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Photosynthetic Response and Protective Regulation To Ultraviolet-B Radiation In Green Pepper (*Capsicum annuum* L.) Leaves

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The deteriorative effect of ultraviolet-B (UV-B) radiation on photosynthesis was assessed by the simultaneous measurement of O_2 evolution and chlorophyll (Chl) fluorescence in green pepper. UV-B was given at the intensity of 1 $W \cdot m^{-2}$, a dosage often encountered in urban area of Seoul in Korea, to the detached leaves. Both Pmax and quantum yield of O_2 evolution was rapidly decreased, in a parallel phase, with increasing time of UV-B treatment. Chl fluorescence parameters were also significantly affected. *Fo* was increased while both *Fm* and *Fv* were decreased. Photochemical efficiency of PS II (*Fv / Fm*) was also declined, although to a lesser extent than Pmax. Both qP and NPQ were decreased similarly with increasing time of UV-B treatment. However, PS I remained stable. The addition of lincomycin prior to UV-B treatment accelerated the decline in *Fv / Fm* to some extent, suggesting that D1 protein turnover may play a role in overcoming the harmful effect of UV-B. The amount of photosynthetic pigments was less affected than photosynthetic response in showing decline in Chl *a* and carotenoids after 24 h-treatment. Presumptive flavonoid contents, measured by changes in absorbance at 270 nm, 300 nm and 330 nm, were all increased by roughly 50% after 8 h-treatment. Among antioxidant enzymes, activities of catalase and peroxidase were steadily increased until 12 h of UV-B treatment whereas ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase did not show any significant change. The results indicate that deteriorative effect of UV-B on photosynthesis precedes the protection exerted by pigment synthesis and antioxidant enzymes.

key words: UV-B radiation, pepper, photosynthesis, chlorophyll fluorescence, flavonoid, antioxidant enzyme

INTRODUCTION

Plants in nature are continuously affected by various natural environmental factors such as light, water and temperature and respond to changes in those factors to their advantage. After rapid industrialization, anthropogenic environmental pollutants such as heavy metals and airborne pollutants presented a new set of adverse factors directly or indirectly affecting plants growth. Among these factors, UV-B (between 280 and 320 nm) has drawn attention in recent years since the heavy usage of Freon (chloroflurocarbon) gas for aerosol propellants and refrigeration resulted in the rapid erosion of stratospheric ozone layer on a global scale [1,2]. In consequence, UV-B level reaching on the ground has greatly increased [3]. For example, UV-B radiation was in the range of 0.9 $W \cdot m^{-2}$ during the midday in Seoul according to a recent report of Korea Meteorological Administration.

Increased UV-B radiation was shown to affect harmfully most physiological and biochemical processes in plants including photosynthesis [4,5], dark respiration [4,5], transpiration [5], biomass allocation [6], and leaf expansion [7,8]. UV-B irradiation could also be damaging when essential UV-absorbing biomolecules, namely proteins and nucleic acids were photomodified [9,10]. In addition, UV-B radiation resulted in changes in pigment composition by reducing Chl content and increasing flavonoid content, which was especially magnified under low PFR [11].

Decrease in photosynthesis due to UV-B radiation was mainly attributed to the inactivation of PS II [12-15]. UV-B treated leaves showed reduction in O_2 evolution and Chl fluorescence originating from PS II [15]. The same results were observed in the isolated chloroplasts from UV-B treated plants [12]. UV-B mostly affected PS II reaction center itself and water oxidizing complex [16,17]. The outcome was due to the denaturation of proteins by UV-B in PS II complex, especially in D1 protein and LHCP II and subsequent damage in Q_A was followed [13,17]. D2 protein, generally thought to be unaffected, was also shown harmed [18]. Destruction of Trp residue in the ATPase protein resulted in the inactivation of ATPase [19]. UV-B reduced RNA transcription of *cab* and *psb* A [20]. On the other hand, PS I and cytochrome b-f complex were hardly affected by UV-B [15].

UV-B affected the dark reaction as well by reducing Rubisco activities [15,20]. Both large (55 kDa) and small (15 kDa) subunits of Rubisco were degraded and synthesis of their mRNA was declined after long exposure to UV-B [20]. The photomodification of Trp residue by UV-B in the large subunit produced 66 kDa subunit instead of 55 kDa subunit

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[21]. All of them led to the decline in Rubisco activities and subsequent reduction in CO_2 assimilation.

