



Nitric oxide counteracts the senescence of detached rice leaves induced by dehydration and polyethylene glycol but not by sorbitol

Fang-yi Cheng, Shih-Yung Hsu and Ching Huei Kao*

Department of Agronomy, National Taiwan University, Taipei, Taiwan, Republic of China; *Author for correspondence (e-mail: kaoch@ccms.ntu.edu.tw; phone: +866-2-23698159; fax: +886-2-23620879)

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Abstract

In the present study, we evaluated the protective effect of nitric oxide (NO) against senescence of rice leaves enhanced by water deficit. Dehydration (DH), polyethylene glycol (PEG) and sorbitol (ST) treatments were used to induce water deficit. Senescence of rice leaves was determined by the decrease of protein content. NO donors [N-*tert*-butyl- α -phenylnitron (PBN), sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1), and ascorbic acid + NaNO₂] were effective in inhibiting senescence of dehydrated and PEG-treated rice leaves, but had no effect on senescence of ST-treated rice leaves. PEG or DH resulted in a marked increase in malondialdehyde (MDA) content and decrease in superoxide dismutase (SOD) activity, but ST had no effect on MDA content and SOD activity. Treatment with NO donors caused a reduction of PEG- and DH-induced increase in MDA content and decrease in SOD activity. The protective effect of NO donors on promotion of senescence, increase in lipid peroxidation and decrease in SOD activity induced by PEG and DH was reversed by 2-(4-carboxy-2-phenyl)-4,4,5,5-tetra-methylinimidazoline-1-oxyl-3-oxide, a NO specific scavenger, suggesting that the protective effect of NO donors is attributed to NO released. The inhibition of PEG- and DH-enhanced senescence of rice leaves by NO is most likely mediated through increasing SOD activity and decrease in lipid peroxidation.

Abbreviations: Asc – ascorbate, c-PTIO – 2-(4-carboxy-2-phenyl)-4,4,5,5-tetra-methylinimidazoline-1-oxyl-3-oxide, DH – dehydration, MDA – malondialdehyde, NO – nitric oxide, PBN – N-*tert*-butyl- α -phenylnitron, PEG – polyethylene glycol, SIN-1 – 3-morpholinosydnonimine, SNP – sodium nitroprusside, SOD – superoxide dismutase, ST – sorbitol

Introduction

Water deficit is known to promote leaf senescence (Smirnoff 1993; Lin and Kao 1998; Lin et al. 1999; Lin and Kao 2000). Lipid peroxidation is considered to be an important mechanism of leaf senescence (Thompson et al. 1987; Strother 1988). Active oxygen species can initiate lipid peroxidation (Kellogg and Fridovich 1975). Drought-induced inhibition of photosynthesis leads to increased active oxygen species in the chloroplasts (Smirnoff (1993, 1998); Dat et al. 2000). Numerous studies report increased oxidative stress and lipid peroxidation during water deficit (Sgherri and Navari-Izzo 1995; Wellburn et al. 1996;

Aziz and Larher 1998; Boo and Jung 1999; Loggini et al. 1999; Lin and Kao 2000). Thus, it is likely that drought-enhanced senescence of leaves is linked to active oxygen-mediated lipid peroxidation (Smirnoff 1993; Lin and Kao 2000).

Nitric oxide (NO) is a labile free radical and is produced from L-arginine by NO synthase in various mammalian cells (Sakuma et al. 1988; Rees et al. 1989). NO is used by mammals to regulate various biological processes of the immune, nervous and vascular systems (Schmidt and Walter 1994). It is now becoming evident that NO is also a ubiquitous signal in plants. NO promotes leaf expansion, seed germination and de-etiolation, but it also inhibits hypocotyl

and internode elongation, induces defense genes and phytoalexin production, potentiates the induction of hypersensitive cell death, and extends post-harvest life (Leshem 2000; Beligni and Lamattina 2001). Recently, several reports demonstrate convincingly that NO is able to counteract the toxicity of paraquat and diaquat, which are known to generate free radicals, in potato and rice leaves (Beligni and Lamattina (1999a, 1999b); Hung et al. 2002). Thus, a possible participation of NO in antioxidant system in plants, as occurs in animals (O'Donnell et al. 1997; d'Ischia et al. 2000), is suggested.

