Tree Physiology 28, 947–957

Ionic homeostasis and reactive oxygen species control in leaves and xylem sap of two poplars subjected to NaCl stress

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Received August 23, 2007; accepted December 4, 2007; published online April 1, 2008

Summary We investigated the effects of increasing soil NaCl concentration on intracellular compartmentalization of salt and on the activities of antioxidant enzymes (superoxide dismutase (SOD), ascorbic peroxidase (APX), catalase (CAT) and glutathione reductase (GR)) and their role in the regulation of reactive oxygen species (ROS; O_2^- and H_2O_2) in leaves and xylem sap of salt-tolerant *Populus euphratica* Oliv. and salt-sensitive *P. popularis* cv. 35-44. Mesophyll cells of *P. euphratica* exhibited a high capacity for NaCl exclusion and compartmentalization of salt in vacuoles compared with *P. popularis*. In *P. popularis*, the salt treatment resulted in large accumulations of Na^+ and Cl^- in leaves that induced significant increases in O_2^- and H_2O_2 production despite marked increases in the activities of antioxidant enzymes in leaves and xylem sap. Separation of the isoforms of leaf SOD, APX and CAT by polyacrylamide gel electrophoresis followed by in-gel activity staining revealed that the salt-induced activities of APX and CAT were the result of increases in activities of all the isoenzymes. Leaf injury and shedding of aged leaves occurred following the oxidative burst in *P. popularis*, indicating that the increased activities of antioxidant enzymes in *P. popularis* were insufficient to counter the harmful effects of ROS at high soil NaCl concentrations. Unlike *P. popularis* plants, *P. euphratica* plants did not exhibit an oxidative burst in response to the NaCl treatments, because of (1) a high salt exclusion capacity and effective compartmentalization of salt in vacuoles, and (2) up-regulation of antioxidant enzymatic activities after the onset of salt stress. We conclude that *P. euphratica* plants subjected to saline conditions control ROS homeostasis through two pathways: (1) by maintaining cellular ionic homeostasis and thereby limiting the NaCl-induced enhancement of ROS production under long-term saline conditions; and (2) by rapidly up-regulating antioxidant defenses to prevent oxidative damage.

Keywords: APX, CAT, GR, H_2O_2, isoenzyme, leaf, Populus euphratica, Populus popularis, SOD, superoxide radical, X-ray microanalysis.

Introduction

In addition to osmotic stress and ion toxicity, salinity induces production of reactive oxygen species (ROS) and causes oxidative stress (Hernández et al. 1993, 1995, 1999, 2000, Gosset et al. 1996, Gómez et al. 1999, Savouré et al. 1999). Plants modulate the amounts of cellular antioxidants and antioxidant enzymes to alleviate the oxidative damage initiated by ROS. The main scavenger of superoxide radicals (O_2^-) is superoxide dismutase (SOD), a group of metallo-enzymes (Mn-SOD, Cu/Zn-SOD and Fe-SOD) that catalyzes the conversion of O_2^- to hydrogen peroxide (Beauchamp and Fridovich 1973). Catalase (CAT) and ascorbate peroxidase (APX) detoxify H_2O_2 to water and oxygen. Glutathione reductase (GR) acts by recycling oxidized glutathione using NADPH as a cofactor. Based on species-specific variations in antioxidant response to salinity, it is suggested that increased activities of antioxidant enzymes represent one component of salt tolerance in plants (Gosset et al. 1994, Hernández et al. 1995, 2000, Gueta-Dahan et al. 1997, Dionisio-Sese and Tobita 1998, Shalata et al. 2001).

There is much variation in salt tolerance among *Populus* species. Comparative investigations have shown that *Populus euphratica* Oliv. is more salt resistant than the hybrid *Populus talassica* Kom. × (*P. euphratica* + *Salix alba* L.) and other hybrids and species, e.g., *P. deltoides* × *P. nigra* (Dode) Guinier cv. I-214 (*P.* cv. I-214), *P. simonii* × (*P. pyramidalis* × *Salix matsudana*) (*P. popularis* cv. 35-44, *P. popularis*) and *P. tomentosa* Carr. (Ma et al. 1997, Fung et al. 1998, Chen et al.

2001, 2003). Salt tolerance of *Populus* spp. largely depends on the ability to exclude salt, and salt-sensitive species usually exhibit a lower capacity to exclude salt than salt-resistant species (Chen et al. 2001, 2002*a*, 2002*b*, 2003, Gu et al. 2004). Recently, we found that an increase in membrane permeability in salt-sensitive *P. popularis* and *P.* cv. I-214 was associated with the accumulation of salt in leaves in response to increasing salt stress (Wang et al. 2006). Furthermore, salt accumulation in leaves and chloroplasts of *P. popularis* induced a decline in antioxidant enzymatic activities, e.g., SOD, POD, leading to lipid peroxidation in turn leading to ion leakage (Wang et al. 2007). However, little is known about the variation in salt-induced ROS production in poplars differing in NaCl sensitivity, or about the role of antioxidant enzymes in controlling ROS production in leaves and xylem sap.

Compartmentalization of salt in plant cells seems to attenuate oxidative stress under saline conditions. There is evidence that NaCl-stressed sorghum and barley plants preferentially partition Cl^- into leaf sheaths relative to blades, and into epidermal vacuoles relative to mesophyll cells (Boursier and Läuchli 1989, Huang and van Steveninck 1989, Dietz et al. 1992, Leigh and Storey 1993). Exclusion of Cl^- from the photosynthetically active mesophyll would lessen the effect of salinity on photosynthetic processes in the leaf blade. High Cl^- concentrations in leaf mesophyll cells of a barley cultivar (cv. Clipper) indicate that the low salt resistance of this cultivar is directly related to the low degree of Cl^- exclusion by the mesophyll cells (Huang and van Steveninck 1989).

Accordingly, we hypothesized that salt-tolerant *P. euphratica* attenuates oxidative stress by excluding NaCl from the mesophyll, thereby reducing the salt-induced perturbation of photosynthetic processes in mesophyll cells, especially the electron transport processes in the chloroplasts, and enabling ROS homeostasis to be maintained under saline conditions. To test this hypothesis we conducted cellular studies. Our primary objective was to investigate the effects of NaCl on ionic homeostasis, and the activities of antioxidant enzymes and their role in ROS (O_2^- and H_2O_2) regulation in leaves and in xylem sap. We also attempted to compare the capacity for ROS control in poplar species differing in salt tolerance.

