

Full Length Research Paper

# Effects of ammonium nitrate, cesium chloride and tetraethylammonium on high-affinity potassium uptake in habanero pepper plantlets (*Capsicum chinense* Jacq.)

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Potassium ( $K^+$ ) is an essential nutrient and the most abundant cation in plant cells. Plants have a wide variety of transport systems for  $K^+$  acquisition that catalyze  $K^+$  uptake across a wide spectrum of external  $K^+$  concentrations and mediate  $K^+$  movement within the plant, as well as its release into the environment. The KUP/HAK/KT transporter family plays a key role in  $K^+$  homeostasis in plant cells. The present study demonstrates that habanero pepper plantlets have a clear pattern of  $K^+$  uptake when re-supplemented with  $K^+$  after  $K^+$  starvation. Habanero pepper plantlets, re-supplemented with a solution containing low concentrations of  $K^+$  after 72, 96 or 120 h of  $K^+$  starvation were able to decrease the amount of  $K^+$  in the solution at different time points. To study the effect of  $NH_4^+$ , we added different concentrations of  $NH_4NO_3$  to the medium solution and demonstrated that  $NH_4^+$  inhibited  $K^+$  uptake in a dose-dependent manner. When the plantlets were subjected to  $K^+$  starvation for 72 h and then re-supplemented with 50 or 100  $\mu M$   $K^+$ , exposure to  $K^+$  channel blockers (10 mM CsCl and 20 mM TEA) decreased their  $K^+$  uptake compared with the control treatment. A model demonstrating the process of  $K^+$  uptake through an  $NH_4^+$ -insensitive component was proposed.

**Key words:** Potassium, high affinity transporters, channel blockers, ammonium.

## INTRODUCTION

Since the work of Knop and Sachs over 130 years ago, it has been known that plants cannot grow in the absence of potassium ( $K^+$ ) (Pfeffer, 1900).  $K^+$  is the second most

abundant inorganic cation in non-halophytes plants. As a major inorganic osmolyte,  $K^+$  is essential for plant growth and consequently for crop production. Although,  $K^+$  concentrations in soil solution are in the range of only 0.1 to 6 mM depending on soil type (Adams, 1971), plants are able to accumulate large amount of this element that constitutes 2 to 10% of plant dry weight (Leigh et al., 1984; Tisdale et al., 1993). Plant roots absorb  $K^+$  at a wide range of external  $K^+$  concentrations ( $[K^+]_{ext}$ ), typically from 0.1 to 10 mM (Hawkesford and Miller, 2004).  $K^+$  plays major biochemical and biophysical roles in plants (Szczereba et al., 2009).  $K^+$  is involved in cell elongation, leaf movement, tropism, metabolic homeostasis, germination, seasonal changes and stomata opening/ closing;

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**Abbreviations:** CsCl, Cesium chloride; HAK, high-affinity potassium transporters; TEA, tetraethylammonium; HATS, high-affinity potassium system.

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it is also associated with numerous biochemical processes (Marschner, 1995; Szczerba et al., 2009).  $K^+$  is present in all compartments of plant cells and enriched in two large pools: vacuole and cytosol. The role of  $K^+$  in enzyme activation and protein biosynthesis is based on its high and stable concentration in the cytoplasm.  $K^+$  homeostasis in the cytoplasm is essential for metabolic processes; therefore, cytosolic  $K^+$  concentrations are strictly controlled and maintained in a narrow range (around 100 mM) that is optimal for the function of cytosolic enzymes (Leigh et al., 1984; Maathuis and Sanders, 1996; Walker et al., 1996; Cuin et al., 2003). Vacuolar  $K^+$  content is more variable, depending on  $K^+$  availability and tissue type and is observed to be in the range of 20 to 200 mM (Leigh et al., 1984; Walker et al., 1996). Two major regulatory mechanisms are involved in maintaining  $K^+$  homeostasis:  $K^+$  flow across the plasma membrane and mobilization of vacuolar  $K^+$  reserve (Fernando and Glass, 1992; Walker et al., 1996). The  $K^+$  transport system in plants consists of low- and high-affinity components (Epstein et al., 1961). At the molecular level, these components conventionally refer to channels and transporters, respectively based on their different properties (Maathuis and Sanders, 1994, 1997). The presence of several types of  $K^+$ -transporting membrane proteins has been reported, including  $K^+$  channels (Shaker-type  $K^+$  channels), two pore channels (the KCO/TPK family), non-selective channels (CNGC),  $K^+$  transporters (the HKT family, the KT/KUP/HAK family),  $K^+/H^+$  antiporters (KEA) and cation/ $H^+$  antiporters (CHX) (Maser et al., 2001, 2002; Ashley et al., 2006). KT/KUP/HAK transporters, together with shaker-type  $K^+$  channels, play a fundamental role in  $K^+$  homeostasis in plant cells, involved in both high- and low-affinity  $K^+$  uptake (Santa-Maria et al., 1997; Rigas et al., 2001; Elumalai et al., 2002; Vallejo et al., 2005). Previous evidence has suggested that these transporters are present in all plants (Kim et al., 1998; Rubio et al., 2000; Ahn et al., 2004) and have functions in the plasma membrane and tonoplast (Senn et al., 2001; Bañuelos et al., 2002). Reports have shown that the *Arabidopsis* genes encoding  $K^+$  channels and transporters are directly regulated by external  $K^+$  concentration, although, many of these genes have also been shown to be induced or repressed by stress and hormones (Pilot et al., 2003; Gierth et al., 2005). Very little is known about how the  $K^+$  transport system and available supply are regulated and coordinated in plants.  $K^+$  starvation is known to activate  $K^+$  uptake in plants (Siddiqi and Glass, 1986; Benlloch et al., 1989; Kochian and Lucas, 1983; Fernando et al., 1990; Fernando and Glass, 1992; Maathuis and Sanders, 1996; Shin and Schachtman, 2004). This activation has been conventionally associated with induction of the expression of high-affinity transporters and considered as a major mechanism of adaptation to  $K^+$  starvation (Drew et al., 1984; Fernando et al., 1990).

Sensitivity to  $NH_4^+$  is an important feature of high-

affinity  $K^+$  uptake; in *Arabidopsis* (Spalding et al., 1999), barley (Santa-Maria et al., 2000) and pepper (Martinez-Cordero et al., 2005), both  $NH_4^+$ -sensitive and  $NH_4^+$ -insensitive components have been identified. The  $NH_4^+$ -sensitive component is probably mediated by HAK1 transporters, whereas, in *Arabidopsis*, the  $NH_4^+$ -insensitive component has been postulated to be mediated by the inward-rectifier  $K^+$  channel AtAKT1, indicating that channels may be involved in high-affinity  $K^+$  uptake in a range of  $K^+$  concentrations (Hirsch et al., 1998; Spalding et al., 1999).  $NH_4^+$  is not only an important tool to study the  $K^+$  transport system in plants but also used in fertilizers. The interactions between  $NH_4^+$  and  $K^+$  are very important for crop management, especially when  $K^+$  concentration decreases or salinity increases.

