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Salt-Stress-Induced Ion Transport Contributes to K^+/Na^+ Homeostasis in Roots of Ping'ou Hybrid Hazelnut

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Abstract: Soil salinity is a worldwide problem that adversely affects plant growth and development. Soil salinization in Xinjiang of China is very serious. Ping'ou hybrid hazelnut, as an important ecological and economic tree species, as well as a salt-tolerant plant, has been grown in Xinjiang for over 20 years. Understanding the salt-tolerance mechanism of Ping'ou hybrid hazelnut is of great significance for the breeding of salt-tolerant varieties and the rational utilization of salinized land. In this study, 'Liaozhen 7', a fine variety of Ping'ou hybrid hazelnut, was selected as test material, and seedlings were treated with 0 (control), 50, 100 and 200 mM NaCl. Subsequently, the pattern of NaCl-induced fluxes of Na^+, K^+ and H^+ in the root meristematic zone and their response to ion transport inhibitors were studied using non-invasive micro-test technology (NMT). Different concentrations of NaCl stress significantly increased the Na^+ concentration in roots, while K^+ concentration decreased first and then increased with the increase of NaCl concentration. Meanwhile, NaCl stress induced a significant decline in K^+/Na^+ ratio. Control and 200 mM NaCl-induced Na^+ and K^+ fluxes in roots exhibited an outward efflux, whereas an inward flux was observed for H^+. Under 200 mM NaCl stress, the average rates of net Na^+ and K^+ efflux, as well as H^+ influx in roots were significantly increased, which were 11.6, 6.7 and 2.3 times higher than that of control, respectively. Furthermore, pharmacological experiments showed that 200 mM NaCl-induced Na^+ efflux; H^+ influx was significantly suppressed by amiloride, an inhibitor of plasma membrane (PM) Na^+/H^+ antiporter, and sodium vanadate, an inhibitor of PM H^+-ATPase. Net Na^+ efflux and H^+ influx induced by NaCl decreased by 89.9% and 135.0%, respectively. The NaCl-induced Na^+ efflux was mediated by a Na^+/H^+ antiporter using energy provided by PM H^+-ATPase. The NaCl-induced K^+ efflux was significantly restricted by tetraethylamine chloride, a K^+ channel inhibitor, and promoted by sodium vanadate, which decreased by 111.2% and increased by 80.8%, respectively, indicating that K^+ efflux was regulated by depolarization-activated outward-rectifying K^+ channels and non-selective cation channels (NSCCs). In conclusion, NMT data revealed that NaCl stress up regulated the root Na^+/H^+ antiporter and H^+ pump (an activity of PM Na^+/H^+ antiport system) of 'Liaozhen 7', which compelled the Na^+/H^+ exchange across the PM and restricted K^+ loss via depolarization-activated K^+ channels and NSCCs simultaneously, thereby maintaining the K^+/Na^+ homeostasis and higher salt tolerance.

Keywords: K^+/Na^+ homeostasis; ion transport; NaCl stress; inhibitor; Ping'ou hybrid hazelnut

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1. Introduction

Soil salinity is a major disaster in nature, and is one of the most serious abiotic stresses threatening plant growth and development [1,2]. Nearly 10% of the world's total land and at least 20% of its irrigated land is affected by salinity [3]; this proportion is still increasing due to global climate change, land clearing and excessive irrigation [4,5]. Soil salinity affects

various stages of plant growth and development (such as seed germination, growth, flowering, fruiting, etc.), as well as various processes of physiological metabolism (such as water transport, photosynthesis, osmotic regulation, enzyme activity, etc.), resulting in a decrease in crop productivity and quality, and in severe cases, even no harvest at all [6–9]. In China, soil salinity is particularly prominent. The salinized area is approximately 1.0×10^8 ha, accounting for 10.3% of the country's total land, mainly located in the northwest, northeast, central-north and eastern coastal areas [3].

When plants are subjected to high saline conditions, external Na^+ establishes a large electrochemical gradient, which is conducive to the passive entry of salt ions through various ion channels or transport proteins in the plasma membrane (PM) [10–12]. As a consequence of salt-stress exposure, excessive Na^+ enters plant cells, leading to cell membrane depolarization and K^+ extrusion from the cytosol, affecting the ion homeostasis and ultimately causing secondary stress [13,14]. Accordingly, sustaining low cytosolic salt concentration is important for the salt adaptation of plants [1,15]. Plants only need to maintain high K^+ and low Na^+ levels in the cytosol to prevent salt damage [16]. The K^+/Na^+ ratio and K^+ levels are considered to be the key factors associated with the salt tolerance of plants [17,18]. Plants have evolved various mechanisms for extruding Na^+ out of cells and organs to maintain their Na^+ homeostasis during salt stress [19]. Upholding a suitable cellular ion homeostasis requires adequately coordinated adjustments of Na^+ uptake, sequestration and extrusion [7,20,21]. Excluding the absorbed Na^+ is among the most important actions [1,3].

Na^+ extrusion to the apoplast or external environment is essential for maintaining ion homeostasis in the cytosol [14]. Na^+/H^+ antiporters in the PM are widely believed to play a crucial role in active Na^+ extrusion under saline environments. The activity of PM Na^+/H^+ antiporter induced by NaCl stress has been reported in many studies. For example, under 100 mM NaCl stress, the activity of PM Na^+/H^+ antiport system in Mangrove (*Bruguiera gymnorrhiza*) roots was significantly increased and therefore decreased the Na^+ accumulation [22]. Zhu et al. [23] also found that the level of Na^+/H^+ antiport system in the PM of Ningxia Wolfberry (*Lycium barbarum*) roots was significantly up regulated after NaCl stress, playing a positive role in plant adaptation to salt stress. However, the Na^+/H^+ antiporters in the PM depend on electrochemical H^+ gradients, which are generated by PM H^+-ATPase [24]. A non-invasive electrophysiological study showed the involvement of PM H^+-ATPase in the Na^+/H^+ antiport according to H^+ kinetics on salt shock [17,25]. Therefore, the H^+ pumping induced by NaCl stress is the foundation of Na^+/H^+ exchange and salt tolerance. In addition, external NaCl stress usually induces PM depolarization, and activates outward-rectifying K^+ channels or non-selective cation channels (NSCCs) and finally leads to K^+ extrusion. Despite all this, plants can inhibit K^+ extrusion through PM repolarization [12]. Lang et al. [22] observed that salt-stress-induced K^+ efflux in *B. gymnorrhiza* was decreased through inhibiting the depolarization-activated K^+ channels.

