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Effects of exogenous salicylic acid on growth and H_2O_2-metabolizing enzymes in rice seedlings under lead stress

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Abstract

Salicylic acid (SA) was an essential component of the plant resistance to pathogens and also plays an important role in mediating plant response to some abiotic stress. The possible effects of SA on the growth and H_2O_2-metabolizing enzymes in rice seedlings under lead stress were studied. When rice seedlings grown in nutrient solution containing Pb^{2+} (0, 0.05, 0.15, 0.25 mmol/L) for 18 d, the plant biomass as well as the chlorophyll content of leaves decreased with increasing Pb concentration. The pre-treatment with SA (treated with 0.1 mmol/L SA for 48 h before Pb stress) partially protected seedlings from Pb toxicity. The chlorophyll contents were significant higher in leaves of Pb-exposed with SA pre-treatment seedlings than in Pb-exposed plants at the same Pb intensity. SA pre-treated alone could significantly increase the length of shoot and root of seedlings but the vigour difference was not marked under long-term exposure to Pb toxicity. SA pre-treated influence the H_2O_2 level in leaves of seedlings by up-regulating the activity of superoxide dismutase (SOD), repressing the activity of catalase (CAT) and ascorbate peroxidase (APX) depending on the concentrations of Pb^{2+} in the growth medium. The results supported the conclusion that SA played a positive role in rice seedlings against Pb toxicity.

Key words: ascorbate peroxidase; catalase; H_2O_2; lead stress; rice (*Oryza sativa* L.); salicylic acid; superoxide dismutase

Introduction

Lead (Pb) is a highly toxic and persistent environmental poison to plants and animals which originates from various sources. Apart from the natural weathering processes, the main sources of Pb pollution are exhaust fumes of automobiles, chimneys of factories sing Pb and fertilizers, pesticides as well as the additives in pigments and gasoline (Eick *et al*., 1999). Its increasing level in soil environment exerts a wild range of adverse effects on plants and even the healthy of human beings by food chains. Like various heavy metals, Pb treatment influences the activity behaviours of a wide range of enzymes of different metabolic pathways. The visual non-specific symptoms of Pb toxicity on plants are: inhibition of root growth, stunted stem growth and chlorosis (Burton *et al*., 1984). Increasing attention has been paid to the action of Pb on plant enzymes. Pb at a concentration range of 1×10^{-5}–20×10^{-5} mol/L may produce about 50% inhibition of activities of many enzymes. This concentration is defined as the inactivation constant (*K*i) (Sharma and Dubey, 2005).

Salicylic acid (SA), considered as a hormone-like substance, plays an important role in the regulation of plant growth and development, such as seed germination, flowering, heat production and fruit ripening (Raskin, 1992; Klessig and Malamy, 1994). A substantial body of evidence indicated that SA was a critical signalling molecule in the pathways leading to local and systemic disease resistance, as well as PR expression (Alvarez, 2000; Enyedi *et al*., 1992; Klessig and Malamy, 1994; Vasudha and George, 2001). It was proposed that one of the mechanisms involved in SA effect on biotic stress was the up-regulation of H_2O_2 (Chen *et al*., 1993; Klessig *et al*., 1997).

Although attention was paid on the roles of SA in biotic stresses, recent studies gave an evidence of SA in modulation of acclimation responses to abiotic stressors such as UV, heat, chilling, drought and salt (Yalpani *et al*., 1994; Dat *et al*., 1998; Janda *et al*., 1999; Senaratna *et al*., 2000). SA application caused a partial protection against cadmium toxicity in barley seedlings (Metwally *et al*., 2003) and alleviated the heavy metal-induced membrane degradation in rice seedlings (Mishra and Choudhuri, 1999). Wang *et al*. (2004) provided evidence that treatment with SA could amend the Al-induced oxidative damage in root tips. In contrast, Rao *et al*. (1997) found that SA might act as a pro-oxidant and phytotoxin if given at concentration higher than 1 mmol/L. Besides, SA increased reactive oxygen species (ROS) generation under salinity and osmotic stress with a bacterial SA-decomposing salicylate hydroxylase expressing in *Arabidopsis* mutants than in wild-type (Borsani *et al*., 2001).

