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Maintenance of K^+/Na^+ Balance in the Roots of *Nitraria sibirica* Pall. in Response to NaCl Stress

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Abstract: Using Non-invasive Micro-test Technology (NMT), the Na^+, K^+ and H^+ flux profiles in the root meristem regions were investigated in *Nitraria sibirica* Pall. seedlings under different NaCl concentrations. NaCl stress increased the K^+ and Na^+ contents in the roots of *N. sibirica* seedlings. NaCl stress significantly increased the steady Na^+ efflux from the *N. sibirica* seedling roots. Steady K^+ effluxes were measured in the control roots (without NaCl) and in the roots treated with 200 mM NaCl, and no significant differences were observed between the two treatments. The steady K^+ efflux from roots treated with 400 mM NaCl decreased gradually. NaCl treatment significantly increased the H^+ influx. Pharmacological experiments showed that amiloride and sodium vanadate significantly inhibited the Na^+ efflux and H^+ influx, suggesting that the Na^+ efflux was mediated by a Na^+/H^+ antiporter using energy provided by plasma membrane H^+-ATPase. The NaCl-induced root K^+ efflux was inhibited by the K^+ channel inhibitor tetraethylammonium chloride (TEA), and was significantly increased by the H^+-ATPase inhibitor sodium vanadate. The NaCl-induced K^+ efflux was mediated by depolarization-activated outward-rectifying K^+ channels and nonselective cation channels (NSCCs). Under salt stress, *N. sibirica* seedlings showed increased Na^+ efflux due to increased plasma membrane H^+-ATPase and Na^+/H^+ antiporter activity. High H^+ pump activity not only restricts the Na^+ influx through NSCCs, but also limits K^+ leakage through outward-rectifying K^+ channels and NSCCs, leading to maintenance of the K^+/Na^+ balance and higher salt tolerance.

Keywords: NaCl stress; Na^+ flux; K^+ flux; H^+ flux; *Nitraria sibirica* Pall.; ICP-OES; NMT

1. Introduction

Intracellular K^+/Na^+ is considered to be a key component of salinity tolerance in plants [1–7]. Shabala et al., suggested that the ability of plant to maintain a high cytosolic K^+/Na^+ ratio was more important than simply maintaining a low Na^+ content and high K^+ absorption [8]. Whether plants can survive in saline environments largely depends on whether they are able to maintain the K^+/Na^+ balance under salt stress [9]. Plants maintain an optimal K^+/Na^+ ratio by restricting Na^+ uptake, increasing Na^+ exclusion, compartmentalizing Na^+ in the vacuole and preventing K^+ loss [2,10,11].

The significance of vacuolar sequestration by tonoplast Na^+/H^+ antiporters has been confirmed by transgenic plants [12–15]. The gene encoding *NtNHX1* and *NsNHX1*, a putative tonoplast Na^+/H^+ antiporter in *Nitraria sibirica* Pall. and *Nitraria tangutorum* Bobr. respectively, have been characterized by Tang [16] and Wang et al. [17] respectively. They found that the expression of *NsNHX1* and *NtNHX1* in *N. sibirica* and *N. tangutorum* was related to the induction and regulation by salt stress.

Previous studies have shown that Na^+ exclusion, long-distance Na^+ transport and intracellular K^+ homeostasis are controlled by the plasma membrane (PM) Na^+/H^+ antiporter [18–21]. The gene

encoding *NtSOS1*, a putative PM Na^+/H^+ antiporter in *N. tangutorum*, has been characterized by Zheng et al. [22]. They found that the level of expressed *NtSOS1* protein in *N. tangutorum* leaves was significantly upregulated in the presence of 200 mM NaCl.

The change of H^+-ATPase activity is directly or indirectly related to plant salt tolerance [23]. H^+-ATPase hydrolysis of ATP to establish H^+ transmembrane electrochemical potential not only provides a driving force for Na^+/H^+ exchange, but also may inhibit the entry of Na^+ by repolarizing the PM, because external NaCl usually depolarizes the PM and causes a massive Na^+ influx via voltage-independent non-selective cation channels (VI-NSCCs) [6,24–27].

