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Photochemical and biochemical changes in wheat seedlings exposed to supplementary ultraviolet-B radiation

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Abstract

The effect of UV-B radiation (312 nm; 1 mW cm^{-2}) was studied on net photosynthesis, chlorophyll fluorescence and changes in flavonoid and carotenoid contents in wheat seedlings. Control plants (without UV-B treatment but identical light and temperature regime as for UV-B treatment) and UV-B treated plants were grown in two separate growth chambers for 15 days. The supplementary UV-B radiation caused a significant decrease in net photosynthesis which was much greater than could be explained by limitation of stomatal conductance. Initial fluorescence (F_o), F_v/F_m and photochemical quenching (q_P) and non-photochemical quenching (q_N) of chlorophyll fluorescence did not change due to 5 days of UV-B treatment, but longer treatment (up to 15 days) increased F_o while decreasing the F_v/F_m ratio. q_P and q_N also decreased after 15 days of UV-B exposure. Changes in UV-B absorbing phenolic compounds such as flavonol (kaempferol), caumarin and anthocyanin were observed, whereas cinnamic acid was only synthesised after 4 days of UV-B treatment. No zeaxanthin formation was observed while neoxanthin showed a decrease in longer UV-B treatment. Violaxanthin showed an increase in early treatment but decreased after 15 days of UV-B exposure. Our results suggest two types of damage, one within 4–5 days of UV-B exposure and another with longer than 5 days exposure.

Keywords: Carotenoid; Chlorophyll fluorescence; Flavonoids; Net photosynthesis; Ultraviolet-B; Xanthophyll

1. Introduction

The measurable decrease in the stratospheric ozone layer has led to an increase in the ultraviolet-B (UV-B 280–320 nm) radiation reaching the earth's surface. This increase is likely to continue into the foreseeable future resulting in a negative impact on biological organisms. Plants use solar radiation for photosynthesis and as a consequence are also exposed to UV-B radiation. UV-B radiation affects plants in several ways. Chloroplast function is impaired [1], protein synthesis is decreased [2] and mRNA levels of photosynthetic

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genes are lowered [3]. UV-B exposure also result in up-regulation of defence genes such as chalcone synthase [3–5].

Numerous investigations have demonstrated that the photosynthetic system is a sensitive component to increased exposure to UV-B. However, there are contrasting reports regarding the effect of UV-B on the photochemistry of photosynthesis. Several studies have demonstrated that photosystem II (PS II) is the most sensitive component of the photosynthetic apparatus to increased UV-B radiation Iwanzik et al. [6] in spinach, Renger et al. [7] in peas, Melis et al. [8] in peas, Jansen et al. [9] in spirodella, Herrmann et al. [10] in Dunaliella, He et al. [11] in pea and rice. However, there are also reports suggesting that UV-B radiation inhibits photosynthesis without an appreciable effect on PS II photochemistry in higher plants (Nogesh and Baker [12] in pea, Allen et al. [13] in brassica, Ziska and Teramura [14] in rice, Middleton and Teramura [15] in soybean and Lesser [16] in algae). Likewise, although there are studies which indicate stomatal limitation to photosynthesis under UV-B exposure [15,17,18], other studies have not implicated stomatal effects as limiting for photosynthesis [13,14,19–21].

Low levels of UV-B have always been present in the environment and adaptive mechanisms that diminish the effects of the damaging UV-B radiation have evolved in plants [22]. Some plants are more tolerant to UV-B than others because they produce a variety of secondary metabolites that effectively absorb UV-B and prevent it from penetrating into the leaf mesophyll cells [23]. Primary UV-B screening pigments in most plant species are flavonoids which are synthesized in response to UV-B [24], accumulate in the upper epidermis cells of leaves [25] and absorb in the UV-B range in methanolic extract [26].

The aim of the present study was to investigate the effect of supplementary UV-B radiation on the photochemistry of photosynthesis and on the defence mechanism against UV-B in wheat seedlings grown under laboratory conditions.

