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Osmotic stress-induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings

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Abstract

The changes in activity of peroxidase (POD) extracted from the cell walls and the level of H_2O_2 in rice seedling roots treated with mannitol and their correlation with root growth were investigated. Increasing concentrations of mannitol from 92 to 276 mM, which is iso-osmotic with 50 to 150 mM NaCl, progressively reduced root growth and increased POD activities extracted from the cell walls of rice roots. The reduction of growth was also correlated with an increase in H_2O_2 level. Both diamine oxidase (DAO) and NADH peroxidase (NADH-POD) are known to be responsible for the generation of H_2O_2. Mannitol treatment increased DAO but not NADH-POD activities in roots of rice seedlings, suggesting that DAO contributes to the generation of H_2O_2 in the cell walls of mannitol-treated roots. An increase in the level of H_2O_2 and the activity of POD extracted from the cell walls of rice roots preceded root growth reduction caused by mannitol. An increase in DAO activity coincided with an increase in H_2O_2 in roots caused by mannitol. Since DAO catalyses the oxidation of putrescine, the demonstration that mannitol increases the activity of DAO in roots is consistent with those that mannitol decreases the level of putrescine. In conclusion, cell-wall stiffening catalysed by POD is possibly involved in the regulation of root growth reduction caused by mannitol.

Abbreviations: DAO – diamine oxidase, DW – dry weight, POD – peroxidase, FW – fresh weight, Put – putrescine, Spd – spermidine, Spm – spermine

Introduction

Osmotic stress, salinity stress, and water stress in the root environment can each result in inhibition of growth in crop plants. An understanding of the mechanisms regulating growth inhibition may facilitate the selection of crop plants which are more stress resistant (Boyer 1982). Cell growth can be limited by the osmotic potential of the growing cell, the conductivity of the water uptake pathway, and the extensibility of growing cell wall. These can affect the cell turgor pressure, which is required for wall expansion. Evidence has been presented to show that growth inhibition in response to salinity, osmotic, and water stress can occur without reduction of turgor pressure (Thiel et al. 1988; Neumann et al. 1994; Nonami and Boyer 1990; Spollen and Sharp 1991; Serpe and Mathews 1992). Inhibition of cell growth could then be associated with a stiffening of expanding cell walls rather than inability to maintain turgor pressure.

There are reports showing that osmotic stress induces cell-wall stiffening of leaves or hypocotyls (Chazen and Neumann 1994; Neumann et al. 1994; Nonami and Boyer 1990). On the other hand, osmotic stress that suppresses cell-wall stiffening in plant tissues has also been reported (Acevedo et al. 1971; Sakurai et al. 1987; Sakurai and Kuraishi 1988; Kutschera 1989; Wakabayashi et al. 1997). One of the processes of cell wall stiffening is related to formation of cross-links between cell wall polysaccharides which is mediated by cell wall-associated peroxidative enzymes (Fry 1986). There are many reports

showing that peroxidase (POD) activity extracted from the cell walls is inversely related to cell growth (Goldberg et al. 1987; MacAdam et al. 1992; Chen and Kao 1995; Hohl et al. 1995; Lee and Lin 1995; Bacon et al. 1997). Recently, we have shown that cell-wall POD is associated with growth inhibition of seedling roots of rice caused by NaCl (Lin and Kao 2001). Thus, it is expected that cell-wall POD may also participate in the regulation of root growth reduction of rice seedlings under mannitol-induced osmotic stress conditions.

H_2O_2 is a necessary substrate for the cell wall stiffening process catalysed by peroxidase (Schopfer 1994; Hohl et al. 1995). Using a sensitive tissue-print assay, Schopfer (1994) has been able to demonstrate that H_2O_2 is localized in the cell wall of pea epicotyls. The formation of H_2O_2 by isolated cell walls from horseradish has been reported (Elstner and Heupel 1976). It has been shown that H_2O_2 inhibits auxin-mediated growth of maize coleoptile segments (Schopfer 1996) and H_2O_2 causes a rapid cross-linking of cell-wall polymers (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 POD activity extracted from the cell walls and H_2O_2 level in roots of mannitol-treated rice seedlings and their correlation with root growth.