On the one hand, the deficiency of K^+ under NaCl stress is because Na^+ and K^+ have similar physicochemical properties, including similar ionic radius and ion hydration energy, and Na^+ competes with K^+ for binding sites on the plasma membrane, leading to reduced K^+ uptake [2,8,28,29]. On the other hand, NaCl also induces membrane depolarization and activates outward-rectifying K^+ channels (KOR), leading to K^+ leakage [8,30]. Studies with barley [10] and wheat [31] crops, as well as the woody plant poplar [32], have shown that salt tolerant strains have higher capacities for Na^+ exclusion or restriction of K^+ leakage.

Nitraria sibirica Pall., a typical perennial woody salt-diluting halophyte, is a deciduous spiny shrub of the *Nitraria* L. genus. It is an important salt-tolerant, sand-fixing plant, which is mainly distributed in the northwestern and northern saline-alkali areas, the northeast soda saline areas and Bohai coastal saline-alkali areas of China, as well as Siberia [33–35]. *N. sibirica* is highly salt tolerant and can grow in habitats with salinities of 8‰–10‰ NaCl [36]. Most of studies have been conducted to investigate the physiological mechanisms of *N. sibirica* to responding to salt stress, which mainly involve the growth, osmotic adjustment of substances, the distribution of Na^+ and K^+ in different organs, and the changes of oxidase activity, among others [37–40]. Previous studies have shown that transient NaCl treatment induces substantial changes in the Na^+ and K^+ concentrations in *N. sibirica* seedlings. NaCl treatment leads to a significant increase in Na^+ and reduction in the K^+/Na^+ ratio in the roots, stems and leaves of *N. sibirica*. NaCl treatment also significantly increases the Na^+ and K^+ concentrations in the roots. Additionally, the Na^+ levels in the roots and stems of *N. sibirica* are substantially lower than in the leaves under NaCl stress [35,41,42]. Moreover, the K^+/Na^+ ration in *N. sibirica* root was higher than that in *Elaeagnus angustifoliain* L. [43] and *Kandelia candel* (L.) Druce [44] under NaCl treatment. These observations prompted us to ask the following questions: Does the root of *N. sibirica* have a high capacity to efflux Na^+ and take up K^+? What is the mechanism underlying the maintenance of K^+/Na^+ balance for *N. sibirica*, which allows it to adapt to a saline environment?

To address these questions, we applied the Non-invasive Micro-test Technology (NMT) to clarify ion homeostasis control by mapping Na^+, K^+ and H^+ flux profiles from the *N. sibirica* roots under different NaCl concentrations. Our goal is to understand the mechanisms underlying salt tolerance of *N. sibirica* through exploring the dynamic ion transport and K^+ and Na^+ balance of the *N. sibirica* roots.

2. Materials and Methods

2.1. Plant Materials and Hydroponic Culture

N. sibirica seeds were collected from Keluke beach saline-alkali land in the Qaidam basin of Qinghai province, China. The seeds were soaked in warm water (50–60 °C) for 24 h and mixed with wet sand to accelerate germination. The seeds selected for sowing were placed in containers filled with vermiculite:perlite (3:1) when they germinated in April. Two-month-old seedlings were transplanted into plastic containers (length × width × height: 40 × 30 × 15 cm) with aerated hydroponic solution for 24 h. We used tap water until the seedlings generated new roots, and then half-strength Hoagland nutrient solution was supplied. The hydroponic solution was renewed every 4 days, and the pH was maintained at 6.0. The seedlings were grown in the greenhouse with 14-h days at 25–30 °C and 10-h nights at 20–25 °C.

2.2. NaCl Treatment

Three treatments were applied to the seedlings: 0 mM NaCl (control), 200 mM NaCl and 400 mM NaCl. The required amount of NaCl was added to the nutrient solution. The control plants were well fertilized but treated without the addition of NaCl. Salt stress began three days after seedlings were transplanted by adding NaCl at a concentration of 50 mM per day. All treatments reached final concentrations at the same time. Each treatment level included three replicate pots, and each pot contained 20 seedlings. Steady fluxes of Na^+, K^+ and H^+ in apical were measured after 24 h of NaCl treatment. The roots were sampled after 24 h of NaCl treatment, oven dried, pulverized and used for ion content analysis by means of an inductively coupled plasma optical emission spectrometer (ICP-OES).

