Chapter 5

Salt Compartmentation and Antioxidant Defense in Roots and Leaves of Two Non-Salt Secretor Mangroves under Salt Stress

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Abstract

The effects of increasing NaCl (100–400 mM) on cellular salt distribution, antioxidant enzymes, and the relevance to reactive oxygen species (ROS) homeostasis were investigated in 1-year-old seedlings of two non-salt secretor mangroves, *Kandelia obovata* and *Bruguiera gymnorhiza*. *K. obovata* accumulated less Na^+ and Cl^- in root cells and leaf compartments under 400 mM NaCl compared to *B. gymnorhiza*. However, *B. gymnorhiza* leaves are notable for preferential accumulation of salt ions in epidermal vacuoles relative to mesophyll vacuoles. Both mangroves upregulated antioxidant enzymes in ASC-GSH cycle to scavenge the salt-elicited ROS in roots and leaves but with different patterns. *K. obovata* rapidly initiated antioxidant defense to reduce ROS at an early stage of salt stress, whereas *B. gymnorhiza* maintained a high capacity to detoxify ROS at high saline. Collectively, our results suggest that salinized plants of the two mangroves maintained ROS homeostasis through (i) ROS scavenging by antioxidant enzymes and (ii) limiting ROS production by protective salt compartmentation. In the latter case, an efficient salt exclusion is favorable for *K. obovata* to reduce the formation of ROS in roots and leaves, while the effective vacuolar salt compartmentation benefited *B. gymnorhiza* leaves to avoid excessive ROS production in a longer term of increasing salinity.

Keywords: *Bruguiera gymnorhiza*, *Kandelia obovata*, reactive oxygen species, antioxidant enzymes, X-ray microanalysis

1. Introduction

Mangrove plants form a dominant ecosystem in tropical and subtropical coastlines [1]. *Bruguiera gymnorhiza* is widely distributed in tropical and subtropical area, from the southeastern coast of Africa through Asia to Australia and the southwestern Pacific [2]. *Kandelia obovata* is distributed mostly in the transition regions from tropical to subtropical coastlines of southern China, Taiwan, and the southern islands of Japan [3]. Climatic factors affecting the vegetative and reproductive phenology of *B. gymnorhiza* and *K. obovata* growing in subtropical regions were assessed in recent years. Temperature, day length, and rainfall are suggested to be the important external controlling factors of leaf initiation in *B. gymnorhiza* [4]. Leaf litterfall of the subtropical mangrove *K. obovata* was correlated to monthly day length and maximum wind speed [5]. Flowering of *K. obovata* was influenced by monthly sunshine hour and monthly mean air temperature [6]. While in *B. gymnorhiza*, flowering phenophase was linked with rainfall and relative humidity [4]. *B. gymnorhiza* and *K. obovata* are two major mangrove species along southern China coastlines. *B. gymnorhiza* is a frontline species and mostly occurs in high-saline zones compared with *K. obovata*, which grows in low-saline creeks in mangrove areas [7].

The most striking feature of mangroves is the capacity to withstand high salinity concentrations [8–11]. In general, secretor and non-salt secretor mangroves both exhibited a high capacity to maintain Na^+ homeostasis under sodium chloride (NaCl) stress [7, 12–15]. Root flux recordings showed that *B. gymnorhiza*, *K. candel* (or *K. obovata*, non-salt secretors), *Aegiceras corniculatum*, and *Avicennia marina* (secretors) retained an obvious Na^+ exclusion under NaCl treatment [7, 13–16]. Hydrogen peroxide (H_2O_2), nitric oxide (NO), and calcium (Ca^{2+}) mediated Na^+/H^+ antiport across the PM, thus contributing to control ionic homeostasis in the two non-salt secretor mangrove species [7]. Recently, multiple signaling networks of extracellular ATP (eATP), H_2O_2, Ca^{2+}, and NO in the mediation of root ion fluxes were established in salt-stressed *K. obovata* and *A. corniculatum* [15]. Salt exclusion by roots is the most important salt-tolerant mechanism in woody plants [17–22] and herbaceous species [23–24]. Although mangrove roots could effectively exclude salt ions under NaCl stress, Na^+ and Cl^- taken up by roots would eventually transport to shoots via the transpiration stream during a long-term salt exposure [16, 20–22]. Jing et al. found that Na^+ extrusion capacity in *K. candel* roots declined with the prolonged duration of salt exposure [16]. As a result, large amount of Na^+ accumulated in roots was transported to shoots [12, 16]. Excessive Na^+ accumulation in leaves leads to oxidative stress by the production of reactive oxygen species (ROS) in trees [25–27]. Similarly, salt-induced oxidative stress has been widely shown in herbaceous species [28–35]. In mitochondria and chloroplasts, superoxide anions (O_2^-) are generated as a by-product of electron transfer to O_2 via photosynthetic and respiratory electron transport chain [36, 37]. The active O_2^- leads to subsequent formation of H_2O_2 and hydroxyl radicals ($OH^{\bullet -}$) through chemical and enzymatic reactions [36, 37]. Salt induced an oxidative stress in chloroplast and mitochondria of pea leaves [28–30, 34]. In poplars, great buildup of Na^+ and Cl^- in chloroplasts may directly cause ion toxicity and induce the subsequent oxidative stress [26, 38]. X-ray microanalysis results showed that the inability for the restriction of Na^+ entry into the chloroplasts leads to an uncontrolled oxidation in *Populus popularis* [26, 38]. Salt-resistant plants may maintain ROS homeostasis through limiting ROS production by a protective salt partitioning. Evidence presented elsewhere suggests that NaCl-stressed sorghum plants preferentially

partition Cl^- into leaf sheaths relative to blades [39]. The preferential accumulation of Cl^- in the sheath would lessen the effect of salinity on photosynthetic processes in the leaf blade. Furthermore, X-ray microanalysis of various cell types in leaf sheaths and blades revealed that Cl^- was preferentially accumulated in epidermal vacuoles, relative to mesophyll vacuoles in salt-tolerant barley and sorghum [39, 40]. The high Cl^- concentration in the leaf blade mesophyll cells of a barley cultivar (cv. Clipper) suggests that the lower salt resistance of this cultivar is directly related to the degree of Cl^- exclusion by these cells [40]. Thus, it can be inferred that compartmentalizing salt ions in cell layers of leaf blade would reduce the perturbation of salt on photosynthetic processes in photosynthetically active mesophyll, especially the electron transport processes in chloroplasts. As a result, ROS is less produced [26, 27]. Although salt increased H_2O_2 in *K. candel* leaves, the ROS-induced necrotic lesions were not seen during the period of stress [16]. In addition to ROS scavenging by both enzymatic and nonenzymatic antioxidants, it is possible that mangrove plants could attenuate oxidative stress by a reasonable salt compartmentation in cells. However, this needs further investigations, e.g., by X-ray microanalysis, to clarify.

Under salt stress, the antioxidant defense system serves to remove reactive oxygen species (e.g., $O_2^{\bullet -}$ and H_2O_2) in the chloroplast, mitochondria, and cytosol. Superoxide dismutases (SODs) are considered to be the first defense line against $O_2^{\bullet -}$ and the reaction product [41, 42]. H_2O_2 is further detoxified through a reaction catalyzed by an ascorbate-specific peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). APX utilizes ascorbate (AsA) as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDAsA), a univalent oxidant of AsA [43]. CAT, an enzyme that splits hydrogen peroxide to yield oxygen and water, is an important part of the antioxidant defense [44]. GPX efficiently catalyzes the reduction of hydrogen peroxide and organic hydroperoxides by glutathione [45, 46]. In addition to these antioxidant enzymes that can directly scavenge toxic oxygen species, glutathione reductase (GR), which regenerates glutathione (GSH) that has been oxidized during ROS scavenging, is also implicated in redox homeostasis control [47]. The contribution of antioxidant defense to salt tolerance has been confirmed in crop species [32, 33, 48, 49] and woody plants, e.g., poplars [25–27] and mangroves [8, 10, 50, 51]. Takemura et al. detected an increased activity of SOD and CAT in *B. gymnorhiza* at high salt [50]. Parida et al. found that the elevation of antioxidant enzymes, APX and guaiacol peroxidase, was able to scavenge salt-induced H_2O_2 in *B. parviflora* [51]. Therefore, the capacity for regulating ROS homeostasis serves as one important component for salt tolerance in mangroves.