Materials and methods

Plant materials

We studied 1-year-old seedlings of *P. euphratica* Oliv. and 1-year-old rooted cuttings of *P. popularis* cv. 35–44 (hereafter *P. popularis*). Hardwood cuttings of *P. popularis* were obtained from the nursery at Beijing Forestry University (China) and seedlings of *P. euphratica* were collected from the Xinjiang Uygur Autonomous Region of China. On April 3, 2005, each plant was transferred to a 10-l pot containing a 2:1 (v/v) mix of nursery soil and sand. The potted plants were placed in a greenhouse and kept well watered and received 1 l of full-strength Hoagland's nutrient solution every 2 weeks. *Populus euphratica* plants showed vigorous growth in May, whereas *P. popularis* plants had a higher growth rate in June. Salt stress was initiated in July when plants of both species exhibited similar growth rates.

Salt treatment and soil analysis

Plants were subjected to 18 days of increasing NaCl stress by top watering with 2 l of 0 (control), 100, 150, 200 and 250 mM NaCl in full-strength Hoagland's nutrient solution on days 1, 5, 10 and 15. Plants were harvested after 2, 7, 13 and 18 days of exposure to the saline treatments. At each harvest, soil Na^+, Cl^-, K^+, Ca^{2+} and Mg^{2+} concentrations were measured as described by Chen et al. (2001). Ion analysis showed that, at each harvest, soil NaCl concentrations in the 100, 150, 200 and 250 mM NaCl treatments were 112, 147, 178 and 250 mM, respectively. Soil NaCl concentration in the control treatment was about 10 mM throughout the experiment. Concentrations of K^+, Ca^{2+} and Mg^{2+} in the soil were not significantly altered by the NaCl treatments.

Leaf harvest

At each sampling time, leaves were sampled from the upper shoot (leaf index number 4-10 from the shoot apex) and immediately frozen in liquid N_2, then stored at −80 °C until analyzed. Three plants were harvested per treatment.

Xylem sap extraction

At each sampling time, xylem sap was collected as described by Liang and Zhang (1997) and Chen et al. (1997, 2001). Briefly, terminal twigs (20 cm to 30 cm long, fresh mass 15–20 g) were excised and immediately enclosed in a pressure chamber. Bark was removed to a height of about 5 cm to avoid xylem sap contamination by phloem exudate. The pressure was slightly increased above the balance pressure (the pressure that just brought the water to the cut surface) and then maintained at 0.5–1.0 MPa above that pressure for 5–10 min to obtain a xylem sap flow rate of 20–40 µl min^{-1}. Xylem sap was collected in vials and the collection was stopped when 200 µl had exuded. Xylem sap was analyzed for ROS (O_2^-, H_2O_2) and activities of antioxidant enzymes (SOD, CAT, APX and GR). For each of the above-mentioned variables, three plants per treatment were harvested at each sampling time. To determine the degree of contamination by cytoplasmic and chloroplastic constituents in the xylem sap, concentrations of reducing sugars, sucrose and soluble sugars were measured. Results showed that there were no detectable sugars in xylem sap of either the control or NaCl-treated plants.

Ion analysis

Leaf samples were oven-dried (65 °C for 4 days) and ground to pass a 1-mm sieve for mineral analysis. Tissue Na^+ and Cl^- were analyzed as described by Chen et al. (2001).

X-Ray microanalysis

Samples were prepared for X-ray microanalysis as described by Fritz (1989) with several modifications. At the final harvest, leaf sections, 2 × 2 mm, were cut with a razor blade along the smaller veins and immediately placed in isopentane that

had been pre-cooled in liquid nitrogen. Afterward, samples were vacuum freeze-dried at –106 °C for 7 days and then allowed to equilibrate at room temperature for 24 h. Thereafter, samples were stored over silica gel until embedded in plastic.

Freeze-dried samples were placed in pressure chambers, vacuum infiltrated with diethyl ether and stored overnight at room temperature before infiltration with plastic (i.e., a 1:1 mixture of styrene and butyl methacrylate containing 1% benzoylperoxide). Infiltration with plastic was carried out as follows: 1:1 ether:plastic for 24 h, 1:2 ether:plastic for 24 h, 1:3 ether:plastic for 24 h, and finally 100% plastic for 24 h (×2). Following infiltration, samples were transferred to gelatine capsules and polymerized at 60 °C for at least 7 days. The polymerized samples were sectioned into 1-µm thick slices with a dry glass knife attached to an ultramicrotome, mounted on copper grids (mesh 50), coated with carbon and stored over silica gel until analyzed.

Sections were examined with a HITACHI-H800 transmission electron microscope equipped assembled with an EDAX-9100 energy dispersive X-ray analyzer. The accelerating voltage was 150 kV with a take-off angle of 25°. Probe measurements were made on mesophyll cells, and the following structures were examined: cell wall, cytoplasm (with chloroplasts) and vacuole. The electron beam was modified according to the shape and size of the targeted cell compartments. Probe measurements of cell walls were taken with a long and narrow electron beam, and measurements of vacuole were taken with a broad electron beam covering all vacuole structures. Ten to 20 measurements were taken from each compartment. The magnification was 6350× and the counting time for each spectrum was 60 s. The X-ray data were expressed as counts per second (cps) of an element peak after subtraction of the background (Fritz 1989).

Leaf enzyme extraction

Leaf tissues (0.5 g) were ground to a fine powder in liquid N_2 and then homogenized in 4 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP), as described by Jiang and Zhang (2002). The homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C and the supernatant was assayed for superoxide radical production rate ($O_2^{\cdot-}$) and activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). For ascorbate peroxidase (APX) measurement, 1 mM ascorbic acid (ASC) was added to the enzyme extraction buffer. Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

Assays of antioxidant enzymes in leaf tissue and in xylem sap

Superoxide dismutase Total SOD activity was measured by monitoring superoxide-radical-induced reduction of nitro blue tetrazolium (NBT) at 560 nm (Giannopolits and Ries 1977). One unit of SOD was defined as the amount of enzyme causing 50% inhibition of the reaction compared with a blank sample. The 3-ml reaction mixture contained 1.75 ml of 50 mM potassium phosphate buffer (pH 7.8), 0.3 ml of 30 mM methionine, 0.3 ml of 750 µM NBT, 0.3 ml of 100 µM EDTA-Na_2, 0.3 ml of 20 µM riboflavin and 50 µl of enzyme extract or 200 µl of xylem sap. For the blank controls, 50 µl of 50 mM potassium phosphate buffer (pH 7.8) was added to the reaction mix instead of enzyme extract. The reaction mixtures with and without tissue extracts were illuminated by cool white fluorescent lamps (30 µmol m^{-2} s^{-1}) for 8 min, and a control set of reaction mixtures that included both blank controls and mixtures with tissue extracts received no illumination. Thereafter, the absorbance (*A*) at 560 nm was recorded and SOD activity was calculated as $A_{enzyme} - A_{control}$.