Materials and methods

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilised 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 in the dark. After 1-day of incubation, uniformly germinated seeds were selected and transferred to Petri dishes (9.0 cm) containing two sheets of Whatman No. 1 filter paper moistened with 10 ml of distilled water or test solutions. Each Petri dish contained 20 germinated seeds. Each treatment was replicated 4 times. The germinated seeds were allowed to grow at 27 °C for 5 days in darkness. To avoid water loss by evaporation and uptake by the seeds, a further 3 ml of distilled water or test solutions was added to each Petri dish on day 3. Fresh weight (FW), dry weight (DW), POD (EC 1.11.1.7), DAO (EC 1.4.3.6), NADH-POD (EC 1.11.1.7), H_2O_2 and polyamines were measured at the times indicated.

Cell walls were prepared by homogenising the roots in ice-cold phosphate buffer (50 mM, pH 5.8) using a pestle and mortar. The homogenate was centrifuged at 1,000 *g*, and washed at least four times with 50 mM phosphate buffer (Lee and Lin 1995). The pellet was collected and used as a cell wall fraction.

POD ionically bound to the cell wall was extracted with 1 M NaCl. Cell walls prepared as described above were incubated in 1 M NaCl for 2 h with shaking at 30 °C and centrifuged at 1,000 *g*. The supernatant was used for enzyme assays.

POD was assayed according to Sanchez et al. (1996). The oxidation of ferulic acid was measured spectrophotometrically following the absorbance decrease at 310 nm in a reaction mixture containing 1.35 ml Na-phosphate buffer (0.2 M, pH 5.8), 0.5 ml ferulic acid (240 μM), 0.5 ml H_2O_2 (0.3 mM) and 0.15 ml enzyme extract. One unit of POD was defined as a decrease of 1 A_{310} per min.

NADH-POD, which catalyses the reduction of O_2 to H_2O_2 using NADH as an electron donor, 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 mM, pH 6.5), 5 mM $MnCl_2$ 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 a decrease of 1 A_{340} per min.

DAO activities in the cell wall fraction were measured by the method of Naik et al. (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 10 mM putrescine (Put), 0.1 mM 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 5,000 *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 intensity was measured at 510 nm. In controls, trichloroacetic acid was 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 homogenising 10 roots with 3 ml of phosphate buffer (50 mM, pH 6.8) containing catalase inhibitor hydroxylamine (1 mM). The homogenate