2.3. Ion Content Analysis

Roots sampled from control and stressed plants were dried separately in an oven at 105 °C for 15 min and then at 80 °C until a constant dry weight (DW) was reached. Approximately 0.10 g DW sample was accurately weighed and placed into a glass beaker (50 mL) containing a di-acid mixture of 10 mL HNO_3 and 1 mL $HClO_4$ and digested overnight. Then, the solution was placed on a hot-plate and evaporated to near dryness at 100 °C. The residues were dissolved in 0.50 mL HNO_3, and the inside walls of the glass beakers were washed 3–5 times using sub-boiling de-ionized water. After cooling, a clear solution was obtained and finally diluted to a 5 mL volume. The Na^+ and K^+ concentrations were determined by using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; Perkin Elmer Dual View Optima 5300DV, Waltham, MA, USA) [43].

2.4. Flux Measurements with NMT

The net K^+, H^+ and Na^+ fluxes in roots of *N. sibirica* were measured by Non-invasive Micro-test Technology (NMT Physiolyzer®, YoungerUSA LLC, Amherst, MA, USA; Xuyue (Beijing) Sci. & Tech. Co., Ltd., Beijing, China). Two-month-old seedlings were treated with 0, 200 and 400 mM NaCl for 24 h. Apices (1–2 cm) were used to measure steady Na^+, K^+ and H^+ flux profiles. Prior to the flux measurement, the roots were equilibrated for 30 min in measuring liquid (0.10 mM KCl, 0.10 mM $MgCl_2$, 0.50 mM NaCl, 0.10 mM $CaCl_2$, 0.30 mM MES, pH 5.7). After equilibration, roots were transferred to the measuring chamber containing 10 mL fresh measuring solution and the roots were immobilized on the bottom. Ion fluxes were measured along the axis at apex (200–250 μm from the tip with a 30 μm measuring interval). Then, a continuous flux recording was taken for 600 s using different ion selective flux microsensor. All of the measurements were repeated at least six samples (roots) independently [45].

For pharmacological experiments, the plants were treated with 200 mM NaCl for 24 h first, then the roots were subjected to 500 μM vanadate (a PM H^+-ATPase inhibitor) [32,45], 100 μM amiloride (a Na^+/H^+ antiporter inhibitor) [45] or 20 mM tetraethylammonium chloride (TEA, a K^+ channel inhibitor) [10,46] for 30 min. Finally, the plants were sampled and used to measure steady-state ion fluxes (i.e., Na^+, H^+ and K^+).

2.5. Data Analysis

Three-dimensional ionic fluxes were calculated using Mageflux software (Younger USA Sci & Tech Corp, Amherst, MA, USA). The data were analyzed using one-way ANOVA and Duncan's multiple range test implemented in SPSS 16 (Chicago, IL, USA). A significant difference was detected at $p < 0.05$. The data are presented as the mean ± standard error (SE) of three independent experiments.

3. Results

3.1. Variations of Na^+ and K^+ in N. sibirica Roots under NaCl Stress

In this study, ICP-OES was used to measure Na^+ and K^+ concentrations in roots. NaCl stress induced the absorption of K^+ by the *N. sibirica* root. Compared with the control, the 200 mM and 400 mM NaCl treatments increased root K^+ contents by 16.72% and 10.50%, respectively (Figure 1A). NaCl stress significantly increased both the Na^+ and K^+ concentrations in the roots, but no significant difference was detected in the root K^+ content between 200 mM and 400 mM NaCl treatments. The Na^+ contents under 200 mM and 400 mM NaCl treatments were 2.22-fold and 2.68-fold that of the control, respectively (Figure 1B). The K^+/Na^+ ratio was significantly reduced under salt stress. Compared with the control, the K^+/Na^+ ratio under 200 mM and 400 mM NaCl treatment was reduced 49.97% and 58.92%, respectively, but no significant difference was found between 200 mM and 400 mM NaCl treatments (Figure 1C).