Analyses of isoforms of antioxidant enzymes showed species differences in antioxidant defense system against salt treatment. Plants generally have three SOD isozymes: Cu/Zn-SOD in the cytosol and chloroplasts, Mn-SOD in mitochondria, and Fe-SOD in chloroplasts [52]. Activity of CuZn-SOD I and CuZn-SOD II, the two dominant SOD proteins in poplar leaves, was not detectable in *P. popularis* (salt-sensitive) after 16 days of salt stress, while there were no marked inhibitory effects of NaCl on the two SOD isoenzymes in *P. euphratica* (salt-resistant) during the observation period [26]. Furthermore, genetic differences were found in the timing of APX and CAT response to increasing salinity. Salt treatments increased activity of CAT and APX isoenzymes in the two poplar species, but their activity increased earlier in *P. euphratica* than in *P. popularis* [27]. In mangrove, a certain number of SOD isoenzymes (Mn-SOD, Fe-SOD),

guaiacol peroxidase isoenzymes, and GR isoenzymes were preferentially elevated by NaCl in *B. parviflora* [51]. The induction of antioxidant enzymes might be the result of salt-induced gene transcription. Northern blot analysis revealed that the transcript level of cytosolic Cu/Zn-SOD was increased after a few days of NaCl treatment [50]. Similarly, NaCl was shown to increase *KcCSD* expression in *K. candel* leaves [16]. Proteomic analysis of *K. candel* leaves revealed that SOD abundance increased in response to high NaCl at 450–600 mM [53]. Furthermore, overexpression of copper/zinc superoxide dismutase from mangrove *K. candel* in tobacco enhances salinity tolerance by the reduction of reactive oxygen species in chloroplast [16].

We have previously shown species differences between secretor and non-salt secretor mangroves in root salt exclusion and leaf gas exchange response to salt treatment [7, 12, 15]. The object of this study is to investigate the effect of NaCl on the pattern of cellular salt compartmentation, variations in antioxidant enzymes, and their contributions to ROS (in particular, $O_2^{\bullet -}$ and H_2O_2) homeostasis maintenance in non-salt secretor mangroves.

2. Salt compartmentation and antioxidant defense

2.1. Plant materials and salt treatment

K. obovata hypocotyls developed from fruits turned into mature propagules, which began to drop in March and continued dropping until May [6]. Mature and developing propagules of *B. gymnorhiza* were found throughout the year, but the abundance of mature propagules was highest in summer and lowest in winter [4]. In early March, 200 of propagules of *K. obovata* and *B. gymnorhiza* were obtained from Dongzhai Harbor in Hainan Province of China (latitude 19°51′N and longitude 110°24′E). Propagules were collected from the surface of soil or seawater during the ebb tide. Single hypocotyls were planted in individual pots (15 cm in diameter and 18 cm in height) containing sand and placed in a greenhouse at Beijing Forestry University, Beijing, China (latitude 39°56′N and longitude 116°20′E). The pots were fertilized with 1000 ml half strength Hoagland nutrient solution every 14 d. Seedlings were raised from March to August under nonsaline conditions. The relative humidity was maintained at 60–70%, and photosynthetically active radiation (PAR) varied from 400 to 1200 μmol m^{-2} s^{-1}. Salt treatment was carried out when the fourth pair of leaves came out from the apex of the growing shoots (mid-August) [12].

NaCl concentration started from 100 mM and increased stepwise by 100 mM [12], reaching 400 mM and remained at this salinity until the terminal of experiment. Increasing NaCl saline was applied at day 1 (100 mM), day 3 (200 mM), day 6 (300 mM), and day 10 (400 mM), respectively. Control plants were kept well watered with no addition of NaCl. PAR was 400–1200 μmol m^{-2} s^{-1}, and air temperature was 20–35°C over the duration of experiment. On day 2, day 5, day 9, and day 14, leaves and roots were sampled for ROS ($O_2^{\bullet -}$ and H_2O_2) determination and total activity measurements of antioxidant enzymes, i.e., superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR). For SOD and CAT isoenzyme analyses, leaves and roots were sampled at day 3, day 6, day 10, and day 15. Three replicated plants per treatment were harvested at each sampling

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time. At the final harvest time, roots and upper mature leaves were sampled from control and stressed plants and used for X-ray microanalysis.

2.2. $O_2^{\bullet -}$ and H_2O_2 levels in roots and leaves

$O_2^{\bullet -}$ production rate was typically higher in roots than in leaves in control plants of the two species (**Table 1**). High salinity (400 mM NaCl) increased root $O_2^{\bullet -}$ production rate by 82 and 83% in *K. obovata* and *B. gymnorhiza*, respectively, but the salt-induced rise of $O_2^{\bullet -}$ was absent in the two mangroves when NaCl concentration was below 300 mM (**Table 1**). The same trend was observed in leaves, but the NaCl-induced increase of $O_2^{\bullet -}$ was only observed in *B. gymnorhiza* leaves at 400 mM NaCl (**Table 1**).

Increasing NaCl stress did not significantly elevate root H_2O_2 levels in either species; rather, a significant reduction of H_2O_2 was observed in *B. gymnorhiza* when NaCl saline ranged from 100 to 300 mM (**Table 1**). An abrupt rise of H_2O_2 occurred in *K. obovata* leaves when plants were subjected to 400 mM NaCl, although H_2O_2 remained less than controls at low salt (100–200 mM, **Table 1**). However, salinized *B. gymnorhiza* maintained a H_2O_2 level similar to control leaves despite of a NaCl increase, from 100 to 400 mM (**Table 1**).

In general $O_2^{\bullet -}$ production and/or H_2O_2 levels in roots and leaves were enhanced by high salinity (400 mM NaCl) in the two mangrove species, although root and leaf ROS levels were usually downregulated after exposure to a lower salinity (100–200 mM NaCl), e.g., O_2^{-} and H_2O_2 in *B. gymnorhiza* roots and H_2O_2 in *K. obovata* leaves (**Table 1**). Similarly, NaCl-induced increase of H_2O_2 was observed in leaves of *B. parviflora* [51] and *K. candel* [16] under hydroponic conditions. In this study, the moderate ROS increment induced by 400 mM NaCl caused no oxidative burst in both species, suggesting that stressed plants of *K. obovata* and *B. gymnorhiza* maintained ROS homeostasis throughout the duration of salt exposure. Our data showed that salt compartmentation and antioxidant enzymes contributed to ROS homeostasis in both species but with different patterns under NaCl stress (see below).

2.3. Salt compartmentation and ROS production

2.3.1. Salt compartmentation within root and leaf cells

In this study, SEM-EDX analysis was performed on cross sections of *B. gymnorhiza* and *K. obovata* roots. Na^+ and Cl^- were detectable in root cells of no-salt controls (**Table 2**). Under salt conditions, Na^+ and Cl^- levels significantly increased in the tested structures, i.e., epidermis, exodermis, cortex, endodermis, and stelar parenchyma (**Table 2**). The long-term salt treatment with increasing NaCl saline (100–400 mM, 15 d) significantly increased the content of salt ions by 0.6–9.6 (Na^+) and 0.5–5.1 fold (Cl^-), although Na^+ and Cl^- levels were typically higher in *B. gymnorhiza* than in *K. obovata* in all measured structures (**Table 2**).

In leaf cells of control plants, TEM-EDX data showed an evident Na^+ and Cl^- in epidermis, mesophyll, and xylem vessels (leaf vascular bundle), but *B. gymnorhiza* exhibited 28–195% higher Na^+ than *K. obovata* in all measured cell compartments, such as xylem vessel, epidermal wall and vacuole, mesophyll wall and vacuole, and chloroplast (**Table 3**). NaCl (400 mM) treatment markedly increased Na^+ and Cl^- concentrations in the apoplastic space and vacuoles

Treatment	*K. obovata*				*B. gymnorhiza*			
	Leaf		Root		Leaf		Root	
	H_2O_2	$O_2^{-\bullet}$	H_2O_2	$O_2^{-\bullet}$	H_2O_2	$O_2^{-\bullet}$	H_2O_2	$O_2^{-\bullet}$
Control	17.63 ± 6.00a	1.16 ± 0.12a	10.04 ± 1.98a	5.77 ± 1.00a	5.93 ± 0.91a	0.61 ± 0.07a	17.50 ± 0.17a	3.44 ± 0.56a
NaCl (100 mM)	2.37 ± 0.60b	1.35 ± 0.11a	7.76 ± 2.56a	6.26 ± 0.99a	11.53 ± 4.96a	0.47 ± 0.08a	1.43 ± 1.91b	2.35 ± 0.22a
Control	16.48 ± 4.10a	1.11 ± 0.07a	4.95 ± 1.86a	4.03 ± 1.35a	3.33 ± 1.12a	0.50 ± 0.29a	31.43 ± 4.79a	3.63 ± 0.58a
NaCl (200 mM)	1.50 ± 0.05b	1.26 ± 0.04a	3.20 ± 1.02a	4.34 ± 1.25a	9.01 ± 4.52a	0.69 ± 0.28a	4.79 ± 1.57b	2.68 ± 0.02a
Control	10.97 ± 1.23a	0.67 ± 0.09a	13.10 ± 2.07a	1.54 ± 0.09a	15.93 ± 4.50a	0.48 ± 0.07a	26.44 ± 4.98a	1.81 ± 0.60a
NaCl (300 mM)	11.47 ± 5.10a	0.84 ± 0.12a	13.93 ± 2.34a	1.78 ± 0.13a	23.13 ± 1.10a	0.69 ± 0.29a	4.66 ± 0.35b	1.89 ± 0.52a
Control	11.60 ± 5.80b	1.76 ± 0.41a	8.05 ± 1.08a	1.69 ± 0.06b	19.12 ± 1.18a	0.71 ± 0.09b	10.39 ± 2.15a	2.65 ± 0.09b
NaCl (400 mM)	41.38 ± 8.97a	1.76 ± 0.31a	11.62 ± 3.09a	3.08 ± 0.46a	22.90 ± 3.40a	1.08 ± 0.13a	16.07 ± 3.31a	4.85 ± 0.52a

