Plant and Soil **230:** 135–143, 2001.

Cell wall peroxidase activity, hydrogen peroxide level and NaCl-inhibited root growth of rice seedlings

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Received 20 August 1999. Accepted in revised form 10 November 2000

Key words: hydrogen peroxide, NaCl, *Oryza sativa*, peroxidase, root growth

Abstract

The changes in cell-wall peroxidase (POD) activity and H_2O_2 level in roots of NaCl-stressed rice seedlings and their correlation with root growth were investigated. Increasing concentrations of NaCl from 50 to 150 m*M* progressively reduced root growth and increased ionically bound cell-wall POD activity. NaCl had no effect on covalently bound cell-wall POD activities. The reduction of root growth by NaCl is closely correlated with the increase in H_2O_2 level. Exogenous H_2O_2 was found to inhibit root growth of rice seedlings. Since ammonium and proline accumulation are associated with root growth inhibition caused by NaCl, we determined the effects of NH_4Cl or proline on root growth, cell-wall POD activity and H_2O_2level in roots. External application of NH_4Cl or proline markedly inhibited root growth, increased cell-wall POD activity and increased H_2O_2 level in roots of rice seedlings in the absence of NaCl. An increase in cell-wall POD activity and H_2O_2 level preceded inhibition of root growth caused by NaCl, NH_4Cl or proline. NaCl or proline treatment also increased NADH-POD and diamine oxidase (DAO) activities in roots of rice seedlings, suggesting that NADH-POD and DAO contribute to the H_2O_2 generation in the cell wall of NaCl- or proline-treated roots. NH_4Cl treatment increased NADH-POD activity but had no effect on DAO activity, suggesting that NADH-POD but not DAO is responsible for H_2O_2 generation in cell wall of NH_4Cl-treated roots.

Abbreviations: DAO – diamine oxidase; DW – dry weight; FW – fresh weight; POD – peroxidase

Introduction

Peroxidase (POD, EC 1.11.1.7) influences plant growth through lignin synthesis (Siegel, 1953), oxidative coupling reactions involving phenolics that are esterified to cell wall polysaccharides (Fry, 1986), formation of isodityrosine bridges that are believed to crosslink structural protein molecules (Fry, 1986). An inverse relationship between growth rate and POD activity has been reported in many plant systems (Carpita and Gilbeaut, 1993; Chen and Kao, 1995; Fry, 1986; Gardiner and Cleland, 1974; Lee and Lin, 1995;). Thus POD is generally believed to be a putative wall rigidification enzyme (Cosgrove, 1997).

The inhibition of plant growth by salinity is a widespread problem in agricultural practice. However, the mechanisms underlying this inhibition are not yet clear (Munns, 1993; Rengel, 1992). Neumann et al. (1994) demonstrated that root growth inhibition caused by salinity was associated with wall stiffening. Recently, we have demonstrated that an increase in ionically bound POD activity is associated with growth inhibition of rice seedling roots caused by NaCl (Lin and Kao, 1999). The ionically bound fraction of POD, removable from homogenized tissues with high ionic strength buffers, has been equated with the cell wall fraction. However, Mader et al. (1986) suggested that at least some ionically bound POD activity might be an artifact of homogenization. Therefore, to test the hypothesis that an increase in cell-wall POD activity is associated with growth in-

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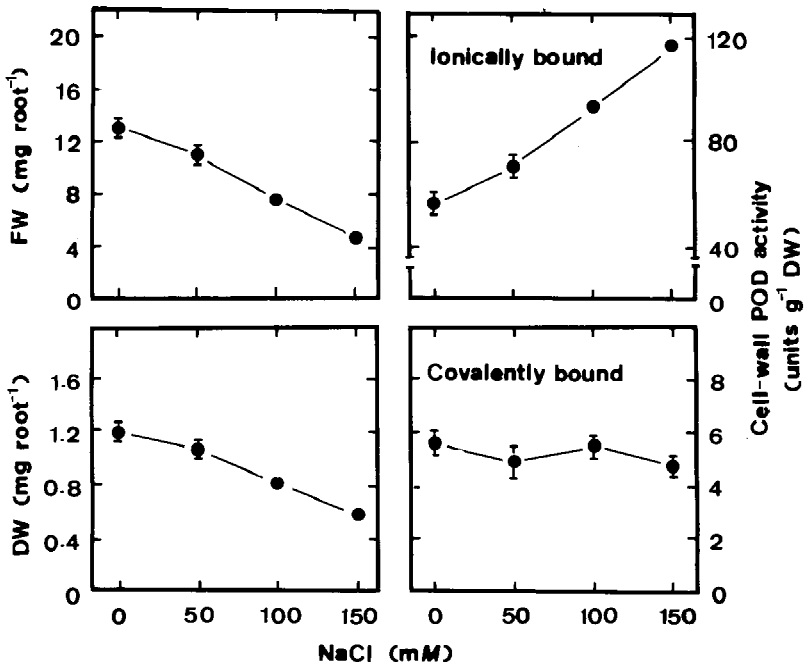