Tetraethylammonium (TEA) is considered a specific blocker of voltage-gated  $K^+$  channels.  $Ba^{2+}$  and  $Cs^+$  inhibit  $K^+$  uptake through most  $K^+$  channels and some other transporters (Hedrich and Schroeder, 1989; Tester, 1990; Hille, 1992; Fu and Luan, 1998). According to the values of electrical distance, blockers can be classified as "surface" if they act at the entrance of the pore (toxins or quaternary ammonium ions in  $K^+$  channels) and as "deep" if they deeply enter the pore by slowly passing a selective filter (for example,  $Na^+$  and  $Cs^+$  in  $K^+$  channels) (French and Shoukimas, 1985). The alkali cation  $Cs^+$  acts as a  $K^+$  analogue and is also toxic to plants (White and Broadley, 2000). It is well known that  $Cs^+$  is a competitive inhibitor of  $K^+$  and acts as a  $K^+$  channel blocker (White and Broadley, 2000; Zhu and Smolders, 2000);  $Cs^+$  accumulation in plants decreases with increasing  $K^+$  concentration (Smolders et al., 1996; Tsukada et al., 2002). However, short-term  $K^+$  starvation can increase  $Cs^+$  influx, indicating the importance of internal and external  $K^+$  status (Zhu and Smolders, 2000). Fu and Luan (1998) reported the inhibition of AtKUP1-mediated  $K^+$  uptake by  $K^+$  channel blockers, such as TEA,  $Cs^+$ , and  $Ba^{2+}$ . Consistent with a possible function in  $K^+$  uptake from the soil, the *AtKUP1* gene is primarily expressed in roots. Therefore, the authors concluded that the *AtKUP1* gene product may function as a  $K^+$  transporter in *Arabidopsis* roots over a broad range of concentration of  $K^+$  in soil. Another possibility is that these inhibitors (including TEA) may block  $K^+$  influx through other  $K^+$  transporters aside from voltage-gated  $K^+$  channels (Hille, 1992).

In pepper (*Capsicum annum*), it has been demonstrated that HAK1 transporters greatly contribute to the high-affinity  $K^+$  uptake in roots (Martinez-Cordero et al., 2004, 2005).  $K^+$  starvation in pepper plants promotes high-affinity  $K^+$  uptake ( $K_m$  of 6  $\mu M$   $K^+$ ) that is very sensitive to ammonium ( $NH_4^+$ ); indeed, the high-affinity  $K^+$  transporter (CaHAK1) is expressed in their roots. When expressed in yeast (*Saccharomyces cerevisiae*), CaHAK1 mediates high-affinity  $K^+$  and  $Rb^+$  uptake with  $K_m$  values of 3.3 and 1.9  $\mu M$ , respectively.  $Rb^+$  uptake

can be competitively inhibited by micromolar concentrations of  $\text{NH}_4^+$  and  $\text{Cs}^+$  and by millimolar concentrations of  $\text{Na}^+$  (Martinez-Cordero et al., 2004, 2005).

To date, functional characterization in pepper plants about structural and regulatory elements involved in  $\text{K}^+$  uptake has only been conducted in *C. annuum* (Martinez-Cordero et al., 2004, 2005; Rubio et al., 2000). Comparative studies in *Capsicum* will support its role as a model system to investigate the physiology of  $\text{K}^+$  uptake in pepper. In addition, it could be important for studying  $\text{K}^+$  nutrition of this crop under  $\text{K}^+$ -limiting conditions and in the presence of abiotic stress. Consequently, intensive studies are necessary to elucidate the nature of the systems contributing to  $\text{K}^+$  transport in pepper plants. Knowledge about the effects of  $\text{K}^+$  starvation,  $\text{K}^+$  re-supplementation and  $\text{K}^+$  uptake in pepper plants will allow more understanding of the roles of the  $\text{K}^+$  transport system. To explore the relative contribution of the components involved in  $\text{K}^+$  transport, we examined  $\text{K}^+$  uptake under the conditions of  $\text{K}^+$  starvation and  $\text{K}^+$  re-supplementation in habanero pepper plantlets, as well as the effects of  $\text{NH}_4^+$ ,  $\text{CsCl}$  and TEA on  $\text{K}^+$  uptake by habanero pepper roots.

## MATERIALS AND METHODS

### Plant materials

Seeds of habanero pepper fruits (*Capsicum chinense* Jacq.) were used in this study. Disinfection was achieved under aseptic conditions. The seeds were surface-sterilized in 80% ethanol for 5 min followed by rinses in sterilized, distilled and deionized water for 4 min. Subsequently, 30% sodium hypochlorite was added and the seeds were imbibed for 15 min with continuous shaking and rinsed in sterile water. Finally, the seeds were left soaking in sterile water for 72 h and germinated in agrolite at 28°C. After seven days, the seedlings were placed in 600 ml plastic containers filled with a modified 1/5 (of its ionic strength) Hoagland solution (H1/5) containing both the macronutrients, including 1.2 mM  $\text{KNO}_3$ , 0.8 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.2 mM  $\text{KH}_2\text{PO}_4$  and 0.2 mM  $\text{MgSO}_4$  and the micronutrients, including 50  $\mu\text{M}$   $\text{CaCl}_2$ , 12.5  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 1  $\mu\text{M}$   $\text{MnSO}_4$ , 1  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.5  $\mu\text{M}$   $\text{CuSO}_4$ , 0.1  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.1  $\mu\text{M}$   $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  and 10  $\mu\text{M}$  Fe-EDDHA. The plantlets were grown under conditions of photoperiod (16 h light/8 h dark) and air temperature of 20 and 25°C, respectively. The relative humidity was 65% (day) and 85% (night). The nutrient solution was replaced weekly with fresh  $\text{K}^+$ -free solution (H1/5-K). In all experiments, 45-days-old plantlets were used.

### Physiological studies of $\text{K}^+$ uptake in pepper plantlets

#### $\text{K}^+$ depletion and $\text{K}^+$ uptake

For  $\text{K}^+$  starvation treatment, plantlets were transferred into a  $\text{K}^+$ -free nutrient solution (H1/5-K) and incubated for different time periods (0, 72, 96 and 120 h). This solution contained 1.4 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.1 mM  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ , 0.2 mM  $\text{MgSO}_4$  and the micronutrients described earlier. After the incubation, the plantlets with a uniform size were selected and homogeneously weighed in groups of 15 plantlets. The plantlets roots were then rinsed with cold H1/5-K solution and at time zero, were transferred to 250 ml containers filled with the

same solution supplemented with 50, 100 or 200  $\mu\text{M}$  of  $\text{KCl}$  ( $\text{K}^+$ ). Medium samples of 1 ml were obtained at intervals of 30 min and  $\text{K}^+$  concentration was determined by atomic absorption using a Perkin-Elmer 5500 spectrophotometer (Boston, MA, USA). Control plantlets were maintained in the  $\text{K}^+$ -containing nutrient solution described earlier (H1/5).