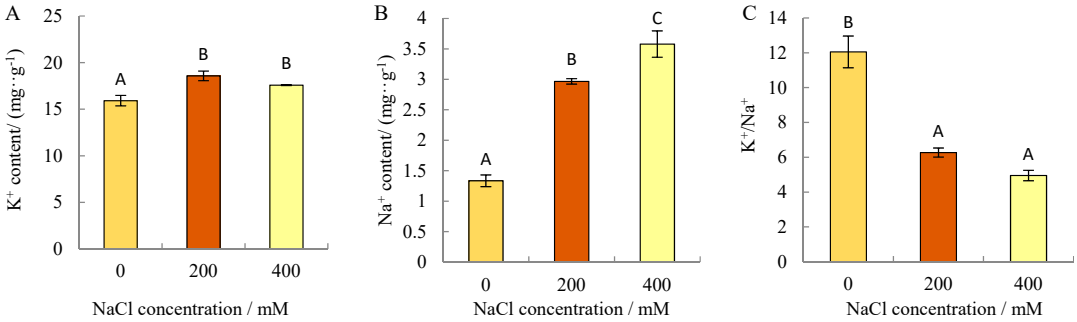

Figure 1. Effect of NaCl on K^+, Na^+ and K^+/Na^+ ratio in roots of *Nitraria sibirica* seedlings. K^+ (**A**), Na^+ (**B**), K^+/Na^+ (**C**). Each column is the mean of three replicates, and bars represent the standard error of the mean. Columns labeled with different letters, A–C, indicate significant difference at $p < 0.05$.

3.2. Steady Ion Fluxes under Salt Treatment

3.2.1. Na^+ Fluxes

As shown in Figure 2A, the Na^+ efflux was detected in the *N. sibirica* seedling roots in the control, 200 mM and 400 mM NaCl treatments. The Na^+ efflux significantly increased with the increase of NaCl concentration. The Na^+ efflux under 400 mM NaCl treatment was 2.34-fold and 1.43-fold that of control and 200 mM NaCl treatments, respectively (Figure 2B). These results indicate that NaCl treatment significantly increases the Na^+ efflux of the *N. sibirica* roots.

3.2.2. K^+ Fluxes

As shown in Figure 2C, the K^+ efflux was measured in controls and 200 mM NaCl treatments with the measure time from 0 to 10 min. The K^+ efflux was significantly reduced under 400 mM NaCl treatment. According to the average flow rates within 0–10 min (Figure 2D), the net K^+ flux was 156 and 159 pmol·cm^{-2}·s^{-1} under control and 200 mM NaCl treatments, respectively, and no significant difference was detected between these two treatments. Importantly, the 400 mM NaCl treatment significantly reduced the K^+ efflux, the net K^+ flux was 116 pmol·cm^{-2}·s^{-1}. The flux rate was significantly different from that of the control and 200 mM NaCl treatments. These results indicate that 200 mM NaCl stress had no substantial influence on the K^+ efflux of the *N. sibirica* seedling roots, whereas 400 mM NaCl stress significantly reduced K^+ efflux.

3.2.3. H^+ Fluxes

As shown in Figure 2E, the H^+ influx was measured in the control, 200 mM and 400 mM NaCl treatments. The H^+ influx significantly increased under 200 mM and 400 mM NaCl treatments.

According to the average flow rates within 0–10 min (Figure 2F), the H^+ influx significantly increased as the concentration of NaCl increased. Compared with the control, the net H^+ fluxes under 200 mM and 400 mM NaCl treatments increased by 20.62% and 78.34%, respectively, and there was a significant difference between these two groups.