Hazelnut (*Corylus*), a member of the Corylaceae family, is one of the most important nut crops and a woody oil plant, widely favored by people because of its extremely high nutritional value. Hazelnut fruit is an energy-rich food that plays a key role in the diet due to its rich content of lipids, proteins, carbohydrates, vitamins, dietary fibers, antioxidant phenolics and Ca, P, K, Fe, etc. [26,27]. Ping'ou hybrid hazelnut (*Corylus heterophylla* × *Corylus avellana*) is a cultivated species independently bred in China, with strong stress resistance, simple management, high productivity and high commercial value [28]. As an important ecological and economic tree species, as well as a salt-tolerant plant, Ping'ou hybrid hazelnut has been grown in Xinjiang since 2001. The fine varieties of Ping'ou hybrid hazelnut mainly include 'Dawei', 'Yuzhui', 'Liaozhen 3', 'Liaozhen 7' and 'Ping'ou 15', etc. After more than 20 years of development, it has achieved large-scale planting and industrial development. However, the ecological environment in Xinjiang is very harsh, especially the serious problem of soil salinization. According to statistics of soil survey in Xinjiang, the salinized area is 2.33×10^6 ha, accounting for 37.72% of the cultivated land in the irrigated area [29]. Among them, the area of mild salinity (3–6 $g \cdot kg^{-1}$) is 1.78×10^6 ha, accounting

for 28.86%; moderate salinity (6–10 g·kg^{-1}) is 4.77 × 10^5 ha, accounting for 7.72% and severe salinity (10–20 g·kg^{-1}) is 7.02 × 10^4 ha, accounting for 1.13% [29]. Utilization of these saline soils for agricultural and forestry production could mitigate the issues of food and wood demand, and improve the ecological environment caused by the ever-increasing human population [30].

Our previous studies have shown that under NaCl stress, Ping'ou hybrid hazelnut can adapt to the saline environment through adjusting biomass allocation, reshaping leaf anatomical structure and root configuration, as well as altering photosynthetic characteristics [31–33]. Moreover, based on the analysis of ion distribution characteristics, the NaCl-induced Na^+ levels in the leaf and stem tissues of different Ping'ou hybrid hazelnut varieties were substantially higher than in the roots. Furthermore, compared with 'Dawei' and 'Yuzhui', NaCl stress-induced Na^+ concentration in the root, stem and leaf tissues of 'Liaozhen 7' was significantly decreased, whereas K^+ concentration was obviously increased. Meanwhile, the K^+/Na^+ ratio in 'Liaozhen 7' tissues was consistently higher than the other two varieties [34]. These findings prompted us to raise the following questions: Does 'Liaozhen 7' have a high capacity to exclude Na^+ and retain K^+? What is the potential mechanism for maintaining K^+/Na^+ homeostasis, thereby enabling it to adapt to saline conditions? Therefore, this needs further investigation, e.g., by electrophysiology, to clarify. To address these questions, the 'Liaozhen 7' seedlings were selected as test materials, and non-invasive micro-test technology (NMT) was used to detect the NaCl-induced Na^+, K^+ and H^+ flux profiles in roots. Moreover, we examined the effects of ion transport inhibitors on root Na^+, K^+ and H^+ fluxes under NaCl stress. The aim was to compare the alternations of root ion flux profiles after NaCl and inhibitor treatments and to explore the salt-tolerance mechanism of 'Liaozhen 7' by studying the ion dynamic transport and K^+/Na^+ homeostasis maintenance.

2. Materials and Methods

2.1. Test Materials and Hydroponic Culture

'Liaozhen 7', a fine variety of Ping'ou hybrid hazelnut, was used as test material. In April 2021, two-year-old clonal seedlings were collected from Chabuchaer County, Yili, Xinjiang Uygur Autonomous Region, China, and were then planted in individual plastic pots containing vermiculite and placed in a greenhouse at Xinjiang Academy of Forestry Science. Potted seedlings were well irrigated according to evaporation demand. Seedlings were raised three months prior to the beginning of hydroponic culture. In July 2021, uniform seedlings were washed free of vermiculite and transferred to cylindrical PVC buckets (diameter × height: 15 cm × 20 cm) containing half-strength Hoagland nutrient solution. Seedlings were continuously aerated by passing air through the solution. The seedlings were grown in the greenhouse at a temperature of 26 °C to 28 °C and a light cycle of 14 h/10 h (light/dark), with a light intensity of 75 μmol·m^{-2}·s^{-1}. Nutrient solutions were renewed every week and seedlings were raised for 30 d under hydroponic conditions prior to salt-stress treatment.

2.2. Salt Treatment and Sampling

The experiment was laid out as a completely randomized design. According to the results of our previous studies [31–34] and Zhang et al. [35], four treatments were applied to the seedlings in this study: 0 (control), 50, 100 and 200 mM NaCl. The required amounts of NaCl were added to the Hoagland nutrient solution. Control plants were cultivated in nutrient solutions without NaCl, while stressed plants were well cultivated with nutrient solutions containing corresponding NaCl concentrations. Each treatment level included five replicates, and each replicate contained 15 seedlings. After 24 h treatment, young roots were collected from each seedling in each replicate. Roots collected from each replicate were mixed to obtain one homogeneous and representative sample. All samples were then immediately transported to the laboratory in duplicate. One was used for analysis of Na^+ and K^+ concentrations, and the other was prepared for measurement of Na^+, K^+ and H^+

fluxes. Based on the analysis results of growth and physiological characteristics [31,32], ion fluxes were only detected for control and 200 mM NaCl stressed plants.

2.3. Ion Concentration Analysis

Na^+ and K^+ concentrations were analyzed according to the method described previously by Tang et al. [36]. Briefly, in the laboratory, the root samples were rinsed three times with deionized water to remove any contaminants. The samples were dried in an oven at 105 °C for 20 min and then at 80 °C until a constant dry weight. After grinding into powder, 0.1 g sample was accurately weighed and digested in a concentrated acid mixture of 10 mL HNO_3 and 1 mL $HClO_4$ at 115 °C for 3 h. Then the digested solution was evaporated to near dryness. The residues were dissolved in 0.5 mL HNO_3, and finally a clear solution was diluted to 5 mL with deionized water. Na^+ and K^+ concentrations were analyzed by inductively coupled plasma-optical emission spectrometer (ICP-OES).