SA has broad effects on plant acclimation to adverse

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stress or on the development of plant. But the effects of SA on rice growth and activities of H_2O_2-metabolizing enzymes after long-term Pb stress still remain discuss. In the present study the experiment was carried out to test whether SA had a protective effect on rice seedlings against the Pb toxicity. The biomass of plants, H_2O_2 content, chlorophyll content and activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) in leaves were determined.

1 Materials and methods

1.1 Plant materials and culture

Rice (*Oryza sativa* L.) cultivar "Huanghuazhan" was used in the experiment. Seeds were surface sterilized with 0.5% NaClO for 20 min, rinsed, and germinated in petri dish (15 cm) containing two sheets filter papers with 15 ml distill water in the dark at 28°C for 2 d. After seedlings grown on a plastic screen floating on distilled water at 28°C for 4 d, uniform size seedlings were selected and transferred to black polyethylene pots containing 6 L of complete nutrient solution. The composition of nutrient solution was: K_2SO_4 0.75 mmol/L, $MgSO_4$ 0.65 mmol/L, KCl 0.1 mmol/L, $Ca(NO_3)_2$ 2.0 mmol/L, KH_2PO_4 0.25 mmol/L, EDTA-Fe 0.1 mmol/L, $MnSO_4$ 1 μmol/L, HBO_3 0.01 mmol/L, $CuSO_4$ 0.1 μmol/L, $ZnSO_4$ 1 μmol/L, $(NH_4)_6Mo_7O_{24}$ 0.005 μmol/L. The nutrient solution was adjusted to pH 5.0–5.1 with 1 mol/L HCl and the solution in each pot was renewed every 7 d. Seedlings were grown in a growth chamber at a day/night cycle of 14 h/10 h, at 32°C/27°C, respectively, at a relative humidity between 55% and 65% and a light intensity of 500 μmol quanta/(m^2·s).

1.2 SA treatment and Pb stress

When developing the seventh leaf, seedlings were selected uniformly for the four treatments as follows: (1) control plants (CK): seedlings were transplanted into the nutrient solution; (2) Pb-exposed plants (+Pb): transplanted into nutrient solution supplemented with Pb^{2+} (0, 0.05, 0.15 and 0.25 mmol/L); (3) SA pre-treated seedlings (+SA): transplanted into nutrient solution supplemented with 0.1 mmol/L SA for 48 h; (4) Pb-exposed with SA pre-treated plants (SA+Pb): 0.1 mmol/L SA pre-treated for 48 h, then transplanted into nutrient solution with Pb^{2+} (0, 0.05, 0.15 and 0.25 mmol/L). In order to prevent precipitation, the nutrient solution was modified slightly: KH_2PO_4 was eliminated and K_2SO_4 concentration increased to 0.875 mmol/L, and 0.25% (w/v) KH_2PO_4 was sprayed on leaves twice a day (Pang *et al*., 2002). After 18 d treatments, the samples were collected respectively and stored at –80°C for further analysis. Eight individual plants were considered as one replicate, each treatment had three replicates.

1.3 Analyse

H_2O_2 determination: the levels of H_2O_2 in leaves were measured by monitoring the absorption of the titanium-peroxide complex at 410 nm, following the method of Jana and Choudhuri (1981). Briefly, we extracted H_2O_2 from 100 mg leaf samples by homogenization with 6 ml of phosphate buffer (50 mmol/L, pH 6.5). The homogenate was centrifuged at 6,000 g for 25 min. To determine the H_2O_2 content, 3 ml of extracted solution was mixed with 1 ml of 0.1% (m/v) titanium chloride in 20% (v/v) H_2SO_4, then the mixture was centrifuged at 6,000 g for 15 min and the supernatant absorbance at 410 nm was read.

