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Function of Photosynthetic Apparatus of Intact Wheat Leaves under High Light and Heat Stress and Its Relationship with Peroxidation of Thylakoid Lipids[1]

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ABSTRACT

Effects of high light and temperature stress on the structure and function of the photosynthetic apparatus of wheat (*Triticum aestivum*) were studied. There was a decrease in the electron transport activity of chloroplasts isolated from photoinhibited and heat-stressed leaves. Chlorophyll fluorescence was measured in photoinhibited and heat-stressed leaves and the decrease in variable fluorescence and variable to maximum fluorescence ratio of the stressed leaves indicated a loss in the quantum yield of photosynthesis. The decrease in electron transport activity was accompanied by an increase in peroxidation of thylakoid lipids. Lipid peroxidation indicated the oxidative degradation of polyunsaturated fatty acyl residues of the thylakoid lipids. A negative correlation was observed between electron transport activity and lipid peroxidation. The electron transport activity was completely lost as the peroxidation level reached a threshold equivalent to 0.6 micromoles malondialdehyde. The threshold of lipid peroxidation for complete loss of activity was the same for both photoinhibition and heat treatment, suggesting that the nature of the environmental stress may be less important with respect to the relationship between electron transport and lipid peroxidation. Thus, it seems likely that lipids are required for sustaining the photosynthetic activity under environmental stress, and a loss in activity is observed as the lipids are degraded either by high light or high temperature stress.

The photosynthetic activity of an organism can be severely reduced after exposure to light intensity in excess of that required to saturate photosynthesis. This phenomenon, referred to as photoinhibition (14, 19, 23), primarily affects PSII (21) and is manifested as a decrease in photosynthetic efficiency. PSII contains a series of redox components and catalyzes electron transport from H_2O to plastoquinone. PSII has long been known to be one of the most susceptible complexes of higher plant chloroplasts (4). Exposure of leaves to temperatures higher than those during growth conditions has also been reported to cause a reduction of PSII activity and a functional separation of the peripheral light harvesting Chl *a*/*b* complex from the PSII complex (26). It has also been shown to result in the release of two of the four manganese atoms of the oxygen-evolving complex (22, 27), denaturation of certain functional proteins (27), and the lateral phase-separation of the nonbilayer-forming galactolipid monogalactosyldiacylglycerol of thylakoid membranes (13).

Although a number of studies have been performed on thylakoid lipids, their exact functional role still remains unclear, probably because lipids, unlike proteins, do not exhibit any catalytic property (17, 24). The lipids are, however, thought to allow the maintenance of appropriate protein conformation that may be required for the optimal chloroplast functioning. In the present paper, we have attempted to characterize the nature and the extent of damage to the photosynthetic electron transport chain in chloroplasts isolated from stressed leaves and correlate them with the peroxidative degradation of the fatty acyl residues of thylakoid lipids.

MATERIALS AND METHODS

Plant Material

Wheat seeds (*Triticum aestivum* var HD-2329, Indian Agricultural Research Institute, New Delhi) were surface-sterilized with 0.1% $HgCl_2$ solution. They were germinated on moist filter paper in dark at 25°C for 48 h. After germination, the seedlings were transferred to a plant growth chamber maintained at 25°C and grown there under 14/10 h light-dark cycles. Light intensity during growth of the seedlings was 75 $W \cdot m^{-2}$. The seedlings used for all the experiments were 8- to 10-d-old. The photoperiod in the growth chamber ended at 7:00 AM and the stress treatments were started at 10:00 AM.

Heat Treatment

Heat treatments to wheat seedlings were given in a plant growth chamber by raising its temperature to desired levels from 25 to 50°C. All the heat treatments were given in the dark. The seedlings were exposed to 35, 40, and 45°C for 6 h, and samples were taken at hourly intervals for isolation of chloroplasts and measurement of electron transport activities. The treatments were given only for 30 min at different temperatures for plotting Chl fluorescence characteristics against temperature.

