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Original Research

Response to NaCl Stress in *Salix matsudana* Koidz Seedlings

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Abstract

The effects of different NaCl concentrations (0.1, 0.2, and 0.4%) on plant growth, the enzymatic antioxidant system, lipid peroxidation, and cell damage were investigated in *Salix matsudana* Koidz to better understand the tolerant mechanism under NaCl stress. The results indicate that cell damage was induced in roots by NaCl stress as early as after just 1 h of exposure, which increased with increasing NaCl concentration and prolonged treatment. The activities of SOD, POD, and CAT in *S. matsudana* under NaCl stress were enhanced except for the SOD activity in leaves under 0.4% NaCl at day 28, and CAT activities in leaves exposed to 0.4% NaCl on days 21 and 28. NaCl exposure caused increasing O_2^- and H_2O_2 contents. The MDA content in roots exposed to 0.2 and 0.4% NaCl increased except for that in 0.2% NaCl on day 14 compared with control. The MDA level in leaves of control was lower than that of all NaCl treatments. The soluble protein contents in roots increased significantly (P<0.05), except for that 0.1% NaCl during days 21 to 28. It increased significantly in leaves exposed to 0.4% NaCl, but decreased sharply at day 28.

Keywords: cell damage, enzymatic antioxidant system, lipid peroxidation, reactive oxygen species (ROS), soluble protein

Introduction

Soil salinity negatively affects agricultural and forestry productivity worldwide [1]. Increased salinization can affect arable land globally, and the loss of arable land is expected to be 30% in the next 25 years and in the year 2050 up to 50% [2]. It is well known that salinity can inhibit the growth and development of plants by excessive accumulation in plants [3].

Salt toxicity in plants has been known for a long time [4-5]. Apoptosis-like DNA degradation, which leads to sequential nuclear degradation, cell death and inhibition of root growth, has been observed after salt stress [6]. At the cellular level, salinity stress leads to an accumulation of reactive oxygen species (ROS) in the root and leaf tissues of plants [7-8]. Plants have evolved a range of complex physiological and metabolic responses to cope with adverse environments, including great stress-responsive genes and the synthesis of various functional proteins through a complex signal transduction network for alleviating the stress caused by salt [9-10]. Salinity can induce an antioxidant defense rising response to oxidative stress in plants. ROS, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), etc., are harmful byproducts of aerobic metabolism in aerobic

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organisms. Coordinated work of ROS-scavenging pathways from different cellular compartments may play a key role in plant salt tolerance by modulating the level of ROS in cells, preventing cellular damage and controlling ROS [11-12].

Plants have a range of antioxidant strategies to restrain these toxic compounds [13]. Under stress conditions, plants can maintain the safety level of ROS by altering the activity of many antioxidant enzymes and metabolites [14-15]. The activity levels of such antioxidant enzymes as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and peroxidase (POX) are important for a plant's response to salinity stress [16-17]. These enzymes and metabolites not only protect plants from cellular damage, but also adjust ROS concentration to keep their functions better in the metabolism process [18]. In addition, many plants adapt to salt stress by enhanced biosynthesis of secondary metabolites, such as soluble solids, sugars, organic acids, proteins, and amino acids [19], which may act as osmolytes or osmoregulators to maintain plant turgor under salt stress.

S. matsudana (Chinese willow), a member of the Salicaceae family planted in northern China, mainly grows in the frigid and temperate regions of the northern hemisphere where low-rainfall and soil water retention, low temperature, and saline soil coexist [1]. As one of the most important landscaping and timber tree species, it has the good qualities of easy breeding, fast growth, resistance to water, and salt tolerance [20]. It was reported that *S. matsudana* could tolerate drought, salt, and heavy metal stress [1]. Salt toxicity in plants is well documented. However, limited information is available about the comprehensive evaluation of the toxic effects of salt on antioxidant enzymes, lipid peroxidation, and cell damage. Therefore, the aim of this work was to comprehensively evaluate the responses of the antioxidant system and the defense mechanisms in the seedlings of *S. matsudana* stressed by NaCl in order to better understand the tolerant mechanism in woody plants.

Materials and Methods

Plant Material and Growth Conditions

Healthy and equal-sized woody cuttings (25 cm long) from 1-year-old shoots of *S. matsudana* were collected before starting the experiments. After being dipped in tap water for 10 d at 25ºC, the plants were cultured with 1/2 Hoagland nutrient solution for a week. Then the plants were randomly divided into four groups and treated with different concentrations of NaCl solution (control, 0.1, 0.2, and 0.4%) for four weeks. The Hoagland nutrient solution consisted of 5 mM Ca $(NO_3)_2$, 5 mM KNO_3, 1 mM KH_2PO_4 , 1 mM $MgSO_4$, 50 μM H_3BO_3 , 10 μM FeEDTA, 4.5 μM $MnCl_2$, 3.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, and 0.1 μM $(NH_4)_6Mo_7O_{24}$, adjusted to pH 5.5. Control seedlings were grown in the nutrient solution alone. The solutions were constantly aerated with an air pump and replaced every week. Root and shoot length were measured every seven days. All treatments were done in three replicates.

