	Zero UV-B
N	=	Natural UV-B
L	=	Low UV-B
H	=	High UV-B

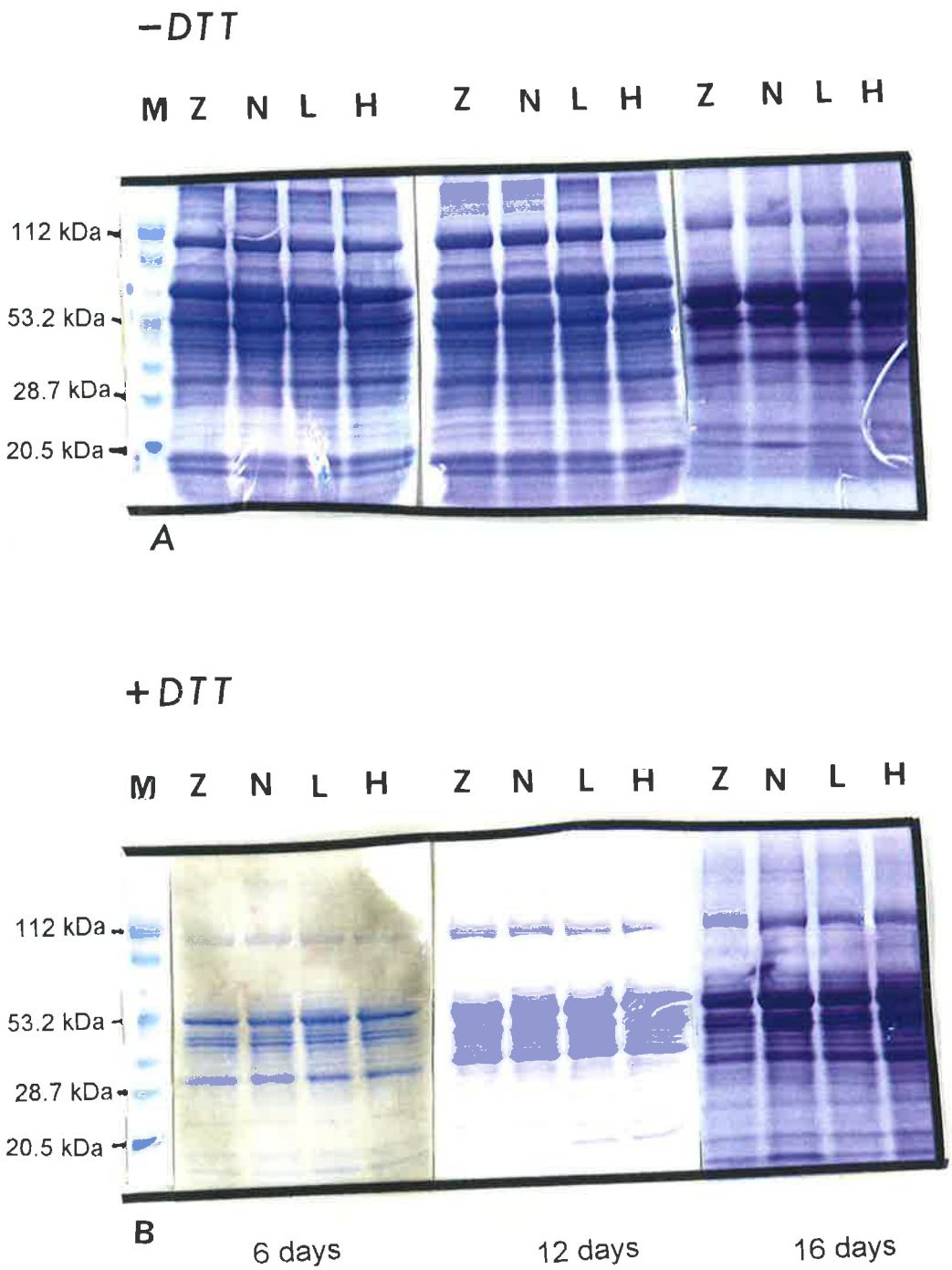

−DTT
M Z N L H Z N L H Z N L H
112 kDa
53.2 kDa
28.7 kDa
20.5 kDa
A
+DTT
M Z N L H Z N L H Z N L H
112 kDa
53.2 kDa
28.7 kDa
20.5 kDa
B
6 days
12 days
16 days

Fig.5.28. SDS-PAGE gels of wheat leaf mitochondrial protein in the absence (A) and presence (B) of DTT in the sample buffer, at different days from wheat plants grown under UV-B treatments. Proteins (40 μg per lane) stained with Coomassie blue. Apparent molecular weights of the markers in kiloDaltons are indicated on the left.

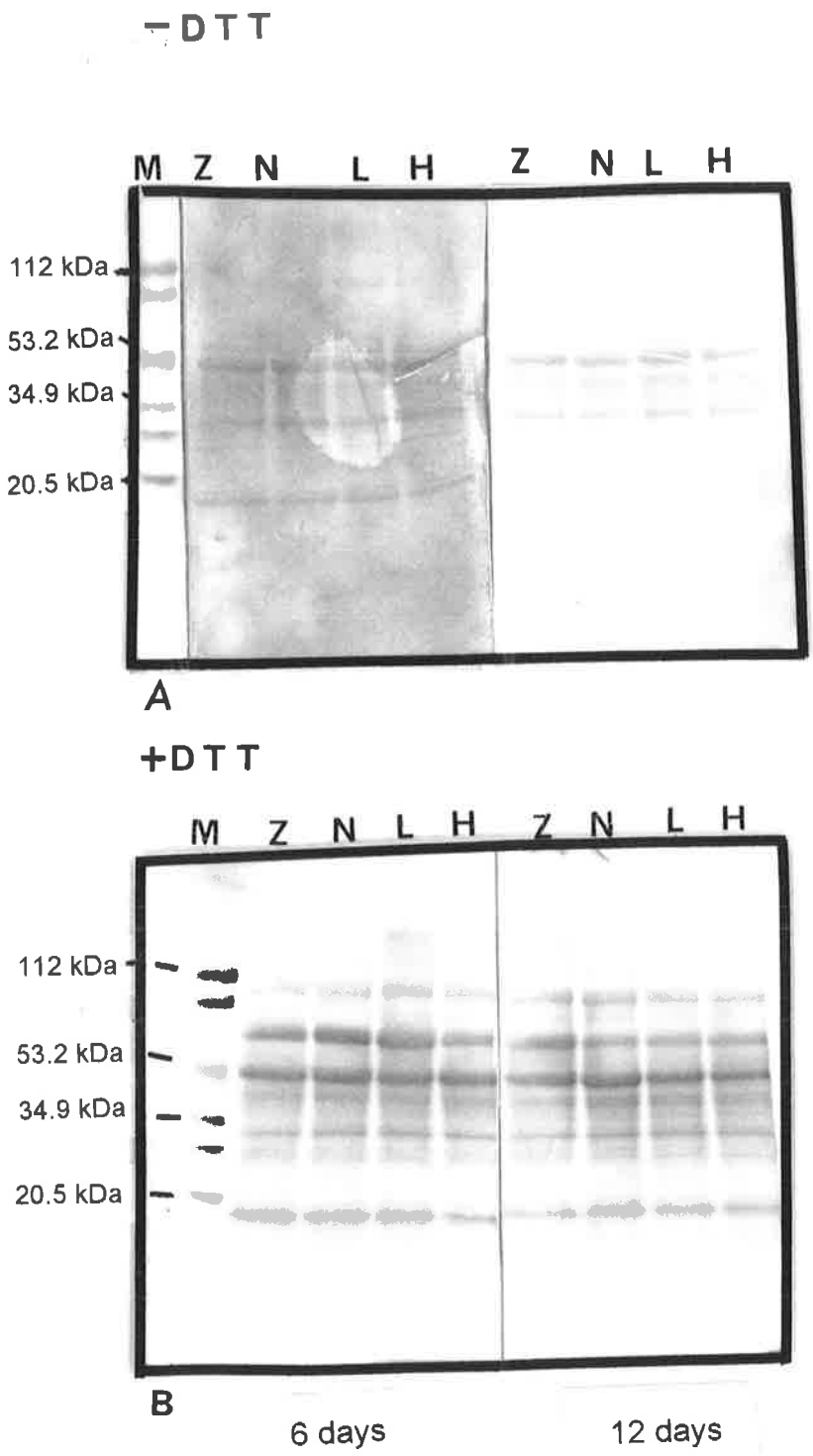
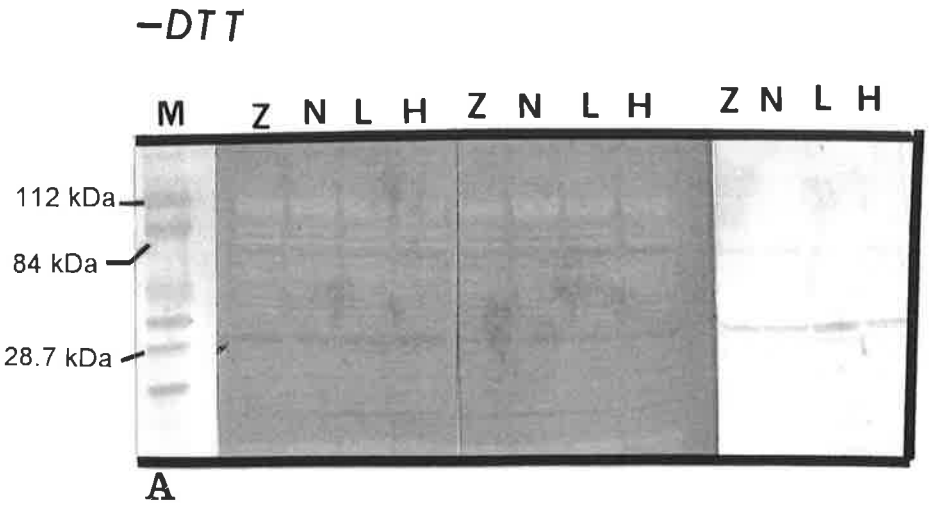

-DTT
M Z N L H Z N L H
112 kDa
53.2 kDa
34.9 kDa
20.5 kDa
A
+DTT
M Z N L H Z N L H
112 kDa
53.2 kDa
34.9 kDa
20.5 kDa
B
6 days
12 days

Fig.5.29. Expression of the alternative oxidase proteins in pea leaf mitochondria grown under UV-B treatments. All samples were prepared in the absence (A) and presence (B) of DTT in the sample buffer. Proteins (40 μM) were probed with a monoclonal antibody to the alternative oxidase. Apparent molecular masses of the markers in kD are indicated on the left.