Staining with diaminobenzidine (DAB): DAB was used for *in situ* detection of H_2O_2 in leaf tissue (Thordal *et al*., 1997). Cut leaves were floated in 0.01% (v/v) TritonX-100 containing 0.5 mg/ml DAB, and vacuum infiltrated for 30 min. DAB incubation was continued for 36 h. At the end of staining, leaves were washed with distilled water and boiled in 8 ml of 95% ethanol for 10 min. H_2O_2 is visualized as a reddish brown coloration.

Enzyme extraction and assays: 0.3 g fresh samples were homogenized in 3 ml of 50 mmol/L K-phosphate buffer (pH 7.0). The homogenate was centrifuged at 15000 g for 20 min. The supernatant was used for enzyme assays. All operations were carried out at 4°C. SOD was assayed by photochemical method described by McCord and Fridovich (1969). 3 ml of the assay mixture contained 50 mmol/L K-phosphate buffer (pH 7.8), 13 mmol/L L-methionine, 63 μmol/L NBT, 0.1 mmol/L EDTA, and 1.3 μmol/L riboflavin. One unit of SOD activity was defined as the amount of enzyme resulting in 50% inhibition of the rate of NBT chloride reduction at 560 nm. CAT was assayed following the protocol of Cakmak (1991). The assay mixture contained 25 mmol/L K-phosphate buffer (pH 7.0), 0.1 mmol/L EDTA, 10 mmol/L H_2O_2 and enzyme extract. The enzymatic activity was expressed by mmol H_2O_2 oxidised/(min·g fw). APX activity was measured in the presence of 0.3 mmol/L ascorbic acid and 0.3 mmol/L H_2O_2 by monitoring the decrease in absorption at 290 nm (Hossain and Asada, 1984).

Chlorophyll content: sections taken from the flag leaves of approximately the same areas were extracted in 5 ml 80% acetone. After centrifugation at 3000 g for 5 min, total chlorophyll contents were determined at 663 nm and 645 nm (Arnon, 1949).

1.4 Statistical analysis

Triplicates biological replications were used for analysis of each parameter. Data were presented as means and theirs standard errors. Mean difference comparison among different treatments was done by analysis of variance analysis (ANOVA), and the significant differences were reported at $P<0.05$ or 0.01.

2 Results

2.1 Effects of SA on the growth of rice seedlings under lead stress

Length changes of shoots and roots are shown in Table 1. Compared Pb-exposed plants (+Pb) to the control plants (CK), the biomass of the studied seedlings in the four

Table 1 Effects of SA on the growth of rice seedlings after 18 d growth in the medium with increasing Pb concentrations

Pb^{2+} (mmol/L)	Length (cm)			
	Shoot		Root	
	–SA	+SA	–SA	+SA
0	$41.9\pm2.2^{a*}$	48.5 ± 1.7^{a}	$26.2\pm1.3^{a*}$	29.2 ± 0.5^{a}
0.05	39.9 ± 2.0^{a}	36.6 ± 1.6^{b}	26.5 ± 1.7^{ab}	24.6 ± 1.4^{a}
0.15	28.7 ± 1.0^{b}	30.5 ± 0.4^{c}	21.2 ± 1.5^{b}	19.0 ± 0.4^{b}
0.25	27.5 ± 1.1^{b}	29.2 ± 1.3^{c}	16.7 ± 1.1^{b}	16.9 ± 1.1^{c}

Data represent mean values±*SD* (n=8) based on three independent determinations; *indicates significant differences at P=0.05 (ANOVA) between SA pre-treated and non SA pre-treated seedlings; a, b, and c indicate significant differences at P=0.05 (ANOVA) between Pb-exposed seedlings and control plants.

treatment groups decreased along with the increase of Pb concentration. The reduction were up to 34.4% in shoot length and 36.3% in root length of 0.25 mmol/L Pb-exposed plants (+Pb) compared with that of control plants (CK), respectively. Compared SA pre-treated seedlings (+SA) to the control plants (CK), SA pre-treated could significant increase the length of shoot and root of seedlings, up to 15.8% (P<0.05) and 11.5% (P<0.05) increase. The biomass of rice seedlings of Pb-exposed plants (+Pb) to Pb-exposed with SA pre-treated seedlings (SA+Pb) decreased along with the increase of Pb concentration, however, the phenotypic differences between the two groups were not significant.

