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Peroxidation of lipids and growth inhibition induced by UV-B irradiation

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Abstract. Cotyledons excised from dark-grown seedlings of cucumber (*Cucumis sativus* L.) were cultured *in vitro* under UV radiation at different wavelengths, obtained by passage of light through cut-off filters with different transmittance properties. Growth and the synthesis of chlorophyll (Chl) in cotyledons were inhibited and malondialdehyde was accumulated upon irradiation at wavelengths below 320 nm. Exogenous application of scavengers of free radicals reversed the growth inhibition induced by UV-B. Measurement of the fluorescence of Chl *a* suggested that electron transfer in photosystems was affected by UV-B irradiation. On the basis of these results, the involvement is postulated of active species of oxygen in damages to thylakoid membranes and the growth inhibition that are induced by UV-B irradiation.

Key words: Active oxygen - cucumber cotyledon - *Cucumis sativus* - growth inhibition - lipid peroxidation - photosystem II photochemistry - ultraviolet-B

Abbreviations: Chl: chlorophyll; F_m: maximal fluorescence (dark); F'_m: maximal fluorescence (light); F_v: variable fluorescence (dark); F'_v: variable fluorescence (light); MDA: malondialdehyde; O_2^-: superoxide radical; PS: photosystem; q_N: non-photochemical quenching of fluorescence; q_P: photochemical quenching of fluorescence; $UV\text{-}B_{BE}$: biologically effective UV-B radiation; WL(T=0.5): wavelength at which 50% transmittance occurs.

Introduction

During the past two decades, considerable attention has been paid to the reduction in the stratospheric concentration of ozone that is due to human activities, such as the production and emission of chlorofluorocarbons (Molina and Rowland 1974). Decreases in the stratospheric concentration of ozone cause increases in the amount of UV-B radiation (290-320 nm) that reaches the earth's surface. Exposure to UV-B irradiation causes reductions in the growth and the development of many plant species (Teramura 1983), but the mode of action of UV-B is not clearly understood.

Shibata et al. (1991) reported recently that a water-soluble fraction prepared from cells of the cyanobacterium *Anacystis nidulans* generated superoxide anion radicals upon UV irradiation, and they suggested that the production of active species of oxygen, as a result of UV irradiation, elicited the synthesis of shock proteins. However, in higher plants, involvement of active oxygen in the growth inhibition that is induced by UV-B has not yet been fully clarified.

Recent improvements in techniques for measuring the fluorescence of Chl *a* yielded an important tool for research in basic and applied plant physiology (Krause and Weis 1991). Such techniques have already been applied to the detection and analysis of the effects of stress on plants (Brüggemann 1992, Krupa et al. 1993, Terashima et al. 1991).

In this study, to clarify the relationship between the peroxidation of lipids and the growth inhibition that is induced by UV-B, we examined the accumulation of malondialdehyde (MDA), an indicator of the peroxidation of lipids, in cucumber cotyledons that had been cultured *in vitro* and exposed to UV-B, as well as the effects of scavengers of free radicals on growth inhibition. We also investigated the effects of UV-B on the properties of photosystem (PS) II by non-destructive measurements of fluorescence. We discuss our results in the context of the mechanism for generation of active species of oxygen in UV-B-irradiated cucumber cotyledons.

Materials and Methods

Preparation and culture of cotyledons. Seeds of cucumber (*Cucumis sativus* L. cv. Hokushin) were

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germinated and seedlings were allowed to develop on wet paper towels for 5 days at 25℃ in darkness. The cotyledons were excised and placed on filter paper on the bottom of a 5-cm-wide, stainless-steel Petri dish. Two ml of 20 mM potassium phosphate buffer (pH 6.0) containing 20 mM KCl and 100 μM zeatin were added to each dish as the growth medium (Takeuchi and Amino 1984, Takeuchi et al. 1985). Each dish was covered with a UV-transmitting filter (5 × 5 cm^2; Hoya Co. Ltd., Tokyo, Japan) and sealed with Parafilm™. In this report, each filter is characterized by reference to the wavelength at which 50% transmittance occurred [WL(T=0.5)] (Takeuchi et al. 1993).