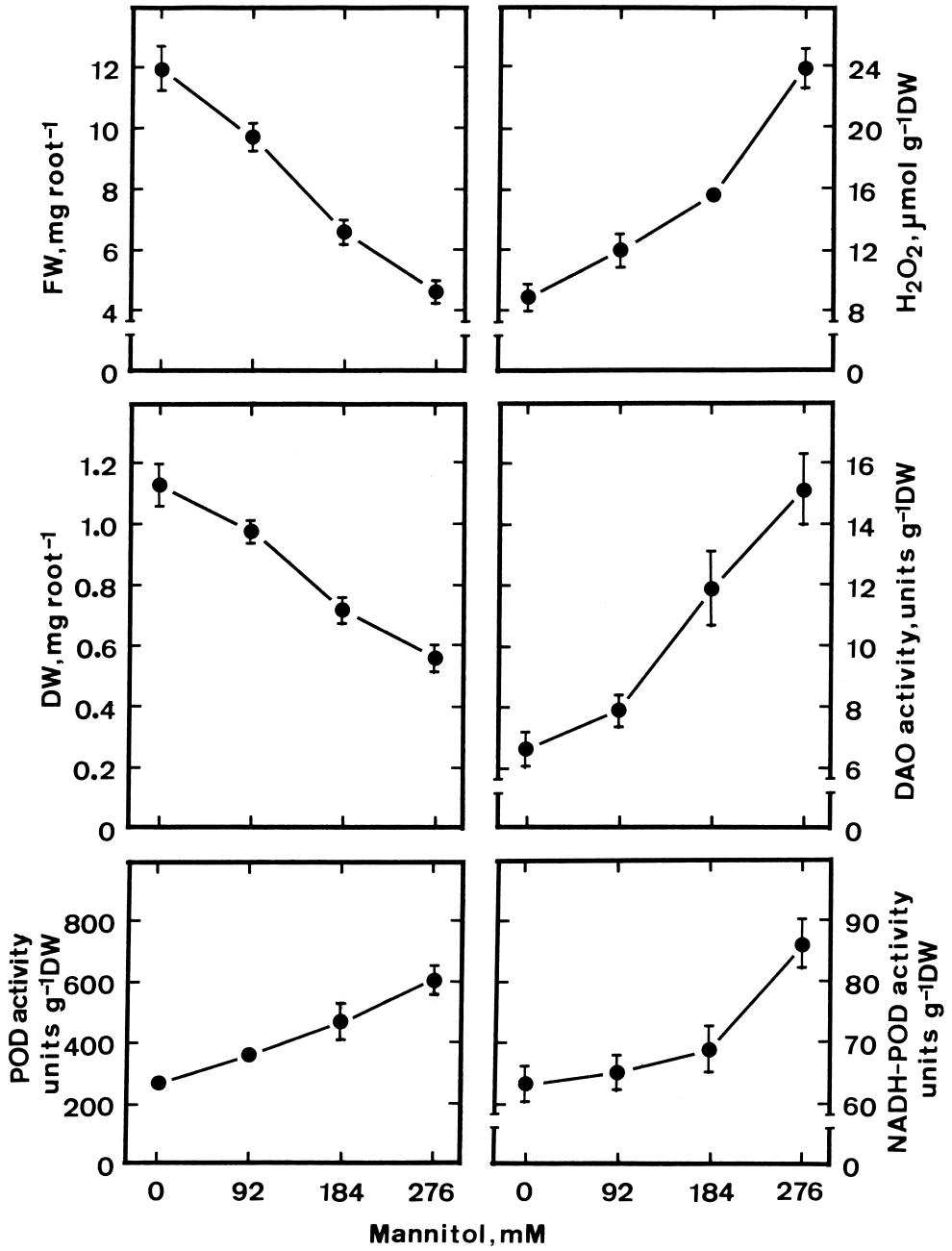

Figure 1. Effects of mannitol on root growth, peroxidase (POD) activity, H_2O_2 level, diamine oxidase (DAO) activity and NADH peroxidase (NADH-POD) activity in roots of rice seedlings. Root growth, H_2O_2 level and enzyme activities were determined after 5 days of treatment. Vertical bars represent standard errors (n = 4).

was centrifuged at 6,000 *g* for 25 min. To determine H_2O_2 levels, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium sulfate in 20% (v/v) H_2SO_4 and the mixture was then centrifuged at 6,000 *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 determinations of Put, spermidine (Spd) and spermine (Spm), roots were homogenized in 5% (v/v) perchloric acid. Levels of Put, Spd and Spm were de-

termined using HPLC after benzolation as described previously (Chen and Kao 1991).

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

In the present investigation, mannitol concentrations at 92, 184, and 276 mM, which are iso-osmotic with 50, 100, and 150 mM NaCl, respectively, were used to induce osmotic stress. Root growth was followed by measuring FW and DW of roots. Increasing concentrations of mannitol from 92 to 276 mM progressively decreased root growth (Figure 1). The results of the present investigation are consistent with the generally accepted idea that osmotic stress reduces growth of plant tissues.

A key role of cell-wall POD in the stiffening of the cell walls through formation of cross-links between wall polymers and, consequently, in the reduction or cessation of cell growth has been proposed (Fry 1986). Guaiacol is commonly used as a substrate for POD. However, guaiacol is not the natural substrate in the formation of cross-links between wall polymers. The polysaccharides of plant cell walls contain hydroxycinnamic acids that are presented as ester-linked side chain. Ferulic acid has been identified as being ester linked to arabinoxylans in cell walls of monocotyledonous plants (Kato and Nevin 1985; Hartley and Ford 1989). A key role in the cell-wall stiffening of dimerization of ferulic acid catalysed by cell-wall POD has been proposed (Sanchez et al. 1996). Thus, ferulic acid appears to be the suitable substrate to establish the relationship between cell-wall POD activity and mannitol-inhibited root growth of rice seedlings. In the present study, we used ferulic acid as a substrate to measure cell-wall POD activity. The data in Figure 1 show that the reduction of root growth with increasing mannitol concentrations is correlated with an increase in POD activity.

