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Stimulation of antioxidant enzymes and lipid peroxidation by UV-B irradiation in thylakoid membranes of wheat

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Abstract

In wheat seedlings (*Triticum aestivum* L. cv. 2329) oxidative stress caused by UV-B radiation led to lipid peroxidation of thylakoid membrane; it was expressed in term of malondialdehyde (MDA) formation. The peroxidation of lipids of thylakoid membrane in isolated chloroplasts was prevented when flavonoids quercetin and rutin were supplied into the incubation medium. The activities of superoxide dismutase, ascorbate peroxidase, and catalase increased during the first hours of UV-B exposure. A comparative study of UV-B and temperature effects showed different profiles of the antioxidant enzymes and MDA, suggesting that these two stresses have distinct sites of action. In addition to quantitative increase in flavonoids, qualitative change in flavonoid composition was also marked during UV-B stress, and a new peak at 330 nm was found as compared to control.

Additional key words: ascorbate peroxidase, catalase, flavonoids, superoxide dismutase, *Triticum aestivum*.

Introduction

The ultraviolet-B (UV-B) radiation directly or indirectly cause damage to photosynthetic apparatus. Such injuries were noticed to relatively higher extent in Photosystem 2 (PS2) as compared to PS1 (Brandle *et al.* 1977, Okada *et al.* 1976). UV radiation also cause increase in the content of active oxygen radicals (Polle *et al.*

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Abbreviations: Chl - chlorophyll; H_2O_2 - hydrogen peroxide; MDA - malondialdehyde; PS - photosystem; SOD - superoxide dismutase; TBA - thiobarbituric acid; UV-B - ultraviolet-B.
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1993) like hydroxyl radical, and superoxide radical and hydrogenperoxide in and around the photoharvesting sites. Superoxide originates more frequently close to PS1 and also PS2 while hydroxyl radical and H_2O_2 arise from the stromal side of thylakoid membrane. The oxygen radicals are known as secondary stress (*e.g.*, Scandalios 1993) and are responsible for peroxidation of membrane lipids of chloroplast and mitochondria. Many reports are available on the effect of UV-B radiations on chloroplast and mitochondrial enzymes (Predieri *et al.* 1993).

In continuation with earlier work of authors laboratory (Kumar and Knowles 1993) concerning production of active oxygen species caused by high irradiance along with high temperature, an attempt has been made to understand the effect of spectral composition of radiation acting alone or in combination with high temperature on oxygen radical generation and lipid peroxidation of chloroplast membranes.

Materials and methods

Plant growth: Wheat (*Triticum aestivum* L. cv. HD 2329, IARI, New Delhi) seeds were surface sterilized with 0.1 % (m/v) $HgCl_2$ solution and germinated on paper at 25 °C, 14-h photoperiod and irradiance was 75 W m^{-2}. Seven-day-old seedlings were used for all experiments.

UV-B irradiation: Wheat seedlings were exposed to UV-B (UV-B lamp with filter system *VL-215, Vilber,* Lourmant, France provided most of UV-B radiation below 315 nm; Chauhan and Singhal 1996). The irradiance at the seedling level was 100 μmol m^{-2} s^{-1}. The treatments were carried out at 25 °C. To see the combined effect of temperature and UV-B, the treatment set was irradiated with UV-B at 40 °C The sampling of UV-B irradiated leaves was carried out at 1-h-intervals for 6 to 8 h The leaves were immediately stored in liquid N_2 and used for the assay of different enzymes. Where indicated 100 μM quercetin (3,3',4',5,7-pentahydroxy flavone dihydrate) and 100 μM rutin (quercetin 3,3-D-rutinoside, trihydrate) were added to chloroplasts during incubation.

Assay of enzyme activities: Washed leaves (10 - 12 g) were homogenized in mortar and pestle in 20 cm^3 of ice-cold 50 mM potassium phosphate buffer, pH 7.0, 0.25 % (m/v) *Triton X-100* and 1 % (m/v) polyvinylpyrrolidone phosphate. The homogenate was filtered through 8 layers of cheese cloth and filtrate used for enzyme assays. Activities of all the enzymes were expressed on the basis of chlorophyll (Chl) content as it remained constant after irradiation.