$O_2^{-\bullet}$ production rate was measured as described by Wang and Luo [54] and Wang et al. [27]. Briefly, leaf and root tissues (ca. 0.5 g) were homogenized in a 3-ml ice-cold buffer [50 mM potassium phosphate, pH 7.0, 1.0 mM EDTA, 1% (w/v) PVP] and then centrifuged at 10,000 × *g* for 20 min at 4°C. A 1.0 ml of extract was mixed with same volume of 50 mM sodium phosphate buffer (pH 7.8) and 10 mM hydroxylammonium chloride. The mixture was kept at 25°C (water bath) for 20 min and then centrifuged at 1500 × *g* for 10 min. Then, 1.0 ml of the mixture was mixed with same volume of 17 mM sulfanilic acid and 7.0 mM 1-naphthylamine. After incubation at 25°C for 20 min, 3.0 ml ethyl ether was introduced to the mixture, shaken to uniform, and centrifuged at 1500 × *g* for 5 min. Absorbance of the water phase at 530 nm was then recorded. For blank controls, the same amount of 50 mM sodium phosphate buffer (pH 7.8) was added into the reaction system instead of the enzyme extract.

Measurement of H_2O_2 content was performed according to Patterson et al. [55], Liu et al. [56], and Wang et al. [27] with modifications. In brief, leaf and root tissues (ca. 0.5 g) were ground to a fine powder in liquid N_2 and then homogenized in 3.0 ml precooled acetone. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C. A 1.0 ml supernatant, 2.5 ml extractant (CCl_4:$CHCl_3$ = 3:1, v/v), and 2.5 ml redistilled water was mixed and centrifuged at 5000 × g for 5 min at 4°C. Then, 2.0 ml water phases in the supernatant were divided into two aliquots of 1.0 ml. One was taken as blank control and the other was used to examine H_2O_2 concentration. For the blank control, catalase was introduced to a concentration of 3.0 U ml^{-1} and then kept at 30°C for 10 min. Same amount of solution with inactivated catalase (by high temperature) was added into the H_2O_2 extract. Then, 1.0 ml of 0.2 M sodium phosphate buffer (pH 7.8) and colorimetric reagent, 1.0 ml of 0.2 mM Ti(IV)-PAR, were introduced to both series [57]. The color was developed at 45°C for 20 min and then allowed to equilibrate with room temperature for 20 min. Finally, the absorbance at 508 nm was recorded, and concentration of H_2O_2 was given based on the established standard curve.

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.

Table 1. Effects of NaCl on H_2O_2 (nmol g^{-1} fw) levels and $O_2^{-\bullet}$ production rates (nmol min^{-1} mg^{-1} pro) in leaves and roots of *K. obovata* and *B. gymnorhiza*.

Compartment	Treatment	*K. obovata*		*B. gymnorhiza*	
		Na^+	Cl^-	Na^+	Cl^-
Epidermis	Control	2.98 ± 0.30b	5.35 ± 1.26b	10.3 ± 4.46b	24.3 ± 1.76b
	NaCl	7.36 ± 2.19a	32.8 ± 3.94a	16.0 ± 3.82a	44.9 ± 1.96a
Exodermis	Control	1.05 ± 0.23b	12.1 ± 2.12b	7.76 ± 1.78b	27.2 ± 4.46b
	NaCl	11.1 ± 1.24a	23.6 ± 4.72a	23.9 ± 3.33a	51.4 ± 2.14a
Cortex	Control	0.70 ± 0.34b	7.71 ± 1.05b	12.6 ± 3.54b	34.2 ± 1.42b
	NaCl	5.39 ± 1.36a	43.7 ± 2.65a	26.3 ± 3.32a	51.2 ± 3.50a
Endodermis	Control	1.39 ± 0.97b	10.2 ± 0.37b	15.8 ± 4.90b	35.9 ± 3.08b
	NaCl	4.84 ± 0.96a	49.3 ± 1.64a	31.0 ± 5.82a	57.3 ± 5.14a
Stelar parenchyma	Control	1.56 ± 0.58b	6.96 ± 1.33b	17.1 ± 2.19b	24.8 ± 2.46b
	NaCl	5.80 ± 1.12a	38.6 ± 2.51a	35.3 ± 7.89a	48.2 ± 4.51a

K. obovata and *B. gymnorhiza* roots were sampled from control and stressed plants after 15 days of increasing NaCl treatment (100–400 mM). Cellular Na^+ and Cl^- contents were measured by SEM-EDX according to Sun et al. [58]. Briefly, roots with tips were washed free of soil particles and rapidly frozen in liquid nitrogen, vacuum freeze-dried at −100 °C for 24 h, and then slowly allowed to equilibrate to room temperature (ca. 22°C) for 24 h. Freeze-dried roots were gold coated in a high vacuum sputter coater and analyzed with a Hitachi S-3400 N scanning electron microscope equipped with an energy-dispersive X-ray detector (EX-250, Horiba Ltd. Kyoto, Japan). Probe measurements of samples were taken with a broad electron beam covering the whole cells that were randomly selected in the epidermis, exodermis, cortex, endodermis, and stele. Na^+ and Cl^- levels were expressed as a percentage of the total atomic number for all the major elements (K^+, Na^+, Ca^{2+}, Mg^{2+}, and Cl^-) detected from the cell samples.
Each value (±SE) is the mean of three plants, and 5–12 measurements (for each compartment) were taken from each root. Values in the same column followed by different letters are significantly different ($P < 0.05$) between control and NaCl treatment.

Table 2. Salt distribution in root cells of *K. obovata* and *B. gymnorhiza*.

of the two species but with the exception of Cl^- in *K. obovata* (**Table 3**). In comparison, the fractions of Na^+ and Cl^- in the xylem vessel, cell wall, and vacuole were 30–196% higher in stressed *B. gymnorhiza* as compared to *K. obovata* (**Table 3**). However, NaCl stress did not significantly increase Na^+ and Cl^- concentrations in the chloroplast of two mangroves (**Table 3**).

Vacuolar compartmentation in mesophyll was clearly seen in stressed *B. gymnorhiza*, in which the Na^+ and Cl^- concentrations were higher in the vacuole than in the chloroplast (**Table 3**). Noteworthy, *B. gymnorhiza* preferentially accumulated 73–94% higher Na^+ and Cl^- in vacuoles of epidermal cells as compared to mesophyll vacuoles (**Table 3**). In contrast to *B. gymnorhiza*, vacuolar fractions of Na^+ and Cl^- in stressed *K. obovata* remained the same as that of chloroplast, and vacuolar Na^+ and Cl^- in epidermis was similar to that in mesophyll vacuole regardless of treatments (**Table 3**).

2.3.2. Salt compartmentation and ROS production in roots and leaves

X-ray microanalysis data show that Na^+ and Cl^- were evident in root and leaf cells of control plants in the two mangroves (**Tables 2** and **3**), presumably originated from hypocotyls as propagules were collected from the surface of soil or seawater in coastal habitats of mangrove

Compartment	Treatment	*K. obovata*		*B. gymnorhiza*	
		Na^+	Cl^-	Na^+	Cl^-
Xylem vessels (leaf vascular bundle)	Control	131 ± 102b	633 ± 48a	309 ± 22b	576 ± 101b
	NaCl	246 ± 4a	636 ± 101a	729 ± 119a	1069 ± 254a
Epidermal wall (abaxial and adaxial)	Control	266 ± 96b	725 ± 181a	403 ± 22b	812 ± 101b
	NaCl	377 ± 110a	945 ± 71a	840 ± 119a	1305 ± 254a
Mesophyll wall (palisade and spongy)	Control	228 ± 35b	669 ± 224a	420 ± 46b	545 ± 67b
	NaCl	336 ± 27a	926 ± 170a	904 ± 287a	1445 ± 418a
Epidermal vacuole (abaxial and adaxial)	Control	75 ± 71b	495 ± 282a	221 ± 42b	646 ± 23b
	NaCl	183 ± 133a	558 ± 285a	509 ± 125a	1148 ± 121a
Mesophyll vacuole (palisade and spongy)	Control	86 ± 14b	510 ± 123a	123 ± 16b	531 ± 33b
	NaCl	134 ± 31a	634 ± 31a	263 ± 6a	664 ± 3a
Chloroplast (palisade and spongy)	Control	103 ± 18a	532 ± 129a	182 ± 69a	640 ± 89a
	NaCl	141 ± 15a	681 ± 45a	145 ± 57a	494 ± 101a