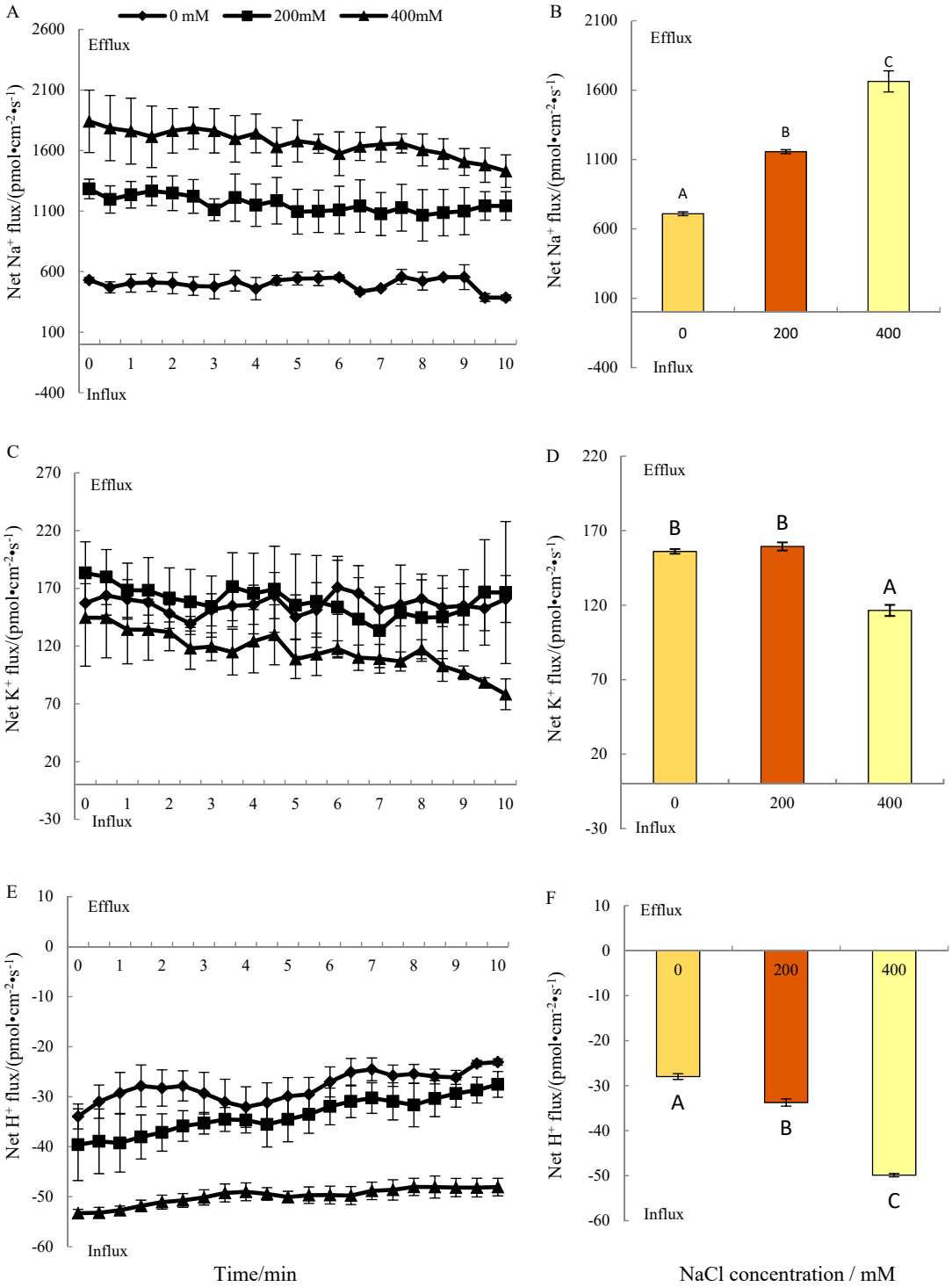

Figure 2. Steady Na^+ (**A**), K^+ (**C**) and H^+ (**E**) fluxes of *N. sibirica* seedling roots under different NaCl concentrations. (**B**,**D**,**F**) Mean of net Na^+, K^+ and H^+ fluxes corresponding to (**A**,**C**,**E**). Note: Each point represents the mean of three to six individual roots. Error bars represent the standard error of the mean. Different capital letters indicate significant differences between treatments ($p < 0.05$).

