Tree Physiology Advance Access published December 20, 2012

Tree Physiology 00, 1–15
doi:10.1093/treephys/tps119

Research paper

Exogenous hydrogen peroxide, nitric oxide and calcium mediate root ion fluxes in two non-secretor mangrove species subjected to NaCl stress

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Received January 9, 2012; accepted October 28, 2012; handling Editor Torgny Näsholm

Using 3-month-old seedlings of *Bruguiera gymnorrhiza* (L.) Savigny and *Kandelia candel* (L.) Druce, we compared species differences in ionic homeostasis control between the two non-secretor mangrove species. A high salinity (400 mM NaCl, 4 weeks) resulted in a decline of the K^+/Na^+ ratio in root and leaf tissues, and the reduction was more pronounced in *K. candel* (41–66%) as compared with *B. gymnorrhiza* (5–36%). Salt-altered flux profiles of Na^+, K^+, H^+ and Ca^{2+} in roots and effects of exogenous hydrogen peroxide (H_2O_2), nitric oxide (NO) and Ca^{2+} on root ion fluxes were examined in seedlings that were hydroponically treated short term with 100 mM NaCl (ST, 24 h) and long term with 200 mM NaCl (LT, 7 days). Short term and LT salinity resulted in Na^+ efflux and a correspondingly increased H^+ influx in roots of both species, although a more pronounced effect was observed in *B. gymnorrhiza*. The salt-enhanced exchange of Na^+ with H^+ was obviously inhibited by amiloride (a Na^+/H^+ antiporter inhibitor) or sodium orthovanadate (a plasma membrane H^+-ATPase inhibitor), indicating that the Na^+ efflux resulted from active Na^+ exclusion across the plasma membrane. Short term and LT salinity accelerated K^+ efflux in the two species, but *K. candel* exhibited a higher flux rate. The salt-induced K^+ efflux was markedly restricted by the K^+ channel blocker, tetraethylammonium chloride, indicating that the K^+ efflux is mediated by depolarization-activated channels, e.g., KORCs (outward rectifying K^+ channels) and NSCCs (non-selective cation channels). Exogenous H_2O_2 application (10 mM) markedly increased the apparent Na^+ efflux and limited K^+ efflux in ST-treated roots, although H_2O_2 caused a higher Na^+ efflux in *B. gymnorrhiza* roots. $CaCl_2$ (10 mM) reduced the efflux of K^+ in salinized roots of the two mangroves, but its enhancement of Na^+ efflux was found only in *B. gymnorrhiza*. Under ST treatment, sodium nitroprusside (SNP) (100 μM, an NO donor) increased Na^+ efflux at the root apex of the two species; however, its inhibition of K^+ loss was seen only in *K. candel*. Of note, NaCl caused an obvious influx of Ca^{2+} in *B. gymnorrhiza* roots, which was enhanced by H_2O_2 (10 mM). Therefore, the salt-induced Ca^{2+} benefits *B. gymnorrhiza* in maintaining K^+/Na^+ homeostasis under high external salinity.

Keywords: *Bruguiera gymnorrhiza, Kandelia candel*, K^+ flux, K^+/Na^+ homeostasis, Na^+/H^+ antiport, root, salt stress, scanning ion-selective electrode technique.

†These authors contributed equally to this work.

Introduction

Mangroves are halophytes thriving in the intertidal zone of tropical and subtropical climates (Huang et al. 2003). The capacity for salt tolerance varies within secretor and non-secretor mangrove species (Mishra and Das 2003, Parida et al. 2004*a*), and the mechanisms by which mangrove plants survive in saline conditions are complex and involve interactions of morphological, anatomical and physiological adaptations (Parida and Das 2005). Salt treatment induces alterations in leaf morphology, ultrastructure, photosynthetic capacity and intensity of low molecular polypeptides (Parida et al. 2003, 2004*a*, 2004*b*, 2004*c*). Activity of antioxidant enzymes increases upon salt stress, allowing scavenging of reactive oxygen species in *Bruguiera parviflora* (Roxb.) Wight & Arn. ex Griff. and *B. gymnorrhiza* (Takemura et al. 2002, Parida et al. 2004*c*). Salt-treated *B. parviflora* increases both reducing and non-reducing sugars in leaves, contributing to osmotic adjustment (Parida et al. 2002). Similarly, polyphenol and proline levels increase significantly in leaves of the non-secretor mangrove *B. parviflora* (Parida et al. 2002). Furthermore, salt causes accumulations of Na^+ and Cl^- in root and shoot tissues but decreases Ca^{2+}, Mg^{2+}, total nitrogen and nitrate uptake in *B. parviflora* (Parida and Das 2004, Parida et al. 2004*a*).

Under natural saline environments, salt exclusion by roots is the most important salt-tolerance mechanism in secretor and non-secretor mangrove species (Wang and Lin 2003). The salt-excluding mangrove species eliminate excess salt by an ultrafiltration mechanism occurring at the root cell membranes of cortical cells (Takemura et al. 2000, Irfan and Ajmal 2001, Wang et al. 2002). Hypocotyls of *B. gymnorrhiza* act as an additional filter to retain salt from transport to the shoot (Lawton et al. 1981). The capacity of salt exclusion in *B. gymnorrhiza* likely depends on the concentration of NaCl in the external solutions and the duration of salt exposure (Takemura et al. 2000). Kura-Hotta et al. (2001) suggested that at the cellular level, Na^+ extrusion occurs only at a high salt concentration while a low salinity causes Na^+ accumulation in *Bruguiera sexangula* (Lour.) Poir. Meanwhile, inhibitors of the proton pump and Na^+/H^+ antiporter reduce the salt extrusion. Osmotic adjustment and selective ion accumulation have been investigated in *K. candel*, and accumulation as well as synthesis of compatible solutes were considered to contribute to salt adaption of this species, although the amount of inorganic ions and organic osmolytes varies under various salinity (Zhu et al. 2011). However, the mechanisms regulating ion homeostasis in these non-secretor mangroves have not been elucidated.

The capacity to maintain K^+/Na^+ homeostasis is crucial for plants to adapt to salinity stress (Shabala and Cuin 2008, Sun et al. 2010*a*, 2010*b*). Increasing evidence suggests that hydrogen peroxide (H_2O_2), nitric oxide (NO) and Ca^{2+} function as intermediates regulating Na^+/K^+ homeostasis under salt stress. In *Arabidopsis*, exogenous H_2O_2 mediates *SOS1* mRNA stability and may contribute to cellular Na^+ detoxification (Chung et al. 2008). Hydrogen peroxide activates the plasma membrane (PM) Ca^{2+}-permeable channels, resulting in an increase of Ca^{2+} in the cytosol (Mori and Schroeder 2004). The elevated $[Ca^{2+}]_{cyt}$ regulates PM Na^+/H^+ antiporter activity through the SOS signaling pathway in *Arabidopsis* and rice (Zhu 2001, 2003, Qiu et al. 2002, Martínez-Atienza et al. 2007). However, H_2O_2 also activates DA-KORCs (depolarization-activated outward rectifying K^+ channels), leading to K^+ loss or programmed cell death under salt stress (Demidchik et al. 2010). Nitric oxide plays a crucial role in plant growth and developmental regulation, such as seed dormancy, germination, hypocotyl elongation, flowering and senescence (Beligni and Lamattina 2000, He et al. 2005, Shapiro 2005, Bethke et al. 2006, Besson-Bard et al. 2008, Giudice et al. 2011). In addition, NO contributes to salt secretion in maintaining ion equilibrium in a mangrove plant, *Avicennia marina* (Forsk) Vierh (Chen et al. 2010) and can increase salt secretion from salt glands and promote Na^+ sequestration into the vacuoles of the epidermis and hypodermal cells (Chen et al. 2010).

Bruguiera gymnorrhiza and *Kandelia candel* are two typical non-secretor mangrove species occurring along the southern China coastline (Li et al. 2008). In this study, the scanning ion-selective electrode technique (SIET) was applied to measure K^+, Na^+, H^+ and Ca^{2+} fluxes from root tissue of the two non-secretor mangrove species. We attempted to investigate species-specific differences in the regulation of ion homeostasis under NaCl stress. To clarify the role of stress signals in ionic homeostasis control, the effects of H_2O_2, NO and $CaCl_2$ on K^+/Na^+ homeostasis were examined in the two mangrove species.

Materials and methods

Plant materials and culture conditions

During December 2008–10, propagules of *Bruguiera gymnorrhiza* (L.) Savigny and *Kandelia candel* (L.) Druce were obtained from Dongzhai Harbor Mangrove Nature Reserve, Haikou, Hainan Province (latitude 19°51′N, longitude 110°24′E). Propagules were planted in pots (15 cm in diameter and 18 cm in height) containing sand and placed in a greenhouse at Beijing Forestry University, Beijing, China. Potted plants were irrigated according to evaporation demand and fertilized with full-strength Hoagland's solution every 2 weeks. In the greenhouse, air temperature was 25–30 °C and relative humidity was 60–70%. A 12-h photoperiod (7:00–19:00) was applied, and photosynthetically active radiation varied from 400 to 800 μmol m^{-2} s^{-1}. Young roots of 3-month-old seedlings were washed and transferred to 200-ml pots containing quarter-strength Hoagland's nutrient solution. Plants were equilibrated to hydroponic culture for 24 h prior to salt, hyperosmotic and inhibitor treatments.