-DTT
M
Z N L H Z N L H Z N L H
112 kDa
84 kDa
28.7 kDa
A

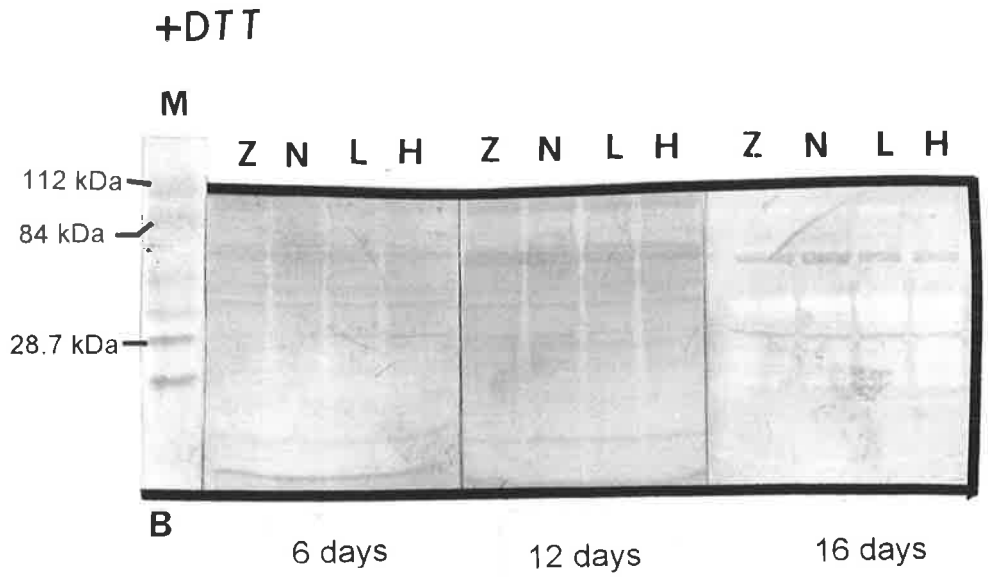
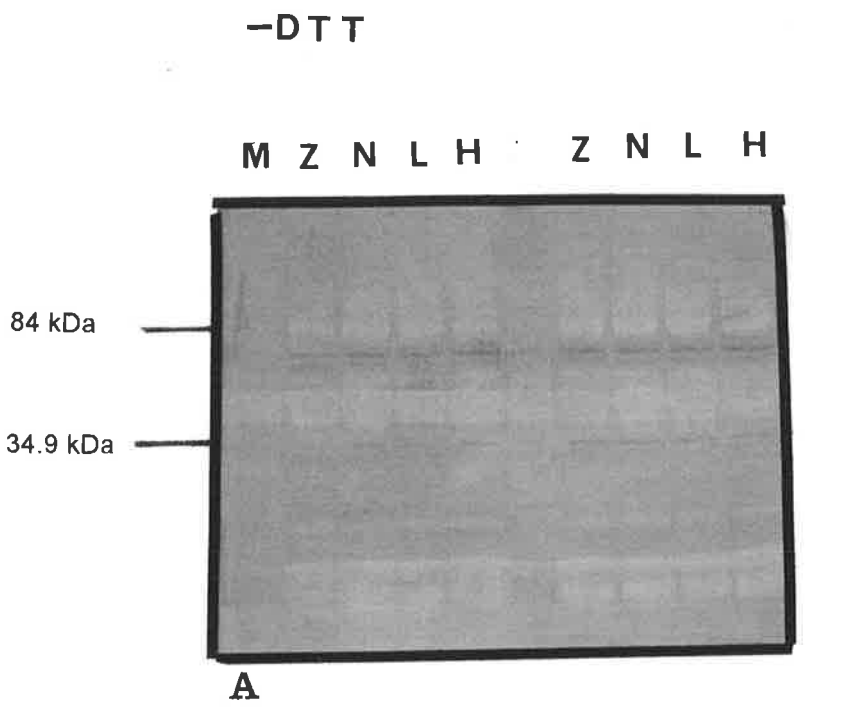

+DTT
M
Z N L H Z N L H Z N L H
112 kDa
84 kDa
28.7 kDa
B
6 days
12 days
16 days

Fig.5.30. Expression of the alternative oxidase proteins in wheat leaf mitochondria grown under UV-B treatments. All samples were prepared in the absence (A) and presence (B) of DTT in the sample buffer. Proteins (40 μM) were probed with a monoclonal antibody to the alternative oxidase. Apparent molecular masses of the markers in kD are indicated on the left.


−DTT
M Z N L H Z N L H
84 kDa
34.9 kDa
A
+ DTT
M Z N L H M Z N L H
84 kDa
34.9 kDa
B
6 days
12 days

The smaller alternative oxidase peptides from both pea and wheat leaf mitochondria are more abundant than the higher molecular weight bands (Fig.5.29A and Fig.5.30A respectively). Reductants such as DTT can be applied to revert the oxidase to the lower molecular weight forms (Day *et al,* 1995) and as such they may show a greater transfer of proteins from the gel matrix to the membrane during blotting. When reductant was omitted from the gel sample buffer, the proteins appeared as the high molecular mass bands (Fig.5.29B and 5.30B).

No differences in polypeptide expression was noticed between UV-B treated and untreated leaves. The amount of alternative oxidase protein was about the same in both species at all ages, and though there was a small increase with leaf age. Umbach and Siedow (1993) showed that the proportion of reduced enzyme increased with time during thermogenesis in *Sauromatum guttatum.* The activity of the alternative oxidase, therefore, depends on the degree of reduction of the alternative oxidase protein. Similar patterns have been observed during the growth of pea seedlings (Lennon *et al.,* 1995).

In this study, it was previously observed that alternative oxidase activity was increased by UV-B treatment. This suggests that the stimulatory effect on the activity of the CN-resistant alternative by UV-B was not due to an increase in the amount of the alternative oxidase protein, but due to an activation of the proteins.

5.3. Conclusion

The total or uninhibited rate of respiration in pea and wheat leaf tissue was affected by UV-B treatment. Pea and wheat seedlings grown under supplementary UV-B radiation showed an increase in the total respiration, even though it was not statistically significant in wheat species. These results indicate that UV-B radiation influences the activity of mitochondria in carrying out the respiration.

The total respiration, as well as the activity of the alternative and the Cyt pathways decreased with age in pea and wheat, but were stimulated by UV-B treatment. Supplementary UV-B radiation applied in this study did increase the sensitivity of pea leaf tissue to KCN and SHAM, but not of wheat leaf tissue. Moreover, the activity of the cyanide-resistant pathway was markedly higher in UV-B treated leaves, suggesting that the increase in uninhibited respiration was due to the contribution of the cyanide-resistant pathway. However, as far as the

participation of the Cyt path was concerned no difference was observed between treated and untreated leaves.

UV-B radiation affected pea and wheat leaf mitochondria. Pea leaf mitochondria displayed an increase in respiration with NADH and TCA intermediates, but a decrease with both glycine and glycine plus malate, as a result of UV-B treatment. Wheat leaf mitochondria were not significantly affected by UV-B radiation. The nature of changes in respiratory activity of mitochondria from UV-B treated pea and wheat leaves is unclear, but there were some possible causes as follows.

UV-B radiation seemed to influence photorespiration in pea leaves possibly as a consequence of reduction in photosynthetic rates. This leads to a decrease in the rate of mitochondrial glycine oxidation.

UV-B radiation increased the resistance of mitochondria to myxothiazol in peas, but not in wheat, suggesting that there was a higher amount or greater activity of alternative oxidase in pea leaf mitochondria. Western blots showed that UV-B radiation did not induce the expression of alternative oxidase proteins in either species, but UV-B activated alternative oxidase in peas.

Increasing alternative oxidase activity can be seen from the rates of oxygen uptake in the presence of myxothiazol and pyruvate and accompanied by increasing the stimulation of alternative oxidase by pyruvate under UV-B radiation

Overall, mitochondrial respiration in pea and wheat leaves during growth under UV-B treatment appears to be regulated by coarse control of the capacities of glycolysis, TCA cycle and photorespiration and the alternative pathway. Pea leaf mitochondria were found to be more sensitive to UV-B radiation than wheat leaf mitochondria. These results suggest that the nature of tissue and plant species to which UV-B treatment was applied has elicited different responses.

Chapter 6. Effect of supplementary UV-B radiation on photosynthesis

6.1. Introduction

6.1.1. Photosynthesis

Photosynthesis is one of the most important physiological plant processes and the most vulnerable to environmental changes. This process, which involves the formation of chemical energy from solar radiant energy via oxidation and reduction reactions, is essential for crop productivity. During the oxidation process, electrons removed from water to produce oxygen, are used to reduce CO_2. The organelle which plays an important role in photosynthesis is the chloroplast, a green plastid found abundantly in leaf cells (Anderson and Beardall, 1991).