UV-B was absorbed directly by DNA to form UV-induced photoproducts such as cyclobutane pyrimidine dimer and pyrimidine (6,4) pyrimidone dimer, which caused serious errors in replication and transcription [9,22]. To ameliorate these deteriorative effects, plants developed two types of protective mechanisms: DNA repair and shielding to reduce DNA damage. Photolyase was shown to be involved in repairing UV-B damaged DNA in plants [23, 24]. Thickening of leaves and formation of cuticular waxes were manifested to prevent the penetration of UV-B [25]. Accumulation of proline, ferulic acid, and flavonoids was observed in the leaves [26,27]. Among them UV-absorbing flavonoids and transcripts involved in flavonoid biosynthesis were increased after long exposure to UV-B [27-29]. Increase in flavonoid content decreased the UV-induced dimer formation [30]. Furthermore, UV-B radiation led to the formation of biologically active oxygenic species that could damage the photosynthetic apparatus and adversely affect the enzyme activities. Antioxidant enzymes such as catalase, ascorbate peroxidase, and glutathione peroxidase were activated against oxidative stresses [31]. Accordingly, activities of various antioxidant genes and enzymes were increased after UV-B treatment [32,33].

In the present study, the multiple effects of UV-B and plants' response against it were studied in green pepper, a major produce in Korea. We first attempted to evaluate the deteriorative effect of realistic UV-B dosage encountered often in Korea, on photosynthesis. Next the lesion sites were identified and assessed by indices of Chl fluorescence parameters. Finally, the physiological protective responses in terms of pigment composition and regulation of antioxidant enzymes were investigated.

MATERIALS AND METHODS

Plant material. Green pepper plants (*Capsicum annuum* L. cv. Saemaeul Kumjang #3) were grown in pots filled with soil (Bioplug #2, Hungnong Seeds Co, Ltd., Korea) for 4 to 5 weeks in a growth chamber maintained at 25 ± 1°C under the photoperiod of 16 h-light and 8 h-dark. Light was provided by True-lite II fluorescent lamps (Durotest, USA) at the intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Randomly selected leaves of similar size were sampled for subsequent UV-B treatment.

UV-B treatment. UV-B, at the intensity of 1 $W \cdot m^{-2}$ was directly cast on the detached leaves floating on DW at RT. The intensity of UV-B was adjusted to be slightly higher than the daily maximal dosage (0.9 $W \cdot m^{-2}$) in urban area of Seoul in May. UV-B radiation was provided by the additional attachment of a UV-B lamp (XX-15B, Spectronics Corporation, USA), covered with cellulose acetate membrane to eliminate UV below 280 nm, to the fluorescent lamps in the growth chamber. UV-B intensity was determined by Digital Radiometer (DRC-100X, Spectronics Corporation, USA).

Measurement of O_2 evolution and Chl fluorescence. Chl fluorescence and O_2 evolution were measured simultaneously using leaf discs of 3.5 cm-diameter, taken from leaves of randomly selected plants, in Hansatech (Kings Lynn, UK) LD2 leaf disc chamber. After UV-B treatment, leaves were dark-adapted for 30 min in a floating state on DW. Each leaf disc was chosen to have approximately equal Chl content, when measured by Minolta Chl meter (SPAD-502, Minolta, Japan) and normalized to have equal Chl content. The measurement of Chl content by SPAD-502 was reliably matched with that of conventional extraction method using acetone. Chl fluorescence was measured using Walz PAM Chl fluorometer (Effeltrich, Germany) while O_2 evolution was polarographically measured with a Clark type electrode at 25°C using Hansatech O_2 electrode control box. To measure Pmax and quantum yield of O_2 evolution, actinic light was given at three different intensities of 30, 50, and 270 $\mu mol \cdot m^{-2} \cdot s^{-1}$ using Schott illuminator (Schott Glass, Stafford, UK). *Fv/Fm*, NPQ (defined as *Fm/Fm'*-1), and qP were calculated as described before [34]. PS I oxidation and reduction kinetics was measured by monitoring changes in absorbance at 820 nm (ΔA_{820}) using PAM Chl fluorometer connected to 830 nm LED (type ED 800T) as described previously [35].

