BIOLOGIA PLANTARUM 46 (4): 617-619, 2003

BRIEF COMMUNICATION

The protective effect of free radical scavengers and metal chelators on polyethylene glycol-treated rice leaves

S.Y. HSU and C.H. KAO*

Department of Agronomy, National Taiwan University, Taipei, 106 Taiwan, Republic of China

Abstract

Effect of free radical scavengers and metal chelators on polyethylene glycol (PEG, osmotic potential -1.5 MPa) induced oxidative damage in detached rice leaves was investigated. PEG treatment resulted in a decrease in relative water content and an increase in proline content, and lipid peroxidation. PEG treatment also decreased chlorophyll and protein contents. Free radical scavengers (ascorbate, sodium benzoate, reduced glutathione, and thiourea) retarded and metal chelators [2,2'-bipyridine (BP), 8-hydroxyquinoline, and 1,10-phenanthroline] prevented PEG-induced oxidative damage. Furthermore, the protective effect of BP was reversed by adding Fe^{2+} and Cu^{2+}, but not by Mn^{2+} or Zn^{2+}. The protective effect of BP is most likely mediated through chelation of iron. It seems that oxidative damage induced by PEG may require the participation of iron.

Additional key words: lipid peroxidation, *Oryza sativa*, oxidative damage.

Drought is an important stress that dramatically limits plant growth and productivity (Boyer 1982). Leaves close their stomata under water stress which limits water loss and the influx of CO_2. Lowered CO_2 influx leads to a decrease in carbon reduction by the Calvin cycle and to a decrease in oxidized $NADP^+$ to serve as an electron acceptor in photosynthesis. As a result, electrons flow to the alternative electron acceptor, O_2, producing superoxide radical (Biehler and Fock 1996, Sgherri *et al.* 1996). Superoxide radical can serve as a source of more active hydroxyl radicals generated by Haber-Weiss and Fenton reactions (Naqui and Chance 1986, Strother 1988, Smirnoff 1993). Transition metals, such as iron and copper, are able to accelerate Haber-Weiss and Fenton reactions (Gutteridge *et al.* 1981). Reactive oxygen species can directly damage chlorophyll, protein, and nucleic acids and cause peroxidation of membrane lipids (Dat *et al.* 2000). Recently, we demonstrated that iron played a major role in methyl jasmonate-promoted senescence (Fang and Kao 2001) and Cd-induced toxicity in rice leaves (Chien *et al.* 2001). In this study, we investigated the effect of free radical scavengers and metal chelators on water stress-induced oxidative damage in detached rice leaves.

Rice (*Oryza sativa* L. cv. Taichung Native 1) was cultured on a stainless net floating on half-strength Johnson's modified nutrient solution (pH 4.2) in a 500-cm^3 beaker (Lin *et al.* 1999). The nutrient solution was replaced every 3 d. Rice plants were grown for 12 d in a greenhouse, under natural light and the day/night temperature of 30/25 °C. The apical 3 cm of the third leaf of 12-d-old seedlings was used for the experiment. A group of 10 segments floated in a Petri dish containing 10 cm^3 of distilled water served as controls. For induction of water stress, leaf segments were exposed to polyethylene glycol (PEG 6000) solution of osmotic potential -1.5 MPa. All samples were kept at 27 °C and irradiance of 40 μmol m^{-2} s^{-1} for 4, 8 and 12 h. RWC, defined as water content of leaf tissue as a percentage of that in the fully water saturated tissue, was determined by the method of Weatherley (1950). Proline was extracted and its concentration determined by the method of Bates

Received 12 February 2002, *accepted* 24 July 2002.

Abbreviations: BP - 2,2'-bipyridine; f.m. - fresh mass; Chl - chlorophyll; GSH - reduced glutathione; HQ - 8-hydroxyquinoline; MDA - malondialdehyde; PA - 1,10-phenathroline; PEG - polyethylene glycol; RWC - relative water content.

Acknowledgements: This work was supported by the National Science Council of the Republic of China, grant NSC 89-2313-B-002-222

*Fax: (+886) 02-23620879, e-mail: kaoch@ccms.ntu.edu.tw

et al. (1973). For protein determination, leaf segments were homogenized in 50 mM sodium phosphate buffer (pH 7.5). 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). Chlorophyll (Chl) *a+b* content was determined according to Wintermans and De Mots (1965) after extraction in 96 % (v/v) ethanol. Malondialdehyde (MDA) was extracted with 5 % (m/v) trichloroacetic acid determined according to Heath and Packer (1986). MDA level is routinely used as an index of lipid peroxidation. H_2O_2 was extracted and determined according to the method described previously (Lin and Kao 2001). Protein, MDA, H_2O_2, and proline contents were expressed on the basis of initial fresh mass (f.m.). All experiments were repeated three times; within each experiment, treatments were replicated 4 times. Similar results and identical trends were obtained in all experiments. The data reported here are from a single experiment.