Photoinhibition

Wheat seedlings grown for 6 to 8 d in the plant growth chamber at 25°C and 75 $W \cdot m^{-2}$ were placed on moist ger-

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mination papers and exposed to white light provided by a 1000 W tungsten lamp. A trough of transparent plexiglass (8 cm depth) with circulating water was placed under the lamp to protect the seedlings from the heat generated by the lamp. Air was circulated around the seedlings with a fan. The temperature in this set-up was maintained at 25 ± 1.5°C. Care was taken to keep the roots moist throughout the treatment. Light intensity was varied by keeping the seedlings at different distances relative to the lamp. Light intensity at the surface of the leaves was measured by a radiometer (Yellow Springs Instrument Co., Yellow Springs, OH). Leaves were photoinhibited at three different light intensities (430, 680, and 850 $W \cdot m^{-2}$) for 6 h. Leaf samples were taken at hourly intervals and used for isolation of chloroplasts that were used for measurement of electron transport activity. For Chl fluorescence recordings, light treatments at different intensities were given for 30 min only.

Chloroplast Isolation

The leaves were cut into pieces with a pair of sharp scissors. The chopped leaves were blended in semi-frozen isolation buffer containing 0.4 M sucrose, 50 mM Hepes (pH 7.6), 15 mM NaCl, and 5 mM $MgCl_2$. The resulting slurry was filtered through eight layers of cheesecloth, and the filtrate was centrifuged in a refrigerated centrifuge (Hitachi model CR20B2, Tokyo, Japan) 250*g* for 1 min to remove the cell debris. The supernatant was again centrifuged at 4000*g* for 5 min. The pellet was washed in the same buffer and resuspended in the isolation buffer. All these steps were carried out in the dark at 0 to 4°C. Chl concentration was determined according to Arnon (3).

Fluorescence Induction Curves

Fluorescence transients of 30 min dark-adapted attached leaves were recorded at room temperature on a Plant Productivity Fluorometer (SF-30, Richard Brancker, Ottawa, Canada) with a timebase of 10 s. The data digitized by an inbuilt A/D converter was transferred to an online IBM-compatible personal computer (PC-XT, Unicomp, New Delhi, India) for further retrieval and analysis. The values of F_m[2] in untreated control leaves were set at 100 and all other readings were normalized accordingly (5).

Electron Transport Activity

Photosynthetic electron transport activity of chloroplasts was measured at 25°C as light-induced reduction of DCIP at 600 nm on a Shimadzu UV-3000 spectrophotometer (Kyoto, Japan) operated in double beam mode. The assay mixture contained 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM Mes, pH 6.5 and chloroplast equivalent to 5 μg $Chl \cdot mL^{-1}$. The activity, excluding water-oxidizing complex, was measured by using 1 mM DPC as electron donor in the assay mixture. The assay mixture was illuminated with saturating white light (50 $W \cdot m^{-2}$) for 30 s and light-induced reduction of the dye was calculated using the molar extinction coefficient of 18 mM^{-1} cm^{-1}.

Assay of Lipid Peroxidation

A number of techniques are available for measuring the peroxidation of membrane lipids (12, 15, 16). Measurement of the MDA is the most widely employed method for determination of lipid peroxidation in biological samples. Two molecules of TBA react stoichiometrically with one molecule of MDA to form the "TBA-MDA adduct," which absorbs at 532 nm in acidic medium. In our experiments, the stress-induced lipid peroxidation was measured spectrophotometrically by determining the amount of MDA forming the adduct with TBA. TBA stock was prepared in 0.20 N HCl and mixed with TCA to give the final concentration of 0.5% TBA and 20% TCA. MDA formation was measured by adding an equal aliquot of this mixture to an aliquot of the incubation mixture containing 175 mM NaCl, 50 mM Tris (pH 8.0), and chloroplasts equivalent to 10 μg $Chl \cdot mL^{-1}$. The solution was incubated at 95°C for 25 min and centrifuged for 1 min at 250*g* in a tabletop centrifuge (Remi, Bombay, India) to clarify the solution by pelleting out the proteins precipitated by TCA. No cloudiness in the supernatant was observed, which could arise due to the presence of carbohydrates and impede the spectrophotometric detection. $A_{532-600\ nm}$ was measured on a Shimadzu UV-3000 spectrophotometer operated in dual wavelength mode using 600 nm as the reference beam. The subtraction of absorbance at 600 nm was done in order to correct for the nonspecific turbidity. The amount of MDA formed was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1} (16).