Catalase Total CAT activity was determined as the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) measured at 240 nm for 3 min at 25 °C (Aebi 1984). The 3-ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 2% H_2O_2 and 30 µl of leaf enzyme extract or 100 µl of xylem sap. Immediately after the enzyme extract or xylem sap was added to the reaction mixture, the initial linear rate of decrease in absorbance at 240 nm was recorded and CAT activity calculated.

Glutathione reductase Total GR activity was determined based on the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 3 min at 25 °C in a 3-ml assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 5 mM GSSG (glutathione disulfide) and 50 µl of leaf enzyme extract or 100 µl of xylem sap. The reaction was initiated by the addition of NADPH. Corrections were made for the background absorbance at 340 nm in the absence of NADPH (Schaedle and Bassham 1977).

Ascorbate peroxidase Total APX activity was determined as the decrease in A_{290} (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 2 min at 25 °C in a 3-ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 15 mM ascorbate, 30 mM H_2O_2 and 30 µl of enzyme extract or 100 µl of xylem sap. The reaction was started by the addition of H_2O_2. Correction was made for the low, non-enzymatic conversion of ascorbate by H_2O_2 (Nakano and Asada 1981).

Assays of SOD, APX and CAT isoenzymes in leaves

Separation of the isoforms of SOD, APX and CAT was performed by native polyacrylamide gel electrophoresis (PAGE) at 4 °C with the Laemmli (1970) buffer systems. Crude leaf protein extracts were mixed with 20% glycerol (v/v) and 0.25% bromophenol blue before loading onto the gels. Thirty µg of protein was applied to each lane.

SOD isoenzymes A 10% separating gel and 3.9% stacking gel were used for native PAGE of SOD. Immediately after electrophoresis at 4 °C, SOD in the gels was visualized by activity staining as described by Beauchamp and Fridovich (1971). Briefly, the gel was immersed in a solution containing 2.45 mM NBT for 20 min in darkness at room temperature. The gel was then rinsed with distilled water and incubated in 36 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED

and 0.028 mM riboflavin for 15 min in darkness. Finally, gels were incubated in 50 mM potassium phosphate buffer (pH 7.8) containing 0.31 mM EDTA and exposed to cool white fluorescent lamps (30 μmol m^{-2} s^{-1}) until SOD bands became visible (SOD bands appeared as light bands on a blue background).

APX isoenzymes For native PAGE of APX, separating gel (10%), stacking gel (3.9%) containing 10% glycerol were prepared, and APX activity was measured as described by Mittler and Zilinskas (1993). In brief, gels were pre-run for 30 min in the carrier buffer containing 2 mM ascorbate to allow the ascorbate present in the gel matrix, both during extraction procedure and throughout the duration of electrophoresis, to sustain APX activity, since APXs are very labile in the absence of ascorbate. Electrophoretic separation was performed at 4 °C for no longer than 6 h. Immediately after electrophoresis, gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) and 2 mM ascorbate for 30 min; the equilibration buffer was renewed every 10 min. Then gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min (the H_2O_2 was added to this solution immediately before incubation of the gel). The gels were subsequently washed with 50 mM potassium phosphate buffer (pH 7.0) for 1 min and submerged and gently agitated in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT. The APX activity appeared as an achromatic band on a purple-blue background (usually within 3–5 min). The reaction was allowed to continue for about 10 min and terminated by washing with deionized water. All steps of APX detection in gels were performed at room temperature (20–25 °C) with the exception of electrophoresis.

CAT isoenzymes Stacking gel (3.9%) and separating gel (7.5%) containing 0.5% soluble starch was used for native PAGE of CAT. The staining procedures for catalase activity described by Thorup et al. (1961) were followed. After electrophoretic separation, the gel was incubated in a solution containing 4.2 mM sodium thiosulfate and 0.15% H_2O_2 for 15 min at room temperature (~25 °C). The gel was then rinsed with distilled water and incubated with 4.5 mM potassium iodide solution acidified with 1% (v/v) glacial acetic acid. Negative bands of CAT enzymes appeared on the blue background of the gel.

O_2^- and H_2O_2 determination in leaf and in the xylem sap

A standard procedure for determination of O_2^- was followed (Wang and Luo 1990). A 1-ml aliquot of crude enzyme extract or 200 μl of xylem sap was mixed with 1 ml of 50 mM sodium phosphate buffer (pH 7.8) and 1 ml of 10 mM hydroxylammonium chloride. The mixture was kept at 25 °C for 20 min and then centrifuged at 1500 *g* for 10 min. One ml of supernatant was mixed with 1 ml of 17 mM sulphanilic acid and 1 ml of 7 mmol l^{-1} 1-naphthylamine. After incubation at 25 °C for 20 min, 3 ml of ethyl ether was added and the mixture shaken and centrifuged at 1500 *g* for 5 min. Absorbance of the water phase at 530 nm was recorded. In blank controls, the same amount of 50 mM sodium phosphate buffer (pH 7.8) was added to the reaction system instead of the enzyme extract or xylem sap.

Concentrations of H_2O_2 in leaves were determined according to Patterson et al. (1984) and Liu et al. (2000) with modifications. In brief, leaf tissue (0.5 g) were ground to a fine powder in liquid N_2 and then homogenized in 3 ml of pre-cooled acetone. The homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C. One ml of supernatant, 2.5 ml of extractant (CCl_4:$CHCl_3$ = 3:1, v/v) and 2.5 ml of redistilled water were mixed and centrifuged at 5000 *g* for 5 min at 4 °C. Then 2 ml of the water phase in the supernatant was divided into two 1-ml aliquots. One aliquot was used as the blank control and another was used to determine H_2O_2 concentration. For the blank, catalase was added to a concentration of 3 Unit ml^{-1} and then kept at 30 °C for 10 min. The same amount of solution with catalase inactivated by high temperature treatment was added to the H_2O_2 extract. Then, 1 ml of 0.2 M sodium phosphate buffer (pH 7.8) and 1 ml of 0.2 mM Ti(IV)-PAR colorimetric reagent (Sellers 1980, Matsubara et al. 1983) were added to both series. The color was developed at 45 °C for 20 min, and then allowed to equilibrate at room temperature for 20 min. Finally, absorbance at 508 nm was recorded and the concentration of H_2O_2 was determined based on the established standard curve.