In our study, we used propagules germinated in sand fertilized with Hoagland solution rather than in salt water, the natural condition. We found that propagules germinated in Hoagland solution were quite as healthy as those germinated in natural salt conditions. Naturally grown *K. candel* (3 months old) and *B. gymnorrhiza* (2 years old) were collected from Dongzhai Harbor Mangrove Nature Reserve, Haikou, Hainan Province. Roots of naturally grown seedlings were carefully dug out with piles of seaside silt and placed in individual pots. The roots were not injured during sampling and transportation. Na^+ and K^+ concentrations in roots and leaves were examined in these saline-acclimated plants. Moreover, fluxes of Na^+ and K^+ were examined in young roots of *K. candel* after plants were subjected to 100 mM NaCl for 1 day. Flux recordings showed that salt treatment did not cause a significant efflux of K^+ and Na^+ in roots of these naturally grown seedlings (see Supplemental Figure S1 available as Supplementary Data at *Tree Physiology* Online).

Treatments

Using 3-month-old seedlings of *K. candel* and *B. gymnorrhiza*, three series of experiments were carried out as described below. For PM transporter studies, sodium orthovanadate (a specific inhibitor of PM H^+-ATPase) and amiloride (an inhibitor of Na^+/H^+ exchange) were used to inhibit the Na^+/H^+ antiport system in the PM (Sun et al. 2009*a*). Tetraethylammonium chloride (TEA), a K^+ channel blocker, was used to reduce the salt-induced K^+ efflux.

Two mangrove species were exposed to short-term (ST) salinity via NaCl solution (100 mM) for 1 day and long-term (LT) salinity via 200 mM NaCl for 7 days. The required amounts of NaCl were added to the full-strength Hoagland's nutrient solution. In a 24-h hyperosmotic treatment, the two species were exposed to 170 mM mannitol instead of iso-NaCl (100 mM). Control plants were raised in full-strength Hoagland's nutrient solution without the addition of NaCl or mannitol. In this study, plants were in good physiological state after long treatment in hydroponics (7 days). Young roots with apices of 1.0–2.0 cm were sampled from the control, salt- and hyperosmotic stress-treated plants of the two species and used for steady flux measurements of Na^+, H^+, K^+ and Ca^{2+}.

The effects of PM transport inhibitors on ion fluxes were examined in salt-treated *B. gymnorrhiza* and *K. candel*. After 24 h of NaCl treatment (100 mM), roots with apices of 1.0–2.0 cm, sampled from *B. gymnorrhiza* and *K. candel*, were subjected to 500 μM sodium orthovanadate (50 min) or 100 μM amiloride (30 min). Then the steady flux of Na^+ and H^+ was measured along the roots treated with or without inhibitors. We also examined the effect of TEA (50 μM, 30 min) on K^+ flux in ST- and LT-treated plants. Prior to the flux measurements, measuring solutions containing sodium orthovanadate were removed slowly with a pipette and a 10-ml fresh solution was then slowly added to the measuring chamber (a Petri dish, 3.5 cm in diameter). Measuring solutions containing amiloride and TEA were not replaced because amiloride and TEA have no obvious effect on the Nernstian slopes of Na^+, H^+ and K^+ electrodes.

We examined the effects of exogenous H_2O_2, SNP (sodium nitroprusside, a donor of NO) and $CaCl_2$ on Na^+ and K^+ fluxes in salt-treated *B. gymnorrhiza* and *K. candel*. Hydroponic-equilibrated plants were subjected to an ST NaCl treatment (100 mM, 24 h) supplemented with or without H_2O_2 (10 mM), SNP (100 μM) or $CaCl_2$ (10 mM). Young roots with apices were sampled and used for steady flux measurements of Na^+ and K^+. The effects of H_2O_2 on Ca^{2+} flux were also examined in salt-stressed plants of the two species.

Ion analysis in tissue

Root and leaf samples were harvested from control and salt-stressed (400 mM NaCl, 28 days) plants of the two species in the greenhouse. In addition, roots and leaves were also sampled from saline-acclimated plants in the natural habitat, Dongzhai Harbor Mangrove Nature Reserve. Samples were oven-dried at 65 °C for 4 days, ground and passed through a 1.0-mm sieve and stored for Na^+ and K^+ measurements. Na^+ and K^+ were quantified using an atomic absorption spectrophotometer (Perkin-Elmer 2280, PerkinElmer, Inc., Wellesley Hills, MA, USA) (Chen et al. 2001).

Measurements of Na^+, H^+, K^+ and Ca^{2+} fluxes with SIET

Net fluxes of Na^+, H^+, K^+ and Ca^{2+} were measured non-invasively using the SIET system (BIO-001A, Younger USA Sci. and Tech. Corp., Amherst, MA, USA; Applicable Electronics Inc., Forestdale, MA, USA; and ScienceWares Inc., East Falmouth, MA, USA) (Sun et al. 2009*a*, 2009*b*). The concentration gradients of the target ions were measured by moving the ion-selective microelectrode between two positions close to the plant material in a pre-set excursion (30 μm for excised roots) at a programmable frequency in the range of 0.3–0.5 Hz. Pre-pulled and silanized glass micropipettes (2–4 μm aperture; Xuyue (Beijing) Sci. and Tech. Co., Ltd., Beijing, China) were treated with a backfilling solution (Na^+: 250 mM NaCl; K^+: 100 mM KCl; H^+: 40 mM KH_2PO_4 and 15 mM NaCl, pH 7.0; Ca^{2+}: 100 mM $CaCl_2$) to a length of 1.0 cm from the tip. Then the micropipettes were front-filled with 15 μm columns of selective liquid ion exchange cocktails (LIXs) (Na: Fluka 71178; K: Fluka 60398, H: Fluka 95293; Ca^{2+}: Fluka 21048; Fluka Chemie GmbH, Buchs, Switzerland). An Ag/AgCl wire electrode holder (XYEH01-1; Xuyue (Beijing) Sci. and Tech. Co., Ltd., Beijing, China) was inserted in the back of the electrode to make electrical contact with the electrolyte solution. The reference electrode was an Ag/AgCl half-cell (DRIREF-2; World Precision Instruments, Inc., Sarasota, FL, USA) connected to the experimental solution by a 0.5% agarose bridge containing

3.0 M KCl. Ion-selective electrodes of the following target ions were calibrated prior to flux measurements:

(i) Na^+: 0.1, 0.5 and 1.0 mM (Na^+ concentration was 0.1 mM in the measuring buffer for root samples, because the fluxes of Na^+ were only recorded at lower Na^+ concentrations using the Na LIX, Fluka 71178; Sun et al. 2009*a*, 2009*b*).
(ii) H^+: pH 5.0, 6.0, 7.0 (pH of the measuring buffer was adjusted to 6.0 with NaOH and HCl for root samples).
(iii) K^+: 0.1, 0.5 and 1.0 mM (K^+ concentration was 0.5 mM in the measuring solution).
(iv) Ca^{2+}: 0.1, 0.5 and 1.0 mM (Ca^{2+} concentration was 0.2 mM in the measuring solution).

Only electrodes with Nernstian slopes >50 mV/decade (Na^+, H^+, and K^+) and >25 mV/decade (Ca^{2+}) were used in our experiments. The flux rate was calculated from Fick's law of diffusion:

$$J = -D\,(dc/dx)$$

where J represents the ion flux in the x direction, dc/dx is the ion concentration gradient and D is the ion diffusion constant in a particular medium. Data and image acquisition, preliminary processing, control of the three-dimensional electrode positioner and stepper-motor-controlled fine focus of the microscope stage were performed with ASET software [Science Wares (East Falmouth, MA, USA) and Applicable Electronics].

Experimental protocols for SIET measurements

After exposure to the saline (ST and LT) treatments, root segments with 1.0–2.0 cm apices were sampled for ion flux measurements. To decrease the effect of salt release on flux recording (pre-loaded Na^+ diffuses from the surface of NaCl-stressed roots in a buffer with lower Na^+ concentrations), roots were rinsed with re-distilled water and immediately incubated in the measuring solution to equilibrate for 30 min.