RESULTS AND DISCUSSION

A decrease in the rate of DCIP photoreduction was observed with an increase in the period of photoinhibition of leaves. The decrease in the electron transport activity was linear at all three light intensities (430, 680, and 850 $W \cdot m^{-2}$) chosen for the investigation (Fig. 1a). This inhibition of electron transport activity has been attributed to photoinhibition damage. There was a small difference in the activities measured in the presence and absence of DPC. DPC is known to donate electrons to the PSII reaction center, bypassing the oxygen-evolving complex. The inhibition of activity determined in the presence of DPC (Fig. 1b) represents the true photoinhibition damage, *i.e.* the damage to the photochemical reaction center of PSII. The difference in activities in the presence and absence of DPC may be explained by damage to the electron transport chain prior to the site of electron donation by DPC, which may not account for photoinhibition damage. However, Wang *et al.* (28) have suggested that photoinhibition in pea PSII particles may produce a lesion on the oxidizing side of PSII. Moreover, there is no agreement in the literature over the site of primary lesion due to photoinhibition and there are several reports of damage at the level of primary charge separation (10), and primary (25) and secondary quinone acceptor in PSII (20). We presume that photoinhibition damage in the photosynthetic apparatus of wheat is not localized

[2] Abbreviations: F_m, maximum fluorescence; F_v, variable fluorescence; MDA, malondialdehyde; DCIP, 2,6-dichlorophenol indophenol; DPC, diphenylcarbazide; TBA, thiobarbituric acid; F_o, initial fluorescence.

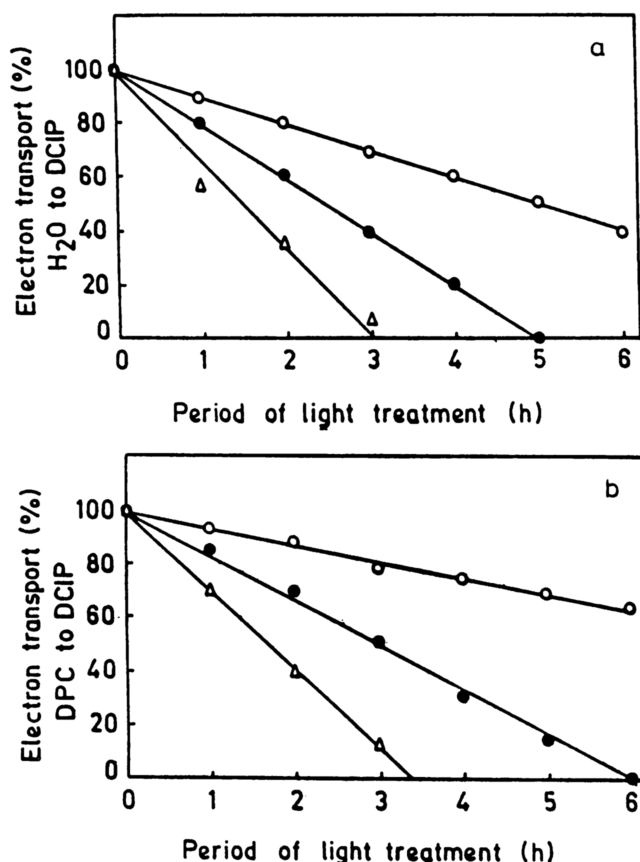

Figure 1. Photosynthetic electron transport activity of chloroplasts isolated from unstressed and photoinhibited leaves. Panels a and b represent activity measured in the absence and presence of exogenously added electron donor (1 mM DPC), respectively. ○, 430 $W \cdot m^{-2}$; ●, 680 $W \cdot m^{-2}$; and △, 850 $W \cdot m^{-2}$. The rates of DCIP photoreduction in chloroplasts isolated from unstressed leaves was 132 ± 2 and 188 ± 4 $\mu mol \cdot mg\ Chl^{-1} \cdot h^{-1}$ measured in the absence and presence of 1 mM DPC, respectively.

only on the donor or the acceptor side of PSII. It seems likely that both sides are damaged by photoinhibition. However, the damage to one side of PSII may be a consequence of the damage to the other side.

Chloroplasts isolated from heat-stressed leaves showed a reduction in photosynthetic electron transport activity. The electron transport activity was inhibited to a lesser extent when 1 mM DPC was used as electron donor (Fig. 2a). The observation that electron transport activity in the presence of DPC was less perturbed as compared with the activity in the absence of DPC (Fig. 2b) supports the suggestion that the oxygen-evolving complex of higher plant chloroplasts is one of the targets for high temperature damage. The inactivation of the oxygen-evolving complex has been suggested to be due to perturbation of the active site by release of two of the four manganese atoms and thermal denaturation of certain proteins (22, 27).

