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Nitric oxide counteracts the senescence of rice leaves induced by hydrogen peroxide

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Abstract. In the present study, we evaluate the protective effect of nitric oxide (NO) against the senescence of rice leaves promoted by hydrogen peroxide (H_2O_2). Senescence of rice leaves was determined by decreases in protein content. H_2O_2 treatment resulted in (1) increases in leaf H_2O_2 content, (2) induction of leaf senescence, (3) increases in lipid peroxidation, (4) decreases in ascorbic acid (AsA) and reduced form glutathione (GSH) contents, and (5) increases in antioxidative enzyme activities (ascorbate peroxidase, glutathione reductase, and peroxidase). NO donors [N-*tert*-butyl-α-phenylnitrone (PBN), sodium nitroprusside, 3-morpholinosydonimine, and AsA + $NaNO_2$] were effective in reducing H_2O_2-induced leaf senescence. PBN prevented H_2O_2-increased H_2O_2 content, H_2O_2-induced lipid peroxidation, H_2O_2-decreased AsA and GSH contents, and H_2O_2-increased antioxidative enzyme activities. The protective effect of PBN on H_2O_2-promoted senescence, H_2O_2-increased H_2O_2 content and lipid peroxidation, H_2O_2-decreased AsA and GSH contents, and H_2O_2-increased antioxidative enzyme activities was reversed by 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, a NO-specific scavenger, suggesting that the protective effect of PBN is attributable to the NO released. Reduction of H_2O_2-induced senescence by NO in rice leaves is most likely mediated through its ability to scavenge H_2O_2.

Keywords: Hydrogen peroxide; Lipid peroxidation; Nitric oxide; *Oryza sativa*.

Introduction

Hydrogen peroxide (H_2O_2) is a constituent of oxidative plant metabolism and is itself an active oxygen species (AOS). H_2O_2 can also react with superoxide radicals to form more active hydroxyl radicals in the presence of trace amounts of Fe or Cu (Van Breusegem et al., 2001). The hydroxyl radicals initiate self-propagating reactions leading to peroxidation of membrane lipids and destruction of proteins (Halliwell and Gutteridge, 1989). H_2O_2 has been shown to promote leaf senescence (Parida et al., 1978; Mondal and Choudhuri, 1981; Sarkar and Choudhuri, 1981; Begam and Choudhuri, 1992; Lin and Kao, 1998), and induction of senescence is accompanied by an increase in endogenous H_2O_2 content (Mondal and Choudhuri, 1981). Lipid peroxidation is considered an important mechanism of leaf senescence (Thompson et al., 1987; Strother, 1988). The peroxidation of lipids can be initiated by active oxygen species (Thompson et al., 1987; Halliwell and Gutteridge, 1989). Thus, H_2O_2-induced leaf senescence is mediated, at least in part, through lipid peroxidation.

In mammalian systems, nitric oxide (NO), a bioactive molecule, is produced mainly from L-arginine by NO synthase (NOS). However, in plants neither the protein nor the appropriate gene for NOS activity have been detected (Lamattina et al., 2003). Recent work demonstrates that the mammalian NOS antibodies recognize many NOS-unrelated plant proteins (Butt et al., 2003), suggesting that inferring the presence of plant NOS using the immunological technique may be inappropriate. These results led to speculation that the plant NOS-like enzyme could be structurally different from the mammalian NOS. Recently, a pathogen-inducible plant NOS has been identified as a variant P protein of the mitochondrial glycine decarboxylase complex (Chandok et al., 2003; Wendehenne et al., 2003). Additionally, Guo et al. (2003) reported the presence of an NOS gene (*AtNOS1*) in *Arabidopsis*. AtNOS1 turned out to be a protein very similar to a group of bacterial proteins with putative GTP-binding or GTPase domains. Neither the variant P protein nor the purified AtNOS1 protein had sequence similarities to any mammalian NOS. It is increasingly evident that plant nitrate reductase also catalyzes a NAD(P)H-dependent reduction of nitrite to NO (Yamasaki et al., 1999; Rockel et al., 2002, Sakihama et al., 2002; Lamattina et al., 2003). Evidence is mounting that NO acts as an important messenger in plant physiological processes, including growth, development, and defense responses (Noritake et al., 1996; Gouvea et al., 1997; Dangl, 1998; Beligni and Lamattina, 2000, 2001; Neill et al., 2002; Pagnussat et al., 2002; Jih et al., 2003; Zhao et al., 2004). NO has been shown capable of counteracting the toxicity of paraquat and diquat, which are known to generate superoxide radicals, in potato leaves (Beligni and Lamattina, 1999a,b; 2002) and block H_2O_2 production induced by jasmonic acid in tomato leaves (Orozco-Cárdenas and Ryan, 2002). NO seems to be a potent antioxidant, and its