Figure 1. Effects of NaCl on root growth and cell-wall POD activities in roots of rice seedlings. Root growth and cell wall POD activities were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

hibition of seedling roots of rice, data on POD activity extracted from the cell walls are required.

H_2O_2 is a necessary substrate for a cell wall stiffening process catalyzed by POD (Elstner and Heupel, 1976; Hohl et al., 1995; Schopfer, 1994). Recently, Schopfer (1996) demonstrated that H_2O_2 inhibited auxin-mediated growth of maize coleoptiles. The formation of H_2O_2 by isolated cell walls from horseradish has been reported (Elstner and Heupel, 1976). Using a sensitive tissue-print assay, Schopfer (1994) demonstrated that H_2O_2 is localized in the cell wall of pea epicotyls. H_2O_2 causes a rapid cross-linking of cell-wall polymers (Bradley et al., 1992; Schopfer, 1996). Therefore, a sufficient supply of H_2O_2 is required for ensuring complete stiffening of the cell wall. The present investigation was designed to study the changes in cell-wall POD activity and H_2O_2 level in roots of NaCl-stressed rice seedlings and their correlation with root growth.

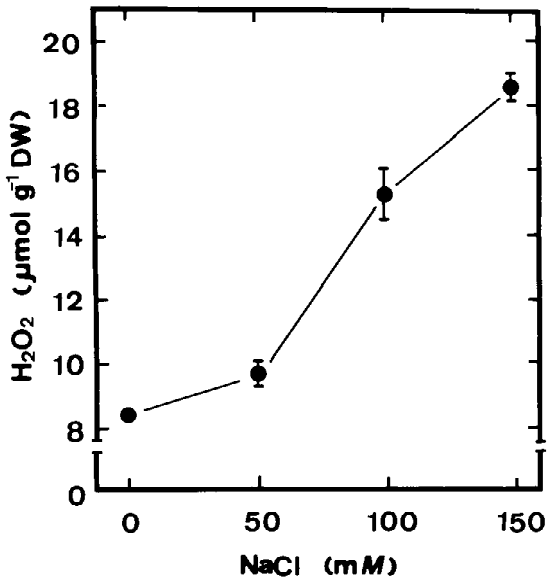

Figure 2. Effects of NaCl on H_2O_2 levels in roots of rice seedlings. H_2O_2 was determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

Materials and methods

Rice (*Oryza sativa L.*, cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in a Petri dish (20 cm) containing distilled water at 37 °C under dark condition. After 1-day incubation, uniformly germinated seeds were selected and transferred to a Petri dish (9.0 cm) containing two sheets of Whatman No.1 fil-

