

# Salinity Effects on Antioxidant Enzymes in Mulberry Cultivar

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**ABSTRACT** To investigate the antioxidant defense system, salt-stress induced changes of antioxidant enzymes were examined in the leaves of mulberry (*Morus sp.*) of the salt-tolerance cultivar Pei. With increasing salinity up to 150 mM NaCl, the hydrogen peroxide content and the activity of guaiacol-specific peroxidase increased markedly. In addition, the activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase slightly increased at 150 mM NaCl. In contrast, catalase activity with increasing salinity was not correlated with hydrogen peroxide content. The results suggest that under increasing salinity, the primarily prominent peroxidase activity appears to play an active role in scavenging reactive oxygen species in this cultivar, whereas the superoxide dismutase/ascorbate-glutathione cycle seem to be important consequently.

**KEYWORDS:** antioxidant enzymes, mulberry, salinity.

## INTRODUCTION

Even under optimal conditions, reactive oxygen species including superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen are metabolic by-products of plant cells. These reactive oxygen species affect lipid peroxidation, protein denaturation and DNA mutation.<sup>1</sup> To remove reactive oxygen species, plant cells possess an antioxidant system consisting of low-molecular-weight antioxidants, such as ascorbate,  $\alpha$ -tocopherol, glutathione and carotenoids, as well as antioxidant enzymes. These include superoxide dismutase for scavenging the superoxide radicals and other key enzymes, ascorbate peroxidase and glutathione reductase, in the ascorbate-glutathione cycle for detoxifying the hydrogen peroxide.<sup>2</sup> Under normal conditions, the production and destruction of these reactive oxygen species is well regulated in plant cells. However, under environmental stress, the balance between the production of reactive oxygen species and the quenching activity of the antioxidant system is upset.<sup>3</sup> Injury to plants due to various environmental stresses, in association with oxidative damage, directly or indirectly occurs through formation of reactive oxygen species. Such effects have been reported for high light intensity, herbicide and air pollutant exposure, pathogen attack, waterlogging, drought and salinity.<sup>2-6</sup>

Qualitative and quantitative changes in the activity of antioxidant enzymes isolated from plants subjected

to salt stress have been reported.<sup>7-9</sup> A regulated balance between oxygen radical production and destruction is required in order to maintain metabolic efficiency and function, either under normal or stress conditions. A constitutively high antioxidant capacity under stress conditions can prevent damage and correlate with the plants' resistance to that particular stress. Hence, metabolic processes that reduce oxidative stress are expected to play an important role in imparting tolerance in plants under saline condition.<sup>7-9</sup>

Soil salinity is an inevitable problem for agriculture in the northeast of Thailand. Better understanding of the mechanisms that enable plants to adapt to salt stress is necessary to make the best use of these saline soils. Cellular mechanisms are especially important to glycophytes, in which physiological and biochemical processes contribute to the adaptation to salt stress.

In our previous studies, we reported not only the elevation of proline as an osmoprotectant in the mechanism of salt-stress adaptation in mulberry, cv. Khonpai, but also the enhancement of ascorbate peroxidase activity.<sup>10</sup> Ascorbate peroxidase and glutathione reductase are the key enzymes in the ascorbate-glutathione cycle, which occurs mainly in the chloroplast and cytosol as the scavenging system of oxygen reactive species.<sup>11</sup> In the present study, we investigate the role of antioxidant enzymes including those of the ascorbate-glutathione cycle as possible

mechanisms for salt-stress adaptation in mulberry, cv. Pei, which is known to be a salt tolerant cultivar, as well as being able to survive well in waterlogged environments.

## MATERIALS AND METHODS

### Plant materials

The salt tolerant mulberry (*Morus sp.*) cultivar Pei was used in this study. It is also known to survive well in a waterlogged environment. The method for developing multiple shoots was basically the same as that reported previously.<sup>10</sup> Buds were surface-sterilized with 0.1% mercuric chloride for 3-5 min and thoroughly washed with sterilized water. Then, they were cultured on modified Murashige and Skoog<sup>12</sup> solid medium, supplemented with 1 ml/L 6-benzylaminopurine. The multiple shoots were subcultured at 40 days interval and cultured under  $150 \mu\text{mol m}^{-2}\text{s}^{-1}$  white light at 25 °C.