Irradiation with UV light. The cotyledons were cultured for 2 days at 20℃ or 25℃ under visible light (photosynthetic photon flux density, 100-120 μmol m^{-2} s^{-1}) and UV radiation. UV light was supplied by one to three fluorescent sunlamps (FL 20SE; Toshiba, Tokyo, Japan), which were suspended 35 cm above the cotyledons. Spectral UV flux densities were measured with a double monochromator spectroradiometer (PGD-25C; Japan Spectroscopic Co. Ltd., Tokyo, Japan). The spectral distributions of UV radiation obtained with the three cut-off filters are shown in Fig. 1. The weighted effective irradiances (biologically effective UV-B radiation, $UV\text{-}B_{BE}$) were integrated between 280 and 400 nm, calculated by reference to the generalized plant spectrum of Caldwell (1971) and normalized at 300 nm. Under two lamps, $UV\text{-}B_{BE}$ after passage through UV-294, UV-307 and UV-325 filters were 1760, 560 and 5 J m^{-2} day^{-1}, respectively.

Determination of levels of Chl and MDA. Cotyledons were homogenized in 80% (v/v) acetone with a glass homogenizer and the homogenate was centrifuged at 2,000×*g* for 10 min. The Chl content of the supernatant was determined from the absorption coefficients of Porra (1989). The amount of MDA in the homogenate was determined by a modified version of the method of Heathand Packer (1968) with 1,1,3,3-tetraethoxypropane as the standard. Two cotyledons were homogenized in 5 ml of 12.5% (w/w) trichloroacetic acid, and then 3 ml of the homogenate were mixed with 5 ml of 20% trichloroacetic acid that contained 0.5% (w/v) 2-thiobarbituric acid and heated at 95℃ for 60 min. After centrifugation at 3,000×*g* for 10 min, the amount of MDA in the supernatant was determined from the difference between the absorbance at 532 and that at 520 nm. The statistical significance of differences was calculated by applying the *t*-test.

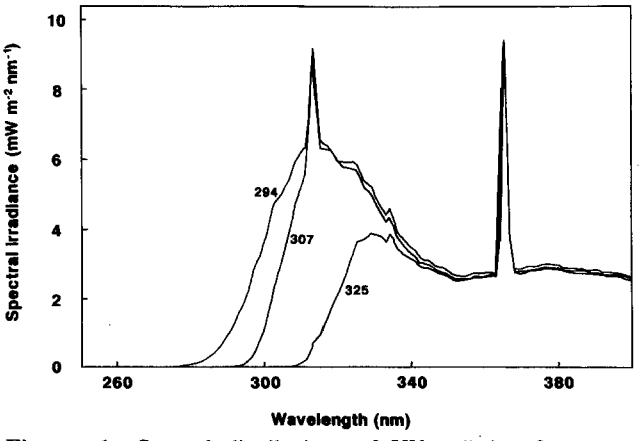

Figure 1. Spectral distributions of UV radiation from two sunlamps after passage through each of the three cut-off filters. Each number in the Figure indicates the wavelength (nm) at which the transmittance of the respective cut-off filter was 0.5 [WL(T=0.5)].

Fluorescence measurements. A cotyledon was positioned on a wet filter paper in a Petri dish and was acclimated to darkness for 15 min. Fluorescence was monitored at 20℃ with a PAM-2000 Chlorophyll Fluorometer (H. Walz, Effeltrich, Germany) that was equipped with 2010-F fiberoptics and DA-2000 data acquisition software (H. Walz). The distance between the fiber and the surface of the cotyledon was 5 mm. The terminology suggested by van Kooten and Snel (1990) was adapted.

Results and Discussion

Effects of UV-B on growth, the synthesis of Chl and the accumulation of MDA in cotyledons

Irradiation with UV-B at wavelengths from 290 to 320 nm inhibited the growth (increases in the fresh weight) of cotyledons and the synthesis of Chl (Table 1). The extent of inhibition increased in each case with increasing intensity of UV-B at 290 - 300 nm at 20℃, but not at higher temperature (25℃). Since UV irradiation at longer than 320 nm (UV-A) had no significant effect on growth or Chl synthesis under the conditions of our experiment (Takeuchi et al. 1993), in the present paper we regarded the cotyledons that were cultured under the UV-325 filter as the control.