Lincomycin treatment. Lincomycin was treated by immersing the petiole of each detached leaf in a Eppendorf tube containing 1.2 mM lincomycin solution for 4 h in the growth chamber. UV-B treatment was subsequently done to the lincomycin-imbibed leaves.

Determination of photosynthetic pigments. The photosynthetic pigments were extracted following the method of Hiscox and Israelstham [36]. In 15 mL tube, leaf segments were put with 5 mL of DMSO and incubated for 1 h at 65°C. The extracted solution was diluted twice with the same volume of DMSO and absorbance was read at 470, 645, and 663 nm, respectively. The amount of Chl *a*, Chl *b*, and total carotenoids was calculated according to the formula of Lichtenthaler [37] as given below.

$$\text{Chl } a = 12.25\ A_{663} - 2.79\ A_{645}$$
$$\text{Chl } b = 21.50\ A_{663} - 5.10\ A_{645}$$

$$\text{Total carotenoid} = (1000\ A_{470} - 1.82\ \text{Chl } a - 85.02\ \text{Chl } b) / 198$$

Measurement of UV-B absorbing pigments. The amount of UV-B absorbing flavonoids was estimated according to Krizek *et al.* [38]. Leaf slices were extracted in 15 mL of solution containing 40% (V/V) methanol and 1% (V/V) HCl and boiled for 3 min in a water bath. The extracted solution was left at RT for 24 h and the absorbance values at 270 nm, 300 nm, and 330 nm were monitored. The absorbance values were calculated based on the mg basis of FW.

Activities of antioxidant enzymes. To measure the activities of antioxidant enzymes, crude extract was obtained by grinding 1 g of leaf slices with 2 mL of solution containing 85 mM K-PO_4 buffer (pH 7.8) and 1% (W/V) polyvinylpyrrolidone in a mortar and centrifuging at 18000 g for 25 min. CAT, GR, and POD were

assayed according to Rao *et al.* [32]. CAT activity was determined spectrophotometrically by the decrease of absorbance at 240 nm in a cuvette with 1 mL of reaction mixture containing 100 mM K-PO_4 (pH 7.0), 40 μL of crude extract, and 10 mM H_2O_2, which was added just prior to the initiation of reaction. GR was assayed in 1 mL of reaction mixture containing 100 mM K-PO_4 (pH 7.8), 0.2 mM NADPH, 0.5 mM GSSG (oxidized glutathione), 2 mM EDTA, and 100 μL of crude extract by monitoring decrease of absorbance at 340 nm. The assay was initiated by the addition of NADPH. POD activity was quantified by measuring the rate of guajacol tetramerization shown as the increase in absorbance at 470 nm in a cuvette containing 1 mL of reaction mixture of 100 mM K-PO_4 (pH 6.5), 16 mM guajacol, 10 mM H_2O_2, and 100 μL of crude extract. The reaction was initiated by adding crude extract and followed for 10 min.

APX and DHAR were measured by following the method of Asada [39]. APX was monitored by the decrease in absorbance at 290 nm in 1 mL of reaction mixture composed of 50 mM K-PO_4 (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2, and 5 μL of crude extract. DHAR was assayed in 1 mL of reaction mixture containing 50 mM K-PO_4 (pH 6.5), 0.5 mM dehydroascorbate, 5 mM GSH (reduced glutathione), 0.1 mM EDTA, and 15 μL of crude extract by monitoring increase of absorbance at 256 nm.

RESULTS AND DISCUSSION

General effect of UV-B on leaf morphology

The detached leaves did not show any significant change in appearance after 24 h of UV-B treatment. However, the leaves became more darkened and glossy after a few h of UV-B treatment. Morphological alteration such as thickening of leaves and formation of cuticular waxes were shown to be the primary response to UV-B in terrestrial plants [25]. In green pepper, the leaves also became glossy and dark, possibly due to the accumulation of waxes and some UV-B absorbing pigments.