For every experiment, multiple shoots were cultured in the medium containing various concentrations of NaCl for 12 days. The control was defined as the multiple shoots without NaCl treatment.

### Extraction of protein

Total protein was extracted by the method of Anderson *et al.*<sup>13</sup> Leaves (0.5 g) were homogenized in 0.75 ml of 0.1 M sodium phosphate, pH 7.8, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulphonyl fluoride (PMSF) and 20 mg of polyvinylpyrrolidone (PVP). For analysis of ascorbate peroxidase, the extraction buffer also contained 5 mM ascorbate. Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4 °C, and glycerol was immediately added up to 40% (v/v) to the supernatant. Total soluble protein content was determined by Lowry's method<sup>14</sup> using bovine serum albumin as standard.

### Enzymatic activity assays

Ascorbate peroxidase (EC 1.11.1.11) activity was determined as described by Yamaguchi *et al.*<sup>15</sup> The reaction mixture (1 ml) contained 50 mM potassium phosphate (pH 7.0), 1.2% sucrose, 1 mM hydrogen peroxide, 0.5 mM ascorbate and 10 mM 3-aminotriazole (an inhibitor of catalase). The hydrogen peroxide-dependent oxidation of ascorbate was followed by monitoring the decrease in absorbance at 290 nm, using the extinction coefficient of  $2.8 \text{ mM cm}^{-1}$ .

Catalase (EC 1.11.1.6) activity was assayed by measuring the initial rate of hydrogen peroxide disappearance using the method of Velikova *et al.*<sup>16</sup> One milliliter of catalase assay reaction mixture contained 10 mM potassium phosphate buffer, pH 7.0 with an appropriate aliquot of enzyme extract and 33 mM

hydrogen peroxide. The decrease in hydrogen peroxide was followed as a decline in optical density at 240 nm, and the activity was calculated using the extinction coefficient of  $40 \text{ mM cm}^{-1}$  for hydrogen peroxide.

Glutathione reductase (EC 1.6.4.2) activity was determined by the oxidation of NADPH at 340 nm with the extinction coefficient of  $6.2 \text{ mM cm}^{-1}$  as described by Lee and Lee.<sup>17</sup> The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM glutathione (oxidized form, GSSG) with an appropriate volume of enzyme extract in a 1 ml volume. The reaction was initiated by the addition of NADPH at 25 °C.

Peroxidase (EC 1.11.1.7) was determined using the method of Srinivas *et al.*<sup>18</sup> by following the formation of tetraguaiacol by measuring the absorbance at 470 nm, and using an extinction coefficient of  $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$  to calculate the amount of tetraguaiacol. The 1 ml reaction mixture contained 20 mM phosphate buffer, pH 6.0, 5 mM 2-methoxy phenol (guaiacol), 1 mM hydrogen peroxide with an appropriate aliquot of enzyme extract. The reaction was carried out for 3 min. One unit of peroxidase activity represents the amount of enzyme catalysing the oxidation of 1  $\mu\text{mol}$  of guaiacol in 1 min.

Superoxide dismutase (EC 1.15.1.1) activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium, according to the method of Yu and Rengel.<sup>7</sup> For total the superoxide dismutase assay, the 1 ml reaction mixture contained 50 mM Hepes (pH 7.6), 0.1 mM EDTA, 50 mM  $\text{Na}_2\text{CO}_3$ , 13 mM methionine, 0.025% (w/v) Triton X-100, 75  $\mu\text{M}$  nitroblue tetrazolium, 2  $\mu\text{M}$  riboflavin and an appropriate aliquot of enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of  $350 \mu\text{mol m}^{-2}\text{s}^{-1}$ . One unit of superoxide dismutase activity is defined as the amount of enzyme required to cause 50% inhibition of nitroblue tetrazolium reduction, which was monitored at 560 nm.

### Determination of hydrogen peroxide content

Hydrogen peroxide levels were determined according to Velikova *et al.*<sup>16</sup> Leaf tissues (500 mg) were homogenized in an ice bath with 5 ml 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the supernatant was measured at 390 nm. The content of hydrogen peroxide was determined using a standard curve.