2.4. Non-Invasive Micro-Test Technology

Net ion fluxes (Na^+, K^+ and H^+ for this study) in roots of 'Liaozhen 7' were detected using non-invasive micro-test technology (NMT, NMT100 Series, Younger USA LLC, Amherst, MA, USA; Xuyue Sci. & Tech. Co., Ltd., Beijing, China) as described previously [3,37]. Prepulled and silanized glass micropipettes (5 ± 1 μm, XY-DJ-01; Younger USA) were first filled with a backfilling solution (K^+, 100 mM KCl; Na^+, 250 mM NaCl; H^+, 15 mM NaCl and 40 mM KH_2PO_4, pH 7.0). The micropipettes were front filled with 40–180 μm (H^+, 40–50 μm; K^+, 180 μm) columns of selective liquid ion-exchange (LIX) cocktails (Na^+ LIX, XY-SJ-Na; K^+ LIX, XY-SJ-K; H^+ LIX, XY-SJ-H; Younger USA). An Ag/AgCl wire electrode holder (XY-DJGD; Younger USA) was inserted into the back of the electrode to make an electrical contact with the electrolyte solution. An electrode (YG003-Y05; Younger USA) was used as the reference electrode. Ion-selective electrodes of the target ions were calibrated prior to flux measurements. Only electrodes with a Nernstian slope > 53 mV per decade (Na^+, K^+, H^+) were used in this study. The ionic fluxes were calculated based on Fick's law of diffusion:

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

where J represents the ion flux (unit: picomoles·cm^{-2}·s^{-1}), dc is the ion concentration gradient, dx is the distance over which the microelectrode moved between two positions above the surface of the specimen and D is the ion diffusion constant. The frequency of the movement was 0.3 Hz, and the travel range was 30 μm (between 5 and 35 μm from the specimen surface). The direction of the flux was derived from Fick's law of diffusion (a positive value represents efflux, and a negative value represents influx).

2.5. Measurements of Ion Flux under Salt Stress

Roots sampled from the control and 200 mM stressed plants were washed 5~6 times with deionized water, and root tips (3~4 cm) were obtained. The root tips then were equilibrated in basic measuring solution (0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 0.3 mM MES, 0.2 mM Na_2SO_4, pH 6.0) for 30 min. After equilibration, the root tips were immobilized at the bottom of a measuring chamber containing 10 mL fresh measuring solution. The dynamic changes of root Na^+, K^+ and H^+ fluxes were detected at the meristematic zone (about 200 μm from the apex) as determined previously [33]. The recording rate for the ion flux was one reading per 6 s. A continuous flux recording was performed at least 8 min with Na^+, K^+ and H^+ selective flux microsensor to ensure steady-state flux values. All of the measurements were repeated for 5 samples (roots) independently. The ion flux rates were expressed as the means of the measured positions of 5 individual roots.

2.6. Measurements of Ion Flux under Transport Inhibitor (Pharmacological Experiments)

Before testing, 4 basic measuring solutions were prepared in advance. Among them, the first one was added with 0.1 mM amiloride (a Na^+/H^+ antiporter inhibitor), the second

was added with 20 mM tetraethylammonium chloride (TEA, a K^+ channel inhibitor), the third was added with 0.5 mM sodium vanadate (a PM H^+-ATPase inhibitor) and the remaining one was used as the basic measuring solution.

Roots were washed 5~6 times with deionized water, and root tips (3~4 cm) were obtained and divided into 4 parts. Subsequently, the root tips were equilibrated in the basic measuring solution and the measuring solution containing different transporter inhibitors for 30 min. Finally, the dynamic changes of Na^+, K^+ and H^+ fluxes were measured at the meristematic zone. Continuous recording was taken at least 8 min with different ion selective flux microsensor. All of the measurements were repeated for 5 samples (roots) independently.

2.7. Statistical Analysis

The measured ion flux data were imported into Microsoft Excel 2007, and then the flux values were calculated using Mageflux software 1.0 (Younger USA Sci. & Tech. Co., Ltd., Amherst, MA, USA). The t-test was used to compare the differences of ion fluxes between control and salt stress. To test if ion fluxes of Na^+, K^+ and H^+ varied with different ion transporter inhibitors, we conducted one-way analysis of variance (ANOVA), followed by Duncan's multiple-range tests with inhibitor as a fixed factor and each attribute of ion fluxes as responses. Additionally, Pearson correlation analysis was applied to test the correlation among Na^+, K^+ and H^+ fluxes. All statistical tests were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Data were tested for normality prior to the statistical analysis. Differences between means were considered to be significant when the p value was less than 0.05. Figures were generated using OriginPro 9.0 (OriginLab Inc., Northampton, MA, USA) and SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA). The data are presented as the mean ± standard error (SE).

3. Results

3.1. NaCl-Induced Ion Cncentration

As shown in Figure 1A, NaCl stress significantly increased the root Na^+ concentration, but no significant difference was found between 50 and 100 mM NaCl treatments. The concentration of Na^+ under 50, 100 and 200 mM NaCl stress was 1.06-, 1.29- and 2.34-fold higher than that of the control, respectively. The root K^+ concentration under 50 and 100 mM NaCl treatments decreased markedly, being 77.9% and 69.6% of the control, respectively, but later showed an obvious increase after 200 mM NaCl treatment (Figure 1B). NaCl stress induced a significant decline in the K^+/Na^+ ratio. Compared with the control, 50, 100 and 200 mM NaCl stress decreased root K^+/Na^+ ratio by 64.1%, 71.7% and 74.4%, respectively, and the K^+/Na^+ ratio was maintained at a relatively stable level under 100 and 200 mM NaCl stress (Figure 1C).