K. obovata and *B. gymnorhiza* leaves were sampled from control and stressed plants after 15 days of increasing NaCl treatment (100–400 mM). Standard procedures required for sample preparation and X-ray microanalysis were followed as described in Fritz [59, 60]. In brief, leaf segments, 2–3 mm long and 1–2 mm wide, were cut with a razor blade along the smaller veins adjacent to the central vein and immediately placed into aluminum sample holders and rapidly frozen in a 3:1 mixture of propane:isopentane at the temperature of liquid nitrogen. Samples were vacuum freeze-dried at −60°C for 72 h and then slowly allowed to equilibrate to room temperature (ca. 22°C) over a period of 24 h. Then, samples were stored over silica gel until infiltration in plastic. Freeze-dried leaf samples were transferred into vacuum-pressure chambers and infiltrated in ether at 27°C overnight before infiltrating with plastic. The plastic used was a 1:1 mixture of styrene (Merck Schuchardt) and butyl methacrylate (Sigma-Aldrich) containing 1% benzoyl peroxide stabilized with 50% phthalate. Infiltration with plastic was carried out in the following steps: 1:1 ether:plastic for 24 h, 1:3 ether:plastic for 24 h, and finally 100% plastic for 24 h. Following infiltration, samples were transferred into gelatin capsules and polymerized at 60°C for 12 h, then transferred into 35°C oven, and polymerized for at least 7 days. After polymerization, agar samples were cut into 1-μm-thick sections using dry glass knife with an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria). The slices were mounted in copper grids (mesh 50), coated with carbon, and stored over silica gel until analysis.

Leaf sections were analyzed in a Phillips EM 420 electron transmission microscope (Eindhoven, the Netherlands) with the energy dispersive system EDAX DX-4 (EDAX International, Mahwah, NJ 07430, USA). The operating parameters were as follows: accelerating voltage was 120 kV; take-off angle was 25°; and the time for collecting X-rays was 60 live seconds. Probe measurements were made on xylem vessels in the bundle, spongy, and palisade mesophyll, adaxial, and abaxial epidermis. The following structures were examined: cell wall, vacuole, and chloroplast (mesophyll), and magnification was at ×6350. Probe measurements of cell walls were taken with a long and narrow electron beam, and measurements of vacuole and chloroplasts were taken with a broad electron beam covering the target structures. For each section, 10–20 measurements were taken from each compartment. The X-ray spectra were processed with EDAX DX-4 software after manual fitting of the background. Concentrations of Na^+ and Cl^- were determined by analytical calibration standard of NaCl that established according to Fritz and Jentschke [61].

Values in the same column followed by different letters are significantly different ($P < 0.05$) between control and NaCl treatment.

Table 3. Salt compartmentation within leaf cell compartments of *K. obovata* and *B. gymnorhiza*.

forest. Mangrove propagules absorbed salt ions when they contacted seawater [7, 12]. Na^+ and Cl^- increased in cell compartments of roots and leaves (**Tables 2** and **3**). This indicates that the salt ions taken up by roots transported to shoots under NaCl stress [12, 16, 22]. Our data show that there were marked differences in the pattern of salt compartmentation in the two mangroves. *K. obovata* exhibited a high capacity to exclude NaCl from root and leaf cells, whereas

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B. gymnorhiza are notable for (1) vacuolar compartmentation in mesophyll cells and (2) preferential accumulation of Na^+ and Cl^- in epidermal vacuoles, relative to mesophyll vacuoles (**Table 3**). The ability to extrude Na^+ from root cells of *K. obovata* likely results from an active Na^+/H^+ antiport driven by H^+ pumping activity of PM H^+-ATPase [7, 15]. Salt compartmentation in vacuoles likely depends on active transport of salt ions across the tonoplast. Salinity may increase the activity of vacuole H^+ pumps, thus making a contribution to the compartmentation of toxic ions into the vacuoles via Na^+/H^+ antiporter systems [62–64]. Mimura et al. found that the elevated concentrations of Na^+ and Cl^- in swelling vacuoles were correlated with the salt-induced activation of tonoplast H^+-ATPase in suspension-cultured cells of *B. sexangula* [65]. We suggest that the two mangrove plants may maintain ROS homeostasis through limiting ROS production by a protective cellular salt compartmentation, in addition to scavenging ROS by antioxidant enzymes in a longer term of increasing salinity (see below). In brief:

a. Salt exclusion and ROS production in *K. obovata*: NaCl treatment increased Na^+ in the leaf apoplast and vacuole of epidermis and mesophyll, but did not elevate Cl^- in *K. obovata* (**Table 3**). Moreover, the absolute values of Na^+ and Cl^- in these measured compartments were lower in *K. obovata* than in *B. gymnorhiza* under 400 mM NaCl (**Table 3**). Result suggests that *K. obovata* plants had a higher capacity for NaCl exclusion, presumably due to the salt uptake and transport restrictions in roots (**Table 2**) [7, 12, 15]. Effective salt exclusion is a benefit for *K. obovata* to reduce ROS production. We have shown that the inability to exclude NaCl favored the formation of $O_2^{\bullet -}$ and H_2O_2, which causes an oxidative burst in leaf cells of a salt-sensitive poplar, *P. simonii* × (*P. pyramidalis* × *Salix matsudana*) (*P. popularis* cv. '35–44') [26, 27, 38].

b. Vacuolar salt compartmentation and ROS production in *B. gymnorhiza*: *B. gymnorhiza* leaves exhibited a more pronounced salt accumulation than *K. obovata* (**Table 3**), resulting from a higher root-to-shoot salt transport [12]. Noteworthy, *B. gymnorhiza* preferentially accumulated Na^+ and Cl^- in epidermal vacuoles, instead of mesophyll vacuoles (**Table 3**). Similar findings were observed in leaf sheaths and blades of sorghum and barley in which Cl^- was preferentially accumulated in most cell layers, particularly the adaxial epidermal cells [39, 40]. The evident Cl^- exclusion from photosynthetically active mesophyll would lessen the effect of salinity on photosynthetic processes, especially the electron transport in chloroplasts in the mesophyll. Moreover, fractions of Na^+ and Cl^- remained higher in mesophyll vacuole than in the cytoplasm (**Table 3**), which may inhibit the enhancement of NaCl on the formation of $O_2^{\bullet -}$ and H_2O_2 in the cytosol, chloroplasts, and mitochondria. NaCl was found to favor the formation of $O_2^{\bullet -}$ and H_2O_2 in chloroplasts and mitochondria of pea cultivars [28, 29]. Accordingly, we hypothesize that the *B. gymnorhiza* limits ROS production by preferential accumulation of Na^+ and Cl^- in epidermal vacuoles, as well as vacuolar compartmentation in mesophyll cells.

2.4. Antioxidant enzymes contributed to ROS homeostasis

2.4.1. Activity of antioxidant enzymes in roots and leaves

Under no-salt control conditions, total activity of measured antioxidant enzymes roots and leaves, SOD, APX, CAT, and GR, varied markedly during the observation period (**Tables 4**

and **5**). This was presumably resulted from genetic difference of seedlings and variations in light intensity and air temperature. In our study, natural PAR was 400–1200 μmol m^{-2} s^{-1}, and air temperature was 20–35°C over the duration of experiment. In general, activities of antioxidant enzymes in roots and leaves were not reduced upon increasing saline (with a few exceptions) but upregulated in both species (**Tables 4** and **5**). Noteworthy, there were species differences in antioxidant defense to increasing salinity. Activity of each component in the measured antioxidant defense system, SOD, APX, CAT, and GR, drastically increased in *K. obovata* roots at 300 mM NaCl, while the same trend was observed in *B. gymnorhiza* roots at 400 mM (**Table 4**). Furthermore, *B. gymnorhiza* leaves showed a higher increase of SOD, APX, and CAT at 400 mM NaCl as compared to *K. obovata* (**Table 5**). SOD of *K. obovata* was upregulated after salt exposure, but the response is quite variable in roots and leaves. Root SOD activity was increased by 100 and 300 mM NaCl, while leaf activity was increased by 200 and 400 mM (**Tables 4** and **5**). SOD activity in roots and leaves of *B. gymnorhiza* did not increase after exposure to 100–300 mM NaCl (**Tables 4** and **5**). The variable response of antioxidant enzymes to salt treatment was also seen in GR. It exhibited a marked elevation in *B. gymnorhiza* leaves at 100 mM NaCl, whereas the steady increase of GR in *K. obovata* was observed at a salt concentration of 300 mM (root) and 400 mM (leaf) (**Tables 4** and **5**).

SOD isoenzymes and CAT isoenzymes in roots and leaves were analyzed by native PAGE. In root extracts, three dominant SOD isoenzymes were detected in *K. obovata* roots, whereas there were two SOD isoforms in *B. gymnorhiza* (**Figure 1A** and **B**). KCN and H_2O_2 inhibited the activity of these isoenzymes in the two species, indicating they were CuZn-SOD isoforms (**Figure 1A** and **B**). NaCl did not restrict activity of all SOD isoforms in *K. obovata* roots during the period of salt treatment (**Figure 1C**), but a marked elevation of CuZn-SODs was observed in *B. gymnorhiza* at 300–400 mM NaCl (**Figure 1D**).