6.1.2. Structure of chloroplast

A transmission electron micrograph of a chloroplast from a higher plant cell is presented in Fig. 6.1. Its general size is about 5x2x1-2 μm. Chloroplasts are self-replicating organelles and are surrounded by an envelope comprised of two membranes. Besides these two membranes, chloroplasts have another three compartments, the intramembrane space between the outer and inner membranes, the stroma (which contains soluble protein, such as Ribulose 1,5-biphosphate carboxylase (Rubisco)), and the thylakoid space which results from the inner membrane extending into the stroma. Thylakoids form flattened disc-shaped membranous sacs called grana and they contain the pigments responsible for absorption of light energy (Newcomb, 1990).

6.1.3. CO_2-dependent O_2 evolution

The final product of photosynthesis is the biosynthesis of carbohydrate as glucose. This process involves the formation of ATP and NADPH molecules by light-driven reactions in the thylakoid membrane, which are then used in the dark reactions to fix CO_2 into organic compounds. The CO_2 fixation occurs in the stroma of the chloroplasts where the soluble enzymes are located (Salisbury and Ross, 1992).

Fig.6.1. Transmission electron micograph of a chloroplast in a mesophyll cell of pea (*Pisum sativum* L.). Shown within the chloroplast are the bounding membrane envelope (small arrow), starch (S) granula, thylakoids (T), and cell wall (CW). X 13.500

The first stable product of CO_2 fixation within chloroplasts of higher plants is 3-PGA. The carboxylation to form 3-PGA occurs via ribulose biphosphate carboxylase located in the chloroplast stroma.

$$\text{Ribulose 1,5-P2} + CO_2 + H_2O \xrightarrow[\textit{Rubisco}]{} 2 \text{ glycerate 3-P} \qquad (6.1)$$

With ATP and NADPH as reductant, glycerate 3-P is reduced to form phosphoglyceraldehyde.

$$2 \text{ glycerate 3-P} + 2 \text{ ATP} \xrightarrow[\textit{phosphoglycerate kinase}]{} 2 \text{ glycerate-1,3-P2} + 2 \text{ ADP} \qquad (6.2)$$

$$2 \text{ glycerate-1,3-P2} + 2 \text{ NADPH} + 2H^+ \xrightarrow[\textit{NADP-glyceraldehyde 3-phosphate dehydrogenase}]{} 2 \text{ glyceraldehyde-3-P} + 2 NADP^+ + 2Pi \qquad (6.3)$$

The overall stoichiometry for the synthesis of one molecule of hexose sugar from CO_2 in the C3 pathway is therefore:

$$6\ CO_2 + 18\ ATP + 12\ NADPH + 12\ H+\ 12\ H_2O \rightarrow glucose + 18\ ADP + 12\ NADP^+ + 18\ Pi \qquad (6.4)$$

Not all of the glyceraldehyde-3-P formed in photosynthesis is net gain. Five-sixths must be recycled to form the original RuBP and one-sixth is equivalent to the amount of CO_2 fixed.

The rate of CO_2 fixation can also be decreased by the competition between O_2 and CO_2 at the active site of Rubisco (Canvin, 1990). Rubisco can act as an oxygenase. The products of this reaction are 3-PGA and P-glycollate.

6.1.4. Electron transport

The path of electron flow in oxygenic photosynthesis has similarities to that of mitochondria (Cramer *et al.*, 1991), in that there are three major supramolecular complexes. These three are arranged within the thylakoid membrane and are necessary in transporting electrons and protons across the bilayer membrane (Fig.6.2). These three are:

1. Photosystem II (PS II)

It consists of light harvesting pigment-protein complexes (LHCII) and a core complex (CCII) which catalyzes electron transfer from water to plastoquinone (PQ) (Fig.6.2). PS II also contain D1 and D2 polypeptides, and another three distinct small hydrophobic polypeptides (Cramer *et al.*, 1991). Only light energy at 680 nm can be absorbed by the reaction centre of PS II.

2. Cyt.*b/f* complex

This complex contains iron-sulfur (Fe-S) proteins as well as cytochromes *b* and *f*. It oxidizes the PQH_2 (plastohydroquinone) and donates electrons to plastocyanin (PC). On the PQH_2 side, it is involved in the transport of H^+ from the stroma into the thylakoid lumen (Fig.6.2).

3. Photosystem I (PS I)

Photosystem I is composed of a light harvesting pigment-protein complex (LHCI) and also a core complex (CCI). This complex oxidizes plastocyanin (PC) and transfers electrons to $NADP^+$ via ferredoxin to generate a reductant NADPH. The reaction centre has an absorption peak at 700 nm.

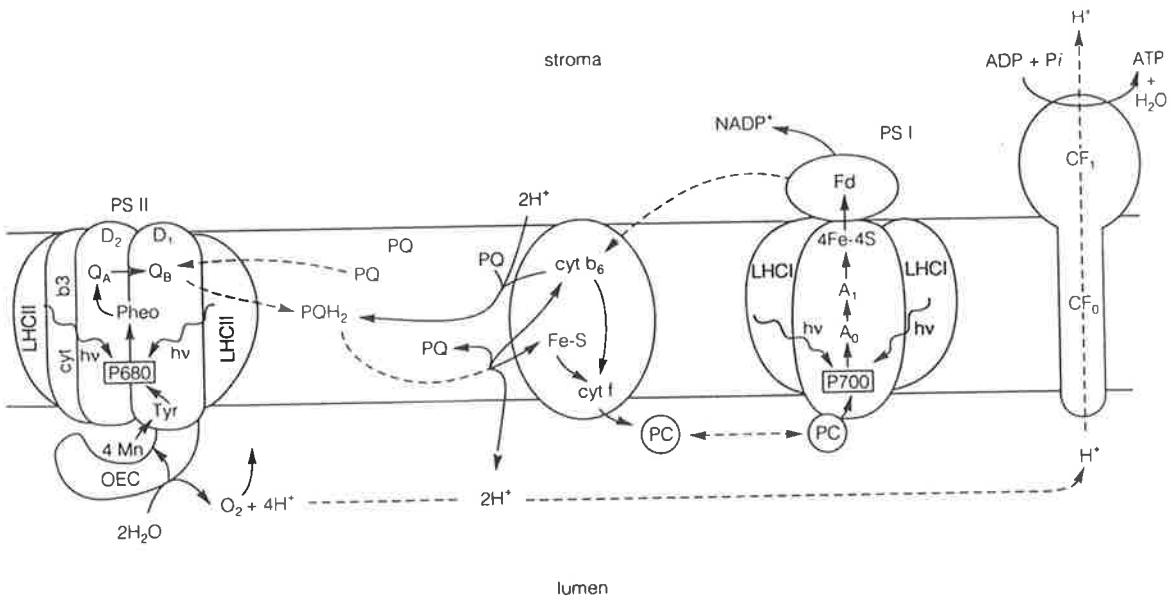


Fig.6.2. The pathways of electron transport and H^+ translocation in noncyclic (H_2O $\rightarrow$ $NADP^+$), cyclic (PS I $\rightarrow$ Fd $\rightarrow$ *bf* [PQ]), and pseudocyclic (ATP formation) electron transport chain of oxygenic photosynthesis along the thylakoid membrane (Salisbury and Ross, 1992, p. 218).

During electron transport from PS II to PS I, mobile electron transport carriers are required, to link the two. Plastoquinone is thought to connect PS II to the cyt.*b/f* complex, while plastocyanin (PC) receives electrons from the cyt.*b/f* complex and then donates them to PS I (Cramer *et al.*, 1991).

Beside these three electron transport complexes, there is another protein complex in the thylakoid membrane required for ATP production, the chloroplast ATPase complex. This complex is arranged as the hydrophobic CF_0 and surface bound CF_1 and is driven by the proton gradient (Salisbury and Ross, 1992). The vectorial location of CF_0CF_1 is such that the CF_o part of the enzyme spans the thylakoid membrane, whereas CF_1 is bound to the stromal side of CF_0. Light-induced electron transport through the PS I and PS II and the cytochrome *bf* complex is coupled to proton pumping across the thylakoid membrane into the lumen. The flow of protons back to the stroma through CF_0CF_1 drives the synthesis of ATP, which is released into the stromal space, where it is used in subsequent dark reactions.

Three different patterns of electron transport occur in the thylakoid membrane of chloroplasts (Salisbury and Ross, 1992).

6.1.4.1. Non-cyclic electron transport

Electrons which flow from water, the primary electron donor at photosystem II, to $NADP^+$, the terminal electron acceptor at photosystem I, do not cycle back to H_2O (Cramer *et al.*, 1991). This sequence is noncyclic electron transport and is known as the Z scheme (Fig.6.3) (Anderson and Beardall, 1991). As one molecule of H_2O is oxidized, two electrons are transported to $NADP^+$ to generate $½O_2$ and one NADPH (reaction 5). Non-cyclic electron transport will then couple ATP formation to oxygen evolution and is called noncyclic photophosphorylation. This process is tightly dependent on light energy, as the oxidation of H_2O is highly unfavourable from a thermodynamic standpoint as is the reduction of $NADP^+$.

$$H_2O + NADP+ + ADP + Pi \rightarrow NADPH_2 + ATP + ½\ O_2 \qquad (6.5)$$

6.1.4.2. Cyclic electron transport

Unlike noncyclic electron flow, cyclic electron transport occurs when electrons cycle from P_{700} (PS I) through ferredoxin to plastoquinone (PQ) and then back again to PS I (Fig.6.2). This process does not generate $NADPH_2$ and no O_2 is

evolved. H^+ transport at PQ will contribute to the pH gradient driving photophosphorylation. The formation of ATP during this pathway is therefore called cyclic phosphorylation (Cramer *et al.*, 1991). It is thought to occur when the NADPH/$NADP^+$ ratio is high.