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ability to directly scavenge AOS may be behind its action (Beligni and Lamattina, 2002).

In rice leaves, we have shown that NO counteracts oxidative stress induced by paraquat, dehydration, and polyethylene glycol (Cheng et al., 2002; Hung et al., 2002). More recently, we have shown that the promotion of rice leaf senescence by abscisic acid and methyl jasmonate, both of which induce H_2O_2 production and lipid peroxidation, can be counteracted by NO donors (Hung and Kao, 2003, 2004). In the present investigation, we examined the effect of NO on the H_2O_2-induced senescence of rice leaves.

Materials and Methods

Plant Material and Chemicals

Rice (*Oryza sativa* L., cv. Taichung Native 1) was sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes with wetted filter paper at 37°C under dark conditions. After a 48-h incubation, uniformly germinated seeds were selected and cultivated in a 500 mL beaker containing half-strength Kimura B solution as described previously (Chu and Lee, 1989). The hydroponically cultivated seedlings were grown for 12 days in a Phytotron (Agriculture Experimental Station, National Taiwan University, Taipei, Taiwan) with natural light 30°C day (12 h)/25°C night (12 h) and 90% relative humidity. The apical 3 cm of the third leaf was used in all experiments. A group of ten segments was floated in a Petri dish containing 10 mL of test solution. Incubation was carried out at 27°C in the dark.

Test solutions included H_2O_2, NO donors, and a NO scavenger [2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, c-PTIO]. N-*tert*-butyl-α-phenylnitrone (PBN), 3-morpholino-sydonimine (SIN-1), and sodium nitroprusside (SNP) were used as NO donors. We also used a solution containing ascorbic acid (AsA) and $NaNO_2$ as another NO donor. All chemicals were purchased from Sigma Co. (St. Louis, MO, USA).

Determinations of Protein, H_2O_2, Lipid peroxidation, GSH, and AsA

Chlorophyll was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein extraction, 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 supernatants were used for determination of protein by the method of Bradford (1976) and antioxidant enzyme activities. The H_2O_2 content was measured colorimetrically as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing leaf tissue with phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. The homogenate was centrifuged at 6,000 *g* for 25 min. To determine H_2O_2 content, extracted solution was mixed with 0.1% (v/v) titanium chloride in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6,000 *g* for 25 min. The absorbance was measured at 410 nm. The H_2O_2 content was calculated using the extinction coefficient 0.28 μmol^{-1} cm^{-1}. Lipid peroxidation in leaf tissue was determined by measuring thiobarbituric acid reactive substances (TBARS). The leaf tissue was extracted with 5% (w/v) trichloroacetic acid and determined according Heath and Packer (1968). GSH in 5% (w/v) sulfosalicylic acid extract and AsA in 5% (w/v) trichloroacetic acid extract were determined as described by Smith (1985) and Laws et al. (1983), respectively.

Enzyme Assays

Peroxidase (POD) activity was measured using a modification of the procedure of MacAdam et al. (1992). Activity was calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm) for tetraguaiacol. 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 absorbance 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 and Asada (1981). The decrease in AsA concentration was followed as the 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 AsA. Glutathione reductase (GR) was determined by the method of Foster and Hess (1980). One unit of activity for CAT, POD, SOD, APOD, and GR was defined as the amount of enzyme which degraded 1 µmol H_2O_2 per min, caused the formation of 1 µmol tetraguaiacol per min, inhibited 50% the rate of NADH oxidation observed in control, degraded 1 µmol of AsA per min, and decreased 1 A_{340} per min, respectively.