Concentrations of H_2O_2 in the xylem sap were determined according to Sergiev et al. (1997). A 0.05- to 0.2-ml aliquot of extruded xylem sap was mixed with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI, then incubated for 5 min. The absorbance at 390 nm was obtained and the concentration of H_2O_2 was determined based on a standard curve established as previously described.

Data analysis

All data were subjected to one-way analysis of variance (ANOVA) and significant differences between means were determined by Duncan's multiple-range test. Unless otherwise stated, differences were considered statistically significant when $P < 0.05$.

Results

Salt ion concentration in leaves

Foliar Na^+ and Cl^- concentrations increased in response to the salt treatments in both species, but salt buildup in leaves occurred earlier in *P. popularis* (Day 13) than in *P. euphratica* (Day 18) (Figure 1). Moreover, salinized *P. popularis* plants had 1.7- and 2.8-fold higher foliar Na^+ and Cl^- concentrations at the end of the experiment than salinized *P. euphratica* plants (Figure 1).

Salt compartmentation in leaf cells

Control *P. euphratica* plants had more salt ions in measured cell compartments of mesophyll than *P. popularis* controls (Table 1). Salt treatment increased Na^+ and Cl^- concentrations in mesophyll cells of both species (Table 1), but the patterns of intracellular salt compartmentalization differed between spe-

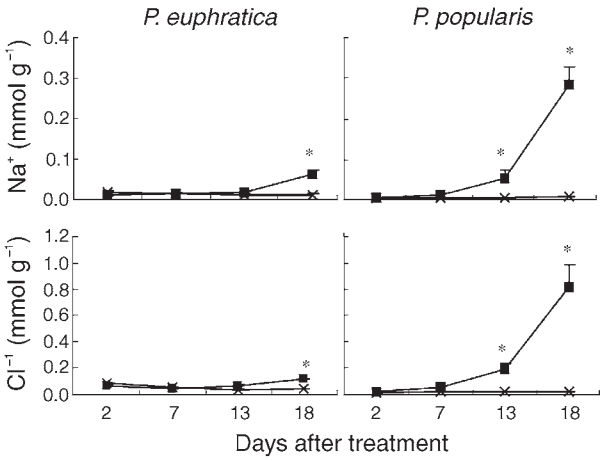

Figure 1. Effects of NaCl on Na^+ and Cl^- concentrations in leaf tissue water of *Populus euphratica* and *P. popularis*. Each value is the mean of three plants and bars represent the standard error of the mean. An asterisk (*) denotes a significant difference at $P < 0.05$ between control (×) and NaCl (■) treatment.

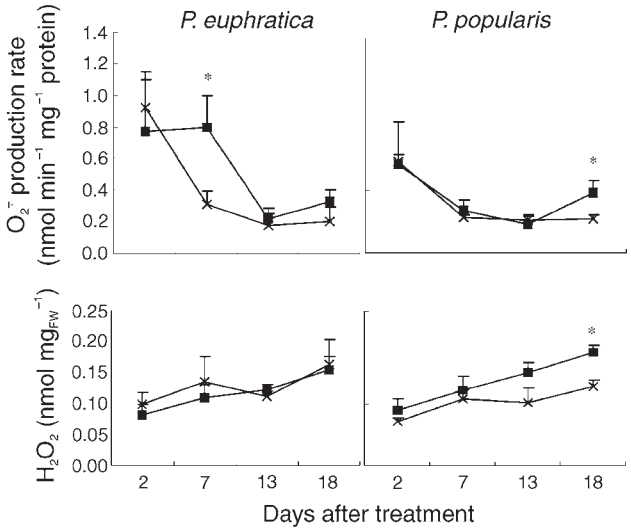

Figure 2. Effects of NaCl on O_2^- production rate and H_2O_2 concentration in leaves of *Populus euphratica* and *P. popularis*. Each value is the mean of three plants and bars represent the standard error of the mean. An asterisk (*) denotes a significant difference at $P < 0.05$ between control (×) and NaCl (■) treatment.

cies. Salinized *P. euphratica* plants accumulated less salt in cell walls and cytoplasm compared with *P. popularis* plants, although Na^+ and Cl^- concentrations in these compartments were typically higher than in control *P. euphratica* plants (Table 1). Moreover, vacuolar compartmentalization was evident in *P. euphratica*, with Na^+ and Cl^- concentrations higher in the vacuole than in the cytoplasm (Table 1). In contrast, in salt-treated *P. popularis,* Na^+ and Cl^- concentrations remained lower in the vacuole than in the cytoplasm (Table 1).

ROS in leaves and in xylem sap

A difference between species in ROS production was observed during the treatment period. Production rates of O_2^- and concentrations of H_2O_2 in leaves and xylem sap of *P. euphratica* remained at control values during the 18-day salt treatment, with the exception of leaf O_2^- at Day 7 (Figures 2 and 3). In contrast, ROS was markedly elevated in leaves and xylem sap of *P. popularis* after 13 days of salt treatment (Figures 2 and 3).

Activities of antioxidant enzymes in leaf

Gradient PAGE analysis of crude leaf extracts revealed two dominant SOD isoenzymes in control plants of both species, but with different patterns (Figure 4). Both KCN and H_2O_2 inhibited activity of both SOD isoenzymes in both species, indicating that these isoenzymes were CuZn-SOD isoforms (Wang et al. 2007). In general, there were no marked inhibitory effects of NaCl on SOD isoenzymes or total SOD activity in either species during the observation period (Figure 4).

Two APX isoenzymes (apx 1 and apx 2) were observed in control plants of both species, but apx 2 was more evident in *P. poplaris* than in *P. euphratica* (Figure 5). A genetic difference was observed in the timing of APX response to increasing salinity: total APX activity in *P. euphratica* increased coincident with the rise in apx 2 on Day 7 (Figure 5). Salt-stressed *P. poplaris* plants exhibited an increase in APX activity, but the

Table 1. Effects of NaCl on Na^+ and Cl^- concentrations in mesophyll cells of *Populus euphratica* and *P. popularis*. Each value (± SE) is the mean of three plants and values in the same column followed by different letters are significantly different ($P < 0.05$) between control and NaCl treatment. Abbreviation: cps = counts per second as determined from X-ray data.

