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Effect of oxidative stress caused by hydrogen peroxide on senescence of rice leaves

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Abstract. Lipid peroxidation is considered to be an important mechanism of leaf senescence. The peroxidation of lipids can be initiated by free radicals. H_2O_2 itself is active oxygen species and can react with superoxide radicals to form more reactive hydroxyl radicals in the presence of trace amounts of Fe or Cu. Thus, it is of interest to investigate the relationship between lipid peroxidation and H_2O_2-promoted senescence in detached rice leaves. H_2O_2 effectively promoted senescence and increased malondialdehyde levels in detached rice leaves under both light and dark conditions. However, the promotion of senescence by the exogenous level of H_2O_2 is not associated with an increase in its endogenous level. Thiourea (a scavenger of hydroxyl radical) almost completely prevented H_2O_2-promoted senescence and the H_2O_2-induced decrease in superoxide dismutase and ascorbate peroxidase activities in light and darkness. It seems that promoted senescence by H_2O_2 in detached rice leaves is associated free radical-induced lipid peroxidation.

Keywords: Free radical scavengers; H_2O_2; Leaf senescence; Lipid peroxidation; *Oryza sativa*; Oxidative stress.

Abbreviations: APOD, ascorbate peroxidase; **ASC,** ascorbic acid; **GR,** glutathione reductase; **GSH,** reduced glutathione; **MDA,** malondialdehyde; **SB,** sodium benzoate; **SOD,** superoxide dismutase; **TU,** thiourea.

Introduction

Hydrogen peroxide (H_2O_2) is a constituent of oxidative plant metabolism. It is a product of peroxisomal and chloroplastic oxidative reactions (Del Rio et al., 1992). H_2O_2 itself is an active oxygen species. H_2O_2 can also react with superoxide radicals to form more reactive hydroxyl radicals in the presence of trace amounts of Fe or Cu (Thompson et al., 1987). The hydroxyl radicals initiate self-propagating reactions leading to peroxidation of membrane lipids and destruction of proteins (Asada and Takahashi, 1987; Bowler et al., 1992; Halliwell, 1987). It has been reported that H_2O_2 promotes senescence of detached leaves (Begam and Choudhuri, 1992; Mondal and Choudhuri, 1981; 1982; Parida et al., 1978; Sarkar and Choudhuri, 1981) and induction of senescence is accompanied by an increase in endogenous H_2O_2 level (Mondal and Choudhuri, 1981). Lipid peroxidation is considered to be an important mechanism of leaf senescence (Dhindsa et al., 1981; 1982; Kunnert and Ederer, 1985; Strother, 1988; Thompson et al., 1987). The peroxidation of lipids can be initiated by free radicals (Girotti, 1985; Thompson et al., 1987). Thus, accelerated senescence in leaves could be the result of an H_2O_2-mediated increase in rates of oxidative reactions. We thus examined the relationship between lipid peroxidation and H_2O_2-promoted senescence in detached rice leaves.

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Materials and Methods

Rice (*Oryza sativa* L. cv. Taichung Native 1) was cultured as previously described (Kao, 1980). The apical 3-cm segments excised from the third leaves of 12-day-old seedlings were used. A group of 10 segments was floated in a Petri dish containing 10 mL of test solutions. Incubation was carried out at 27°C in light (40 μmol m^{-2} s^{-1}) or in darkness.

For protein determination, leaf segments were homogenized in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 *g* for 20 min, and the supernatant liquids were used for determination of protein by the method of Bradford (1976). Protein levels were expressed as mg g^{-1} fresh weight. Malondialdehyde (MDA) was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968). MDA level is routinely used as an index of lipid peroxidation and was expressed as nmol g^{-1} fresh weight.

The H_2O_2 level was colorimetrically measured as described by Jena and Choudhuri (1981). H_2O_2 was extracted by homogenizing 50 mg leaf tissue with 3 mL of phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged at 6,000 *g* for 25 min. To determine H_2O_2 level, 3 mL of extracted solution was mixed with 1 mL of 0.1% titanium sulphate in 20% H_2SO_4 (v/v), and the mixture was then centrifuged at 6,000 *g* for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient (0.28 $\mu mol^{-1} cm^{-1}$).

