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BRIEF COMMUNICATION

Involvement of cation channels and NH_4^+-sensitive K^+ transporters in Na^+ uptake by cowpea roots under salinity

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Abstract

Na^+ accumulation was investigated in the roots of 11-d-old cowpea [*Vigna unguiculata* (L.) Walp.] plants. The relative contribution of different membrane transporters on Na^+ uptake was estimated by applying Ca^{2+}, K^+, NH_4^+, and pharmacological inhibitors. Na^+ accumulation into the root symplast was decreased by half in the presence of 1 mM Ca^{2+} and it was almost abolished by 100 mM K^+. The inhibitory effect of external NH_4^+ on Na^+ accumulation was more pronounced in the roots of NH_4^+-free growing plants. Na^+ accumulation was reduced about 73 % by 0.1 mM flufenamate and it was almost blocked by 2 mM quinine. In addition, 20 mM tetraethylammonium and 1.0 mM Cs^+ decreased Na^+ accumulation by 28 and 30 %, respectively. These results evidenced that low-affinity Na^+ uptake by cowpea roots depends on Ca^{2+}-sensitive and Ca^{2+}-insensitive pathways. The Ca^{2+}-sensitive pathway is probably mediated by nonselective cation channels and the Ca^{2+}-insensitive one may involve K^+ channels and to a lesser extent NH_4^+-sensitive K^+ transporters.

Additional key words: Ca^{2+}-sensitive and Ca^{2+}-insensitive pathways, K^+ channels, Na^+ accumulation, nonselective cation channels, salt stress, *Vigna unguiculata*.

The agricultural losses caused by high soil salinity have been partially attributed to the specific effects of Na^+ on plant nutrition and metabolism, especially on the uptake, distribution and utilization of K^+ (Apse and Blumwald 2007, Silva *et al*. 2008). Although Na^+ uptake by the roots is a critical step to trigger ionic toxicity in plants, the pathways implicated in Na^+ entry into the root symplast are still poorly characterized. Low-affinity Na^+ uptake in glycophytes is generally mediated by Ca^{2+}-sensitive and Ca^{2+}-insensitive pathways (Essah *et al.* 2003, Rubio *et al.* 2003). Nonselective cation channels (NSCCs; Essah *et al.* 2003, Tester and Davenport 2003) and the low-affinity cation transporter (LCT1) (Schachtman *et al.* 1997) may play a role in the Ca^{2+}-sensitive pathway. In addition, the Ca^{2+}-insensitive pathway probably involves K^+ inwardly-rectifying channels (KIRCs) from the *Arabidopsis* K^+ transporters family (AKT/KAT; Amtmann and Sanders 1999), as well as K^+ transporters from the KT/HAK/KUP (K^+ transporter/high-affinity K^+ transporter/K^+ uptake; Santa-María *et al.* 2000, Rubio *et al.* 2003) and HKT (high-affinity K^+ transporter) families (Uozumi *et al.* 2000, Horie *et al.* 2001). Nevertheless, the contribution of NSCCs to the Ca^{2+}-insensitive pathway may not be ruled out as Na^+ transport mediated by NSCCs are not completely blocked by Ca^{2+} (Davenport and Tester 2000).

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Abbreviations: AKT/KAT - *Arabidopsis* K^+ transporter family, Flu - flufenamate; HAK, HKT - high-affinity K^+ transporters; KIRCs - K^+ inwardly-rectifying channels; KT - K^+ transporter; KUP - K^+ uptake; LCT - low-affinity cation transporter; NSCCs - nonselective cation channels; Qui - quinine.
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In glycophytes, NSCCs have been considered the main pathways for Na^+ entry into the root symplast (Essah *et al.* 2003, Tester and Davenport 2003). The kinetic characterization of $^{22}Na^+$ influx into maize roots demonstrates the involvement of voltage-insensitive cation channels in Na^+ uptake (Jacoby and Hanson 1985). Na^+ accumulation in pepper roots also depends on a main pathway attributed to NSCCs as it is partially inhibited by Ca^{2+} and a cGMP membrane-permeable analogue (Rubio *et al.* 2003). In *Arabidopsis thaliana* roots, $^{22}Na^+$ unidirectional influx is mediated by different types of NSCCs as it is stimulated by glutamate, it is reduced by cGMP and unspecific cation channel inhibitors, and it is not affected by Ca^{2+} and K^+ channel blockers (Essah *et al.* 2003). Conversely, in the roots of the halophyte *Suaeda maritima*, Na^+ accumulation does not depend on NSCCs as it is not inhibited by Ca^{2+} and cAMP. Two distinct pathways for low-affinity Na^+ uptake have been proposed in this species. The pathway 1 plays a role under mild salinity (25 mM NaCl) and may depend on K^+ transporters from the HKT family, as it is sensitive to Ba^{2+} and insensitive to tetraethylammonium (TEA^+) and Cs^+. The pathway 2, in turn, operates under severe salinity (150 mM NaCl) and may be mediated by K^+ channels from the AKT/KAT family as it is inhibited by Ba^{2+}, TEA^+ and Cs^+ (Wang *et al.* 2007).