2. Material and methods

2.1. Material

Wheat seeds *(Triticum aestivum* L. cv HD2380) were obtained from the Indian Agricultural Research Institute, New Delhi, India and grown in 10×10 cm plastic pots containing vermiculite and bottom-irrigated routinely using half strength Hoagland's solution. Plants were grown in a growth chamber illuminated with incandescent bulbs and fluorescent tubes (Fig. 1A) for a 12 h photoperiod having a non-photoinhibitory level of radiation of 300 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), measured with a radiometer (Model LI-189, Licor, USA). The day/night temperature was maintained at 25 ± 2°C.

2.2. UV-B irradiation

Pots containing 4-day-old wheat seedlings were transferred to another growth chamber with the identical PAR, photoperiod and temperature as described above but supplemented with UV-B radiation. The source of UV-B was a Vilbour-Lourmat (France) T-6M source with intensity of 6 mW cm^{-2} with a long term glass filter (Fig. 1B).

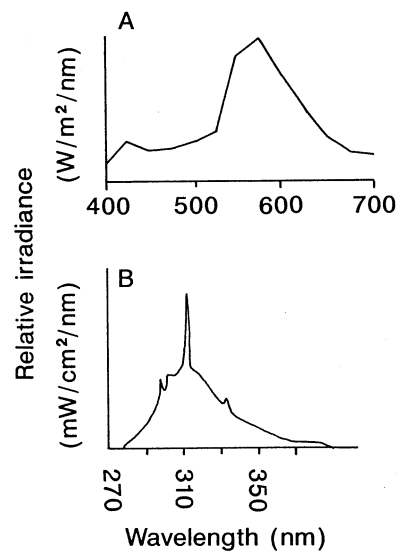

Fig. 1. Spectrum of fluorescent and incandescent light used for plant growth (A) and ultraviolet-B (B).

The UV-B radiation was measured using a UV-B radiometer from the same-manufacturer. The intensity was measured at the base, middle and top of the seedlings by placing the sensor of the UV-B radiometer in close contact with the leaves. The average intensity of UV-B radiation was 1 mW cm^{-2}. Care was taken to maintain the average level of UV-B irradiance at 1 ± 0.2 mW cm^{-2} during the treatment while plants were growing by changing the distance between source and seedlings and by placing the bandage cloth in-between. The average UV-B radiation level in the environment in Goa, India, was ≈ 0.55 mW cm^{-2}.

2.3. Gas exchange measurements

Net CO_2 assimilation rate and stomatal conductance was measured with a pair of leaves at 1200 μmol m^{-2} s^{-1} PAR using an open type gas analyzer as described by Long et al. [27].

2.4. Chlorophyll fluorescence

In vivo chlorophyll fluorescence was measured with a pulse amplitude modulation fluorometer (PAM 101, Walz, Effelrich, Germany), as described by Schreiber et al. [28]. Leaves were dark adapted for 15 min. F_o was measured at very low irradiance (0.6 μmol m^{-2} s^{-1}; modulated light). F_m was determined by application of a saturating pulse of white light (3000 μmol m^{-2} s^{-1}). Variable fluorescence (F_v) was determined as $F_m - F_o$. After measurement of F_m the leaf was exposed to actinic light (1200 μmol m^{-2} s^{-1}) and allowed to reach a steady state fluorescence (F_s). Another burst of saturating light was used to measure F'_m. F'_o was measured by exposing the leaf to infra-red radiation (15 μmol m^{-2} s^{-1}). q_P is defined as $(F'_m - F_s)/(F'_m - F'_o)$ and q_N as $1 - (F'_m - F'_o)/(F_m - F_o)$.

2.5. Analysis of UV-B absorbing (flavonoid) compounds

Fresh leaves (2 g) were excised and homogenised in 20 ml of 80% methanol with 1% (w/v) HCl in mortar. The homogenate was incubated for 2 h at room temperature in the dark and centrifuged at $1600 \times g$ for 20 min. The supernatant was collected for absorption spectra, paper chromatography and HPLC analysis (Spectraphysics, UK).

Spectral scan of UV-B absorbing compound was carried out with extract diluted to 1:5 ratio in 80% methanol with 1% (v/v) HC1, using spectrophotometer (Shimadzu UV240).

For paper chromatography and HPLC analysis 5 ml of each sample was fully dried by flushing with N_2 and re-dissolved in 200 μl of methanol.