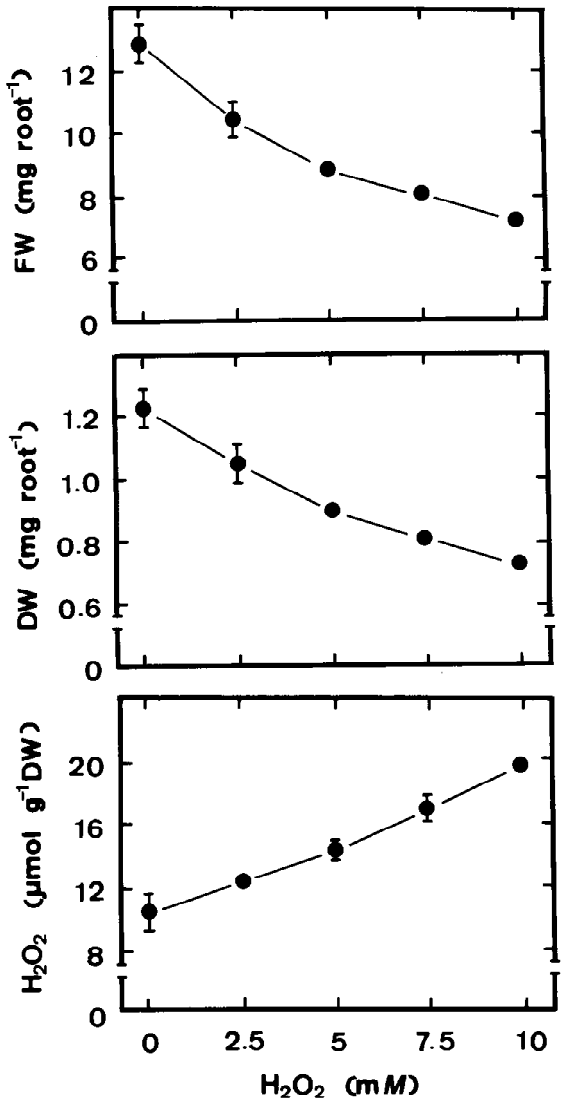

Figure 3. Effects of exogenous application of H_2O_2 on root growth and H_2O_2 levels in roots of rice seedlings. Root growth and H_2O_2 levels were determined after 5 days of treatment. Vertical bars represent standard errors (*n*=4).

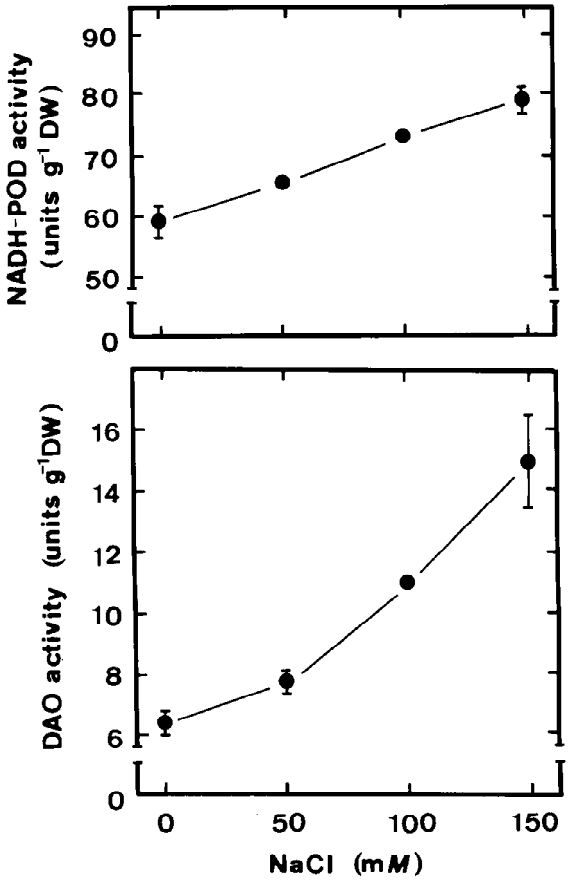

Figure 4. Effects of NaCl on NADH-POD and DAO activities in roots of rice seedlings. NADH-POD and DAO activities were determined after 5 days of treatment. Vertical bars represent standard errors (*n*=4).

ter paper moistened with 10 ml of distilled water or test solutions. Each Petri dish contained 20 germinated seeds. Each treatment was replicated four times. The germinated seeds were allowed to grow at 27 °C in darkness and 3 ml of distilled water or test solutions was added to each Petri dish on day 3 of the growth. Fresh weight and dry weight of roots were measured at the times indicated.

Cell walls were prepared by homogenizing roots in ice-cold phosphate buffer (50 m*M*, pH 5.8) using a pestle and mortar. The homogenate was centrifuged at 1000 *g*, and washed at least four times with 50 m*M* phosphate buffer (Lee and Lin, 1995). The pellet was collected and used as a cell wall fraction.