Statistical Analysis

Statistical differences between measurements (n = 4) on different treatments or on different times were analyzed following Duncan's multiple range test.

Results and Discussion

The most obvious character of leaf senescence is yellowing. Chlorophyll loss has long been considered the principal criterion of senescence. The protein breakdown that occurs during leaf senescence has been recognized since the earliest studies performed. We have shown that protein breakdown precedes chlorophyll loss during rice leaf senescence (Kao, 1980). Thus, senescence of rice leaves in the present investigation was followed by measuring the decrease of protein. The changes in protein and TBARS content in detached rice leaves treated with 10 mM H_2O_2 in the dark are shown in Figures 1A and 1B. The decease in protein and increase in TBARS were evident at 2 days after H_2O_2 treatment. Clearly, H_2O_2 is effective in promoting the senescence of rice leaves. To be sure that the described effect of H_2O_2 on leaf senescence

was related to an increase in the leaf H_2O_2 content, H_2O_2 concentration was determined in detached rice leaves treated with either water or 10 mM H_2O_2 (Figure 1C). H_2O_2 content in control leaves remained unchanged during 3 days of incubation in the dark. The increase in H_2O_2 content in H_2O_2-treated detached rice leaves was clearly evident at 2 days after treatment.

H_2O_2 treatment resulted in a marked increase in TBARS content, indicating that H_2O_2 brings about lipid peroxidation (Figure 1B). In previous work, when free radical scavengers such as AsA, GSH, sodium benzoate, and thiourea were used together with H_2O_2, it was found that they prevented H_2O_2-promoted senescence and lipid peroxidation (Lin and Kao, 1998). All these results suggest that H_2O_2 treatment caused an oxidative stress.

Plant cells are equipped with several AOS detoxifying enzymes and antioxidants to protect them against oxidative damage. Antioxidant enzymes include SOD, APOD, GR, CAT, and POD (Foyer et al., 1997). AsA and GSH are two main water-soluble antioxidants (Foyer et al., 1997). The striking increase in lipid peroxidation seen in rice leaves treated with H_2O_2 (Figure 1B) may reflect the change in the activities of antioxidative enzymes and in the contents of antioxidants. It was observed that, H_2O_2-treated rice leaves had higher activities of APOD (Figure 2B), GR (Figure 2C), and POD (Figure 2E) than the controls at 2 days after dark incubation. Increased CAT activity by H_2O_2 was observed at 3 days after treatment (Figure 2D). However, H_2O_2 had no effect on SOD activity in rice leaves (Figure 2A). Treatment with H_2O_2 significantly decreased AsA and GSH contents compared with the control leaves, with the increase occurring 2 day after treatment (Figures 3A and 3B). The increased activities of APOD, GR, and POD and the decreased contents of AsA and GSH in rice leaves in response to H_2O_2 further suggest a strong induction of oxidative stress.

Figure 1B and 1C show that no changes in TBARS or H_2O_2 contents were observed in the control leaves. Lipid peroxidation and dark-induced senescence of rice leaves appear to have no direct relationship under dark