2.2 Effects of SA on chlorophyll content in leaves of rice seedlings under lead stress

Chlorophyll content in the second leaves of rice seedlings is shown in Fig.1, chlorophyll content in both SA pre-treated and non SA pre-treated seedlings significantly decreased in accordance with the increasing Pb concentrations. In the absence of Pb, chlorophyll content of SA pre-treated seedlings (+SA) reduced by about 10.6% compared to CK. However, SA pre-treatment prevented the sharp decline of chlorophyll content in rice seedlings under Pb stress. SA pre-treatment led to about 23.1% (P<0.05) and 31.3% (P<0.01) increase in chlorophyll content compared to Pb-exposed seedlings (+Pb) at 0.15 mmol/L and 0.25 mmol/L Pb^{2+} in the growth medium, respectively. At the lowest concentration of 0.05 mmol/L Pb^{2+}, the chlorophyll content of Pb-exposed with SA pre-treated plants (SA+Pb) was notably higher, up to 23.3% (P<0.01) than Pb-exposed plants (+Pb).

2.3 Effects of SA on H_2O_2 content in leaves of rice seedlings under lead stress

H_2O_2 accumulated with increasing Pb concentrations in Pb-exposed seedlings (Fig.2). The H_2O_2 contents were 27.8% and 64.9% higher in the Pb-exposed seedlings (+Pb) compared to that of CK at 0.05 and 0.25 mmol/L Pb^{2+}, respectively. SA pre-treatment had significant effects on H_2O_2 accumulation in the leaves of seedlings grown in the absence of Pb and 0.25 mmol/L Pb^{2+}. The H_2O_2 content increased significantly by about 29.7% (P<0.01) in leaves of SA pre-treated seedlings (+SA) compared to control plants (CK). While, at the highest concentration of 0.25 mmol/L Pb^{2+}, the level of H_2O_2 increased by 14.4% (P<0.05) in Pb-exposed with SA pre-treated seedlings (SA+Pb) compared to the Pb-exposed seedlings (+Pb). The H_2O_2 levels in leaves induced by SA pre-treatment were different along with the Pb concentration. The H_2O_2 levels of Pb-exposed with SA pre-treated seedlings (SA+Pb) grown in the 0.05 mmol/L Pb^{2+} was 9.1% (P<0.05) decrease compared to that of Pb-exposed plants (+Pb).

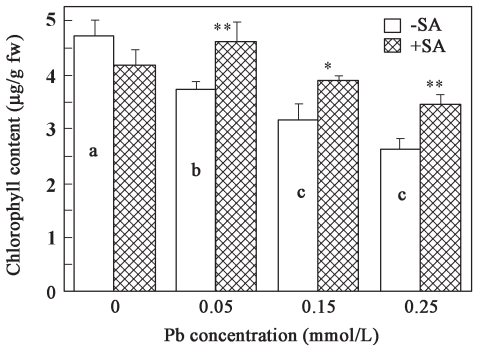

Fig. 1 Effects of SA on chlorophyll content in leaves of rice seedlings in non SA pre-treated and SA pre-treated seedlings after 18 d Pb stress. The different letters (a, b, c) mean the significant differences at 0.05 level. **P<0.01; *P<0.05.

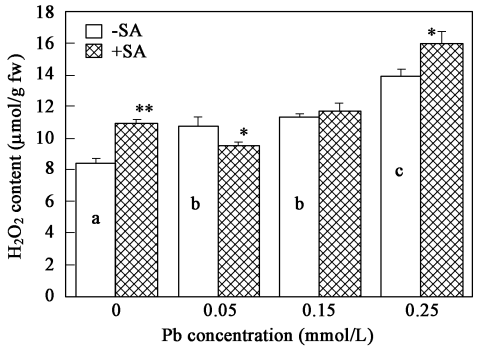

Fig. 2 Effects of SA on H_2O_2 content in leaves of rice seedlings in non SA pre-treated and SA pre-treated seedlings after 18 d Pb stress. The letters a, b, and c are the same as Fig.1. **P<0.01; *P<0.05.