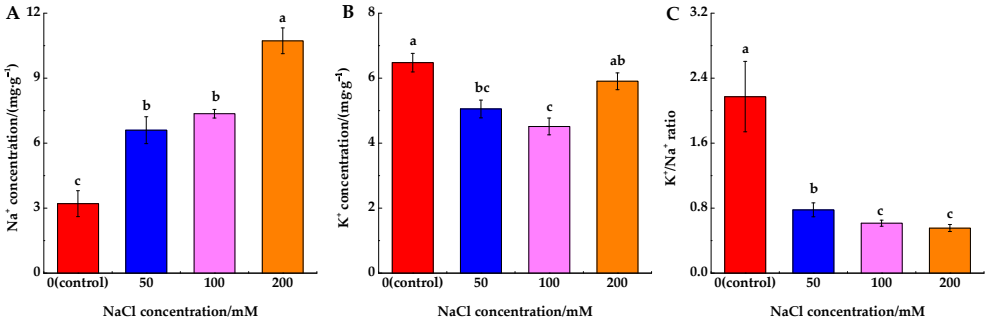

Figure 1. Na^+ (**A**), K^+ (**B**) and K^+/Na^+ ratio (**C**) in roots of 'Liaozhen 7' under NaCl stress. Each column is the mean of five replicates, and bars represent the standard error of the mean. Columns labeled with different lowercase letters indicate significant difference at $p < 0.05$.

3.2. NaCl-Induced Ion Fluxes

3.2.1. Na^+ Fluxes

As shown in Figure 2A, the Na^+ flux was measured at meristematic zone of 'Liaozhen 7' roots. A Na^+ efflux was observed in roots under the control and 200 mM NaCl stress. With the prolongation of scanning time, the Na^+ efflux decreased gradually under the control, while it increased in fluctuation under the 200 mM NaCl stress. According to the average flux rates within 0−8 min (Figure 2B), the net Na^+ efflux under the 200 mM NaCl stress (1893.15 pmol·cm^{-2}·s^{-1}) was 11.6 times higher than that of control (163.20 pmol·cm^{-2}·s^{-1}), indicating that NaCl stress significantly induced Na^+ efflux.

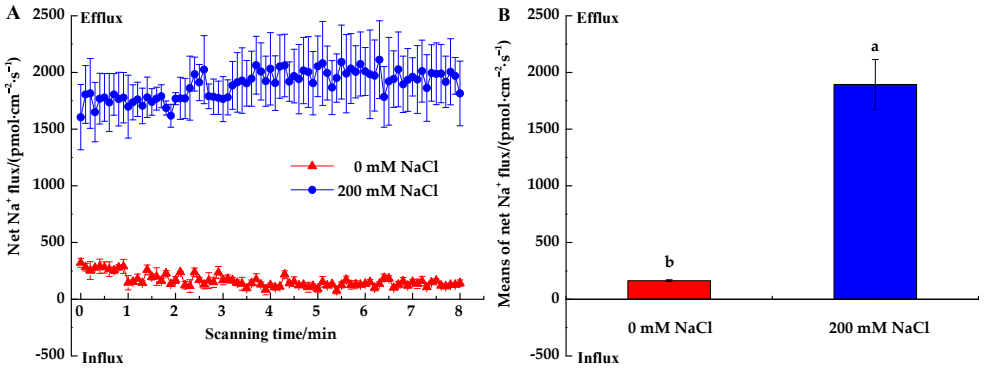

Figure 2. Net Na^+ fluxes in roots of 'Liaozhen 7' under NaCl stress. (**A**): net Na^+ fluxes; (**B**): means of net Na^+ fluxes. Each point represents the mean of five individual roots. Each column is the mean during the measurement period. Error bars represent the standard error of the mean. Different lowercase letters indicate statistically significant differences ($p < 0.05$). The same below.

3.2.2. K^+ Fluxes

Fluxes of K^+ were recorded in roots under the control and 200 mM NaCl stress (Figure 3A). Control and NaCl-induced K^+ fluxes all showed an outward efflux. During the period of recording, the net K^+ flux under the control remained stable with minor fluctuations at a flux rate of 3.91~18.67 pmol·cm^{-2}·s^{-1}, while a decrease-increase-decease type was found for 200 mM NaCl stress. According to the average flux rates during the recording periods (Figure 3B), the net K^+ efflux under the 200 mM NaCl stress (76.81 pmol·cm^{-2}·s^{-1}) was 6.7 times higher than that of control (11.43 pmol·cm^{-2}·s^{-1}), indicating that NaCl induced a marked K^+ efflux.

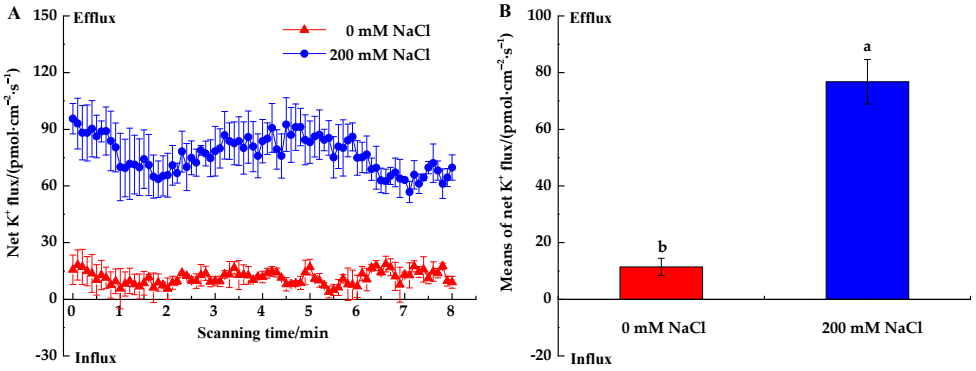

Figure 3. Net K^+ fluxes in roots of 'Liaozhen 7' under NaCl stress. (**A**): net K^+ fluxes; (**B**): means of net K^+ fluxes. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

3.2.3. H^+ Fluxes

In contrast to Na^+ or K^+, control and NaCl-induced H^+ fluxes all showed an inward flux over the course of 0–8 min (Figure 4A). The H^+ influx did not show an obvious change during the initial 6 min, followed by an increase in control roots and a decrease in stressed roots to-

wards the end of the recording. According to the mean flux rates (Figure 4B), an accelerated net H^+ influx was observed in stressed roots, where the influx rate (-2.60 pmol·cm^{-2}·s^{-1}) was 2.3 times higher than that of control (-1.11 pmol·cm^{-2}·s^{-1}), indicating that NaCl stress significantly induced H^+ influx.