RWC of detached rice leaves exposed to PEG under light, decreased considerably during 12-h treatment (Table 1). Proline accumulates in leaves under water stress (*e.g.* Hanson and Hitz 1982). Under our experimental condition, we also observed that the content of proline increased markedly in detached rice leaves exposed to PEG (Table 1). Based on the results of RWC and proline, we conclude that PEG treatment in our study did indeed cause water stress in detached rice leaves.

Table 1. Relative water content (RWC) [%] and contents of proline [μmol g^{-1}(f.m.)], protein [mg g^{-1}(f.m.)], chlorophyll *a+b* [mg g^{-1}(f.m.)], MDA [nmol g^{-1}(f.m.)], and H_2O_2 [μmol g^{-1}(f.m.)] in detached rice leaves floating on water (H_2O) or PEG solution (osmotic potential -1.5 MPa), for 4, 8, and 12 h. Means ± SE, $n = 4$.

Parameter	0 h	4 h		8 h		12 h	
		H_2O	PEG	H_2O	PEG	H_2O	PEG
RWC	95.1 ± 1.1	100.0	81.1 ± 1.1	100.0	76.0 ± 0.7	100.0	76.4 ± 1.9
Proline	0.96 ± 0.02	0.94 ± 0.02	2.42 ± 0.10	0.95 ± 0.05	5.72 ± 0.4	1.03 ± 0.01	6.63 ± 0.29
Protein	59.4 ± 1.7	59.1 ± 0.6	53.4 ± 1.5	57.1 ± 1.0	39.5 ± 0.5	57.4 ± 0.8	31.8 ± 0.8
Chl *a+b*	4.51 ± 0.24	4.33 ± 0.09	4.09 ± 0.10	4.54 ± 0.09	3.32 ± 0.11	4.57 ± 0.19	3.29 ± 0.09
MDA	17.8 ± 1.5	16.2 ± 0.9	19.2 ± 0.8	14.5 ± 0.1	24.2 ± 0.5	14.3 ± 0.1	29.3 ± 1.8
H_2O_2	42.8 ± 0.6	41.8 ± 1.1	43.0 ± 1.1	41.5 ± 1.7	42.7 ± 0.6	43.1 ± 0.6	48.0 ± 1.7

The decline of protein and chlorophyll contents during 12 h was faster in PEG-treated detached rice leaves than in control leaves (Table 1). MDA content in PEG-treated detached rice leaves was observed to be greater than that in water-treated controls, throughout the entire duration of incubation (Table 1). This indicates that PEG-induced loss of chlorophyll and protein in detached rice leaves is linked to lipid peroxidation. H_2O_2 content remained unchanged in control leaves throughout the entire duration of incubation. However, H_2O_2 content was higher in PEG-treated detached rice leaves than the water-treated controls only at 12 h treatment.

Table 2. Effect of free radical scavengers on protein and chlorophyll contents [mg g^{-1}(f.m.)] in detached rice leaves treated with PEG (-1.5 MPa). Detached rice leaves were pretreated with either water or 1 mM ascorbate, 5 mM sodium benzoate, 5 mM reduced glutathione (GSH), or 5 mM thiourea for 6 h in the light and then treated with either water or PEG for 12 h in the light. Means ± SE, $n = 4$.

Pretreatment	Treatment	Protein	Chl *a+b*
H_2O	H_2O	45.2 ± 1.0	4.47 ± 0.14
H_2O	PEG	34.0 ± 0.2	3.09 ± 0.09
Ascorbate	PEG	37.3 ± 0.8	3.81 ± 0.03
Na-benzoate	PEG	40.0 ± 1.0	4.03 ± 0.01
GSH	PEG	44.1 ± 1.5	3.98 ± 0.03
Thiourea	PEG	38.2 ± 0.9	3.84 ± 0.08

When free radical scavengers such as ascorbate, sodium benzoate, reduced glutathione (GSH), and thiourea were treated together with PEG, it was found that they partially prevented the decrease in protein and chlorophyll contents (Table 2), indicating that PEG treatment resulted in oxidative damage in detached rice leaves.

When metal chelators 2,2'-bipyridine (BP), 8-hydroxyquinoline (HQ), and 1,10-phenathroline (PA) were treated together with PEG, it was found that they prevented the decrease in protein and chlorophyll

Table 3. Effect of metal chelators on the contents of protein and chlorophyll [mg g^{-1}(f.m.)] in detached rice leaves treated with PEG (-1.5 MPa). Detached rice leaves were treated with 3 mM metal chelators 2,2'-bipyridine (BP), 8-hydroxyquinoline (HQ), and 1,10-phenathroline (PA) and PEG for 12 h. Means ± SE, $n = 4$.