Propidium Iodide Staining

To observe the cell damage in root tip cells of *S. matsudana* stressed by NaCl, the intact root tips from the woody cuttings exposed to different concentrations of NaCl (0, 0.1, 0.2, and 0.4%) for 1, 3, 6, 9, 12, and 24 h were stained with propidium iodide (PI) in darkness at room temperature, and then they were washed three times (3 × 10 min) with the sodium phosphate buffer (PBS) (pH 7.8), according to Koyama et al. [21] modified by Jones and Senft [22]. The fluorescence density was measured to analyze distribution in 10 intact root tips of *S. matsudana* woody cuttings under NaCl stress using the analysis and measure function of Image J software (NIH, Bethesda, MD, USA). The immunofluorescent specimens were examined with a confocal laser scanning microscope (ECLIPSE 90i, Nikon Corporation, Tokyo, Japan) with excitation maximum at 535 nm and fluorescence emission maximum at 617 nm.

Determining Antioxidant Enzyme Activities

The fresh roots or leaves from each treatment were homogenized in a pestle and mortar with 0.05 M PBS (pH 7.8) at the end of each time interval (7 d). The homogenate was centrifuged at 12,000 × g for 20 min, and the supernatant was used for analyzing the activities of SOD, POD, and CAT. The above steps were carried out at 4ºC. More details are described by Wei et al. [23].

Determining MDA Content

The fresh roots and leaves (0.15 g) in each treatment were homogenized in 5 mL of 10% TCA (trichloroacetic acid) with a pestle and mortar at the end of each time interval (7 d). The homogenates were centrifuged at 12,000 × g for 15 min. 2 mL of 0.6% thiobarbituric acid (TBA) in 10% TCA was added to 2 mL aliquot of the supernatant. The mixture was heated at 100ºC for 15 min and then quickly cooled in an ice bath. The absorbance of the supernatant was recorded at 532 and 450 nm. Lipid peroxidation was expressed as MDA content in l M kg^{-1} fresh weight.

Determining Soluble Protein Concentration

Measuring content of soluble proteins in this investigation was carried out according to Bradford's method [24] using bovine serum albumin (BSA) solution as a standard. The fresh roots and leaves from each treatment (six seedlings) were washed in distilled water, dried, and put in a mortar with 5 mL 0.05 M PBS (pH 7.8) at the end of each time interval (7 d) of the NaCl treatment. The homogenate was centrifuged at 10,000 × g for 20 min and the supernatant was used for

analyzing soluble protein contents. The soluble protein content was expressed as mg per g fresh weight.

Determining O_2^- and H_2O_2 Concentrations

H_2O_2 content was determined based on Velikova [25]. Leaf and root fresh tissues (0.5 g) were homogenized by 5 mL of acetone into an ice bath at the end of each time interval (7 d). The mixture was centrifuged at 10,000 × g for 20 min at 4ºC, and then 1 mL of the extract was mixed with 0.15 mL of 5% titanium sulfate and 0.2 mL of aqua ammonia. After precipitating, the mixture was centrifuged at 10,000 × g for 20 min at 4ºC. After the sediments were rinsed by acetone three times 5 mL of 2 M sulphuric acid was added. Until sediments dissolved, the absorbance of the solution was recorded at 415 nm and H_2O_2 contents were measured using a standard curve.

O_2^- production rate was determined by monitoring the nitrite formation form hydroxyl-amine in the presence of O_2^- according to Wang and Luo [26]. About 0.2 g frozen leaves were extracted with 2 mL of 50 mM PBS (pH 7.8) into an ice bath. The mixture was centrifuged at 12,000 × g for 20 min at 4ºC, and 0.5 mL supernatant was mixed with 0.5 mL of 50 mM PBS (pH 7.8) and 1 mL of 1 mM hydroxylamine hydrochloride. The mixture was incubated in a water bath for 20 min at 25ºC, and then 1 mL of 17 mM sulfaniclic acid and 1 mL of 7 mM 1-aminonaphthalene were added. The solution was then incubated for 20 min in a water bath at 25ºC, equal 4 mL volumes of chloroform were added, and the solution was centrifuged for 3 min at 12,000 × g at 25ºC. The absorbance of the supernatant was measured at