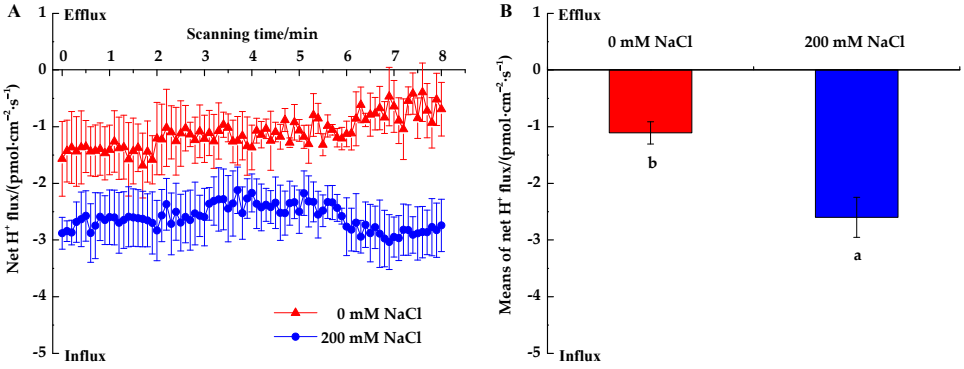

Figure 4. Net H^+ fluxes in roots of 'Liaozhen 7' under NaCl stress. (**A**): net H^+ fluxes; (**B**): means of net H^+ fluxes. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

3.3. Effects of Transport Inhibitors on Ion Fluxes under NaCl Stress

3.3.1. Inhibitor-Induced Na^+ Fluxes under NaCl Stress

The inhibitor-induced Na^+ fluxes under NaCl stress were detected at meristematic zone of 'Liaozhen 7' roots (Figure 5A). Na^+ fluxes under NaCl and transport inhibitor treatments all showed an outward efflux. During the recording periods, the root Na^+ efflux gradually decreased after treatment with amiloride (a Na^+/H^+ antiport inhibitor), TEA (a K^+ channel inhibitor) and vanadate (a PM H^+-ATPase inhibitor). The NaCl treatment showed the largest Na^+ efflux. All three inhibitors suppressed Na^+ efflux, and the smallest Na^+ efflux with a flux rate of 93.74–358.82 pmol·cm^{-2}·s^{-1} was detected in the presence of amiloride. According to the mean flux rates, amiloride (182.16 pmol·cm^{-2}·s^{-1}), TEA (290.73 pmol·cm^{-2}·s^{-1}) and vanadate (749.27 pmol·cm^{-2}·s^{-1}) treatments significantly decreased by 89.9%, 83.8% and 58.3% compared with NaCl stress, respectively (Figure 5B).

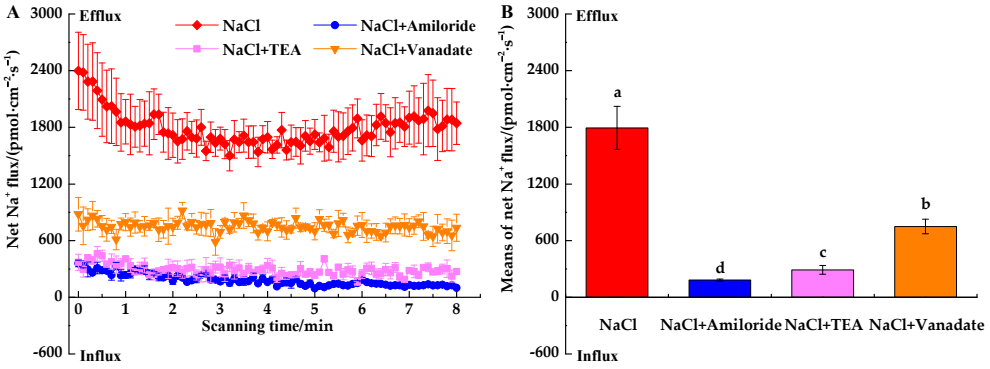

Figure 5. Effects of ion transporter inhibitors on net Na^+ fluxes in roots of 'Liaozhen 7'. (**A**): net Na^+ fluxes; (**B**): means of net Na^+ fluxes. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

3.3.2. Inhibitor-Induced K^+ Fluxes under NaCl Stress

As shown in Figure 6A, the K^+ fluxes under NaCl, amiloride and vanadate treatments all showed an outward efflux. However, TEA treatment did not cause a net K^+ efflux in the roots; instead, an enhanced K^+ influx was seen. Under NaCl and vanadate treatments, the K^+ efflux decreased with time. While the amiloride-induced K^+ efflux exhibited a notable decrease initially, staying relatively constant thereafter. According to the mean flux rates, amiloride (27.01 pmol·cm^{-2}·s^{-1}) and TEA (-8.55 pmol·cm^{-2}·s^{-1}) significantly decreased

by 64.6% and 111.2%, while vanadate (138.08 pmol·cm^{-2}·s^{-1}) significantly increased by 80.8%, compared with NaCl stress, respectively (Figure 6B).

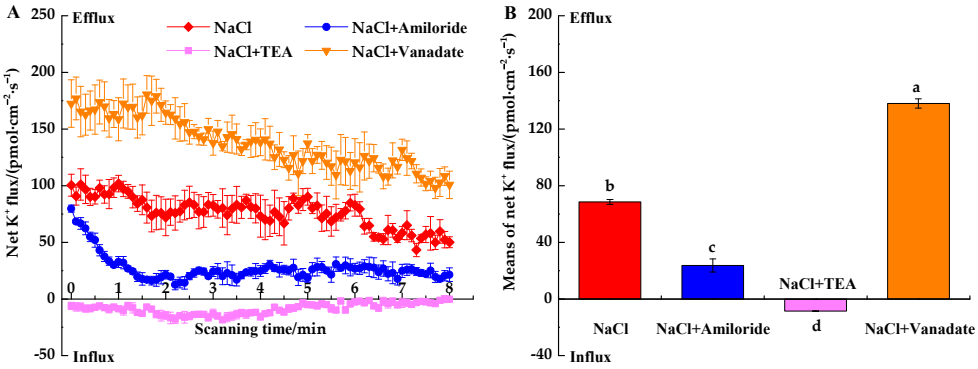

Figure 6. Effects of ion transporter inhibitors on net K^+ fluxes in roots of 'Liaozhen 7'. (**A**): net K^+ fluxes; (**B**): means of net K^+ fluxes. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

3.3.3. Inhibitor-Induced H^+ Fluxes under NaCl Stress

The NMT results showed that (Figure 7A), the H^+ fluxes under NaCl and TEA treatments all showed an inward flux. However, amiloride and vanadate treatments did not cause a net H^+ influx in the roots; instead, an enhanced H^+ efflux was observed. NaCl-stressed roots exhibited a stable and constant H^+ influx with a mean value of −2.57 pmol·cm^{-2}·s^{-1}. After being subjected to TEA, 'Liaozhen 7' roots exhibited a decreased H^+ influx, although the flux rates oscillated during the period of recording. After being subjected to vanadate, the H^+ efflux in roots increased first and then gradually decreased with time. According to the mean flux rates, the net H^+ influx under amiloride (0.32 pmol·cm^{-2}·s^{-1}), TEA (−1.77 pmol·cm^{-2}·s^{-1}) and vanadate (0.90 pmol·cm^{-2}·s^{-1}) treatments significantly decreased by 112.5%, 31.1% and 135.0% compared with NaCl stress, respectively (Figure 7B).