Treatment	Protein	Chl *a+b*
H_2O	49.2 ± 1.8	4.24 ± 0.09
PEG	32.3 ± 0.4	3.12 ± 0.04
PEG + BP	47.8 ± 1.1	3.90 ± 0.15
PEG + HQ	37.4 ± 0.4	3.64 ± 0.04
PEG + PA	40.3 ± 0.9	3.87 ± 0.02

Table 4. Reversal of BP-reduced decrease in protein and chlorophyll contents [mg g^{-1}(f. m.)] of detached rice leaves induced by PEG (-1.5 MPa). Detached rice leaves were pretreated with either water or 3 mM BP or 3 mM BP plus 10 mM metal (sulfate salt) for 6 h in the light and then treated with either water or PEG for 12 h in the light. Means ± SE, $n = 4$.

Pretreatment	Treatment	Protein	Chlorophyll
H_2O	H_2O	42.6 ± 0.2	5.05 ± 0.19
H_2O	PEG	32.6 ± 1.5	3.98 ± 0.18
BP	PEG	39.8 ± 1.0	4.73 ± 0.03
BP + Fe^{2+}	PEG	29.1 ± 1.6	4.20 ± 0.18
BP + Cu^{2+}	PEG	28.4 ± 1.0	4.16 ± 0.08
BP + Mn^{2+}	PEG	38.8 ± 1.7	4.86 ± 0.19
BP + Zn^{2+}	PEG	41.2 ± 0.8	4.80 ± 0.12

contents (Table 3). Among the metal chelators used, BP was found to be the most effective.

The reversal of the protective effect of BP against PEG-induced loss of chlorophyll and protein was studied using divalent metal ions such as Fe^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+} (Table 4). The effect of BP could be reversed by Fe^{2+} and Cu^{2+}. However, Mn^{2+} and Zn^{2+} had no effect. These results indicate that the protection of BP is mediated through chelation of iron and copper, suggesting that iron and copper play a major role in induction of chlorophyll and protein loss in detached rice leaves caused by PEG. It is currently accepted that *in vivo* copper is unlikely to initiate the Haber-Weiss reaction in the way that has been demonstrated for iron (Halliwell and Gutteridge 1988). Thus, PEG-induced oxidative damage in detached rice leaves may require the participation of iron.

References

Bates, L.S., Waldren, S.P., Teare, I.D.: Rapid accumulation of free proline for water stress studies. - Plant Soil **39**: 205-207, 1973.

Biehler, K., Fock, H.: Evidence for the contribution of Mehler-peroxidase reaction in dissipating excess electrons in drought-stressed wheat. - Plant Physiol. **112**: 265-272, 1996.

Boyer, J.S.: Plant productivity and environment. - Science **218**: 443-448, 1982.

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

Chien, H.-F., Wang, J.-W., Lin, C.C., Kao, C.H.: Cadmium toxicity of rice leaves is mediated through lipid peroxidation. - Plant Growth Regul. **33**: 205-213, 2001.

Dat, J., Van den Abeele, S., Vranova, E., Van Montagu, M., Inze, D., Van Breusegem, F.: Dual action of the active oxygen species during plant stress responses. - Cell. mol. Life Sci. **57**: 779-795, 2000.

Fang, W.-C., Kao, C.H.: Inhibition of methyl jasmonate-promoted senescence in rice leaves by a metal chelators, 2,2'-bipyridine. - Plant Growth Regul. **33**: 87-93, 2001.

Gutteridge, J.M.C., Rowley, D.A., Halliwell, B.: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. - Biochem. J. **199**: 263-265, 1981.

Halliwell, B., Gutteridge, J.M.C.: Free Radicals in Biology. - Clarendon Press, Oxford 1988.

Hanson, A.D., Hitz, W.D.: Metabolic responses of mesophytes to plant water deficits. - Annu. Rev. Plant Physiol. **33**: 163-203, 1982.

Heath, R.L., Packer, L.: Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichometry of fatty acid peroxidation. - Arch. Biochem. Biophys. **125**: 189-198, 1986.

Lin, C.C., Kao, C.H.: Cell wall peroxidase activity, hydrogen peroxide level and NaCl-inhibited root growth of rice seedlings. - Plant Soil **230**: 135-143, 2001.

Lin, J.-N., Wang, J.-W., Kao, C.H.: Effect of abscisic acid and water stress on the senescence of detached rice leaves. - Biol. Plant. **42**: 313-316, 1999.

Naqui, A., Chance, B.: Reactive oxygen intermediates in biochemistry. - Annu. Rev. Biochem. **55**: 127-166, 1986.

Sgherri, C.L.M., Pinzino, C., Navari-Izzo, F.: Sunflower seedlings subjected to increasing stress by water deficit: Changes in $O_2^{\cdot}$ production related to composition of thylakoid membranes. - Physiol. Plant. **96**: 446- 452, 1996.

Smirnoff, N.: The role of active oxygen in the response of plants to water deficit and dessication. - New Phytol. **125**: 27-58, 1993.

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

Weatherley, P.E.: Studies in the water relation of cotton plant. I. The field measurement of water deficit in leaves. - New Phytol. **49**: 81-97, 1950.

Wintermans, J. F. G. M., De Mots, A.: Spectrophotometric characteristics of chlorophyll *a* and *b* and their pheophytins in ethanol. - Biochim. biophys. Acta **109**: 448-453, 1965.