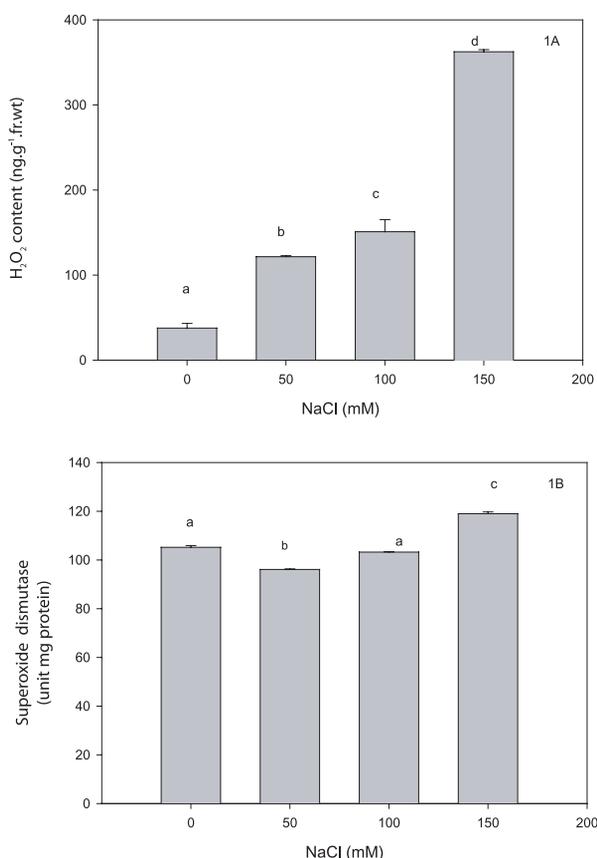
### Data analysis

Data presented are means  $\pm$  standard deviation for three replicates. The data means were compared using

Duncan's multiple range test (with significance at  $P < 0.05$ ).

## RESULTS AND DISCUSSION

Mulberry is a suitable plant-model to study the mechanisms involved in salt-stress adaptation, as it able to grow well in the salty and dry area of the northeast of Thailand. We chose the salt-tolerant Pei cultivar of mulberry. In addition to its salt-tolerance, cv. Pei is also recognized as waterlogging-tolerant. Our focus was to observe the response of antioxidant enzymes to increasing salinity. In order to clarify the protective mechanism of the antioxidant enzymes against salt-stress, we determined the changes in hydrogen peroxide content together with changes in the activation and inactivation of antioxidant enzymes in the leaves of mulberry plants subjected to salt stress.

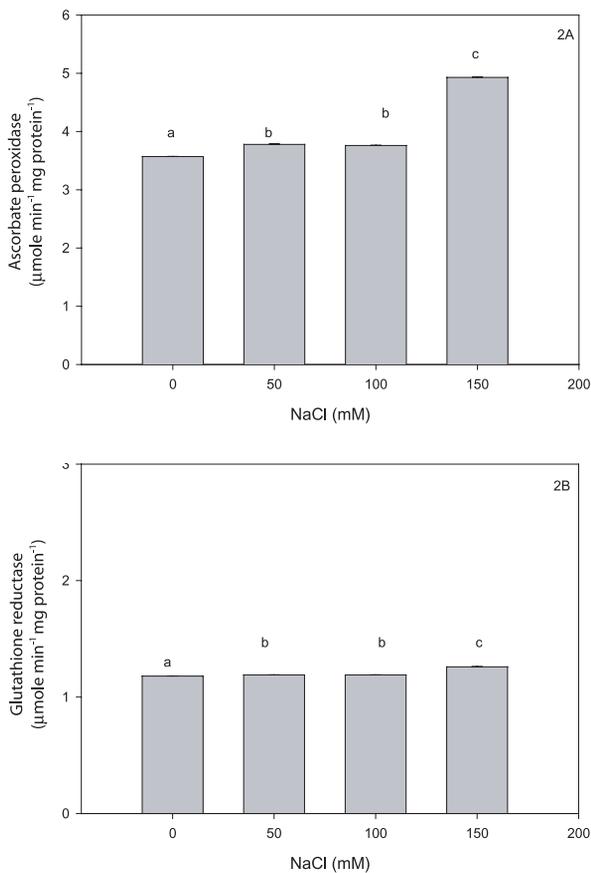


**Fig 1.** (A) Changes in the contents of hydrogen peroxide in the leaves of mulberry subjected to increasing salinity. (B) Superoxide dismutase activity in the leaves of mulberry subjected to increasing salinity. One unit of superoxide dismutase is defined as the amount of enzyme causing 50% decrease of superoxide dismutase-inhibitable nitroblue tetrazolium reduction. Data are mean  $\pm$  SD ( $n = 3$ ). Different letters indicate that the mean value is significantly different ( $P < 0.05$ ).