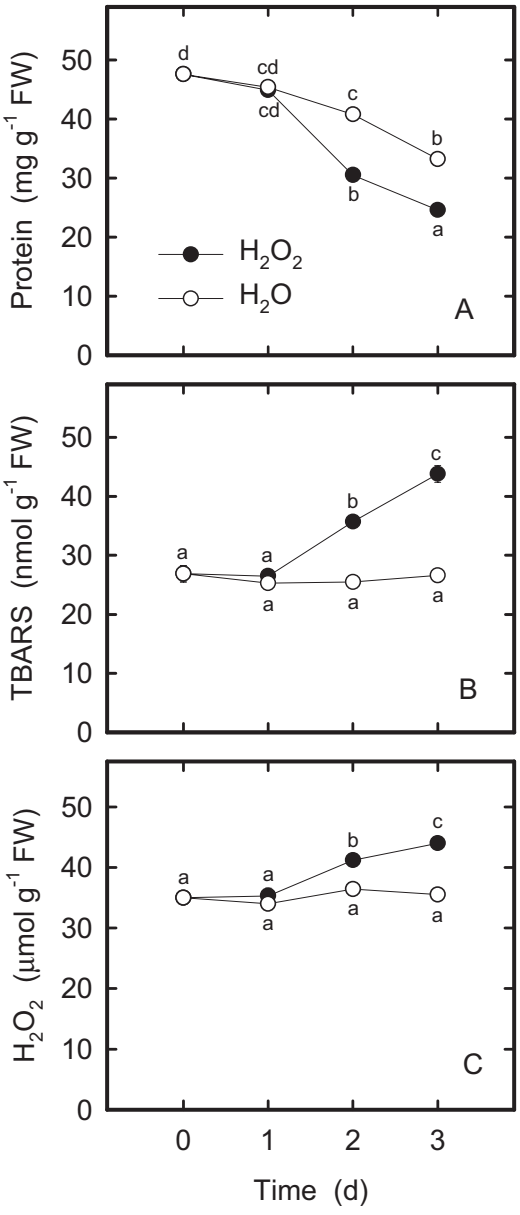

Figure 1. Changes in the contents of protein (A), MDA (B), and H_2O_2 (C) in rice leaves treated with H_2O_2. Detached rice leaves were treated with either water or 10 mM H_2O_2 in the dark. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

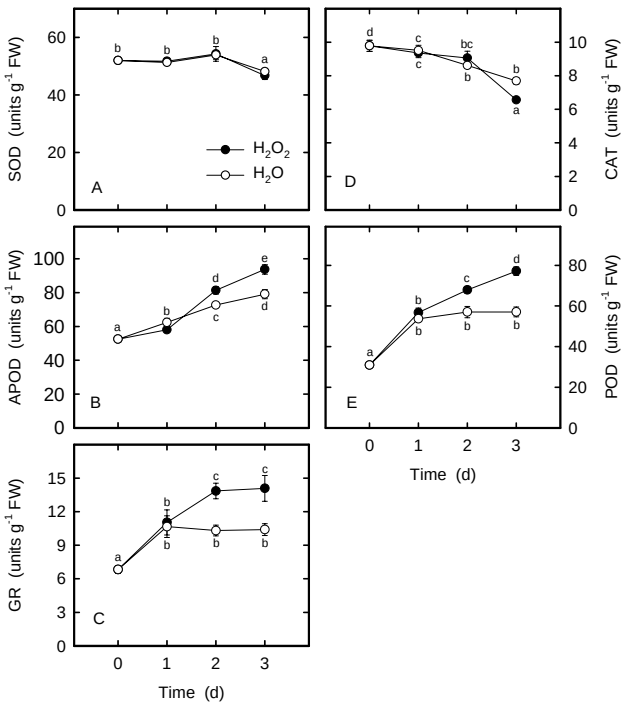

Figure 2. Changes in the activities of SOD (A), APOD (B), GR (C), CAT (D) and POD (E) in rice leaves treated with either water or 10 mM H_2O_2 in the dark. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

conditions. The changes in the activities of SOD (Figure 2A), APOD (Figure 2B), GR (Figure 2C), CAT (Figure 2D), and POD (Figure 2E) and the contents of AsA (Figure 3A) and GSH (Figure 3B) which occurred in detached rice leaves after incubation in water may be a manifestation of changes in metabolism associated with excision and consequent senescence.

In the present investigation, we show that the promotion of senescence in detached rice leaves by H_2O_2 is associated with lipid peroxidation or oxidative stress. NO is known to counteract oxidative stress in plants (Beligni and Lamattina, 1999a,b; 2002; Beligni et al., 2002; Cheng et al., 2002; Hung et al., 2002; Hung and Kao, 2003, 2004). Thus, it is of great interest to know whether the protective power of NO is also active in the H_2O_2-promoted senescence of rice leaves.