2.6. Paper chromatography

A 20 μl sample was loaded on a Whatman filter paper no. 1 and resolved using *n*-butanol: acetic acid: water (6:1:2) at room temperature and observed under UV photoilluminator. The flavonoids were identified according to Swain [29].

2.7. High performance liquid chromatography

Identification and separation of flavonoids was carried out using HPLC (Spectraphysics, UK) with a C_{18} reverse phase column (ET 250/4 Nucleosil 100-5C_{18} ODS), Spectraphysics SP ternary HPLC pump with SP 4270 integrator and Spectra 100 variable wavelength detector. The 100 μl of re-dissolved sample was diluted in 1 ml methanol and 25 μl of this was injected on to the HPLC column at room temperature. The separation was carried out using an isocratic system (0.5% phosphoric acid and methanol in 1:1 ratio) over 20 min with a 1.0 ml min^{-1} flow rate at 280 nm for flavonoid and 418 nm for anthocyanin.

2.8. Analysis of carotenoids

This was carried out according to Buch et al. [30]. Leaf tissue (100 mg fresh weight) from the apex region (5 cm) was extracted in 2 ml acetone. The extract was centrifuged at $5000 \times g$ for 15 min at 4°C. The supernatant was dried using N_2 gas and samples stored in a -70°C freezer for HPLC analysis of carotenoids including xanthophylls.

Identification and separation was carried out using high pressure liquid chromatography (for detail of machine and its specification see above). The dried extract was dissolved in 200 μl of acetone and 100 μl of this suspension injected on to the HPLC column and detected at 445 nm. A mobile phase gradient of increasing acetone in aqueous buffer (1 mM Hepes, pH 7.0 was run at 30°C with the following time schedule; 0–5 min 60–72.5% acetone, 5–7 min 72.5–75% acetone, 7–8 min 75–80% acetone and 815 min 80–98% acetone. The flow rate was 1.2 ml min^{-1}. The pigments were identified by comparing their retention time and their spectral characteristics with known standards.

3. Results

The effect of supplementary UV-B radiation resulted in a large decrease in net photosynthesis and a relatively smaller decrease in stomatal conductance (Fig. 2A and B, respectively). The UV-B treatment up to 3 days resulted in 12% decrease in net photosynthesis as compared to its respective control followed by a 66% decrease after 15 days of UV-B treatment (Fig. 2A). Stomatal conductance, however, showed only 30% decrease even after 15 days of UV-B exposure as compared to its respective control (Fig. 2B).

UV-B treatment for 6 h caused no change in initial fluorescence (F_o) as well as in F_v/F_m ratio compared to control (data not shown). Even longer duration of UV-B exposure of 5 days showed negligible effect on initial fluorescence as well as on F_v/F_m ratio. However, exposure for 15 days caused a 79% increase in initial fluorescence and a 16% decrease in the F_v/F_m ratio (Fig. 3A) compared to control.

q_P and q_N of chlorophyll fluorescence also remained relatively unchanged for up to 5 days of UV-B treatment (Fig. 3B). However, longer treatment resulted in decreases in q_P and q_N. Seven days of UV-B treatment caused 34 and 18% decreases in q_P and q_N, respectively with respect to controls. This was decreased to 48% q_P and 28% q_N, respectively after 15 days of UV-B treatment (Fig. 3B).