In Vivo Chl Fluorescence

Chl fluorescence has been used as a noninvasive probe of photochemical events taking place in intact leaves under high light and heat stresses. F_v and F_v/F_m ratio have been suggested as quantitative measures of the photochemical efficiency of the PSII complex and the photon yield of oxygen evolution under different environmental stresses (1, 6, 11).

A decrease in F_v/F_m ratio of leaves was observed with an increase in the intensity at which the leaves were illuminated (Fig. 3a). The reduction in F_v/F_m ratio indicates a loss in the efficiency of primary photochemistry of photoinhibited leaves. This reduction was brought about by a high light-induced decrease in the variable Chl fluorescence resulting from a reduction in F_m (Fig. 3b).

The reduction of $P680^+$ has been reported to slow down considerably due to an inactivation of Z, which is identified as tyrosine-161 of the D1 protein (9). The persisting $P680^+$ has been reported to act as a fluorescence quencher (18), which may explain the lowering of F_m observed under photoinhibitory conditions. High light-induced changes in the photosynthetic apparatus have been divided into two categories, reversible and irreversible (2). The reversible change is suggested to result from a destabilization of the semiquinone

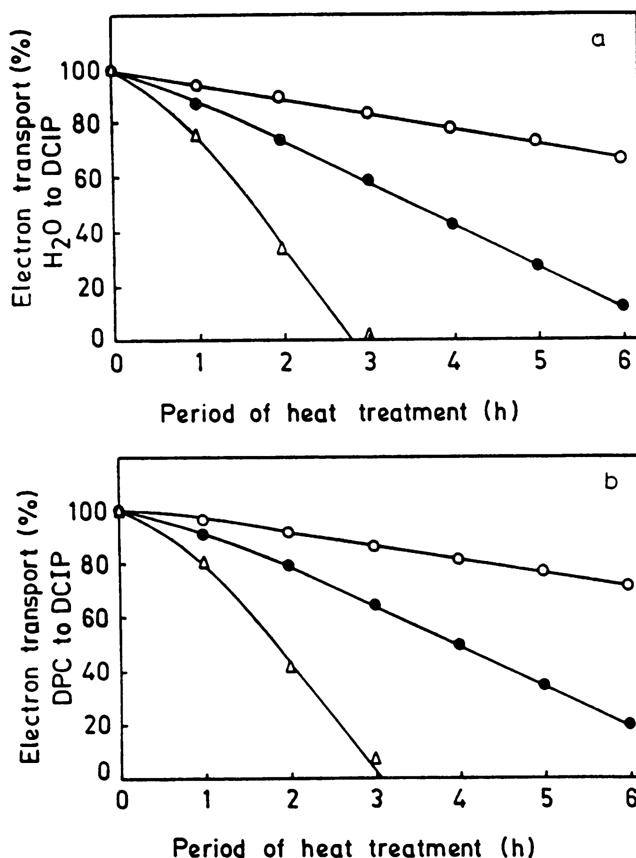

Figure 2. Photosynthetic electron transport activity of chloroplasts isolated from unstressed and heat-stressed leaves. Panels a and b represent activity measured in the absence and presence of exogenously added electron donor (1 mM DPC), respectively. ○, 35°C; ●, 40°C; and △, 50°C. The rates of DCIP photoreduction in chloroplasts isolated from unstressed leaves was 126 ± 4 and 179 ± 12 $\mu mol \cdot mg\ Chl^{-1} \cdot h^{-1}$ measured in the absence and presence of 1 mM DPC, respectively.