To study the role of NO, various donors were employed, with the assumption that they release NO. Typically, NO is applied to plant tissues via a NO-donor, that is, a molecule that will generate NO, sometimes after passage into cells. Chamulitrat et al. (1993) postulated that PBN can undergo oxidative decomposition to release NO. SIN-1 is known to generate both superoxide anion and nitric oxide which spontaneously form peroxynitrite (Spiecker et al., 1993). Peroxynitrite has been shown to react with H_2O_2 to yield nitrite ion and oxygen (Martinez et al., 2000). This reaction has been suggested to be the mechanism of NO cytoprotective actions in animals (Wink et al., 1993). NO is released from SNP mainly by decomposition of pure solutions due to photochemical reactions or by the various reducing metabolites, including thiols, in biological organelles (Rochelle et al., 1994; Rao and Cederbaum, 1995; Ioannidis et al., 1996). As shown in Table 1, all NO donors, such as PBN, SIN-1, SNP, and a mixture of AsA and $NaNO_2$, are effective at inhibiting H_2O_2-promoted senescence of rice leaves.

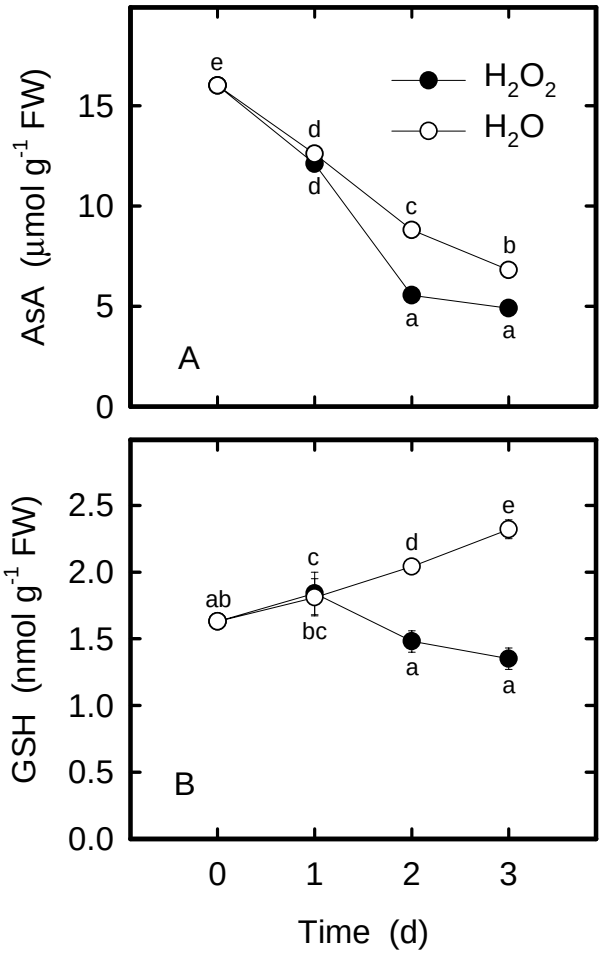

Figure 3. Changes in the contents of AsA (A) and GSH (B) in rice leaves treated with either water or 10 mM H_2O_2 in the dark. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

To investigate whether the protective effect induced by PBN treatment was the result of the production of NO, 100 μM c-PTIO, a NO-specific scavenger, was applied along with 100 μM PBN. The effect of PBN on H_2O_2-promoted protein loss and H_2O_2-induced increase in lipid peroxidation and H_2O_2 content could be reversed by c-PTIO (Table 2). We also observed that PBN counteracted H_2O_2-induced increases in antioxidant enzyme activities, and c-PTIO reversed the effect of PBN-decreased enzyme activities (Table 3). Furthermore, the effect of PBN on H_2O_2-decreased AsA and GSH contents could be reversed by c-PTIO (Table 4). Clearly, the effect of NO donor PBN would then be attributable to NO released.