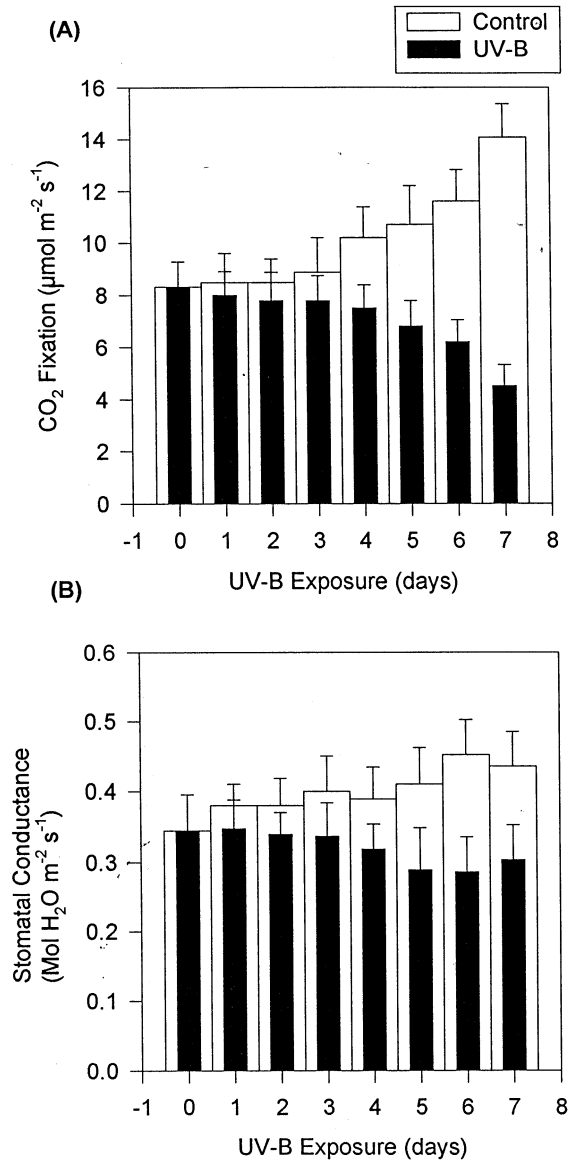

Fig. 2. Effect of supplementary UV-B radiation (1 mW cm^{-2}) on net CO_2 fixation (A) and stomatal conductance (B), $n = 4$. Standard errors are shown.

Plants treated with UV-B showed changes in flavonoid contents (Fig. 4). Absorption spectra of 80% methanolic extract showed an increase in phenolic compounds with absorption peaks at 210 nm (caumarin), 280 nm (Kaempferol) and 320 nm (flavone).

Paper chromatogram of methanolic extract showed qualitative changes in flavonoids. UV-B exposure up to 3 days to wheat plants did not cause changes in phenolic compounds, however, further increase in the duration of UV-B exposure resulted in induction of cinnamic acid ($R_f = 59$) as well as a different anthocyanin ($R_f = 56$; Fig. 5). Identification of flavonoids was determined on the basis of R_f value, the colour characterization under visual and ultraviolet transilluminator (with and without NH_3 fumes) and their absorption spectra according to Swain [29]. HPLC analysis of methanolic extract was carried out to further characterize flavonoid changes under UV-B exposure. The analysis showed that UV-B exposure caused increases in Kaempferol, caumarin, flavone (Fig. 6ITable 1) and anthocyanin (Fig. 6IITable 1). A few small peaks seen in Fig. 6II may indicate different types of anthocyanin.

Changes in carotenoids in response to supplementary UV-B radiation were also studied (Table 2). It was observed that violaxanthin increased up to 30% after 7 days of UV-B treatment, however, it decreased by 12% after 15 days of UV-B treatment compared to control (plants grown without

