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Adaptive Responses of Birch-Leaved Pear (*Pyrus betulaefolia*) Seedlings to Salinity Stress

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Abstract.

One-year-old birch-leaved pear (*Pyrus betulaefolia* Bunge) seedlings were subjected to 0, 50, 100, 150, and 200 mmol/L NaCl solutions for 27 days in order to study the effects of salinity stress on photosynthesis, ion accumulation and enzymatic and non-enzymatic scavenging of reactive oxygen species in the seedlings. The research was performed in a greenhouse using potted trees. Salinity stress reduced photosynthetic rates, stomatal conductance and water use efficiency of leaves of the pear seedlings, but increased transpiration rates and leaf temperature. Hydrogen peroxide and superoxide anion radical contents increased with increasing NaCl concentrations, a phenomena also observed for malondialdehyde, suggesting that leaves of the pear seedlings suffered from oxidative injury. Superoxide dismutase (SOD) and catalase (CAT) activities quickly responded by increasing when the pear seedlings were subjected to salinity stress. Total protein content in leaves of the seedlings was restrained by salinity stress, whereas ascorbate content increased. Salinity stress reduced glutathione content once the birch-leaved pear seedlings were exposed to a low level (50 or 100 mmol/L) of NaCl, whereas a high level (150 or 200 mmol/L NaCl) of salinity stress stimulated the accumulation of glutathione. Salinity stress increased the accumulation of Na+, Cl-, K+ and Mg2+ in the seedlings, but reduced Ca2+ levels and the ratio of other ions to Na+ except K+/Na+ under 50 mmol/L NaCl conditions. This suggests that leaves of birch-leaved pear seedlings possess the capacity for salt exclusion only under 50 mmol/L NaCl conditions, and Ca2+ does not play a fundamental role as a secondary messenger under salinity stress conditions.

Key words: birch-leaved pear; Ca2+; photosynthesis; reactive oxygen species; salinity stress

Introduction

Soil salinization is a serious problem in the entire world, and it has grown substantially causing loss in crop productivity (da Silva *et al*., 2008). It is estimated that almost 109 ha of land around the world, which corresponds to 7% of the global land surface, are already salinized (Szabolcs, 1994). In addition, about 5% of cultivated land is affected by salinity and about 20% of irrigated land is suffering from secondary salinization due to inappropriate treatment of irrigation systems (Miyake *et al*., 2006). Thus, salinity stress imposes a major environmental threat to agriculture, so it is an important research subject for increasing global agricultural production. Understanding the basic physiological and biochemical responses of plants to salinity stress is crucial agricultural productivity.

Pear (*Pyrus* spp.) belongs to the *Rosaceae*, subfamily *Pomoideae*, the pome fruits (Jackson, 2003). Pear trees are generally sensitive to soil salinity (Francois and Maas, 1994), and are damaged by exposure to relatively low salinity for long periods (Okubo *et al*., 2000). The productivity of pear trees in China, Japan and Korea is frequently restricted by soil salinity, especially in arid and semi-arid areas (Myers *et al*., 1995). Westwood and Lombard (1983) reported a large variation among *Pyrus* species in soil adaptation. Birch-leaved pear (P. *betulaefolia* Bunge) is native to northeast China and is now used as a rootstock for European and Japanese pear cultivation (Okubo and Sakuratani, 2000). However, information about the adaptive responses of birch-leaved pear to salinity stress is limited. In birch-leaved pear, only two experiments on growth and mineral uptake responses to salinity stress have been conducted under potted conditions (Okubo and Sakuratani, 2000; Matsumoto *et al*., 2006), but salinity effects on photosynthesis, scavenging of reactive oxygen species (ROS) and ion accumulation have attracted little attention. Therefore, in this work, an attempt was made to understand the adaptive responses of birch-leaved pear by evaluating photosynthesis, enzymatic and non-enzymatic scavenging of ROS, and ion accumulation to salinity stress.