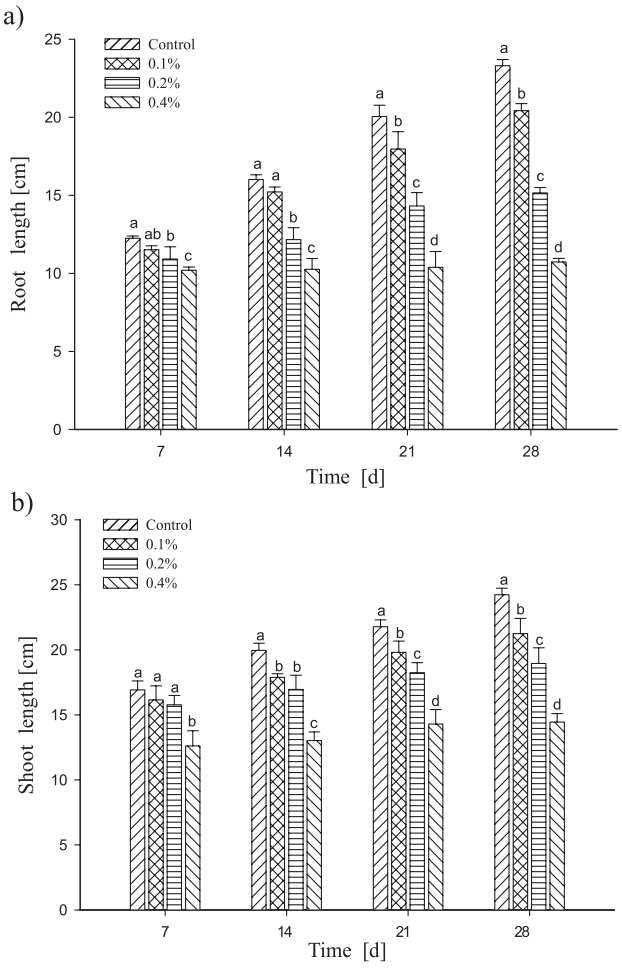

Fig. 1. Effects of different concentrations of NaCl (0.1, 0.2, and 0.4%) and treatment times (7, 14, 21, and 28 days) on root and shoot length of *S. matsudana*. Vertical bars denote SE. Values with different letters differ significantly from each other (n = 10, $P<0.05$).

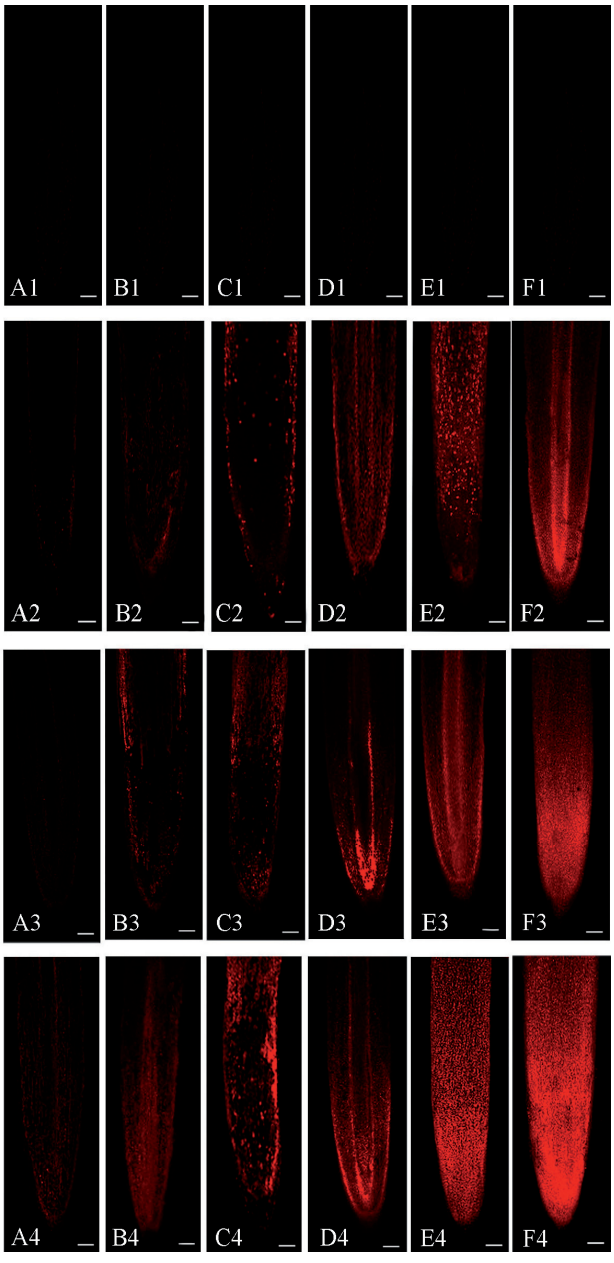

Fig. 2. Micrographs of roots from *S. matsudana* exposed to different NaCl concentrations using PI. Red fluorescence due to PI entering cells is an indicator of cell damage, A1-F1: control; A2-F2: 0.1% NaCl, for 1, 3, 6, 9, 12, and 24 h; A3-F3: 0.2% NaCl, for 1, 3, 6, 9, 12, and 24 h; A4-F4: 0.4% NaCl, for 1, 3, 6, 9, 12, and 24 h. Scale bar = 1 mm.

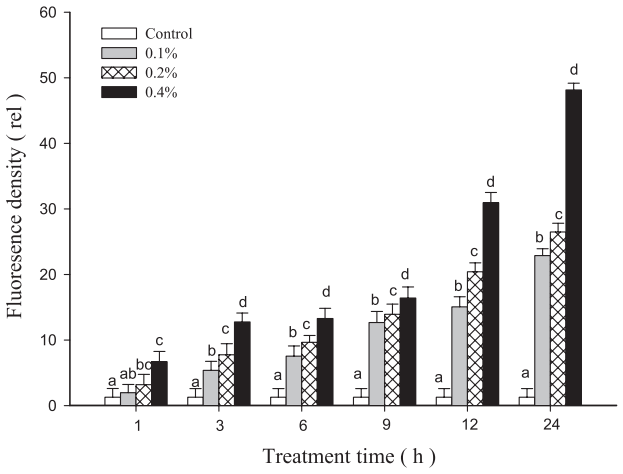

Fig. 3. Distribution of PI fluorescence density in roots of *S. matsudana* treated with 0, 0.1, 0.2, and 0.4% NaCl concentrations for different treatment times (1, 3, 6, 9, 12, and 24 h). Values followed by the same letters are not significantly different (P<0.05); means ± standard error, n = 15.