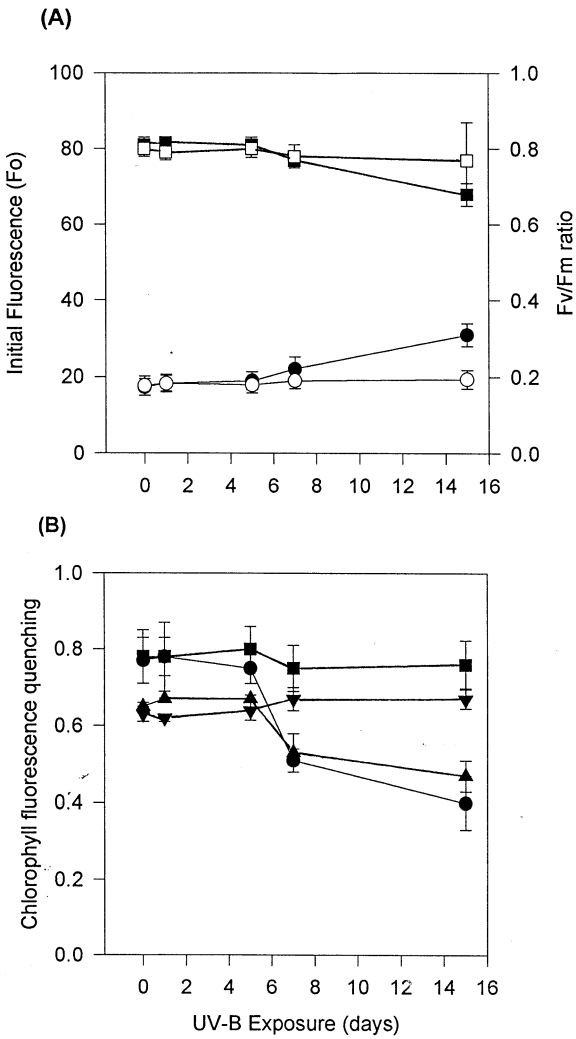

Fig. 3. (A) Effect of supplementary UV-B radiation (1 mW cm^{-2}) on initial fluorescence in control plants (–○–) and UV-B treated plants (–●–) and on F_v/F_m ratio in control plants (–□–) and UV-B treated plants (–■–), $n = 4$. Standard errors are shown and (B) on photochemical quenching (q_P) in control (–■–) and treated plants (–●–) and on non-photochemical quenching of chlorophyll fluorescence (q_N) in control (–▼–) and in treated plants (–▲–), $n = 4$. Standard errors are shown.

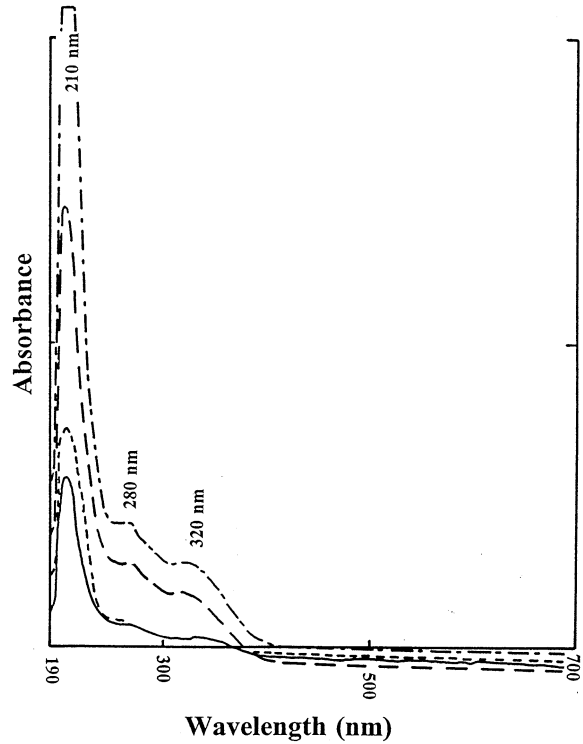

Fig. 4. Effect of supplementary UV-B radiation (1 mW cm^{-2}) on changes in flavonoid contents in wheat seedlings. Control (—), 3 days UV-B (----), 7 days UV-B (– – –) and 15 days UV-B (-·-·-) treatment.