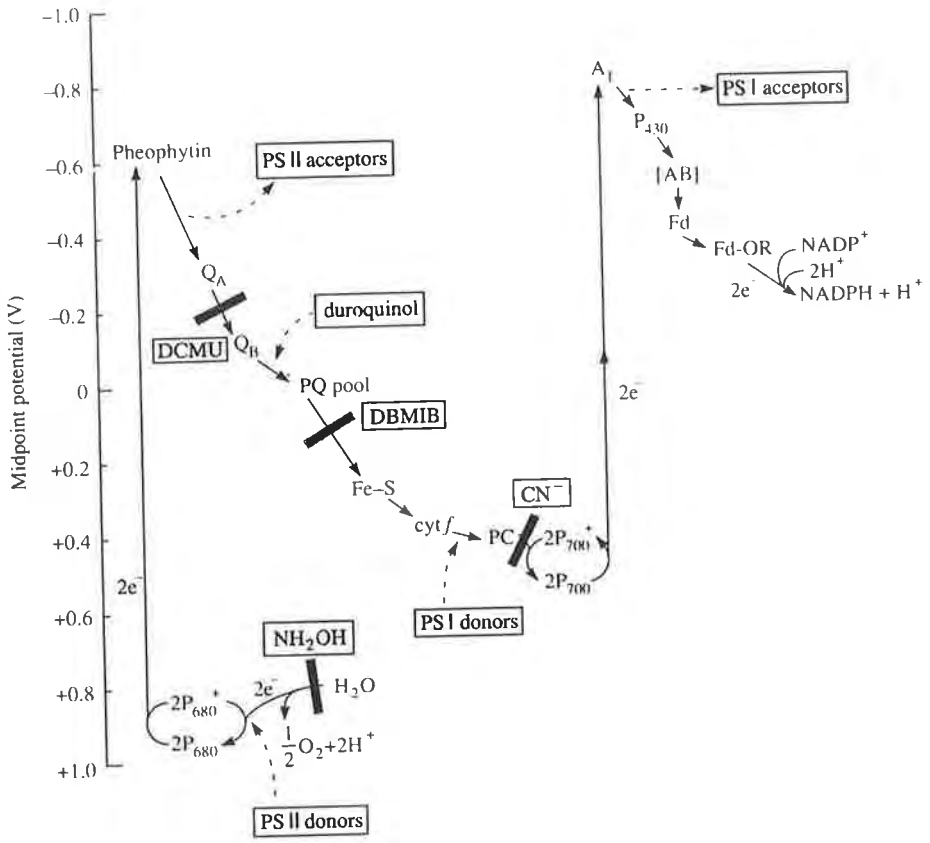


Fig.6.3. The noncyclic electron transport chain (from Anderson and Beardall, 1991, p. 150).

6.1.4.3. Pseudocyclic electron transport

During this process, only ATP is made and although no net change in oxygen occurs, oxygen is involved. Ferredoxin is thought to be the natural mediator (reactions 6.6-6.9). Reduced ferredoxin reacts with O_2 to produce hydrogen peroxide and catalase present in the chloroplast hydrolyzes this to H_2O and oxygen. In general, light absorbed by chlorophyll in the photosystems functions in driving electrons from H_2O to reduce ferredoxin and also in providing energy to phosphorylate ADP (Salisbury and Ross, 1992). This process, like cyclic photophosphorylation is considered to be a way of producing ATP without producing a reduced product.

$$H_2O + 2\ MV + ADP + Pi \rightarrow MVH_2 + \tfrac{1}{2}O_2 + ATP \quad (6.6)$$

$$MVH_2 + 2\ H^+ + O_2 \rightarrow MV + H_2O_2 \quad (6.7)$$

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2}\ O_2 \quad (6.8)$$

$$\text{Sum : } ADP + Pi \rightarrow ATP \quad (6.9)$$

6.1.5. Effect UV-B on photosynthesis

Light is the driving force for photosynthesis and regulates the development of the photosynthetic apparatus (Smith, 1983). However, light is an elusive substrate that is not easily managed at the molecular level, and can be harmful to the photosynthetic apparatus. When plants are exposed to light intensities exceeding the capacity of assimilatory electron flow, regulatory mechanisms are effective which lead to an increase of non-radiative energy dissipation, accompanied by a loss in energy transformation efficiency of the two photosystems in photosynthesis (Walker, 1992). This provides protection against photoinhibition, a term used to refer to the decrease in photosynthetic activity that occurs when plant is exposed to high radiances of visible light or UV-B radiation (Schnettger *et al.*, 1994).

Many studies have been done on the effects of UV-B radiation on either the rate of net photosynthesis or of its partial reactions (Jordan *et al.*, 1993; Bornman, 1989). A reduction in net photosynthetic oxygen evolution was caused by a lower amount of chlorophyll content under UV-B radiation (Ekelund, 1994). The photosynthetic oxygen exchange in pea and rice leaf discs were also observed (He *et al.*, 1993, 1994). The inhibition of partial reactions of photosynthesis under UV-B radiation may be due to impairment of electron transport in chloroplasts of crop plants (Nooruden and Kulandaivelu, 1982; Nedunchezhian and Kulandaivelu, 1991b; Eichhorn *et al.*, 1993), loss of enzyme activity and changes in the composition of chloroplast pigments (Strid *et* al., 1990; Nedunchezhian and Kulandaivelu, 1991a; He *et* al., 1993, 1994; Lingakumar and Kulandaivelu, 1993) and damage to the photosynthetic reaction centre (Nedunchezhian and Kulandaivelu, 1991b; Friso *et al.*, 1994). The effects of UV-B on photosynthesis are summarized in Table 1.1 (Chapter 1).

The chloroplast PSII reaction centre is the main target of UV-B light damaging effects (Renger *et al.*, 1989; Eckert *et al.*, 1992), leading to impairment of electron transport and irreversible damage to the reaction centre subunits. In particular, the D1 protein becomes degraded with the formation of photochemically

1994). UV-B light inhibits electron transport and destroys structural components of PS II with an associated decrease in chlorophyll content and damage to chloroplast structure (Chapter 4).

A number of studies have also investigated the role of UV-B light in reducing the photosynthetic activity, however, only a few experiments used leaf slices and/or isolated chloroplasts. Therefore, oxygen evolution and electron transport activity were investigated in leaf slices and isolated chloroplast from UV-B treated plants.

6.2. Results

6.2.1. Chlorophyll content

For chlorophyll assays, leaf discs were taken from the third pair of pea leaves and the second wheat leaf. It meant that the tissues were comparable in their physiological properties. Table 6.1. shows the chlorophyll content in pea and wheat leaves after irradiation with UV-B. With radiation, the total chlorophyll content tended to decrease in both species. In peas, there was a tendency to partial reduction under UV-B treatment (low and high UV-B light). The total chlorophyll content per unit area in low UV-B was decreased almost 10% and 25% at 8 and 12 days treatment, while in high UV-B, reductions were 12% and 32% at 8 and 12 days exposure, respectively. This drop in total chlorophyll was mainly due to the substantial lowering of the Chl *a* (30%) after 12 days under high UV-B. Chl *a* declined to a greater extent than Chl *b,* the the latter seeming to be constant under UV-B treatment. Taken together, UV-B treated plants displayed a slight reduction of Chl *a*/Chl *b* ratio (Table 6.1) by 15% (8 days after high UV-B treatment). The reduction of the Chl *a*/Chl *b* ratio in the control could be due to the shading of the third leaf pair. In wheat, however, chlorophyll content did not change in control or treated leaves over 8 days treatment, but decreased after 12 days.

Table 6.1. Chlorophyll content and Chl *a*/Chl *b* ratios in pea (A) and wheat (B) leaves as a function of the duration of the UV-B treatment. Each value is the mean of 4 replications. Standard errors of the mean ranged from 0.0001-0.03 (for chl *a*), 0.001-0.02 (for Chl *b*), 0.002-0.03 for Chl *a*/Chl *b* ratios.

Species	Treatment	Chlorophyll content (μg mm^{-2})									Chl *a*/*b* ratio		
		Chl *a*			Chl *b*			Chl *a* and *b*					
		4	8	12	4	8	12	4	8	12	4	8	12
A. Peas	Zero UV-B	0.40	0.39	0.32	0.11	0.12	0.13	0.51	0.51	0.45	3.6	3.3	2.5
	Natural UV-B	0.41	0.38	0.25	0.12	0.12	0.11	0.53	0.49	0.40	3.4	3.2	2.3
	Low UV-B	0.39	0.35	0.24	0.12	0.11	0.10	0.51	0.46	0.34	3.3	3.2	2.4
	High UV-B	0.37	0.33	0.23	0.10	0.12	0.10	0.47	0.45	0.31	3.7	2.8	2.3
B.Wheat	Zero UV-B	0.56	0.62	0.33	0.21	0.27	0.14	0.77	0.89	0.47	2.7	2.3	2.4
	Natural UV-B	0.61	0.56	0.29	0.26	0.21	0.13	0.87	0.77	0.41	2.3	2.7	2.2
	Low UV-B	0.61	0.60	0.29	0.26	0.23	0.11	0.87	0.83	0.39	2.3	2.6	2.6
	High UV-B	0.63	0.63	0.28	0.27	0.28	0.11	0.89	0.91	0.39	2.3	2.3	2.5

6.2.2. Net photosynthesis of intact leaf

The influence of UV-B radiation on net photosynthesis and stomatal aperture in pea leaves was estimated using a LI-6200 IRGA (Infra Red Gas Analyzer) consisting of a Portable Photosynthesis system (Table 6.2).

Table 6.2. Effect of UV-B radiation on the net photosynthesis, CO_2 concentration and stomatal conductivity of intact pea leaves after 4 days of exposure. Measurements were performed using a LI-6200 IRGA (Infra Red Gas Analyzer) consisting of a Portable Photosynthesis system with light intensity around 1850 µmol photons m-2 s-1, CO2 concentration inside the chamber was in the range of approximately 350 - 400 ppm and air temperature was 30-34 °C. Third leaf pairs were always used to give uniform samples. Values are means of 6 replicates from at least 3 individual plant leaves.