(i) Na^+ measuring solutions: basic solution (0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.5 mM KCl), pH 6.0 was adjusted with KOH and HCl.
(ii) H^+ measuring solutions: basic solution (0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.5 mM KCl), pH 6.0 was adjusted with KOH and HCl.
(iii) K^+ measuring solutions: basic solution (0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.5 mM KCl) supplemented with 100 mM NaCl (for ST-treated roots) or 200 mM NaCl (for LT-treated roots). pH 6.0 was adjusted with choline and HCl.
(iv) Ca^{2+} measuring solutions: basic solution (0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 0.5 mM KCl) supplemented with 100 mM NaCl (for ST-treated roots). pH 6.0 was adjusted with choline and HCl.

Afterwards, roots were transferred to the measuring chamber containing 10 ml of a fresh measuring solution. When the roots were immobilized on the bottom of the chamber, ion flux measurements were started 200 μm from the apex and conducted along the root axis until 2700 μm. The measured positions of roots could be visualized and defined under the SIET microscope because young roots were semi-transparent under light. A 2-min continuous recording was performed at each measuring point in apical regions. Although buffered solutions may affect the actual magnitude of the H^+ flux, the qualitative tendency of NaCl- or mannitol-induced H^+ flux is not altered.

Data analysis

Ionic fluxes were obtained using MageFlux developed by Yue Xu (http://www.youngerusa.com). All mean data were subjected to analysis of variance. Significant differences between means were determined using Duncan's multiple range test, and unless otherwise stated, differences were considered statistically significant when $P < 0.05$.

Results

Salt accumulation in roots and leaves

After 7 days of salt treatment (200 mM NaCl), young white roots of *K. candel* turned slightly brown at the tip. However, no salt injury was seen in *B. gymnorrhiza* during the period of salt stress. We examined Na^+ and K^+ concentrations in roots and leaves of the two species.

Control plants contained 0.098–0.125 mmol g^{-1} dry weight (DW) Na^+ and 0.287–0.600 mmol g^{-1} DW K^+ in roots of the two mangroves (Table 1). Salt ions in root and leaf tissues in part originate from hypocotyls, which receive salts from the parent tree. Furthermore, mangrove propagules absorbed Na^+ ions from seawater because they were collected from the surface of soil or seawater in coastal habitats of mangrove forest. A high salinity of 400 mM NaCl increased root Na^+ but reduced K^+ in both species, leading to a decline in the K^+/Na^+ ratio (Table 1). Compared with *B. gymnorrhiza*, the salt-induced increase in Na^+ and reduction in K^+ were more pronounced in *K. candel* roots (Table 1), and the decrease in root K^+/Na^+ was thus higher in *K. candel* than in *B. gymnorrhiza* (Table 1).

As in the roots, Na^+ was evident in leaves of control plants in the two mangroves (Table 1). Leaf Na^+ showed a tendency to increase in both species, but a significant rise in Na^+ was found only in *K. candel* at 400 mM NaCl (Table 1). The salt-induced reduction of K^+ was evident in *K. candel* but not in *B. gymnorrhiza* (Table 1). As a result, salinized *K. candel* exhibited a greater decrease in K^+/Na^+, similar to the finding in roots (Table 1).

Na^+ and K^+ concentrations in roots and leaves were also examined using naturally grown *K. candel* and *B. gymnorrhiza*.

Table 1. Effects of NaCl on Na^+ and K^+ concentrations (mmol g^{-1} DW) and ratio of K^+/Na^+ in roots and leaves of *B. gymnorrhiza* and *K. candel*.

		Root		Leaf	
	[NaCl] (mM)	0	400	0	400
Na^+	*B. gymnorrhiza*	0.098 ± 0.016b	0.128 ± 0.004b	0.108 ± 0.009b	0.113 ± 0.006b
	K. candel	0.125 ± 0.017b	0.266 ± 0.067a	0.096 ± 0.004b	0.153 ± 0.028a
K^+	*B. gymnorrhiza*	0.287 ± 0.014c	0.250 ± 0.009c	0.235 ± 0.029b	0.233 ± 0.021b
	K.candel	0.600 ± 0.021a	0.416 ± 0.007b	0.277 ± 0.014a	0.246 ± 0.008b
K^+/Na^+	*B. gymnorrhiza*	3.050 ± 0.382b	1.948 ± 0.040c	2.175 ± 0.150b	2.069 ± 0.077b
	K. candel	4.932 ± 0.904a	1.671 ± 0.349c	2.878 ± 0.122a	1.702 ± 0.409c

Plants were subjected to 4 weeks of increasing NaCl stress (100–400 mM); NaCl concentrations started from 100 mM and increased stepwise by weekly 100 mM, reaching 400 mM in the fourth week. Control plants were well fertilized but were treated without additional NaCl. Each value (±SE) is the mean of three plants, and values followed by different letters (a, b, c) are significantly different at $P < 0.05$.

Compared with greenhouse plants treated with Hoagland solution, root and leaf Na^+ was much higher in naturally grown plants from saline habitats (Table 1, see Supplemental Table S1 available as Supplementary Data at *Tree Physiology* Online). Mangrove plants adapt to hyposaline environments by accumulating salt ions as inorganic osmotica (Zhao et al. 1999). To avoid disruption of ion balance, mangrove species growing near sea shores have developed a balance between Na^+ exclusion and increase in K^+ uptake mechanisms (Joshi et al. 1972, Wang et al. 2002). Salt exclusion by roots is crucial to maintain a low salt content in above-ground organs in secretor (*A. marina* and *Aegiceras corniculatum* (L.) Blanco) and non-secretor mangrove species (*K. candel*, *B. sexangula* and *Rhizophora stylosa* Griffith) (Wang and Lin 2003). Naturally and greenhouse-grown seedlings exhibited similar features in controlling K^+/Na^+ homeostasis; i.e., *K. candel* accumulated typically high K^+ in roots and leaves, and *B. gymnorrhiza* roots limited uptake of Na^+ under different saline conditions (Table 1, see Supplemental Table S1 available as Supplementary Data at *Tree Physiology* Online).

NaCl and osmotic stress-induced alterations of ion fluxes in roots

Steady flux profiles of K^+, Na^+, H^+ and Ca^{2+} were measured along the root axis at the apical zones (200–2700 μm from the root tip) at an interval 200 or 300 μm.

Na^+ fluxes

Under non-salt conditions, a typically higher Na^+ efflux was recorded in *B. gymnorrhiza* roots compared with *K. candel* (Figure 1). The two mangrove species exhibited a stable and constant Na^+ efflux along the root axes after ST (24 h) exposure to 100 mM NaCl and LT (7 days) exposure at a salinity of 200 mM NaCl (Figure 1). We note that LT-stressed roots of *B. gymnorrhiza* displayed a higher flux rate than those of *K. candel* (Figure 1).

Fluxes of Na^+ upon hyperosmotic stress differed from the response to salt stress in *B. gymnorrhiza*. An iso-osmotic stress caused by 170 mM mannitol (24 h, osmotic potential of 170 mM mannitol = 100 mM NaCl) did not change the Na^+ flux profile along the root axis in *B. gymnorrhiza* (Figure 1). However, the hyperosmotic stress caused a drastic Na^+ efflux along the root axis in *K. candel*, similar to the finding in ST-stressed roots (Figure 1).

Inhibitor experiments were also carried out in ST-stressed plants of the two species. Sodium orthovanadate (500 μM), the specific inhibitor of PM H^+-ATPase, significantly reduced the salt-induced efflux of Na^+ along the root axis in the two species (Figure 2). Amiloride (100 μM), the specific inhibitor of Na^+/H^+ antiporter, caused a drastic shift of Na^+ efflux towards an influx, and a more pronounced effect was seen in *K. candel* (Figure 2).

H^+ fluxes

NaCl induced a marked H^+ influx in the measured regions of *B. gymnorrhiza* roots (200–2700 μm from the apex), and the effect was more pronounced in LT-stressed roots (Figure 3). Compared with *B. gymnorrhiza*, the salt-increased influx of H^+ was lower in *K. candel* and observed only at the regions of 200–500 μm (ST, LT) and 2000–2300 μm (ST) (Figure 3). The hyperosmotic stress (170 mM mannitol) did not significantly change the H^+ flux profile along the root axis in either species, although a drastic H^+ efflux was detected in *K. candel* roots, at the region of 700–1300 μm (Figure 3).

Amiloride and sodium orthovanadate both significantly reduced the NaCl-induced H^+ influx along *B. gymnorrhiza* roots (Figure 2). However, the inhibitory effect of the two PM transport inhibitors was less pronounced in *K. candel* roots (Figure 2).

