BIOLOGIA PLANTARUM 46 (1): 149-152, 2003

BRIEF COMMUNICATION

Aluminum effects on lipid peroxidation and antioxidative enzyme activities in rice leaves

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Abstract

The effects of aluminum on lipid peroxidation and activities of antioxidative enzymes were investigated in detached rice leaves treated with 0 to 5 mM $AlCl_3$ at pH 4.0 in the light. $AlCl_3$ enhanced the content of malondialdehyde but not the content of H_2O_2. Superoxide dismutase activity was reduced by $AlCl_3$, while catalase and glutathione reductase activities were increased. Peroxidase and ascorbate peroxidase activities were increased only after prolonged treatment, when toxicity occurred. The results give evidence that Al treatment caused oxidative stress and in turn, it caused lipid peroxidation.

Additional key words: *Oryza sativa*, oxidative stress.

Aluminum is the most abundant metal and the third most common element in the earth's crust and Al toxicity is the major growth-limiting factor for crop cultivation on acid soils. Al ions interact with lipid components of the plasma membrane (Akeson *et al.* 1989). The binding of Al to the membrane lipids causes the rigidification of the plasma membrane (Deleers *et al.* 1986), which seems to facilitate Fe-mediated free radical chain reaction (Oteiza 1994). In fact, the Al-enhanced Fe-mediated peroxidation of lipids has been reported in root tips of soybean (Cakmak and Horst 1991, Horst *et al.* 1992) and cultured tobacco cells (Ono *et al.* 1995, Yamamoto *et al.* 1997, Yamaguchi *et al.* 1999, Ikegawa *et al.* 2000). Recently, Yamamoto *et al.* (2001) reported that Al enhanced the peroxidation of lipids without contribution of iron ions during Al treatment of pea roots. In *Arabidopsis* and cultured tobacco cells, Al induced the expression of several genes (*e.g.* for POX and SOD) that are induced by oxidative stress (Ezaki *et al.* 1995, 1996, Richards *et al.* 1998). Thus a possible induction of oxidative stress by Al was suggested (Snowden and Gardner 1993, Ezaki *et al.* 1995, 1996, Richards *et al.* 1998). In more recent work, Sakihama and Yamasaki (2002) also suggested that Al had the potential to induce oxidative stress in plants by stimulating the pro-oxidant nature of endogenous phenolic compounds. In the present paper, we have studied the effect of $AlCl_3$ on rice leaf oxidative stress and the activities of some antioxidative enzymes.

Rice (*Oryza sativa* L. cv. Taichung Native 1) was cultured as described previously (Lin *et al.* 1999). The apical 3-cm segments excised from the third leaves of 12-d-old seedlings were used. A group of 10 segments was floated in a Petri dish containing 10 cm^3 of 0 - 5 mM $AlCl_3$ (pH 4.0). The pH was adjusted by using 0.1 M HCl. Incubation was carried out at 27 °C under irridance of 40 $\mu mol\ m^{-2}\ s^{-1}$.

Chlorophyll was determined according to Wintermans and De Mots (1965) after extraction in 96 % (v/v) ethanol using spectrophotometer (*U-2000*, *Hitachi*, Tokyo, Japan). For protein extraction, leaf segments were homogenised 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). Malondialdehyde (MDA) was extracted with 5 % (m/v) trichloroacetic acid and determined according to Heath and Packer (1968).

Received 5 December 2001, *accepted* 25 April 2002.
Abbreviations: APX - ascorbate peroxidase; CAT - catalase; d.m.- dry mass; f.m. - fresh mass; GR - glutathione reductase; MDA - malondialdehyde; POX - peroxidase; SOD - superoxide dismutase.
Acknowledgements: This work was supported by the National Science Council of the Republic of China (NSC 89-2313-B-002-222).
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For the determination of Al, leaf segments were dried at 65 °C for 48 h. Dried material was ashed at 550 °C for 20 h. Ash residue was incubated with 31 % HNO_3 and 17.5 % H_2O_2 at 70 °C for 2 h, and dissolved in 0.1 M HCl. Al was then quantified using an atomic absorption spectrophotometer (*Model AA-680*, *Shimadzu*, Kyoto, Japan).