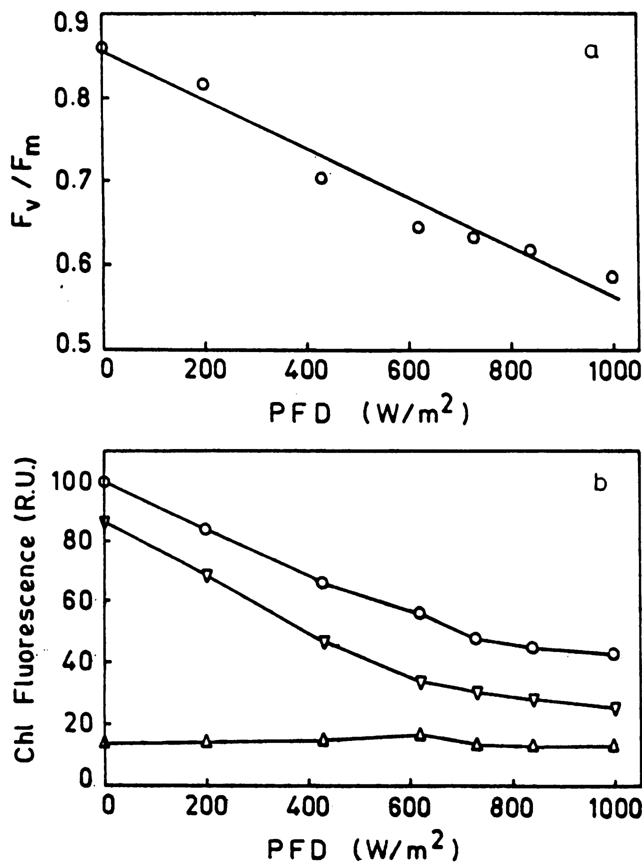

Figure 3. a, F_v/F_m ratio; b, F_m (O), F_o (△), and F_v (▽) of intact wheat leaves after photoinhibition at different light intensities for 30 min each. The leaves were dark-adapted for 30 min before recording the fluorescence induction curves.

(2), causing an increase in the F_o. However, no significant change in F_o was observed after photoinhibition in our experiments, which could be because of recovery of the reversible changes in the period of dark adaptation.

High temperature treatment of wheat leaves also resulted in a decrease in the F_v/F_m ratio. This decrease, however, was slow from 25 to 39°C, beyond which it decreased sharply and the ratio remained only about 15% of the control at 43.5°C (Fig. 4a). The reduction in F_v/F_m ratio was mainly due to a decrease in the variable fluorescence at higher temperatures, which resulted from a decrease in F_m and a gradual increase in F_o (Fig. 4b). Physiologically, the decrease in F_v/F_m ratio and in F_v indicate a reduction in the photochemical efficiency of the PSII complex, which could be due to inefficient energy transfer from the light-harvesting Chl *a/b* complex to the reaction center. However, damage to the reaction center may not be ruled out (7). The decrease in F_m could be due to structural alterations in the PSII complex, causing a decrease in the photochemistry of PSII, an increase in the decay of excitation energy as fluorescence, an increase in the radiationless decay, or the transfer of excitation energy in favor of PSI.

Lipid Peroxidation

The lipid composition of the thylakoid membrane is conserved over a wide range of plant species that might be required for a specific function. The characteristic feature of the thylakoid membranes is the abundance of polyunsaturated fatty acids that are prone to oxidative degradation (12, 15). Lipid peroxidation has been defined as oxidative degradation of polyunsaturated lipids that contain more than two carbon-carbon double covalent bonds (12). Oxygen, which is always abundant in the vicinity of the functioning PSII units of higher plants, could be a powerful mediator of free radical reactions (15, 20) and might be involved in the peroxidative degradation of polyunsaturated fatty acyl residues of the thylakoid lipids.

A linear increase in lipid peroxidation with increasing period of photoinhibition was observed at all three light intensities under study (Fig. 5). It is also evident that the peroxidation process was regulated by the intensity of light treatment as the rate of MDA formation increased with the increase in light intensity. Because lipids do not have catalytic properties like proteins, the peroxidation of chloroplast lipids might not be directly related to the functioning of the photosynthetic apparatus. Peroxidation of thylakoid lipids on pho-