Catalase (CAT) activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu, 1987). The decrease in H_2O_2 was followed as the decline in optical density at 240 nm, and activity was calculated using the extinction coefficient (40 mM^{-1} cm^{-1} at 240 nm) for H_2O_2 (Kato and Shimizu, 1987). Superoxide dismutase (SOD) was determined according to Paoletti et al. (1986). Ascorbate peroxidase (APOD) was determined according to Nakano ans Asada (1981). The decrease in ascorbate concentration was followed as a decline in optical density at 290 nm and activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1} at 290 nm) for ascorbate. Glutathione reductase (GR) was determined by the method of Foster and Hess (1980). Enzyme activity was expressed on the basis of gram fresh weight.

All experiments were performed three times, and within each experiment, treatments were replicated 4 times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

Results and Discussion

The senescence of detached rice leaves was followed by measuring the decrease of protein. Effects of H_2O_2 on the levels of protein, malondialdehyde (MDA), and endogenous H_2O_2, and the activity of catalase of detached rice leaves are shown in Figure 1. H_2O_2 was found to promote protein degradation under both light and dark conditions. The MDA level in H_2O_2-treated detached rice leaves was higher than that in water-treated controls under both light and dark conditions. After incubation in darkness, protein levels in water- and H_2O_2-treated leaves were lower than those in the light. The MDA level in water- and H_2O_2-treated detached rice leaves incubated in darkness was higher than that incubated in light. This shows that H_2O_2-promoted senescence of detached rice leaves is linked to lipid peroxidation. Our results are consistent with those obtained by previous investigators (Mondal and Choudhuri, 1981; Parida et al., 1978; Sarkar and Choudhuri, 1981), who showed that H_2O_2-promoted the senescence of detached leaves.

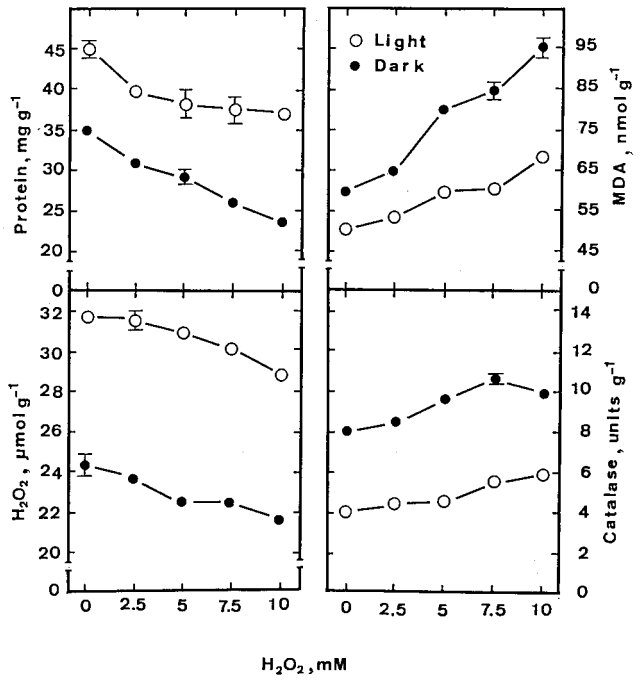

Figure 1. Effects of H_2O_2 on the levels of protein, MDA, and endogenous H_2O_2, and the activity of catalase of detached rice leaves under light and dark conditions. Protein, MDA, and endogenous H_2O_2 levels and catalase activity were determined at 4 d after treatment. Vertical bars represent standard errors (n=4). Only those standard errors larger than symbol size are shown.