Compartment	Treatment	*P. euphratica*		*P. popularis*	
		Na^+ (cps)	Cl^- (cps)	Na^+ (cps)	Cl^- (cps)
Mesophyll wall	Control	7.1 ± 2.1b	21.7 ± 2.9b	0 ± 0b	12.5 ± 4.4b
	NaCl	49.6 ± 3.8a	90.9 ± 5.6a	78.3 ± 2.8a	92.6 ± 4.0a
Mesophyll cytoplasm	Control	14.7 ± 3.0b	21.2 ± 3.9b	0 ± 0b	15.5 ± 3.0b
	NaCl	40.7 ± 4.3a	30.7 ± 2.2a	81.4 ± 3.8a	102.2 ± 5.8a
Mesophyll vacuole	Control	12.7 ± 2.7b	22.5 ± 4.7b	0 ± 0b	14.0 ± 4.3b
	NaCl	98.2 ± 9.1a	89.6 ± 4.4a	71.4 ± 3.5a	91.1 ± 3.6a

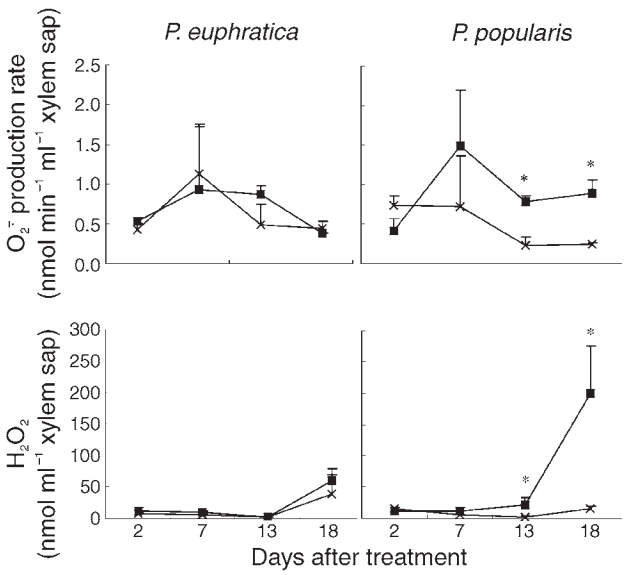

Figure 3. Effects of NaCl on O_2^- production rate and H_2O_2 concentration in xylem sap of *Populus euphratica* and *P. popularis*. Each value is the mean of three plants and bars represent the standard error of the mean. An asterisk (*) denotes a significant difference at $P < 0.05$ between control (×) and NaCl (■) treatment.

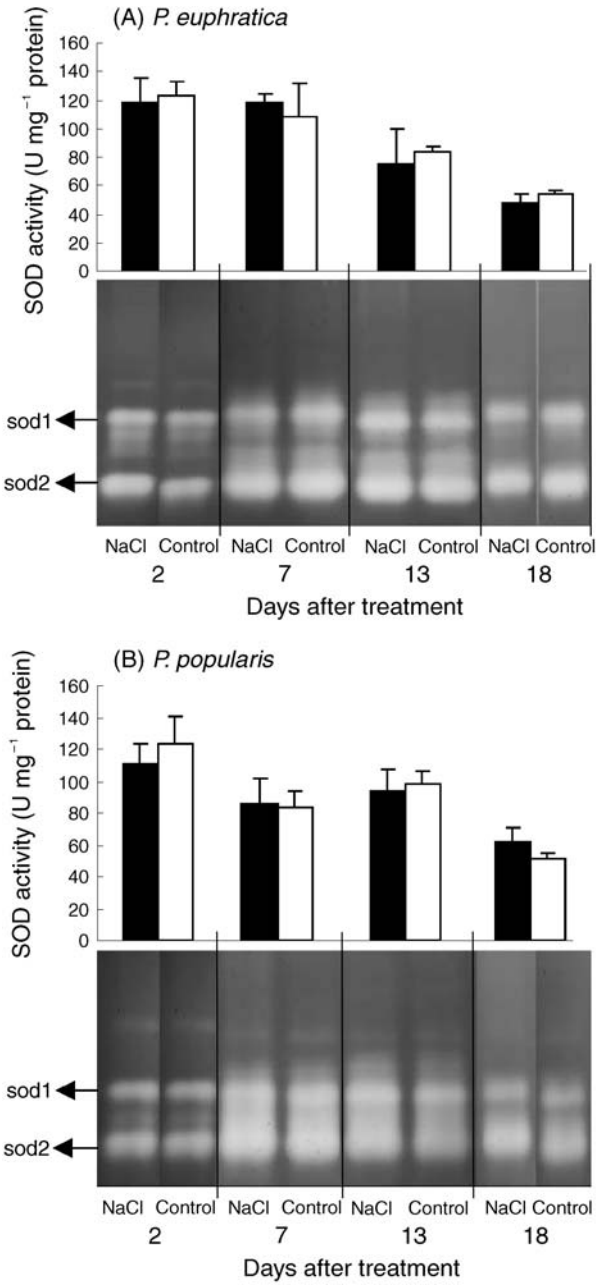

Figure 4. Effects of NaCl on total superoxide dismutase (SOD) activity and SOD isoenzymes in leaves of *Populus euphratica* and *P. popularis*. Each value in the columns of total SOD activity is the mean of three plants and bars represent the standard error of the mean. In each lane, 30 μg of soluble protein was applied to the native polyacrylamide gel and electrophoresis was performed at 4 °C. Filled bars represent NaCl and open bars represent the control.

increase occurred 11 days later than in *P. euphratica* (Day 18 versus Day 7; Figure 5).

The pattern of activity of CAT enzymes differed significantly between the species, although four CAT isoenzymes were detected in control plants (Figure 6). The salt treatments increased total CAT activity in both species as a result of increases in the activities of all isoenzymes, but CAT activity increased earlier in *P. euphratica* than in *P. popularis* (Day 7 versus Day 18) (Figure 6).

Populus euphratica plants exhibited no significant change in GR activity during the salt treatment, whereas GR activity in *P. popularis* increased markedly after Day 13 (Figure 7).