Effect of UV-B on Pmax and quantum yield of O_2 evolution

To evaluate the extent of deteriorative effect of UV-B on the photosynthetic capacity, Pmax and quantum yield of O_2 evolution were measured after direct treatment of UV-B on the detached leaves. Pmax was determined under saturating PFR (270 $\mu mol \cdot m^{-2} \cdot s^{-1}$) while quantum yield was determined under low PFR (30 and 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). As shown in Fig. 1, Pmax was decreased about 28% after 1 h, 47% after 2h, 49% after 3 h, and 64% after 4 h while quantum yield was decreased about 18%, 42%, 46%, and 59%, respectively. Although decrease in Pmax preceded decrease in quantum yield, both Pmax and quantum yield were linearly decreased in a parallel phase in relation to the increased time of UV-B treatment, demonstrating both maximal photosynthetic activity and efficiency in the photosynthetic apparatus were similarly affected by UV-B. Parallel decrease in Pmax and quantum yield implies that UV-B treatment may not make green pepper more susceptible to photoinhibition. Pmax was decreased more rapidly than quantum yield after UV-B treatment in coleus [40].

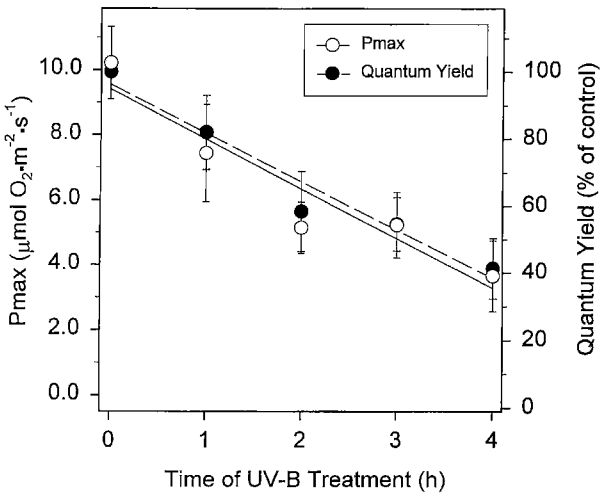

Figure 1. Changes in the Pmax and quantum yield of O_2 evolution in the UV-B treated leaves. Data presented are mean values ± S.E. for 5 measurements.

Changes in Chl fluorescence parameters

The initial fluorescence (*Fo*) and maximal photochemical efficiency (*Fv/Fm*) are general indicators for PS II functionality. Damages to PS II are often accompanied with rise in *Fo* level and drop in *Fv/Fm*. Changes in *Fo* reflect structural alterations at the antenna of PS II while those in *Fv/Fm* indicate varied energy capturing efficiency in PS II [41]. As shown in Fig. 2A, after UV-B treatment *Fo* was increased whereas both *Fm* and *Fv* were decreased rapidly in the initial 2 h. Photochemical efficiency of PS II, represented by *Fv/Fm*, was linearly declined, although to a lesser extent than Pmax, showing 12%, 23%, 29% and 39% reduction after 1, 2, 3 and 4 h of UV-B treatment (Fig. 2B). The linear decrease in *Fv/Fm* was also observed in *Dunaliella* after UV-B treatment [42].

After onset of illumination, the maximal Chl fluorescence declines as photosynthetic reaction proceeds due to the photosynthetic electron flow represented as qP, and nonradiative energy dissipation and ΔpH formation manifested as NPQ [34]. Both qP and NPQ were decreased linearly in a parallel phase by the increased time of UV-B treatment (Fig. 3). Decreased qP indicates that the acceptor side of PS II (Q_A) is overly reduced by hindered electron flow [34]. NPQ usually increases when qP is decreased [44]. Decreased NPQ accompanied with decreased qP may reflect the damage in ATPase by UV-B, which would block DpH formation by increased back pressure. APTase was shown harmed in earlier report [15,19].

Changes in absorbance at 820 nm reflects the oxidation and reduction state of P-700 that reflects electron flow from PS II to PS I and from PS I to $NADP^+$ [43]. After 4 h of UV-B