Treatment	Net photosynthesis (μmol CO_2 m^{-2} sec^{-1})	Stomatal conductivity (mol H_2O m^{-2} sec^{-1})	Internal CO_2 concentration (ppm)
Zero UV-B	0.75 ± 0.11 (100)	0.013 ± 0.003 (100)	272 ± 2.5 (100)
Natural UV-B	0.49 ± 0.16 (65)	0.010 ± 0.002 (77)	277 ± 16 (102)
Low UV-B	0.47 ± 0.06 (63)	0.009 ± 0.001 (70)	266 ± 23 (98)
High UV-B	0.23 ± 0.08 (31)	0.009 ± 0.002 (70)	303 ± 23 (111)

Note : Numbers in brackets indicate the percentage (%) of the control without UV-B

Supplementary UV-B radiation had significant effects on the rate of net photosynthesis of intact pea leaf after 4 days exposure. Table 6.2 shows that UV-B radiation tended to reduce the rate of net photosynthesis. The magnitude of photosynthetic inhibition was considerably greater in high UV-B treated leaves (70%) with smaller inhibition (35%) being observed under either natural or low UV-B treatment.

The changes in stomatal conductivity of intact pea leaves are also shown in Table 6.2. In all UV-B treated leaves (natural, low and high UV-B), there was a rapid and similar reduction in stomatal conductivity by 30%.

The internal CO_2 concentration, however, was found to be slightly enhanced under high UV-B (11% increase), but was unchanged in the other treatments.

6.2.3. Oxygen evolution of leaf slices

Measurements of leaf photosynthesis using an oxygen electrode and leaf slices and expressed on a fresh weight basis are presented in Table 6.3. In general, it was found that oxygen evolution tended to decrease with age in pea leaves (Table 6.3A), but remained relatively constant in wheat (Table 6.3B).

In peas, control leaf slices showed a higher rate of oxygen evolution than did treated leaves. Natural and low UV-B plants displayed a similar photosynthetic activity, but lower than control, and with no apparent change in the degree of inhibition relative to control over the period of irradiation. In contrast, high UV-B treatment caused a rapid decrease (40%) in oxygen evolution, which was a reduced further as the period of radiation increased. This resulted in a reduction of 47% by 16 days of treatment.

In contrast, the oxygen evolution of wheat leaf slices under natural and low UV-B treatment (Table 6.3B) remained the same as in control leaf slices. Under high UV-B radiation, the rates of oxygen evolution were slightly lower by 14% and 25% respectively for 4 and 8 days after treatment, and showed no change after 12 days.

Table 6.3. The rate of oxygen evolution from pea (A) and wheat (B) leaf slices on fresh weight basis as a function of days treatment. Values are means of 8 replications from at least 2 independent experiments.

Treatment	Oxygen evolution (nmol O_2 g^{-1} fwt s^{-1})		
	4 days	8 days	16 days
A. Peas			
Zero UV-B	3.9 ± 0.2 (100)	3.5 ± 0.0 (100)	3.1 ± 0.3 (100)
Natural UV-B	2.7 ± 0.6 (71)	2.4 ± 0.1 (69)	2.6 ± 0.4 (83)
Low UV-B	2.8 ± 0.4 (73)	2.4 ± 0.9 (70)	2.3 ± 0.1 (73)
High UV-B	2.4 ± 0.5 (62)	2.3 ± 0.4 (67)	1.6 ± 0.1 (53)
B. Wheat			
Zero UV-B	5.0 ± 0.7 (100)	5.5 ± 0.2 (100)	4.4 ± 0.3 (100)
Natural UV-B	4.7 ± 0.3 (94)	5.2 ± 0.6 (96)	4.9 ± 0.3 (110)
Low UV-B	5.1 ± 0.4 (101)	4.6 ± 0.3 (85)	4.7 ± 0.5 (106)
High UV-B	4.3 ± 0.3 (86)	4.1 ± 0.7 (75)	4.0 ± 0.3 (90)

Note : Numbers in brackets indicate the percentage (%) rates of the control.without UV-B

It should be noted that the size of the leaves was affected by UV-B treatment, hence it was considered necessary to use another reference.

Table 6.4 shows the effect of UV-B radiation on the rates of oxygen evolution as expressed on a chlorophyll weight basis. Pea leaves showed a decline in oxygen evolution both with increasing time of UV-B radiation and with age (Table 6.4A). It appeared that a decrease in photosynthesis was greater when plants received a longer period of radiation. This pattern was found under natural, low and high UV-B treatments. However, high UV-B treatment still gave the greatest inhibition compared to other treatments.

Changes in oxygen evolution on a chl. weight basis in wheat species were observed only at 4 days, when oxygen evolution under high UV-B was reduced by 30%. For larger treatment, the rates were relatively constant, and UV-B radiation obviously had no effect on oxygen evolution.

Table 6.4. The rate of oxygen evolution from pea (A) and wheat (B) leaf slices on chlorophyll weight basis as a function of days treatment. Values are means of 8 replications from at least 2 independent experiments.

Treatment	Oxygen evolution (nmol O_2 mg^{-1} chl. s^{-1})		
	4 days	8 days	16 days
A. Peas			
Zero UV-B	30.5 ± 0.8 (100)	29.1 ± 0.6 (100)	27.6 ± 2.5 (100)
Natural UV-B	24.0 ± 2.9 (83)	23.7 ± 1.2 (86)	18.9 ± 1.1 (62)
Low UV-B	22.8 ± 0.9 (78)	20.9 ± 3.9 (68)	14.8 ± 1.1 (54)
High UV-B	21.9 ± 1.7 (72)	17.1 ± 3.5 (59)	12.2 ± 1.5 (44)
B. Wheat			
Zero UV-B	15.6 ± 3.1 (100)	10.5 ± 1.8 (100)	9.3 ± 0.4 (100)
Natural UV-B	15.7 ± 3.6 (100)	11.1 ± 2.0 (106)	9.9 ± 0.5 (106)
Low UV-B	15.3 ± 1.9 (98)	9.8 ± 1.4 (93)	8.9 ± 1.2 (96)
High UV-B	11.0 ± 0.4 (71)	10.4 ± 3.1 (99)	10.1 ± 0.6 (108)

Note : Numbers in brackets indicate the percentage (%) rates of the control without UV-B.

6.2.4. Oxygen evolution in pea chloroplasts

Chloroplasts isolated from control and treated pea leaves showed an effect of UV-B on oxygen evolution. Fig.6.4. shows the plot of CO_2 fixation-dependent oxygen evolution as estimated in leaf chloroplasts versus time of radiation.

Obviously, oxygen evolution rates declined with increasing leaf age or time of exposure. It also can be seen from the figure that a reduction of the oxygen evolution was observed under low, natural and high UV-B treatment, even though there was no difference between natural and low UV-B treatments. The pattern of reduction of photosynthesis during treatment was identical between natural UV-B and low UV-B treatments. In contrast, high UV-B showed a greater reduction in oxygen evolution than the other treatments.

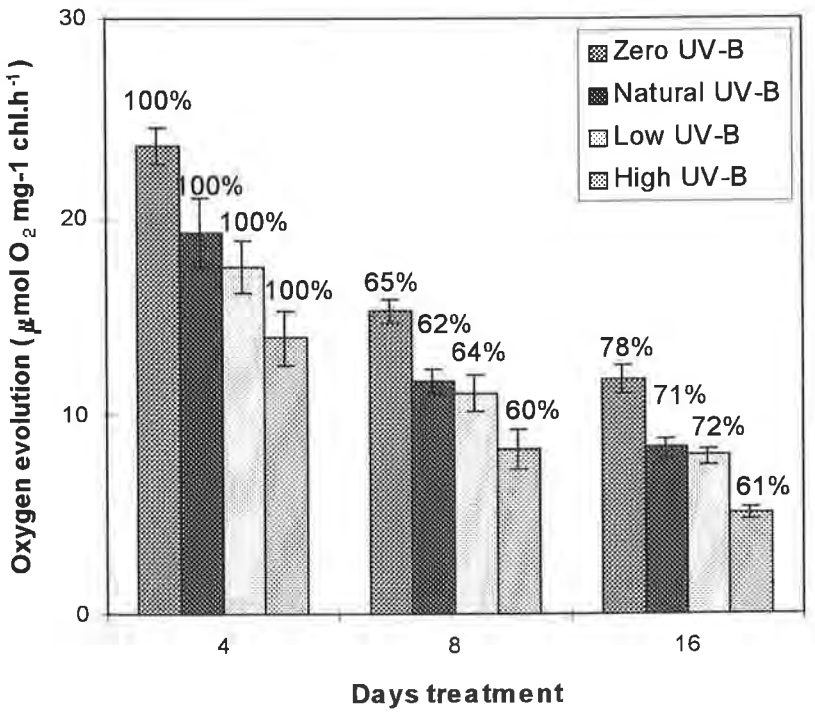


Fig.6.4. The rate of CO_2-dependent oxygen evolution from pea leaf chloroplasts. Each value is a mean of 4-6 replications from at least 2 independent experiments. The percentages indicate the proportional reduction of the 4 day with time of exposure.

6.2.5. Electron transport

6.2.5.1. Non-cyclic electron transport flow

Table 6.5 shows the effect of UV-B radiation on non-cyclic electron transport as measured by O_2 evolution. It was found that high UV-B treatment gave the lowest rate of electron flow from water to NADP under state 3 conditions. Oxygen evolution under low UV-B radiation was not affected, while natural UV-B treatment appeared to stimulate the rate of electron flow (Table 6.5).

Electron transport-dependent O_2 evolution under state 4 was not significantly affected by UV-B radiation (Table 6.5). However, photosynthetic control ratios were considerably lower in the chloroplast fragments from high UV-B treated leaves. This decrease in PCR was due to the reduced state 3 rate.