Levels of MDA in the etiolated cotyledons and the controls were negligible, being less than 10 nmol $cotyledon^{-1}$. This level was the limit of measurements of levels of MDA in cucumber cotyledons cultured under the conditions of the present study. MDA was formed during irradiation by UV-B and its level increased with increases in the dose of UV-B, especially at wavelengths below 300 nm (Table 1). Less MDA accumulated in the cotyledons cultured at 25℃ than in those cultured at 20℃.

MDA contents have also been reported to be increased by UV-B irradiation in cucumber leaves (Kramer et al. 1991), but not in the leaves of sugar beet (Panagopoulos et al. 1992). Due to the large differences in growth conditions including the intensities of UV-B and visible light among these studies, it is hard to conclude whether these differential responses are species specific or not.

As shown in Fig. 2, the MDA content was correlated

Table 1. Effects of UV-B on growth, Chl content and MDA content of cucumber cotyledons

Time (days)	Temp. (℃)	No. of UV lamps	WL(T=0.5) (nm)	fresh weight (mg cotyledon^{-1})	Chl (μg cotyledon^{-1})	MDA (nmol cotyledon^{-1})
0	—	—	—	27.4 ± 1.6	1.9 ± 0.1	tr [a]
2	20	1	294	47.7 ± 6.1 *	45.0 ± 4.0 *	98 ± 38 *
			307	52.4 ± 7.7 *	72.8 ± 1.1	14 ± 13
			325	58.2 ± 7.4	62.5 ± 4.2	tr
		2	294	51.9 ± 4.8 *	23.4 ± 2.5 *	176 ± 22 *
			307	59.2 ± 6.3 *	48.5 ± 4.4 *	30 ± 8 *
			325	72.0 ± 7.9	75.4 ± 4.6	tr
		3	294	46.5 ± 5.0 *	18.2 ± 3.8 *	495 ± 53 *
			307	61.4 ± 5.8 *	49.8 ± 2.6 *	79 ± 16 *
			325	71.6 ± 9.9	82.9 ± 10.3	tr
	25	2	294	85.9 ± 8.9 *	37.4 ± 3.8 *	116 ± 4 *
			307	120.2 ± 9.6	75.8 ± 7.1 *	tr
			325	110.6 ± 12.6	114.2 ± 22.7	tr
		3	294	66.5 ± 7.4 *	32.7 ± 5.0 *	298 ± 30 *
			307	73.2 ± 11.0	53.4 ± 9.9 *	30 ± 7 *
			325	80.1 ± 13.7	94.5 ± 9.5	tr

Each value is the average of results of 10 cotyledons (fresh weight) and 5 separate samples (for Chl and MDA contents) ± standard deviation. Cotyledons were irradiated with UV-B from 1-3 sunlamps and cultured at 20℃ or 25℃. Asterisks denote statistically significant differences from the control value [WL(T=0.5), 325 nm] at $P<0.01$.
[a] tr, Trace (less than 10 nmol cotyledon^{-1}).

with the inhibition of growth and of the synthesis of Chl, suggesting that the peroxidation of lipids participates in the inhibition of both growth and the synthesis of Chl that is induced by UV-B irradiation. The peroxidation of lipids, which is indicative of damage to cellular membranes and interference with their functions, has been reported to be accelerated in plants that are subjected to various environmental stresses including UV-B (Kramer et al. 1991, Panagopoulos et al. 1990) and sometimes to be accompanied by the bleaching of Chl (Elstner 1982). In many reported cases, peroxidation of lipids appeared to be initiated by active oxygen species (Bowler et al. 1992, Monk et al. 1989). Among the active oxygen species, the superoxide anion (O_2^-) plays a central role in the peroxidation of lipids via the formation