POD ionically bound to the cell walls was extracted with 1 *M* NaCl. Cell walls were prepared as described above and incubated in 1 *M* NaCl for 2 h with shaking at 30 °C and centrifuged at 1000 *g*. The supernatant was considered as the ionic cell wall fraction. POD covalently bound to the cell walls was extracted as follows: Cell walls previously subjected to the saline extraction procedure were incubated in an enzyme preparation containing 0.5% cellulase (EC 3.2.1.4, Sigma) and 2.5% pectinase (EC 3.2.1.15, Sigma), both from *Aspergillus niger*, in 0.1 *M* sodium acetate buffer, pH 5.0 (Sanchez et al., 1989). The incubation was carried out for 24 h with shaking at 25 °C. The suspension was then centrifuged at 1000 *g*

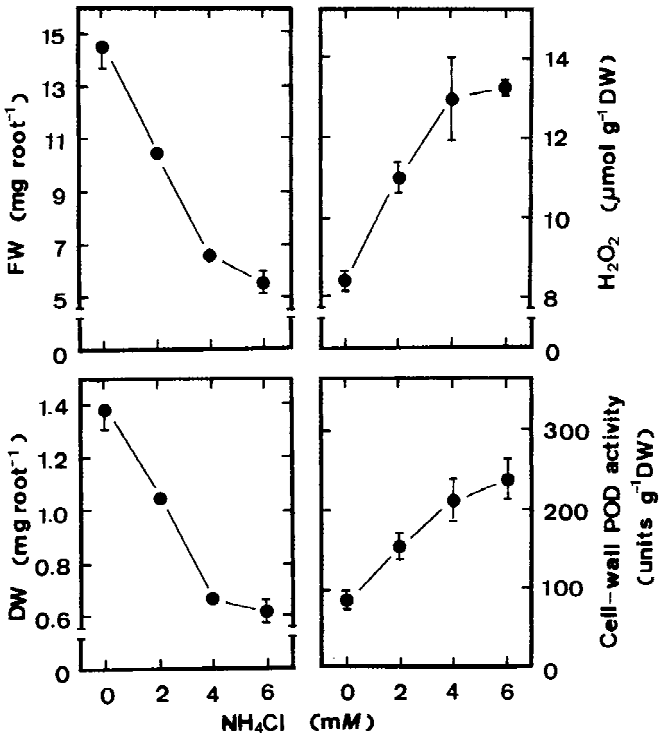

Figure 5. Effects of NH_4Cl on root growth, cell-wall POD activities and H_2O_2 levels in roots of rice seedlings. Root growth, cell-wall POD activities and H_2O_2 levels were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

for 10 min. The supernatant was considered to be the covalently bound cell wall fraction.

POD activities were measured using a modification of the procedure described by Curtis (1971). The assay medium contained 0.05 *M* phosphate buffer (pH 5.8), 7.2 m*M* guaiacol, 11.8 m*M* H_2O_2 and 0.1 ml enzyme extract in a final assay volume of 3.0 ml. The reaction was initiated by the addition of H_2O_2 and the change in absorbance at 470 nm was measured. Activity was calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm) for tetraguaiacol. One unit of POD was defined as the amount of enzyme that causes the formation of 1 μmol tetraguaiacol per min.

NADH-POD activities in the ionic cell wall fraction were determined according to the method of Ishida et al. (1987). The assay mixture contained 50 μM NADH in Na-acetate buffer (30 m*M*, pH 6.5), 5 m*M* $MnCl_2$ and 20 μM *p*-coumaric acid. The reaction was started by adding the enzyme, and the decrease of absorbance at 340 nm by oxidizing NADH was measured at 25 °C. One unit of NADH-POD was defined as 1 nmol NADH oxidized per min.

Diamine oxidase (DAO), which is involved in polyamine catabolism, oxidizes putrescine with the formation of Δ^1-pyrrolinetogether with H_2O_2 and ammonia (Smith, 1985). DAO activities in the ionic cell wall fraction were measured by the method of Naik et al. (1981). The incubation mixture contained 50 m*M* phosphate buffer (pH 7.8), 10 m*M* putresine, 0.1 m*M* pyridoxal phosphate and enzyme extract in a total volume of 4 ml. After incubation at 30 °C for 1 h, the reaction was terminated using 1 ml 20% (w/v) trichloroacetic acid. After 30 min, the incubation mixture was centrifuged at 5000 *g* for 15 min. One ml of ninhydrin mixture (250 mg ninhydrin in 6 ml acetic acid and 4 ml phosphoric acid) was added to the supernatant. Colour was developed at 100 °C for 30 min. After adding 1 ml of acetic acid absorbance was measured at 510 nm. In controls, trichloroacetic acid added prior to the enzyme solution. One unit of DAO was defined as an increase of 1 A_{510} per h.