Three dominant SOD isoenzymes were detected in control leaves of both species but with different patterns (**Figure 2A** and **B**). KCN and H_2O_2 inhibited activity of two SOD isoenzymes in both genotypes, indicating that these were CuZn-SOD isoforms (**Figure 2A** and **B**). Another SOD isoform was defined as Mn-SOD since it was resistant to both inhibitors (**Figure 2A** and **B**). Activity of SOD isoenzymes in *K. obovata* leaves was increased by a lower salt, e.g., Mn-SOD at 100 and 200 mM NaCl and Cu/Zn-SOD1 and Cu/Zn-SOD2 at 200 NaCl mM (**Figure 2C**). *B. gymnorhiza* upregulated both Mn-SOD and Cu/Zn-SODs at a higher salt, 300–400 mM NaCl (**Figure 2D**).

Native PAGE of root extract showed three CAT isoenzymes in *K. obovata* and two in *B. gymnorhiza* (**Figure 3**). Increasing NaCl, from 100 to 400 mM, did not restrict activity of all CAT isoforms in both species, although activity of CAT isoforms in control roots fluctuated over the observation period (**Figure 3**). Compared with *K. obovata*, *B. gymnorhiza* exhibited typically a higher activity of CAT1 and CAT2 regardless of treatments (**Figure 3**).

Three and four CAT isoenzymes were identified in *K. obovata* and *B. gymnorhiza* leaves, respectively (**Figure 4**). Salt markedly enhanced the activity of CAT2 in *K. obovata* at 200 mM; however, the enhancement of NaCl on CAT2, CAT3, and CAT4 in *B. gymnorhiza* was observed at 300–400 mM NaCl (**Figure 4**).

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Treatment	*K. obovata*				*B. gymnorhiza*			
	SOD	**CAT**	**APX**	**GR**	**SOD**	**CAT**	**APX**	**GR**
Control	246.9 ± 25.0b	138.1 ± 21.2a	270.0 ± 22.3a	33.7 ± 15.2a	133.3 ± 9.0a	27.8 ± 4.8a	130.2 ± 7.8a	12.9 ± 2.9a
NaCl (100 mM)	406.4 ± 21.5a	136.2 ± 11.1a	272.3 ± 22.2a	15.7 ± 3.3a	125.2 ± 7.3a	45.1 ± 19.0a	144.1 ± 18.6a	20.7 ± 3.1a
Control	108.0 ± 20.2a	152.9 ± 21.4a	249.7 ± 46.5a	49.9 ± 8.0a	149.0 ± 31.3a	43.3 ± 2.9a	377.1 ± 34.1a	19.1 ± 5.6a
NaCl (200 mM)	112.2 ± 61.2a	151.9 ± 27.2a	181.4 ± 15.4a	77.6 ± 12.2a	148.6 ± 17.9a	46.7 ± 10.1a	369.4 ± 94.6a	30.4 ± 13.8a
Control	148.4 ± 1.7b	28.9 ± 6.2b	119.8 ± 16.4b	17.4 ± 6.0b	98.4 ± 17.4a	26.4 ± 1.8a	120.8 ± 27.7a	30.0 ± 9.4a
NaCl (300 mM)	377.4 ± 32.9a	60.71 ± 8.0a	343.7 ± 96.4a	49.4 ± 11.4a	121.8 ± 28.1a	27.8 ± 1.2a	178.1 ± 47.7a	29.8 ± 6.5a
Control	193.8 ± 88.8a	35.1 ± 6.3a	62.2 ± 5.7a	17.9 ± 3.6a	132.6 ± 11.8b	109.0 ± 16.3a	218.1 ± 55.6b	23.7 ± 2.3b
NaCl (400 mM)	244.4 ± 64.1a	54.6 ± 18.6a	77.8 ± 4.5a	26.1 ± 0.5a	232.4 ± 19.5a	132.6 ± 1.7a	361.6 ± 80.7a	59.8 ± 15.0a

K. obovata and *B. gymnorhiza* plants were subjected to increasing NaCl treatment. NaCl saline was applied at day 1 (100 mM), day 3 (200 mM), day 6 (300 mM), and day 10 (400 mM). Roots of control and salinized plants were harvested at day 2, day 5, day 9, and day 14, respectively. Three replicated plants per treatment were harvested at each sampling time. Roots (ca. 0.5 g) were ground in cold mortars using liquid nitrogen and homogenized in a 3.0-ml ice-cold extraction buffer [50 mM potassium phosphate, pH 7.0, 1.0 mM EDTA, 1%(w/v) PVP]. The extracts were centrifuged at 10,000 × *g* for 20 min at 4°C and used for the assays of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR). For ascorbate peroxidase (APX) measurement, 1.0 mM ascorbic acid (ASC) was added into the enzyme extraction buffer [27]. Protein concentration in the supernatant was determined according to Bradford [66] using bovine serum albumin as standard.

Total SOD activity was assayed as described in Giannopolits and Ries [67] with modifications. The reaction mixture contained 1.8 ml potassium phosphate (50 mM, pH 7.8), 0.3 ml L-methionine (130 mM), 0.3 ml nitroblue tetrazolium salt (750 μM), 0.3 ml Na_2EDTA (100 μM), 30 μl enzyme extract, and 0.3 ml riboflavin (20 μM). The cocktail was mixed and then illuminated by cool white fluorescent lamps (30 μmol m^{-2} s^{-1}) for 6 min. For blank controls, a 30 μl potassium phosphate buffer (50 mM, pH 7.8) was added into the reaction system instead of the enzyme extract. The increase in absorbance at 560 nm, due to the formation of formazan, was recorded. SOD activity was calculated as $A_{enzyme} - A_{control}$. One unit of SOD is defined as the amount of enzyme that causes a 50% inhibition of the aforementioned reaction in comparison with a blank sample.

Total CAT activity was determined spectrophotometrically by measuring the rate of H_2O_2 consumption (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min [44]. The reaction mixture (3.0 ml) contained 50 mM potassium phosphate (pH 7.0) and 2% H_2O_2. Immediately, after the addition of 30 μl enzyme extract, the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity. One unit is defined as the amount of catalase decomposing 1.0 μmol of hydrogen peroxide per minute at pH 7.0 at 25°C. In blank controls, the same amount of 50 mM sodium phosphate buffer (30 μl, pH 7.0) was added into the reaction system instead of the enzyme extract.

Total APX activity was assayed as described in Mishra et al. [68]. The reaction mixture (3.0 ml) contained 50 mM potassium phosphate (pH 7.0), 15 mM ascorbic acid, and 30 mM H_2O_2 and 30 μl enzyme extract. The reaction at 25°C was initiated by the addition of H_2O_2. APX activity was immediately measured by recording the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 2 min. One unit of APX is defined as the amount of enzyme required to consume 1.0 μmol of ascorbate per min. Correction was done for the low, nonenzymatic oxidation of ASC by H_2O_2 [69]. In blank controls, a 30 μl potassium phosphate buffer (50 mM, pH 7.0) was added into the reaction system instead of the enzyme extract.

Total GR activity was determined at 25°C by measuring the rate of NADPH oxidation [47]. The reaction mixture (3.0 ml) contained 50 mM potassium phosphate (pH 7.8), 2.0 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), and 50 μl of enzyme extract. NADPH was added to start the reaction, and the decrease in absorbance at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) was recorded as soon as the reaction began. Corrections were made for the background absorbance at 340 nm, without the addition of NADPH. One unit of GR is defined as the amount of enzyme that oxidizes 1.0 μmol of NADPH per min. For blank controls, a 50 μl potassium phosphate buffer (50 mM, pH 7.8) was added into the reaction system instead of the enzyme extract.

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.

Table 4. Effects of increasing NaCl on activity of SOD, CAT, APX, and GR in roots of *K. obovata* and *B. gymnorhiza*.