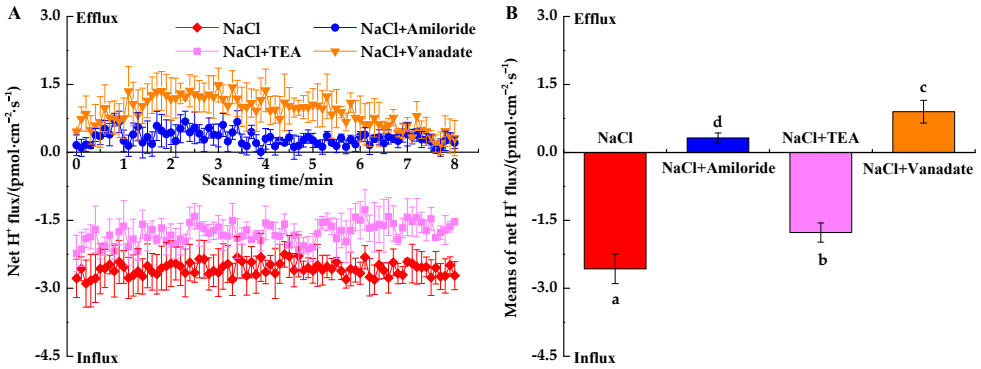

Figure 7. Effects of ion transporter inhibitors on net H^+ fluxes in roots of 'Liaozhen 7'. (**A**): net H^+ fluxes; (**B**): means of net H^+ fluxes. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

3.4. Relationships among Na^+, K^+ and H^+ Fluxes

Net H^+ influx increased with net Na^+ efflux and decreased with net K^+ efflux in roots under the control (Figure 8B,C). Similarly, after being subjected to NaCl, net H^+ influx decreased with net K^+ efflux (Figure 8F), but the relationship turned to that net H^+ influx declined with increases in net Na^+ efflux (Figure 8E). Net H^+ uptake increased with net Na^+ efflux in roots exposed to 200 mM NaCl in the absence of ion transporter inhibitors (Figure 8H). After inhibition of Na^+/H^+ antiporter, net K^+ efflux enhanced when net Na^+ efflux increased in the measuring period for roots (Figure 8J). No significant correlation

was found among net Na^+, K^+ and H^+ fluxes in roots exposed to K^+ channel inhibitors (Figure 8M–O). Net K^+ and H^+ efflux increased with net Na^+ and K^+ efflux, respectively, when roots were exposed to vanadate (Figure 8P,R).

