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H_2O_2 Distribution and its Relationship with Antioxidant Enzymes during Germination in *Dianthus chinensis* L. under Long-Term Salt Stress

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Abstract: *Dianthus chinensis* L. is a very ornamental plant and widely used in landscaping. It can be tolerant to some level of salt stress. In order to study H_2O_2 distribution and activities of antioxidant enzymes under salt stress, seeds of *Dianthus chinensis* L. were treated with different NaCl concentrations. The results suggested that H_2O_2 initially produced in root and then transferred to leaf via stem xylem and leaf vein during seed germination under long-term salt stress. Same phenomena were observed in seedlings under short-term salt stress. However, the distribution varied with time under different NaCl. H_2O_2 was quickly scavenged with 100 mM NaCl. POD and CAT obviously dropped, as well as SOD significantly rose under NaCl. In the meantime, APX and GR seemed to be no associated with salt stress.

Keywords: Antioxidant enzymes, *Dianthus chinensis* L., H_2O_2 balance, H_2O_2 distribution, salt stress

INTRODUCTION

The distribution of plant species in saline environments is closely associated with soil water potentials and other factors influencing the level of salinity stress, including precipitation and depth of the water table (Ungar *et al*., 1979). Soil salinization results in decrease of plant diversity, as well as increase of difficulties in urban landscaping. Therefore, selection and usage of salt-tolerant ornamental plants are the good way to achieve the aim of economic landscaping. *Dianthus chinensis* L. is a very ornamental plant and widely used in urban landscaping due to its salt-tolerance (He *et al*., 2009).

The Reactive Oxygen Species (ROS) increases as a response to most biotic stresses including salinity (Sudhakar *et al*., 2001; Tsai *et al*., 2004). Among different ROS, only Hydrogen peroxide (H_2O_2) can cross plant membranes and it has been increasingly proved to be one of the most important signals in plant cell signaling, especially in elicitor-induced defence responses (Vanderauwera *et al*., 2005; Gadjev *et al*., 2006). Avsian-Kretchmer *et al*. (1999, 2004) had proved that salt or oxidative stress raised H_2O_2 in citrus cell on transcriptional level.

ROS-scavenging enzymes include Superoxide Dismutase (SOD), Peroxidase (POD), Ascorbate Peroxidase (APX), Glutathione Reductase (GR) and Catalase (CAT) (Mittler *et al*., 2004). Some results showed that salt-tolerant plants had positive association with antioxidant enzymes activities. Compared to cultivated tomato, increased activities of SOD, CAT and APX were found in salt-stressed roots of wild salt-tolerant relative (Shalata *et al*., 2001). Additionally, increased activities of total SOD, APX and several is forms of non-specific Peroxides (POD) were found in chloroplasts of salt-treated wild salt-tolerant tomato (Mittova *et al*., 2002). But up till now, researches on roles of H_2O_2 and the link between H_2O_2 and antioxidant enzymes were poor in salt-tolerant plant under salt stresses.

In view of this, the distribution of H_2O_2 during seed germination and activities of antioxidant enzymes of seedlings were examined under different concentrations of NaCl in *Dianthus chinensis* L.

MATERIALS AND METHODS

Plant materials and treatments: After being sterilized with 2.5% sodium hypochlorite for 10 min and repeatedly washed with distilled water, the seeds of *Dianthus chinensis* L. were placed in Petri dishes with sterilized water under light at 22°C to germinate. After 1 day, germinated seeds with more than 2 mm radical were selected and placed in Petri dish containing discs of filter paper soaked in 0, 50, 100 and 150 mM NaCl, respectively. Each Petri dish contained 50 germinated seeds and each treatment was replicated 3 times. Discs of filter paper soaked with different solutions were changed every 24 h. The seedlings grew at 22°C in lightness.