K^+ fluxes

Bruguiera gymnorrhiza roots exhibited a higher K^+ efflux than *K. candel* under no-salt control conditions (Figure 4). Salt stress caused a stable and constant K^+ efflux with mean values of 377 (ST) and 265 pmol cm^{-2} s^{-1} (LT) in the measured regions of *K. candel* roots (200–2700 μm from the apex) (Figure 4). However, the ST- and LT-induced K^+ loss in *B. gymnorrhiza* roots was significantly less than that observed

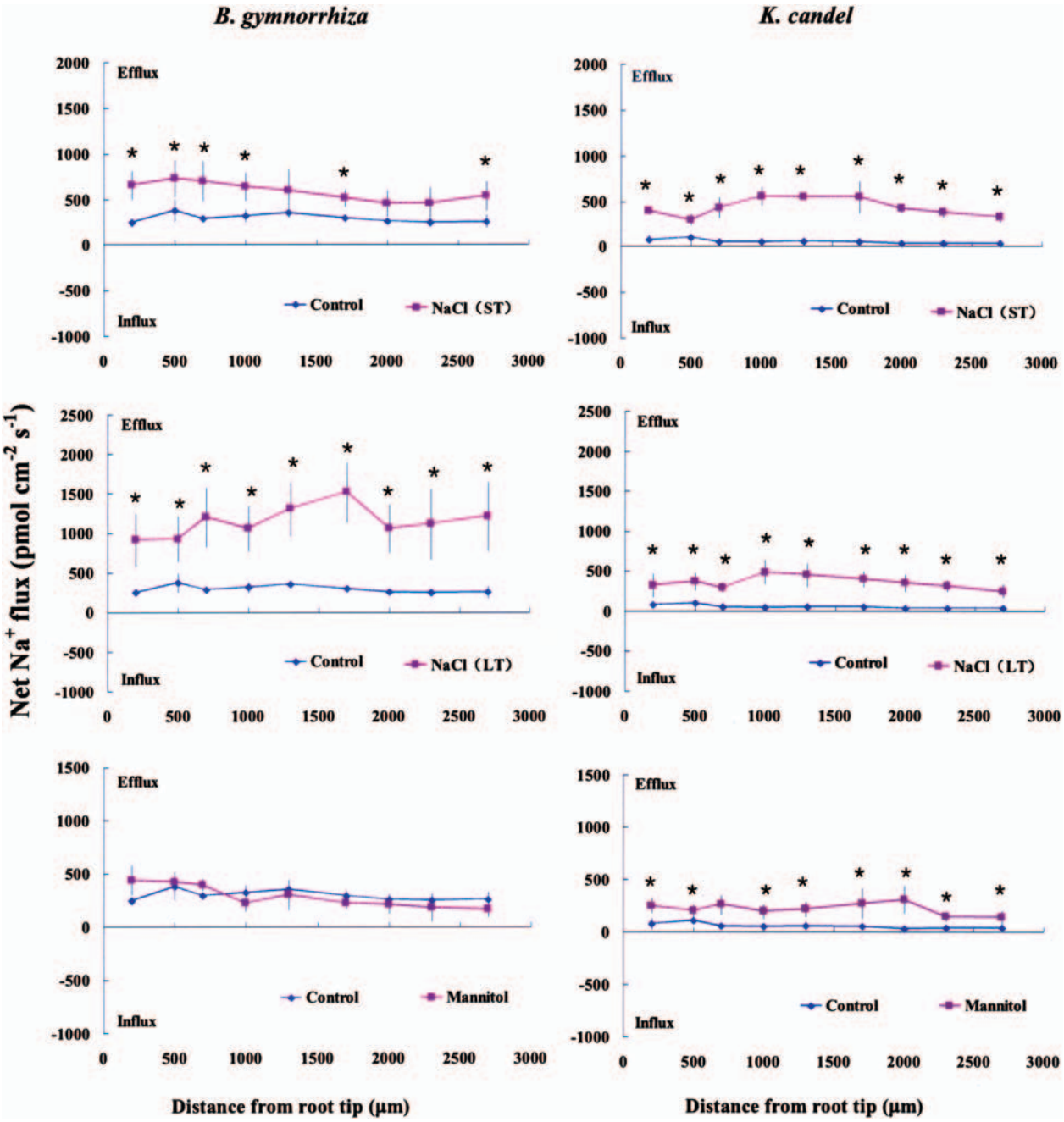

Figure 1. Effects of ST salinity (100 mM NaCl, 24 h), LT salinity (200 mM NaCl, 7 days) and hyperosmotic stress (170 mM mannitol, 24 h) on net Na^+ fluxes in roots of *B. gymnorrhiza* and *K. candel*. Control roots were well fertilized but treated without NaCl or mannitol. Each point is the mean of five to six individual plants, and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

in *K. candel* (Figure 4). K^+ flux in the two mangrove species was not significantly altered by a hyperosmotic stress (170 mM mannitol, 24 h) with a few exceptions (Figure 4). Inhibitor experiments showed that the salt-induced K^+ efflux in the two species was evidently inhibited by the K^+ channel blocker TEA, regardless of NaCl concentrations and exposure durations (Figure 5).

Effects of H_2O_2, $CaCl_2$ and NO on salt-induced ion fluxes in roots

Na^+ fluxes

Under ST stress, exogenously applied H_2O_2 (10 mM) significantly accelerated the efflux of Na^+ along the root axis in the two species (Figure 6). More pronounced effects were observed in *B. gymnorrhiza* with a mean value of 1958 pmol cm^{-2} s^{-1},

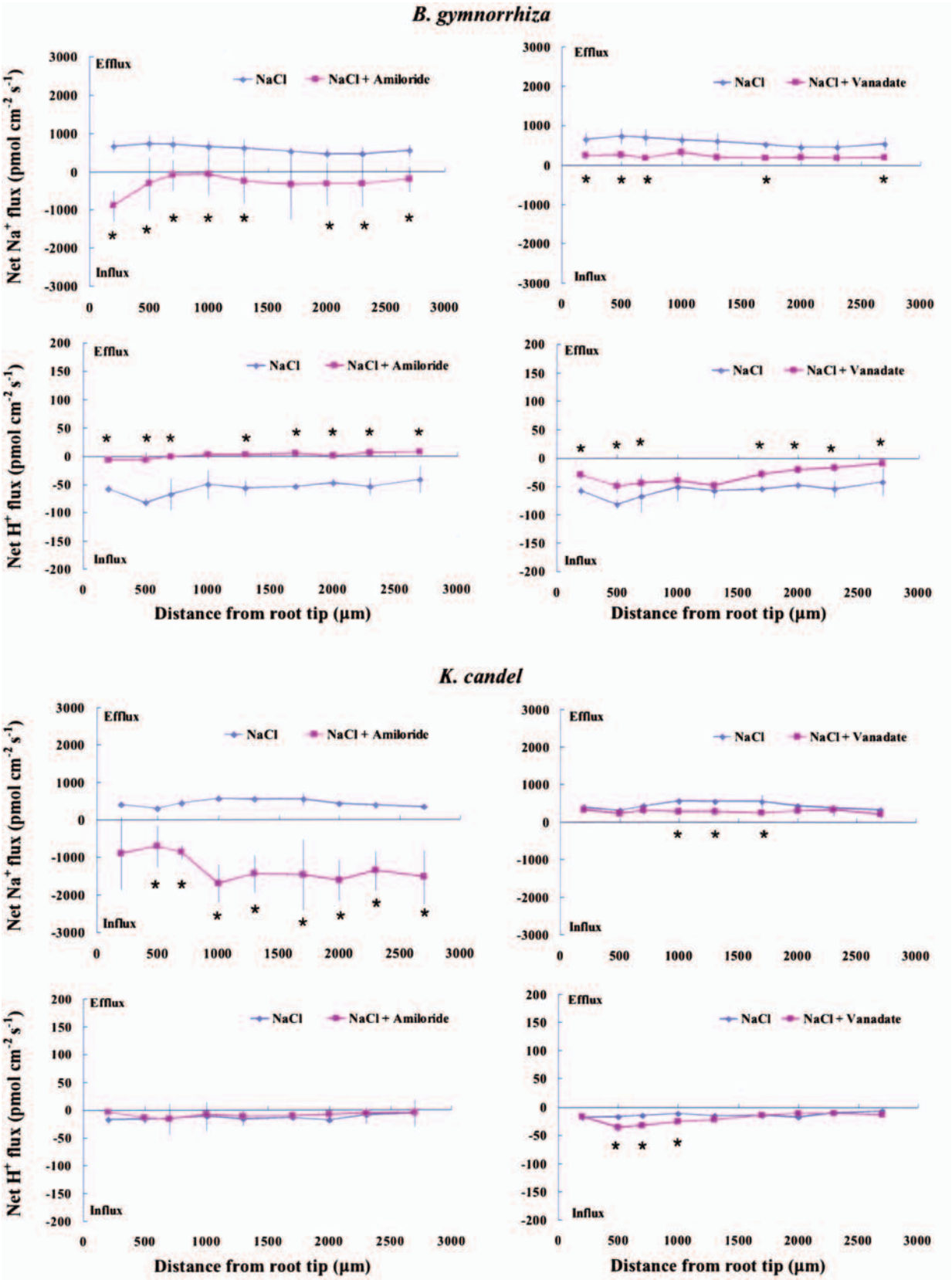

Figure 2. Effects of amiloride (100 μM) and sodium orthovanadate (500 μM) on net Na^+ and H^+ fluxes in ST-stressed roots of *B. gymnorrhiza* and *K. candel*. Short-term-stressed roots were pretreated with 100 μM amiloride or 500 μM sodium orthovanadate for 30–50 min prior to flux measurements. For NaCl controls, roots were not subjected to inhibitor treatment. Each point is the mean of five to six individual plants, and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