García Mata and Lamatina (2001) reported that NO is able to confer an increased tolerance to severe drought stress through induction of stomatal closure, decrease in transpiration rate and lowering of ion leakage. We have recently demonstrated that NO donors reduced oxidative stress of rice leave through increases in antioxidant enzyme activities and decreases in lipid peroxidation (Hung et al. 2002). In the present investigation, we examined the effect of NO donors on water deficit-enhanced senescence of rice leaves.

Materials and methods

Rice (*Oryza sativa* L., cv. Taichung Native1) was cultured as described previously (Lin et al. 1999). The apical 3-cm segments excised from the third leaves of 12-day-old seedlings were used. Induction of water deficit was achieved by dehydration (DH), and treating with osmotica [polyethylene glycol (PEG) and sorbitol (ST)]. For leaf DH, the method of Stewart (1971) was adopted. Groups of ten leaf segments were exposed to the vapour above a 0.5 M NaCl solution, by spreading the leaf segments on water-proof paper boats, which were in turn kept over 20 ml NaCl solution in Petri dishes. Comparable leaf samples were kept turgid by floating them in 20 ml of distilled water to serve as the control. For osmotically-induced water deficit, leaf segments were exposed to PEG-6000 or ST solution of osmotic potential -1.5 MPa. All NO donors and scavenger used were obtained from Sigma (St. Louis, USA) and dissolved in distilled water.

RWC, defined as water content of leaf tissue as a percentage of that of the fully turgid tissue, was determined by the method of Weatherley (1950). Proline was extracted and its concentration determined following the method of Bates et al. (1973). Leaf

segments were homogenised with 3% (w/v) sulphosalicylic acid and centrifuged. The supernatant was treated with acetic acid and acid-ninhydrin, boiled for 1 h, and extracted with toluene. Then, its absorbance at 520 nm was read. For protein extraction, leaf segments were homogenised in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,000 g for 20 min, and the supernatants were used for determinations of both protein by the method of Bradford (1976) and superoxide dismutase (SOD) activity.

Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968). SOD was determined according to Paoletti et al. (1986). One unit activity for SOD was defined as the amount of enzyme which inhibited 50% the rate of NADH oxidation observed in control.

Proline, protein and MDA contents were expressed on the basis of initial fresh weight. SOD activity was expressed as units per mg protein. Absolute contents of each measurement varied among experiments because of seasonal effects. However, the patterns of responses to water deficit or water deficit plus NO donors were reproducible. For biochemical analysis, four independent extractions were performed for each treatment. All experiments described here were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

Results and discussion

In the present study, leaf segments exposed to the vapour above a 0.5 M solution of NaCl were considered as DH treatment. PEG and ST solutions of osmotic potential -1.5 MPa were also used to induce water deficit in the leaves. RWC of detached rice leaves subjected to DH, and exposed to PEG or ST solution of -1.5 MPa decreased during 12-h of incubation (Figure 1), suggesting that DH, PEG, and ST treatments in our study did indeed cause water deficit in the leaves. The decreases in RWC in dehydrated and PEG-treated rice leaves were greater than that in ST-treated rice leaves (Figure 1). Proline is known to accumulate in leaves under water deficit (Hanson and Hitz 1982). Under our experimental condition, we also observed that proline accumulation in dehydrated and PEG-treated rice leaves was more pronounced

than that in ST-treated rice leaves (Figure 1). Based on the results of RWC and proline content we conclude that water deficit in rice leaves induced by DH and PEG treatments was more severe than that induced by ST treatment. In previous study, O'Toole and Cruz (1980) reported a linear relationship between leaf rolling and water potential and concluded that leaf rolling in rice could be used as an estimate of water deficit. Apparently, leaf rolling in PEG-treated leaves was greater than in ST-treated leaves (Figure 2). Since ST molecules can be absorbed by plant cells (Flores and Galston 1984), a lower degree of water deficit induced by ST appears to result from a certain amount of osmotic adjustment, due to the accumulation of ST.