Treatment	*K. obovata*				*B. gymnorhiza*			
	SOD	**CAT**	**APX**	**GR**	**SOD**	**CAT**	**APX**	**GR**
Control	49.1 ± 8.6a	107.4 ± 6.5b	62.0 ± 2.2a	16.5 ± 3.1a	38.1 ± 3.0a	69.3 ± 24.7a	58.7 ± 1.1a	5.8 ± 0.6b
NaCl (100 mM)	67.5 ± 17.6a	138.9 ± 7.5a	76.4 ± 5.4a	20.0 ± 3.1a	32.6 ± 4.5a	37.5 ± 9.0a	64.9 ± 7.5a	10.4 ± 1.5a
Control	61.2 ± 5.9b	223.9 ± 3.3a	102.9 ± 1.9a	25.8 ± 3.4a	52.2 ± 4.2a	88.7 ± 8.2a	181.2 ± 19.5a	22.5 ± 0.2a
NaCl (200 mM)	83.8 ± 5.0a	205.7 ± 22.8a	90.7 ± 16.5a	32.0 ± 2.6a	42.2 ± 7.9a	89.1 ± 9.9a	179.0 ± 10.1a	9.7 ± 1.6b
Control	66.2 ± 8.6a	135.6 ± 21.8a	83.6 ± 6.4a	22.1 ± 6.4a	32.0 ± 4.9a	118.0 ± 1.4a	130.5 ± 12.4a	18.9 ± 3.3a
NaCl (300 mM)	64.4 ± 10.9a	150.5 ± 29.1a	47.3 ± 4.7b	16.0 ± 1.0a	46.4 ± 6.6a	125.2 ± 2.1a	60.4 ± 11.7b	12.9 ± 3.3a
Control	52.2 ± 13.9b	331.3 ± 4.0a	111.9 ± 6.4a	19.9 ± 1.2b	30.6 ± 9.4b	75.9 ± 5.1b	116.1 ± 15.7b	9.5 ± 1.4a
NaCl (400 mM)	74.8 ± 4.2a	356.6 ± 15.1a	129.8 ± 2.5a	36.8 ± 4.1a	59.7 ± 8.6a	250.7 ± 29.6a	213.9 ± 16.3a	7.4 ± 2.1a

K. obovata and *B. gymnorhiza* plants were subjected to increasing NaCl treatment. NaCl saline was applied at day 1 (100 mM), day 3 (200 mM), day 6 (300 mM), and day 10 (400 mM). Roots of control and salinized plants were harvested at day 2, day 5, day 9, and day 14, respectively. Three replicated plants per treatment were harvested at each sampling time. Leaf tissues (ca. 0.5 g) were ground in cold mortars using liquid nitrogen and homogenized in a 3.0-ml ice-cold extraction buffer [50 mM potassium phosphate, pH 7.0, 1.0 mM EDTA, 1%(w/v) PVP]. The extracts were centrifuged at 10,000 × *g* for 20 min at 4°C and used for the assays of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR). For ascorbate peroxidase (APX) measurement, 1.0 mM ascorbic acid (ASC) was added into the enzyme extraction buffer [27]. Protein concentration in the supernatant was determined according to Bradford [66] using bovine serum albumin as standard. Methodologies for total activity of SOD, CAT, APX, and GR are shown in **Table 4** legend.

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.

Table 5. Effects of increasing NaCl on activity of SOD, CAT, APX, and GR in leaves of *K. obovata* and *B. gymnorhiza*.

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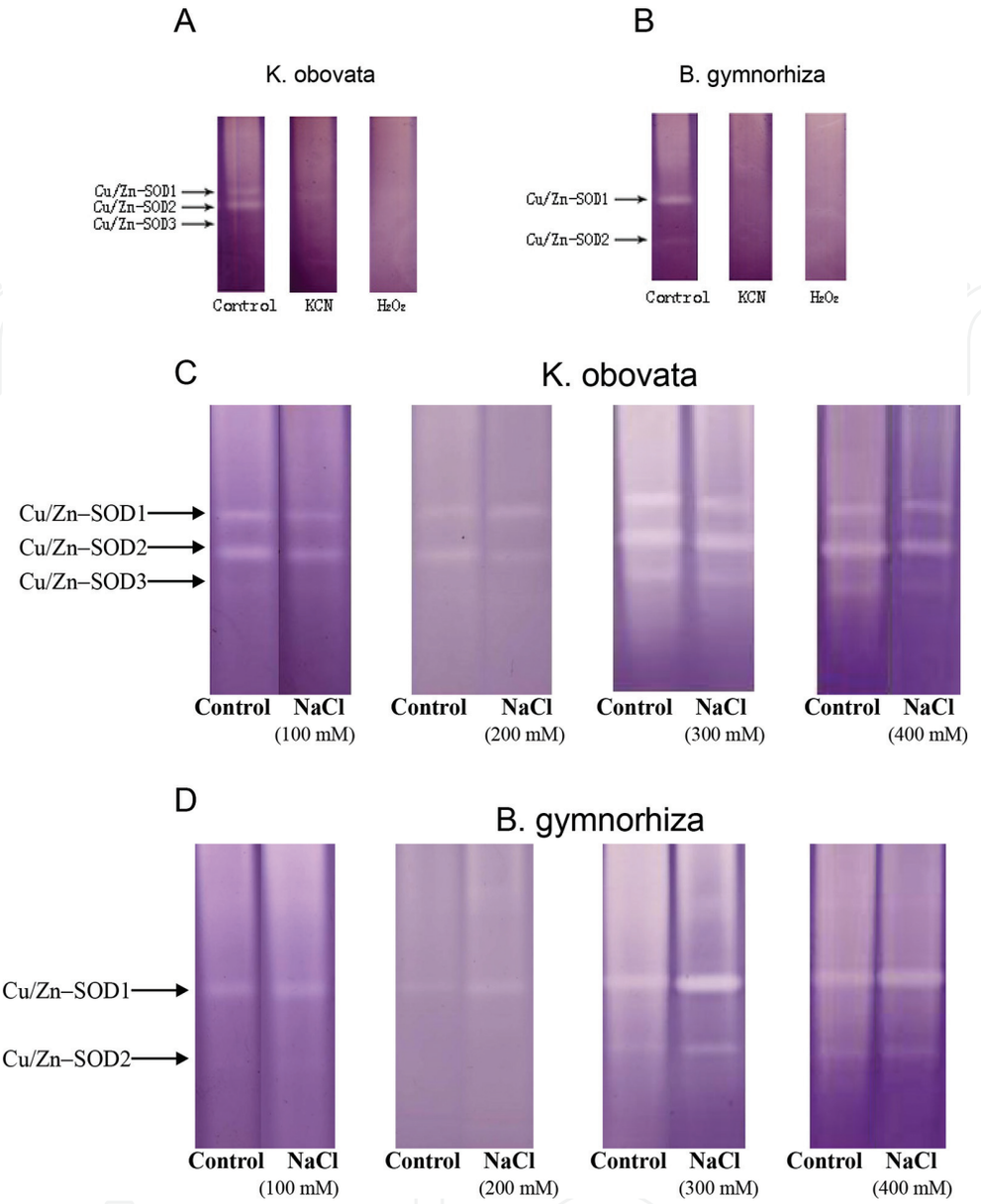

Figure 1. Identification of root SOD isoenzymes and effect of increasing NaCl on SOD isoforms in roots of *K. obovata* and *B. gymnorhiza*. (A and B) Identification of root SOD isoenzymes. Different isoforms of SOD in *K. obovata* and *B. gymnorhiza* were determined by incubating the gels with 5 mM H_2O_2 to inhibit both Cu/Zn-SOD and Fe-SOD or with 5 mM KCN to inhibit only Cu/ZnSOD [70]. Meanwhile, Mn-SOD activity was obtained since it is resistant to both inhibitors, H_2O_2 and KCN. (C and D) NaCl effects on SOD isoforms. *K. obovata* and *B. gymnorhiza* plants were subjected to increasing NaCl treatment. Increasing NaCl was applied at day 1 (100 mM), day 3 (200 mM), day 6 (300 mM), and day 10 (400 mM). Control plants were kept well watered with no addition of NaCl. Roots of control and salinized plants were harvested at day 3, day 6, day 10, and day 15, respectively. Three replicated plants per treatment were harvested at each sampling time. The three replicates were extracted independently and ran on three different gels, a representative one of which is shown in the figure. Electrophoretic separation for CAT and SOD was performed at 4°C using the Laemmli (1970) buffer systems [71]. Prior to loading onto the gels, crude protein extracts were mixed with 10% glycerol (v/v) and 0.25% bromophenol blue. Separating gel (10%) and stacking gel (3.9%) were used for native PAGE of SOD isoenzymes. SOD isoenzymes were visualized by the activity staining [72]. In each track 20 μg of soluble protein was applied to native polyacrylamide gel electrophoresis at 4°C. The gels were run at a constant current, 35 mA at 4°C for no longer than 6 h. Immediately, after electrophoretic separation, gels were incubated in staining buffer (50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, 28 mM TEMED, 0.003 mM riboflavin, and 0.25 mM NBT) for 30 min in the dark at room temperature. Thereafter, gels were exposed to two fluorescent tubes (20 W each) until the SOD bands became visible (SOD bands appeared as light bands on a blue background).