Materials and methods

Plant materials and experimental design

This trial was carried out in a plastic greenhouse at the College of Horticulture and Gardening, Yangtze University, China, between May and August, 2007, where no temperature controlling equipment was available. The photo flux density ranged from 550 to 850 μmol/m2/s2; the min/

max day temperature was 20/37 °C. The mature seeds of birch-leaved pear previously held in wet sand storage and for 9 weeks of cold stratification were sown in washed sand trays in March, 2007. Two five-leaved seedlings, uniform in size, were transferred into a plastic pot (16 cm in depth and 20 cm in diameter) filled with 3.56 kg of soil (pH 7.6, available phosphorus 26.25 mg/kg) on May 17, 2007.

The seedlings acclimated for 60 days and then were subjected to salinity stress. The salinity gradients were developed by adding 400 mL of 50, 100, 150, 200 mmol/L NaCl solutions (EC values were 4.9, 9.7, 13.6, and 17.7 dS/m, respectively), and the control (0 mmol/L NaCl treatment) seedlings were irrigated with 400 mL of distilled water (EC 0.1 dS/m). The soil was salinized stepwise to avoid osmotic shock using 50 mmol/L NaCl per day. Each of the five treatments was replicated three times in a completely randomized design, leading to a total of 15 pots. The seedlings were harvested 27 days after starting salinity stress. A fraction of leaf tissues were oven dried at 75 °C for 48 h to constant weight. The other leaf tissues were frozen and stored at -70 °C for assays.

Parameter measurement

Stomatal conductance (gs), transpiration rates (E), photosynthetic rates (Pn) and leaf temperature (LT) were measured using an infrared gas analyzer (Li-6400, Li-Cor, Lincoln, USA) on six replicated leaves randomized from three replicate pots of each treatment from 10:00 to 11:00 am in the greenhouse at the day of harvest, using the fourth mature leaf from the apex. Water use efficiency (WUE) was calculated as WUE=Pn/E.

About 100 mg of dry leaf tissue were used for extracting inorganic ions. The samples were incubated in 20 mL distilled water at 100 °C for 2 h, and kept cold until assayed. The cations K+, Na+, Ca2+ and Mg2+ contents were determined directly using an Atomic Emission Spectrometer (AA670, Shimadzu, Japan). Cl- was assessed with the method of silver ion titration (Bao, 2000).

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation according to the method of Sudhakar *et al*. (2001). Superoxide anion radical ($O2^{-}$) assay was performed as described by Wang and Luo (1990). Hydrogen peroxide (H2O2) was determined according to the method of Velikova *et al*. (2000).

Leaves (0.5 g) were homogenized in 5 mL of 0.1 mol/L phosphate buffer, pH 7.8, containing 0.1 mmol/L EDTA, 1 mmol/L ascorbate, 1 mmol/L 1,4-dithiothreitol and 2% (w/v) polyvinylpyrrolidone. Insoluble material was removed by centrifugation at 4,000 g for 10 min, with the resulting supernatant used for the assays of superoxide dismutase (SOD), catalase (CAT) and total total protein. SOD (EC 1.15.1.1) activity was measured using the method of Giannopolitis and Ries (1977). CAT (EC 1.11.1.6) activity was assayed as described by Wang (2006). CAT activity was defined as the consumption of KMnO4 (0.1 mol/L) for 1 min from 1 g fresh sample. Total protein was evaluated by the method of Bradford (1976) using bovine serum albumin as the standard.

Approximately, 0.4 g of leaf tissues were homogenized by the addition of 5 mL 5% trichloroacetic acid in an ice bath. The homogenates were centrifuged at 15,000 g at 4 °C for 15 min. The supernatant was collected for determination of ascorbate (ASC) and glutathione (GSH) using the previously described method by Wu *et al*. (2006).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS Institute Inc., Cary, N.C.) and Fisher's Protected least significant difference (LSD, $P<0.05$) was used to compare means.

Results

Salinity affected gas exchange of birch-leaved pear seedlings. The reduction in gs, Pn and WUE values and the increase in E and LT values occurred in stressed seedlings during salinity periods (Tab. 1). Salinity stress significantly reduced Pn and WUE. Salinity stress significantly increased E and LT of the seedlings when above the 50 mmol/L NaCl treatment. High-salinity treatments decreased gs of the seedlings.