Beligni and Lamattina (1999a, 1999b) reported that NO counteracts cellular damage caused by diquat and paraquat in potato leaves. Based on their evidence, they proposed that NO acts as an antioxidant in reducing cellular damage in potato leaves. Our recent work also demonstrated that the reduction of paraquat toxicity by NO in rice leaves is mediated through an increase in antioxidative enzyme activities and a decrease in lipid oxidation (Hung et al. 2002). More recently, García Mata and Lamatina (2001) reported that exogenous NO donors are able to withstand water deficit, probably through reducing ion leakage, a consequence of oxidative stress. We have previously reported that the promotion of senescence (the decrease of protein content) of detached rice leaves in the light by DH was linked to lipid peroxidation (Lin and Kao 2000). If NO also plays a protective role in water deficit-enhanced senescence of rice leaves, it is expected that exogenous NO donors would be effective in inhibiting senescence of rice leaves enhanced by water deficit.

In the present study, PBN, SIN-1, SNP, and a mixture of Asc and NaNO_2 were used as NO donors. For DH experiments, detached rice leaves were pretreated with distilled water or NO donors for 6 h in the light and then treated with distilled water or subjected to DH for 12 h. As indicated in Table 1, DH clearly promoted senescence of rice leaves and all the NO donors used were effective in inhibiting DH-promoted senescence in rice leaves. For PEG and ST experiments, NO donors were applied together with PEG or ST solution to detached rice leaves for 12 h. Both PEG and ST promoted senescence of rice leaves (Tables 2 and 3). NO donors were also observed to be effective in inhibiting PEG-enhanced senescence of rice leaves (Table 2). However, NO donors had no

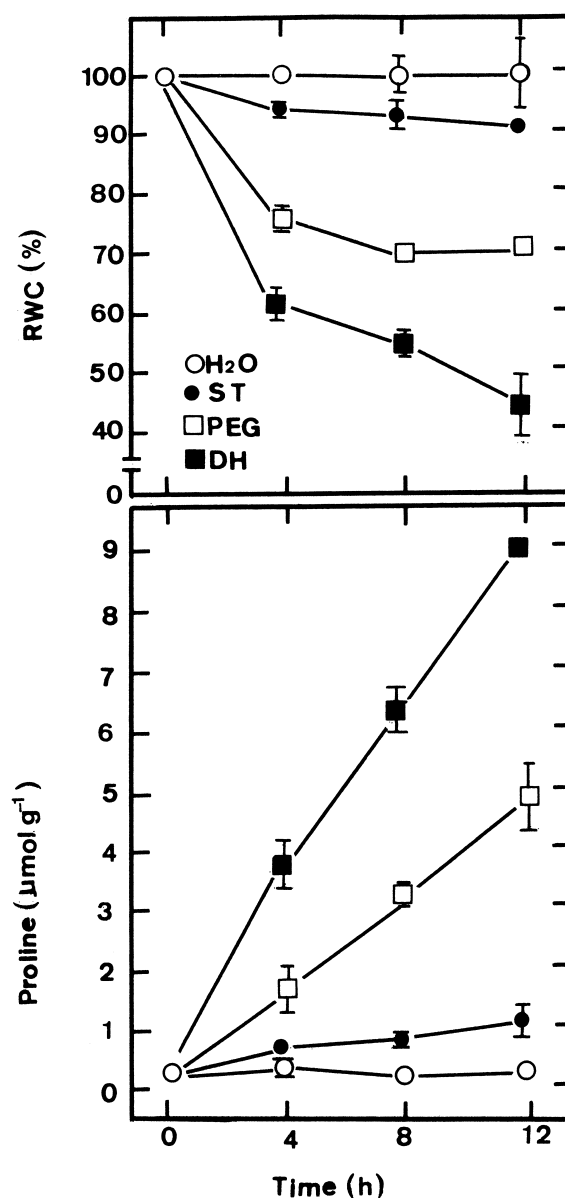


Figure 1. Changes in RWC and proline content in rice leaves treated with sorbitol (ST), polyethylene glycol (PEG), and dehydration (DH). Vertical bars represent standard errors ($n = 4$). Only those standard errors larger than the symbol are shown.

effect on ST-enhanced senescence of rice leaves (Table 3).