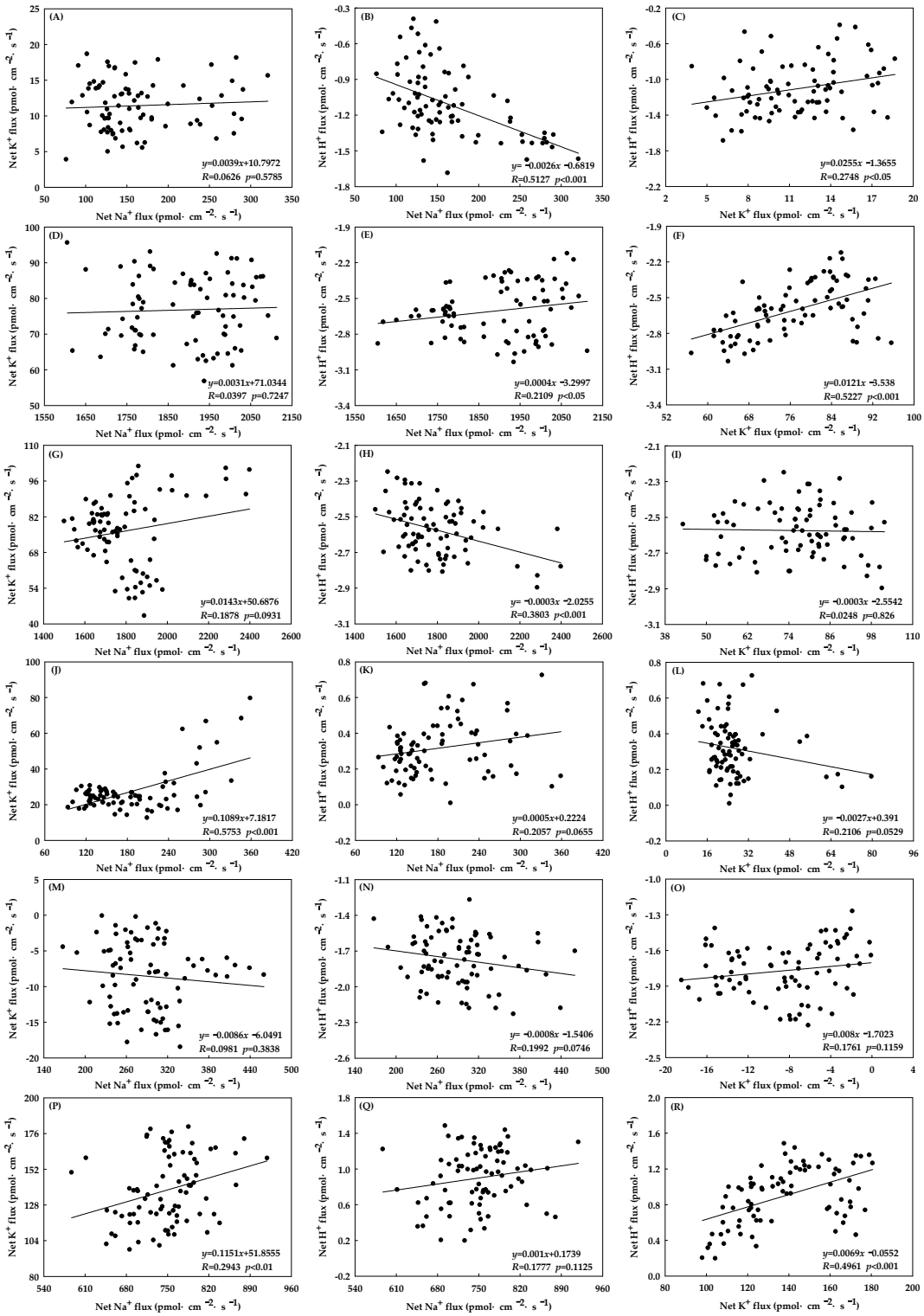

Figure 8. The relationships among Na^+, K^+ and H^+ fluxes of 'Liaozhen 7' roots (n = 81). (**A**–**C**): basic measuring solution; (**D**–**F**): basic measuring solution + 200 mM NaCl; (**G**–**I**): 200 mM NaCl + no ion transporter inhibitor; (**J**–**L**): 200 mM NaCl + amiloride; (**M**–**O**): 200 mM NaCl + TEA; (**P**–**R**): 200 mM NaCl + vanadate.

4. Discussion

4.1. Salinity Effects on Root Ion Concentration and Ratio

Under NaCl stress, the Na^+ concentration in roots was significantly higher than that in the control, while K^+ concentration decreased first and then increased (Figure 1A,B). In a previous study, we found that Ping'ou hybrid hazelnut mainly sequestrate Na^+ into leaf and stem tissues under NaCl stress [34]. Combined with the results of this study, we speculated that the root, as the earliest locus and most directly affected site that senses salt-stress signals, absorbs and retains a large amount of Na^+ first and then transports it to the leaf and stem tissues. On the one hand, this regulatory mechanism reduces the toxicity of Na^+ on the roots and ensures the absorption of other nutrient ions, such as K^+ and Ca^{2+}, by the plants. On the other hand, Na^+ accumulation in leaf and stem tissues, involved in osmotic regulation, increases the osmotic potential difference between aboveground and underground, thereby promoting the water absorption and upward transport to dilute the high-salt environment in the body [36]. Yang et al. [7] suggested that maintaining a suitable cytosolic K^+/Na^+ ratio in plants is an important adaptive trait for salt-tolerant plants to respond to excessive salt ions. Therefore, the K^+/Na^+ ratio is usually considered as an important indicator of salinity tolerance in plants [17,38]. In the current study, NaCl stress induced a significant decline in the root K^+/Na^+ ratio; however, the K^+/Na^+ ratio was maintained at a relatively stable level under 100 and 200 mM NaCl stress (Figure 1C), indicating that the roots had a strong ability to maintain the K^+/Na^+ homeostasis under NaCl stress. This is similar to the results of Nitraria (*Nitraria sibirica*) [36]. These changes implied that, when accumulation Na^+ under NaCl stress, 'Liaozhen 7' root could maintain a re-established ion balance in the body by enhancing the Na^+ transportation capacity between tissues and K^+ selective absorption capacity in roots, so as to alleviate the damage of the salinity and maintain the normal physiological and metabolic activities under salt stress.

Plants maintain a suitable K^+/Na^+ ratio primarily through increasing Na^+ exclusion or compartmentalization and reducing K^+ loss [39]. Studies in poplar [37] and wheat [40] have shown that salt-tolerant strains have higher capacities in Na^+ exclusion or restriction of K^+ leakage or switching from K^+ efflux to K^+ influx. In our study, 200 mM NaCl stress significantly enhanced the Na^+ exclusion in roots of 'Liaozhen 7' (Figure 2A), a significant increase was also found for K^+ efflux (Figure 3A). However, according to the average flux rates during the recording periods, the NaCl-induced Na^+ and K^+ effluxes were 11.6 and 6.7 times higher than those of control, respectively (Figures 2B and 3B), indicating that Na^+ efflux was faster than K^+ efflux. These results indicate that 'Liaozhen 7' seedling roots maintain K^+/Na^+ homeostasis through high capacities of Na^+ exclusion and retaining K^+. This may be a strategy for 'Liaozhen 7' to adapt to salt stress.

4.2. Salinity Effects on Root Na^+, K^+ and H^+ Fluxes

NMT has been reported as an effective method for studying ion transport in plants and animals. The different ion fluxes (such as Na^+, K^+, NH_4^+, Ca^{2+}, Cd^{2+}, Pb^{2+}, NO_3^-, etc.) can be determined by NMT in real time under different physiological conditions [37,41,42]. Ion transport is crucial for understanding the ion homeostasis mechanisms in plants. Along with the Na^+ accumulation in plants, salt stress usually induces K^+ efflux, disrupting the original K^+/Na^+ homeostasis in the body [43,44]. In the present study, the NMT results showed that 200 mM NaCl-induced Na^+ and K^+ fluxes in roots showed an obvious extrusion during the period of recording (Figures 2 and 3), which is consistent with previous studies [36,45]. However, after 21 days of 50 mM NaCl stress, poplar (*Populus euphratica*) roots showed a decrease in Na^+ efflux and an increase in K^+ retention capacity [46]. Su et al. [47] found that under NaCl stress, high-salt-tolerant wheat (*Triticum aestivum*) exhibited a reduction in K^+ efflux or a replacement of K^+ efflux by influx. The underlying cause for this phenomenon may be that high-NaCl concentration activates inward-rectifying K^+ channels and HAK/KUP/KT (high-affinity K^+/K^+ UPtake/K^+ Transporters) transporters, which in turn inhibits outward-rectifying K^+ channels. In the study of *N. sibirica*, 200 mM NaCl treatment had no significant effect on the K^+ efflux in roots, while 400 mM NaCl

treatment significantly reduced the K^+ efflux [36]. It is believed that the differences in H^+-ATPase activity and depolarization of PM induced by different NaCl concentrations were the main reasons for the significant differences in the K^+ efflux. Tang et al. [36] suggested that high concentrations of NaCl disrupt the cell membrane structure of roots, resulting in a decrease in the K^+ efflux. In our study, 200 mM NaCl-induced Na^+ and K^+ effluxes in 'Liaozhen 7' roots were 11.6 and 6.7 times higher than those of the control, respectively (Figures 2 and 3), indicating that NaCl stress significantly induced Na^+ and K^+ efflux. Similar results were observed in *L. barbarum* when studying the salinity effects on root Na^+ and K^+ transport [23]. The reason may be that under the initial NaCl stress, Na^+ entering the cell induces depolarization of PM potential, activates the outward-rectifying K^+ channels and NSCCs, and then causes the increase of K^+ efflux [48,49]. However, with the continuation of NaCl stress, we found that K^+ efflux in 'Liaozhen 7' roots exhibited a downward trend (Figure 3). As the time of prolonged salt stress, root H^+-ATPase reduces the depolarization of PM, reducing the K^+ loss through the depolarization-activated channels, and enhancing K^+ retention, thus improving the salt tolerance of 'Liaozhen 7' seedlings. These findings suggest that different experimental results in different studies may be related to plant materials, stress types or stress intensity.