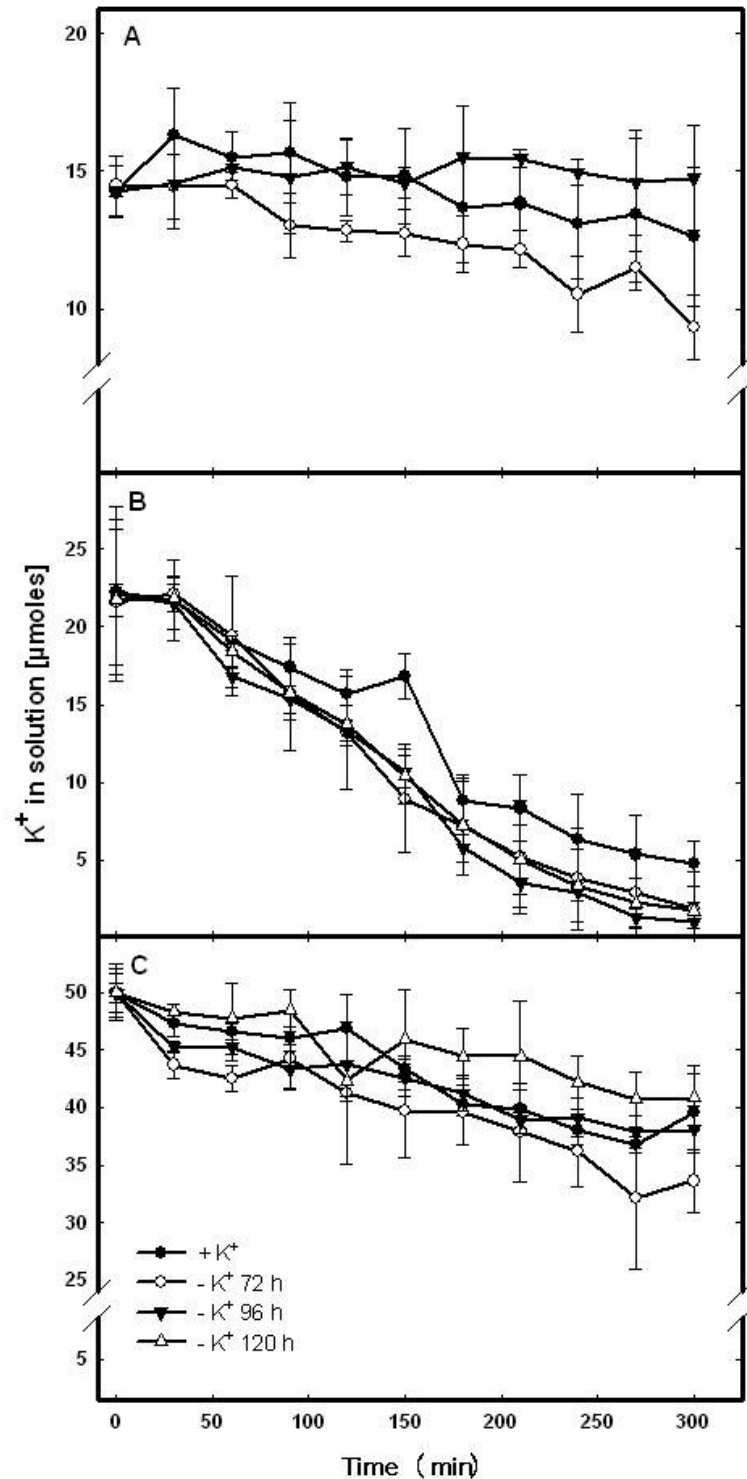
#### Effect of the presence of $\text{NH}_4^+$ or other $\text{K}^+$ channel blockers on $\text{K}^+$ uptake

$\text{NH}_4\text{NO}_3$  was used as an  $\text{NH}_4^+$  source. Plantlets were subjected to  $\text{K}^+$  starvation for three days. Subsequently, the plantlets were transferred to the H1/5-K solution immediately re-supplemented with 50 or 100  $\mu\text{M}$   $\text{KCl}$  ( $\text{K}^+$ ) and maintained in the presence of 0, 250, 500 or 1,000  $\mu\text{M}$   $\text{NH}_4^+$ . For channel blocker treatment, after  $\text{K}^+$  re-supplementation, the plantlets were maintained in the presence of 10 mM  $\text{CsCl}$  or 20 mM TEA. Medium aliquots (1 ml) were obtained at intervals of 30 min for 5 h and their  $\text{K}^+$  concentrations were determined using a Perkin-Elmer 5500 spectrophotometer (Boston, MA, USA).

## RESULTS AND DISCUSSION

### $\text{K}^+$ uptake by habanero pepper roots

To evaluate the effect of  $\text{K}^+$  starvation on  $\text{K}^+$  uptake by pepper roots, the plantlet roots grown in the H1/5-K solution and maintained for different time periods were rinsed with the same cold solution and, at time zero, were transferred to containers filled with 250 ml of the H1/5-K solution supplemented with 50, 100 or 200  $\mu\text{M}$   $\text{K}^+$ . The analysis of  $\text{K}^+$  uptake in a solution containing 50  $\mu\text{M}$   $\text{K}^+$  showed a decrease of the  $\text{K}^+$  concentration in the solution, indicating that the plantlet roots were capable of absorbing external  $\text{K}^+$ ; this uptake was dependent on the duration of the  $\text{K}^+$  starvation treatment. The plantlets subjected to  $\text{K}^+$  starvation for 72 h exhibited an increased  $\text{K}^+$  uptake, whereas the plantlets subjected to  $\text{K}^+$  starvation for 96 h showed low  $\text{K}^+$  uptake that was not significantly different from that in control plantlets (Figure 1a). The net  $\text{K}^+$  uptake, calculated as the difference between total uptake (the amount of  $\text{K}^+$  depleted from the solution) and total release (the amount of  $\text{K}^+$  increased in the solution), was approximately five times higher in the plantlets treated with  $\text{K}^+$  starvation for 72 h than that in the control plantlets growing in the  $\text{K}^+$ -containing medium, when they were both supplemented with 50  $\mu\text{M}$   $\text{K}^+$  (Table 1). However, there was no net  $\text{K}^+$  uptake in the plantlets treated with  $\text{K}^+$  starvation for 96 h. Regardless of the duration of  $\text{K}^+$  starvation that the plantlets were subjected to, the total  $\text{K}^+$  uptake under these conditions was between 5 and 8  $\mu\text{M}$ ; there were no significant differences among the treatments (Table 1). The net adsorption in the plantlets treated with  $\text{K}^+$  starvation for 96 h and that in the control plantlets were much less than the total uptake, suggesting that  $\text{K}^+$  was released into the medium solution under these conditions. This phenomenon was not observed in the plantlets treated with  $\text{K}^+$



**Figure 1.** Effect of  $K^+$  starvation on high-affinity  $K^+$  uptake. Plantlets were grown in the H1/5 solution (1.4 mM  $K^+$ ) for 45 days and then transferred to a  $K^+$ -free solution for 0 h (close circles), 72 h (open circles), 96 h (close triangles) or 120 h (open triangles). Subsequently, KCl was added to the medium solution as a  $K^+$  source to a concentration of 50  $\mu\text{M}$  (A), 100  $\mu\text{M}$  (B) or 200  $\mu\text{M}$  (C). Samples of medium solution were obtained at different time points;  $K^+$  content was determined. Data represent the mean  $\pm$  standard deviations of three independent experiments,  $n = 3$ .

**Table 1.** Total and net K<sup>+</sup> uptake in habanero pepper plantlets. The plantlets were grown in the H1/5 solution (1.4 mM K<sup>+</sup>) for 45 days and transferred to the H1/5-K solution for 0, 72, 96 and 120 h.