The low-affinity Na^+ uptake is probably mediated by K^+ channels and K^+ transporters in the roots of the *S. maritima* (Wang *et al.* 2007), while the involvement of these transporters in Na^+ entry into the root symplast in glycophytes is still uncertain. Although K^+ channels display high K^+/Na^+ selectivity, it has been suggested that they may mediate Na^+ transport under high salinity (Amtmann and Sanders 1999). Na^+ uptake and/or accumulation is partially inhibited by external K^+ in the roots of some glycophytes (Cramer *et al.* 1987, Rubio *et al.* 2003), but pharmacological inhibition of Na^+ unidirectional influx as well as insertional mutants has failed to prove that K^+ transporters contribute to Na^+ uptake in *A. thaliana* roots (Essah *et al.* 2003). Accordingly, the aim of this work is to investigate the relative contribution of NSCCs, K^+ channels and K^+ transporters in the different pathways for low-affinity Na^+ uptake in cowpea roots under salinity. Cowpea is utilized as the experimental model because it is a glycophyte wildly cultivated in arid and semi-arid regions often submitted to salt stress (Ehlers and Hall 1997).

Cowpea [*Vigna unguiculata* (L.) Walp.] salt resistant cv. Pitiúba seeds were germinated in paper rolls as previously described (Vieira and Carvalho 1994). After 4 d, the seedlings were transferred to plastic containers with 20 dm^3 of modified Hoagland nutrient solution [625 μM $Ca(NO_3)_2$, 250 μM NH_4Cl, 125 μM K_2HPO_4, 125 μM $MgSO_4$, 500 μM KNO_3, 25 μM Fe-EDTA, 10 μM HBO_3, 2.25 μM $MnCl_2$, 0.75 μM $CuSO_4$, 1.75 μM $ZnSO_4$ and 0.025 μM Na_2MoO_4, pH 6.0]. To obtain NH_4^+-free growing plants, this solution was changed to 750 μM $Ca(NO_3)_2$, 125 μM K_2HPO_4, 125 μM $MgSO_4$, 500 μM KNO_3, 25 μM Fe-EDTA and micronutrients, pH 6.0. Plants were grown in greenhouse for 7 d (day/night temperature of 32/24 °C, relative humidity of 72 %, maximum photosynthetic photon flux density of 750 μmol m^{-2} s^{-1} and 12-h photoperiod).

Na^+ uptake experiments were carried out according to (Huang *et al.* 1992). The roots of 11-d-old plants were excised and rinsed in 0.1 mM $CaCl_2$ at room temperature for 4 min. Then, roots were gently blotted onto towel paper and transferred to Erlenmeyer flasks containing 50 cm^3 of different solutions (2.0 mM MES-Tris, pH 6.0, 0.1 mM $CaCl_2$, 50 mM NaCl and further additions, as fully described in the table legends) and were incubated at 30 °C for 60 min. At the end of all experiments, roots were rinsed in ice-cold (2 - 4 °C) 0.1 mM $CaCl_2$ during 4 min for apoplastic Na^+ displacement and dried at 70 °C for 48 h for tissue Na^+ determination by flame photometry. Roots incubated in uptake solution without NaCl were used to assess the background content of Na^+. Na^+ accumulation into the roots was calculated by subtracting the background content of Na^+ from that determined for each treatment.

The experiments were performed according to a completely randomized design with 5 replicates per treatment. The results were submitted to analysis of variance (*ANOVA*) followed by the Tukey test at 5 % of significance.

Increasing external Ca^{2+} concentrations decreased Na^+ accumulation in excised cowpea roots (Table 1). The addition of 0.1, 0.5 and 1.0 mM Ca^{2+} in the uptake solution reduced Na^+ accumulation by 15, 45 and 50 %, respectively, in comparison with the control. Additional increments of the external Ca^{2+} concentration did not cause further decrease in the root Na^+ content (data not shown). These results indicated that a Ca^{2+}-sensitive and a Ca^{2+}-insensitive pathways contributed similarly to Na^+ uptake by cowpea roots. The Ca^{2+}-sensitive pathway was almost blocked by external Ca^{2+} concentrations above 1 mM.

Na^+ accumulation in excised cowpea roots was progressively diminished by increasing external K^+ concentrations (Table 1). In the presence of 0.1 mM Ca^{2+}, the addition of 25, 50 and 100 mM K^+ in the uptake solution reduced Na^+ accumulation by 50, 70 and 99 %, respectively, relative to the control. Additionally, 25 and 50 mM K^+ in the presence of 1 mM Ca^{2+} decreased Na^+ accumulation by 63 and 80 %, respectively. In this way, high external K^+ concentrations could affected both the Ca^{2+}-sensitive and Ca^{2+}-insensitive pathways involved in Na^+ uptake by cowpea roots.