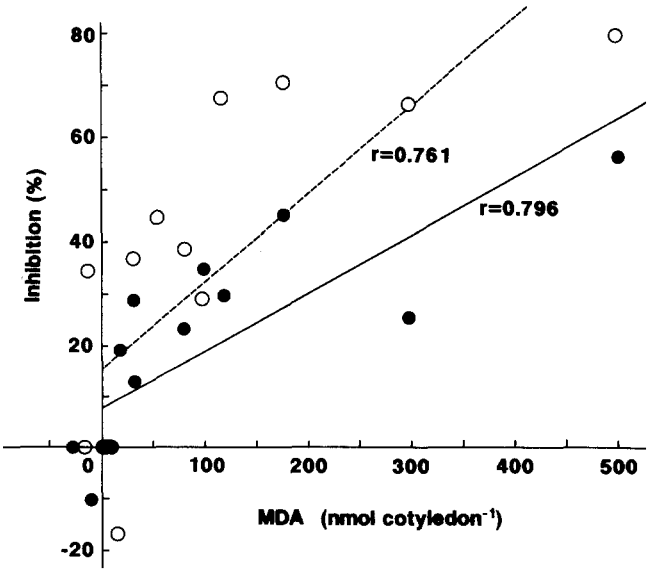

Figure 2. Correlation between the level of MDA and the inhibition of growth (fresh weight increment, solid line, ●) and of the synthesis of Chl (dashed line, ○). Correlation coefficients are indicated in the Figure.

of more active species, such as the hydroxyl radical and singlet oxygen, that react directly with unsaturated fatty acids to generate lipid peroxides.

Effects of scavengers of free radicals on the inhibition of growth of cotyledons

To confirm the possibility that active oxygen species might participate in the inhibition of growth that is induced by UV-B, cucumber cotyledons were cultured on medium that contained scavengers of O_2^- (Sakaki et al. 1983, Shimazaki et al. 1980). Appropriate concentrations of the scavengers were determined from the results of preliminary experiments. Exogenous application of scavengers at the concentrations shown in Table 2 did not affect the growth of control cotyledons [WL(T=0.5), 325 nm], but at higher concentrations it tended to inhibit the growth of the cotyledons (data not shown). Ascorbic acid and tiron (1,2-dihydroxybenzene-3,5-disulfonate) were slightly effective in reversing the inhibition of growth that was induced by irradiation at 300-320 nm. Hydroquinone was more effective; growth inhibition induced by irradiation at 290-300 nm or at 300-320 nm was completely reversed (Table 2). These results confirm the suggestion that active oxygen species or free radicals are involved in the inhibitions of growth and of the synthesis of Chl that are due to UV-B.

Then we attempted to examine the effects of the scavengers on the levels of MDA. However, since the scavengers interfered with the measurement, we failed to obtain the levels of MDA in the cotyledons cultured with the scavengers. More direct evidences for the participation of active oxygen species will be reported in the near future.

Effects of UV-B on the properties of PS II photochemistry

Table 2 Effects of scavengers of free radicals on the inhibition of growth that was induced by UV-B

Scavenger	fresh weight (mg cotyledon^{-1})		
	WL(T=0.5) (nm)		
	294	307	325
no addition	45.2 ± 4.5 * (75.9)	50.1 ± 6.7 * (84.1)	59.5 ±7.5 (100)
Ascorbic acid (50 mM)	44.0 ± 6.4 * (77.4)	55.1 ± 7.3 (97.0)	56.8 ± 9.7 (100)
Tiron[a] (2.5 mM)	45.2 ± 5.5 * (78.6)	51.7 ± 10.9 (89.7)	57.6 ± 5.9 (100)
Hydroquinone (0.25 mM)	60.1 ± 7.7 (92.7)	64.8 ± 13.6 (100)	64.8 ± 6.5 (100)

[a] 1,2-dihydroxybenzene-3,5-disulfonate
Each value is the average of results from 10 cotyledons ± standard deviation. Cotyledons were irradiated with UV-B from two sunlamps and cultured at 20℃ with or without scavengers. Values in parentheses are percentages of the control value [WL(T=0.5), 325 nm] and asterisks denote statistically significant differences from the control value at $P<0.01$.