3.3. The Effects of Ion Transport Inhibitors on the Ion Fluxes of N. sibirica Seedling Roots under NaCl Treatment

As shown in Figure 3A, the Na^+ effluxes were measured in the meristematic zone of *N. sibirica* seedling roots under all treatment conditions. The NaCl treatment showed the largest Na^+ efflux. All three inhibitors suppressed Na^+ efflux, and the smallest Na^+ efflux with a flow rate of 130–168 pmol·cm^{-2}·s^{-1} was detected in the presence of sodium vanadate, a plasma membrane H^+-ATPase inhibitor. During the 0–2 min time period, the Na^+ efflux in the meristematic zone of *N. sibirica* seedling roots remained stable after treatment with amiloride, a Na^+/H^+ antiporter inhibitor, or TEA, a K^+ channel inhibitor. The inhibition of the Na^+ efflux increased over time under amiloride treatment. Sodium vanadate and amiloride had stronger effects on Na^+ efflux than TEA, suggesting that the Na^+ efflux is significantly associated with the Na^+/H^+ antiporter and plasma membrane H^+-ATPase.

Figure 3B shows that TEA and amiloride inhibited the K^+ efflux of the *N. sibirica* seedling roots to different extents, whereas the plasma membrane H^+-ATPase inhibitor sodium vanadate increased K^+ efflux. The K^+ flux rate of the meristematic zone of *N. sibirica* seedling roots was 133–183 pmol·cm^{-2}·s^{-1} under NaCl treatment, and it was gradually reduced to 107–160 pmol·cm^{-2}·s^{-1} as a result of sodium vanadate and amiloride treatments. TEA treatment induced the greatest inhibition of the K^+ efflux, and this change remained stable over the course of 0–10 min.

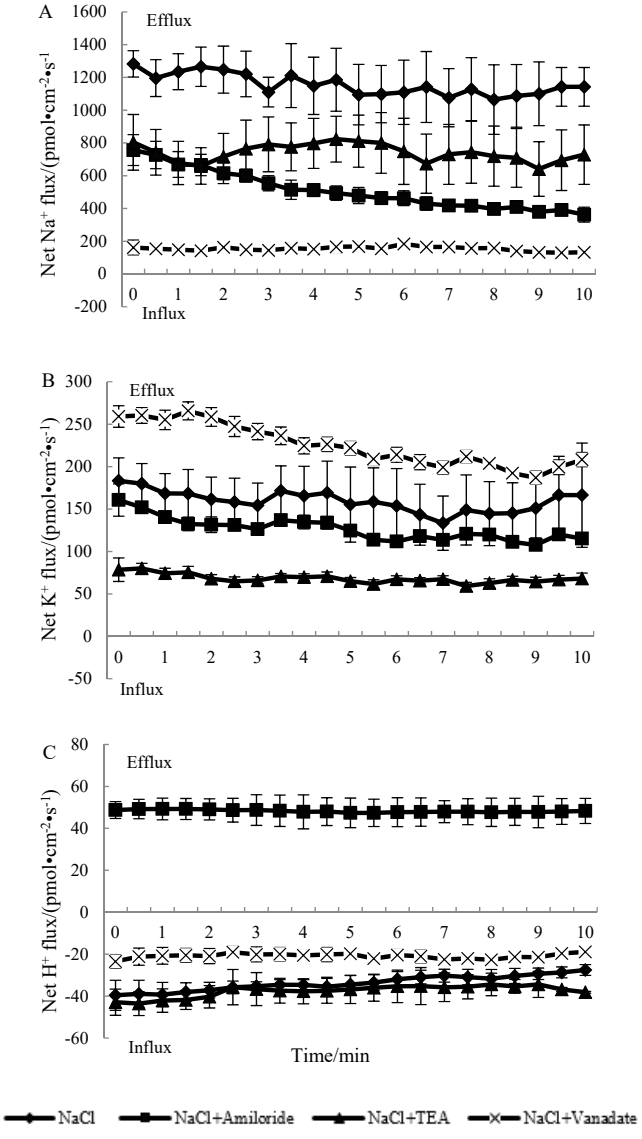

Figure 3. Effects of ion inhibitors on net Na^+ (**A**), K^+ (**B**) and H^+ (**C**) flux at *N. sibirica* seedlings roots.

