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Lipid Peroxidation Is an Early Event in Necrosis of Wheat Hybrid

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We previously reported enhanced superoxide anion generation in an F1 necrotic hybrid produced from normal parents (Khanna-Chopra *et al., Biochem. Biophys. Res. Commun.* (1998) 248, 712–715). Further investigation of the mechanism of necrosis shows the possibility of lipid peroxidation as an early event in the death of necrotic leaves. Lipid peroxidation resulting from the inability of free radical scavenging is often associated with cell death. In this study the accumulation of malondialdehyde, an end product of lipid peroxidation, was measured in hybrid leaves and those of the parents. Lipid peroxidation was higher in the hybrid leaves through out the leaf ontogeny. This was accompanied by increased membrane permeability. Cell viability measured by a TTC reduction test showed a significant correlation with conductivity. There was no apparent effect on photosynthetic pigments and maximum efficiency of PSII (Fv/Fm) until the appearance of necrotic lesions on the hybrid leaf. There seems to be a close relationship among lipid peroxidation, membrane permeability, and cell viability in the leaves undergoing necrosis. This suggests the possibility of a genetic mechanism whereby the scavenging of free radical is impaired, leading to enhanced lipid peroxidation and membrane permeability, resulting in necrosis and death of the hybrid leaves in wheat.

Key Words: **active oxygen species; necrosis; lipid peroxidation; TTC; *T. aestivum;* wheat.**

Lipid peroxidation is an integral feature of membrane deterioration leading to cell death. This is evident from studies on cellular membranes that the physiochemical properties of membranes are deleteriously altered during senescence (1). These changes have been attributed to peroxidation of lipids. Involvement of membrane lipid peroxidation has also been demonstrated in the development of tissue necrosis in hypersensitive response (2). Moreover lower level of damage to membranes has been related with tolerance to drought (3).

The role of active oxygen species has been implicated in inducing peroxidation of membrane lipids during natural as well as stress induced cell death in plants (4, 5). Increased level of superoxide anion has been reported in the leaves of necrotic wheat hybrid (6). Active oxygen species can damage various macromolecules and cellular components. Unsaturated fatty acids are especially prone to attack by these species. Superoxide being a nucleophile, is more reactive in hydrophobic environment such as interior of lipid bilayer (7). Superoxide can induce lipid peroxidation leading to loss of membrane integrity and finally to development of tissue necrosis (8). Therefore in the present study, relationship between lipid peroxidation, membrane integrity and cell viability have been studied. Since loss of chlorophyll is a symptom of visible necrosis, changes in photosynthetic pigments and maximum efficiency of PSII were also examined.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* L.) cv. C306 was crossed with cv. WL711. The F1 seeds of WL711 × C306 along with its parents, were sown in the field of Water Technology Center, Indian Agriculture Research Institute, India, on 15th November 1998. The plants were allowed to grow under natural field conditions and recommended agronomic practices were followed. For all the measurements, fourth leaf from the base of the plant from hybrid and its parents were sampled on 4, 8, 10, 12, 14 and 16 days after leaf emergence. The 10th day represents full expansion stage in both hybrids and parents. On the visual scale, F1 hybrid leaves were 25% necrotic on the 12th day, which progressed to 50 and 75% necrosis on the 14th and 16th days after leaf emergence.

Lipid peroxidation was determined according to Heath and Packer (9). Leaf samples were homogenized in 0.1% TCA and the extract was centrifuged at 10,000g for 5 min. The supernatant was used for further analysis. An extinction coefficient of 155 mM^{-1} cm^{-1} was used for calculation of MDA content.

The cell viability was measured by two tests, the TTC reduction test and conductivity test. TTC reduction test was done according to Chen *et al.* (10). Leaves of F1 hybrid at 10, 12, 14 and 16 days after emergence were taken for TTC reduction test. Leaves were cut into

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Abbreviations: MDA, malondialdehyde; PSII, photosystem II; TTC, 2,3,5-triphenyl tetrazolium chloride; TCA, trichloro acetic acid.