The results presented in Figure 1 seem to suggest that mannitol-induced inhibition of root growth of rice seedlings is due to a cell-wall stiffening process catalysed by POD. If POD regulates cell-wall stiffening by catalysing the oxidative cross-linking of cell wall polymers, there must be a sufficient supply of H_2O_2. Thus, it is a great interest to know whether or not mannitol increases the level of H_2O_2 in the roots of rice seedlings. We found that increasing concentrations of mannitol from 92 to 276 mM progressively increased H_2O_2 level in roots (Figure 1). Schopfer (1994) reported that in hypocotyls of sunflower and

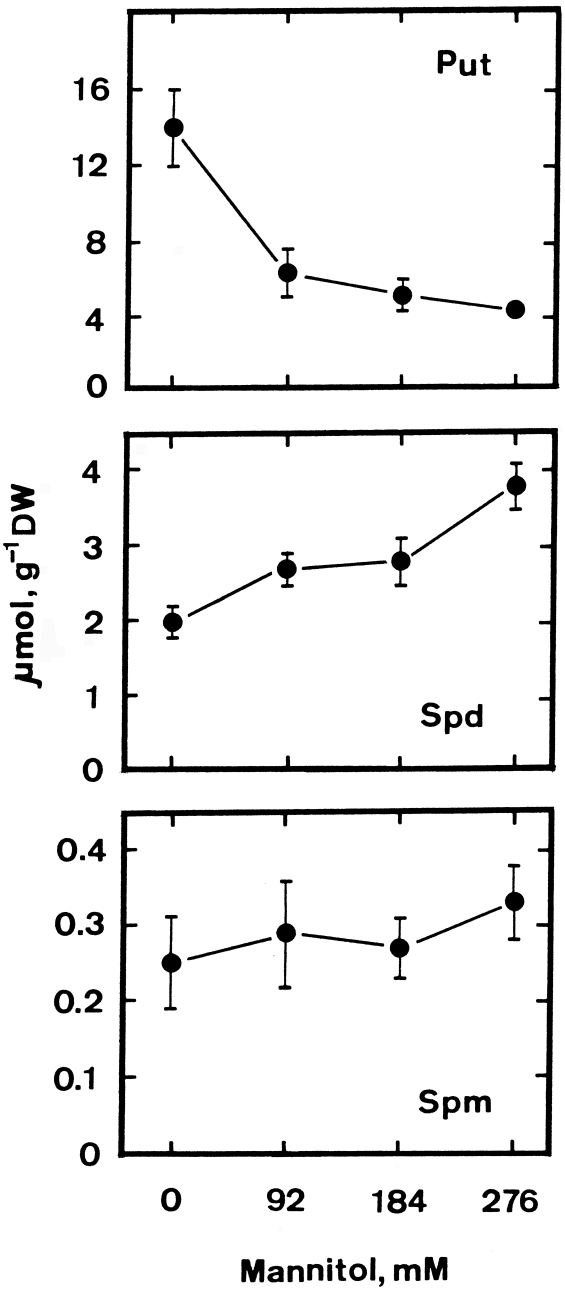

Figure 2. Effects of mannitol on the levels of polyamines in roots of rice seedlings. Putrescine (Put), spermidine (Spd) and spermine (Spm) were determined after 5 days of treatment. Vertical bars represent standard errors (n = 4).

Table 1. Changes in root growth, peroxidase (POD) activity, H_2O_2 level, and diamine oxidase (DAO) activity in roots of rice seedlings treated with mannitol. Rice seeds were germinated in distilled water for 2 days and then transferred to distilled water and mannitol (276 mM), respectively. The data represent mean values ± standard errors, n = 4