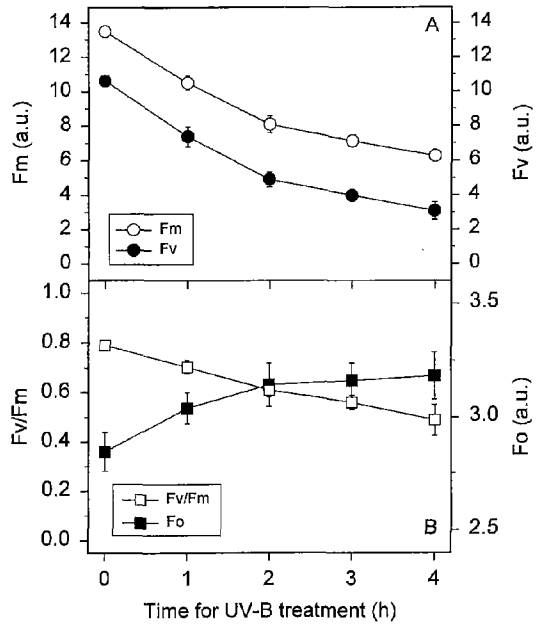

Figure 2. Changes in various Chl fluorescence parameters in the UV-B treated leaves: (A) the maximal (*Fm*) and variable (*Fv*) fluorescence, (B) the initial (*Fo*) and the maximal photochemical efficiency of PSII (*Fv/Fm*). *Fo*, *Fm*, and *Fv* are given in arbitrary units. Data presented are mean values ± S.E. for 5 measurements.

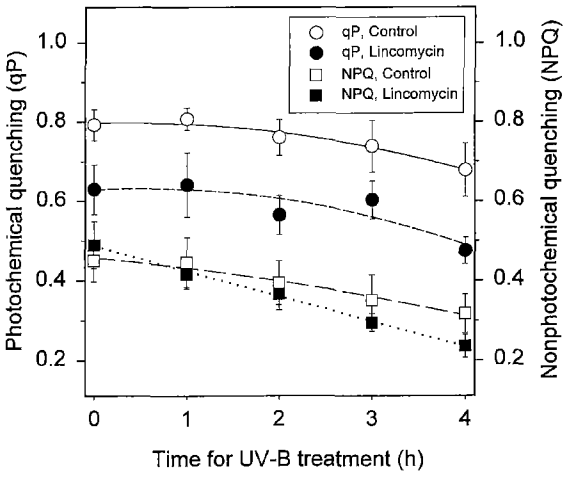

Figure 3. Changes in the photochemical quenching (qP) and nonphotochemical quenching (NPQ) in the control and lincomycin-treated leaves after UV-B treatment. Data presented are mean values ± S.E. for 5 measurements.

treatment absorbance at 820 nm was not changed in P-700 oxidation-reduction kinetics (data not shown). Therefore, PS I appeared to be resistant to UV-B damage as previously reported [15].

Effect of lincomycin

Turnover of D1 protein plays a significant role in overcoming photoinhibition as lincomycin-infiltrated leaves show exacerbating

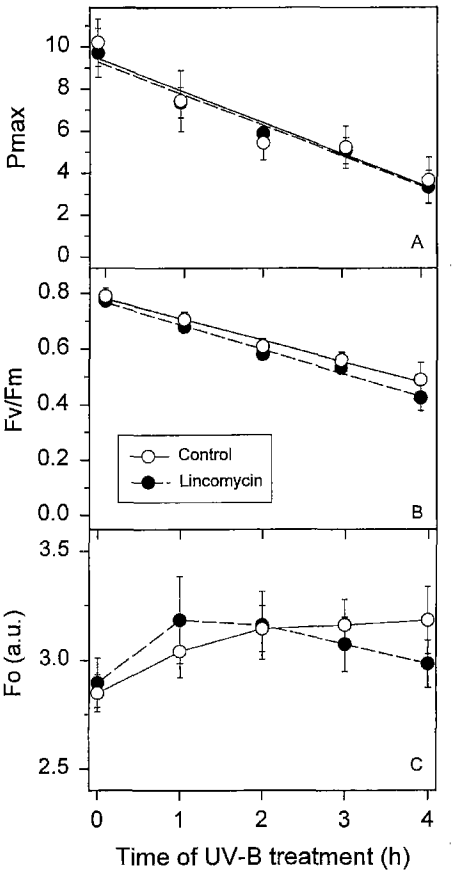

Figure 4. Changes in the Pmax (A), *Fv/Fm* (B), and *Fo* (C) in the control and lincomycin-treated leaves after UV-B treatment. Data presented are mean values ± S.E. for 5 measurements.

effect in photoinhibition. Similarly, UV-B treatment damaged D1 proteins [17]. In view of these, UV-B was treated to the lincomycin-infiltrated leaves to test the role of D1 protein turnover in alleviating the adverse effect of UV-B. As shown in Fig. 4A, decrease in Pmax was identical in control and lincomycin-treated leaves. However, decrease in *Fv/Fm* and increase in *Fo* was accelerated in lincomycin-treated leaves (Fig. 4B and 4C). The decrease in qP and NPQ was also accelerated in lincomycin-treated leaves (Fig. 3). The results implicate that D1 protein turnover would play a role in protection against UV-B.