Changes in the rate of O_2 evolution after the addition of NH_4Cl and nigericin, uncouplers of photosynthetic electron transport are also presented in Table 6.5. Both stimulated the rate well in excess of state 3 rates. Nigericin was more effective than NH_4Cl in stimulating the rate of oxygen evolution. Assuming the nigericin-stimulated rate to be the maximum rate possible, it appears that high UV-B treatment has affected the rate of electron flow. The effect of low and natural UV-B treatments is not clear after 4 days of treatment but appears to be inhibiting after 8 days.

6.2.5.2. Pseudocyclic electron transport flow

Table 6.6 shows the rates of pseudocyclic electron transport-dependent oxygen consumption by pea leaf chloroplasts. Methylviologen and azide were initially included in the reaction mixture, while ADP and uncoupler were added after a steady rate of O_2 consumption had been achieved. In general, the state 3 rate of O_2 consumption decreased with leaf age and time of radiation. However, it appeared that the percentage of reduction by UV-B was relatively constant after 4 days of treatment.

Oxygen uptake under state 4 conditions, when ADP has run out, displayed as similar pattern to that found under state 3 condition. Compared to the control, the percentage of O_2 uptake tended to be lowered by supplementray UV-B radiation. The phototsynthetic control ratios are also shown in Table 6.6. There was no change in the photosynthetic control ratio observed during these experiments and UV-B radiation had no effect.

Table 6.6 also shows the effect of NH_4Cl on the rate of pseudocyclic-dependent-O_2 consumption by pea chloroplasts. The addition of NH_4Cl activated the rates of electron transport under state 3. This stimulation was found to be much greater under UV-B treatment, and was relatively constant with period of radiation. Chloroplasts isolated from high UV-B treated plants showed the greatest stimulation.

Table 6.5. The rate of non-cyclic electron transport from broken pea chloroplasts expressed as nmol O_2 $min^{-1}mg^{-1}$chl. All assays were measured as described in MAterials and Methods and contained 1.3 mM FeCN, 0.05 mM ADP, 5 mM NH4Cl and 1.7 mM nigericin . Numbers in brackets indicate the % of rates from control. Values are the mean of 4-8 replications from at least 4 independent experiments ± se.

A. 4 days after treatment

Treatment	O_2 evolution (nmol O_2 mg^{-1} chl. min^{-1})		photosynthetic control ratio	In the addition of NH_4Cl	Stimulation by NH_4Cl (%)	In the addition of nigericin	Stimulation by nigericin (%)
	state 3	state 4					
Zero UV-B	235 ± 5 (100)	66 ± 1 (100)	3.6 ± 0.0 (100)	565 ± 10 (100)	140	807 ± 29 (100)	243
Natural UV-B	259 ± 2 (110)	61 ± 2 (92)	4.2 ± 0.2 (94)	530 ± 13 (94)	105	810 ± 59 (100)	213
Low UV-B	213 ± 4 (91)	58 ± 4 (88)	3.8 ± 0.4 (104)	569 ± 12(101)	167	496 ± 26 (61)	133
High UV-B	191 ± 8 (81)	64 ± 0 (98)	3.0 ± 0.1 (83)	514 ± 10(91)	169	642 ± 17 (79)	236

B. 8 days after treatment

Treatment	O_2 evolution (nmol O_2 mg^{-1} chl. min^{-1})		photosynthetic control ratio	In the addition of NH_4Cl	Stimulation by NH_4Cl (%)	In the addition of nigericin	Stimulation nigericin (%)
	state 3	state 4					
Zero UV-B	225 ± 22 (100)	68 ± 4 (100)	3.5 ± 0.1 (100)	415 ± 11 (100)	72	605 ± 16 (100)	188
Natural UV-B	273 ± 25 (121)	85 ± 0 (126)	3.7 ± 0.6 (106)	527 ± 30 (127)	96	462 ± 21 (76)	76
Low UV-B	226 ± 0 (100)	62 ± 1 (92)	3.7 ± 0.1 (104)	629 ± 13 (152)	178	427 ± 3 (71)	71
High UV-B	183 ± 16 (81)	66 ± 2 (97)	2.8 ± 0.1 (80)	491 ± 1 (118)	187	499 ± 20 (82)	82

Table 6.6 . The rates of pseudocyclic electron flow from broken chloroplasts from pea leaves. Values are the mean of 4-8 replications from at least 4 independet experiments.

A. 4 days after treatment

Treatment	O_2 uptake (nmol O_2 mg^{-1} chl. min^{-1})		photosynthetic control ratio	In the addition of NH_4Cl	Stimulation by NH_4Cl (%)
	state 3	state 4			
Zero UV-B	276 ± 41 (100)	95 ± 8 (100)	3.2 ± 0.4 (100)	259 ± 23 (100)	28
Natural UV-B	243 ± 32 (88)	77 ± 7 (81)	3.2 ± 0.5 (100)	343 ± 25 (67)	41
Low UV-B	204 ± 27 (74)	59 ± 3 (62)	3.6 ± 0.5 (113)	326 ± 35 (92)	60
High UV-B	192 ± 23 (67)	57 ± 6 (60)	3.2 ± 0.5 (100)	381 ± 28 (108)	98

B. 8 days after treatment

Treatment	O_2 uptake (nmol O_2 mg^{-1} chl. min^{-1})		photosynthetic control ratio	In the addition of NH_4Cl	Stimulation by NH_4Cl (%)
	state 3	state 4			
Zero UV-B	251 ± 12 (100)	72 ± 12 (100)	2.8 ± 0.4 (100)	391 ± 2 (100)	56
Natural UV-B	206 ± 13 (82)	78 ± 20 (108)	3.0 ± 0.5 (107)	439 ± 13 (112)	113
Low UV-B	179 ± 8 (71)	53 ± 10 (74)	3.0 ± 0.4 (107)	299 ± 13 (59)	67
High UV-B	166 ± 8 (66)	57 ± 5 (79)	3.2 ± 0.4 (114)	333 ± 22 (85)	101

6.3. Discussion

Exposure of pea and wheat plants to supplementary UV-B radiation for 12 days caused a decline in several photosynthetic properties, including net photosynthetic rates, oxygen evolution by leaf slices, CO_2-dependent oxygen evolution by isolated chloroplasts (peas), and chlorophyll content as well as electron transport activities (peas). The effect of UV-B radiation on net photosynthesis and photosynthetic electron transport was observed soon after exposure and was not worsened by longer periods of exposure.

In this study, photosynthetic rates under UV-B were more affected in pea than in wheat leaves. Wheat leaves seemed to maintain their photosynthetic activity. The different response of pea and wheat leaves could be due to differences in leaf morphology, development of protective pigments, (chapter 3), leaf anatomical structure, chloroplast number (chapter 4), their photosynthetic pathway, internal cellular organization and biochemistry, and chlorophyll distribution. It may be that growth habit of wheat (nearly vertical) may helps to protect the photosynthetic apparatus. By screening a number of crops plants, Strid *et al* (1990) also demonstrated that peas were more sensitive to UV-B radiation than wheat.

The rates of photosynthesis of attached leaves and detached leaf slices in pea were also lowered by UV-B treatment. This reduction was more pronounced under high UV-B radiation. With attached leaves, these reductions were primarily due to decreases in stomatal conductivity which was found to be lower under UV-B treatment. However, CO_2 concentration inside these leaves was not reduced, suggesting that alterations to the photosynthetic activity was not due to a lowered availability of CO_2. When measurements on leaf slices were performed with saturating CO_2 and high light intensity, leaves harvested from UV-B treated plants showed a reduction in oxygen evolution both on a leaf area and chlorophyll weight basis. Thus, this reduction could be due to a decrease in chloroplast numbers within palisade cells or to a reduction in chlorophyll as photosynthetic pigments. In this study, there was a slight reduction of total chlorophyll content. It was found that UV-B radiation decreased the amount of chlorophyll pigments (Strid *et al.*, 1990, He *et al.*, 1993, 1994). The total chlorophyll decreased by 40% over 8 days of UV-B treatment compared to control plants (Strid *et al.*, 1990). The reduction of chlorophyll content could be related to a decline in pigment synthesis, mRNA transcription for chlorophyll, increase in pigment degradation or all of them. Jordan *et al.*(1991) found that there was an inhibition in the gene expression for the chlorophyll *a*/*b* binding protein. A further analysis has been made by Strid and

Porra (1992) who analyzed tetrapyrrolic pigments to examine the changes in biosynthesis and degradataion of chlorophylls. They concluded that the decrease in chlorophyll was due to an increase in its degradation (an increase in chlorophyllides *a* and *b* resulting from chlorophyll degradation), but not a decrease in its their synthesis (no increase in chlorophyll intermediates). However, it was also found that UV-B increased chlorophyll *a*/*b* ratios (Deckmen *et al.*, 1994). This finding indicates that different species and the conditions of the experiments will result in different responses.

The effects of UV-B radiation on the partial reactions of photosynthesis have been extensively studied and clearly demonstrate multiple sites of inhibition (Bornman, 1989; Strid *et al.*, 1990). In this study, UV-B exposure for 8 days caused CO_2-dependent oxygen evolution, non cyclic electron and pseudocyclyc electron transport by isolated chloroplasts to decline. These changes increased progressively and appear to be a response to the cumulative UV-B dosage.