It has been reported that reactive oxygen species, including superoxide and hydrogen peroxide, are elevated with increased salinity, due to the imbalance in the production and destruction of reactive oxygen species.<sup>11</sup> Fig 1A shows that the hydrogen peroxide level is increased with increasing salinity in this cultivar. This result is similar to reports of plant responses to other unfavourable conditions, such as induced chilling stress in cucumber (*Cucumis sativus* L.)<sup>17</sup> and acid rain-treated bean (*Phaseolus vulgaris* L.).<sup>16</sup> Lee and Lee<sup>17</sup> reported that hydrogen peroxide content increased over time during chilling stress. However, during the poststress period, the level of hydrogen peroxide was significantly higher than the level during chilling stress. In addition, after 24 hours poststress, the leaves showed visible injury symptoms such as leaf yellowing starting at the tip of the leaf due to the breakdown of chlorophyll. The metabolism of active oxygen species, such as hydrogen peroxide, is dependent on various functionally interrelated antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase and peroxidase. In comparison to the control, chilling stress induced a significant increase of superoxide dismutase activity, whereas after 12 hours poststress, the superoxide dismutase of chilled plants decreased to almost the same activity level as the control.

Total superoxide dismutase activity is enhanced under salinity at 150 mM NaCl in mulberry cv. Pei, the salt-tolerant and waterlog-tolerant cultivar (Fig 1B). Although it is unlikely that superoxide dismutase would play a major protective role under anoxic conditions, it may have a critical role in the survival of the plant, when oxygen levels increase as the flooding stress abates. Anaerobic tissue has a very high redox potential and the soil environment surrounding the roots contains highly reduced forms of metal ions such as iron, which could readily reduce atmospheric oxygen to superoxide.<sup>19</sup>

The levels of ascorbate peroxidase and glutathione reductase activity in cv. Pei at various NaCl concentrations are shown in Fig 2. At 150 mM NaCl, the activity of the two enzymes was increased. Since these two enzymes are the key enzymes of the ascorbate-glutathione cycle, the cycle may be a potential mechanism of mulberry adaptation to salinity. It was reported that in the stress conditions of intense light, high temperature, flood and salinity, free radicals and reactive oxygen molecules are formed.<sup>1, 4, 6, 11, 19</sup> Therefore, a scavenging system should be active. Our results showed that cv. Pei possessed an effective superoxide dismutase/ascorbate-glutathione cycle under high salinity (150 mM NaCl). Benavides *et al*<sup>9</sup> also showed a close relationship between the antioxidant defense system and salt tolerance in two clones of