The H_2O_2 level was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 10 roots with 3 ml of phosphate buffer (50 m*M*, pH 6.8). The homogenate was centrifuged at 6000 *g* for 25 min. To determine H_2O_2 levels, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium chloride (Aldrich) in 20%

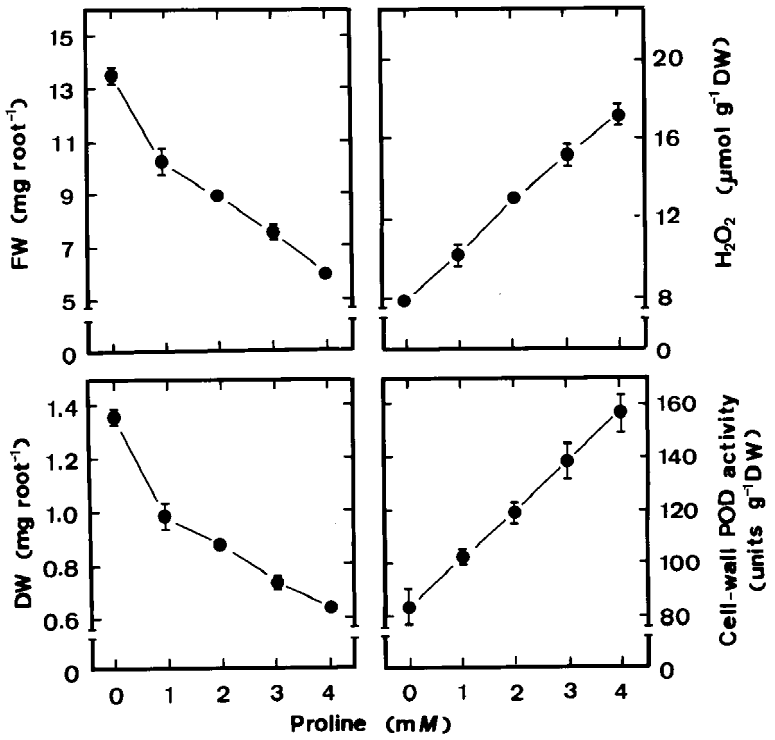

Figure 6. Effects of proline on root growth, cell-wall POD activities and H_2O_2 levels in roots of rice seedlings. Root growth, cell wall-POD activities and H_2O_2 levels were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

(v/v) H_2SO_4 and the mixture was then centrifuged at 6000 *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 μmol^{-1}cm^{-1}.

For all measurements, each treatment was repeated four times. 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

Root growth was followed by measuring FW and DW of roots. Figure 1 shows the effect of NaCl on root growth of rice seedlings. Increasing concentrations of NaCl from 50 to 150 m*M* progressively decreased root growth.

Both ionically bound and covalently bound cell-wall PODs were present in cell walls of rice roots (Figure 1). The reduction of root growth with increasing NaCl concentrations is correlated with an increase in ionically bound cell-wall POD activity (Figure 1). The effect of different concentrations of NaCl on covalently bound cell- wall POD activities was also examined. No appreciable changes in covalently bound cell-wall POD activities occurred between 0 and 150 m*M* NaCl. Thus, ionically bound POD in the cell wall preparations was assayed in all subsequent experiments.

It has been postulated that the action of POD located in the cell walls would be to confer rigidity to the cell walls and prevent later expansion involved in growth (Carpita and Gilbeaut, 1993; Fry, 1986; Gardiner and Cleland, 1974; MacAdam et al., 1992). Thus, NaCl-induced inhibition in root growth of rice seedlings is likely due to cell wall stiffening process related to the formation of cross-linking among cell wall polymers. This process appears to involve oxidative coupling, dependent on H_2O_2 (Fry, 1986). In fact, H_2O_2 has been demonstrated to cause a rapid cross-linking of cell wall polymers (Bradley et al., 1992; Schopfer, 1996). If POD regulates cell wall stiffening by catalyzing the oxidative cross-linking of cell wall polymers, there must be a sufficient supply of H_2O_2. Thus, it is of great interest to know whether NaCl increases the level of H_2O_2 in roots of rice seedlings.