It has been shown that NO is a potent antioxidant in plants and that its action may, at least in part, be explained by its ability to directly scavenge AOS, H_2O_2 and $O_2^{\cdot-}$ (Beligni and Lamattina, 2002). If NO acts as an antioxidant, it may reduce H_2O_2 content in H_2O_2-treated rice leaves. Since NO reduces H_2O_2-increased H_2O_2 content (Table 2), it appears that it indeed has the ability to scavenge AOS. Orozco-Cárdenas and Ryan (2002) also reported that NO blocked the H_2O_2 production that was induced by jasmonic acid. We also observed that NO donors blocked abscisic acid- and methyl jasmonate-induced H_2O_2 production in rice leaves (Hung and Kao, 2003, 2004).

Table 1. Effect of NO donors on protein content in rice leaves treated with H_2O_2. The concentrations of H_2O_2, PBN, SIN-1, SNP, AsA, and $NaNO_2$ were 10 mM, 100 μM, 100 μM, 100 μM, 100 μM, and 200 μM, respectively. Protein content was determined 2 days after treatment in the dark. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

Treatment	Protein (mg g^{-1} FW)
H_2O	40.9 ± 0.37 (c)
H_2O_2	29.8 ± 0.29 (a)
H_2O_2 + PBN	36.6 ± 0.54 (b)
H_2O_2 + SIN-1	34.5 ± 1.1 (b)
H_2O_2 + SNP	36.8 ± 0.67 (b)
H_2O_2 + AsA+ $NaNO_2$	35.8 ± 0.78 (b)

Table 2. Effect of PBN on the contents of protein, MDA, and H_2O_2 in H_2O_2-treated rice leaves in the presence or absence of c-PTIO. The concentrations of H_2O_2, PBN, and c-PTIO were 10 mM, 100 µM, and 100 µM, respectively. Protein, MDA, and H_2O_2 contents were determined 2 days after treatment in the dark. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

Treatment	Protein (mg g^{-1} FW)	TBARS (nmol g^{-1} FW)	H_2O_2 (µmol g^{-1} FW)
H_2O	41.9 ± 0.68 (c)	26.0 ± 0.73 (a)	35.1 ± 0.48 (b)
H_2O_2	31.3 ± 0.41 (a)	36.0 ± 0.44 (b)	42.1 ± 0.44 (d)
H_2O_2 + PBN	36.8 ± 0.41 (b)	27.5 ± 0.35 (a)	32.4 ± 0.30 (a)
H_2O_2 + PBN + c-PTIO	31.9 ± 1.0 (a)	38.4 ± 0.97 (b)	39.3 ± 0.79 (c)

Table 3. Effect of PBN on the activities of antioxidant enzymes in H_2O_2-treated rice leaves in the presence or absence of c-PTIO. The concentrations of H_2O_2, PBN, and c-PTIO were 10 mM, 100 µM, and 100 µM, respectively. Enzyme activities were determined 2 days after treatment in the dark. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

Treatment	APOD (units g^{-1} FW)	POD (units g^{-1} FW)	GR (units g^{-1} FW)
H_2O	72.7 ± 0.92 (a)	55.5 ± 0.97 (a)	10.6 ± 0.23 (a)
H_2O_2	81.9 ± 0.98 (c)	74.2 ± 0.86 (c)	13.9 ± 0.14 (c)
H_2O_2 + PBN	75.3 ± 0.55 (b)	60.9 ± 1.6 (b)	11.4 ± 0.15 (b)
H_2O_2 + PBN + c-PTIO	82.0 ± 1.2 (c)	74.7 ± 1.7 (c)	14.0 ± 0.17 (c)

Table 4. Effect of PBN on the contents of AsA and GSH in H_2O_2-treated rice leaves in the presence or absence of c-PTIO. The concentrations of H_2O_2, PBN, and c-PTIO were 10 mM, 100 µM, and 100 µM, respectively. AsA and GSH contents were determined 2 days after treatment in the dark. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