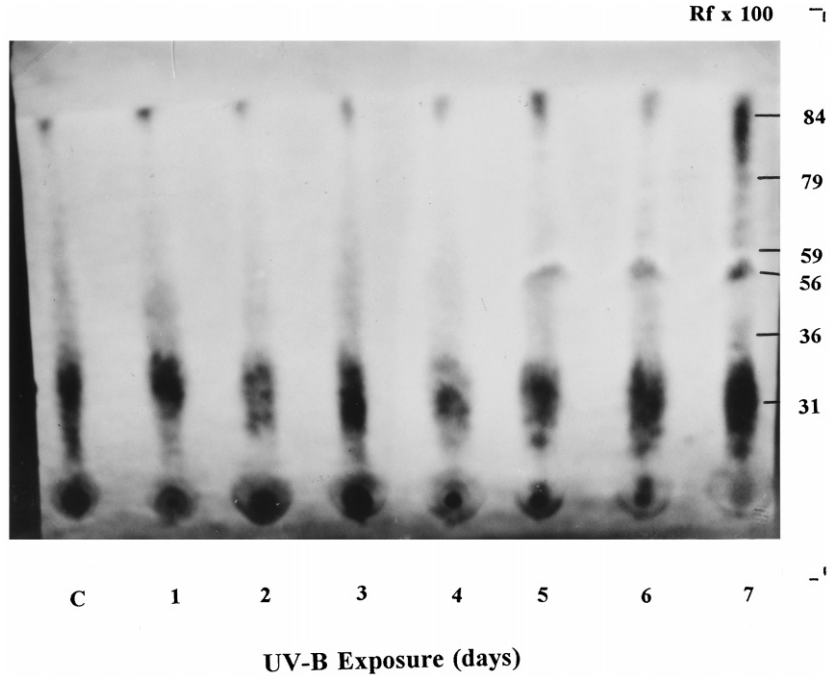

Fig. 5. Effect of supplementary UV-B radiation (1 mW cm^{-2}) on flavonoid contents in wheat seedlings. C (control are the plants which were kept in growth conditions (no UV-B) for 15 days. Identification of flavonoids was determined on the basis of R_f value, the colour characterization under visual and ultraviolet transilluminator (with and without NH_3 fumes) according to Swain [29].

UV-B for 19 days). Zeaxanthin showed an increase of 8% after 15 days of UV-B treatment compared to control. Neoxanthin level declined by 40% in 15 days UV-B treated plants. Chlorophyll *a* and *b* also showed decreases due to the UV-B exposure. β-carotene, however, increased after 7 days of UV-B treatment (Table 2).

4. Discussion

Our results indicate a decrease in net CO_2 assimilation due to the UV-B exposure (Fig. 2A and B). Initially (up to 5 days of UV-B treatment) stomatal limitation may be responsible for the decrease in net CO_2 fixation since the extent of decrease in the CO_2 fixation is similar to the extent of decrease in the stomatal conductance. However, the longer UV-B treatments indicate that factors other than stomatal limitation such as metabolic limitation due to degradation/inactivation of enzymes of the Calvin cycle may be responsible for the decrease in the net photosynthesis.

Middleton and Teramura [15] evaluating the effects of UV-B on photosynthesis with stomatal limitation removed, have demonstrated an increase in photosynthesis suggesting a role for stomatal conductance in limiting photosynthesis. However, the role may be limited and a major decrease in photosynthesis may be due to other factors than stomatal limitation. Gorton and Vogelmann [31] and Krauss et al. [32] have reported that photosynthesis may also be indirectly affected due to changes in leaf thickness or anatomy, which may alter the penetration of visible radiation into the leaf and thus indirectly impair photosynthesis. Allen et al. [13] showed the reduction in photosynthesis may also be due to a large decrease in Rubisco activity which was seen to be due to the reduction in the amount of Rubisco present and not due to the deactivation of the Rubisco under UV-B exposure.