Compared with the control, all levels of salinity stress increased O2.- and MDA contents of the seedlings, which did not elevate significantly under 50-150 mmol/L NaCl conditions (Tab. 2). O_2.- content of the seedlings grown under 200 mmol/L NaCl conditions was significantly higher than the other concentrations of NaCl. Low-salinity treatments did not significantly affect H_2O_2 content of the seedlings. However, after exposure to 150 mmol/L NaCl,

Tab. 1. Effect of salinity stress on leaf photosynthesis and gas exchange of birch-leaved pear seedlings

Salinity (mmol/L)	Pn (μmol/m2/s)	E (mmol/m2/s)	g_s (mmol/m2/s)	LT(°C)	WUE
0	6.64a	2.28d	0.035a	40.21c	2.92a
50	6.04b	2.55cd	0.032a	40.31c	2.38b
100	5.87bc	2.78bc	0.032a	40.74b	2.12bc
150	5.48cd	3.01ab	0.023b	41.31a	1.84cd
200	5.11d	3.14a	0.019b	41.58a	1.63d

Different letters following the data within each column mean significant difference at 0.05 level

H_2O_2 content abruptly increased to the highest level, up to 5.72 μg/g, and then slightly decreased under increasing salinity. Increasing salinity stress induced an increasing trend of SOD and CAT activity (Tab. 3). CAT activity of the seedlings did not significantly increase at 50 mmol/L NaCl, but was markedly elevated at 100-200 mmol/L NaCl. Increasing salinity stress induced a decrease in total protein content. Stressed seedlings presented markedly higher ASC content, with ASC content highest at 100 mmol/L NaCl, and decreasing notably above that. Salinity reduced GSH content of the seedlings at 100 mmol/L and increased it at 200 mmol/L/.

Tab. 4 shows that Cl- and Na+ levels increased with increasing NaCl concentrations in leaves of birch-leaved pear seedlings. All of the NaCl treatments significantly elevated K+ concentration but markedly reduced Ca2+ concentration. Mg2+ concentration was increased notably by 100-200 mmol/L NaCl. Means comparisons showed that the highest K+ (23.97 mg/g) and Mg2+ (5.89 mg/g) and the lowest Ca2+ (2.51 mg/g) concentrations each appeared under 150 mmol/L NaCl conditions.

Compared with the controls, the Ca2+/Na+ ratio was significantly reduced by NaCl treatments (Tab. 5). K+/Na+ ratio was notably enhanced only at 50 mmol/L NaCl conditions, and other NaCl concentrations did not alter

Tab. 2. Effects of salt stress on leaf H2O2, O2.- and MDA contents of leaves of birch-leaved pear

Salinity (mmol/L)	O2.- (μmol/g)	H2O2 (μg/g)	MDA (μmol/g)
0	86.97c	4.26c	20.90c
50	120.70b	3.92c	26.57b
100	128.97b	4.35bc	27.30ab
150	137.57b	5.72a	28.74ab
200	180.56a	4.86b	31.81a

Different letters following the data within each column mean significant difference at 0.05 level

Tab. 3. Effect of salt stress on activities of antioxidant enzymes and contents of antioxidants of leaves of birch-leaved pear

Salinity (mmol/L)	SOD (U/g)	CAT (mg/g/min)	Total protein (mg/g)	ASC (mmol/g)	GSH (mmol/g)
0	524.01d	4.72d	7.40a	1.46c	0.74bc
50	742.82c	5.61cd	6.98ab	2.69b	0.68cd
100	760.1c	6.49c	6.61b	5.93a	0.66d
150	1271.44b	8.26b	5.43c	2.64b	0.78b
200	1812.72a	9.44a	4.33d	2.49b	1.09a

Different letters following the data within each column mean significant difference at 0.05 level

Tab. 4. Effects of salt stress on foliar concentrations of inorganic ions of birch-leaved pear