H_2O_2 localization: From 4 to 8 days after treatment, hydrogen peroxide was localized in seedlings using DAB according to an adapted procedure from Thordal-

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Christensen *et al*. (1997) and Orozco-Cardenas and Ryan (1999). Seedlings infiltrated for 30 min in 0.5 mg/mL^{-1} of DAB and then boiled for 10 min in 80% ethanol. Intensity and pattern of DAB staining were assessed visually under stereomicroscope.

Extraction and assay of enzymes: After treatment for 8 days, seedling was homogenized with 0.1 M sodium phosphate buffer (pH 6.8), containing 2 mM ascorbate in a chilled pestle and mortar. The homogenate was centrifuged at 12000 g for 20 min and the resulting supernatant was used for determination of enzymes activity. The whole extraction procedure was carried out at 4°C. APx was determined according to Nakano and Asada (1981). POD was determined following Kuroda *et al*. (1990). The assays of CAT, SOD and GR were conducted according to Aebi (1983), Beyer and Fridovich (1987) and Rao *et al*. (1996), respectively.

Protein contents were determined according to the Bradford (1976) method using Bovine Serum Albumin (BSA) as standard.

RESULTS

H_2O_2 distribution in seedling of *Dianthus chinensis* L. under long-term salt stress: H_2O_2 production was visualized by staining the seedling with 3'3-Diaminobenzidine (DAB) and turned brown in the presence of H_2O_2 (Thordal-Christensen *et al*., 1997; Orozco-Cardenas and Ryan, 1999).

The staining in root tip after being treated for 4 days was visible with or without NaCl. The staining under NaCl was stronger than the control in root tip and increased with NaCl concentration (Fig. 1a). Vascular tissue in stem emerged after five days under 0 and 50 mM NaCl, so the staining was visible in it. Consequently, no staining located when no vascular tissue was in stems with 100 and 150 mM NaCl (Fig. 1b). After 6 days leaf vein emerged under 0, 50 and 100 mM NaCl and the staining was visible in leaves under 50 and 100 mM NaCl. No staining was visible under 0 and 150 mM NaCl (Fig. 1c). Seven days later vascular tissue in stem and leaf emerged with 150 mM NaCl and H_2O_2 was detected in it. Meanwhile staining in leaves was visible under 0 and 50 mM NaCl, but invisible under 100 and 150 mM NaCl (Fig. 1d). No staining in leaves and stems under 0, 50 and 100 mM NaCl, but dark staining showed that H_2O_2 accumulated in whole seedling under 150 mM NaCl after 8 days (Fig. 1e).

H_2O_2 distribution in *Dianthus chinensis* L. seedling under short-term salt stress: *Dianthus chinensis* L. seeds germinated and grew under normal condition. On the 8^{th} day seedlings were collected and treated with

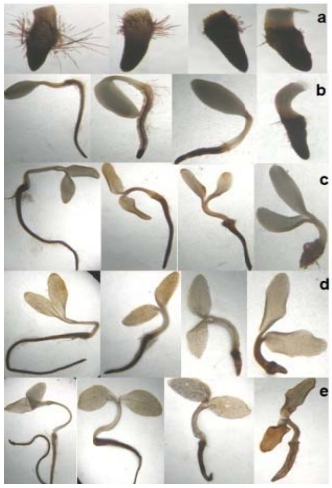

Fig. 1: H_2O_2 accumulation in seedling of *Dianthus chinensis* L. under long-term NaCl. a-e 4, 5, 6, 7 and 8 days under NaCl; from left to right 0, 50, 100, 150 mM

Fig. 2: H_2O_2 accumulation in seedlings of *Dianthus chinesis* L. under short-term NaCl from left to right 50, 100, 150 mM

different NaCl for 4, 12 and 24 h, respectively. After 4 h, staining was visible in all roots and stems under NaCl. Meanwhile, weak staining was also visible in leaves under 150 mM NaCl (Fig. 2). Compared with weak staining in leaves with 50 mM NaCl after 12 h, the staining was strong under 100 and 150 mM NaCl (Fig. 2). With time staining in leaves became dark with 50 mM NaCl. However, the staining in leaves became weak with 100 and 150 mM NaCl after 24 h (Fig. 2). There was no difference between staining in roots under NaCl.