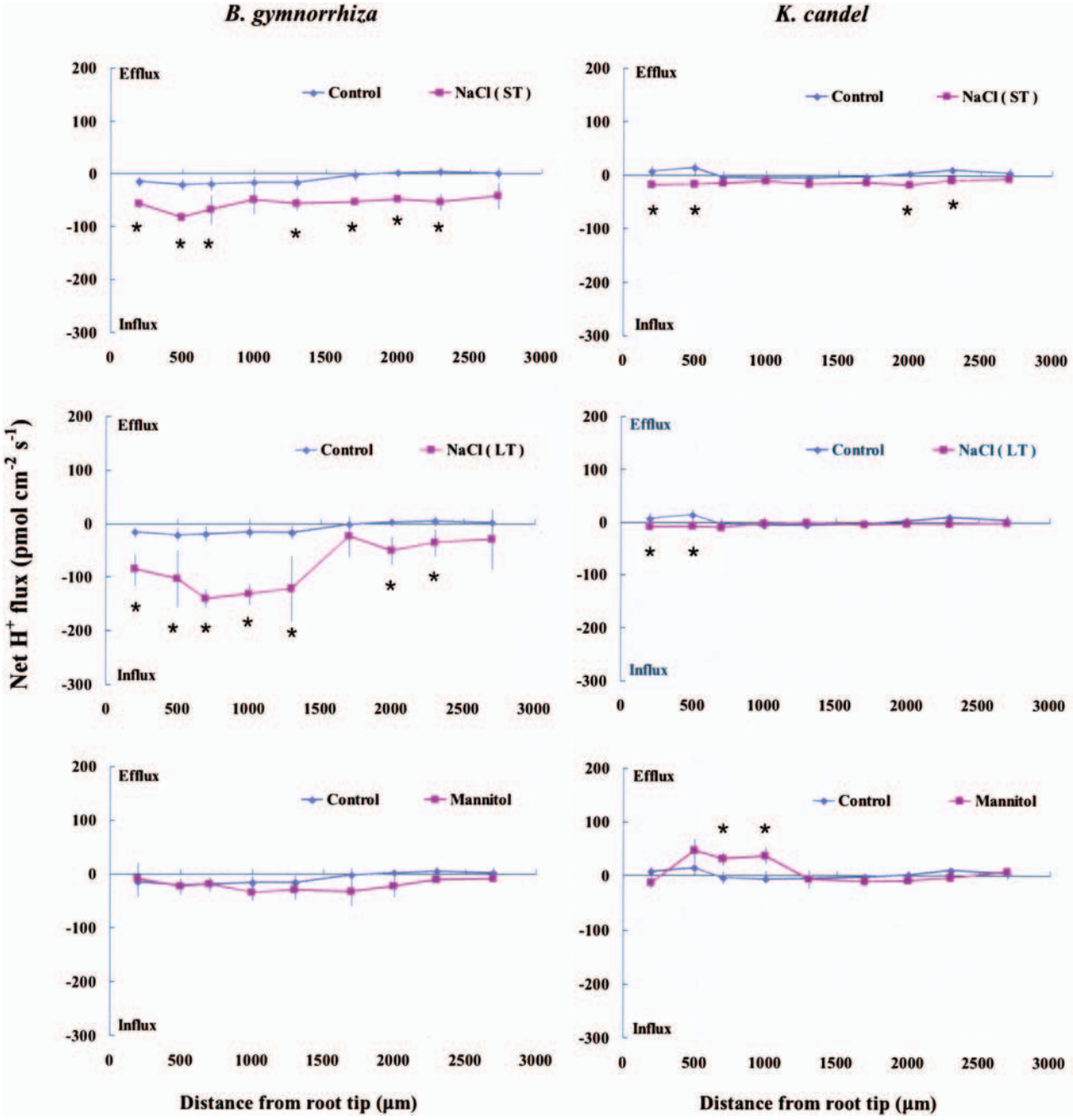

Figure 3. Effects of ST salinity (100 mM NaCl, 24 h), LT salinity (200 mM NaCl, 7 days) and hyperosmotic stress (170 mM mannitol, 24 h) on net H^+ fluxes in roots of *B. gymnorrhiza* and *K. candel*. Control roots were well fertilized but treated without NaCl or mannitol. Each point is the mean of five to six individual plants, and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

compared with *K. candel* (920 pmol cm^{-2} s^{-1}) (Figure 6). The enhancement effect of $CaCl_2$ (10 mM) on Na^+ efflux was found in salinized *B. gymnorrhiza* but absent in *K. candel* (Figure 6). Application of SNP (100 μM) increased Na^+ efflux in the two mangrove species, but Na^+ fluxes in roots varied within the apical zone and elongation region (Figure 6). SNP induced a net Na^+ efflux (696–1104 pmol cm^{-2} s^{-1}) at the region of 200–700 μm (apical zone), but a less pronounced effect was seen at the region of 1000–2700 μm (elongation zone) (Figure 6).

K^+ fluxes

H_2O_2 (10 mM) and $CaCl_2$ (10 mM) markedly reduced K^+ efflux in NaCl-stressed roots of *B. gymnorrhiza* and *K. candel* (Figure 7). However, the response of K^+ flux to SNP varied between

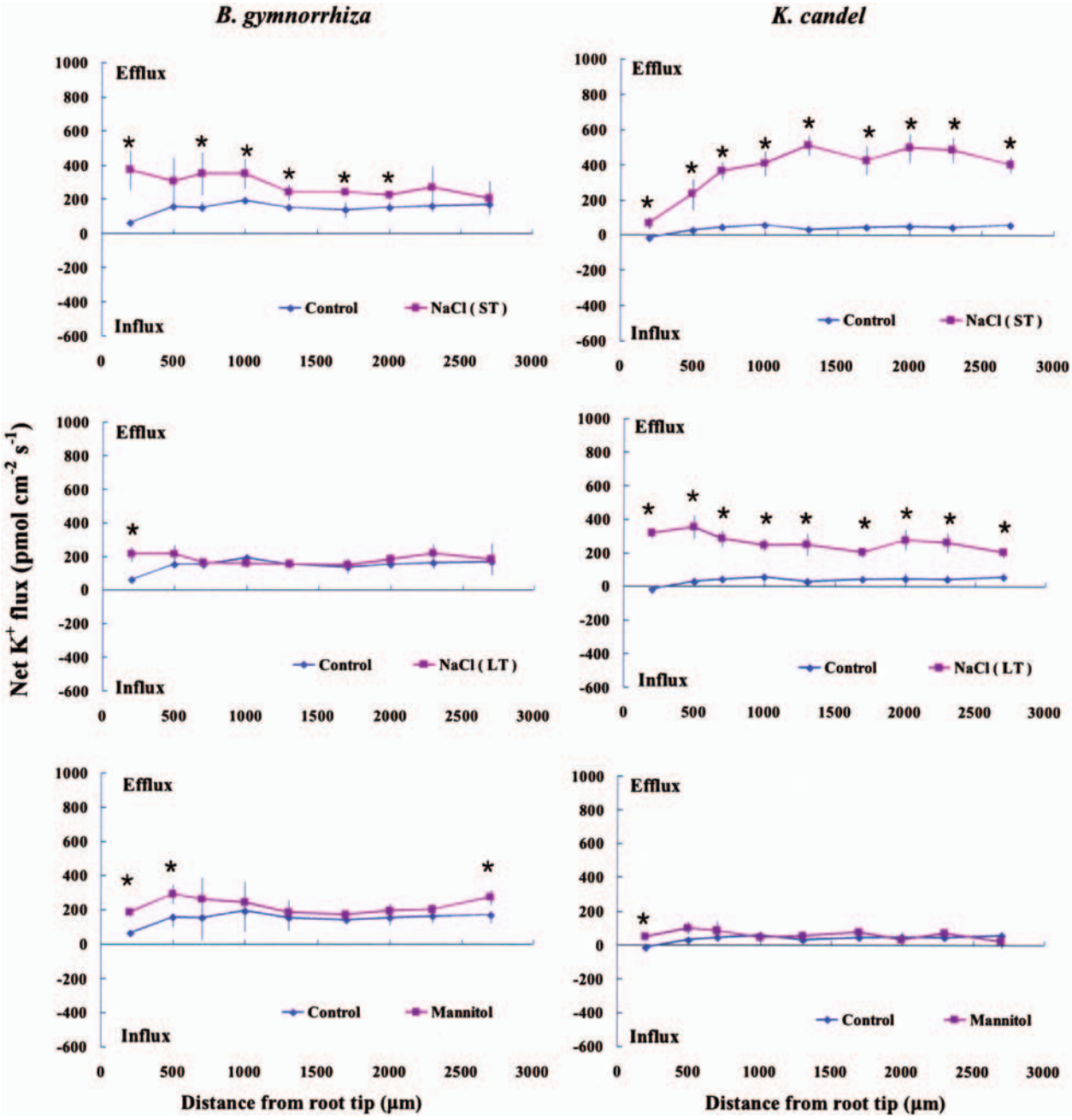

Figure 4. Effects of ST salinity (100 mM NaCl, 24 h), LT salinity (200 mM NaCl, 7 days) and hyperosmotic stress (170 mM mannitol, 24 h) on net K^+ fluxes in roots of *B. gymnorrhiza* and *K. candel*. Control roots were well fertilized but treated without NaCl or mannitol. Each point is the mean of five to six individual plants, and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

the two species. SNP significantly decreased the K^+ efflux in salt-treated roots of *K. candel*, whereas there were no corresponding changes in salinized *B. gymnorrhiza* (Figure 7).