Superoxide dismutase (SOD; E.C. 1.15.1.1.) was assayed at 25 °C following the reduction of cytochrome *c* (change in absorance at 550 nm) according to McCord and Fridovich (1969) with the modification by Schoner and Krause (1990). The reaction was performed in a total volume of 3 cm^3 containing 50 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 18 mM cytochrome *c*, 0.1 mM xanthine and leaf homogenate

equivalent to 20 µg of Chl. One unit of SOD was defined as the amount of enzyme that inhibited the cytochrome *c* reduction by 50 % under the specified conditions.

Ascorbate peroxidase (E.C.1.11.1.7) activity was determined by following the oxidation of ascorbate as a decrease in absorbance at 290 nm (Nakano and Asada 1981). Ascorbate was added to the extraction medium (2 mM) to prevent the inactivation of the enzymes. The assay was carried out at 20 °C in a reaction mixture containing 50 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 0.5 mM sodium ascorbate, 0.1 mM H_2O_2 and leaf homogenate equivalent to 20 µg of Chl. The change in A_{290} was recorded from 10 to 30 s after addition of H_2O_2. Correction was done for the oxidation of ascorbate by H_2O_2 in the absence of leaf homogenate.

Catalase (E.C.1.11.1.6.) was assayed at 25 °C in 3 cm^3 of reaction mixture containing potassium phosphate buffer, pH 7.0, 11 mM H_2O_2 and leaf homogenate equivalent to 20 µg of Chl. Activity was determined by UV spectrophotometer at 240 nm according to Aebi (1983).

Chloroplast isolation: Chloroplasts were isolated from 6 to 8-d-old wheat seedlings (Mishra *et al.* 1991, Mishra and Singhal 1992) at 0 - 4 °C in the dark. Chopped leaves were homogenized in a chilled buffer containing 0.4 M saccharose, 20 mM Hepes, pH 7.6, 15 mM NaCl and 5 mM $MgCl_2$. The slurry was filtered through eight layers of cheese cloth. The filtrate was centrifuged in a refrigerated centrifuge (*Hitachi CR 20B2*, Japan) at 250 *g* for 1 min to remove the cell debris. The supernatant was again centrifuged at 400 *g* for 5 min. The pellet was washed once and resuspended in the same buffer.

Assay of lipid peroxidation: Peroxidation of thylakoid lipids in isolated wheat chloroplasts in the presence of quercetin and rutin was determined spectrophotometrically by monitoring the absorbance of thiobarbituric acid (TBA)-malondialdehyde (MDA) adduct at 532 nm (Mishra and Singhal 1992). An aliquot of 0.5 % TBA in 20 % trichloroacetic acid was added to an equal aliquot of the incubation mixture containing 175 mM NaCl, 50 mM Tris (pH 8.0) and chloroplast equivalent to 15 µg (Chl) cm^{-3}. The solution was incubated at 95 °C for 25 min and then centrifuged for 1 min at 250 *g* in a table top centrifuge (*Remi*, India) to clarify the solution. Absorbance was measured at 532 nm on a *Shimadzu UV-3000* spectrophotometer (Japan) operated in dual wavelength mode with 600 nm as the reference wavelength. The subtraction of absorbance at 600 nm was done for correction of the non-specific increase in turbidity. The amount of MDA formed was calculated using an absorbance coefficient of 155 $mmol^{-1}$ cm^{-1} at pH 8.0.

Photosynthetic pigments were extracted from leaves in 80 % (v/v) aqueous acetone and the content of chlorophyll was determined spectrophotometrically by reading absorbance at 645, 663 and 700 nm as described by Arnon (1949).