530 nm and the content of O_2^- was calculated based on the standard curve.

Statistical Analysis

Each treatment was replicated five times for statistical validity. Analysis of variance of the data was done with SPSS and SigmaPlot 10.0 software. For statistical analysis, one-way analysis of variance (ANOVA) and *t*-test were used to determine the significance at P<0.05.

Results

Effects of NaCl on Seedling Growth

The effects of NaCl on the root and shoot growth of *S. matsudana* varied with the different concentrations and duration of treatment (Figs 1a-b). NaCl had no toxic effect on root growth at 0.1% concentration during days 7 and 14 of treatment when compared with control. With the increasing NaCl concentration and prolonged treatment time, root growth was inhibited significantly (P<0.05). Under 0.4% NaCl, root growth almost completely stopped during the whole course of treatment (Fig. 1a). At 0.1% and 0.2% concentrations, the obvious inhibited effects in shoots appeared after 14 d treatment (P<0.05) (Fig. 1b). The shoots exposed to 0.4% NaCl showed significant reduction during the whole NaCl treatment period (P<0.05) versus control and the other treatment groups.

Effects of NaCl on Cell Damage

To investigate the cell damage affected by NaCl, the root tips of *S. matsudana* exposed to 0.1, 0.2, and 0.4% NaCl for 0, 1, 3, 6, 9, 12, and 24 h were stained with PI (Figs 2-3). Red fluorescence is an indicator of cell damage. It is difficult to observe fluorescence in control root tips (Fig. 2A1-F1). The root tip cells of *S. matsudana* exposed to 0.1, 0.2, and 0.4% NaCl for different periods of treatment exhibited different fluorescence intensities (Figs 2A2-F2, A3-F3, A4-F4). The low level of fluorescence intensity was observed in the root tips exposed to NaCl for 1 h, showing that NaCl could induce cell damage as early as 1 h. More and more red fluorescence appeared with increasing NaCl concentrations and prolonged exposure. The data from the fluorescence density analysis (Fig. 3) by software *Image J* also confirmed the findings above (Fig. 2).

Effects of NaCl on the Activities of SOD, POD, and CAT

As shown in Figs 4(a-b), the effects of NaCl on SOD activities in roots and leaves of *S. matsudana* varied with different NaCl concentrations and duration of treatment time. The SOD activity in roots of *S. matsudana* exposed to NaCl during the 21-day period increased significantly (P<0.05) versus control (Fig. 4a), and there was no obvious effect at day 28 in comparison with control. The SOD activity in leaves treated with NaCl increased significantly (P<0.05) during the whole experiment except for the group exposed to 0.4% NaCl at day 28 when compared with control (Fig. 4b). Higher SOD activities were observed in roots than in leaves in control and all the NaCl treatments. The POD activities in roots and leaves of *S. matsudana* are shown in Figs. 4(c-d). In comparison with control, significantly higher POD activity in roots and leaves was induced during the whole NaCl treatment time (P<0.05) except for the leaves exposed to 0.1% NaCl on days 7 and 14. Information on CAT activity in *S. matsudana* exposed to NaCl was given in Figs. 4(e-f). In roots, CAT activities increased significantly (P<0.05) during the whole experiment except for the group exposed to 0.1% NaCl at day 28 (Fig. 4e). In comparison with control, the activities of CAT increased significantly (P<0.05) in leaves exposed to 0.1 and 0.2% NaCl during days 21 and 28. The CAT activity in leaves at concentrations of 0.4% NaCl increased significantly (P<0.05) at days 7 and 14 of treatment when compared with control and the other treatment groups, then it decreased sharply at days 21 and 28 (Fig. 4f).

Effects of NaCl on Lipid Peroxidation

The effects of NaCl on MDA contents of *S. matsudana* are presented in Fig. 5. The content of MDA in roots exposed to 0.4% NaCl was the highest during the whole experiment time except for the group treated with 0.4% NaCl on day 7 when compared with control and the other treatment groups (Fig. 5a). 0.2% NaCl induced high levels of MDA in roots during the entire period of the experiment except for the treatment group at day 14. In leaves, the MDA contents increased significantly with increasing NaCl concentrations and prolonged treatment times (Fig. 5b).