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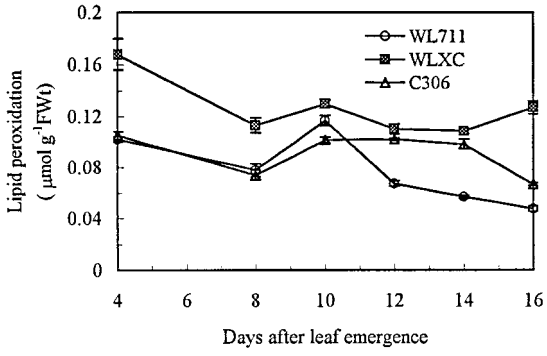

FIG. 1. Changes in lipid peroxidation measured as MDA content in the F1 hybrid and its parents after 4 days of leaf emergence. Error bars indicate SE (n = 3).

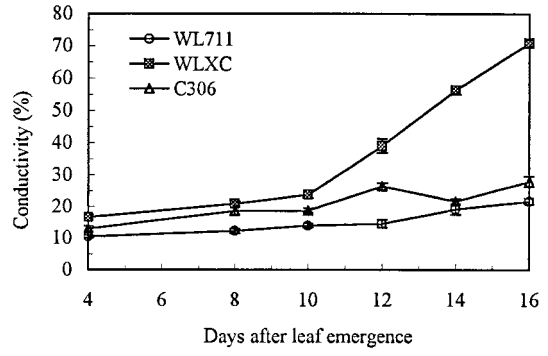

FIG. 2. Changes in conductivity in the F1 hybrid and its parents after 4 days of leaf emergence. Error bars indicate SE (n = 4).

8 mm pieces and transferred to 0.08% TTC in phosphate buffer (pH 7.5). After vacuum infiltration, these were incubated for 18–20 h in the dark. The leaf pieces were removed and rinsed with distilled water and were placed in 95% ethanol followed by evaporation of ethanol in a boiling water bath. The leaf pieces were cooled to room temperature and 95% ethanol was added. The reduction of TTC was estimated at 485 nm (Perkin Elmer, Lambda 2S, UV/VIS Spectrometer).

For conductivity measurement (11), leaves of F1 hybrid and its parents were cut in to 10 mm pieces. These were washed with three changes of deionized water. Leaf pieces were then put in to vials containing 10 ml of deionized water. Vials were kept at 10°C for 20–24 h and then were brought to 25°C and initial conductance was determined. The contents of the vials were autoclaved at 0.10 MPa for 10 min and cooled to 25°C and final conductance was measured. Following formula was used for calculation:

$$\text{Conductivity (\%)} = [(C1/C2) \times 100]$$

where C1 is initial conductivity and C2 is final conductivity after autoclaving.

Chlorophyll and carotenoids were estimated according to Arnon (12) and Lichtenthaler (13) respectively. Chlorophyll fluorescence was measured in the tip, mid and base of intact dark-adapted leaves with a portable fluorometer (MINI-PAM, Walz, Effeltrich, Germany). The fluorometer was connected with a fibreoptic, provided with the instrument. The ratio of Fv/Fm, termed as maximum photosynthetic efficiency was calculated according to Genty *et al.* (14).

RESULTS

The appearance of necrotic symptoms on the apical portion of the hybrid leaf was associated with dehydration and consequently shrinkage of the affected area. Lipid peroxidation was higher in the hybrids, at all stages with a significant increase preceding death while parents showed a decreasing trend after expansion (Fig. 1). Conductivity was higher in the hybrid leaf since the time of emergence and increased very significantly after the onset of necrosis (Fig. 2). There was a decrease in the TTC reduction with a simultaneous increase in conductivity of the hybrid leaves as the necrosis advanced. Conductivity showed a significant correlation with cell viability as measured by TTC reduction (Fig. 3).