Under 200 mM NaCl stress, H^+ in Arabidopsis (*Arabidopsis thaliana*) roots exhibits significant influx [15]. In this study, NaCl-induced root H^+ of 'Liaozhen 7' seedlings showed obvious influx (Figure 4); this is consistent with previous studies in *N. sibirica* [36]. Meanwhile, according to the mean flux rates, an accelerated net H^+ influx in 'Liaozhen 7' roots was observed in stressed roots, where the influx rate was 2.3 times higher than that of control (Figure 4B), indicating that NaCl stress significantly induced H^+ influx. In addition, previous studies have shown that under salt stress, regulated by PM Na^+/H^+ antiporters, there is a strong correlation between Na^+ efflux and H^+ influx [37,50]. Similarly, our correlation analysis showed that Na^+ fluxes were significantly correlated with H^+ fluxes in roots of 'Liaozhen 7' (Figure 8B,E,H). Different from the above results, Yang et al. [45] observed that under control conditions, the H^+ in roots of Russian olive (*Elaeagnus angustifolia*) showed a weak influx, but under salt stress, H^+ changed from influx to weak efflux, and the H^+ influx did not increase with the increase of Na^+ efflux. Accordingly, they proposed that the Na^+ and H^+ transport in roots of salt-tolerant *E. angustifolia* is either not or less regulated by Na^+/H^+ antiporters.

4.3. Responses of Ion Fluxes to Inhibitors under NaCl Stress

Studies have shown that the PM Na^+/H^+ antiporter not only regulates the Na^+ exclusion and long-distance Na^+ transport in plant roots, but also affects intracellular ion homeostasis, pH and Ca^{2+} signal transduction [51,52]. ATP hydrolysis through PM H^+-ATPase generates H^+ electrochemical gradient that provides energy for Na^+ extrusion [36]. In our work, the Na^+ efflux and H^+ influx in 'Liaozhen 7' roots were significantly increased after 200 mM NaCl stress (Figures 2 and 4), indicating that Na^+ efflux and H^+ influx in roots were mainly the results of an active Na^+/H^+ antiport across the PM [37]. Moreover, the pharmacological experiments showed that, under NaCl stress, the Na^+ efflux and H^+ influx in 'Liaozhen 7' roots were all significantly inhibited after being subjected to the amiloride (inhibitor of the PM Na^+/H^+ antiporter) and sodium vanadate (inhibitor of the PM H^+-ATPase), and Na^+ efflux and H^+ influx significantly decreased by 89.9% and 135.0% (Figures 5 and 7), respectively. This agrees with the results of Sun et al. [37], who found that the PM transport inhibitors, amiloride and sodium orthovanadate, simultaneously decrease Na^+ efflux and H^+ influx along the root axis of stressed plants. Previous studies in *B. gymnorrhiza* [22] and *L. barbarum* [23] have shown that the levels of PM Na^+/H^+ antiporters in roots were significantly increased after NaCl stress. Moreover, the PM H^+-ATPase pumps protons and maintains electrochemical H^+ gradients, thus promoting the active Na^+/H^+ antiport in the PM [14]. Shabala et al. [17,25] also claimed the involvement of PM H^+-ATPase in Na^+/H^+ antiport. Given these results, we conclude that in 'Liaozhen 7' roots, NaCl stress increases the activity of PM H^+-ATPase and generates electrochemical H^+

potential gradients in the PM, which activates the Na^+/H^+ antiporters and finally promotes H^+ entering the intracellular and the Na^+ extrusion from intracellular.

Under salt stress, the depolarization of plant cells enhances, thus increasing the K^+ efflux through outward-rectifying K^+ channels and NSCCs [16]. In the present study, the K^+ efflux in 'Liaozhen 7' roots was significantly increased after 200 mM NaCl stress for 24 h (Figure 3). However, the K^+ efflux was suppressed by the K^+ channel inhibitor tetraethylamine chloride (TEA) and promoted by the PM H^+-ATPase inhibitor vanadate, and K^+ efflux was significantly decreased by 111.2% and increased by 80.8% (Figure 6), respectively. This further indicates that NaCl-induced K^+ efflux is mediated by depolarization-activated PM outward-rectifying K^+ channels or NSCCs. The conclusion has also been proved in *B. gymnorrhiza* [22], *N. sibirica* [36] and *P. euphratica* [46].

5. Conclusions

Our data show that under NaCl stress, the K^+/Na^+ homeostasis in 'Liaozhen 7' roots can be maintained through promoting Na^+ extrusion and slowing down K^+ loss. Under NaCl stress, NaCl-induced Na^+ and K^+ efflux, as well as H^+ influx in roots were significantly increased. Furthermore, pharmacological evidence suggested that the inhibition of Na^+/H^+ antiporter by amiloride (a plasma membrane (PM) Na^+/H^+ antiporter inhibitor) and sodium vanadate (a PM H^+-ATPase inhibitor) verified the involvement of Na^+/H^+ antiporters and a H^+ pump (PM H^+-ATPase) in Na^+ extrusion. Meanwhile, NaCl-induced K^+ efflux was significantly suppressed by tetraethylamine chloride (a K^+ channel inhibitor) and promoted by sodium vanadate, indicating that K^+ efflux was mediated by depolarization-activated outward-rectifying K^+ channels and non-selective cation channels (NSCCs). As a result, Na^+ efflux mediated by Na^+/H^+ antiporter and H^+ pump, an activity of PM Na^+/H^+ antiport system, was promoted, while K^+ efflux regulated by depolarization-activated outward-rectifying K^+ channels and NSCCs was slowed down, thus maintaining the root K^+/Na^+ homeostasis.

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