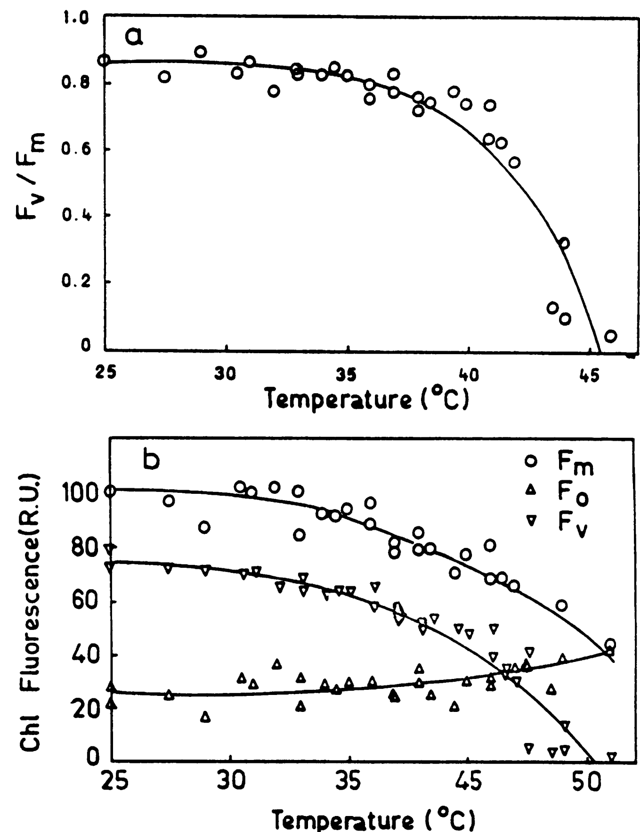

Figure 4. a, F_v/F_m ratio; b, F_m (O), F_o (△), and F_v (▽) of intact wheat leaves after exposure to heat stress at different temperatures for 30 min each. The leaves were dark-adapted at 25°C for 30 min before recording the fluorescence induction curves.

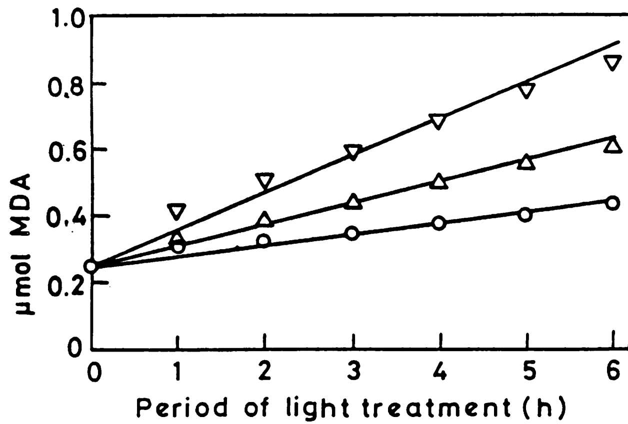

Figure 5. Peroxidation of thylakoid lipids on photoinhibition of wheat leaves at 430 $W \cdot m^{-2}$ (○), 680 $W \cdot m^{-2}$ (△), and 850 $W \cdot m^{-2}$ (▽) at 25°C for different periods of time.

toinhibition of thylakoids and intact chloroplasts of *Codium fragile* has also been reported by Cobb *et al.* (8).

The electron transport activities of chloroplasts were plotted as a function of MDA production to have an understanding of the structure-function relationship of the photosynthetic apparatus under high-light stress (Fig. 6). A negative correlation was observed between electron transport activity and lipid peroxidation, which may suggest a role for lipids in the functioning of the chloroplast. The oxidative degradation of thylakoid lipids also suggests the involvement of active oxygen species in the photoinhibition damage of intact wheat leaves, as has been proposed by several workers (8, 12). Although the functional implication of thylakoid lipid peroxidation is not clearly understood, the degradation of lipids could make the

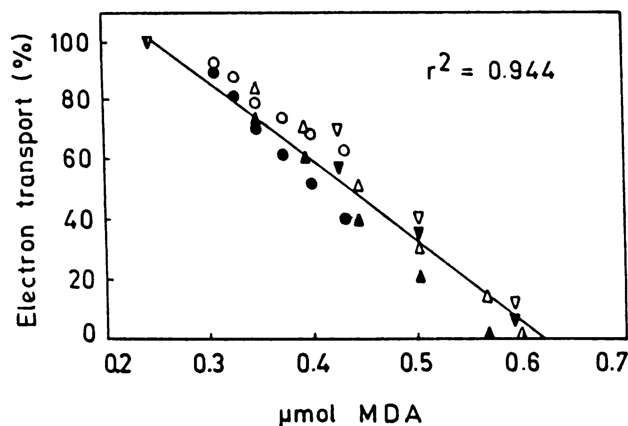

Figure 6. Relationship between photosynthetic electron transport activity and thylakoid lipid peroxidation on exposure of wheat leaves to high-light stress for different periods. Open and filled symbols represent electron transport activity measured in the presence and absence of 1 mM DPC, respectively. Circles, 430 $W \cdot m^{-2}$; triangles, 680 $W \cdot m^{-2}$; and inverse triangles, 850 $W \cdot m^{-2}$ at 25°C; r^2 = correlation coefficient.