The reduction of CO_2-dependent oxygen evolution observed in chloroplasts isolated from UV-B-exposed leaves of pea plants (Fig.6.6) could have been caused by damage to the chloroplast structure (chapter 4). Damaged chloroplasts would have a reduced capacity for photosynthetic activity, and may also have a decrease in Rubisco content and activity . Rubisco is the most abundant protein in leaves and also the primary enzyme of CO_2 fixation in C3 plants (Spreitzer, 1993). As a consequence, Rubisco becomes the major resource of carbon and nitrogen within the plant. Although the content and activity of Rubisco, has not been assayed in this study, it has been found to decline in pea leaves exposed to supplementary UV-B radiation (Strid *et al.*, 1990; Jordan *et al.*, 1992). The activity of Rubisco declined more rapidly than did the amount of Rubisco protein at any time point during UV-B exposure. For example, the large subunits (LSU) and small subunits (SSU) of Rubisco decreased by 10-15% after 1 day and by 56% after 3 days of UV-B treatment, whereas the enzyme activity declined by 38% and 71% over the same period. SDS-PAGE analysis showed UV-B radiation reduced the 55 kDa polypeptides, the large subunits of Rubisco, in chloroplasts isolated from UV-B treated leaves of *Vigna sinensis* L.cv.Walp (Nedunchezhian and Kulandaivelu, 1991a; Lingakumar and Kulandaivelu, 1993). They also observed that the reduction of SSU was greater than was that of LSU. The reduction of Rubisco protein and activity may be associated with the cessation of gene transcription, such as the slow degradation of protein and decreases in the level of mRNA transcripts for both rbc L and rbc S as previously observed (Jordan *et al.*, 1992). Any reduction in rbc S mRNA levels with UV-B radiation can be partially

ameliorated by higher PAR during the period of UV-B exposure (Jordan *et al.*, 1992). Whatever the mechanism of damage, supplementary UV-B radiation appeared to have a strong impact in enzymes associated with carboxylation, light absorption and light transduction.

6.3.1. Non-cyclic photosynthetic electron transport

Inhibition of the movement of electrons along the transport chain of thylakoid membranes of pea chloroplast as a consequence of high supplementary UV-B radiation was evident. It was previously found that UV-B light was more effective than visible light in inhibiting the electron transport capacity of isolated thylakoids (Jones and Kok, 1966). In the present study, pea leaf chloroplasts isolated from high UV-B treated pea leaves showed a marked decrease in the activity of non-cyclic electron transport from PS II to PS I at 4 and 8 days after treatment. This result indicates that the loss of the electron transport-dependent O_2 evolution reflected an inhibition site in the PS complexes and reflects alterations in the chloroplast membrane organization (chapter 4). Previous studies showed that supplementary UV-B decreased the PS I and PS II content of pea leaves on leaf-area basis (Strid *et al.*, 1990). The PS II complex was found to be more vulnerable to many environmental stresses, including UV-B (Renger *et al.*, 1989; Strid *et al.*, 1990). It was also found that after 7 days treatment, the oxygen yield per single turnover flash expressed as the number of functional PS II reaction centres dramatically decreased by 50% and 80% on a chlorophyll and leaf area basis respectively (Chow *et al.*, 1989). There were many different primary sites of inhibition in PS II. Using isolated thylakoids from spinach leaf, UV-B radiation has been found to induce inducing damage to the water-oxidizing complex which will inhibit the electron flow from water to the electron acceptor and decrease the number of centres with reduced Q_B (Renger *et al.*, 1989) or the primary quinone acceptor Q_A (Melis *et al.*, 1992). It has also been shown that UV-B light may reflect both donor (such as DPC, diphenylcarbazide) and acceptor (such as DCIP, 2,6-dichlorophenol indophenol) sites of PSII as well as the degradation of D1 polypeptide (Renger *et al.*, 1989; Hideg *et al.*, 1993). Greeberg *et al.* (1989) reported that the quantum efficiency of degradation of the D1 polypeptide was greatest in the UV-B region and that plastoquinone was the photoreception responsible for the degradation. All these studies indicate the clear sensitivity of PSII to UV-B radiation and that multiple sites within the complex may be inhibited (Jordan *et all.,* 1995, in press). The loss of D2 protein after UV-B irradiation of

isolated thylakoids has also been reported (Friso *et al.*, 1994). Furthermore, chloroplasts isolated from leaves treated with UV-B for 30 min showed a 50% loss of PS II activity (Nedunchezhian and Kulandaivelu, 1991b). This decrease was primarily due to a loss of 23 and 33 kDa extrinsic polypeptides. It is assumed that the structural changes of polypeptides are responsible for the inhibition of the oxidation of water.

The addition of NH_4Cl increased the rate of O_2 evolution. This uncoupler destroys the pH gradient across the thylakoid membrane (Krieger and Weis, 1993). Therefore, the H^+ ions released from water oxidation in the thylakoid lumen are being translocated into the matrix. In addition, Cl^- from NH_4Cl enhances the rate of electron flow as found in spinach thylakoids (Jajoo and Bharti, 1993). Any increase in the rate of uncoupled oxygen evolution rate does not depend on ATP synthesis. Here, thylakoids isolated from UV-B treated leaves were more stimulated by NH_4Cl.

The efficiency of nigericin in stimulating electron flow decreased after irradiation. Nigericin, a mobile ionophore, acts as a carrier of univalent cations across thylakoid membrane without a net transfer of charge. It is an antiporter permiting the entry of H^+ in electroneutral exchange of K^+. The presence of nigericin will increase the permeability of thylakoid membrane to ions, the translocation of H^+ ions will acidify the medium outside the thylakoid and increase the amount of K^+ and Mg^+ in the lumen. Thus, the addition of nigericin increases the rate of dissipation of the proton motive gradient. As a consequence, ATP formation via CF_0-CF_1 on the ATPase is inhibited. If UV-B destroys some of the components of the electron transport chain, e.g. PS II, then the uncoupled rate of electron flow is likely to be slower in UV-B treated plants.

6.3.2. Pseudocyclic electron transport

During pseudocyclic electron flow, methylviologen was added to generate hydrogen peroxide (H_2O_2, reaction 7). This compound acts as an acceptor of electrons from PS I (Anderson and Beardall, 1991). These electrons will then react with oxygen to produce H_2O_2. The addition of azide to the reaction medium will inhibit the activity of catalase which is abundant in chloroplasts, and effectively prevent the hydrolysis of H_2O_2.

Pseudocyclic electron transport activity was inhibited by UV-B radiation, but this reduction did not appear to increase with time of radiation. It was previously found that UV-B inhibited photophosphorylation and CF_0CF_1-ATPase activity in pea leaves (Strid *et al.*, 1990; Zhang, *et al.*, 1994). Almost half of the

photophosphorylation was lost after 8 days of exposure (Strid *et al.*, 1990). It was also found that the content of CF_1-ATPase protein decreased after 4 days of UV-B irradiance (Zhang *et al.*, 1994).

In the presence of the uncoupler NH_4Cl, the rate of oxygen consumption increased. Ammonium released from NH_4Cl will produce H^+ and NH_3, and the neutral NH_3 penetrates thylakoid membranes. The NH_3 binds protons on the inside of the thylakoid membrane to decrease the proton gradient.

6.4. Conclusion

Based on photosynthetic characteristic parameters, this present study showed that pea, a temperate species, is much more sensitive to UV-B radiation than wheat. The photosynthesis activity by intact leaves or leaf slices was greatly reduced by high UV-B radiation, while natural and low UV-B appeared to have a similar effect. The reduction in photosynthetic activity is associated with an inhibition of photosynthetic electron transport.

Chapter 7. General discussion

7.1. The aims

The aims of this study were to examine the different responses to UV-B radiation of sensitive and tolerant species. The major differences observed between tolerant (wheat) and sensitive (peas) species, related to morphological, anatomical, physiological and biochemical characteristics, were investigated with respect to UV-B. In the case of morphological structures, there was evidence for qualitative differences in UV-B radiation effects in the species tested. However, morphological changes under UV-B radiation alone could not explain the differences in UV-B tolerance between these two species. There was however evidence for other changes in response to UV-B radiation, especially with respect to alternative oxidase activity in respiration.

7.2. Environmental condition of experiment

Compared to other studies, there were some differences in setting up the experiments, describe here: (1) the intensity of UV-B radiation used in this study was much lower than most previous studies; (2) the distance between plant canopy and UV-B sunlamps was greater; (3) neither cellulose acetate nor Mylar film were applied, allowing a small percentage of UV-A and UV-C to penetrate into plant foliage and (4) seedlings were 8-10 days old before being subjected to UV-B radiation.

Different environment conditions during treatment may contribute to the different plant responses. In glasshouse or in the field, cellulose acetate plastic films have been used to shape the spectral distribution of radiation produced by low-pressure florescent lamps, by eliminating harmful UV-C radiation. In this study, two species of *Pisum sativum* L. cv. Massey Gem and *Triticum aestivum* L. *var.* Machetti were grown under natural photosynthetic active radiation and supplementary UV-B radiation from unfiltered fluorescent UV-B lamps in an unshaded glasshouse. This artificial UV-B radiation contained some (no more than 5%) UV-C radiation. Control plants did not receive UV-B radiation at all.

7.3. Plant sensitivity

Peas and wheat were both affected by low and high UV-B radiation under natural PAR; however, pea was found to be more sensitive than wheat in response to UV-B radiation based on their morphological, anatomical, physiological and biochemical changes. The differential sensitivities to supplementary UV-B radiation by these two species could be governed by their differing responses to UV-B, since UV-B radiation effects seemed to be dependent upon the species. This explanation is in agreement with previous studies reported by Sinclair *et al.* (1990) and Sullivan *et al.* (1992). They suggested that beside interspecific differences among species, greenhouse and growth chamber studies have shown large intraspecific response differences among cultivars of a single species.

The reasons for this difference in UV-B sensitivity may be largely due to the different in their morphological, internal cellular organization, physiological and biochemistry process. It is also dependent on the development of leaf pigments which can absorb UV-B. Wheat produced more UV-B absorbing compounds than did pea. However, UV-B tolerance can not be described simply to the presence of the UV-B absorbing compounds (see previous discussion). The response measured in pea and wheat plants subjected to natural, low and high UV-B radiation conceivably reflects the interaction of morphological and anatomical features, pigment content and photosynthesis.