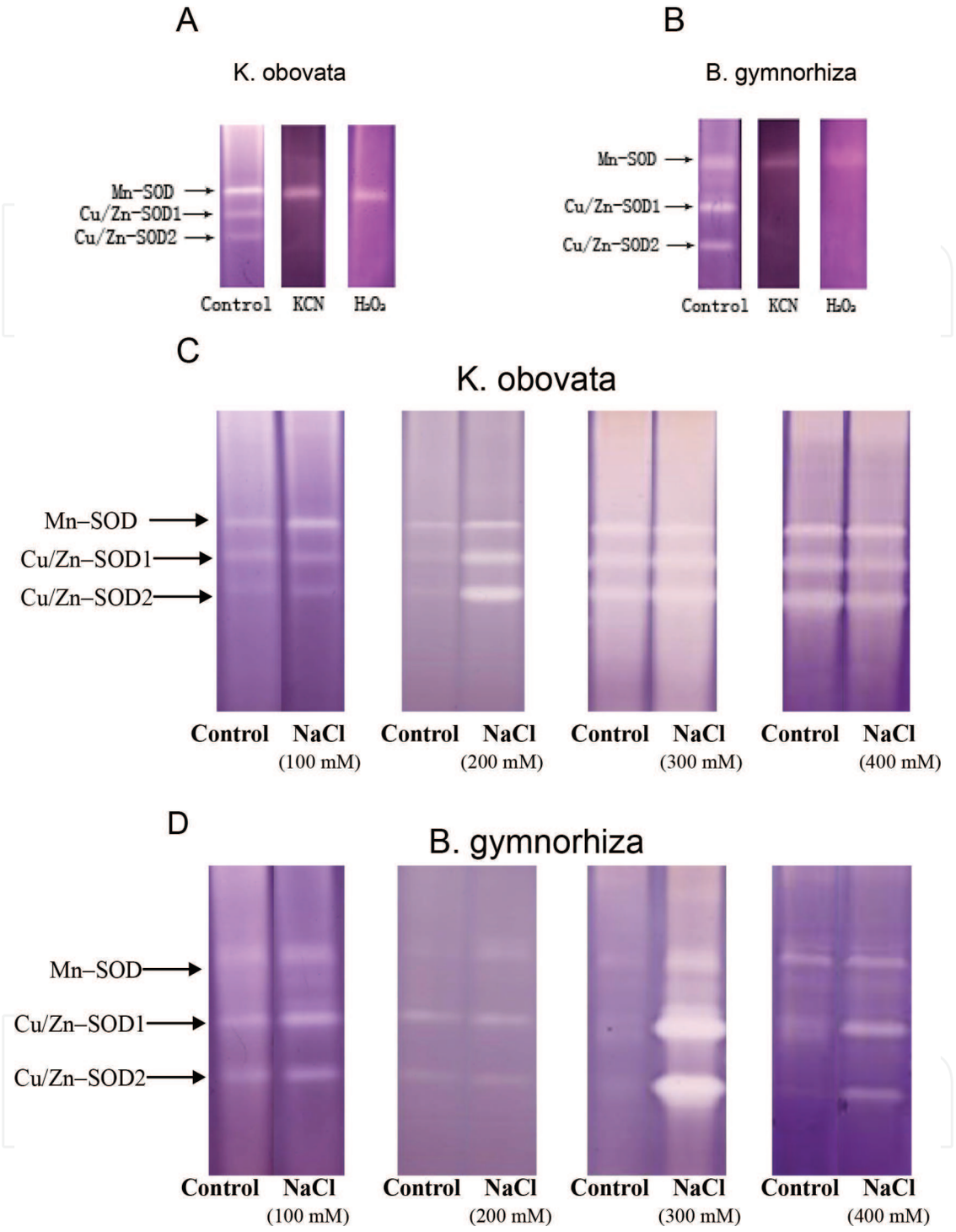

Figure 2. Identification of leaf SOD isoenzymes and effect of increasing NaCl on SOD isoforms in leaves of *K. obovata* and *B. gymnorhiza*. (A and B) Identification of leaf SOD isoenzymes. (C and D) NaCl effects on SOD isoforms. *K. obovata* and *B. gymnorhiza* plants were subjected to increasing NaCl treatment. Increasing NaCl was applied at day 1 (100 mM), day 3 (200 mM), day 6 (300 mM), and day 10 (400 mM). Control plants were kept well watered with no addition of NaCl. Leaves of control and salinized plants were harvested at day 3, day 6, day 10, and day 15, respectively. Three replicated plants per treatment were harvested at each sampling time. The three replicates were extracted independently and ran on three different gels, a representative one of which is shown in the figure. In each track 40 μg of soluble protein was applied to native polyacrylamide gel electrophoresis at 4°C. Electrophoretic separation for SOD isoforms is shown in **Figure 1** legend.

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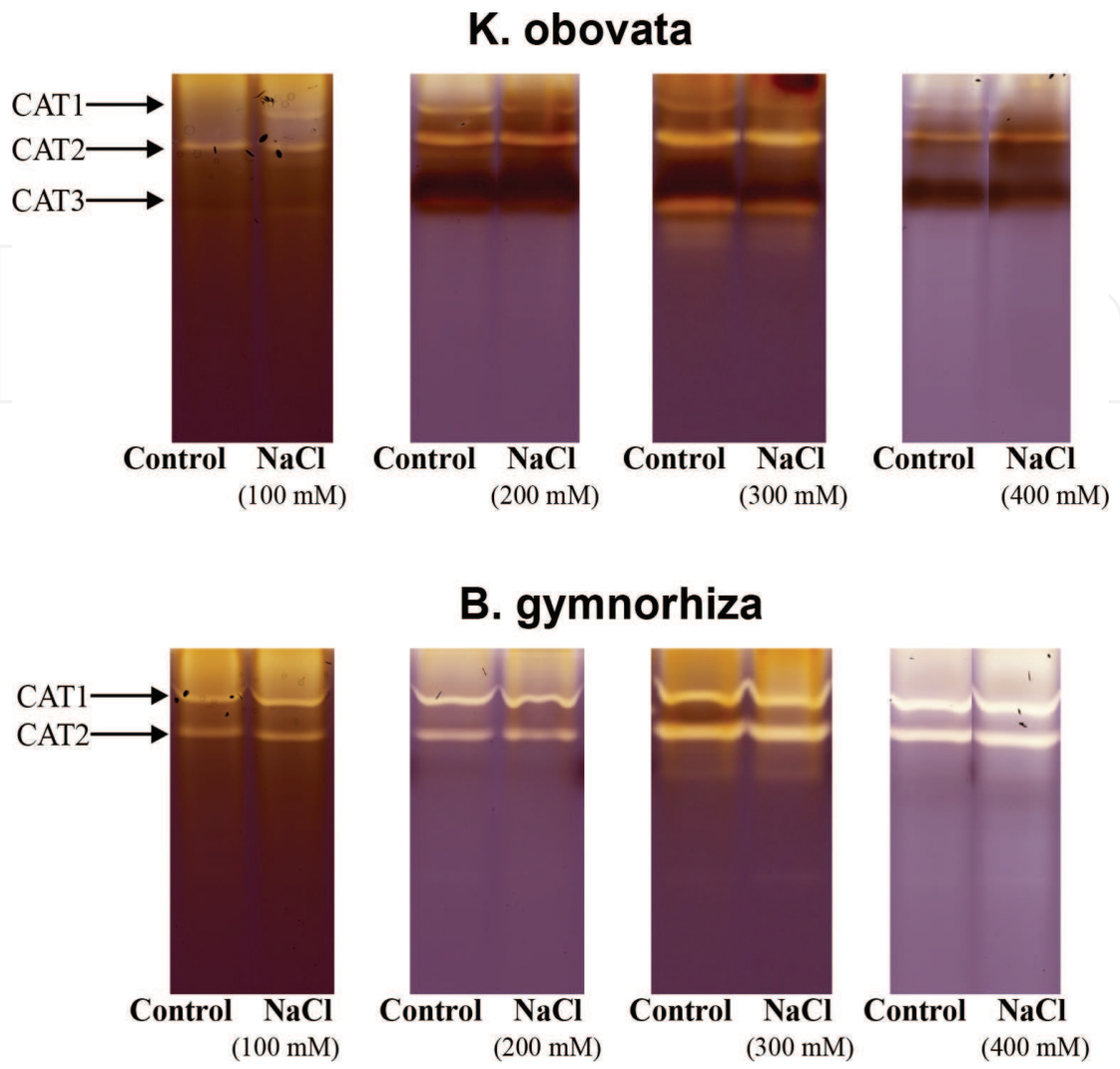

Figure 3. Effect of increasing NaCl on CAT isoforms in roots of *K. obovata* and *B. gymnorhiza*. *K. obovata* and *B. gymnorhiza* plants were subjected to increasing NaCl treatment. Increasing NaCl was applied at day 1 (100 mM), day 3 (200 mM), day 6 (300 mM), and day 10 (400 mM). Control plants were kept well watered with no addition of NaCl. Roots of control and salinized plants were harvested at day 3, day 6, day 10, and day 15, respectively. Three replicated plants per treatment were harvested at each sampling time. The three replicates were extracted independently and ran on three different gels, a representative one of which is shown in the figure. Stacking gel (3.9%) and separating gel (7.5%) containing 0.5% soluble starch were used for native PAGE of CAT isoforms. In each track 20 μg of soluble protein was applied to native polyacrylamide gel electrophoresis at 4°C. The activity staining procedure for catalase was followed as per Thorup et al. [73] with modifications. Immediately, after electrophoresis as described above, the gel was incubated in a solution containing 18 mM sodium thiosulphate and 679 mM H_2O_2 for 30 s at room temperature (25°C). The gel was then rinsed with distilled water and incubated in 90 mM potassium iodide solution acidified with 0.5% glacial acetic acid. Finally, negative bands, representing CAT enzymes, appeared on the blue background of the gel.