Salinity (mmol/L)	Cl- (mg/g)	K+ (mg/g)	Na+ (mg/g)	Mg2+ (mg/g)	Ca2+ (mg/g)
0	0.32d	16.17c	4.76d	4.94c	4.49a
50	0.38bc	21.71ab	5.57c	5.09bc	3.63b
100	0.44b	21.05b	6.07bc	5.54ab	3.17b
150	0.54a	23.97a	6.55b	5.898a	2.51c
200	0.60a	21.14b	7.43a	5.58ab	3.08b

Different letters following the data within each column mean significant difference at 0.05 level

Tab. 5. Effects of salt stress on ratio of various ions and Na+ of leaves of birch-leaved pear

Salinity (mmol/L)	K+/Na+	Ca2+/Na+	Mg2+/Na+
0	3.40b	0.47a	1.04a
50	3.90a	0.33b	0.92a
100	3.48ab	0.26c	0.92a
150	3.68ab	0.19d	0.91ab
200	2.84c	0.20d	0.75b

Different letters following the data within each column mean significant difference at 0.05 level

or significant decreased K+/Na+ ratio. Mg2+/Na+ ratio was markedly reduced only at 200 mmol/L NaCl.

DISCUSSION

Under salinity stress, leaf photosynthetic capacity is limited by the electron transport capacity of thylakoid proteins, the activity of Rubisco and mesophyll resistance (Searson *et al*., 2004). Salinity stress reduced significantly Pn and gs of birch-leaved pear seedlings, and the reduction was proportional to the increase in NaCl level. The reduction in gs of the seedlings under salinity stress effectively limited CO2 influx (Nandy *et al*., 2007). Similar results were reported by Liao *et al*. (1997) with Vitis and Ashraf (2001) with *Brassica* species where both Pn and gs showed significant decreasing trends with increasing salt concentrations in the rooting medium. According to Bañuls and Primo-Millo (1992), the negative effects of salinity in *Citrus* leaves are caused by accumulation of not Cl- but Na+. It is unclear whether the same phenomena occurs in the pear seedlings. Since E and LT of the seedlings were elevated by salinity stress, WUE naturally decreased. The enhanced WUE of nonhalophytes under salinity stress is generally regarded as a sodium avoidance mechanism (Brugnoli and Bjorkman, 1992). Ashraf (2001) recorded increasing WUE of the salt tolerant *Brassica* species with increasing external salt concentration. Thus, birch-leaved pear seedlings are salt-sensitive plants.

MDA, a product of lipid peroxidation, could reflect the degree of lipid peroxidation (Lacan and Baccou, 1998) and has been considered an indicator of oxidative damage (de Azevedo Neto *et al*., 2006). In our experiment, salinity stress increased the MDA content of the seedlings, with increases related to the production of H_2O_2 and O_2.-. The H_2O_2 and O_2.- contents of the seedlings were increased by NaCl treatments, implying that ROS metabolism of the seedlings is broken, initiating destructive oxidative processes such as chlorophyll bleaching, lipid peroxidation, protein oxidation, and damage to nucleic acids (Herbinger *et al*., 2002). Our results are in agreement with those of Panda and Upadhyaya (2003) in *Lemna minor*, de Azevedo Neto *et al*. (2006) in maize, and Mandhania *et al*. (2006) in wheat.

Under salinity stress, because ROS are toxic, plant cells present a mechanism to regulate their intracelluar ROS concentrations by enzymatic (SOD, CAT, etc) and non-enzymatic (ascorbate, glutathione, etc) scavenging of ROS (Mittler, 2002). Once the pear seedlings were subjected to salinity stress, enzymatic scavenging of ROS indicated by increased SOD and CAT activities occurred. The two enzymatic activities increased with the increase in NaCl level, implying the existence of an effective scavenging mechanism to remove ROS. Similar results are shown in previous work with *Catharanthus roseus* (Jaleel *et al*., 2007), wheat (Esfandiari *et al*., 2007), *Elaeagnus angustifolia* (Wang and Yao, 1993), pea (Hernandez *et al*., 2000), and mulberry (Sudhakar *et al*., 2001). A correlation of these enzyme levels and salt tolerance was demonstrated (Parida and Das, 2005). The scavenging capacity of these enzymes was limited and was not sufficient for the complete scavenging of ROS. As a result, the production of H_2O_2 and O_2.- were maintained at a high level in the seedlings.