Flavonoids: Analysis of tissue extracts for the content of flavonoids was done by descending paper chromatography. The run in a mixture of butanol, acetic acid and water 3:1:1 (v/v/v) in the first direction. The second direction of the run was done with 15 % (v/v) acetic acid. The chamber was equilibriated with the solvent

overnight before starting the run. High pressure liquid chromatography for analysis of flavonoid compounds was done with fluorescence detection and integrator (*Hewlett Packard*). Leaf tissue (100 mg) was ground to a fine suspension in 0.4 cm^3 of 80 % (v/v) methanol in a small ground-glass homogenizer. The extracts were clarified at 14 000 *g* for 3 min. The supernatant (0.02 cm^3) was injected into a C18 reverse phase column (4.00 × 250 mm *Hibar RT cartridge* containing 10-μm diameter *Lichrosorb RP-18*). Samples were eluted at a flow rate of 15 cm^3 min^{-1} with increasing concentrations of HPLC grade acetonitrile. Elution was monitored at 330 nm. Spectrophotometric measurements were done on a *Shimadzu UV 3000* spectrophotometer operated in dual wavelength mode.

Results and discussion

During the first 3 h after UV-B irradiation the activities of SOD, catalase and ascorbate increased (Fig. 1). The activity of SOD after 3 h was 2.4 fold and that of catalase 1.7 fold as compared to at the beginning. Prolonged exposure to UV radiation caused a decrease in SOD and catalase activity. The stimulation in activity of peroxidase was meagre during UV-B radiation. The increase in the activities of SOD and catalase during the first hours of exposure to UV-B radiation was probably induced by an increased generation of active oxygen species. In order to overcome this injury plants have defence mechanisms and produce SOD, catalase and peroxidases (Polle *et al.* 1993).

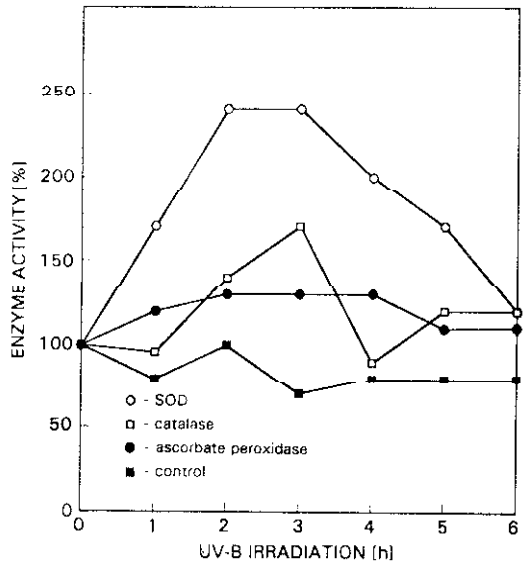

Fig. 1. Changes in SOD, catalase and ascorbate peroxidase activity of wheat leaf during UV-B treatment. Each point represents the mean of three separate sets of experiments. The activities are expressed in percentage of the initial rate. No significant change in activity of these enzymes was observed in controls.

When the wheat plants were exposed to UV-B and temperature stress simultaneously, decrease in SOD activity was noticed (Fig. 2). After 6-h-treatment with UV-B at 40 °C there was no stimulation in the production of SOD activity (heat inactivation probably occurred).

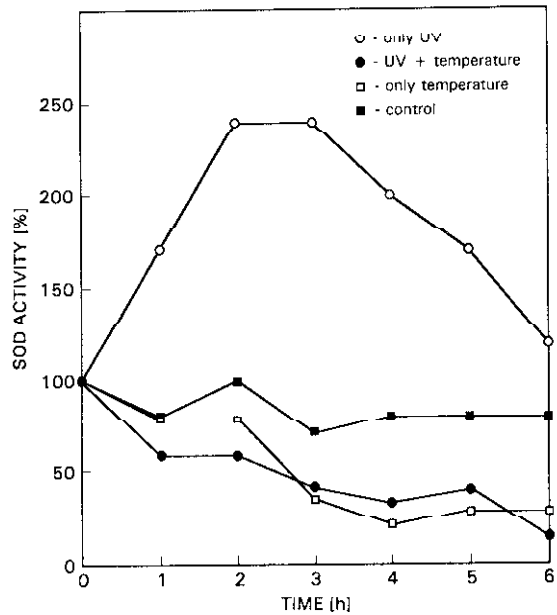

Fig. 2. Changes in SOD activity in wheat leaf under UV-B alone and in combination with high temperature (40 °C). For detail see Fig. 1.