The effects of SA on accumulation of H_2O_2 in leaves of rice seedlings under lead stress could also be testified from the result of DAB staining (Fig.3). The staining was obviously reddish with increasing Pb concentration in Pb-exposed seedlings, more stains in the leaves of Pb-exposed seedlings at 0.05 and 0.25 mmol/L Pb^{2+} compared to control plants, respectively (Fig.3a). SA pre-treatment increased a staining depth depending on the Pb concentration (Fig.3b). Less staining was observed in leaves of control plants (CK) than in SA pre-treated seedlings (SA) in the absence of Pb. In the medium of 0.25 mmol/L Pb^{2+}, the SA pre-treatment increase the accumulation of H_2O_2 in the vascular tissue on a conspicuous level, with a strong reddish brown stains seen in the veins. On the other hand, under 0.05 mmol/L Pb^{2+} stress, the staining in the leaves of Pb-exposed with SA pre-treated seedlings (SA+Pb) was relative lower intensity compared

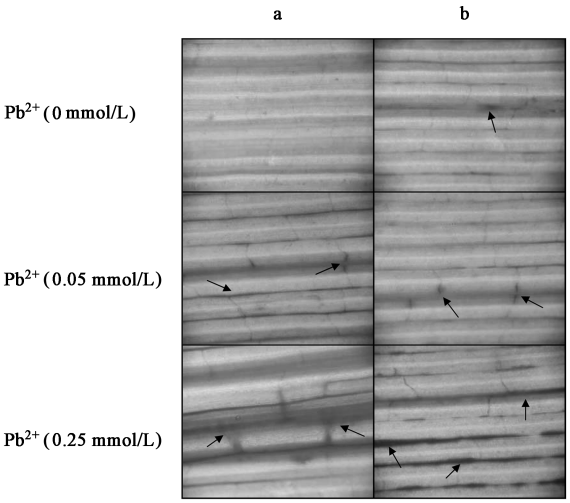

Fig. 3 Changes in H_2O_2 staining in leaves of rice after 18 d growth under Pb stress. a. non SA pre-treated seedlings; b. SA pre-treated seedlings.

to Pb-exposed seedlings (+Pb).

2.4 Effect of SA on the activities of H_2O_2-metabolizing enzymes in leaves of rice seedlings under lead stress

Fig.4 shows the activities of SOD in leaves of rice seedlings in the Pb-exposed (+Pb) and Pb-exposed with SA pre-treated plants (SA+Pb). After long-term exposure to lead stress, the activities of SOD in the leaves of Pb-exposed seedlings (+Pb) maintained at the steady level. SA pre-treatment markly up-regulated the SOD activities in the studied seedlings. It was significantly increased by 69.5% (P <0.01) in the SA pre-treated seedlings compared to the control plants in the absence of Pb. The up-regulation extent was up to 58.9% (P<0.05) in the Pb-exposed with SA pre-treated plants (SA+Pb) than in Pb-exposed seedlings (+Pb) at 0.15 mmol/L Pb^{2+}.

Fig.5 shows the activities of CAT in leaves of seedlings over a period of 18 d Pb stress. The CAT activities were significantly restrained in both Pb-exposed (+Pb) and Pb-exposed with SA pre-treated seedlings (SA+Pb) exposed to a series of Pb concentrations in this study. SA pre-treatment significantly decreased the CAT activity by approximately 40.3% (P<0.01) in SA pre-treated seedlings