Activity of antioxidant enzymes in xylem sap

We detected SOD, APX and GR activities in xylem sap in both species, whereas CAT activity was undetectable (Table 2). Marked increases in SOD, APX and GR activities occurred in *P. popularis* after 7 or 13 days of salt treatment, and activities peaked on Day 18, and were up to 18.0-, 12.1- and 10.0-fold higher than in control plants (Table 2). *Populus euphratica* exhibited a 2.0-fold increase in SOD activity at the end of the salt treatment, whereas activities of APX and GR remained at control values throughout the salt treatment (Table 2).

Discussion

NaCl exclusion, oxidative burst and salt resistance

After 18 days of treatment, increasing soil NaCl concentration caused large increases in Na^+ and Cl^- concentrations in *P. popularis* leaves (Figure 1), which were associated with the production of ROS. The salt-treated *P. popularis* plants exhibited visible leaf injury (i.e., chlorosis or necrosis at the edges of leaves in the upper shoot) and shedding of old leaves following the marked increases in O_2^- and H_2O_2 production, suggesting that the ROS burst was the result of salt accumulation in the leaves. Similarly, Hernández et al. (2001) observed a correlation between ROS production and salt-induced necrotic lesions in pea leaves.

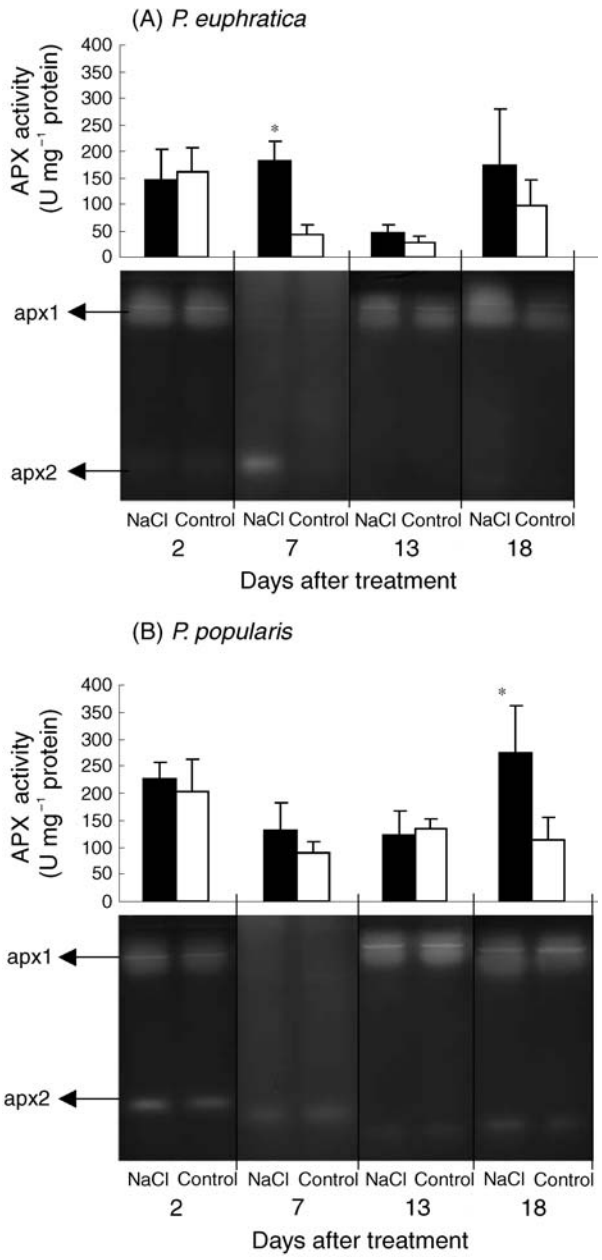

Figure 5. Effects of NaCl on total ascorbate peroxidase (APX) activity and APX isoenzymes in leaves of *Populus euphratica* and *P. popularis*. Each value in the columns of total APX activity is the mean of three plants and bars represent the standard error of the mean. An asterisk (*) denotes a significant difference at $P < 0.05$ between control and NaCl treatment. In each lane, 30 µg of soluble protein was applied to the native polyacrylamide gel and electrophoresis was performed at 4 °C. Filled bars represent NaCl and open bars represent the control.

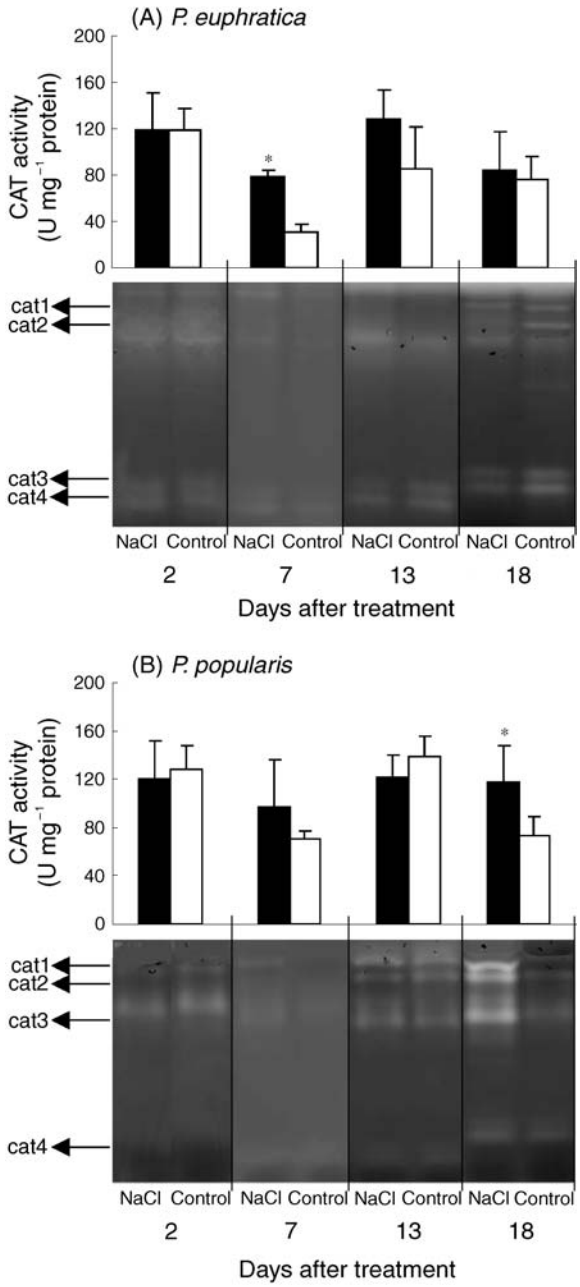

Figure 6. Effects of NaCl on total catalase (CAT) activity and CAT isoenzymes in leaves of *Populus euphratica* and *P. popularis*. Each value in the columns of total CAT activity is the mean of three plants and bars represent the standard error of the mean. An asterisk (*) denotes a significant difference at $P < 0.05$ between control and NaCl treatment. In each lane, 30 µg of soluble protein was applied to the native polyacrylamide gel and electrophoresis was performed at 4 °C. Filled bars represent NaCl and open bars represent the control.