Ca^{2+} fluxes

Short-term treatment caused a net Ca^{2+} influx in *B. gymnorrhiza* roots, ranging from 34 to 354 pmol cm^{-2} s^{-1} at the region of 200–2000 μm from the apex (Figure 8). The salt-induced Ca^{2+} influx was enhanced by H_2O_2 (10 mM); its mean flux rate increased by approximately threefold (Figure 8). However, the NaCl-induced Ca^{2+} influx was much less in *K. candel*, and the enhancement effect of H_2O_2 on Ca^{2+} influx was not significant in salinized *K. candel* (Figure 8).

Discussion

Under natural conditions, *B. gymnorrhiza* is a front line species and mostly occurs in high-saline zones compared with *K. candel*,

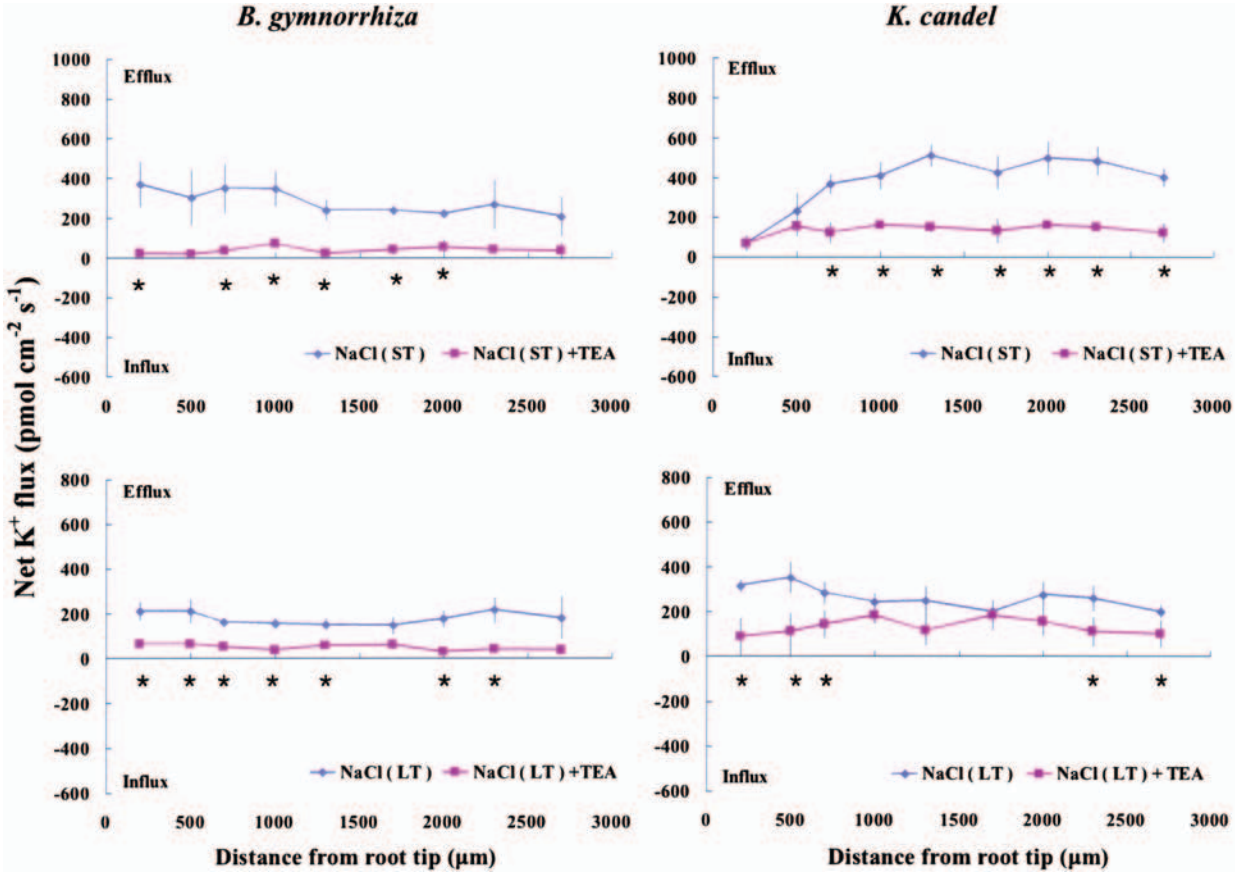

Figure 5. Effects of TEA (50 μM) on net K^+ fluxes in ST- and LT-stressed roots of *B. gymnorrhiza* and *K. candel*. Short-term- and LT-stressed roots were pretreated with 50 μM TEA for 30 min prior to flux measurements. For NaCl controls, roots were not subjected to inhibitor treatment. Each point is the mean of five to six individual plants, and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

which grows in low-saline creeks in mangrove areas. Tissue ion analysis showed that *B. gymnorrhiza* exhibited better salt management compared with *K. candel*, which is consistent with findings from our previous study showing that *B. gymnorrhiza* is more effective than *K. candel* at salt exclusion under high salinity (Li et al. 2008). We also note that salinized *B. gymnorrhiza* plants retained a higher capacity to reduce the loss of K^+ compared with *K. candel*. These findings are similar to previous results showing that K^+ concentration in the *B. sexangula* callus decreases slightly and remains relatively high in the presence of 300 mM NaCl (Mimura et al. 1997). Our flux data indicate that the ability to maintain K^+/Na^+ homeostasis in *B. gymnorrhiza* roots accounted for the greater Na^+ extrusion and the lower K^+ efflux under NaCl stress in this species.

B. gymnorrhiza roots exhibited a more pronounced Na^+ efflux along the root axis under ST and LT treatments. Similarly, a salt-resistant poplar, *Populus euphratica* Oliver, shows a net Na^+ efflux in NaCl-stressed roots (Sun et al. 2009*a*, 2009*b*). The two mangrove species also manifested a net H^+ influx corresponding to the Na^+ efflux under ST and LT salinity. NaCl-induced H^+ influx has been observed in the root apex of a wild-type *Arabidopsis* (Shabala et al. 2005) and *P. euphratica* (Sun et al. 2009*a*). Under ST treatment, the PM transport inhibitor amiloride (an inhibitor of the Na^+/H^+ antiporter) simultaneously decreased Na^+ efflux and H^+ influx along the mangrove roots, especially in *B. gymnorrhiza*. Given these results, we conclude that the Na^+ efflux in roots of the two species mainly resulted from an active Na^+/H^+ antiport at the PM (Sun et al. 2009*a*, 2009*b*). Of note, salt-stressed *B. gymnorrhiza* showed a higher rate of Na^+/H^+ exchange, suggesting that its PM Na^+/H^+ antiport system (H^+-ATPase and Na^+/H^+ antiporters) is more effective than that of *K. candel* for Na^+ extrusion, especially under LT conditions. In accordance with this inference, the efflux of Na^+ in *B. gymnorrhiza* roots was correspondingly reduced when H^+ influx was restricted by sodium orthovanadate (an inhibitor of the PM H^+-ATPase). The strict correlation between Na^+ efflux and H^+ influx indicates that the Na^+ extrusion in *B. gymnorrhiza* is highly dependent on the PM H^+-ATPase, which pumps protons to promote the secondary active Na^+/H^+ antiport at the PM (Blumwald et al. 2000, Zhu 2003). H_2O_2, NO and Ca^{2+} enhanced Na^+ extrusion in the two