2.4.2. Salt-elicited antioxidant enzymes contributed to ROS homeostasis

Salt-elicited antioxidant enzymes contributed to ROS homeostasis in the two mangroves but with different patterns. Salinized *K. obovata* exhibited an early and rapid antioxidative defense as compared to *B. gymnorhiza*. After exposure to 100–200 mM NaCl, total SOD activity in *K. obovata* leaves marked increased coincident with the increase of Cu/Zn-SOD1, Cu/Zn-

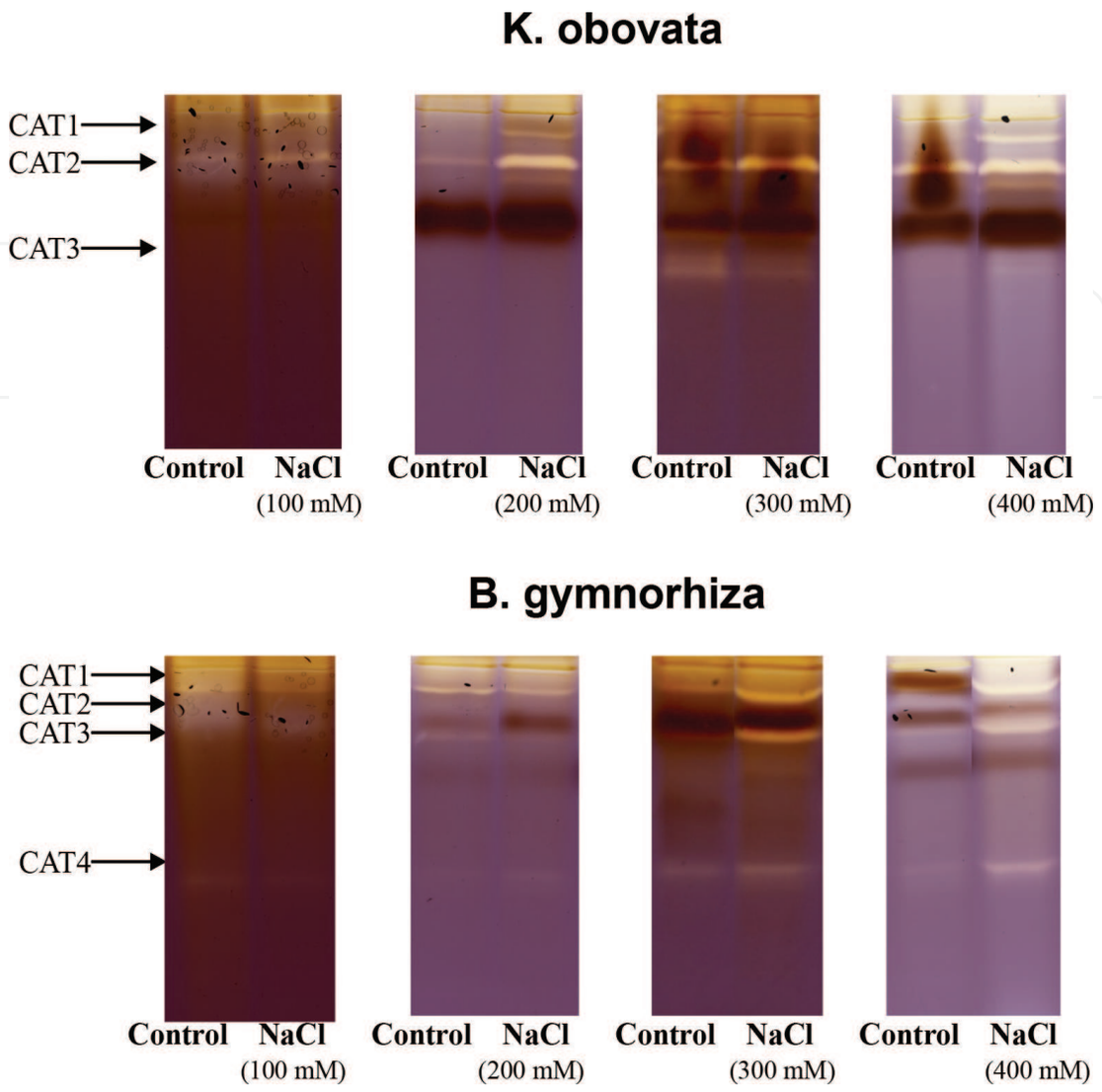

Figure 4. Effect of increasing NaCl on CAT isoforms in leaves of *K. obovata* and *B. gymnorhiza*. *K. obovata* and *B. gymnorhiza* plants were subjected to increasing NaCl treatment. Increasing NaCl was applied at day 1 (100 mM), day 3 (200 mM), day 6 (300 mM), and day 10 (400 mM). Control plants were kept well watered with no addition of NaCl. Leaves of control and salinized plants were harvested at day 3, day 6, day 10, and day 15, respectively. Three replicated plants per treatment were harvested at each sampling time. The three replicates were extracted independently and ran on three different gels, a representative one of which is shown in the figure. In each track 40 μg of soluble protein was applied to native polyacrylamide gel electrophoresis at 4°C. Electrophoretic separation for CAT isoforms is shown in **Figure 3** legend.

SOD2, and Mn-SOD (**Table 5**, **Figure 2**), even though Fe-SOD was not detected as that reported in other mangroves [51]. CAT in *K. obovata* leaves resembles the trend of SOD, and the increased activity was presumably due to the rise of CAT2 (**Table 5**, **Figure 4**). This is inconsistent with a previous report conducted on *B. parviflora* in which NaCl induced a decrease of CAT activity [51]. In the present study, the coincident increase of CAT with SOD in *K. obovata* reveals an elevated capacity to detoxify both $O_2^{\bullet -}$ and H_2O_2 that is caused by NaCl, which is required for rapid removal of ROS and thus avoids oxidative damage. Likewise,

we found that a salt-resistant *Populus* species, *P. euphratica*, was able to enhance active oxygen detoxification by increasing antioxidant enzymes at an early stage of salt stress, thus preventing an oxidative burst [26]. Protein abundance of SOD in *K. obovata* leaves might increase under a high level of NaCl [53]. Furthermore, Jing et al. showed that NaCl increased *KcCSD* transcription in *K. candel* leaves [16]. Thus, it could be inferred that *K. obovata* would upregulate the gene expression of antioxidant enzymes to deal with a long-term saline stress.

Salt-induced elevation of antioxidant enzymes in *B. gymnorhiza* was usually found at high salinity. SOD, APX, and CAT in roots and leaves of *B. gymnorhiza* were all upregulated by 400 mM NaCl (**Tables 4** and **5**). Native PAGE analyses showed that the elevation of leaf SOD in salinized *B. gymnorhiza* resulted from the increase of all detected SOD isoforms, Mn-SOD, Cu/Zn-SOD1, and Cu/Zn-SOD2 (**Table 5** and **Figure 2**), whereas the rise of SOD activity in roots was mainly the result of Cu/Zn-SODs (**Table 4** and **Figure 1**). A similar trend was found in salt-stressed *B. parviflora* in which a significant enhancement of SOD was observed in leaves, mainly due to an increase in Mn-SOD and Fe-SOD2 [51]. NaCl-induced activity of CAT in *B. gymnorhiza* leaves was due to the increase of CAT2, CAT3, and CAT4 (**Table 5** and **Figure 4**).

Noteworthy, both *K. obovata* and *B. gymnorhiza* maintained evident activity of each CAT isoform in root tissues at 400 mM NaCl (**Figure 3**), showing a constant and stable capacity to control H_2O_2 levels. This may partly explain the finding that root $O_2^{\bullet -}$ increased by 82–83%, whereas there was no corresponding changes in H_2O_2 when *K. obovata* and *B. gymnorhiza* were subjected to 400 mM NaCl (**Table 1**).

3. Conclusions

We conclude that both *K. obovata* and *B. gymnorhiza* maintained ROS homeostasis as external NaCl saline increased from 100 to 400 mM but via different pathways:

i. *K. obovata* restricted the increase of salt influx, which is necessary to avoid abrupt increase of ROS. Moreover, *K. obovata* was sensitive to lower salt stress and rapidly initiated antioxidant defense to scavenge active oxygen species by, at least in part, components of the ASC-GSH cycle, e.g., SOD, APX, CAT, and GR. The Na^+/H^+ antiport system and proton pumps, which accelerate the salt exclusion across the plasma membrane, need to be further investigated.

ii. *B. gymnorhiza* maintained higher capacity to detoxify ROS at high salinity; furthermore, the effective vacuolar salt compartmentation in mesophyll cells and the preferential accumulation of Na^+ and Cl^- in epidermal vacuoles may benefit *B. gymnorhiza* plants to reduce ROS production in the mesophyll. Together with antioxidant mechanisms, both enzymatic and nonenzymatic, the critical balance between ROS production and ROS detoxification is remained under salt stress. To elucidate the mechanism underlying the vacuolar compartmentation, critical ion channels and transporters in the vacuolar membranes need to be identified in future investigations.

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Conflict of interest

The authors declare that there is no conflict of interest.

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