Because of effective salt exclusion, salt-treated *P. euphratica* plants showed no evidence of leaf necrosis. The capacity of *P. euphratica* plants to exclude NaCl is likely associated with restricted root-to-shoot salt transport caused by the blocking of root apoplastic salt transport and sequestration of Cl^- in root cortical vacuoles (Chen et al. 2002*a*, 2003). The salt treatments had little effect on O_2^- and H_2O_2 production in *P. euphratica* plants (Figures 2 and 3), and no symptoms of oxidative damage were found.

Salt compartmentalization and ROS control in leaf

Hernández et al. (1993, 1995) demonstrated that NaCl stress

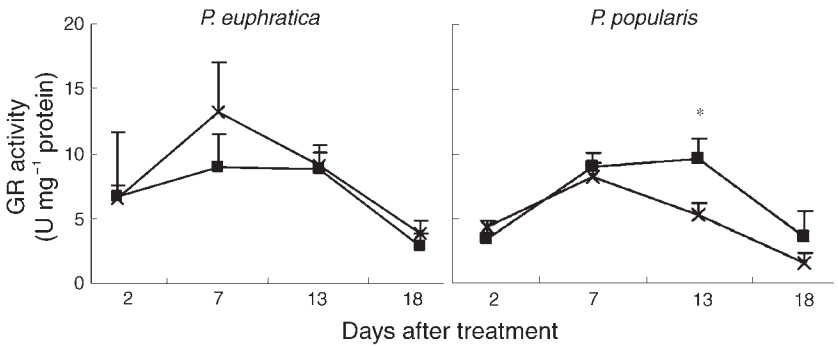

Figure 7. Effects of NaCl on total glutathione reductase (GR) activity in leaves of *Populus euphratica* and *P. popularis*. Each value is the mean of three plants and bars represent the standard error of the mean. An asterisk (*) denotes a significant difference at $P < 0.05$ between control (×) and NaCl (■) treatment.

favored the formation of O_2^- and H_2O_2 in chloroplasts and mitochondria in two pea cultivars differing in NaCl sensitivity. Generally, in plants subjected to saline conditions, the activities of foliar antioxidant enzymes, e.g. SOD, APX, CAT and GR, increase to enhance oxygen-scavenging activity and thereby confer tolerance to salt stress (Takahashi and Asada 1983, Fridovich 1986, Asada and Takahashi 1987, Elstner 1987, Hassan and Scandalios 1990, Bowler et al. 1992, Asada 1994, Hernández et al. 1995, Alscher et al. 2002, Shigeoka et al. 2002). In our study, an 18-day salt treatment did not inhibit SOD activity but enhanced the activities of APX and CAT (both isoenzymatic activity and total activity) and GR in *P. popularis* leaves (Figures 4–7). However, the occurrence of oxidative damage in the *P. popularis* leaves indicates that the rate of ROS production exceeded the oxygen-scavenging capacity of the antioxidant enzymes (Figure 2). The enormous accumulation of Na^+ and Cl^- in the chloroplasts of *P. popularis* leaves likely inhibited both the dark and light reactions (Wang et al. 2007) and thereby favored the formation of O_2^- and H_2O_2 in leaf cells (Table 1). Moreover, excessive production O_2^- and H_2O_2 may result in the generation of the even more highly reactive hydroxyl radicals (·OH) by a metal-catalyzed site-specific Haber-Weiss reaction (Halliwell and Gutteridge 1989), and ultimately to oxidative damage of the leaf tissue.

In contrast to salt-treated *P. popularis*, a salt-induced oxidative burst did not occur in leaves of salt-treated *P. euphratica* (Figure 2). This may be because *P. euphratica* leaves can (1) effectively compartmentalize the salt in the vacuole, and (2) rapidly increase the activities of antioxidant enzymes at low soil salinities. The foliar concentrations of Na^+ and Cl^- in salt-treated *P. euphratica* plants remained higher in the vacuole than in the cytoplasm throughout the treatment period (Table 1). Compartmentalization of Na^+ and Cl^- in the vacuole may inhibit the NaCl-induced formation of O_2^- and H_2O_2 in the cytosol, chloroplasts and mitochondria. In addition, the marked increases in the activities of APX and CAT after 7 days of salt treatment (Figures 5 and 6) may have maintained the H_2O_2 concentration at control values throughout the experiment (Figure 2).

Our result indicate that *P. euphratica* plants are able to tolerate saline conditions and prevent an oxidative burst by two main mechanisms—salt exclusion and enhanced active oxygen detoxification in response to low salt concentrations. The rapid increase in the activities of antioxidant enzymes in *P. euphratica* is presumably associated with salt-induced production of ABA. In our *P. euphratica* plants, xylem ABA con-

Table 2. Effects of NaCl on activities (U ml^{-1} xylem sap) of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) in xylem sap of *Populus euphratica* and *P. popularis*. Each value (± SE) is the mean of three plants and values in the same column followed by different letters are significantly different ($P < 0.05$) between control and NaCl treatment.