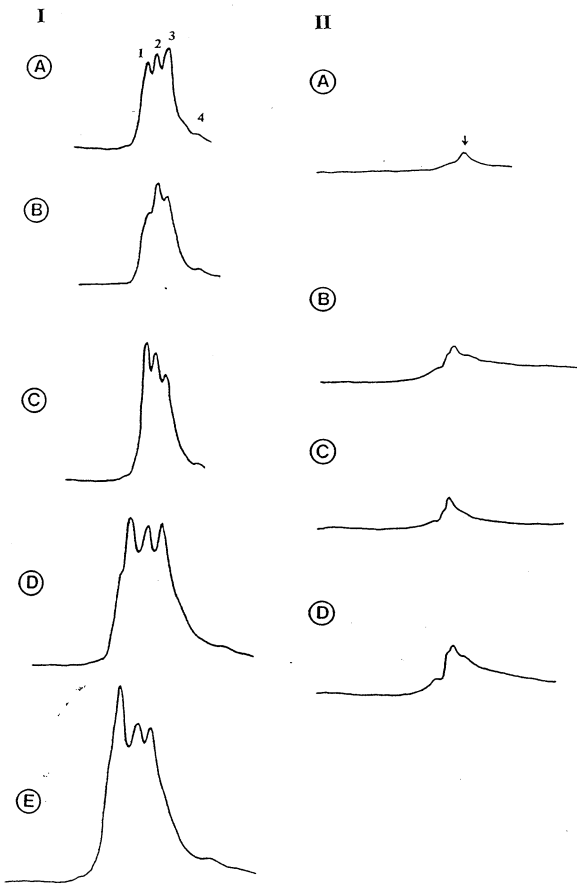

Fig. 6. (I) Effect of supplementary UV-B radiation (1 mW cm-2) on HPLC analysis at 280 nm showing quantitative changes in flavonoids content in (A) control; (B) 1 day; (C) 7 days; (D) 11 days; and (E) 15 days UV-B treatment. Peaks are identified based on retention time. 1, Kaempferol; 2, caumarin; 3, flavone; and 4, anthocyanin. (II) HPLC analysis at 418 nm showing quantitative changes in anthocyanin content in (A) Control; (B) 5 days; (C) 11 days; and (D) 15 days UV-B treatment. Other peaks seen in 15 days UV-B treatment are unidentified but may be different types of anthocyanin [40].

The unchanged F_v/F_m ratio indicates no effect of supplementary UV-B radiation on PS II even after 5 days of UV-B (Fig. 3A). Photochemical quenching and non-photochemical quenching also remained relatively unaffected for up to 5 days of treatment. These results may indicate that early effect of UV-B radiation is not on PS II. Hideg and Vass [33] have reported that UV-B does not result in the production of singlet oxygen, a primary cause of photoinhibitory damage to PS II. Rather it induces free radicals such as hydroxyl and carbon centred (methyl-like) one by direct action of UV-B on H_2O_2. This may cause damage to photosynthesis through peroxidation of membrane lipids and proteins [34]. Hideg and Vass [33] also suggested that unlike photoinhibition UV-B exposure does not involve chlorophyll triplet formation. The UV-B induced production of free radicals is independent of the presence of oxygen which suggest that they are not derived from molecular oxygen. Van Hasselt et al. [35] have shown that low oxygen (2%) and high oxygen (66%) during UV-B exposure did not change level of peroxidation of thylakoid membrane suggesting that light induced oxygen radicals were not involved in lipid peroxidation and peroxidation of membrane may have been a result of breakdown of H_2O_2 by UV-B exposure. The decrease in the q_P, q_N and F_v/F_m ratio seen in our results in longer exposed plants (longer than 5 days; Fig. 3A and B) could be due to this damage to thylakoid membrane by peroxidation of lipids and proteins. Strid et al. [36] have also reported that integrity of thylakoid membrane was more susceptible to UV-B exposure than the activities of photosynthetic components bound within.

In this study we observed changes in flavonol (Kaempferol), caumarin, flavone and anthocyanin and cinnamic acid in response to supplementary UV-B radiation (Figs. 4–6Table 1) which also indicate that these flavonoids are synthesized in response to UV-B treatment and accumulate in the epidermal layer to provide protection by absorbing UV-B radiation and thus preventing it reaching the more sensitive sites such as DNA and protein in mesophyll tissue.

Lois and Buchanan [37], Cullen and Neale [38], Landry et al. [39] and Strid et al. [35] have all suggested that flavonoids and phenyl derivatives are involved in the protection against UV-B in higher plants. Gorton and Vogelmann [31], Krauss et al. [32] suggested that UV-B induces morphological and anatomical changes in order to accumulate increasing amount of flavonoids such as flavones, isoflavonoids and anthocyanin which provide selective attenuation of UV-B radiation. Sarma et al. [40] reported that anthocyanin not only chelates metal ions but also forms ascor-

Table 1
Effect of supplementary UV-B radiation (1 mW cm^{-2}) on flavonoid contents

Flavonoid	Control		UV-B treatment				
	A	B	1 day	5 days	7 days	11 days	15 days
Kampferol	0.326 ± 0.08	0.344 ± 0.07	0.302 ± 0.08	—	0.697 ± 0.07	1.078 ± 0.14	1.461 ± 0.26
Caumarin	0.655 ± 0.09	0.791 ± 0.08	0.564 ± 0.08	—	0.585 ± 0.07	0.693 ± 0.1	0.810 ± 0.17
Flavone	0.693 ± 0.09	0.740 ± 0.09	0.653 ± 0.09	—	0.675 ± 0.08	1.151 ± 0.20	1.300 ± 0.21
Anthocyanin	0.036 ± 0.01	0.048 ± 0.01	—	—	—	0.141 ± 0.02	0.292 ± 0.04

Controls are 9 days (A) and 19 days (B) old plants grown without UV-B. Data are expressed as μg mg^{-1} fresh weight using flavone as an external standard, $n = 4–5$.

bic acid (co-pigment)-metal-anthocyanin complex, which could scavenge hydro-peroxy radicals. Hallbroke et al. [41] have shown an increased expression of genes encoding enzymes such as phenylalanine amonialyase (PAL), 4-coumarate: Co-A ligase (4 Cl) and Chalcone synthetase (CHS) of the phenyl propanoid and flavonoid biosynthesis.