Using antisensor lines of *Arabidopsis* plants with reduced content in xanthophyll, the effect of UV-B treatment was also tested. Antisensor plants showed more rapid decline in *Fv/Fm*. In addition, decrease in qQ and NPQ occurred faster (data not shown). These results indicate that xanthophyll cycle is also involved in relieving the deteriorative effect of UV-B. Therefore, photoprotective mechanisms appear to be beneficiary for reducing the adverse effect of UV-B.

Effect on the contents of photosynthetic pigments

The adverse effect of UV-B on photosynthesis may result in

Table 1. Changes in the amount of photosynthetic pigments after UV-B treatment.

Time (h)	Pigment amount (mg·g FW^{-1} leaf)		
	Chl *a*	Chl *b*	Carotenoids
0	1.77 ± 0.11	0.27 ± 0.03	0.54 ± 0.04
4	1.78 ± 0.08	0.24 ± 0.02	0.52 ± 0.04
8	1.77 ± 0.09	0.24 ± 0.02	0.49 ± 0.05
12	1.71 ± 0.10	0.24 ± 0.02	0.50 ± 0.04
24	1.32 ± 0.07	0.23 ± 0.01	0.30 ± 0.03

*Data presented are mean values ± S.E. for 5 measurements.

part from the declined photosynthetic pigments as UV-B was shown to degrade Chl. As shown in Table 1, UV-B irradiance had negligible effect on the content of photosynthetic pigments up to 12 h of treatment. Total amounts in Chl *a*, Chl *b*, and carotenoids all remained unchanged. At this time point the photosynthesis presumably stops completely. Thus it appears that initial decline in photosynthesis after UV-B treatment is independent of contents in photosynthetic pigments. However, after 24 h of treatment Chl *a* was declined about 25% and carotenoids were decreased about 40% (Table 1). In contrast, Chl *b* stayed little changed. In *Pisum sativum*, decrease in Chl *a* and *b* accompanied with formation of chlorophyllides *a* and *b* was observed [44]. Decrease in carotenoids was contradictory to the result in *Pisum sativum* in which carotenoid content was increased relative to the Chl content [44]. The increased carotenoids provide photoprotection under high light from photoinhibitory damage. In our experiment, UV-B was supplemented with relatively low light (200 μmol·m^{-2}·s^{-1}) where photoinhibition was presumably lacking. Our results implicate that UV-B *per se* may photooxidize carotenoids to some extent, resulting in their degradation.

Effect on the contents of UV-B absorbing pigments

Plants protect themselves against UV light by accumulating screening pigments. Colorless flavonoids were accumulated in the epidermal tissues of UV-treated plants and reduced damage from UV radiation [27,28]. In view of this, changes in presumptive flavonoid contents after UV-B treatment were investigated by monitoring changes in absorbance values at 270, 300, and 330 nm. As shown in Fig. 5, absorbance values at three wavelengths did not change until 4 h after UV-B treatment. However, on the 8th h, the absorbance values at all wavelengths were roughly 50% increased and stayed on the same level on the 12th h. The induction of flavonoid accumulation seemed to occur far after significant decline of photosynthesis, which implied that flavonoid accumulation would not help protecting the photosynthetic apparatus. Time course of induction was correlated with transcription of enzymes involved in flavonoid synthesis [29]. Transcription of chalcone synthase and 4-coumaroyl-CoA ligase reached to maximal level after 6 h and 12 h of UV-B treatment [45].