Figure 1 shows that increasing concentrations of PBN from 50 to 200 μM slightly decreased protein content. When applied together with PEG, PBN concentrations between 50 and 200 μM produced a clear protection against protein loss (Figure 3). The optimal concentration of PBN in inhibiting PEG-en-

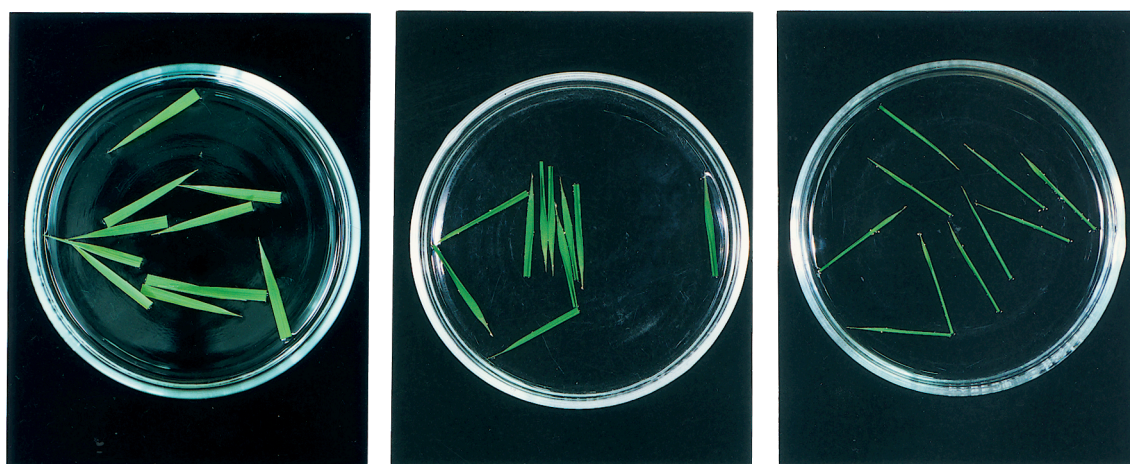
**H₂O****ST****PEG**

Figure 2. Effect of sorbitol (ST) and polyethylene glycol (PEG) on leaf rolling of rice. Picture was taken after 8 h of treatment.

Table 1. Effect of NO donors on protein content in dehydrated rice leaves in the presence or absence of c-PTIO

Treatment	Protein, mg g ⁻¹
H ₂ O	59.8 ± 1.2
DH	47.6 ± 2.3
DH + PBN	55.8 ± 1.2
DH + SIN-1	53.2 ± 0.5
DH + SNP	55.2 ± 0.7
DH + Asc + NaNO ₂	53.2 ± 0.5
DH + PBN + c-PTIO	49.6 ± 0.6
DH + SIN-1 + c-PTIO	48.1 ± 0.6
DH + SNP+ c-PTIO	48.3 ± 2.6
DH + Asc + NaNO ₂ + c-PTIO	48.5 ± 3.0

Detached rice leaves were pretreated with distilled water or NO donors in the presence or absence of c-PTIO for 6 h in the light and then treated with distilled water and subjected to dehydration (DH) for 12 h. The concentrations of PBN, SIN-1, SNP, Asc, NaNO₂, and c-PTIO were 100, 100, 100, 100, 200, and 100 μM, respectively. Data are the means ± standard errors (n = 4).

hanced senescence of rice leaves was observed to be 100 μM (Figure 3). It was also observed that the optimal concentration of PBN pretreatment in protecting against protein loss induced by DH was 100 μM (Figure 3). The protective effect of PBN on senescence of rice leaves induced by DH and PEG was evident at 4 and 8 h, respectively, after treatment (Figure 4).