The addition of H_2O_2 is expected to result in an accumulation of endogenous H_2O_2 in detached rice leaves. Contrary to our expectation, endogenous H_2O_2 did not accumulate in H_2O_2-treated detached rice leaves (Figure 1). H_2O_2 treatment resulted in a decrease in endogenous H_2O_2 level when compared with water-treated controls.

The fact that the addition of H_2O_2 did not result in an accumulation of endogenous H_2O_2 in detached rice leaves can be explained considering that H_2O_2 is being used in metal-catalyzed reactions such as lipid peroxidation. This suggestion is supported by the observation that light incubation resulted in a higher endogenous H_2O_2 level and lower lipid peroxidation level compared to dark (Figure 1).

Catalase converts H_2O_2 to form oxygen and water. H_2O_2 treatment increased in catalase activity (Figure 1). Endogenous H_2O_2 level was observed to be lower and catalase activity was higher in dark-treated detached rice leaves than in light-treated ones. Thus, the possibility that the increase in catalase activity is associated with the decrease in endogenous H_2O_2 level in H_2O_2-treated detached rice leaves cannot be excluded. The addition of exogenous H_2O_2 has been shown to stimulate the expression of catalase (Prasad et al., 1994). Recent work by Willekens et al. (1997) clearly demonstrated that catalase can effectively remove exgenous H_2O_2.

Since the promotion of senescence of detached rice leaves by exogenous H_2O_2 is not associated with an increase in endogenous H_2O_2, the possibility that a factor other than H_2O_2 *per se* may affect the senescence of detached rice leaves cannot be excluded. It has been shown that H_2O_2 can react with superoxide to form more reactive hydroxyl radicals (Thompson et al., 1987). Since lipid peroxidation is generally considered to be induced by free radicals (Girotti, 1985; Thompson et al., 1987), it is possible that the effects of exogenous H_2O_2 on the senescence of detached rice leaves is mediated through them, especially hydroxyl radicals. If this suggestion is correct, then the promotive effect of exogenous H_2O_2 on the senescence of detached rice leaves should be prevented by the addition of free radical scavengers such as ascorbic acid (ASC, destruction of active oxygen species, particuallary H_2O_2), reduced glutathione (GSH, scavenger of free radicals), and thiourea (TU, scavenger of hydroxyl radicals). This is essentially what we see in Figures 2 and 3. TU (10 mM) almost completely prevents H_2O_2-promoted senescence in

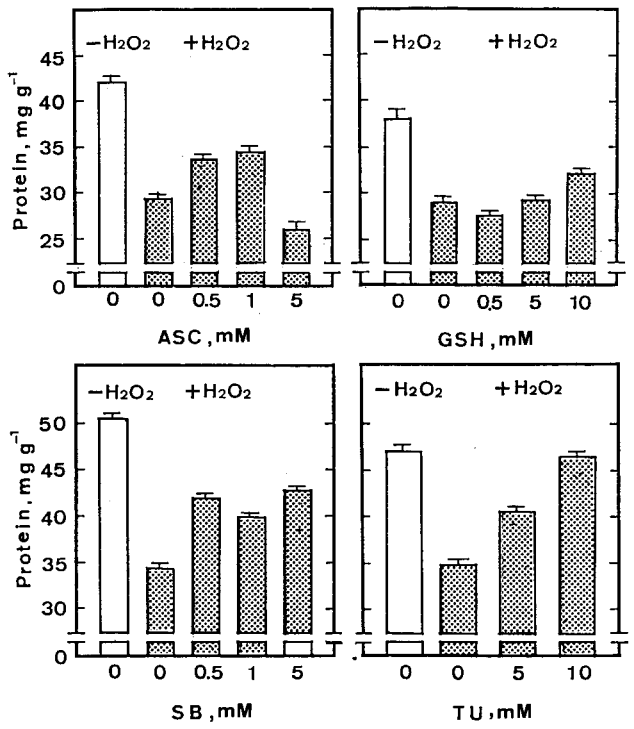

Figure 2. Effect of ascorbic acid (ASC), reduced glutathione (GSH), sodium benzoate (SB), and thiourea (TU) on protein levels of detached rice leaves treated with H_2O_2 (10 mM) in the light. Protein was determined at 4 d after treatment. Vertical bars represent standard errors (n=4). Only those standard errors larger than symbol size are shown.