Effects of NaCl on Soluble Protein Content

As could be seen from Fig. 6a), the contents of soluble proteins in *S. matsudana* roots of all the NaCl treatments increased significantly (P<0.05) during the whole experiment, except for the group exposed to 0.1% NaCl at days 21 and 28 in comparison with control. Fig. 6b) showed that the soluble protein contents in leaves exposed to 0.4% NaCl increased significantly during the 21-day treatment (P<0.05), except for the content in the group exposed to 0.2% NaCl at day 7 when compared with control and the other treatment groups, but it decreased sharply at day

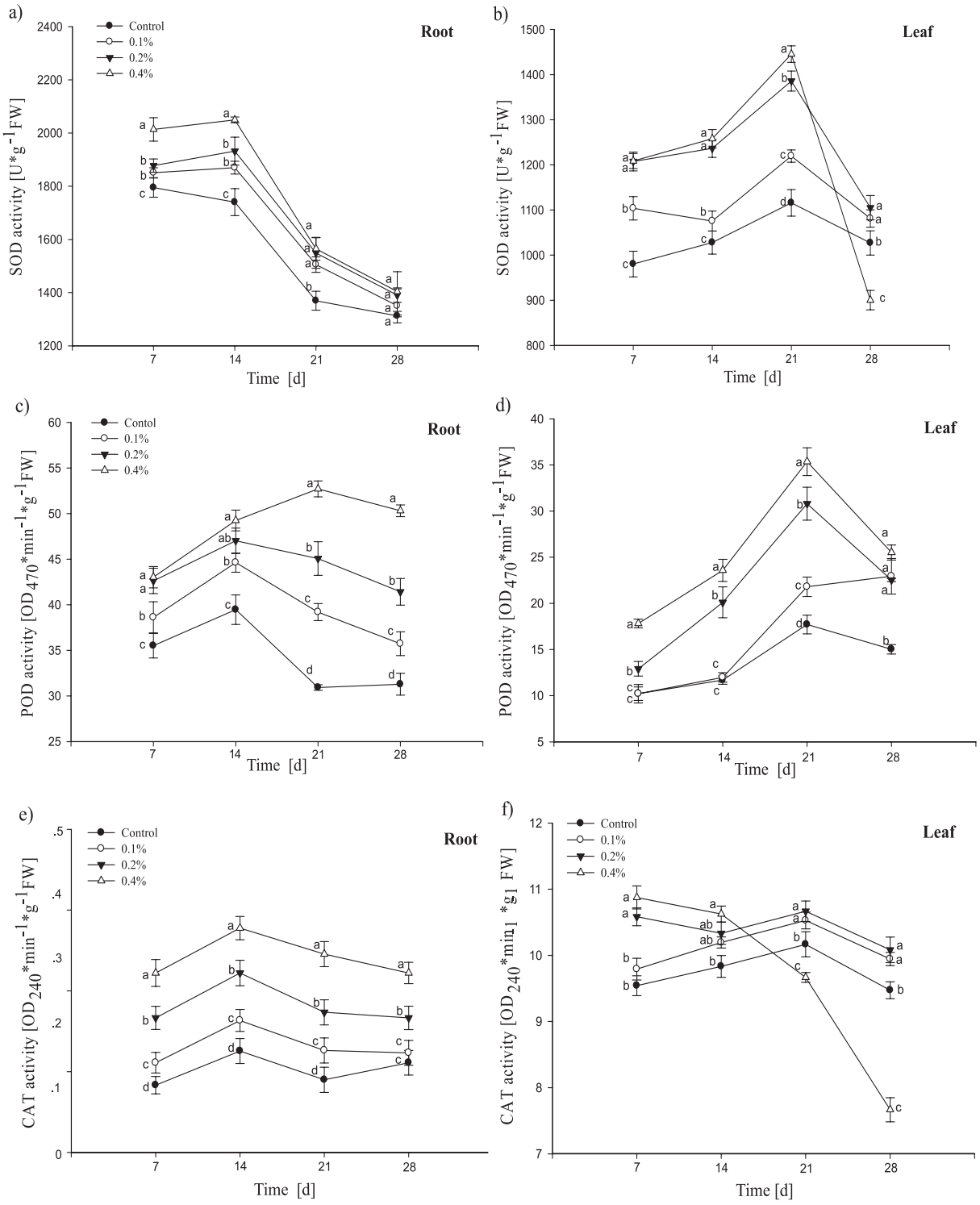

Fig. 4. Effects of different concentrations of NaCl on the activities of SOD, POD, and CAT in *S. matsudana* for 7, 14, 21, and 28 days: a) SOD in roots, b) SOD in leaves, c) POD in roots, d) POD in leaves, e) CAT in roots, and f) CAT in leaves. Vertical bars denote SE. Values with different letters differ significantly from each other ($P<0.05$, t-test).