The non-enzymatic antioxidative mechanisms like GSH, ASC, and total protein responded differently to NaCl treatment. In the pear seedlings, total protein content decreased with increasing salinity stress levels. Ramagopal (1987) also reported that salinity inhibits the synthesis of a majority of shoot proteins in barley seedlings. The present study showed that ASC content of the seedlings notably increased upon salinity stress treatments, especially 100 mmol/L NaCl. This result also was reported by Yu *et al*. (2003) in wild soybean. The increased ASC content is a stress-protecting mechanism of plants under salinity conditions (Shalata *et al*., 2001). ASC is an important antioxidant, which reacts not only with H2O2 but also with O2.- (Reddy *et al*., 2004). ASC can also be used as the terminal antioxidant because the redox potential of the ASC/monodehydroascorbate pair is lower than that of most other bioradicals (Jaleel *et al*., 2007). A high level of endogenous ASC is essential for maintaining the non-enzymatic scavenging system that protects plants from oxidative damage due to salinity stress (Shigeoka *et al*., 2002). We observed that GSH content was enhanced only under 200 mmol/L NaCl, and other NaCl treatments did not alter or significantly decreased GSH content, suggesting that under severe stress conditions, GSH of the pear seedlings plays a protective role in salinity tolerance. The decrease in GSH found in the study might be due to its oxidation under salinity conditions (Jaleel *et al*., 2007).

In the present study, Cl- and Na+ levels increased with increasing NaCl concentrations in leaves of the pear seedlings. Similar observations were made in young umbu plants by da Silva *et al*. (2008) and broad bean plants by Gadallah (1999). Leaves are more vulnerable than roots to Na+ simply because Na+ and Cl- accumulate to higher levels in shoots than in roots. More Na+ and Cl- accumulation results in Na+-specific damage, inducing necrosis of older leaves (Parvaiz and Satyawati, 2008).

Where Na+ is deleterious for plant growth, K+ is one of the essential elements and is required by the plant in large quantities (Mahajan and Tuteja, 2005). Maintenance of a high cytosolic K+/Na+ ratio is a key feature of plant salt tolerance (Cuin *et al*., 2008; Kronzucker *et al*., 2008). Under 50 mmol/L NaCl conditions, K+/ Na+ ratio significantly increased, implying that leaves of the pear seedlings possess the capacity for salt exclusion under 50 mmol/L NaCl conditions. However, under 100-200 mmol/L NaCl conditions, excess Na+ suppressed K+ influx across the plasma membrane and induced K+ leakage, resulting in the decrease of K+/Na+ ratio. The salinity-induced K+ loss from cells is a result of NaCl-induced membrane depolarization, leading to the activation of depolarization-

activated outward-rectifying K+ channels (Shabale *et al*., 2006).

It is well-known that Ca2+ alleviates the adverse effects of salinity on many plant species (Gul and Khan, 2006; Munns, 2002; Ebert *et al*.; 2002). The salt tolerance of species that exclude salts is achieved by changes between Na+ and Ca2+, rather than changes in osmotic potential, since adsorption of Ca2+ on membranes of root cells leads to reduced penetration of monovalent cations (Dajic, 2006). High salinity results in increased cytosolic Ca2+ that is transported from the apoplast as well as the intracellular compartments (Knight *et al*., 1997). This transient increase in cytosolic Ca2+ initiates the stress signal transduction leading to salt adaptation (Mahajan and Tuteja, 2005). In the present study, NaCl treatments significantly reduced the Ca2+ concentrations of birch-leaved pear seedlings and Ca2+/ Na+ ratio, implying that Ca2+ does not perform the signal transduction when the seedlings are exposed to salinity stress. High Na+ levels of plants can replace bound Ca2+ of plasma membrane and intracellular membrane system (Reddy and Reddy, 2002), thus inducing the decrease of Ca2+/Na+ ratio, as a result of the damage to membrane structural integrity and function.

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