The results from the NMT test revealed that the H^+ influx at the meristematic zone of *N. sibirica* Pall. seedling roots under NaCl treatment, and this change remained stable over the course of 0–10 min at a flow rate of −27 to −39 pmol·cm^{-2}·s^{-1} (Figure 3C). The sodium vanadate treatment reduced H^+ influx, whereas TEA treatment had no significant effect on H^+ influx. Strikingly, the amiloride treatment changed the direction of H^+ from influx to efflux. These results suggest that the Na^+/H^+ antiporter inhibitor amiloride inhibits H^+ influx greatly in the meristematic zone of *N. sibirica* seedling roots, with the plasma membrane H^+-ATPase inhibitor sodium vanadate following behind.

4. Discussion

The ICP-OES results showed that the *N. sibirica* roots had a strong ability to maintain the K^+/Na^+ balance under NaCl stress (Figure 1C). Under 200 mM and 400 mM NaCl treatment, the K^+ and Na^+ contents in the *N. sibirica* roots were significantly higher than those in the control and the K^+/Na^+ ratio was significantly decreased, but the K^+/Na^+ ratio in the roots was not significantly changed under 200 and 400 mM NaCl stress. Previous studies have found that *N. sibirica* sequestrate Na^+ into leaves under NaCl stress [37,38]. Combined with the experimental results, we speculated that the root, as the first locus that reacts to NaCl stress, absorbs a large amount of Na^+ and transports Na^+ to the leaves. On the one hand, the damage by Na^+ on the root system is reduced; on the other hand, Na^+ accumulation in the leaf, involved in osmotic regulation, increases the difference in osmotic potential between aboveground and underground parts, thus, enhancing the plant water absorption to dilute salt ions in the body. These changes indicated that under NaCl stress, *N. sibirica* increased absorption of K^+ and increase leaf Na^+ content to reduce the relative Na^+ concentration in the root system, thereby reducing the toxicity of the single salt and maintaining the relative balance of root ions to improve NaCl tolerance in the plants. In addition, the K^+/Na^+ ratio in *N. sibirica* root was maintained at a relatively stable level under 200 and 400 mM NaCl. This indicated that the *N. sibirica* root can maintain a re-establish K^+/Na^+ balance under NaCl stress. This may be a way for *N. sibirica* to adapt to salt stress.

Under salt stress, the ability of a plant to maintain a high K^+/Na^+ ratio is critical to plant salt tolerance [47,48]. Plants maintain an optimal cytosolic K^+/Na^+ ratio by increasing Na^+ exclusion or compartmentalization and reducing K^+ efflux. Studies in wheat [31,49] and poplar [32] 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 the current study, 200 mM and 400 mM NaCl treatments significantly increased the steady Na^+ efflux in *N. sibirica* seedling roots. A significant difference was also found between these two treatments; 400 mM NaCl treatment reduced K^+ efflux, while 200 mM NaCl had no effect on the steady K^+ flux in *N. sibirica* seedling roots. These results indicate that *N. sibirica* seedling roots maintain K^+/Na^+ balance through high capacities of Na^+ exclusion and retaining intracellular K^+.