The H_2O_2 content was measured colorimetrically as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenising 50 mg leaf tissue with 3 cm^3 of 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, 3 cm^3 of extracted solution was mixed with 1 cm^3 of 0.1 % titanium sulphate in 20 % (v/v) H_2SO_4. The mixture was then centrifuged at 6 000 g for 15 min. The absorbance was measured at 410 nm.

For extraction of enzymes, leaf tissues were homogenised with 0.1 M phosphate buffer (pH 6.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12 000 *g* for 20 min and the resulting supernatant was used for determination of enzyme activity. The whole extraction procedure was carried out at 4 °C. 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. One unit (U) of CAT was defined as the amount of enzyme which breaks down 1 nmol H_2O_2 per min. POX activity was measured using a modification of the procedure of MacAdam *et al.* (1992) with guaiacol as a substrate. Activity was measured at 470 nm. One U of POX was defined as the amount of enzyme which produces 1 μmol tetraguaiacol per min. SOD was determined according to Paoletti *et al.* (1986). One U of SOD was defined as the amount of enzyme which inhibits by 50 % the rate of NADH oxidation observed in blank. APX was determined according to Nakano and Asada (1981). The decrease in ascorbate concentration was followed at 290 nm. One U of APX was defined as the amount of enzyme which breaks down 1 μmol of ascorbate per min. GR was determined by the method of Foster and Hess (1980). One U of GR was defined as the amount of enzyme which decreases A_{340} (1 unit per min).

All experiments described here were repeated at least three times; 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.

The toxicity of $AlCl_3$, was judged by the loss of chlorophyll and protein in detached rice leaves (Table 1). Chlorophyll and protein contents in detached rice leaves were not affected by 1 mM $AlCl_3$, but they decreased at 3 and 5 mM $AlCl_3$. Al content in detached rice leaves increased with the increase of $AlCl_3$ concentrations. Al toxicity in rice leaves occurred only when Al content was 500 μg g^{-1}(d.m.) or higher (Table 1). The loss of chlorophyll and protein was evident 2 d after the addition of 5 mM $AlCl_3$ (Table 2).

Table 1. Effects of $AlCl_3$ (pH 4.0) on the contents of Al^{3+}, protein, and chlorophyll in detached rice leaves in the light. All measurements were made after 3 d of treatment. Means ± SD (*n* = 4).

$AlCl_3$ [mM]	Al^{3+} [μg g^{-1}(d. m.)]	Protein [mg g^{-1}(f. m.)]	Chlorophyll [mg g^{-1}(f. m.)]
0	26 ± 5.0	33.8 ± 1.2	2.62 ± 0.04
1	273 ± 20.3	34.3 ± 0.7	2.64 ± 0.08
3	514 ± 31.1	30.5 ± 0.2	2.33 ± 0.13
5	605 ± 11.6	22.3 ± 0.8	1.38 ± 0.15

Table 2. Time courses of chlorophyll and protein contents in detached rice leaves treated with $AlCl_3$ (5 mM, pH 4.0) or distilled water (pH 4.0) in the light. Means ± SD (*n* = 4).

Time	Chlorophyll [mg g^{-1}(f. m.)]		Protein [mg g^{-1}(f. m.)]	
[d]	H_2O	$AlCl_3$	H_2O	$AlCl_3$
0	3.72 ± 0.08	3.72 ± 0.08	58.1 ± 1.3	58.1 ± 1.3
1	3.65 ± 0.02	3.62 ± 0.03	57.0 ± 1.7	56.5 ± 1.5
2	3.58 ± 0.22	2.98 ± 0.07	49.8 ± 2.7	43.7 ± 1.1
3	2.78 ± 0.19	1.31 ± 0.06	31.5 ± 1.7	22.2 ± 1.0

MDA is routinely used as an indicator of lipid peroxidation. MDA content in $AlCl_3$-treated detached rice leaves was higher than in the controls throughout the entire duration of incubation (Table 3). The increase in MDA content in detached rice leaves preceded the decrease of both chlorophyll and protein contents (Tables 2 and 3). H_2O_2 content increased significantly in detached rice leaves incubated in water (Table 3). However, H_2O_2 content in $AlCl_3$-treated detached rice leaves remained unchanged during the first 2 d of incubation and increased slightly at day 3 of incubation (Table 3). Clearly, $AlCl_3$ resulted in a lower content of H_2O_2 than controls. No accumulation of H_2O_2 was also observed in drought-, excess Cd-, excess Fe-, and NaCl-treated leaves

Table 3. Time courses of MDA and H_2O_2 content in detached rice leaves treated with $AlCl_3$ (5 mM, pH 4.0) or distilled water (pH 4.0) in the light. Means ± SD (*n* = 4).