Treatment	K <sup>+</sup> uptake ([KCl] μmole)					
	50		100		200	
	Total uptake	Net uptake	Total uptake	Net uptake	Total uptake	Net uptake
+ K <sup>+</sup>						
0 h	6.01±0.53	1.54±0.22	22.61±8.76	14.01±6.72	14.67±4.01	11.33±0.74
· K <sup>+</sup>						
72 h	8.36±0.73	7.22±0.21	22.19±1.89	19.70±3.86	20.04±1.00	13.89±0.49
96 h	5.08±1.19	0.64±2.01	22.70±2.57	20.99±0.73	11.22±1.83	2.57±1.16
120 h	N.A	N.A	20.13±0.53	20.04±0.51	17.64±2.93	5.99±2.27

At time zero, KCl was added to the medium solution to a concentration of 50, 100 or 200 μM. K<sup>+</sup> content was measured as described in materials and methods. K<sup>+</sup> content is expressed in micromole. Data represent the mean ± standard deviations of three independent experiments, n = 3.

starvation for 72 h (Table 1). Maathuis and Sanders (1996) reported K<sup>+</sup> uptake at concentrations less than 1 μM in several species, whereas Sheahan et al. (1993) noted K<sup>+</sup> uptake at 50 to 100 μM concentrations. Borges et al. (2006) suggested a demand for K<sup>+</sup> uptake when habanero pepper plantlet roots were imbibed in a solution at a K<sup>+</sup> concentration higher than 89 μM. To prevent endogenous K<sup>+</sup> released from the plants to external solution, K<sup>+</sup> concentrations should be above 89 μM. In this study, we observed K<sup>+</sup> uptake at 50 μM K<sup>+</sup> without K<sup>+</sup> releasing into the medium solution, particularly with the 72-h K<sup>+</sup> starvation treatment. This result contradicts a previous finding reported by Borges et al. (2006). Our work is in agreement with the report of Martinez-Cordero et al. (2005) showing that *C. annuum* plants treated with K<sup>+</sup> starvation for 2 to 8 h and subsequently re-supplemented with 50 μM K<sup>+</sup> did not exhibit K<sup>+</sup> uptake, whereas in the plants treated with K<sup>+</sup> starvation for more than 24 h, K<sup>+</sup> uptake was observed; furthermore, with K<sup>+</sup> starvation for a longer time, more K<sup>+</sup> uptake was noted.

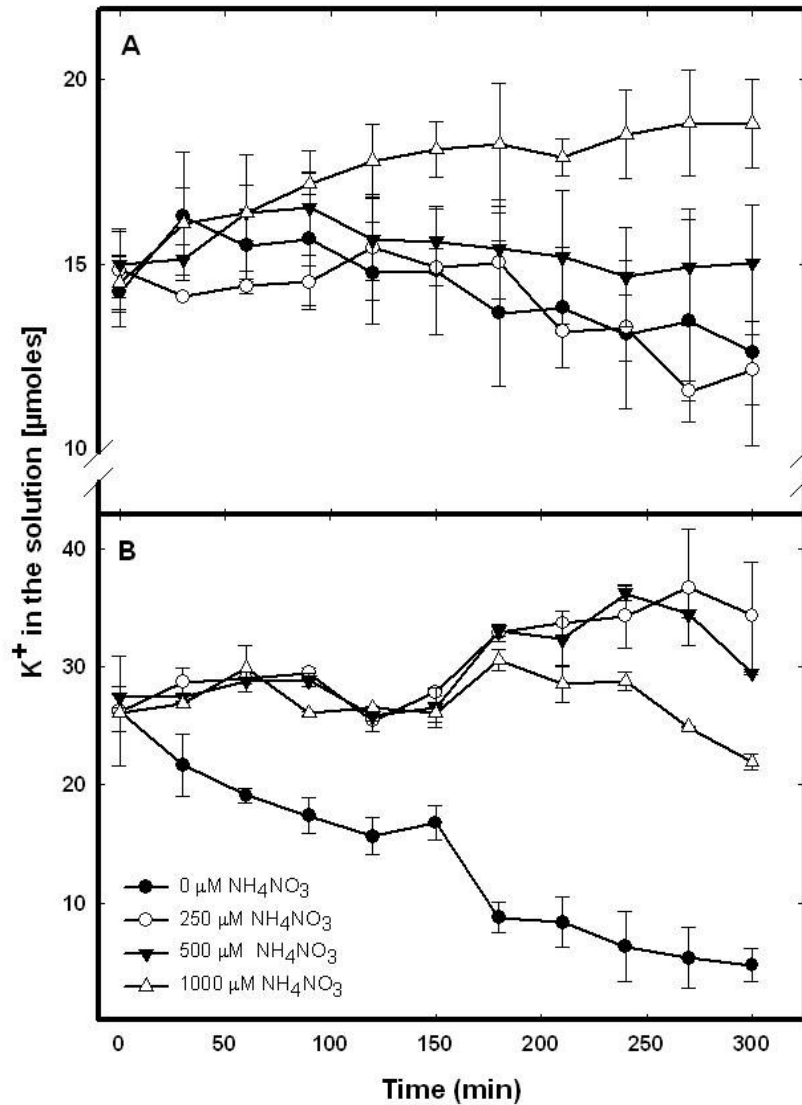
When the seedlings treated with K<sup>+</sup> starvation for different time periods were placed in the presence of 100 μM K<sup>+</sup>, a significant reduction of K<sup>+</sup> was observed in the medium solution, suggesting a K<sup>+</sup> uptake by the plantlets roots (Figure 1b). There was a tendency for higher K<sup>+</sup> uptake in the plantlets subjected to K<sup>+</sup> starvation after 90 min of K<sup>+</sup> exposure (Figure 1b). There were no significant differences between the net K<sup>+</sup> uptake by the K<sup>+</sup>-re-supplemented plantlet roots and the control plantlets subjected to K<sup>+</sup> starvation (20 and 14 μM, respectively; Table 1). Under this condition (100 μM K<sup>+</sup>), we did not observe a significant K<sup>+</sup> release into the medium solution, evidenced by the result that the net uptake was similar to the total uptake in all the treatments (Table 1). The K<sup>+</sup> uptake in the plantlets exposed to 100 μM K<sup>+</sup> was higher compared with that in the plantlets exposed to 50 μM K<sup>+</sup> (Figure 1a). Similar results have been reported in *C. annuum* plants (Martinez-Cordero et al., 2005). Furthermore, the plantlets treated with K<sup>+</sup> starvation for 72 h

showed an increased K<sup>+</sup> uptake when transferred to the medium solution with 200 μM K<sup>+</sup> (Figure 1c). The net K<sup>+</sup> uptake was higher with 72-h K<sup>+</sup> starvation (13.89 ± 0.49 μM), significantly different from that in the control plantlets (11.33 ± 0.74 μM) (Table 1). It is noteworthy that with the 96 and 120 h K<sup>+</sup> starvation treatments, the net K<sup>+</sup> uptake was significantly reduced compared with that in the control plantlets and this value was much lower than the total K<sup>+</sup> uptake value under every condition, indicating that K<sup>+</sup> was released into the medium solution (Table 1). In general, with 200 μM K<sup>+</sup> re-supplementation, the K<sup>+</sup> uptake decreased compared with 100 μM K<sup>+</sup> re-supplementation, suggesting that external K<sup>+</sup> concentration may affect the activity of K<sup>+</sup> high-affinity uptake.

### Effect of NH<sub>4</sub><sup>+</sup> on K<sup>+</sup> uptake

We used 45-day-old habanero pepper plantlets to study the effect of NH<sub>4</sub><sup>+</sup> on K<sup>+</sup> uptake. The experimental conditions were 72 h K<sup>+</sup> starvation followed by a re-supplementation with 50 or 100 μM K<sup>+</sup>. These conditions were chosen because with 72 h K<sup>+</sup> starvation and the re-supplementation with 50 μM K<sup>+</sup>, the net K<sup>+</sup> uptake by the roots was significantly higher than that with the control treatment, and this was the only treatment without inducing a K<sup>+</sup> release, whereas the 72 h K<sup>+</sup> starvation followed by 100 μM K<sup>+</sup> re-supplementation caused a K<sup>+</sup> release into the solution. Under these conditions (100 μM), the net K<sup>+</sup> uptake was higher than that with the other treatments (50 and 200 μM). Thus, habanero pepper plantlets treated with 72 h K<sup>+</sup> starvation and subsequently placed in a solution containing 50 μM K<sup>+</sup> in the presence of 250, 500 or 1,000 μM of NH<sub>4</sub><sup>+</sup> showed a significant reduction in K<sup>+</sup> uptake (Figure 2a). Exposure of the plantlets to a low concentration (250 μM) of NH<sub>4</sub><sup>+</sup> reduced the K<sup>+</sup> content in the solution during the first 30 min, indicating a K<sup>+</sup> uptake during this period (Figure 2a).