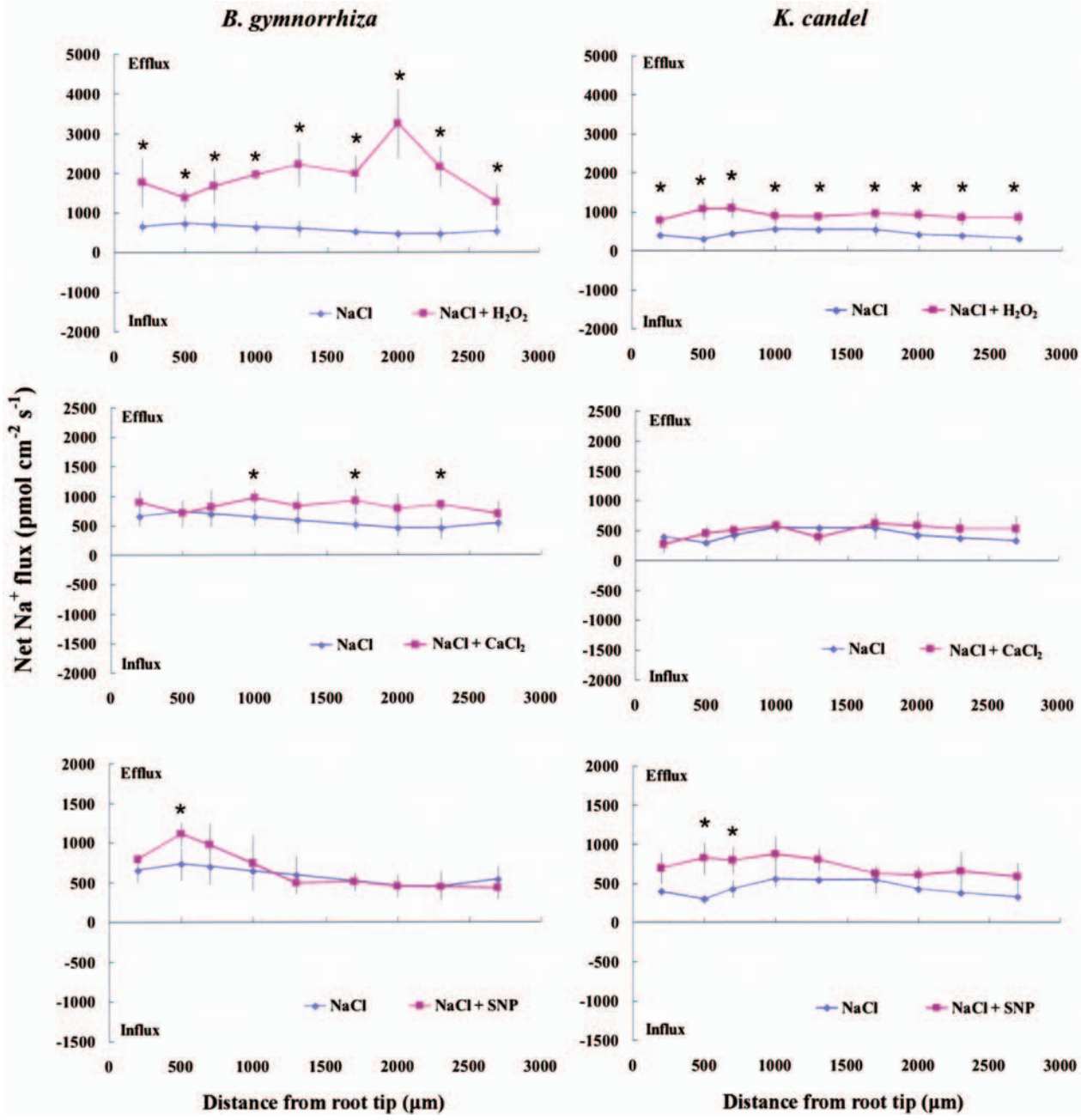

Figure 6. Effects of H_2O_2 (10 mM), $CaCl_2$ (10 mM) and SNP (100 μM) on net Na^+ fluxes in ST-stressed roots of *B. gymnorrhiza* and *K. candel*. Roots were subjected to 100 mM NaCl for 24 h supplemented with 10 mM H_2O_2, 10 mM $CaCl_2$ or 100 μM SNP, respectively. For NaCl controls, roots were subjected to salt treatment but without the addition of H_2O_2, $CaCl_2$ and SNP. Each point is the mean of five to six individual plants, and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

mangrove species, presumably via modulations of H^+-ATPase and the Na^+/H^+ antiporter in the PM. Exogenous H_2O_2 mediates *SOS1* mRNA stability in *Arabidopsis* (Chung et al. 2008) and increases the activity of PM H^+-ATPase in NaCl-stressed calluses of *P. euphratica* (Zhang et al. 2007). In a secretor mangrove, *A. marina*, NO enhances salt secretion and Na^+ sequestration through increasing the expression of H^+-ATPase and the Na^+/H^+ antiporter under high salinity (Chen et al. 2010). Ca^{2+} not only restricts the entry of Na^+ through permeable NSCCs (non-selective cation channels; Demidchik and Tester 2002, Tester and Davenport 2003, Demidchik and Maathuis 2007) but also activates the Na^+/H^+ antiport in *Arabidopsis* via the SOS pathway (Zhu 2003). Noteworthy, NaCl induced a net Ca^{2+} influx in *B. gymnorrhiza*, and the influx was markedly strengthened by exogenous H_2O_2. Mori and Schroeder (2004) suggest that reactive oxygen species activate hyperpolarization-dependent Ca^{2+}-permeable cation channels in the PM. Therefore, the greater capacity for

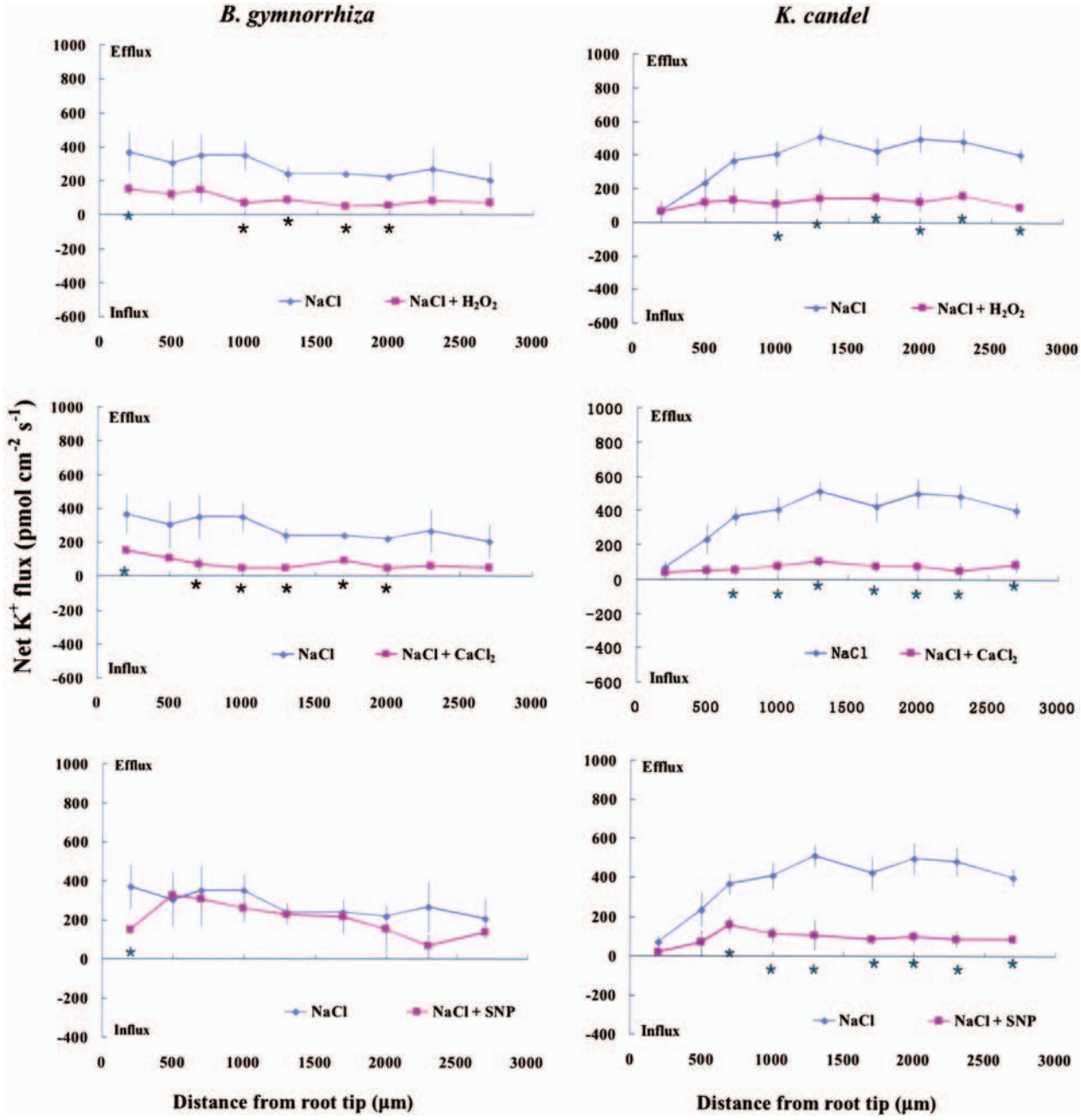

Figure 7. Effects of H_2O_2 (10 mM), $CaCl_2$ (10 mM) and SNP (100 μM) on net K^+ fluxes in ST-stressed roots of *B. gymnorrhiza* and *K. candel*. Roots were subjected to 100 mM NaCl for 24 h supplemented with 10 mM H_2O_2, 10 mM $CaCl_2$ or 100 μM SNP, respectively. For NaCl controls, roots were subjected to salt treatment but without addition of H_2O_2, $CaCl_2$ and SNP. Each point is the mean of five to six individual plants, and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

Na^+/H^+ antiport in *B. gymnorrhiza* is related to the salt-induced Ca^{2+} influx, which presumably increases Ca^{2+} concentration in the cytosol, thus mediating the exchange of Na^+ with H^+ at the PM.