Time	MDA [nmol g^{-1}(f. m.)]		H_2O_2 [nmol g^{-1}(f. m.)]	
[d]	H_2O	$AlCl_3$	H_2O	$AlCl_3$
0	28.0 ± 1.4	28.0 ± 1.4	35.7 ± 1.3	35.7 ± 1.3
1	25.2 ± 1.4	36.6 ± 2.3	44.4 ± 0.7	38.6 ± 1.4
2	42.6 ± 1.3	50.1 ± 3.0	47.9 ± 1.7	38.1 ± 0.6
3	50.9 ± 2.1	76.6 ± 4.9	52.3 ± 0.9	40.7 ± 0.8

(Moran *et al.* 1994, Lin and Kao 2000, Chien *et al.* 2001, Fang *et al.* 2001).

The greater increase in MDA content in $AlCl_3$-treated detached rice leaves may be a reflection of a change in the activities of antioxidative enzymes. $AlCl_3$-treated detached rice leaves had lower activity of SOD than the controls. However, CAT and GR activities were observed to be higher in $AlCl_3$-treated detached rice leaves than control leaves (Table 4). $AlCl_3$ treatment also resulted in a higher POX and APX antitivities than control leaves at the later stage of incubation (Table 4). It has been reported that Al increased SOD and POX activities and decreased CAT activity in soybean root tips (Cakmak and Horst 1991). Ezaki *et al.* (1996) demonstrated that a moderately anionic POX (pI 6.7) and two cationic POX (pI 9.2 and 9.7) were activated by Al treatment and Pi starvation in tobacco cells.

SOD converts superoxide radicals to H_2O_2. The lower activity of SOD in $AlCl_3$-treated rice leaves (Table 4) would result in an increase in superoxide radicals. The superoxide radicals can react with H_2O_2 to form more powerful hydroxyl radicals in the presence of endogenous Fe (Thompson *et al.* 1987). It is possible that H_2O_2 is being utilized by reacting with superoxide radicals in $AlCl_3$-treated detached rice leaves. This would explain why H_2O_2 did not accumulate in detached rice leaves exposed to $AlCl_3$. CAT, POX, and APX are enzymes responsible for elimination H_2O_2. Since $AlCl_3$ treatment resulted in a greater CAT, POX, and APX activities than the control, the possibility that H_2O_2 is being removed by CAT, POX, and APX in $AlCl_3$-treated detached rice leaves cannot be excluded.

Table 4. Time course of antioxidative enzyme activities [U mg^{-1}(protein)] in detached rice leaves treated with $AlCl_3$ (5 mM, pH 4.0) or H_2O (pH 4.0) in the light. Means ± SD (n = 4).

Time	SOD		APX		GR		CAT		POX	
[d]	H_2O	$AlCl_3$	H_2O	$AlCl_3$	H_2O	$AlCl_3$	H_2O	$AlCl_3$	H_2O	$AlCl_3$
0	2.58±0.10	2.58±0.10	1.08±0.07	1.08±0.07	0.12±0.01	0.12±0.01	0.22±0.04	0.22±0.04	0.21±0.01	0.21±0.01
1	3.50±0.03	1.01±0.12	1.32±0.16	1.50±0.14	0.14±0.02	0.22±0.02	0.18±0.02	0.25±0.02	0.41±0.01	0.52±0.05
2	5.21±0.20	0.92±0.02	1.76±0.09	2.46±0.13	0.20±0.01	0.26±0.01	0.20±0.02	0.24±0.01	0.63±0.02	0.91±0.15
3	4.49±0.15	1.07±0.05	2.22±0.19	3.06±0.24	0.22±0.01	0.33±0.03	0.19±0.02	0.24±0.02	1.03±0.01	4.51±0.67

References

Akeson, M.A., Munns, D.N., Burau, R.G.: Adsorption of Al^{3+} to phosphatidylcholine vesicles. - Biochim. biophys. Acta **986**: 33-40, 1989.

Bradford, M.M.: 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, 1976.

Cakmak, I., Horst, W.J.: Effect of aluminum on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). - Physiol. Plant. **83**: 463-468, 1991.