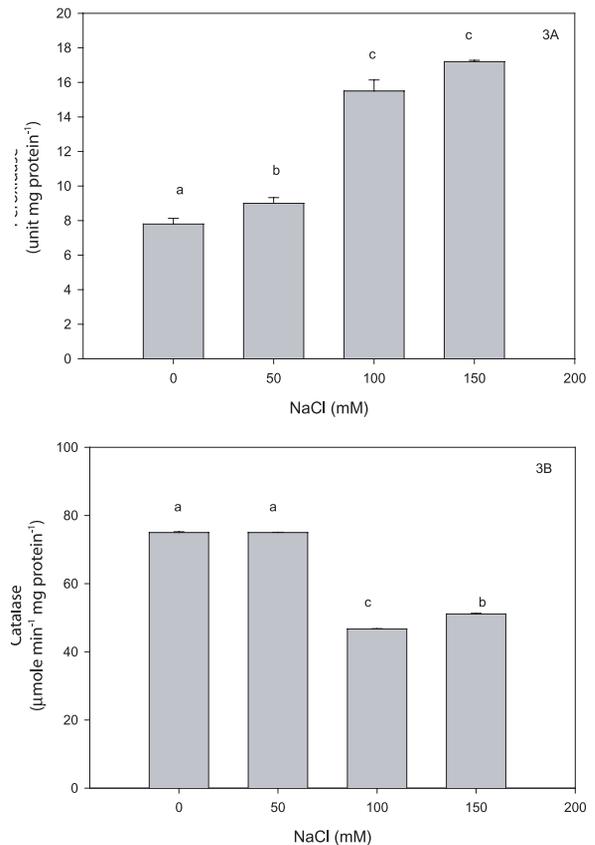


**Fig 2.** (A) Ascorbate peroxidase activity and (B) glutathione reductase activity in the leaves of mulberry subjected to increasing salinity. Data are mean  $\pm$  SD (n = 3). Different letters indicate that the mean value is significantly different (P < 0.05)

potato (*Solanum tuberosum*) differing in salt sensitivity.

In the chloroplast, reactive oxygen species may be produced from triplet chlorophyll in the light-harvesting complex, the oxidizing (water-splitting) side of PSII and by ferredoxin on the reducing side of PSII.<sup>11</sup> Under salinity, the imbalance of production and scavenging of reactive oxygen species occurs, even though the isoenzymes of superoxide dismutase, ascorbate peroxidase and glutathione reductase which are localized in the chloroplast are activated.<sup>3</sup>

Plants also possess other hydrogen peroxide scavenging enzymes: peroxidase and catalase. The increased total peroxidase activities in response to salinity have been reported.<sup>19-21</sup> Sancho *et al*<sup>19</sup> indicated that the increased total peroxidase activity in the medium of salt adapted cells reflected the changing mechanical properties of the cell wall, which, in turn, could be related to the salt adaptation process, since cell wall properties are known to be modified by salt stress. Earlier reports<sup>20-21</sup> showed a relationship between total peroxidase activity and changes in cell wall and



**Fig 3.** (A) Peroxidase activity and (B) catalase activity in the leaves of mulberry subjected to increasing salinity. Data are mean  $\pm$  SD (n = 3). Different letters indicate that the mean value is significantly different (P < 0.05)

membrane integrity under salt stress. Dhindsa and Matowe<sup>3</sup> reported that enhanced production of oxygen free radicals are responsible for stress-dependent peroxidation of membrane lipids. Increased peroxidation of membrane lipids is known to occur during salinity stress. It has been reported that salinity stress could modify the membrane structure, and may stimulate oxygen radical production which facilitates lipid peroxidation.<sup>3,8</sup> Fig 3A reveals the response of the activity of peroxidase in cv. Pei to the elevation in salinity. These collective results confirmed that increasing NaCl concentration activated the expression of peroxidase activity in mulberry cv. Pei.

Catalase consists of isoenzymes localized in the microbody, mitochondria and cytosol. The pattern of change in catalase activity differs from that of ascorbate peroxidase and glutathione reductase activity, as shown in Fig 3B. The catalase activity did not respond to increasing salt concentration. This result reflects the importance of peroxidase, as well as the superoxide dismutase/ascorbate-glutathione cycle, as an oxygen-

reactive species scavenging system in cv. Pei. Benevides *et al*<sup>9</sup> reported the enzymes responsible for hydrogen peroxide detoxification in *Solanum tuberosum* to be ascorbate peroxidase and catalase. However, they suggested that ascorbate peroxidase was likely to be more important than catalase in the detoxification. Since hydrogen peroxide was also involved in peroxidase-mediated oxidative polymerization, which results in cell wall strengthening, the activation of peroxidase may have a protective role. However under abiotic stress causing hydrogen peroxide accumulation, this may be one of the factors that results in the inactivation of catalase.<sup>16</sup>

In conclusion, the adaptive ability of mulberry cv. Pei (salt tolerant and waterlog- tolerant phenotype) allows it to respond to high levels of salinity (150 mM NaCl). Our study indicates that its acquisition of salt tolerance may be a consequence of improved resistance to oxidative stress via increased activities of peroxidase and the superoxide dismutase/ascorbate-glutathione cycle. In addition, the activities of these antioxidant enzymes in this cycle may be a good indicator for selecting salt tolerance genotypes of plants.

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