However, from 30 min to 3 h, there was a slight increase



**Figure 2.** Effect of  $\text{NH}_4^+$  on high-affinity  $\text{K}^+$  uptake. Plantlets were grown in the H1/5 solution (1.4 mM  $\text{K}^+$ ) for 45 days and then transferred to a  $\text{K}^+$ - and nitrogen-free solution supplemented with 50  $\mu\text{M}$   $\text{K}^+$  (A) or 100  $\mu\text{M}$   $\text{K}^+$  (B), together with 250  $\mu\text{M}$   $\text{NH}_4^+$  (open circles), 500  $\mu\text{M}$   $\text{NH}_4^+$  (close triangles) or 1 mM  $\text{NH}_4^+$  (open triangles), using  $\text{NH}_4\text{NO}_3$  as a nitrogen source. Samples of medium solution were obtained at different time points;  $\text{K}^+$  content was determined. Data represent the mean  $\pm$  standard deviation of three independent experiments,  $n = 3$ .

in  $\text{K}^+$  concentration in the solution, indicating a  $\text{K}^+$  release from the roots. After 3 h, there was a more significant  $\text{K}^+$  decrease in the solution. With the treatment of 500 or 250  $\mu\text{M}$   $\text{NH}_4^+$ , the observation was similar, at early time points,  $\text{K}^+$  concentration in the solution increased (indicating a  $\text{K}^+$  release), whereas at later time points,  $\text{K}^+$  concentration decreased or remained constant, indicating a small  $\text{K}^+$  influx (Figure 2a). When  $\text{NH}_4^+$  concentration increased to 1,000  $\mu\text{M}$ , an increase in  $\text{K}^+$  concentration was observed in the solution for 5 h, indicating a  $\text{K}^+$  release. As shown in Table 2, there was a reduction in the total  $\text{K}^+$  uptake in those plantlets exposed to 50  $\mu\text{M}$   $\text{K}^+$

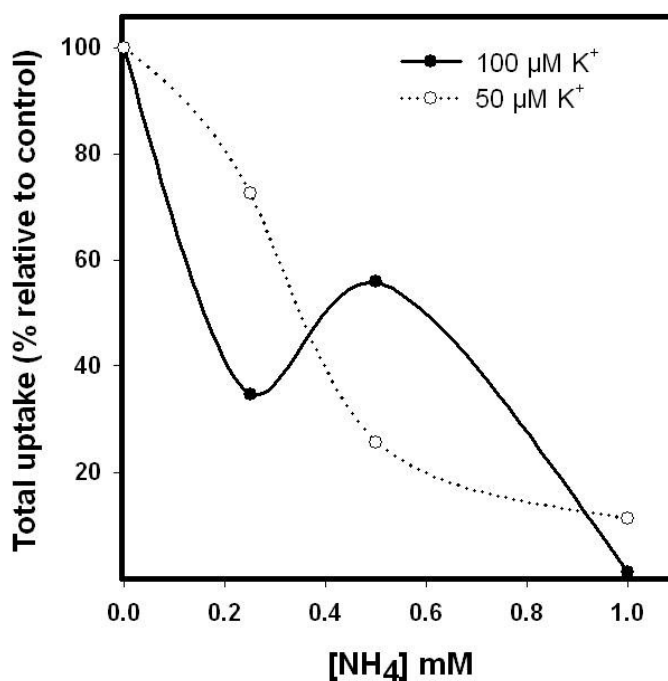
in the presence of  $\text{NH}_4^+$  compared with that in controls. This reduction was  $\text{NH}_4^+$  dose-dependent. The net  $\text{K}^+$  uptake was reduced by over 60% in the plantlets exposed to 250  $\mu\text{M}$   $\text{NH}_4^+$  compared with that in controls and higher doses of  $\text{NH}_4^+$  led to a 100% inhibition of the net  $\text{K}^+$  uptake, significantly favoring a  $\text{K}^+$  release from the roots.

When plantlets were treated with  $\text{K}^+$  starvation for 72 h followed by 100  $\mu\text{M}$   $\text{K}^+$  re-supplementation in the presence of  $\text{NH}_4^+$  at different concentrations, the effect of  $\text{K}^+$  uptake inhibition was significant compared with the control plantlets (Figure 2b). There was a  $\text{K}^+$  release into

**Table 2.** Total and net K<sup>+</sup> uptake in habanero pepper plantlets in the presence of NH<sub>4</sub>NO<sub>3</sub>, CsCl and TEA. Plantlets were grown in the H1/5 solution (1.4 mM K<sup>+</sup>) for 45 days and transferred to the H1/5-K solution for 72 h.

Parameter	K <sup>+</sup> uptake ([KCl] μmole)			
	50		100	
	Total uptake	Net uptake	Total uptake	Net uptake
Control	8.36±0.73	7.22±0.21	22.19±1.89	19.70±3.86
[NH <sub>4</sub> NO <sub>3</sub> ] μM				
250	6.07±1.92	2.69±2.07	7.69±1.57	-5.43±0.01
500	2.14±0.45	-0.04±0.70	12.39±3.48	-0.91±0.81
1000	0.94±0.59	-4.29±0.47	0.28±0.15	-0.03±0.02
CsCl 10 mM	2.21±1.73	-16.19±2.39	8.35±5.9	-5.52±3.89
TEA 20 mM	4.44±0.70	-6.42±0.47	21.36±0.25	9.72±2.11

At time zero, KCl was added to the medium solution to a concentration of 50, 100, or 200 μM. K<sup>+</sup> content was measured as described in "Materials and Methods". K<sup>+</sup> content is expressed in micromole. Data represent the mean ± standard deviations of three independent experiments, n = 3.



**Figure 3.** Dose-response curve of total K<sup>+</sup> uptake to NH<sub>4</sub><sup>+</sup> treatment. Roots treated with K<sup>+</sup> starvation for 72 h were exposed to different concentrations of NH<sub>4</sub><sup>+</sup> (250, 500, or 1,000 μM) in the presence of 50 μM K<sup>+</sup> (○) or 100 μM K<sup>+</sup> (●). Medium aliquots (1 ml) were obtained after 5 h; K<sup>+</sup> content was determined. Data represent the mean ± standard deviation of three independent experiments, n = 3.

the solution at the end of the evaluation period, which was increased in the presence of 250 or 500 μM NH<sub>4</sub><sup>+</sup>. Also, the plantlets exposed to 100 μM K<sup>+</sup> in the presence of NH<sub>4</sub><sup>+</sup> for 5 h showed a significant reduction in total K<sup>+</sup> uptake; this reduction was observed with both doses of NH<sub>4</sub><sup>+</sup> treatment (250 and 1000 μM) (Table 2). Under these conditions, a net K<sup>+</sup> uptake did not occur; on the

contrary, we observed a significant K<sup>+</sup> release into the solution.