Activities of antioxidant enzymes in *Dianthus chinensis* L. seedling under long-term salt stress: The H_2O_2 distribution in seedling varied with time under different NaCl, suggesting that some

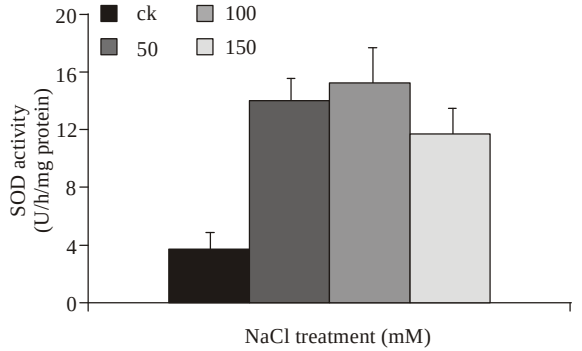

Fig. 3: SOD activity in seedlings of *Dianthus chinensis* L. under NaCl after 8 days

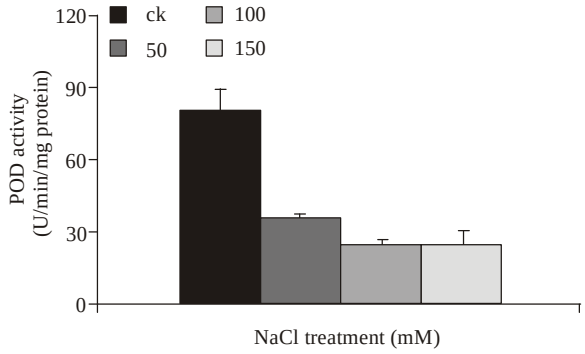

Fig. 4: POD activity in seedlings of *Dianthus chinensis* L. under NaCl after 8 days

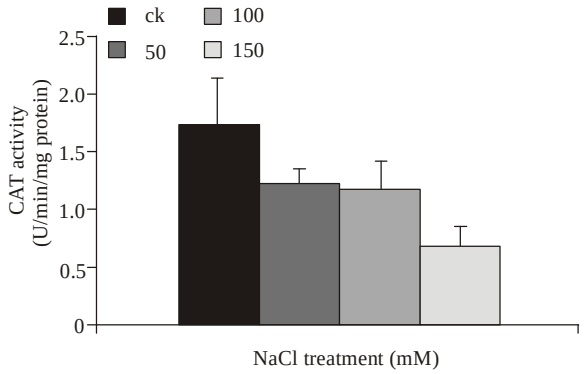

Fig. 5: CAT activity in seedlings of *Dianthus chinensis* L. under NaCl after 8 days

detoxification system was involved. Consequently, treated seedlings for 8 days were used as plant materials to analysis activities of antioxidant enzymes.

Being an H_2O_2-producer enzyme, activity of SOD was significantly different between salt stress and the control (Fig. 3). Compared to the control, SOD activities increased significantly under NaCl, about 250, 280 and 100%, respectively. However, there was no significant difference among different NaCl.

As far as scavenge enzymes related to H_2O_2 were concerned, 4 enzymes were assayed. Activity of POD

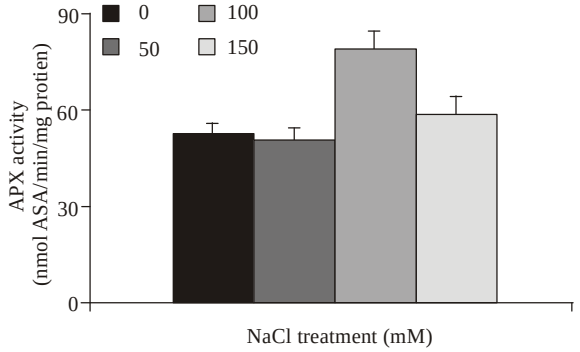

Fig. 6: APX activity in seedlings of *Dianthus chinensis* L. under NaCl after 8 days