To investigate whether the protective effect induced by treatment with NO donors was the result of the production of NO, 100 μM c-PTIO, a NO-specific scavenger, was applied along with NO donors.

Table 2. Effect of NO donors on protein content in PEG-treated rice leaves in the presence or absence of c-PTIO

Treatment	Protein, mg g ⁻¹
H ₂ O	56.8 ± 0.5
PEG	47.2 ± 1.7
PEG + PBN	51.1 ± 1.6
PEG + SIN-1	51.7 ± 1.4
PEG + SNP	52.0 ± 1.6
PEG + Asc + NaNO ₂	51.9 ± 1.5
PEG + PBN + c-PTIO	46.4 ± 0.7
PEG + SIN-1 + c-PTIO	46.2 ± 1.7
PEG + SNP+ c-PTIO	46.0 ± 0.4
PEG + Asc + NaNO ₂ + c-PTIO	47.7 ± 1.0

The concentrations of PBN, SIN-1, SNP, Asc, NaNO₂, and c-PTIO were 100, 100, 100, 100, 200, and 100 μM, respectively. Protein content was determined 12 h after treatment in the light, data are the means ± standard errors (n = 4).

The protective effect of NO donors on PEG- and DH-induced senescence of rice leaves could be reversed by c-PTIO (Tables 1 and 2). Clearly, the protective effect on PEG- and DH-induced rice leaf senescence by NO donors is due to NO.

Table 4 shows that PEG or DH treatment resulted in a marked increase in MDA content, but ST treatment had no effect on MDA content. Treatment with PBN caused a reduction of the PEG- and DH-induced increase in MDA content (Table 4). The effect of PBN on PEG- and DH-induced MDA content could also be reversed by c-PTIO (Table 4). MDA is routinely used as an indicator of lipid peroxidation. The protective

Table 3. Effect of NO donors on protein content in sorbitol (ST)-treated rice leaves in the presence or absence of c-PTIO

Treatment	Protein, mg g ⁻¹
H ₂ O	55.2 ± 0.6
ST	49.8 ± 0.9
ST + PBN	50.0 ± 1.6
ST + SIN-1	49.4 ± 3.3
ST + SNP	47.1 ± 1.7
ST + Asc + NaNO ₂	48.8 ± 1.4

The concentrations of PBN, SIN-1, SNP, Asc, and NaNO₂ were 100, 100, 100, 100, and 200 μ M, respectively. Protein content was determined 12 h after treatment in the light, data are the means $\pm$ standard errors (n = 4).

effect of PBN on senescence of rice leaves induced by PEG and DH is clearly associated with the decrease in lipid peroxidation (Table 4). Plant cells are equipped with several free radical detoxifying enzymes to protect them against oxidative damage. These enzymes include SOD, ascorbate peroxidase, glutathione reductase, catalase, and peroxidase (Foyer et al. 1997; Nakano and Asada 1981). The striking changes in lipid peroxidation seen in leaves treated with PEG or DH alone may be a reflection of the inability of the scavenging enzymes to cope with the increased levels of oxidant species and/or their deg-

radation by these species. Previously, we found that DH-induced enhancement of rice leaf senescence coincided with an elevated lipid peroxidation and reduced SOD activity in detached rice leaves (Lin and Kao 2000). In the present study, the PEG- and DH-induced loss of SOD activity was found to be protected by PBN (Table 4). Table 4 also showed that c-PTIO arrested PBN-mediated SOD protection (Table 4). It should be noted that ST treatment had no effect on SOD activity and MDA content (Table 4).