Plotting total K<sup>+</sup> uptake versus NH<sub>4</sub><sup>+</sup> concentration, we observe that the total K<sup>+</sup> uptake of the plantlets treated with 50 μM K<sup>+</sup> reduced completely in a dose-dependent manner (Figure 3); treatment of 340 μM NH<sub>4</sub><sup>+</sup> induced an approximately 50% inhibition of K<sup>+</sup> uptake. However, a

different effect was shown with the treatment of 100  $\mu\text{M}$   $\text{K}^+$ : in this case, we observed a more significant reduction (over 60% inhibition) in total  $\text{K}^+$  uptake in the plantlets exposed to 250  $\mu\text{M}$   $\text{NH}_4^+$ , whereas  $\text{NH}_4^+$  treatment between 250 and 500  $\mu\text{M}$  did not cause an increased inhibition, suggesting the presence of alternative transportation systems that are not sensitive to  $\text{NH}_4^+$ . However, with an even higher dose of  $\text{NH}_4^+$  (1,000  $\mu\text{M}$ ), the total  $\text{K}^+$  uptake were completely inhibited (Figure 3). It is necessary to note that with the 50  $\mu\text{M}$   $\text{NH}_4^+$  treatment, we did not observe a reduction in  $\text{K}^+$  uptake (Figure 3).

Many studies have reported that  $\text{K}^+$  influx mediated by high-affinity transport systems can be severely inhibited by  $\text{NH}_4^+$  (Scherer et al., 1984; Vale et al., 1987; 1988a, b; Wang et al., 1996; Spalding et al., 1999; Santa-Maria et al., 2000; Bañuelos et al., 2002; Kronzucker et al., 2000; Martinez-Cordero et al., 2004; Szczerba et al., 2006, 2008 a, b; Nieves-Cordones et al., 2007). The mechanism by which  $\text{NH}_4^+$  inhibits  $\text{K}^+$  influx through high-affinity transporters has not been firmly established. However, it could be due to the direct competition between  $\text{NH}_4^+$  and  $\text{K}^+$  transport systems (Vale et al., 1987; Wang et al., 1996; White, 1996; Britto and Kronzucker, 2002, 2008).

The plantlet response to 100  $\mu\text{M}$   $\text{K}^+$  observed in this work could be explained by the results from previous studies. Buschmann et al. (2000) reported an increase in the *AKT1* gene transcript when  $\text{K}^+$  was eliminated in wheat plants, suggesting that  $\text{K}^+$  channels may play an important role in  $\text{K}^+$  uptake. Electrophysiological studies have shown that a  $\text{NH}_4^+$ -insensitive component is specific for Shaker  $\text{K}^+$  channel (Bertl et al., 1995; White, 1996; Hirsch et al., 1998; Moroni et al., 1998; Spalding et al., 1999; Szczerba et al., 2008b). Finally, the differential  $\text{NH}_4^+$  susceptibility between high- and low-affinity transport systems demonstrates the ability of *AKT1* to mediate high-affinity  $\text{K}^+$  transport because high-affinity  $\text{K}^+$  transport systems can be inhibited by  $\text{NH}_4^+$  treatment in *Arabidopsis thaliana*; additionally, an *Akt1* mutant exhibited growth inhibition at low  $\text{K}^+$  concentrations, whereas wild-type plants were less affected, indicating that *AKT1* acts in response to  $\text{K}^+$  concentration change through high-affinity  $\text{K}^+$  transport systems (Hirsch et al., 1998; Spalding et al., 1999). According to our results, we propose a model to explain the behavior of  $\text{K}^+$  uptake by habanero pepper roots in the presence of  $\text{NH}_4^+$ . In the presence of 50  $\mu\text{M}$   $\text{K}^+$  and 250 or 500  $\mu\text{M}$   $\text{NH}_4^+$ ,  $\text{K}^+$  uptake by the roots is inhibited by  $\text{NH}_4^+$ . Thus, high-affinity  $\text{K}^+$  transporters are highly sensitive to  $\text{NH}_4^+$ , whereas the low-affinity  $\text{K}^+$  transport system is inactive when  $\text{K}^+$  concentration is too low; the low-affinity  $\text{K}^+$  transport system requires  $\text{K}^+$  concentrations higher than 50  $\mu\text{M}$  to be activated (Figure 4a, b). In fact, when  $\text{K}^+$  concentrations are increased to 100  $\mu\text{M}$  in the presence of 250 or 500  $\mu\text{M}$  of  $\text{NH}_4^+$ , an increase in  $\text{K}^+$  uptake occurs through a  $\text{NH}_4^+$ -insensitive  $\text{K}^+$  transport system as shown in Figure 4c. When  $\text{NH}_4^+$  concentration increased (500  $\mu\text{M}$ ),  $\text{K}^+$  uptake increased (Figure 4d); previous

evidence also suggest the presence of a  $\text{NH}_4^+$ -insensitive  $\text{K}^+$  transport system mediated by *AKT1* channels (Gierth and Masser, 2007).

### Effect of CsCl and TEA on $\text{K}^+$ uptake

To study the mechanisms of high-affinity  $\text{K}^+$  uptake in habanero pepper plantlet roots, we used two compounds: CsCl and TEA, which have been commonly used as  $\text{K}^+$  channel blockers in animal and plant cells (Tester, 1990; Hille, 1992; Fu and Luan, 1998; Hong-Yan et al., 2006).

Plantlets treated with  $\text{K}^+$  starvation for 72 h were then transferred to the solution containing 50  $\mu\text{M}$   $\text{K}^+$  and 10 mM CsCl. The  $\text{K}^+$  uptake was higher during the first 30 min compared with that in the control plantlets. However, the  $\text{K}^+$  content increased in the solution, indicating a  $\text{K}^+$  release from the roots. This release peaked at 5 h (Figure 4a). Total  $\text{K}^+$  uptake by the roots during this period was inhibited by over 70%; no net uptake was observed and a high  $\text{K}^+$  release into the solution was observed (Table 2).