The quantitative changes in MDA formation brought about during UV-B treatment to thylakoid membranes (Fig. 3) is taken as parameter directly linked with lipid peroxidation (Halliwell and Gutteridge 1989). The increase in MDA was about

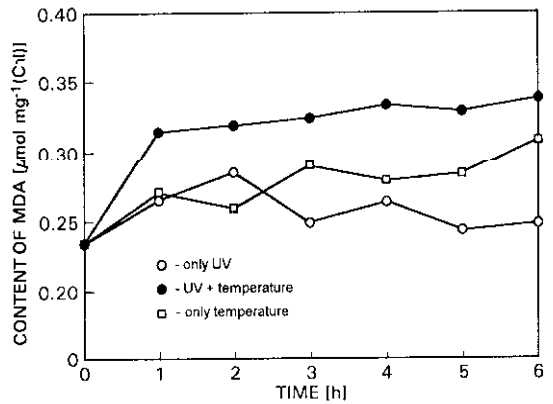

Fig. 3. Peroxidation of lipids of thylakoid membrane measured as change in MDA content *vs* time in wheat leaf under UV-B alone and in combination with high temperature. For detail see Fig. 1.

1.2 fold after first hour of UV-B exposure at 40 °C. But when the sample was exposed to UV-B under normal temperature slight decrease in the MDA level was noticed. High temperature alone also caused an increase in MDA level. However, this increase was smaller as compared to that under combined stress of UV-B and temperature.

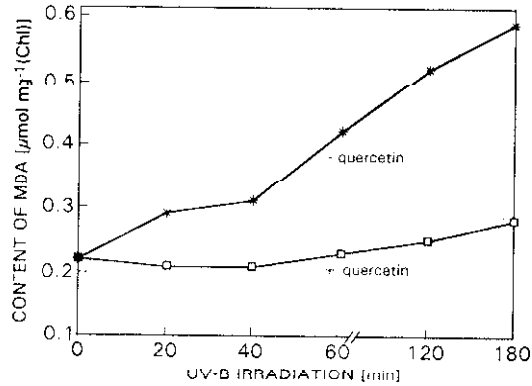

Fig. 4. Peroxidation of lipids of thylakoid membrane in the presence and absence of quercetin.

The reason for immediate suppression of antioxidant enzyme activity during joint treatment of UV-B and temperature is not clearly understood. On the other hand, the continuous increase in the level of MDA (Fig. 3) during our experiment has created certain queries regarding biological connection between rise in lipid peroxidation and reduction in the activity of SOD (Fig. 2).

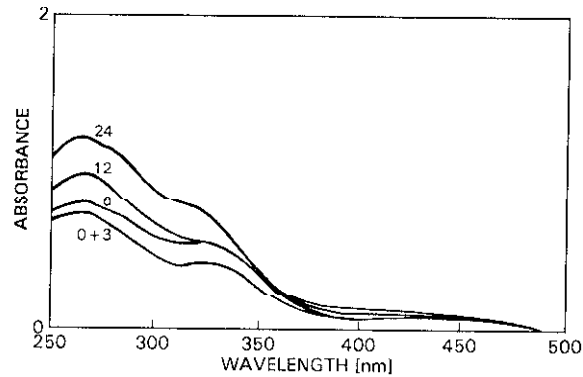

Fig. 5. Absorption spectra of crude methanol extracts (flavonoids) of wheat seedlings during 24-h UV-B treatment. Figure shows the absorbances at 0 h and 3 h (no significant change was observed in absorbance of 3 h UV-B irradiated sample as compared to the 0 h), 6 h, 12 h, and 24 h, respectively.