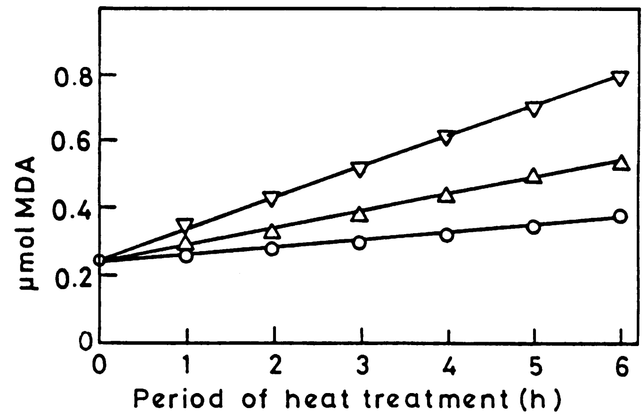

Figure 7. Peroxidation of thylakoid lipids on heat treatment of wheat leaves at 35°C (○), 40°C (△), and 50°C (▽) for 0 to 6 h.

electron transport components more susceptible to photoinhibition damage.

The mechanism of thylakoid lipid peroxidation is not clear, but an abundance of oxygen and its active species, such as superoxide radicals and singlet oxygen, have been reported to be toxic to the thylakoid membrane and may be involved in lipid peroxidation. Chl present in the chloroplasts acts as a photosensitizer and may enhance lipid peroxidation (16) by the formation of singlet oxygen, which is particularly damaging to the membrane lipids. In addition to lipid peroxidation and the formation of reactive aldehydes by peroxide decomposition, there is actual hydrolysis of lipids to release fatty acids, which may cause membrane damage and result in an inhibition of photosynthesis.

An appreciable degree of peroxidation of thylakoid lipids was observed in chloroplasts isolated from wheat leaves heat treated at 35, 40, and 50°C for different periods of time (Fig.

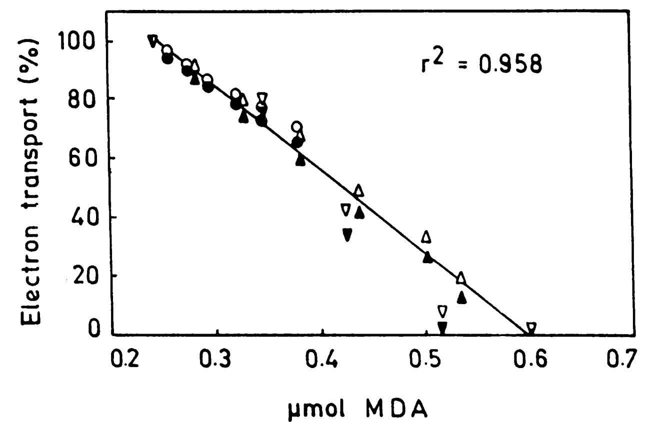

Figure 8. Relationship between photosynthetic electron transport activity and thylakoid lipid peroxidation on exposure of wheat leaves to high temperature stress for different periods. Open and filled symbols represent electron transport activity measured in the presence and absence of 1 mM DPC, respectively. Circles, 35°C; triangles, 40°C; and inverse triangles, 50°C; r^2 = correlation coefficient.

7). The peroxidation of lipids was linear with increasing periods of heat treatment. Peroxidation of thylakoid lipids was also observed to be high at higher temperatures. Furthermore, as in the case of photoinhibition, there was a negative correlation between the peroxidation of thylakoid lipids and the rate of photosynthetic electron transport (Fig. 8). The mechanism of high temperature-induced initiation of peroxidation, however, is not clear. An interesting point to be mentioned here is that the electron transport activity in the case of either heat or light treatment seems to be completely inhibited when the lipid peroxidation reaches the level of 0.6 μmol MDA (Figs. 6 and 8). Thus, it seems likely that there is a threshold of lipid peroxidation, and restrictions on photosynthetic electron transport may be imposed if peroxidation of thylakoid lipids exceeds this threshold.

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