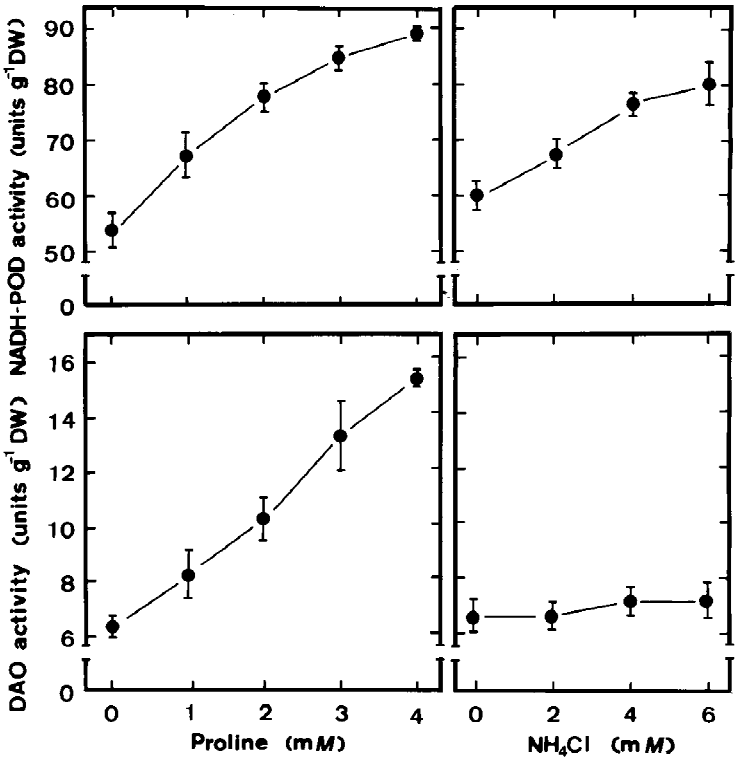

Figure 7. Effects of NH_4Cl or proline on NADH-POD and DAO activities in roots of rice seedlings. NADH-POD and DAO activities were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

Figure 2 shows the effect of NaCl concentrations on H_2O_2 levels in the roots of rice seedlings. Increasing concentrations of NaCl from 50 to 150 m*M* progressively increased H_2O_2 levels in roots. If H_2O_2 plays a role in regulating NaCl-inhibited root growth of rice seedlings, exogenous application of H_2O_2 is expected to inhibit root growth. It is indeed the case (Figure 3). Increasing concentrations of H_2O_2 from 2.5 to 10 m*M* progressively increased endogenous H_2O_2 levels in roots of rice seedlings and decreased FW and DW. Clearly, an increase in H_2O_2 levels is important in regulating NaCl-inhibited root growth of rice seedlings.

Generation of H_2O_2 in the cell walls has been previously proposed since it is a necessary substrate for the formation of cross-linking among cell wall polymers (Elstner and Heupel, 1976) and cell wall-bound malate dehydrogenase and NADH-POD activities devoted to H_2O_2 production have been detected (Goldberg et al., 1987; Gross, 1977; Mader et al., 1980). As expected, increasing NADH-POD activities were found to increase with increasing NaCl concentrations in rice seedling roots (Figure 4). Concerning the origin of the NADH required in the formation of H_2O_2, it has been postulated that a cell wall-bound malate dehydrogenase provides this electron donor (Gross, 1977). However, we have not succeeded in detecting cell wall-bound malate dehydrogenase activity in roots of rice seedlings. Frahry and Schopfer (1998) also failed to detect cell wall-bound malate dehydrogenase activity in intact soybean roots.

DAO is widespread in the Leguminosae family (Smith, 1985) and has been reported in barley (Cogoni et al., 1990) and maize (Suzuki and Hagiwara, 1993). This enzyme, which is involved in polyamine catabolism, oxidizes putrescine with the formation of Δ^1-pyrroline together with H_2O_2 and ammonia (Smith, 1985). DAO activity is mainly located in the cell wall (Angelini and Federico, 1989; Angelini et al., 1990) and perhaps plays a role in regulating putrescine levels (Smith, 1985) or providing H_2O_2 required for peroxidative reactions that occur in the cell walls for the formation of cross-linking (Angelini and Federico, 1989; Angelini et al., 1990). It is most likely that DAO is another source leading to H_2O_2 generation in NaCl-inhibited root growth of rice seedlings. To test this,