The hybrid leaves exhibited a significant decrease in chlorophyll and carotenoid content beyond 25% necrosis stage. In case of parents, chlorophyll content increased during leaf expansion and beyond, while the carotenoid content remained stable at all stages (Fig. 4). The maximum efficiency of PSII photochemistry (Fv/Fm) was measured in the tip, mid and base of the hybrid and its parent after dark adaptation. Fv/Fm in the hybrid was similar to that of parents up to full expansion. Significant changes were observed in hybrid, only after the onset of necrosis. On 12th day, the tip of the hybrid leaves showed both, loss of photosynthetic pigments and decrease in maximum photosynthetic efficiency, yet mid and base portion of the leaf did not show any change in the above parameters (Table 1).

DISCUSSION

Lipid peroxidation was higher in hybrid leaves since the beginning of the leaf development and increased subsequently with the progress of necrosis. Increased lipid peroxidation is known to occur during senescence

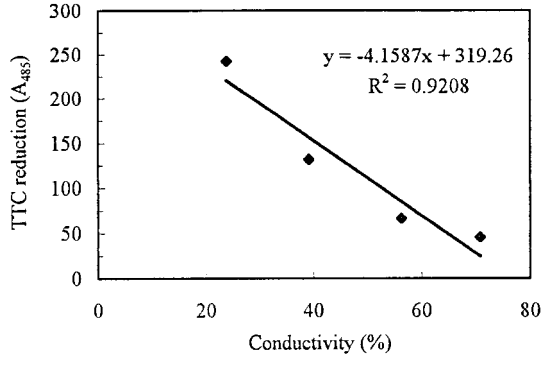

FIG. 3. Correlation between conductivity and TTC reduction by the hybrid cells at different stages after 10 days of leaf emergence.

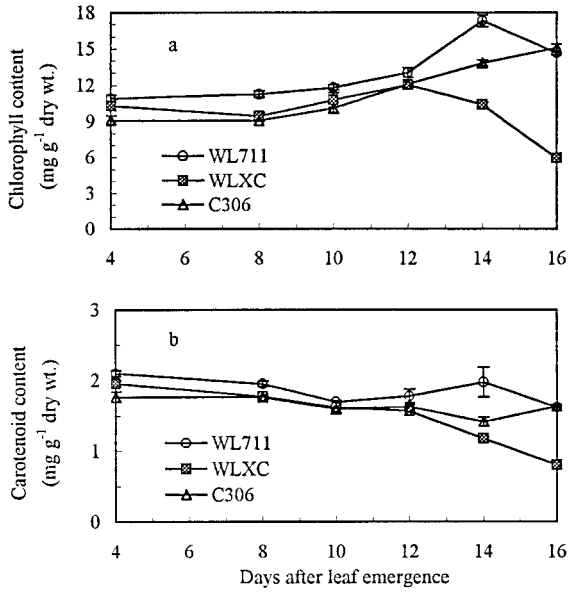

FIG. 4. Changes in the total chlorophyll (a) and carotenoid content (b) in the F1 hybrid and its parents after four days of leaf emergence. Error bars indicate SE (n = 3).

(1). Similarly lipid peroxidation is found to be a characteristic symptom of pathogen or ozone induced necrosis (2, 15) and could also be associated with apoptosis (16). Higher lipid peroxidation has been correlated with membrane permeability and electrolyte leakage (17). In the present study also, hybrid leaves exhibited a significant increase in conductivity, even before the appearance of necrosis lesions on the leaf (Fig. 2). However accumulation of malondialdehyde which is an end product of lipid peroxidation, slightly preceded the increase in electrolyte leakage (18, Fig. 1, 2). Whereas conductivity test showed increased membrane permeability and electrolyte leakage, TTC test measured the capability of plant tissue to carry out electron transport (19) and presumably, the inhibition of TTC reduction also indicated enzyme inactivation (10). As necrosis progressed, there was increase in conductivity and decrease in TTC reduction. This shows that with the increase in membrane damage, there was loss of cell viability in the hybrid leaves. Thus higher rate of lipid peroxidation in hybrids may have altered membrane permeability resulting in solute leakage, dehydration and ultimately loss of physiological functions of the cells.