Oxidative stress or lipid peroxidation was generally considered to be generated as a consequence of water deficit in plants. Baisak et al. (1994) reported that enhancement of lipid peroxidation was observed in leaves subjected to a higher degree of water deficit but not in those subjected to mild stress. Parallel to these observations, we also noticed an increase in lipid peroxidation in detached rice leaves treated with PEG and DH. On the other hand, detached rice leaves did not exhibit an increase in lipid peroxidation when exposed to ST (Table 4). Our results show that the promotion of rice leaf senescence caused by PEG and DH, which induced lipid peroxidation, can be counteracted by NO donors, but the senescence caused by ST, which had no effect on lipid peroxidation, cannot be counteracted by NO donors.

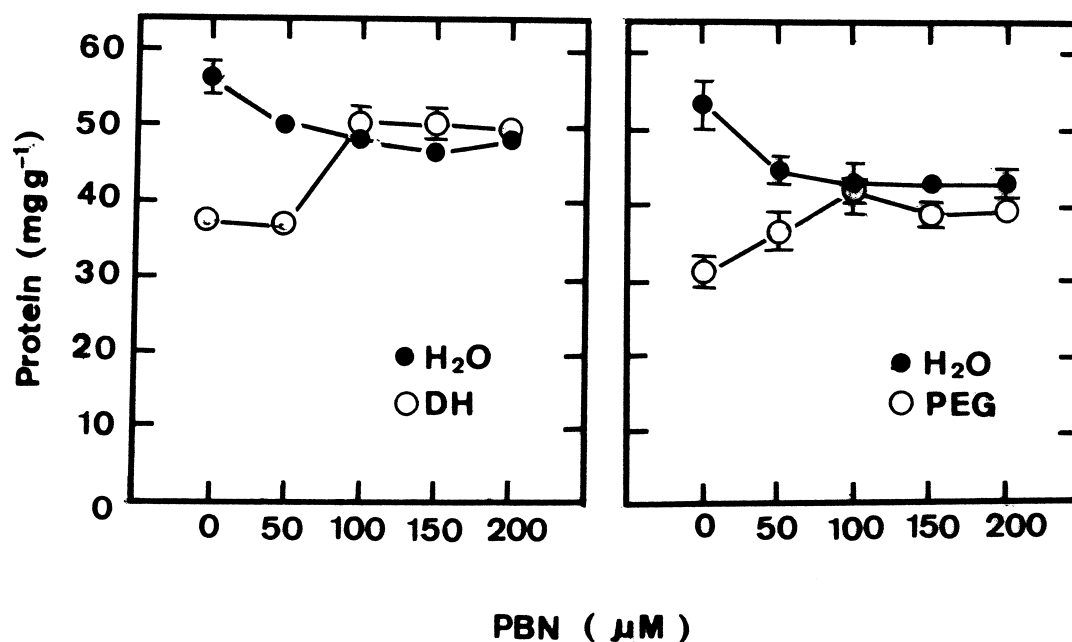


Figure 3. Effect of PBN concentrations on protein content in rice leaves treated with either distilled water, dehydration (DH) or polyethylene glycol (PEG). For the PEG experiment, protein was determined 12 h after treatment in the light. For the DH experiment, detached rice leaves were pretreated with distilled water or various concentrations of PBN for 6 h in the light and then were treated with distilled water or subjected to dehydration for 12 h. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol are shown.