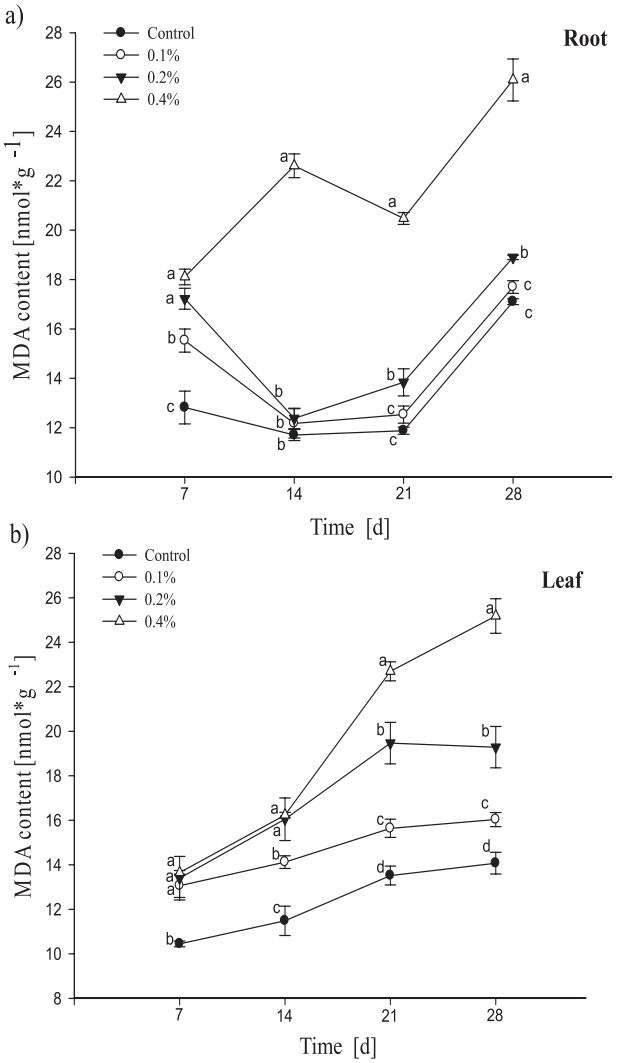

Fig. 5. Effects of different concentrations of NaCl on MDA content in *S. matsudana* for 7, 14, 21, and 28 days: a) roots and b) leaves. Vertical bars denote SE. Values with different letters differ significantly from each other ($P<0.05$, *t*-test).

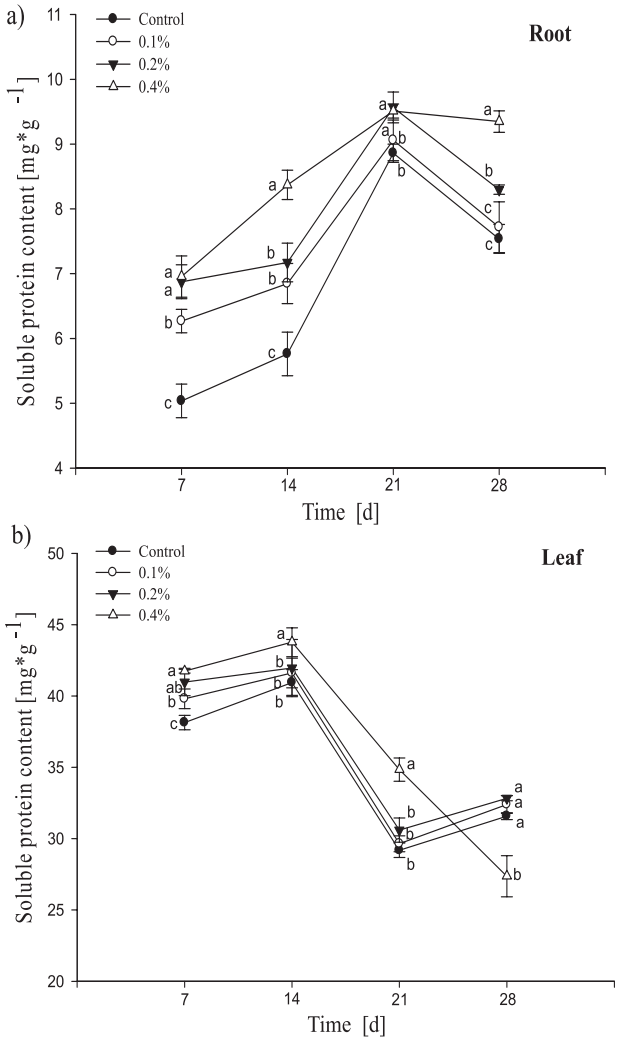

Fig. 6. Effects of different concentrations of NaCl on soluble protein content in *S. matsudana* for 7, 14, 21, and 28 days: a) roots and b) leaves. Vertical bars denote SE. Values with different letters differ significantly from each other ($P<0.05$, *t*-test).

28 and was lower than the other groups. When compared with control, the soluble protein contents in the 0.1% and 0.2% NaCl groups have no obvious difference during the 28-day treatment except those at day 7.

Effects of NaCl on O_2^- and H_2O_2 Content

The effects of NaCl stress on O_2^- and H_2O_2 content in *S. matsudana* are shown in Fig. 7. The O_2^- contents in roots of all the NaCl treatment groups were raised significantly ($P<0.05$) during the 14-28-day period when compared with control (Fig. 7a). The O_2^- contents reached the highest level at day 21 and then decreased sharply. The O_2^- contents in leaves of all the NaCl treatments increased during the whole treatment time in comparison with the control, except for the 0.1% NaCl group at day 28 (Fig. 7b). Effects of NaCl on H_2O_2 content in roots and leaves of *S. matsudana* varied with the different concentrations of NaCl and the duration of treatment. In roots, 0.2 and 0.4% NaCl induced significantly high H_2O_2 content ($P<0.05$) during the whole experiment when compared with control, except for 0.2% NaCl at day 7 (Fig. 7c). The H_2O_2 content in leaves treated with 0.4% NaCl increased significantly ($P<0.05$) during the whole treatment time when compared with control and the other treatment groups (Fig. 7d). The H_2O_2 contents in leaves exposed to 0.1 and 0.2% NaCl increased significantly ($P<0.05$) during the 21-28-days period of treatment compared with control.