The loss in chlorophyll was apparent in the necrotic portion of the hybrid leaves yet the chlorophyll and carotenoid content showed a significant decrease only at 50% necrosis stage in the hybrids (Fig. 4). It is known that pigments bound to the thylakoids are stable while free pigments are labile and sensitive to oxidative degradation (20). The process of membrane damage and disintegration might have resulted in loosening of membrane bound pigments leading to massive destruction of pigments at advanced stages of necrosis. Likewise, PSII activity, measured as Fv/Fm, seemed to be affected only after the visible appearance of necrosis (Table 1). Similar results have been observed in wheat during natural and ozone induced senescence (21, 22). In our study, the decrease in fluorescence was either simultaneous or followed by decrease in photosynthetic pigments (Fig. 4, Table 1).

There are increasing number of evidences, implicating free radical mediated lipid peroxidation and membrane damage as the probable cause of aging or senescence (1, 23). Association of active oxygen species with membrane lipid peroxidation have been demonstrated in hypersensitive response and also in stress induced responses (2, 3, 8). Higher level of superoxide anion generation has been observed in this necrotic hybrid (6). The superoxide anion appeared to play a vital role in the necrosis of leaves in the hybrid, as the gradient in superoxide anion from leaf tip to the base was parallel with the progression of necrosis. It suggests that increased superoxide radical might have induced peroxidation of membrane lipids further leading to more generation of free radicals (7). These reactions, being autocatalytic and nonreversible in nature, continued and caused membrane damage, thus disturbing the

TABLE 1

Maximum Photochemical Efficiency of PSII (Fv/Fm) in the Tip, Mid, and Base Portions of the Dark Adapted Leaves of F1 Hybrid and Its Parents

Days after leaf emergence	WL711			WL711 × C306			C306		
	Base	Mid	Tip	Base	Mid	Tip	Base	Mid	Tip
8	—	0.76 (±0.01)	0.79 (±0.01)	—	0.76 (±0.01)	0.80 (±0.01)	—	0.76 (±0.01)	0.80 (±0.01)
10	0.76 (±0.01)	0.78 (±0.01)	0.78 (±0.01)	0.62 (±0.01)	0.80 (±0.00)	0.77 (±0.01)	0.78 (±0.01)	0.80 (±0.01)	0.82 (±0.00)
12	0.80 (±0.01)	0.82 (±0.01)	0.81 (±0.01)	0.77 (±0.02)	0.80 (±0.00)	0.32 (±0.10)	0.79 (±0.01)	0.80 (±0.01)	0.80 (±0.01)
14	0.83 (±0.01)	0.82 (±0.01)	0.77 (±0.00)	0.78 (±0.02)	0.57 (±0.04)	0.02 (±0.02)	0.80 (±0.01)	0.81 (±0.01)	0.82 (±0.00)
16	0.83 (±0.01)	0.75 (±0.01)	0.76 (±0.03)	0.78 (±0.01)	0.00 (±0.01)	0.00 (±0.01)	0.80 (±0.02)	0.80 (±0.01)	0.78 (±0.01)

Note. On the 8th day the leaves were not fully expanded. Hence reading for base portion of the leaf could not be taken. Values are means ± SE (n = 3).

homeostasis required for the normal functioning culminating in the death of the cells. Inspite of higher lipid peroxidation and membrane damage, delayed effect on photosynthetic pigments and PSII, might be due to differential sensitivity of chloroplast membranes and PSII to superoxide radical (24). The source and the cause of this increased superoxide generation in the necrotic hybrids are being investigated.

In conclusion, lipid peroxidation seems to be an early event in necrosis of leaves in hybrid necrosis. Membrane damage as measured by lipid peroxidation and conductivity exhibited a close relationship with cell viability. Thus lipid peroxidation appears to be a common pathway shared by various cell death mechanisms in plants. This also suggests the possibility of a genetic mechanism whereby the scavenging of free radical is impaired, leading to enhance lipid peroxidation and membrane permeability resulting in necrosis and death of the hybrid leaves in wheat.

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