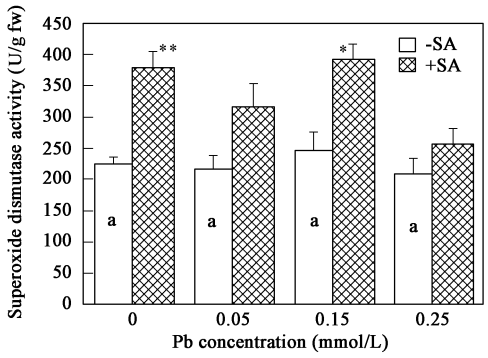

Fig. 4 Effects of SA on activity of superoxide dismutase (SOD) in leaves of rice seedlings in non SA pre-treated and SA pre-treated seedlings after 18 d Pb stress. The letters a, b, and c are the same as Fig.1. **P<0.01; *P<0.05.

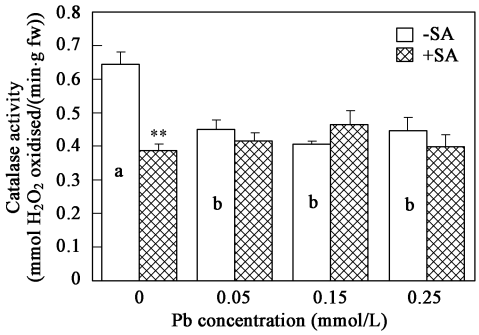

Fig. 5 Effects of SA on activity of catalase (CAT) in leaves of rice seedlings in non SA pre-treated and SA pre-treated seedlings after 18 d Pb stress. The letters a, b, and c are the same as Fig.1. **P<0.01; *P<0.05.

(+SA) compared to the control plants in the absence of Pb. However, SA has no influence on the inhibition of CAT activities induced by Pb stress. The difference of CAT activities between Pb-exposed seedlings (+Pb) and Pb-exposed with SA pre-treated seedlings (SA+Pb) was not significant.

Fig.6 shows the changes of APX activities of rice seedlings after 18 d treatment. Pb treatment increased the APX activity of Pb-exposed seedlings (+SA) along with increasing Pb concentrations. Compared to control plants (CK), the APX activities were up to 25.9% and 22.4% increase in the Pb-exposed seedlings (+Pb) at 0.15 and 0.25 mmol/L Pb^{2+}, respectively. APX activity in SA pre-treated seedlings (SA) decreased compared to control plants (CK). SA pre-treatment did not affect the Pb-dependent increase of APX activity, but had a mild elevation of APX activities in Pb-exposed with SA pre-treated seedlings (SA+Pb) along with Pb concentration gradient compared to Pb-exposed seedlings (+Pb).

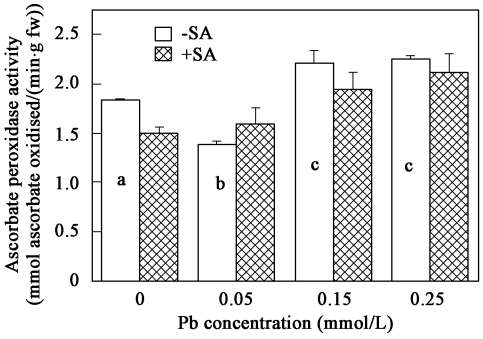

Fig. 6 Effects of SA on activity ascorbate peroxidase of (APX) in leaves of rice seedlings in non SA pre-treated and SA pre-treated seedlings after 18 d Pb stress. The letters a, b, and c are the same as Fig.1.

3 Discussion

This experiment described here analyzes the effects of SA on rice seedlings exposed to toxic Pb concentration in long-term experiments. The visual non-specific symptoms of Pb toxicity on plants are chlorosis and rapid growth inhibition (Sharma and Dubey, 2005; Burton *et al*., 1984). This study conducted a range of Pb concentrations from 0.05 to

0.25 mmol/L as low and moderately toxic concentrations based on the previous observations. The results indicated the decrease in vigour (length and weight) of the studied seedlings along with the increase of Pb concentrations. That is due to that Pb could possibly be attributed to the interference with metabolic and biochemical processes associated with normal growth and development of the plants (Verma and Dubey, 2003). 0.1 mmol/L SA pre-treatment alone could significantly increase the length of shoot and root of seedlings which should attribute to that SA could be involved in the regulation of cell enlargement and division in synergy during plant development (Li, 1995). However, the phenotypic differences between the Pb-exposed with SA pre-treatment (Pb+SA) and Pb-exposed seedlings (+Pb) were not statistically significant. Pb played much influence on the restraining the development of seedlings than 0.1 mmol/L SA pre-treatment.