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**: 205-313, 2001.

Deleers, M., Servais, J.P., Wülfert, E.: Neurotoxic cations induce membrane rigidification and membrane fusion at micromolar concentrations. - Biochim. biophys. Acta **855**: 271-276, 1986.

Ezaki, B., Yamamoto, Y., Matsumoto, H.: Cloning and sequencing of the cDNAs induced by aluminum treatment and Pi starvation in cultured tobacco cells. - Physiol. Plant. **93**: 11-18, 1995.

Ezaki, B., Tugita, S., Matsumoto, H.: Expression of a moderately anionic peroxidase is induced by aluminum treatment in tobacco cells: possible involvement of peroxidase isozymes in aluminum ion stress. - Physiol. Plant. **96**: 21-28, 1996.

Fang, W.-C., Wang, J.-W., Lin, C.C., Kao, C.H.: Iron induction of lipid peroxidation and effects on antioxidative enzyme activities in rice leaves. - Plant Growth Regul. **35**: 75-80, 2001.

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

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

Horst, W.J., Asher, C.J., Cakmak, I., Szulkiewica, P., Wissemeier, A.H.: Short-sterm responses of soybean roots to aluminum. - J. Plant Physiol. **140**: 174-178, 1992.

Ikegawa, H., Yamamoto, Y., Matsumoto, H.: Responses to aluminum of suspension-cultured tobacco cells in a single cadmium solution. - Soil Sci. Plant Nutr. **46**: 503-514, 2000.

Jana, S., Choudhuri, M.A.: Glycolate metabolism of three submerged aquatic angiosperm during aging. - Aquat. Bot. **12**: 345-354, 1981.

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

Lin, C.C., Kao, C.H.: Effect of NaCl stress on H_2O_2 metabolism in rice leaves. - Plant Growth Regul. **30**: 151-155, 2000.

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**: 85-88, 1999.

MacAdam, J.W., Nelson, C.J., Sharp, R.E.: Peroxidase activity in the leaf elongation zone of tall fescue. I. Spatial distribution of ionically bound peroxidase activity in genotypes differing in length of elongation zone. - Plant Physiol. **99**: 872-878, 1992.

Moran, J.F., Becana, M., Iturbe-Ormaetxe, I., Frechilla, S., Klucas, R.V., Aspariciv-Tejo, P.: Drought induces oxidative stress in pea plants. - Planta **194**: 346-352, 1994.

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

Ono, K., Yamamoto, Y., Hachiya, A., Matsumoto, H.: Synergistic inhibition of growth by aluminum and iron of tobacco (*Nicotian tobacum* L.) cells in suspension culture. - Plant Cell Physiol. **36**: 115-125, 1995.

Oteiza, P.I.: A mechanism for the stimulatory effect of aluminum on iron-induced lipid peroxidation. - Arch. Biochem. Biophys. **308**: 374-379, 1994.

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

Richards, K.D., Schott, E.J., Sharma, Y.K., Davis, K.R., Gardner, R.C.: Aluminum induces oxidative stress genes in *Arabidopsis thaliana*. - Plant Physiol. **116**: 409-418, 1998.

Sakihama, Y., Yamasaki, H.: Lipid peroxidation induced by phenolics in conjunction with aluminum ions. - Biol. Plant. **45**: 249-254, 2002.

Snowden, K.C., Gardner, R.C.: Five genes induced by aluminum in wheat (*Triticum aestivum* L.) roots. - Plant Physiol. **103**: 855-861, 1993.

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

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.

Yamaguchi, Y., Yamamoto, Y., Matsumoto, H.: Cell death process initiated by a combination of aluminum and iron in suspension-cultured tobacco cells (*Nicotiana tabacum*): apoptosis-like cell death mediated by calcium and proteinase. - Soil Sci. Plant Nutr. **45**: 647-657, 1999.

Yamamoto, Y., Hachiya, A., Matsumoto, H.: Oxidative damage to membranes by a combination of aluminum and iron in suspension-cultured tobacco cells. - Plant Cell Physiol. **38**: 1333-1339, 1997.

Yamamoto, Y., Kobayashi, Y., Matsumoto, H.: Lipid peroxidation is an early symptom triggered by aluminum, but not the primary cause of elongation inhibition in pea roots. - Plant Physiol. **125**: 199-208, 2001.