	Treatment	Time, h				
		0	4	8	12	16
FW (mg root^{-1})	H_2O	6.5 ± 0.21	7.1 ± 0.38	7.9 ± 0.14	8.9 ± 0.21	10.2 ± 0.08
	Mannitol		6.8 ± 0.15	7.5 ± 0.35	8.1 ± 0.21	8.5 ± 0.05
DW (mg root^{-1})	H_2O	0.62 ± 0.02	0.68 ± 0.04	0.75 ± 0.01	0.84 ± 0.02	0.96 ± 0.01
	Mannitol		0.65 ± 0.01	0.71 ± 0.04	0.77 ± 0.02	0.81 ± 0.01
POD (units g^{-1} DW)	H_2O	188 ± 21	212 ± 18	205 ± 9	231 ± 21	247 ± 15
	Mannitol		244 ± 22	312 ± 15	344 ± 5	388 ± 14
H_2O_2 (μmol g^{-1} DW)	H_2O	6.1 ± 0.41	6.5 ± 0.26	6.8 ± 0.24	7.0 ± 0.31	7.4 ± 0.52
	Mannitol		6.6 ± 0.32	7.5 ± 0.21	8.0 ± 0.13	8.5 ± 0.38
DAO (units g^{-1} DW)	H_2O	5.9 ± 0.37	5.9 ± 0.49	6.3 ± 0.14	6.5 ± 0.37	6.9 ± 0.29
	Mannitol		6.2 ± 0.19	7.0 ± 0.28	7.4 ± 0.32	7.8 ± 0.18

cucumber seedlings, the light-mediated inhibition of elongation growth was correlated with a strong increase in H_2O_2 in the epidermis and in the vascular bundle. We have demonstrated recently that exogenous application of H_2O_2 resulted in an inhibition of root growth of rice seedlings (Chen et al. 2000). These results are in agreement with those of Schopfer (1996) who demonstrated that H_2O_2 inhibited auxin-mediated growth of maize coleoptile segments. Our results seem to suggest POD-mediated cell-wall stiffening of rice roots is involved in mannitol-inhibited root growth.

One of potential sources of H_2O_2 in plant cells is cell wall- or membrane-bound NADH-POD which is responsible for H_2O_2 generation (Elstner and Heupel 1976). However, increasing cell-wall NADH-POD activities were found to increase only in rice seedling roots treated with 276 mM mannitol (Figure 1). It is unlikely that NADH-POD is the source leading to H_2O_2 in mannitol-inhibited root growth of rice seedlings. Diamine oxidase (DAO) is widespread in the Leguminosae family (Smith 1985) and has been reported in barley (Cogonii et al. 1990) and maize (Suzuki and Hagiwara 1993). This enzyme, which is involved in polyamine catabolism, oxidizes Put with the formation of Δ^1-pyrroline together with H_2O_2 and ammonia (Smith 1985). DAO activity is mainly localized in the cell walls (Angelini and Federico 1989) and perhaps plays a role in regulating Put levels (Smith 1985) or providing H_2O_2 required for peroxidative reactions that occur in the cell walls for the formation of cross-links (Angelini and Federico 1989). It seems that DAO is an alternative source leading to H_2O_2 generation in mannitol-inhibited root growth of rice seedlings. To test this, we determined the activity of DAO in rice seedling roots in response to various concentrations of mannitol (Figure 1). As expected, increasing concentrations of mannitol from 92 to 276 mM progressively increased DAO activities. This result is consistent with the observations that mannitol treatment decreases Put level but slightly increases Spd level and has no effect on Spm level (Figure 2). 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.

To test the causal relationship between root growth, cell-wall POD activity, H_2O_2 level, and cell-wall DAO activity caused by mannitol, 2-day-old seedlings were transferred to distilled water and mannitol, respectively, for 4, 8, 12, and 16 h. Changes in root growth, cell-wall POD activity, H_2O_2 level, and cell-wall DAO activity were then monitored. As indicated in Table 1, an increase in cell-wall POD activity and H_2O_2 level (which occurred at 8 h after treatment) preceded inhibition of root growth (which occurred at 12 h after treatment) caused by mannitol. Clearly, the links among mannitol treatment, cell-wall POD, H_2O_2 and root growth are well established. The observations that an increase in DAO activities coincides with an increase in H_2O_2 level in roots caused by mannitol (Table 1) suggest that DAO is the source for generation of H_2O_2 in the cell walls.

In conclusion, mannitol-induced osmotic stress inhibits root growth, increases cell-wall POD activity, and increases the H_2O_2 level. The results from the

present investigation support the involvement of cell-wall stiffening in growth reduction in rice seedling roots exposed to mannitol.

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