Antioxidant enzymes	Species	Treatments	Days after treatment			
			2	7	13	18
SOD	*P. euphratica*	Control	5.7 ± 1.0a	3.1 ± 0.7a	2.9 ± 0.4a	0.8 ± 0.5b
		NaCl	3.8 ± 0.3a	3.2 ± 0.4a	3.4 ± 0.7a	2.4 ± 0.1a
	P. popularis	Control	4.5 ± 0.5a	4.5 ± 1.5a	0.6 ± 0.2b	0.4 ± 0.1b
		NaCl	3.1 ± 0.5a	2.3 ± 1.2a	5.2 ± 1.0a	7.6 ± 0.5a
APX	*P. euphratica*	Control	3.4 ± 0.3a	2.0 ± 0.1a	2.7 ± 0.7a	2.7 ± 2.0a
		NaCl	2.8 ± 0.4a	2.5 ± 0.2a	5.7 ± 0.3a	4.0 ± 0.1a
	P. popularis	Control	4.6 ± 1.8a	5.8 ± 1.2a	2.3 ± 0.3b	3.0 ± 0.3b
		NaCl	2.7 ± 1.4a	7.3 ± 4.7a	25.0 ± 1.0a	39.3 ± 9.3a
GR	*P. euphratica*	Control	0.5 ± 0.2a	1.3 ± 0.4a	2.4 ± 0.2a	2.8 ± 0.6a
		NaCl	0.4 ± 0.1a	2.6 ± 1.7a	2.4 ± 0.2a	2.1 ± 0.1a
	P. popularis	Control	1.1 ± 0.2a	1.1 ± 0.2b	1.7 ± 0.1a	1.7 ± 0.4b
		NaCl	2.4 ± 1.8a	10.7 ± 5.7a	2.8 ± 0.6a	18.9 ± 5.9a

centration increased by 1–2-fold during the first week of the salt treatment (data not shown), indicating that salt-tolerant *P. euphratica* could sense soil salt stress and increase ABA synthesis, which triggers adjustments that confer salt tolerance (Chen et al. 2001, 2002*b*). Xu et al. (2003) observed that ABA increased the activities of SOD and POD (peroxidase) in *Medlar* callus, which help to maintain membrane stability under saline conditions. External application of ABA via the transpiration stream increased antioxidant defense in leaves of *P. euphratica* (authors' unpublished data). The rapid increase in APX and CAT activities that we observed in *P. euphratica* leaves at an early stage of the salt treatment might have been induced by ABA that originated from roots. Compared with *P. euphratica*, *P. popularis* was less sensitive to soil NaCl concentration and had a low capacity to increase ABA synthesis in response to the salt treatment, which may account for the inability of *P. popularis* plants to trigger antioxidant defense in leaves, thus resulting in ROS overproduction during the 18-day salt treatment.

Our spectrophotometric determinations of total SOD activity did not correspond to SOD activity observed by in-gel activity staining of native PAGE (Figure 4). Although bright bands were visible in the polyacrylamide gel, there was no correspondingly higher SOD activity in extracts from salt-treated plants (Figure 4). This was presumably because SOD activity was inhibited by the naturally occurring phenolics in the leaf extracts and their oxidation products, the quinines. Phenolics are present in high concentrations in poplar leaves and SOD activity was rarely detected when PVP was omitted from the grinding medium. We conclude that the inclusion of 1% PVP in the extraction buffer was insufficient to absorb all of the phenolics present in the extract and to protect the extract from browning.

ROS control in the xylem sap

It is proposed that membrane NAD(P)H oxidase is responsible for the generation of ROS in response to pathogens (Levine et al. 1994) and salinity (Hernández et al. 2001). Salt induced a marked increase in O_2^- and H_2O_2 production in xylem sap of *P. popularis*, and the increase in production occurred earlier than in leaf tissue (Day 18) (Figures 2 and 3). Activities of SOD, APX and GR in the xylem sap of *P. popularis* increased by 10–18-fold at the end of the salt treatment (Table 2), although their absolute values in xylem sap of control plants were much less than in control leaves (Table 2; Figures 4, 5 and 7). However, our result differs from other reports documenting the absence of APX and GR activities in the apoplast (Polle et al. 1990, Durán-Carril and Rodriguez Bujan 1998, Hernández et al. 2001). The presence of APX and GR activities in the xylem sap of both poplar genotypes agrees with the results of Vanacker et al. (1998*a*, 1998*b*), who found all components of the ascorbate (ASC)-glutathione (GSH) cycle in the apoplasts of both barley and oat leaves.

In the xylem sap, increased activities of SOD, APX and GR at Days 13 and 18 of the salt treatment may be a result of the increased production of O_2^- and H_2O_2, because ROS are considered secondary messengers that induce antioxidant defenses (Desikan et al. 2001, Vranová et al. 2002). Salt-induced oxidative burst in the xylem was clearly seen in *P. popularis* after 18 days of salt treatment, even though SOD, APX and GR activities were markedly elevated (Figure 3; Table 2). Based on these results we conclude that the enhanced oxygen-scavenging capacity remained insufficient to detoxify all of the ROS produced, thus resulting in ROS accumulation and subsequent membrane peroxidation of the salinized *P. popularis* plants. Hernández et al. (2001) showed that NaCl-induced oxidative stress in apoplasts is related to ROS-induced necrotic lesions in the minor veins in NaCl-treated pea plants. Compared with *P. popularis*, the antioxidant enzymes (SOD, APX and GR) in the xylem sap of *P. euphratica* were able to control the ROS balance. Salinity increased SOD activity in the xylem sap of our poplar species, which differed in salt tolerance (Table 2), contrasting with the results of a study by Hernandez et al. (2001) showing that apoplastic SOD activity is induced by NaCl in the salt-tolerant pea cv. Puget but is decreased by NaCl in the salt-sensitive pea cv. Lincoln.

In conclusion, salt treatment caused a buildup of Na^+ and Cl^- in leaves of *P. popularis*. Because of the low capacity of *P. popularis* plants for NaCl exclusion and vacuolar salt compartmentalization, the salt treatment resulted in significant increases in ROS in leaves and xylem sap, which caused oxidative damage and leaf-injury, despite increases in the activities of antioxidant enzymes in both leaves and xylem sap. In contrast, salt-treated *P. euphratica* plants avoided excessive ROS production by (1) effective salt exclusion and vacuolar salt compartmentalization in mesophyll cells, and (2) up-regulation of the activities of antioxidant enzymes at low soil salt concentrations. Salt exclusion and vacuolar salt compartmentalization are necessary to limit ROS production and marked increases in antioxidant enzymatic activities in response to low salt concentrations enables *P. euphratica* plants to initiate effective antioxidant defense in response to saline conditions.

Acknowledgments

The research was supported jointly by the Alexander von Humboldt-Stiftung/Foundation (Germany), German Science Foundation through Poplar Research Group Germany (PRG), the key project of National Natural Science Foundation of China (30430430), the HI-TECH Research and Development Program of China (863 Program, 2006AA10Z131), a Foundation for the Author of National Excellent Doctoral Dissertation of PR China (200152), and the Teaching and Research Award Program for Outstanding Young Teachers in Higher Education Institution of MOE, PRC (2002-323). We thank Dr. Jinchi Zhou for valuable help with ion analysis.

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