Discussion

Abiotic stresses such as high salinity, extreme temperature, drought, and heavy metal pollution are major limiting factors for growth and development of forest trees [27]. Salt stress negatively affects plant growth, morphology, and physiology as well as biochemical characteristics in plants [28-30]. The results from our

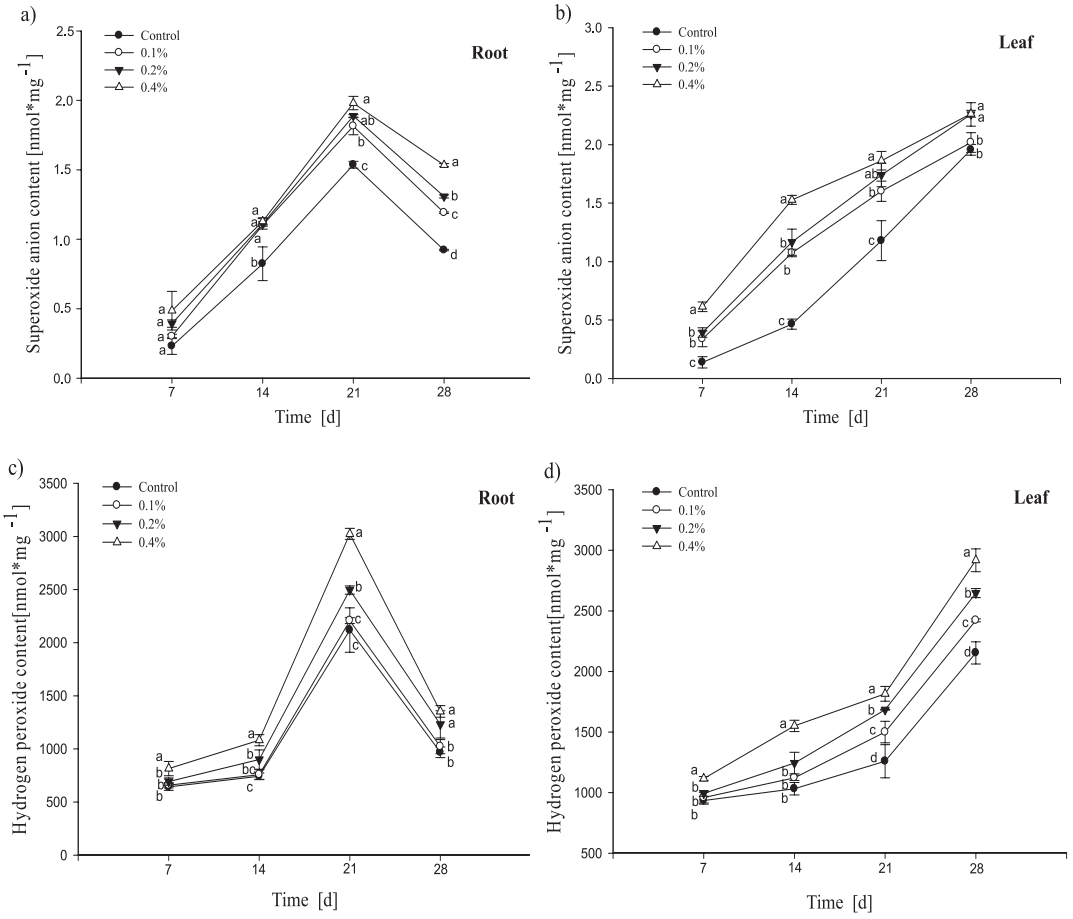

Fig. 7. Effects of different concentrations of NaCl on ROS content in *S. matsudana* for 7, 14, 21, and 28 days: a) O_2^- content in roots, b) O_2^- content leaves, c) H_2O_2 content in roots, and d) H_2O_2 content in leaves. Vertical bars denote SE. Values with different letters differ significantly from each other ($P<0.05$, *t*-test).

physiological and biochemical investigation revealed several features in the seedlings of *S. matsudana* after short-term salt stress.

0.4% NaCl can inhibit seedling growth of *S. matsudana* severely, leading to near complete stoppage. Hence 0.4% NaCl is the lethal concentration for seedling growth of *S. matsudana* under salt stress.

PI is an intercalating agent and a fluorescent molecule that can be used to stain DNA, which is an alternative agent to study cell membrane damage [31]. Morphological changes in the cells may be related to damage to the integrity of cell membranes [32]. Here, the toxic effect of NaCl stress on the cell membrane in the root tips of *S. matsudana* was confirmed by PI staining results. The lower level of fluorescence intensity was noted in the cells exposed to NaCl for 1 h, showing that NaCl could induce cell damage as early as 1 h. This toxic effect increased with increasing NaCl concentration and prolonged treatment time. Katsuhara [33] found that salt stress induced DNA degradation and cell death in barley root tips. When salt stress is temporary or adjustable at the seedling level, nucleotides could be produced by DNA degradation and relocated for shoots and new root formations. In this study, cell death was observed after salt stress. This cell death may lead to inhibition of root growth. A high concentration of NaCl can inhibit seedling growth of *S. matsudana* severely, leading to the growth almost being stopped completely.