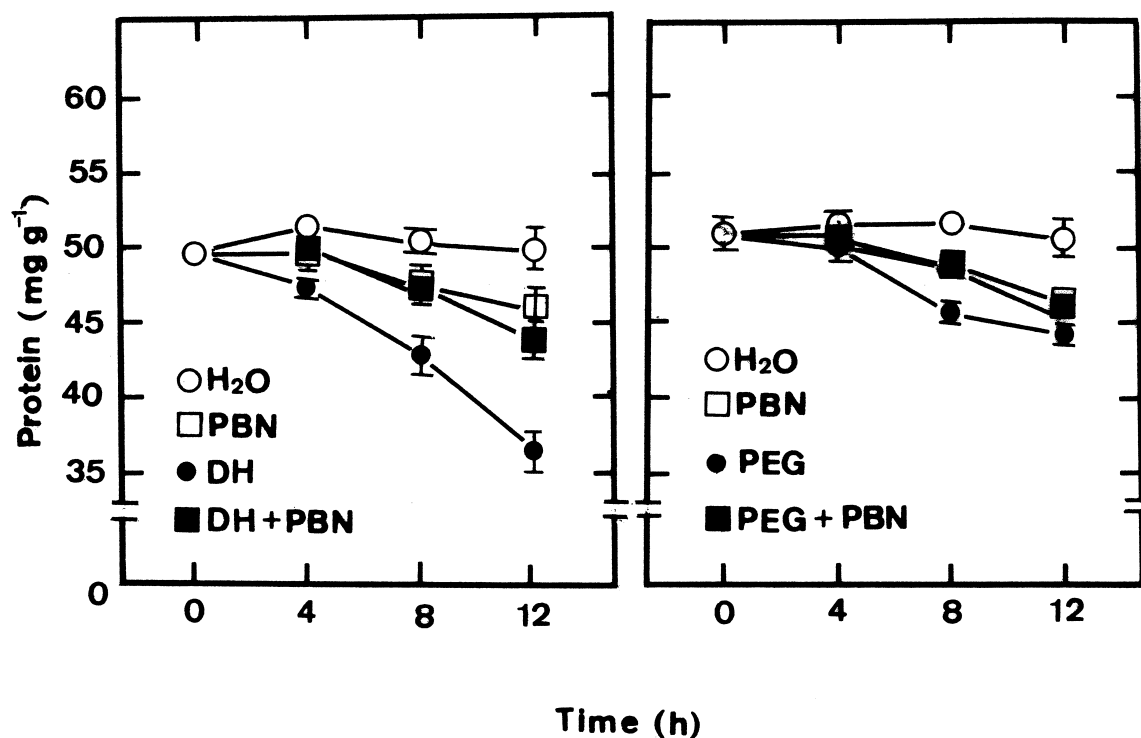


Figure 4. Changes in protein content in rice leaves treated with distilled water, polyethylene glycol (PEG) or dehydration (DH) in the presence or absence of PBN in the light. PBN concentration was 100 μ M. Vertical bars represent standard errors ($n = 4$). Only those standard errors larger than the symbol are shown

Table 4. Effect of PBN on MDA content and SOD activity in PEG-, ST-treated and dehydrated rice leaves in the presence or absence of c-PTIO

Treatment	MDA, nmol g ⁻¹	SOD, units mg ⁻¹ protein
H ₂ O	40.3 $\pm$ 0.9	1.70 $\pm$ 0.03
ST	43.4 $\pm$ 2.1	1.51 $\pm$ 0.09
PEG	67.3 $\pm$ 1.6	1.04 $\pm$ 0.14
PEG + PBN	53.1 $\pm$ 1.4	1.59 $\pm$ 0.10
PEG + PBN + c-PTIO	68.2 $\pm$ 2.1	0.99 $\pm$ 0.14
H ₂ O	40.3 $\pm$ 0.4	1.70 $\pm$ 0.04
DH	93.4 $\pm$ 1.2	0.73 $\pm$ 0.02
DH + PBN	76.5 $\pm$ 2.3	1.17 $\pm$ 0.03
DH + PBN + c-PTIO	91.1 $\pm$ 1.5	0.71 $\pm$ 0.01

The concentrations of PBN and c-PTIO were 100 and 100 μ M, respectively. For sorbitol (ST) and polyethylene glycol (PEG) experiments, MDA and SOD were determined at 12 h after treatment in the light. For dehydration (DH) experiment, detached rice leaves were pretreated with distilled water or PBN in the presence or absence of c-PTIO for 6 h in the light and then treated with distilled water or subjected to dehydration for 12 h. Data are the means $\pm$ standard errors ($n = 4$).

The present study extends our previous results concerning the putative physiological roles of NO in rice leaves (Hung et al. 2002). We now demonstrate the potential ability of NO to withstand water deficit. The inhibition of PEG- and DH-enhanced senescence of rice leaves by NO is most likely mediated through

an increase in SOD activity and a decrease in lipid peroxidation.

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