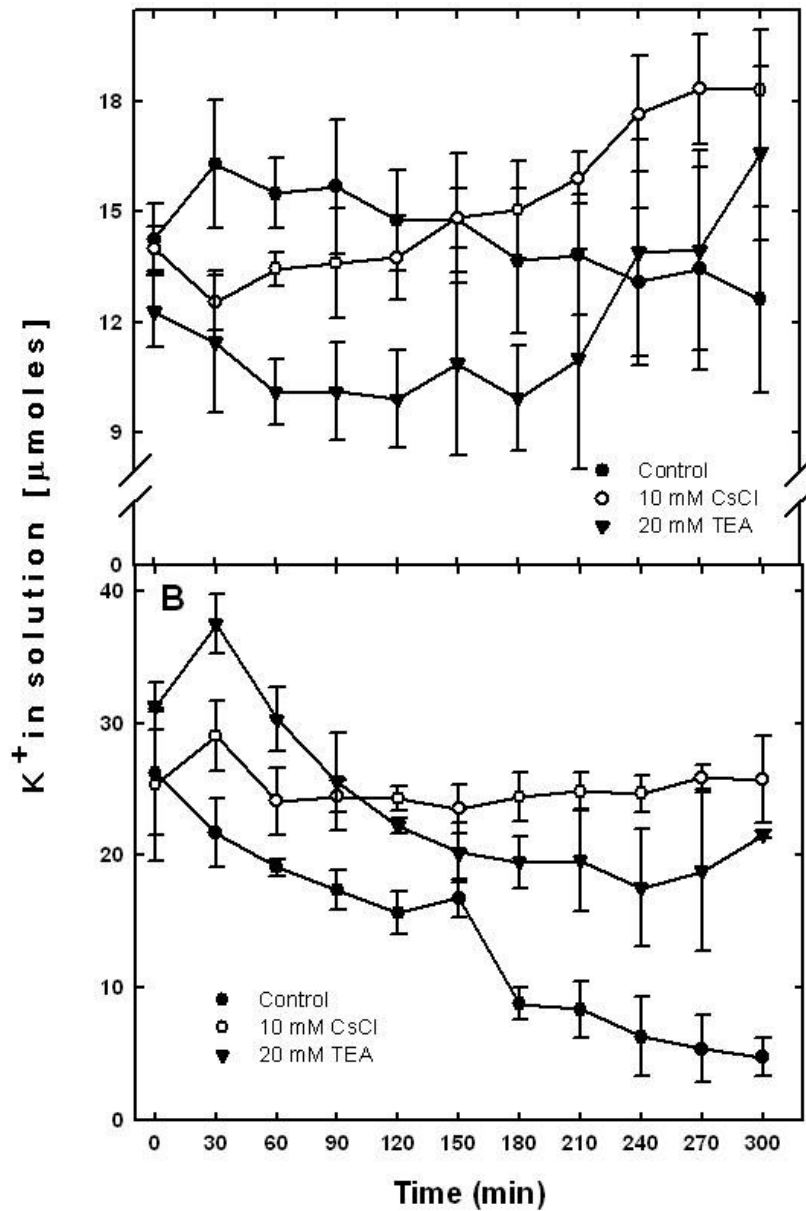
These results suggest that in the plantlets treated with 10 mM CsCl, a  $\text{K}^+$  uptake occurred 150 min after the treatment and the plantlets released endogenous  $\text{K}^+$  that they contained before the treatment. Our results are in agreement with the previous observation by Hong-Yan et al. (2006) showing that in the rice roots treated with 30 mM CsCl and 0.25 mM  $\text{K}^+$  for 3 h, the  $\text{K}^+$  content decreased from  $5.41 \times 10^{-4}$  mol.  $\text{g}^{-1}$  dry weight to  $4.99 \times 10^{-4}$  mol.  $\text{g}^{-1}$  dry weight.

By analyzing  $\text{K}^+$  uptake in the solution containing 100  $\mu\text{M}$   $\text{K}^+$  in the presence of 10 mM CsCl, we observed that  $\text{K}^+$  was initially released and then  $\text{K}^+$  uptake was maintained at a stable level (Figure 4b); total  $\text{K}^+$  uptake in the treated plantlets was inhibited by 62% ( $8.35 \pm 5.90$   $\mu\text{M}$   $\text{K}^+$ ) compared with that in the control plantlets ( $22.19 \pm 1.89$   $\mu\text{M}$   $\text{K}^+$ ) and the net  $\text{K}^+$  uptake value was negative because of the observed  $\text{K}^+$  release into the solution ( $5.52 \pm 3.89$   $\mu\text{M}$ ; Figure 4b; Table 2). The  $\text{K}^+$  release of the 100  $\mu\text{M}$   $\text{K}^+$ -treated plantlets was lower than that of the 50  $\mu\text{M}$   $\text{K}^+$ -treated plantlets.

Regarding the effect of TEA on high-affinity  $\text{K}^+$  uptake, we observed an initial  $\text{K}^+$  uptake in the plantlets treated with  $\text{K}^+$  starvation for 72 h and subsequently, transferred to the solution with 50  $\mu\text{M}$   $\text{K}^+$  in the presence of 20 mM TEA; however, after 3 h, there was a significant  $\text{K}^+$  release peaking at 5 h (Figure 4a). Total  $\text{K}^+$  uptake was reduced by almost 50% compared with that in the control; the release was higher than the influx; therefore, negative values were obtained for the net  $\text{K}^+$  uptake (Table 2).

Furthermore, when the solution with 100  $\mu\text{M}$   $\text{K}^+$  and 20 mM TEA was used, a  $\text{K}^+$  release was observed during the first 30 min, as well as an increased  $\text{K}^+$  uptake in relation to time; this pattern sustained for 150 min and  $\text{K}^+$  uptake was maintained at the same level until 300 min (Figure 4b). The TEA-treated plantlets exhibited a 4% inhibition in total  $\text{K}^+$  uptake ( $21.36 \pm 0.25$   $\mu\text{M}$  of  $\text{K}^+$ ) compared with the