When photosensitizers absorb UV light, they become excited. They transfer their excess energy to O_2, with the resultant production of active species of oxygen. In microorganisms, components of nucleic acids and related compounds have been reported to be the photosensitizers that absorb UV light (Kramer et al. 1988, Peak et al. 1984) and, in addition, flavins have been proposed to be photosensitizers in *Anacystis nidulans* (Shibata et al. 1991). In higher plants, plastoquinone has been suggested to mediate the degradation of the 32-kDa PS II reaction center protein under UV radiation (Greenberg et al. 1989). However, photosensitizers that correlate to the growth inhibition induced by UV-B irradiation have not been well defined.

Many kinds of environmental stresses are known to induce the generation of active species of oxygen via photosynthetic systems (Elstner 1982) and, although there are large differences in sensitivity among species, UV-B has generally been reported to reduce photosynthetic activities (Brandle et al. 1977, Iwanzik et al. 1983, Renger et al. 1986, Sisson 1986, Takeuchi et al. 1989). Since these results suggest that active oxygen species might be generated in photosynthetic systems that are affected by UV-B, we examined the effects of UV-B on PS II photochemistry by measurements of Chl fluorescence.

Recently, Sullivan and Teramura (1994) reported the small but statistically significant reductions in photochemical efficiency of PS II in the leaves of loblolly pine in response to UV-B irradiation. Furthermore, a similar decline in photochemical efficiency has been reported in pea leaves irradiated with UV-B (Chow et al. 1992). However, in the present study, the photochemical efficiency of PS II, measured as F_v/F_m, did not appear to be affected by UV-B at 20℃ or 25℃ (Table 3). At 20℃, UV-B at wavelengths below 300 nm decreased the photochemical quenching (q_P) and the quantum yield of PS II electon transport, expressed as $q_P \times F'_v/F'_m$ (Genty et al. 1989), but no marked effect was observed at 25℃. These results support the hypothesis that, at least at 20℃, UV-B affects the capacities of plant photosystems.

Values of non-photochemical quenching (q_N), which provides a measure of the energization of thylakoids, were also decreased by UV-B at wavelengths of 290-300 nm and 300-320 nm at 20℃, and at wavelengths of 290-300 nm at 25℃. It seems likely that the peroxidation of lipids might be responsible for the damage to thylakoid membranes, as assessed in terms of q_N. The damages to membranes induced by UV-B irradiation have been reported in the leaves of sugar beet and pea with electron microscopic observations (Bornman et al. 1983, Brandle et al. 1977). However, there is also a possibility that membrane damage, such as the uncoupling of thylakoids that was reported in pea leaves irradiated with UV-B (Chow et al. 1992) and in cucumber leaves exposed to low temperatures (Terashima et al. 1991), might also be caused by UV-B in cucumber cotyledons.

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Table 3. Effects of UV-B on the properties of PS II photochemistry

Temp. (℃)	WL(T=0.5) (nm)	F_v/F_m	q_P	q_N	Yield[a]
20	294	0.758 ± 0.011	0.518 ± 0.090 *	0.290 ± 0.031 *	0.352 ± 0.074
	307	0.763 ± 0.014	0.790 ± 0.017	0.315 ± 0.038	0.543 ± 0.025
	325	0.750 ± 0.022	0.819 ± 0.045	0.428 ± 0.059	0.528 ± 0.062
25	294	0.818 ± 0.004 *	0.769 ± 0.034	0.239 ± 0.032 *	0.583 ± 0.024
	307	0.797 ± 0.005	0.786 ± 0.052	0.310 ± 0.038	0.573 ± 0.048
	325	0.797 ± 0.008	0.774 ± 0.015	0.321 ± 0.005	0.560 ± 0.010

[a] Quantum yield of PS II electon transport expressed as $q_P \times F'_v/F'_m$ (Genty et al. 1989).
Each value is the average of results from 3 separate samples ± standard deviation. Cotyledons were irradiated with UV-B from 2 sunlamps and cultured at 20℃ or 25℃. Asterisks denote statistically significant differences from the control value [WL(T=0.5), 325 nm] at $P<0.05$.

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