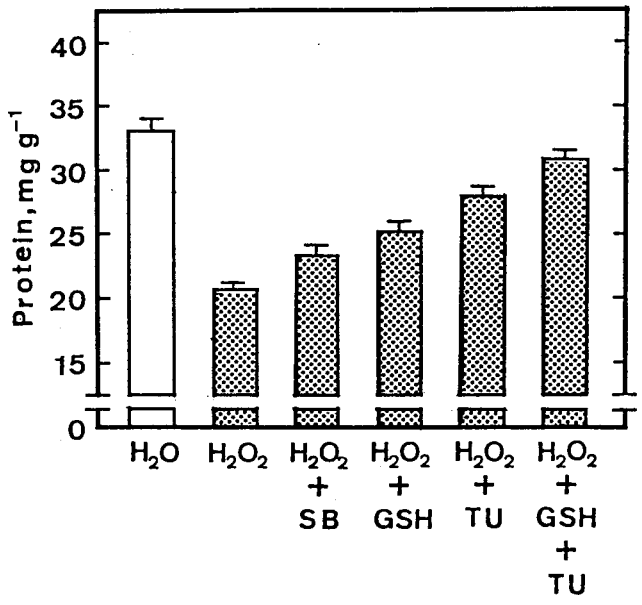

Figure 3. Effect of sodium benzoate (SB), reduced glutathione (GSH), and thiourea (TU) on protein levels of detached rice leaves treated with H_2O_2 (10 mM) in the dark. The concentration of SB, GSH, and TU was 0.5, 0.5 and 10 mM, respectively. Protein was determined at 4 d after treatment. Vertical bars represent standard errors (n=4). Only those standard errors larger than symbol size are shown.

light and darkness, respectively. In darkness, TU and GSH have syngeristic effect on preventing H_2O_2-promoted senescence.

Superoxide dismutase (SOD), ascorbate peroxidase (APOD), and glutathione reductase (GR) have protective roles in scavenging free radicals. Treatment with H_2O_2 decreased SOD and APOD activities in detached rice leaves incubated in light and darkness (Table 1). H_2O_2 had no effect on GR activity in either light- or dark-incubated detached rice leaves. TU was observed to be able to prevent the decrease in SOD and APOD activities by H_2O_2.

In conclusion, the result of the present investigation suggest that H_2O_2-promoted senescence is correlated with free radical-induced lipid peroxidation.

Table 1. Effect of thiourea (TU) on superoxide dismutase (SOD), ascorbate peroxidase (APOD), and glutathione reductase (GR) activities in detached rice leaves treated with H_2O_2.

Treatment[a]	Enzymes[b]		
	SOD	APOD	GR
Light			
H_2O	40.6 ± 1.2	112.6 ± 1.0	8.4 ± 0.6
H_2O_2	24.5 ± 0.6	90.7 ± 0.9	7.3 ± 0.4
H_2O_2 + TU	37.7 ± 1.0	98.2 ± 0.8	7.3 ± 0.6
Dark			
H_2O	27.3 ± 1.6	65.8 ± 0.3	6.5 ± 0.5
H_2O_2	17.3 ± 1.0	58.4 ± 0.7	6.0 ± 0.1
H_2O_2 + TU	22.0 ± 0.9	63.1 ± 0.6	6.5 ± 0.7

[a]The concentration of H_2O_2 and TU was 10 mM.
[b]Enzyme activities were determined after 4 d in light or in darkness.

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Literature Cited

Asada, K. and M. Takahashi. 1987. Production and scavenging of active oxygen in photosynthesis. *In* D. J. Kyle, C. B. Osmond, and C. J. Arntzen (eds.), Photoinhibition, Elsevier, Amsterdam, pp. 227–287.