Treatment	AsA (µmol g^{-1} FW)	GSH (nmol g^{-1} FW)
H_2O	8.53 ± 0.24 (c)	1.93 ± 0.01 (d)
H_2O_2	5.86 ± 0.10 (a)	1.40 ± 0.02 (a)
H_2O_2 + PBN	7.65 ± 0.19 (b)	1.70 ± 0.02 (c)
H_2O_2 + PBN + c-PTIO	5.91 ± 0.15 (a)	1.54 ± 0.01 (b)

In the present investigation, we found that NO reduced H_2O_2-increased lipid peroxidation (Table 2) and antioxidant enzyme activities (Table 3) in rice leaves. These results are in agreement with our previous work, in which we demonstrated that NO counteracted paraquat-increased lipid peroxidation and antioxidant enzyme activities (Hung et al., 2002). Because lipid peroxidation and the increase in antioxidant enzyme activities are the consequence of AOS (Thompson et al., 1987) and NO acts as an AOS scavenger, the reduction of lipid peroxidation and antioxidant enzyme activities could be a result of low levels of H_2O_2 in rice leaves treated with NO and H_2O_2 (Table 1). The fact that NO counteracts H_2O_2-decreased AsA and GSH (Figures 3A and 3B) should result in an increased capacity to scavenge H_2O_2 in rice leaves treated with NO and H_2O_2 compared to rice leaves treated with H_2O_2 alone and might account in part for the lower contents of H_2O_2 observed in rice leaves treated with both NO and H_2O_2 (Table 2).

NO has been shown to act as iron ligand in haemoproteins (Stamler et al., 1992). Ferrer and Barceló (1999) found that the NO donor SNP (5 mM) and NO (55 µM) itself were able to inhibit POD activity in the xylem of *Zinnia elegans*. Clark et al. (2000) also demonstrated that NO donor (0.8 mM) inhibited the activities of tobacco CAT and APOD, heme-containing enzymes. However, our results show that PBN alone had no effect on the lipid peroxidation and activities of POD and APOD in rice leaves (data not shown). Thus, the reduction of H_2O_2-induced increase in antioxidant enzyme activities by NO is not likely due to a direct NO-mediated inhibition of the enzymes. Why is PBN able to counteract H_2O_2-induced senescence, but not able to inhibit the activities of POD, CAT, and APOD in rice leaves? In the present investigation, we used a µM concentration range of PBN and did not measure the concentration of NO released by PBN in rice leaves. It is possible that the concentration of NO released by PBN was high enough to scavenge H_2O_2, but not sufficient to inhibit the activities of heme-containing enzymes.

In conclusion, the results presented in this paper provide evidence that NO acts as an antioxidant in inhibiting H_2O_2-promoted rice leaf senescence.

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E.P.S.

一氧化氮可克服過氧化氫所促進的水稻葉片老化

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本研究主要是探討一氧化氮對過氧化氫所促進水稻葉片老化之影響。水稻葉片老化以蛋白質含量降低之程度做為指標。過氧化氫處理導致（一）葉片內過氧化氫含量增加，（二）葉片老化，（三）脂質過氧化作用增加，（四）還原態 glutathione（GSH）與 ascorbic acid（AsA）含量降低與（五）抗氧化酵素（ascorbate peroxidase, glutathione reductase 與 peroxidase）活性增加。一氧化氮釋放劑 [N-*tert*-butyl-α-phenylnitrone（PBN），sodium nitroprusside，3-morpholinosydonimine 以及 AsA 與 $NaNO_2$ 之混合液] 能有效的降低過氧化氫所促進的葉片老化，過氧化氫所誘導的過氧化氫與 MDA 含量之增加，過氧化氫所誘導的 GSH 與 AsA 含量之降低以及過氧化氫所誘導的抗氧化酵素活性之增加。一氧化氮清除劑 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethyl- imidazoline-1-oxyl-3-oxide 處理可使 PBN 之保護作用消失。該結果顯示，PBN 之保護作用確實是由於釋放出的一氧化氮所造成。一氧化氮延緩過氧化氫所促進的葉片老化很可能是由於其能清除過氧化氫所造成。

關鍵詞： 過氧化氫；脂質過氧化作用；一氧化氮；水稻。