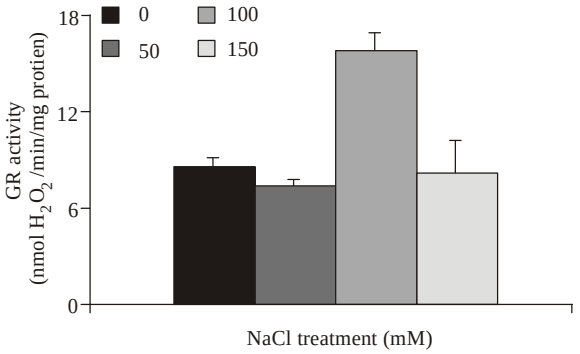

Fig. 7: GR activity in seedlings of *Dianthus chinensis* L. under NaCl after 8 days

decreased with increase of NaCl, but no significant difference under NaCl (Fig. 4). POD activity was obviously inhibited by NaCl. With NaCl increasing activity of CAT dropped. Activity of CAT with 50 mM NaCl was no different with 100 mM NaCl and they were significantly higher than 150 mM NaCl (Fig. 5). Under NaCl activity of APX had the same situation as GR, which the highest was in 100 mM NaCl (Fig. 6). However, no significant difference was observed between 50 and 150 mM NaCl (Fig. 7). It seemed that APX and GR had no association with NaCl.

DISCUSSION

Among the various biotic stress factors, soil salinization is the biggest threat to inland agriculture. Salt stress affects the integrity of cellular membranes, activities of enzymes and the functioning of the plant photosynthetic apparatus (Serrano *et al*., 1999). Oxidative stress are common secondary stress occurring after many kind of biotic or biotic stresses which can change the plant internal redox environment and subsequently disturb its growth processes, metabolism and existence. In order to improve plant tolerance to salinity, it is necessary to reveal physiological mechanisms that allow plants adapts to salinity.

With DAB staining H_2O_2 production can be visualized (Thordal-Christensen *et al*., 1997; Orozco-Cardenas and Ryan, 1999). Weather with or without NaCl, staining was firstly visible in root during germination and then in stem and leaf of *Dianthus chinensis* L. The result revealed that H_2O_2 initially produced in root tip and then transferred to stem and leaf following the formation of vascular tissues. Dat *et al*. (2003) found that H_2O_2 produced along the vein and then in most of leaf sections in catalase-deficient plant of tobacco under high light with time. However, H_2O_2 distributed in seedling varied with time under different NaCl which suggested that some detoxification system was triggered by perturbation of H_2O_2 homeostasis in *Dianthus chinensis* L.

Being an H_2O_2-producer enzyme SOD activity increased significantly under NaCl, but there was no significant difference among different NaCl. POD, CAT, APX and GR are scavenging enzymes related to H_2O_2. CAT is the predominant enzyme controlling the H_2O_2 level and CAT1 expression was induced by applied H_2O_2 in Arabidopsis (Xing *et al*., 2007). Although some studies were tried to elucidate the relation between H_2O_2 and scavenging enzymes, so far it is still unclear (Vansuyt *et al*., 1997; Lin and Kao, 2001; Chang *et al*., 2004). In our study activities of POD and CAT were inhibited by NaCl and significantly dropped. Although the highest activity of APX and GR was in 100 mM NaCl among the treatments, it seemed that these two enzymes had no association with NaCl. We postulated it might be some other detoxification system involved in H_2O_2 scavenging of salt-tolerant *Dianthus chinensis* L.

CONCLUSION

H_2O_2 initially produced in root and then transferred to leaf via xylem and vein under long-term or short-term salt stress. But the distribution in seedling varied with time under different NaCl. SOD significantly rose under NaCl. In contrast, POD and CAT obviously dropped under NaCl. APX and GR seemed to be no associated with salt stress.

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