7.4. Morphological characteristics

7.4.1. Peas

The primary visible effect of UV-B radiation is often bronzed leaves, shorter plant height resulting from shorter internodes, reduction in leaf area and weight, and high absorbance at 300 nm caused by increasing the activity of enzymes for the biosynthesis of UV-B absorbing compounds. In treated leaves, a higher accumulation of UV-B absorbing compounds is usually associated with protection mechanism. Therefore it has been suggested that these plants increase accumulation of UV-B phenolic compounds in the leaves for attenuating the coming UV-B radiation and maintaining biomass production (Murali and Teramura, 1986; Day *et al.*, 1992). In the case of pea species used in this study, the plants were deformed in their morphological structures under UV-B radiation. UV-B treated plants restricted the cell elongation process in the

stem, and consequently the length of internodes and plant height were affected. However, the reduction of these two parameters could also occur in plants grown under the natural sun. Leaf fresh weight and area were reduced and the proportion of reduction was greater under high UV-B radiation, suggesting that the expansion and elongation of leaf cells were inhibited.

7.4.2. Wheat

Wheat plants exhibited lesser effects to UV-B radiation than did peas. Wheat, which are grass plants, have a higher ability to attenuate UV-B radiation penetration than peas even at high UV-B level. Unlike peas, wheat plants produced more UV-B absorbing compounds, even in the control. This production could contribute to wheat tolerance to UV-B radiation, possibly protecting the plants from bronzing and further effects. However wheat plants were shorter under UV-B treatments, including natural UV-B plants, suggesting that light from the sun is sufficient to produce shorter plants.

7.2. Anatomical structure

Wheat leaves are naturally thicker than pea leaves. Since UV-B radiation must penetrate into the leaf to produce any damage, a thicker leaf which has a greater proportion of sensitive organelles, such as chloroplasts, located in deeper, more protected tissue layers would be more resistant to UV-B radiation damage than would a thinner leaf without the benefit of these anatomical screens (Mirecki and Teramura, 1984). In this study, UV-B irradiated plants respond to reduced leaf area with increased leaf thickness as has been reported for soybean leaves (Mirecki and Teramura, 1984). Thicker leaves can mitigate UV-B radiation through additional tissue absorbance, thereby preventing a greater proportion of UV from reaching sensitive organelles in mesophyll tissue. The thickness of leaves decreased in midrib area of pea leaves, contrary to reports for other species (Mirecki and Teramura, 1984; Cen and Bornman, 1990). This reduction was the results of the collapse of the adaxial epidermal cells or also due to the decrease in water content (Santos *et al.*, 1993).

An ultrastructural study also found that the adaxial epidermis damaged under UV-B radiation. UV-B radiation altered the adaxial epidermal cells of pea leaves to the extent of collapse, and this effect was larger in high UV-B

radiation. UV-B treated pea leaves not only showed the damage to epidermal cells, but also to mesophyll cells to some extent. That UV-B can cause collapse of epidermal cell has been reported for *Phaseouls vulgaris* (Cen and Bornman, 1990). Thus a combination of anatomical/morphological responses provides increased protection mechanisms from further damage to deeper tissues. Furthermore, anatomical differences, such as sclerification of tissue and nearly vertical orientation of leaves with protective based sheaths, narrow-leaved plants (wheat) were less sensitive to UV-B radiation than broad-leaved plants (peas) (Van and Garrard, 1975).

There was no evidence of a similar collapse in wheat leaves. If the morphology leaf was not affected, and UV-B absorbing compounds did inhibit UV-B penetration, then it is possible that the epidermis is the critical protective layer. This hypothesis is supported by evidence that the epidermis is considered to be a UV-B radiation filter and usually attenuates transmission to lower-lying tissues (Day *et al.*, 1993). This attenuation is assumed to be due to flavonoid pigments, as mentioned above.

Further mesophyll cells of UV-B treated leaves of peas were found to have chloroplasts that appeared damaged, with some degree of alteration to their thylakoid membranes. This data indicates that UV-B radiation may interfere with the normal differentiation proplastids, and has been reported in peas (Brandle *et al.*, 1977; He *et al.*, 1994) and maize (Santos *et al.*, 1993). In this study, UV-B damage to chloroplasts appeared to accumulate with an increase in UV-B dose. Structural damage to thylakoid membranes was observed to be correlated with decreased activity of the electron transport chain which in turn contributed to lower photosynthetic rates in UV-B treated plants. This analysis is similar to that previously found in pea (Brandle *et al.*, 1977; Strid *et al.*, 1990; He *et al.*, 1994). Structural damage in the chloroplasts envelopes and thylakoid membranes observed in this study have resulted in the inhibition of PS II activity, as previously reported by Strid *et al.* (1990).

7.5. Increased respiration

Measurements of respiration rates by both leaf slices and isolated mitochondria were performed. The results of this study indicated that oxygen uptake was always higher in UV-B treated pea or wheat leaf slices and in their isolated mitochondria. There was evidence that the increased respiration, may have been due to increased alternative activity.

The rates of uninhibited oxygen uptake by pea and wheat leaves slices provided evidence that UV-B had stimulated respiration. The stimulation was greater in the high UV-B treatment. The primary reason for an increase in respiration appeared to be a marked increase in the alternative pathway (CN-resistant pathway). Further no increased respiration by the cytochrome pathway could be detected.

An increased participation of the alternative pathway in UV-B treated pea leaf tissue, may be due to pyruvate stimulation or an increased expression of the alternative oxidase proteins. High levels of ADP strongly disengage the alternative path (Hoefnagel *et al.*, 1994) because they would increase the rate of the cytochrome pathway.

In isolated mitochondria, increased oxygen uptake from under UV-B treated plants was observed with TCA intermediates, whereas rates of oxidation of the photorespiratory substrate, glycine tended to decrease. One possible mechanism for this inhibition is the reduction of photosynthesis activity (Chapter 6) leading to reduction of photorespiration and of glycine synthesis. When glycine synthesis associated with photosynthesis by UV-B treated plants is inhibited, the transfer of glycine into mitochondria from peroxisome is decreased. The relationship between the photorespiration and photosynthesis should also be taken into account, as UV-B radiation produced lower rates of CO_2-dependent oxygen evolution. If this is a reflection of lowered Rubisco activity under UV-B radiation, photorespiration would not be as rapid as without treatment. On the other hand, the production of carbon skeletons within the TCA cycle may remain unchanged. The addition of malate stimulated the rate of oxygen uptake, with glycine. However, UV-B treatment still gave lower the oxygen uptake rates with malate plus glycine that did the isolated mitochondria of control leaves.

Whitecross *et al.* (1994) obtained evidence for increased oxygen uptake by mitochondria isolated from UV-B treated plants. This study also observed a stimulation in the rates of uninhibited state 3 oxygen uptake of mitochondria from UV-B treated pea leaves. However, it seemed in wheat, the mitochondria were not affected by UV-B radiation, as was found for their chloroplasts.

High levels of myxothiazol resistance under UV-B radiation is assumed to be associated with a greater contribution of the alternative pathway to the total respiration. It was observed that stimulation of the alternative pathway is a result of an increase in the alternative pathway activity rather than its protein content, as no change in the alternative oxidase polypeptides was found.

Pyruvate stimulated the rate of oxygen uptake markedly in mitochondria isolated from low and high UV-B-treated leaves. Probably, mitochondria from treated leaves have a lower level of intramitochondrial pyruvate than did mitochondria isolated from control leaves. Therefore, the addition of pyruvate in the presence of myxothiazol to mitochondria with a low concentration of pyruvate will activate the alternative oxidase to a greater extent.

7.6. Reduced photosynthesis

A further consequence of UV-B radiation effect was an inhibition of the photosynthesis rates. In peas, the photosynthesis rates by attached leaves was lower in UV-B treated leaves than in the control. Since peas are sensitive to UV-B radiation, clearly photosynthesis rates by detached leaf slices were dramatically affected by low and high UV-B radiation. However the rate of photosynthesis by leaf slices of wheat whose leaves were species with undamaged was obviously not affected by UV-B radiation.

The lower rates of photosynthesis in UV-B treated peas is thought to be associated with bronzed leaves, yellowing, chlorophyll degradation, a reduction of stomata number and the disruption of both palisade and chloroplasts structure. This conclusion agrees with previous finding in peas (Brandle *et al.*, 1977; Strid *et al.*,1990; He *et al.*, 1993, 1994). High levels of leaf damage under UV-B radiation are assumed to result in ineffective physiological and biochemical processes in leaves, such as a decrease in Rubisco activity (Jordan *et al.*, 1990; He *et al.*, 1993). Decreased Rubisco has been shown to lower photosynthetic rates, in intact leaves or in UV-B treated isolated chloroplasts (Jordan *et al.*, 1992; He *et al.*, 1993; Nedunchezhian and Kulandaivelu, 1991a). This was not only due to decrease in Rubisco protein, LSU and SSU polypeptides, but also to starch accumulated in the chloroplasts in the later stage of supplementary UV-B treatments (He *et al.* 1994). Besides that, reduction in photosynthesis rates was due to an inhibition of PS II expressed as an inhibition in non-cyclic and pseudocyclic electron transport. This may be associated with UV-B-induced disruption of the structural integrity of the lamellar membrane system in the chloroplasts. Electron transport inhibition may be due to many factors, including degradation of the D1 polypeptide within PS II (Greenberg *et al.*, 1989). Many studies have concluded that a major location for UV-B radiation target is PS II (Renger *et al.*, 1989; Melis *et al.*, 1989).

Inhibition of photosynthesis as a result of starch accumulation in chloroplasts (He *et al.*, 1994) is due to a reduced rate of starch breaking down. Because of the inability of such severally damaged cells to mobilize starch, more starch grain is deposited in the UV-B chloroplast.

7.7. Concluding remarks

It was found that UV-B radiation interfered with growth, flavonoids levels, morphology and anatomy of the leaves, chloroplast structure, respiration and photosynthesis electron transport activities in peas. However, the observations pointed out to a tolerance of UV-B by wheat plants.

Peas proved to be a more sensitive species for studying UV-B radiation effects than wheat. The different sensitivity between pea and wheat are caused by their differences in leaf morphology and anatomical structure, UV-B absorbing compounds accumulation, with further influence their respiration and photosynthesis activity.

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