The ability to sustain K^+ is crucial for plant salt adaptation and can be used for salt tolerance screening (Chen et al. 2005, Cuin et al. 2008). NaCl treatments (ST and LT) caused an evident K^+ efflux in the two species, which was significantly reduced by the K^+ channel blocker TEA. This result indicates that salt-induced K^+ efflux is mediated by the depolarization-activated channels, e.g., KORCs and NSCCs (Shabala et al. 2005, 2006, Shabala and Cuin 2008). *Bruguiera gymnorrhiza* roots exhibited a typically lower K^+ efflux than *K. candel* under ST and LT salinity. The capacity for *B. gymnorrhiza* to retain K^+ is likely the result of its high activity of PM H^+-ATPase, one component of the PM Na^+/H^+ antiport system. Our previous studies in *P. euphratica* have shown that salt-induced K^+ efflux depends on the membrane potential, which relies on the

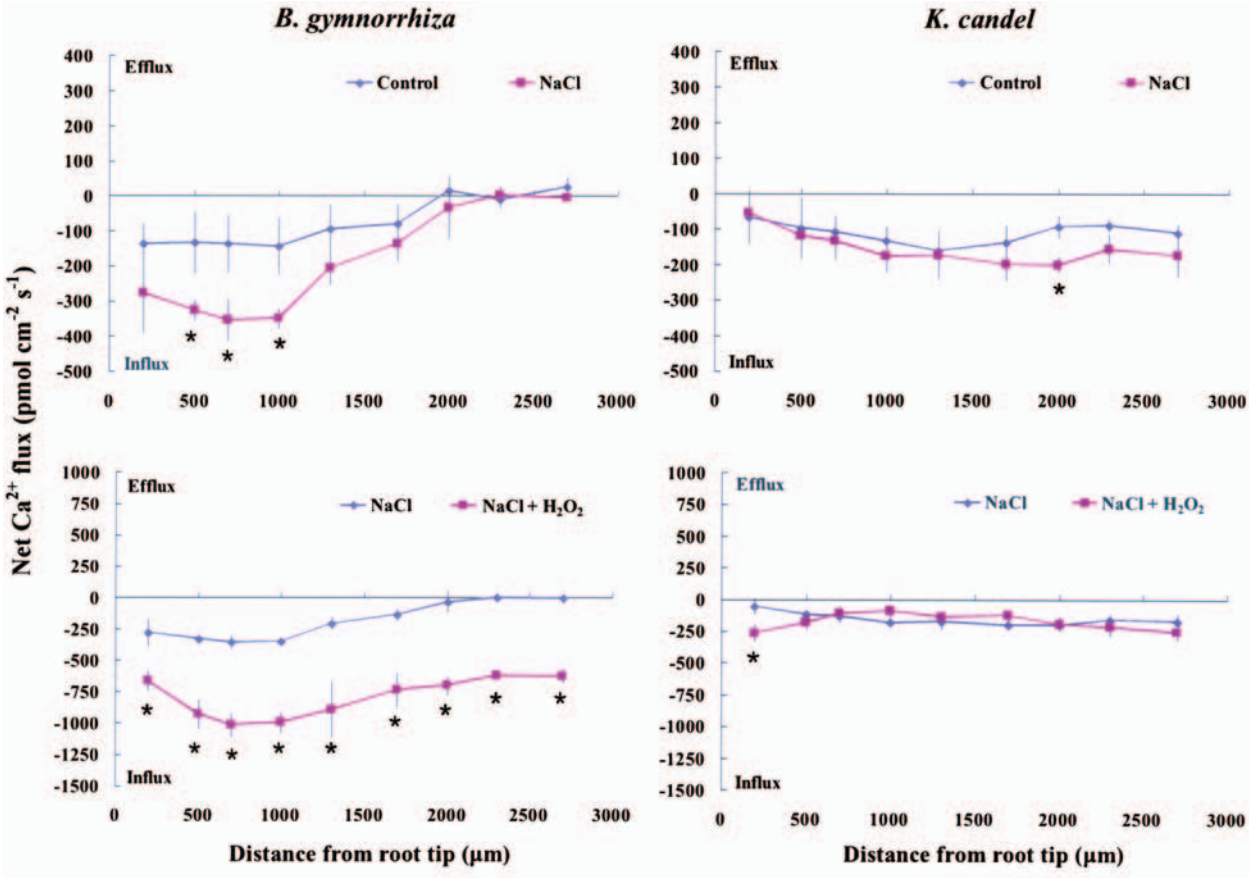

Figure 8. Effects of ST salinity (100 mM NaCl, 24 h) and H_2O_2 (10 mM) on net Ca^{2+} fluxes in roots of *B. gymnorrhiza* and *K. candel*. Control roots were well fertilized but treated without NaCl. For H_2O_2 treatment, roots were subjected to 100 mM NaCl for 24 h supplemented with or without 10 mM H_2O_2. Each point is the mean of five to six individual plants, and bars represent the standard error of the mean. Asterisks denote significant difference at $P < 0.05$ between treatments.

activity of the PM pumps (Sun et al. 2009*b*, 2010*b*). Up-regulation of PM H^+-ATPase can reduce NaCl-induced depolarization of the membrane potential, leading to a small K^+ loss through DA-KORCs and DA-NSCCs (Chen et al. 2007, Shabala and Cuin 2008). H_2O_2, NO and Ca^{2+} markedly reduced K^+ efflux in NaCl-stressed roots of the two mangrove species, although the response of K^+ flux to SNP was not evident in *B. gymnorrhiza*. In addition to the Ca^{2+}-reduced K^+ loss through NSCCs and KORCs under salt stress (Shabala 2000, Demidchik and Tester 2002, Shabala et al. 2006, Sun et al. 2009*b*), the reduction of salt-induced K^+ efflux is likely the result of the less depolarized membrane potential maintained by the activated PM H^+-ATPase (Zhang et al. 2007, Chen et al. 2010).

Ion fluxes upon salt stress differ from the response to hyperosmotic stress in *B. gymnorrhiza* roots. NaCl-induced Na^+, K^+ and H^+ fluxes were not seen in the hyperosmotic stress condition. These results indicate that ionic responses of roots to a hyperosmotic treatment were highly stress specific in this mangrove species (Shabala 2000, Sun et al. 2009*a*). However, for *K. candel*, the isotonic mannitol treatment caused Na^+ efflux along the root axis, which is similar to the response with the NaCl treatment. This result implies that the salt-induced Na^+ efflux in *K. candel* is partly attributable to osmotic stress. There was no H^+ influx corresponding to the Na^+ efflux in *K. candel*, suggesting that hyperosmotic stress-accelerated Na^+ efflux is not likely an active extrusion across the PM. Accordingly, our results indicate marked differences between the two species in mediating ion fluxes under osmotic stress.

In conclusion, salinized *B. gymnorrhiza* seedlings exhibited a greater capacity to maintain K^+/Na^+ homeostasis than *K. candel*, which is accounted for by a greater Na^+ extrusion and lower K^+ efflux in *B. gymnorrhiza* roots. Under NaCl stress, H_2O_2, NO and Ca^{2+} mediated K^+ flux and Na^+/H^+ antiport across the PM, thus contributing to ionic homeostasis control in the two mangrove species tested. Therefore, the high influx of Ca^{2+} that NaCl treatment induced may benefit *B. gymnorrhiza* in regulating the K^+/Na^+ balance under saline conditions.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

Funding

The research was supported jointly by Fundamental Research Funds for the Central Universities (JC2011-2), Foundation for the Supervisors of Beijing Excellent Doctoral Dissertations (YB20081002201), National Natural Science Foundation of China (31170570, 31160150, 31270654), Beijing Natural Science Foundation (6112017), the Program of Introducing Talents of Discipline to Universities (111 Project, B13007) and Key Projects of the Ministry of Education, PR China (209084).

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