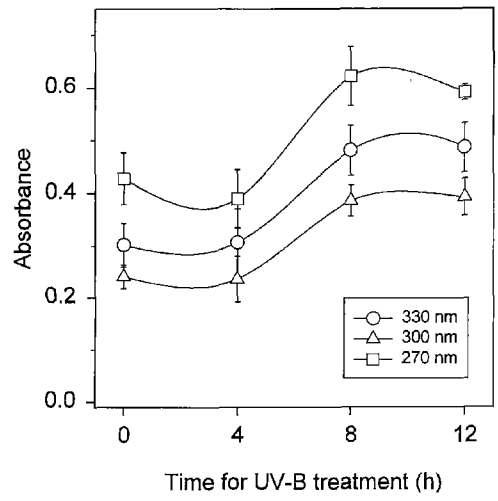

Figure 5. Changes in the contents of UV-B absorbing flavonoids after UV-B treatment. Changes in flavonoid contents were estimated by measuring the absorbance values at 270, 300, and 330 nm of pigment solution extracted in ethanol. Data presented are mean values ± S.E. for 5 measurements.

Effect on the activities of antioxidant enzymes

Under UV-B radiation, high amounts of free radicals are produced, generating, in turn, active oxygenic species such as H_2O_2 although the exact mechanism by which they are generated is unknown. Under such oxidative stresses, plants invoke the antioxidant defense systems [29,31]. One consists of low molecular weight antioxidants such as ascorbate, glutathione, and carotenoids. The other is composed of various antioxidant enzymes that are activated to scavenge the potentially dangerous active species [32,33]. We tested 5 representative antioxidant enzymes, namely APX, CAT, DHAR, GR, and POD under UV-B treatment. Activities of CAT and POD

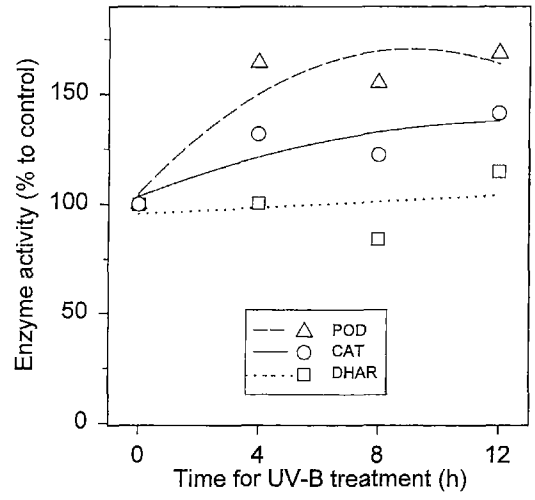

Figure 6. Changes in the activities of various antioxidant enzymes after UV-B treatment: CAT, catalase; DHAR, dehydroascorbate reductase; POD, peroxidase. Data presented are mean values ± S.E. for 3 measurements.

increased with increasing time of UV-B treatment whereas that of DHAR did not change (Fig. 6). POD activity was induced more rapidly and in a higher degree. APX showed a similar tendency as DHAR, but GR did not exhibit any consistent trend (data not shown). The H_2O_2 scavenging system composed of GR, DHAR, and APX (ascorbate-glutathione cycle) did not seem to be a main system to remove active oxygenic species generated by UV-B in green pepper. It was previously shown that enzymes of the ascorbate-glutathione cycle were less affected by UV-B than by ozone [32].

CONCLUSION

Treatment of UV-B radiation, on a dosage often encountered in Korea, to the detached leaves of green pepper led to the rapid reduction in photosynthesis manifested as parallel drop in both Pmax and quantum yield of O_2 evolution. Chl fluorescence parameters were similarly affected to bring in the increase in *Fo*, but decrease in *Fm*, *Fv*, and *Fv/Fm*. Both qP and NPQ were decreased after UV-B treatment. The results would indicate that UV-B primarily affected PS II, but other sites were also affected as shown by decrease in both qP and NPQ. However, PS I remained unaffected. D1 protein turnover and xanthophylls cycle played a role in alleviating the UV-B induced inhibition as in photoinhibition. The Chl *a* and carotenoids were declined far after the decline of the photosynthesis. Flavonoid contents and activities of some antioxidant enzymes were increased, but in a slower pace than reduction in photosynthesis. Our results confirm that earlier reports showing that UV-B was deteriorative on photosynthesis, especially in PS II, and invoked the flavonoid synthesis and activated antioxidant enzymes, are valid in green pepper. The results would also suggest that under UV-B the photosynthetic apparatus is rapidly damaged but the protective mechanisms including synthesis of flavonoids to shield UV and antioxidant enzymes to remove active oxygenic species are set to operate behind.

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