Begam, H.H. and M.A. Choudhuri. 1992. H_2O_2 metabolism during senescence of two submerged angiosperms *Hydrilla* and *Ottelia*: Changes in enzyme activities in light and darkness. Biochem. Physiol. Pflanzen **188:** 105–115.

Bowler, C., M. Van Montagu, and D. Inze. 1992. Superoxide dismutase and stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43:** 83–116.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.

Del Rio, L.A., L.M. Saudalio, J.M. Palma, P. Bueno, and F.J. Corpas. 1992. Metabolism of oxygen radicals in peroxi-

somes and cellular implications. Free Rad. Biol. Med. **13:** 557–580.

Dhindsa, R.S., P. Plumb-Dhindsa, and T.A. Thorpe. 1981. Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. **32:** 93–101.

Dhindsa, R.S., P. Plumb-Dhindsa, and D.M. Reid. 1982. Leaf senescence and lipid peroxidation: Effects of some phytohormones, and scavengers of free radicals and singlet oxygen. Physiol. Plant. **56:** 453–457.

Foster, J.G. and J.L. Hess. 1980. Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere enriched in oxygen. Plant Physiol. **66:** 482–487.

Girotti, A.W. 1985. Mechanism of lipid peroxidation. J. Free Rad. Biol. Med. **1:** 87–95.

Halliwell, B. 1987. Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. Chem. Phys. Lipids **44:** 327–340.

Heath, R.L. and L. Packer. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. **125:** 189–198.

Jena, S. and M.A. Choudhuri. 1981. Glycolate metabolism of three submerged aqutatic angiosperms during aging. Aquat. Bot. **12:** 345–354.

Kao, C.H. 1980. Senescence of rice leaves IV. Influence of benzyladenine on chlorophyll degradation. Plant Cell Physiol. **21:** 1255–1262.

Kato, M. and S. Shimizu. 1987. Chlorophyll metabolism in higher plants. VII. Chlorophyll degradation in senescing tobacco leaves: phenolic-dependent peroxidative degradation. Can. J. Bot. **65:** 729–735.

Kunnert, K.J. and M. Ederer. 1985. Leaf aging and lipid peroxidation: the role of the antioxidant vitamin C and E. Physiol. Plant. **65:** 85–88.

Mondal, R. and M.A. Choudhuri. 1981. Role of hydrogen peroxide in senescence of excised leaves of rice and maize. Biochem. Physiol. Pflanzen **176:** 700–709.

Mondal, R. and M.A. Choudhuri. 1982. Regulation of excised leaves of some C_3 and C_4 species by endogenous H_2O_2. Biochem. Physiol. Pflanzen **177:** 403–417.

Nakano, Y. and K. Asada. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. **22:** 867–880.

Paoletti, F., D. Aldinucci, A. Mocali, and A. Capparini. 1986. A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. Anal. Biochem. **154:** 536–541.

Parida, R.K., M. Kar, and D. Mishra. 1978. Enhancement of senescence in excised rice leaves by hydrogen peroxide. Can. J. Bot. **56:** 2937–2941.

Prasad, T.K., M. Anderson, B.A. Martin, and C.R. Stewart. 1994. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell **6:** 65–74.

Sarkar, U. and M.A. Choudhuri. 1981. Effect of some oxidants and antioxidants on senescence of isolated leaves of sunflower with special reference to glycolate content, glycolate oxidase, and catalase activities. Can. J. Bot. **59:** 392–396.

Strother, S. 1988. The role of free radicals in leaf senescence. Gerontology **34:** 151–156.

Thompson, J.E., R.L. Legge, and R.F. Barber. 1987. The role of free radicals in senescence and wounding. New Phytol. **105:** 317–344.

Willekens, H., S. Chamnogpol, M. Davey, M. Schraudner, C. Langebartels, M. Von Montagu, D. Inze, and W. Van Camp. 1997. Catalase is a sink for H_2O_2 and is indispensable for stress defence in C_3 Plants. EMBO J. **16:** 4806–4816.