Table 1. Changes in root growth, cell-wall POD activity, H_2O_2 level, DAO activity and NADH-POD activity in roots of rice seedlings treated with NaCl. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and NaCl (150 mM), respectively. The data represent mean value ± standard errors, *n*=4

	Treatment	Time, h				
		0	4	8	12	16
FW	H_2O	7.1±0.42	7.6±0.71	8.1±0.35	8.6±0.10	9.4±0.23
(mg root^{-1})	NaCl		7.5±0.28	7.7±0.47	8.1±0.20	8.5±0.36
DW	H_2O	0.67±0.04	0.72±0.03	0.77±0.03	0.82±0.01	0.89±0.02
(mg root^{-1})	NaCl		0.71±0.03	0.73±0.05	0.77±0.02	0.80±0.03
Cell-wall POD	H_2O	38.3±0.82	36.7±1.8	38.9±2.5	38.0±1.1	39.0±1.4
(units g^{-1}DW)	NaCl		41.1±5.9	49.6±3.8	57.3±2.0	65.1±3.4
H_2O_2	H_2O	6.6±0.39	6.7±0.62	6.6±0.16	7.7±0.95	7.7±0.20
(μmol g^{-1} DW)	NaCl		7.0±0.58	8.7±0.72	9.2±0.46	10.4±0.12
DAO	H_2O	6.6±0.66	5.9±0.82	6.6±0.71	7.3±0.76	7.6±0.57
(units g^{-1} DW)	NaCl		7.0±0.68	8.2±0.47	8.6±0.20	9.5±0.36
NADH-POD	H_2O	54.3±3.2	52.3±1.7	54.0±2.4	55.6±0.52	59.8±3.7
(units g^{-1} DW)	NaCl		54.1±2.9	58.5±0.55	60.3±1.7	66.4±4.1

Table 2. Changes in root growth, cell-wall POD activity, H_2O_2 level and NADH-POD activity in roots of rice seedlings treated with NH_4Cl. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and NH_4Cl (4 mM), respectively. The data represent mean values ± standard errors, *n*=4

		Time, h				
		0	4	8	12	16
FW (mg root^{-1})	H_2O	6.8±0.60	7.1±0.31	7.6±0.16	8.9±0.36	9.7±0.32
	NH_4Cl		7.2±0.19	7.6±0.09	7.9±0.09	8.2±0.45
DW (mg root^{-1})	H_2O	0.64±0.06	0.67±0.03	0.72±0.01	0.84±0.03	0.92±0.03
	NH_4Cl		0.68±0.02	0.72±0.01	0.75±0.01	0.78±0.04
Cell-wall POD (unit g^{-1}DW)	H_2O	45.1±5.4	45.9±4.2	45.9±3.0	43.6±5.9	46.4±1.3
	NH_4Cl		47.8±1.7	53.7±2.1	60.9±3.2	68.9±3.8
H_2O_2 (μmol g^{-1} DW)	H_2O	6.7±0.49	7.0±0.72	6.9±0.12	7.5±0.73	8.3±0.49
	NH_4Cl		7.5±0.32	8.4±0.21	10.1±0.65	11.6±0.31
NADH-POD (units g^{-1} DW)	H_2O	50.3±3.4	51.3±3.8	52.3±2.1	53.1±1.8	56.1±1.4
	NH_4Cl		53.2±2.9	57.2±0.38	58.2±1.4	61.4±2.1

we determined the activities of DAO in rice seedling roots in response to various concentrations of NaCl (Figure 4). As expected, increasing concentrations of NaCl from 50 to 150 mm progressively increased DAO activities. This result is consistent with our previous work in which we showed that NaCl treatment resulted in a decline in the levels of putrescine in roots of rice seedlings (Lin and Kao, 1995).