Under salt stress, ROS such as O_2^-, hydroxyl radicals (−OH), and H_2O_2 are generally elevated [34]. ROS interfere with other cellular structures, resulting in oxidative damage like lipid peroxidation, protein, DNA damage, etc. [35]. The present investigation showed that NaCl exposure caused increasing O_2^- and H_2O_2 contents of *S. matsudana*, associated with impaired integrity of the plasma membrane in *S. matsudana*. The plant plasma membrane is considered to be the first living structure affected by heavy metal and salt toxicity [36]. ROS can damage biomolecules and induce lipid peroxidation of the cell membranes [37]. Accordingly, the selectivity of cell membranes decreased due to damage to the cell membranes [38-39]. MDA formation is used as the general indicator of the extent of lipid peroxidation resulting from oxidative stress. The results in the present investigation showed that MDA content in the roots and leaves of *S. matsudana* under NaCl stress increased with increasing time, reflecting accumulating damage. Salinity can lead to oxidative stress, which is accompanied by the accumulation of lipid peroxidation products, including MDA. The results obtained here are consistent with earlier

findings [1]. The results here showed that NaCl induced oxidative stress in both roots and leaves of *S. matsudana* as indicated by the increase of MDA, and O_2^- and H_2O_2 levels. By analyzing the tendency of H_2O_2 and O_2^-, we found that the contents of H_2O_2 and O_2^- rose first and then fell in the root, but they always increase in leaves. The roots are the first sensitive plant organ to make contact with the stresses and pollutants [39]. Hence, physiological and metabolic disorders and toxic symptoms appeared in the roots at first.

Plants have effective scavenging mechanisms to respond to damage [39]. The antioxidative enzymatic system plays a protective role by stabilizing the amounts of ROS in plant cells [40-41]. SOD is a cell's first line of defense against ROS as the superoxide radical, which is a precursor to several other highly reactive species, so that controlling the steady state of superoxide concentration by SOD constitutes an important protective mechanism [42]. POD activity reflects the modified mechanical properties of the cell wall and cell membrane integrity of plant leaves under stress conditions [43]. CAT is the most universal oxidoreductase, which scavenges H_2O_2 to O_2 and H_2O. An adapted ROS scavenging system, including SOD, POD, CAT, ascorbate peroxidase (APX), and glutathione reductase (GR), could provide some protection against oxidative damage under salt-stressed conditions [44]. In the present investigation, the activity of SOD, CAT, and POD in roots and leaves of *S. matsudana* under NaCl stress was enhanced except for the SOD activity in leaves exposed to 0.4% NaCl on day 28, and CAT activities in leaves exposed to 0.4% NaCl on days 21 and 28, indicating their active involvement in scavenging ROS generated by NaCl toxicity, and suggesting that the plants have a high ability to tolerate salt stress due to an efficient antioxidant system. The effects of NaCl on SOD and POD were greater in roots than in leaves, explained by the fact that NaCl is in direct contact with the roots and is taken up mostly through roots. Of most high-concentration NaCl accumulated in roots, only small amounts penetrate the leaves. Thus roots suffer from more oxidative stress than the leaves. The decline in CAT activity in leaves at high NaCl concentration (0.4%) is supposedly due to inhibition of enzyme synthesis or a change in assembly of enzyme. Possibly CAT is more sensitive to high concentrations of NaCl stress than SOD and POD.

Under NaCl stress, soluble protein contents in roots of *S. matsudana* increased significantly ($P<0.05$), except for the groups exposed to 0.1% NaCl during days 21 to 28. The high-soluble protein content induced by NaCl may be explained by the following aspect. Salt induces a high expression of multiple genes and increases the synthesis of several primitive proteins. The results in the present investigation showed that soluble protein contents of *S. matsudana* subjected to salt stress were greater than those of plants under non-saline conditions. Soluble protein contents in leaves were greater than those in roots. High salinity levels induce osmotic stress, which is an important mechanism for plants to tolerate salt stress [45-46]. It is an important mechanism through increased net concentrations of solutes or osmotic adjustment to lower cell water potential for maintaining cell water content [47]. Decreasing the osmotic potential of the cell and stabilizing membranes and macro-molecular structure are two possible physiological responses dealing with the accumulation of osmolyte under stress [48].

Conclusions

Plants have evolved complex mechanisms for minimizing the damage from exposure to non-essential high NaCl. Once NaCl concentration in the cell is too high, a defence mechanism is activated, protecting the cells against oxidative stress, which may result in cell death and stress-induced adaptation and survival.

In view of the present investigation, with increasing NaCl concentration, the number of broken and dead cells was obviously increasing. Root growth was seriously inhibited, which might be mainly caused by the death of massive root cells under high concentration and long treatment time. With increased NaCl concentration, ROS showed a significant increasing trend. Meanwhile, with the excess ROS aggravating, the plant cell membrane system was hurt, which leads to the content of MDA and osmotic regulation substances being changed. The alterations of antioxidant enzymes, lipid peroxidation, and cell damage changes in *S. matsudana* can serve as useful biomarkers to estimate the extent of damage a plant suffered with NaCl. Data obtained here may be important for understanding the tolerant mechanism under NaCl stress in woody plants.

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