Lois and Buchanan [37] working with an *Arabidopsis* mutant deficient in flavonoid accumulation found that the mutant displayed a dramatic increase in the sensitivity to UV-B radiation compared with wild type suggesting a protective role for flavonoids against UV-B radiation. Similarly Landry et al. [39] and Li et al. [42] also showed that *Arabidopsis thaliana* mutants defective in ability to synthesize UV-B absorbing compounds (flavonoids and sinnapic esters) were more sensitive to UV-B than wild type indicating a role for flavonoids in protection against UV-B damage.

Our results showed an initial increase in violaxanthin and no decrease in zeaxanthin suggesting no over energization of PS II which is also indicated by decrease in q_N. This may indicate that UV-B may have a different mechanism of damage to photosynthesis than seen under excess visual radiation. However, 15 days of UV-B exposure caused a decrease in neoxanthin as well as in violaxanthin. The decrease in violaxanthin and neoxanthin could be due to its being diverted to the ABA synthesis probably because of oxidative damage by UV-B [33]. Kende and Zeevaart [43] have suggested that *trans*-violaxanthin is isomerized to *cis*-violaxanthin or *trans*-neoxanthin, both of which are finally isomerized to *cis*-neoxanthin which is oxidised to ABA through an intermediate, xanthoxin. We have observed an increase in ABA due to UV-B exposure (data not presented; work at early stage). The observed decrease in the amount of chlorophyll in the longer treatment may suggest that UV-B alters synthesis of chlorophyll molecules resulting in decreases in the CO_2 fixation as well as in the photochemistry.

In summary our results indicate that UV-B radiation causes limitation to net photosynthesis without major changes in PS II photochemistry up to 5 days of UV-B treatment, however, longer (7–15 days) treatment resulted in a decrease in photochemistry of PS II measured as F_v/F_m ratio and q_P. The changes in q_P and q_N in longer UV-B treatment may be a secondary effect of UV-B damage probably due to peroxidation (breaking down of H_2O_2 by UV-B) of lipids and proteins in thylakoid membrane and degradation of chlorophyll molecules rather than direct effect on PS II reaction centre as seen under photoinhibition. Flavonoids are accumulated in response to UV-B, which absorb in UV-B range (< 400 nm; Fig. 4) and may provide protection from UV-B damage.

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Table 2
Effect of supplementary UV-B radiation (1 mW cm^{-2}) on carotenoid contents

Carotenoids	Control		UV-B treatment		
	A	B	5 days	7 days	15 days
Neoxanthin	5.82 ± 0.9	5.86 ± 1.1	5.72 ± 0.9	5.41 ± 1.6	3.50 ± 0.9
Violxanthin	11.01 ± 1.0	10.41 ± 0.8	11.33 ± 1.2	14.37 ± 0.7	9.16 ± 0.3
Antheraxanthin	0.43 ± 0.1	0.73 ± 0.2	0.86 ± 0.1	0.99 ± 0.1	0.97 ± 0.1
Zeaxanthin	0.23 ± 0.0	0.25 ± 0.0	0.25 ± 0.0	0.24 ± 0.0	0.27 ± 0.0
β-carotene	5.90 ± 0.9	5.75 ± 1.6	6.01 ± 0.9	8.74 ± 1.3	5.06 ± 1.0
Chlorophyll a	16.11 ± 1.8	15.26 ± 3.1	18.86 ± 2.1	14.37 ± 3.9	10.65 ± 2.6
Chlorophyll b	36.77 ± 2.3	38.70 ± 4.2	40.35 ± 3.2	38.86 ± 3.7	30.03 ± 3.2

Control are 9 days (A) and 19 days (B) plants grown without UV-B. Data are expressed as μg mg^{-1} fresh weight, $n = 3$.

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