To test the causal relationship between root growth reduction, cell-wall POD activity, H_2O_2 level, DAO activity and NADH-POD activity caused by NaCl, 2-day-old seedlings were transferred to distilled wa-

Table 3. Changes in root growth, cell-wall POD activity, H_2O_2 level, DAO activity and NADH-POD activity in roots of rice seedlings treated with proline. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and proline (4 mM), respectively. The data represent mean values ± standard errors, n=4

		Time, h				
		0	4	8	12	16
FW (mg root^{-1})	H_2O	6.8±0.60	7.1±0.31	7.6±0.16	8.9±0.36	9.7±0.32
	proline		7.4±0.21	7.9±0.36	7.7±0.17	7.9±0.20
DW (mg root^{-1})	H_2O	0.64±0.06	0.67±0.03	0.72±0.01	0.84±0.03	0.92±0.03
	proline		0.70±0.02	0.75±0.03	0.73±0.0.2	0.75±0.02
Cell-wall POD (units g^{-1}DW)	H_2O	45.1±5.4	45.9±4.2	45.9±3.0	43.6±5.9	46.4±1.3
	proline		52.2±5.3	60.0±2.1	69.3±2.1	80.2±3.5
H_2O_2 (μmol g^{-1} DW)	H_2O	6.7±0.49	7.0±0.72	6.9±0.12	7.5±0.73	8.3±0.49
	proline		7.2±0.18	8.2±0.35	9.2±0.35	10.8±0.66
DAO (units g^{-1} DW)	H_2O	6.2±0.55	6.3±0.49	6.7±0.68	7.2±0.56	7.7±0.37
	proline		6.4±0.59	8.0±0.23	8.3±0.37	9.0±0.44
NADH-POD (units g^{-1} DW)	H_2O	54.3±3.8	55.3±2.6	55.0±2.6	58.1±2.4	60.5±3.2
	proline		56.2±2.3	59.2±0.77	64.3±2.8	67.2±2.3

ter and NaCl, respectively, for 4, 8, 12 and 16 h. Changes in root growth, cell-wall POD activity, H_2O_2 level, DAO activity and NADH-POD activity were then monitored. As indicated in Table 1, an increase in cell-wall POD activity and H_2O_2 level preceded inhibition of root growth caused by NaCl. The observations that an increase in DAO and NADH-POD activities coincides with an increase in H_2O_2 level in roots caused by NaCl (Table 1) suggest that DAO and NADH-POD are the sources for the generation of H_2O_2 in the cell walls. Since H_2O_2 can rapidly pass from the cytoplasm to the cell wall (Allan and Fluhr 1997), a cytoplasmic origin of released H_2O_2 cannot be excluded.

It is known that ammonium strongly inhibits the growth of many plants (Haynes and Goh, 1978). Exogenous application NH_4Cl was also found to reduce root growth of rice seedlings (Lin and Kao, 1996a). It has also been shown that NaCl was effective in stimulating the accumulation of ammonium in roots of rice seedlings and that accumulation of ammonium in roots preceded inhibition of root growth caused by NaCl (Lin and Kao, 1996a). If the increases in cell-wall POD activity and H_2O_2level are important in regulating growth reduction of roots caused by NaCl, the exogenous application of NH_4Cl would be expected to increase cell-wall POD activity and H_2O_2level in roots of rice seedlings. Figure 5 shows that addition of NH_4Cl inhibits root growth, increases cell-wall POD activity and increases H_2O_2 level in roots.

In previous work, we have shown that proline accumulation is associated with root growth inhibition of rice seedlings caused by NaCl (Lin and Kao, 1996b). In the present study, we also demonstrated that proline treatment resulted in an inhibition of root growth, an increase in cell-wall POD activity and an increase in H_2O_2 level (Figure 6). The data in Figure 7 clearly show that DAO and NADH-POD are responsible for the generation of H_2O_2 in the cell wall of proline-treated roots, whereas NADH-POD but not DAO is the source for the generation of H_2O_2 in NH_4Cl-treated roots. Furthermore, we also observed that an increase in cell-wall POD activity and H_2O_2 level preceded inhibition of root growth caused by NH_4Cl or proline and an increase in H_2O_2level coincided with an increase in NADH-POD or DAO activity (Tables 2 and 3).

The observations that rice seedlings treated with NH_4Cl or proline, which resulted in an increase in cell-wall POD activity and H_2O_2 level in roots, reduced root growth in the same way that NaCl did, further support our suggestion that cell-wall POD and H_2O_2 are likely participated in the regulation of root growth reduction of rice seedlings under NaCl condition.

Acknowledgement

This work was supported by the National Science Council of the Republic of China (NSC 89-2313-B-002-009).

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Section editor: H. Lambers