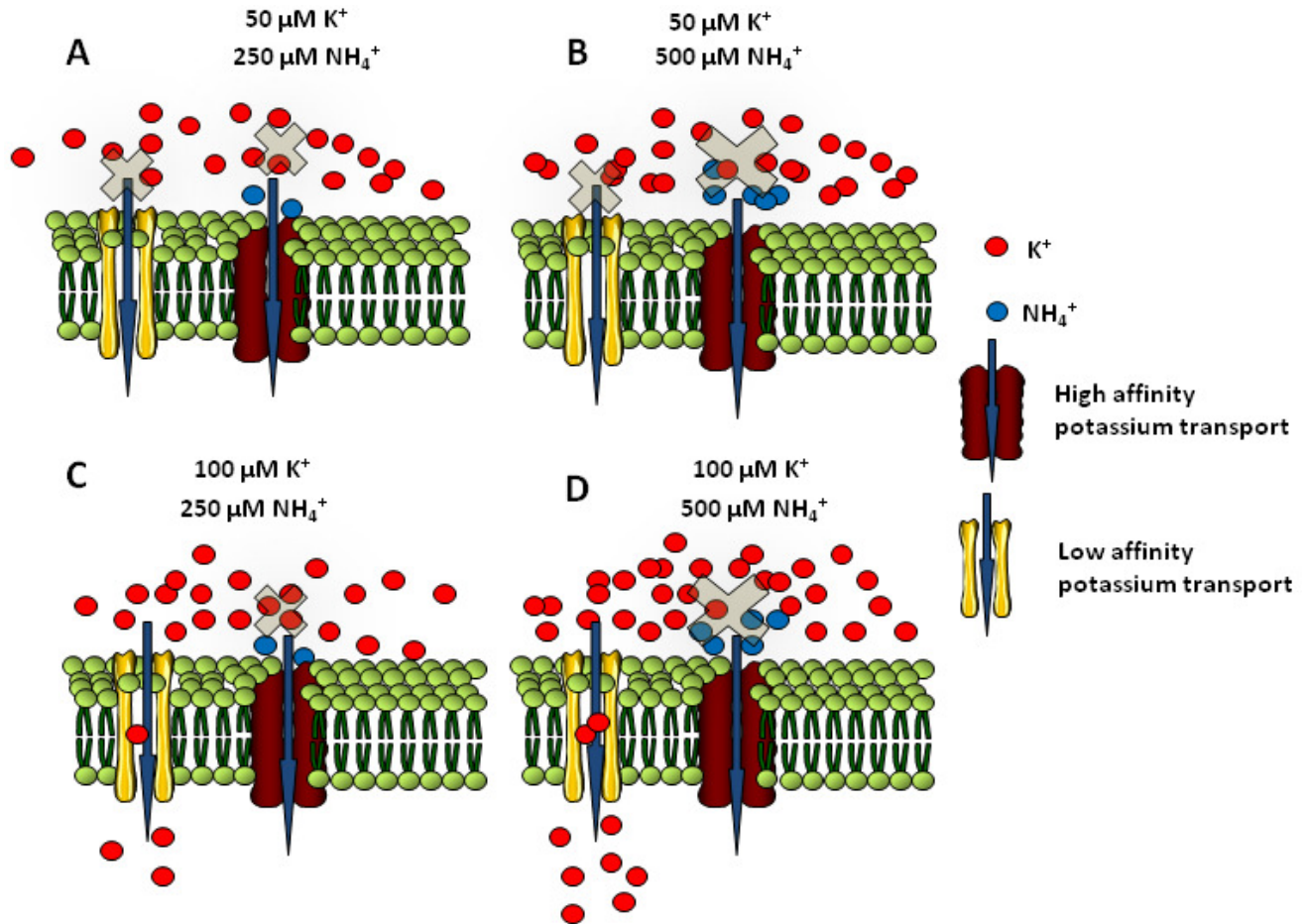




**Figure 4.** Effect of K<sup>+</sup> transport blockers on K<sup>+</sup> uptake. Plantlets were grown in the H1/5 solution (1.4 mM K<sup>+</sup>) for 45 days and then were transferred to the H1/5-K solution re-supplemented with 50 μM K<sup>+</sup> (A) or 100 μM K<sup>+</sup> (B), together with 10 mM CsCl (open circles) or 20 mM TEA (closed circles). Samples of medium solution were taken at different time points, K<sup>+</sup> content was determined. Data represent the mean ± standard deviation of three independent experiments, n

control plantlets ( $22.19 \pm 1.89 \mu\text{M}$  of K<sup>+</sup>); the net K<sup>+</sup> uptake of the treated plantlets was  $9.72 \pm 2.11 \mu\text{M}$ , 51% lower than that of the control plantlets ( $19.70 \pm 3.86 \mu\text{M}$ ) (Figure 4b; Table 2). Ketchum and Poole (1990) previously mentioned that TEA, apparently, is a very ineffective K<sup>+</sup> blocker in plant cells. This view has not changed in recent years. In agreement with this view, Hong-Yan et al., (2006) did not find any significant difference in K<sup>+</sup> uptake in the rice plants exposed to 0.25

mM K<sup>+</sup> and 30 mM TEA; however, there are reports about electrophysiological studies on plant cells demonstrating an inhibition of K<sup>+</sup> uptake in the plants exposed to TEA (Wegner et al., 1994). Our results are different from the reports from Ketchum and Poole (1990) and Hong-Yan et al. (2006), in habanero pepper plantlets, an inhibition of K<sup>+</sup> uptake in the presence of TEA was observed. This inhibition could occur through a dual uptake system. It was reported that AKT1 channels may mediate K<sup>+</sup> trans-



**Figure 5.** Model illustrating the behavior of  $K^+$  uptake by habanero pepper roots in the presence of  $NH_4^+$ . In roots exposed to 50  $\mu M$   $K^+$  in the presence of 250  $\mu M$   $NH_4^+$  (A) or 500  $\mu M$   $NH_4^+$  (B),  $K^+$  uptake was inhibited by  $NH_4^+$ . High-affinity  $K^+$  transporters were highly sensitive to  $NH_4^+$  and the low-affinity  $K^+$  transport system was inactive when  $K^+$  concentration was too low. In roots exposed to 100  $\mu M$   $K^+$  in the presence of 250  $\mu M$   $NH_4^+$  (C) or 500  $\mu M$   $NH_4^+$  (D),  $K^+$  uptake increased through a  $NH_4^+$ -insensitive  $K^+$  transport system. When  $NH_4^+$  concentration increased (500  $\mu M$ ),  $K^+$  uptake also increased.

port at the similar level as a high-affinity transporter does (Hirsch et al., 1998, Spalding et al., 1999).

In conclusion, we proposed a model to explain the behavior of  $K^+$  uptake by habanero pepper roots in the presence of  $NH_4^+$ . When the roots were exposed to 50  $\mu M$   $K^+$ , together with 250 or 500  $\mu M$   $NH_4^+$ ,  $K^+$  uptake was inhibited by  $NH_4^+$  at both concentrations, due to the fact that high-affinity  $K^+$  transporters are highly sensitive to  $NH_4^+$  and this  $K^+$  concentration was too low to activate the low-affinity  $K^+$  uptake system (Figure 5a, b). However, when  $K^+$  concentration increased to 100  $\mu M$  in the presence of the same concentrations of  $NH_4^+$ ,  $K^+$  uptake increased (Figures 5c, d and 3), possibly by activating a  $K^+$  uptake system that is insensitive to  $NH_4^+$  and to  $K^+$  concentrations above 50  $\mu M$ . When the  $NH_4^+$  concentration increased to 500  $\mu M$ ,  $K^+$  uptake also increased, indicating the presence of a  $NH_4^+$ -insensitive  $K^+$  uptake system mediated by AKT1 channels. These results are in agreement with the results from Santa-Maria et al. (2000) showing that at low external  $Rb^+$  concentrations, an  $NH_4^+$ -

sensitive component dominates  $Rb^+$  uptake in plants grown in the absence of  $NH_4^+$ , whereas  $Rb^+$  uptake preferentially occurs through an  $NH_4^+$ -insensitive pathway in plants grown at high external  $NH_4^+$  concentrations. Previous studies have suggested that members of three alkali cation transporter families are likely to be involved in  $K^+$  transport into the root cytoplasm from micromolar  $K^+$  concentrations: AKT1 (Sentenac et al., 1992), HKT1 (Schachtman and Schroeder, 1994; Rubio et al., 1995) and the HAK-Kup transporters HvHAK1 and At-Kup1 (Santa-Maria et al., 1997; Fu and Luan, 1998; Kim et al., 1998).

An insertional mutant line of *AKT1* has been identified in *Arabidopsis*; it exhibits a conditional capacity to grow at micromolar  $K^+$  concentrations (Hirsch et al., 1998). This finding indicates that at least in some environments, the AKT1 inward-rectifier  $K^+$  channel could be involved in  $K^+$  transport into *Arabidopsis* from low  $K^+$  concentration environment. Interestingly, *AKT1* plants are unable to grow at low external  $K^+$  concentrations unless  $NH_4^+$  is

present at millimolar concentrations in the growth medium, indicating the presence of other parallel  $\text{NH}_4^+$ -sensitive  $\text{K}^+$  transport pathways.

$\text{NH}_4^+$ -resistant  $\text{K}^+$  transport through channels may occur at a low external concentration of  $\text{K}^+$  in rice. It has been shown by Spalding et al. (1999) that in *Arabidopsis*, 55 to 63% of  $\text{K}^+$  permeability in the high affinity transport systems HATS range can be mediated by AKT1, a channel believed to be the dominant mediator of low-affinity  $\text{K}^+$  transport (Gierth and Maser, 2007). This contribution may be even higher in rice, particularly under the conditions in the presence of  $\text{NH}_4^+$ , as suggested by Rodriguez-Navarro and Rubio (2006). Moreover, it has been shown that membrane potentials in rice are typically much less negative than those in *Arabidopsis*, particularly when rice is grown in the presence of  $\text{NH}_4^+$ , which causes permanent membrane depolarization in rice (Britto et al., 2001). Furthermore,  $\text{NH}_4^+$  may promote gene expression of high-affinity  $\text{K}^+$  transporters in rice, as previously shown with LeHAK5 in tomato plants (Nieves-Cordones et al., 2007). Conversely,  $\text{NH}_4^+$  may reduce the expression of HAK/KUP/KT transporters in rice, as previously observed in *Arabidopsis* and pepper plants (Martinez-Cordero et al., 2005).

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