Plant Na^+ exclusion and long-distance transportation are regulated by plasma membrane Na^+/H^+ antiporter [19,50]. ATP hydrolysis by plasma membrane H^+-ATPase generates the H^+ electrochemical membrane potential that provides energy for Na^+ efflux. A previous study on the regulation of poplar root K^+/Na^+ balance shows that Na^+/H^+ antiporters PeNhaD1 and PeSOS1 driven by plasma membrane H^+-ATPase are responsible for Na^+ exclusion from poplar cells [9]. Additionally, Ma et al., found that the plasma membrane antiporter ZxSOS1 plays an important role in the transportation and spatial distribution of Na^+ and K^+ in Zygophyllum xanthoxylon (Bunge) Maxim [51]. In our current study, we found that the Na^+ efflux and the H^+ influx in the meristematic zone of *N. sibirica* seedling roots significantly increased following an increase in the concentration of NaCl. Our results indicate that the Na^+ efflux in the *N. sibirica* seedling roots is mediated by the Na^+/H^+ antiporter, whose activity is significantly increased as a result of increased NaCl concentration. Additionally, the pharmacological experiments show that the Na^+ efflux and the H^+ influx in the *N. sibirica* seedling roots were significantly suppressed in the presence of the Na^+/H^+ antiporter inhibitor amiloride or the plasma membrane H^+-ATPase inhibitor sodium vanadate, which

further indicates that NaCl treatment increases plasma membrane H^+-ATPase activity. Increased inner and outer membrane H^+ concentration gradient eventually promotes Na^+ efflux through the Na^+/H^+ antiporter.

A subtle change in the K^+ efflux in the *N. sibirica* seedling roots was detected under 200 mM NaCl treatment. This K^+ efflux was inhibited by the K^+ channel inhibitor TEA, and it was increased by the plasma membrane H^+-ATPase inhibitor sodium vanadate (Figure 3B), indicating that K^+ efflux under NaCl treatment is mediated through depolarization-activated outward-rectifying K^+ channels and NSCCs [8,18,52,53]. This finding is consistent with previous studies in poplar [46] and mangrove [54]. In addition, the K^+ flux rate in *N. sibirica* seedling roots differs significantly in the presence of different concentrations of NaCl, which may be due to differences in plasma membrane H^+-ATPase activities and depolarization induced by different concentrations of NaCl. Strikingly, the K^+ efflux in the *N. sibirica* seedling roots was significantly reduced under the 400 mM NaCl treatment. Similar results were observed in wheat [31,49], with highly salt tolerant strains showing reduced K^+ efflux or K^+ efflux replaced by influx. The underlying cause of this phenomenon may be the high concentration of NaCl treatment activates inward-rectifying K^+ channels and HAK/KUP/KT (High-Affinity K^+/K^+ UPtake/K^+ Transporter) transporters, which in turn inhibit outward-rectifying K^+ channels [55]. Another possibility is that the high NaCl concentration damages the structures of cell membranes, leading to K^+ influx. Further research is needed to clarify this intriguing phenomenon. Nevertheless, the capability of *N. sibirica* seedling roots to retain intracellular K^+ is critical in maintaining K^+/Na^+ balance.

5. Conclusions

In summary, the results indicate that the maintenance of K^+/Na^+ homeostasis in *N. sibirica* roots is achieved by preventing K^+ loss and promoting Na^+ exclusion under NaCl treatment. The pharmacological experiments suggest that the strong Na^+ efflux and H^+ influx are inhibited by the Na^+/H^+ antiporter inhibitor amiloride and the plasma membrane H^+-ATPase inhibitor sodium vanadate, indicating high activities of the Na^+/H^+ reverse transport and H^+ pump. This represents a key mechanism by which the *N. sibirica* roots maintain K^+/Na^+ homeostasis. NaCl treatment also induces K^+ efflux in the *N. sibirica* roots, which is mediated by depolarization-activated outward-rectifying K^+ channels (KOR) and non-selective cation channels (NSCCs). Additionally, increased H^+ influx as a result of increased NaCl concentration increases the H^+ electrochemical membrane potential, which not only promotes Na^+/H^+ reverse transport but also suppresses membrane depolarization. As a result, K^+ efflux mediated by depolarization-activated outward-rectifying K^+ channels and NSCCs, as well as Na^+ influx through NSCCs, is restricted, thus maintaining root K^+/Na^+ homeostasis.

Author Contributions: X.T. and H.Z. conceived and designed the experiments; X.T. and X.Y. performed the experiments, analyzed the data, and drafted the manuscript; H.L., X.Y. aided in analyzing the data and performing the experiments; all authors have read and approved this version of the manuscript.

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