Addition of quercetin to incubation media caused suppression in MDA as gained during UV-B treatment (Fig. 4). The absorption spectra of methanolic extract obtained from wheat seedlings continuously exposed to UV-B light (Fig. 5) revealed

that an increse in total flavonoid occurred only after prolonged and continuous exposure to UV-B. Methanol extracts of both the control and UV-B treated plants showed peak at 265 nm and shoulder at 325 nm. With the increase of exposure time to UV-B radiation a sharp increase in the band at 265 nm was noticed. In order to confirm the composition of methanol extract obtained from UV-B exposed plant material, HPLC and paper chromatography studies of the same were undertaken. HPLC showed appearance of a new peak at 330 nm (Fig. 6) in after 12-h exposure to UV-B. The paper chromatography data confirmed the flavonoid nature of plant extract, as evidenced by earlier workers (Alston *et al.* 1963).

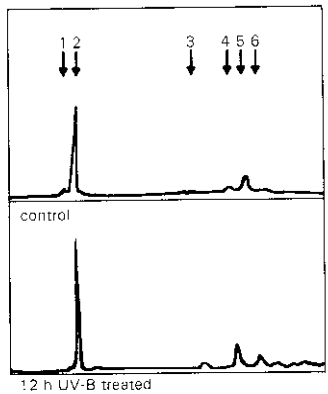

Fig. 6. Separation of flavonoids by HPLC detected at 330 nm. 1 - 6 represent the major flavonoids resolved in wheat seedlings (control plants and plants after 12-h UV-B stress.

Flavonols are widely present in vascular plants. Their localization has been investigated in many plants and glycosides of kaempferol and quercetin have been found to be localized in chloroplasts (Saunders *et al.* 1976). The binding of kaempferol to chloroplast coupling factor has already been confirmed by Cantley and Hammes (1976). In addition, the increase in amount and appearance of new flavonoids after longer exposure to UV-B was reported also (Tevini *et al.* 1991, Santos *et al.* 1993). Even though increased synthesis of secondary metabolites is usual under stress, the specific induction of flavonoid synthesizing enzymes (Li *et al.* 1993) is characteristic for UV-B stress. The increase in the content of flavonoids

Table 1. Inhibition of cytochrome *c* reduction during UV-B treatment of wheat seedlings. Quercetin and rutin were supplemented with the enzyme in the xanthine/xanthine oxidase assay. Control is taken as the reaction medium without enzyme.

Treatment	Cytochrome *c* reduction [%]	Superoxide scavenging activity [%]
Control	100	100
Enzyme	30	70
+ Quercetin	40	60
+ Rutin	46	54

could be responsible for suppressing the activities of antioxidant enzymes after about 3 h of exposure to UV-B. The reduction in the activity of antioxidant enzymes and accumulation of flavonoids starts after 3 h of UV-B treatment. The injury caused by oxygen species might be suppressed by supplying ascorbate, flavonoids or carotenoids exogenously during exposure of plants to various physical stresses (Wagner *et al.* 1988, Young *et al.* 1990). This has been also confirmed in our present investigation while observing quantitative changes in MDA formation in presence/absence of quercetin and rutin (not shown) (Fig. 4). The aforesaid findings support the earlier views related to scavenging role of flavonoids, so as to overcome the injuries caused by superoxide radicals produced *in vitro* by xanthine/xanthine oxidase system (Takahama 1983) (Table 1).

The present investigation suggests that at least two different factors are at work to prevent the oxidative damage under UV-B stress: rise in the activitites of antioxidant enzymes such as SOD, catalase and ascorbate peroxidase in the first hour of exposure and increase in flavonoid level at longer durations of UV-B. Flavonoids were known to protect against lipid peroxidation under photoinhibition (Chauhan *et al.* 1992). Flavonoids were suggested to have active oxygen scavenging properties similar to superoxide dismutase (Takahama 1983). They also stimulate certain peroxidases by acting as substrates (Reign *et al.* 1973). Among the various groups of secondary metabolities flavonoids have the maximum number of the hydroxyl groups that are responsible for the oxygen radical scavenging property (Halliwell and Gutteridge 1985). Flavonoids could prevent oxidative stress either by acting as singlet oxygen scavenger or as hydroxyl radical scavengers.

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