Pb inhibits chlorophyll synthesis by causing impaired uptake of essential elements such as Mg and Fe by plants (Burzynski, 1987) and even accelerates the decomposition of chlorophyll (Saichu, 1982; Yan *et al*., 1998). The result presented in this article showed that exogenous SA pre-treatment played an effect on young rice seedlings growth from Pb toxicity at the view point of chlorophyll content in which the chlorophyll contents were significantly higher in leaves of Pb-exposed with SA pre-treatment seedlings (SA+Pb) than in Pb-exposed plants (+Pb) at the same Pb concentration in growth medium. These results were in conformity with the observation of SA in barley seedlings from Cd toxicity (Metwaly *et al*., 2003).

Pb has been reported to cause oxidative damage due to production ROS (Verma and Dubey, 2003), including superoxide radical ($O_2^{\cdot -}$) and H_2O_2. A certain level of H_2O_2 may play a role in signal transfer processes and activate many tolerance-related genes in both plant and animal organisms exposed to the abiotic stress, while excess content of H_2O_2 accumulation would be toxic to plants tissue (Schreck *et al*., 1991; Prasad *et al*., 1994). The present study indicated that H_2O_2 accumulated with increasing Pb concentrations in Pb-exposed seedlings. SA pre-treatment induced the H_2O_2 accumulation depending on the Pb intensity in the growth medium. At high intensity of 0.25 mmol/L Pb^{2+} and in the absence of Pb, the H_2O_2 level in Pb-exposed with SA pre-treated seedlings (SA+Pb) were markly higher than Pb-exposed seedlings (+Pb). These results corroborate with the hypothesis of SA effect on plant exposed to biotic stress that SA serves as a signal substance by up-regulating the H_2O_2 level, which could then serve as second messenger in the defense signaling pathway (Chen *et al*., 1993; Lamb and Dixon, 1997; Klessig *et al*., 1997).

SOD along with CAT and APX are considered as key enzymes in H_2O_2-metabolizing, which directly determine the cellular concentration of H_2O_2 (Asada, 1992; Monk *et al*., 1989). The observation by Slooten *et al*. (1995) reported transgenic plants over-expressing SOD showed increased tolerance of plants subjected to oxidative stress. The present results show that SA pre-treatment markly up-regulated the SOD activities in Pb-exposed with SA pre-treated plants (SA+Pb) than Pb-exposed seedlings (+Pb) after long-term exposure to Pb toxicity (Fig.4). The early propose mode of SA in plant defense response after pathogen attack includes the H_2O_2-scavenging enzymes CAT and APX (Klessig *et al*., 1997). CAT is the first SA binding protein (originally termed SABP) (Chen *et al*., 1993) that becomes a form of inactivation when was bonded with SA. The results show that SA pre-treatment alone significantly decreased the CAT activity in the absence of Pb, and it maintained at an identical level in both Pb-exposed seedlings (+Pb) and Pb-exposed with SA pretreated seedlings (SA+Pb). Pb treatment increased the APX activity of Pb-exposed seedlings (+Pb) along with increase of Pb concentrations, while the elevation extent in Pb-exposed with SA pretreated seedlings (SA+Pb) was relatively mild.

In summary, our results suggest that exogenous SA pre-treatment played an effect on young rice seedlings growth from Pb toxicity at the view point of chlorophyll content. SA pre-treated influence the H_2O_2 level in leaves of seedlings through up-regulation the activity of SOD and repressing the activity of CAT and APX depending on the concentration of Pb^{2+} in the growth medium.

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