The inhibitory effect of NH_4^+ on Na^+ accumulation in excised cowpea roots was tested in uptake experiments utilizing NH_4^+-free and NH_4^+-growing plants (Table 1). In the absence of NH_4^+, Na^+ accumulation did not differ between the NH_4^+-free and the NH_4^+-growing roots. However, 2.5 and 5.0 mM NH_4^+ diminished Na^+ accumulation by 28 % in the roots grown in the presence of NH_4^+, with regard to the respective control. The same

external NH_4^+ concentrations decreased Na^+ accumulation by 52 % in the roots of NH_4^+-free growing plants. In this manner, NH_4^+ notably inhibited Na^+ uptake by cowpea roots, especially in NH_4^+-free growing plants.

Table 1. Effect of external cations on Na^+ accumulation in excised cowpea roots. When Ca^{2+} was tested, excised roots were washed in distilled-deionized water (control) or 0.1, 0.5, and 1.0 mM $CaCl_2$ for 4 min. To test K^+ and NH_4^+, detached roots were rinsed in 0.1 mM $CaCl_2$ containing increasing concentrations of these cations at the same conditions. After that, roots were transferred to 2.0 mM MES-Tris, pH 6.0, 50 mM NaCl and the same concentrations of Ca^{2+}, K^+ or NH_4^+ used in the washings. Roots were incubated at 30 °C for 60 min and rinsed with ice-cold (2 - 4 °C) 0.1 mM $CaCl_2$ during 4 min. Values are means ± SD of five replicates. Values marked with the same letter do not differ significantly according to the Tukey test at 5 % of significance.

Growth	Treatment	Na^+ accumulation
+ NH_4^+	Control	429.7 ± 19.6a
	0.1 mM Ca^{2+}	364.1 ± 12.0b
	0.5 mM Ca^{2+}	237.5 ± 15.8d
	1.0 mM Ca^{2+}	211.8 ± 14.1e
	0.1 mM Ca^{2+} + 25 mM K^+	215.5 ± 12.4e
	0.1 mM Ca^{2+} + 50 mM K^+	132.0 ± 11.3f
	0.1 mM Ca^{2+} + 100 mM K^+	4.9 ± 6.9h
	1.0 mM Ca^{2+} + 25 mM K^+	157.0 ± 18.1f
	1.0 mM Ca^{2+} + 50 mM K^+	86.0 ± 16.1g
	0.1 mM Ca^{2+} + 2.5 mM NH_4^+	307.8 ± 21.7c
	0.1 mM Ca^{2+} + 5.0 mM NH_4^+	307.3 ± 19.2c
- NH_4^+	0.1 mM Ca^{2+}	357.2 ± 27.0b
	0.1 mM Ca^{2+} + 2.5 mM NH_4^+	202.6 ± 16.0e
	0.1 mM Ca^{2+} + 5.0 mM NH_4^+	207.0 ± 18.7e

In order to verify the involvement of cation channels in Na^+ absorption by cowpea roots, uptake experiments were carried out using flufenamate (Flu), an inhibitor of NSCCs, and quinine (Qui), an unespecific cation channel blocker (Table 2). The addition of 0.05 and 0.10 mM Flu in the uptake solution decreased Na^+ accumulation by 56 and 72 % relative to the control. Moreover, 1 and 2 mM Qui dropped Na^+ accumulation by 68 and 99 %, respectively, in comparison with the control. In this way, Na^+ absorption by excised cowpea roots was reduced by NSCC inhibition and it was roughly abolished by cation channel blockage.

The K^+ channel blockers tetraethylammonium (TEA^+) and Cs^+ were utilized to verify the role of K^+ channels on Na^+ uptake (Table 2). Na^+ accumulation was increased by 5 % in the presence of 10 mM TEA^+, but it decreased about 28 % by 20 mM TEA^+, with regard to the control. In comparison, 0.1 and 1.0 mM Cs^+ reduced Na^+ accumulation by 23 and 31 %, respectively, relative to the control. Thus, the blockage of K^+ channels unexpectedly inhibited Na^+ uptake by cowpea roots.

The interactive effects of the inhibitors Flu, TEA^+ and Cs^+ on Na^+ accumulation was also verified (Table 2). While 0.1 mM Flu plus 20 mM TEA^+ almost abolished Na^+ accumulation, 1 mM Flu plus 1 mM Cs^+ caused only 55 % of decrease, in comparison with the control. These results demonstrated that Flu and TEA^+ showed synergistic effects on Na^+ uptake, probably inhibiting different pathways. Conversely, Cs^+ and Flu displayed antagonistic effects, as the inhibitory effect of Flu on Na^+ accumulation was alleviated in the presence of Cs^+.

Table 2. Effect of pharmacological inhibitors on Na^+ accumulation in excised cowpea roots. Excised roots were rinsed in 0.1 mM $CaCl_2$ without inhibitors or with increasing concentrations of these compounds during 4 min. After that, roots were transferred to 2.0 mM MES-Tris, pH 6.0, 0.1 mM $CaCl_2$, and 50 mM NaCl containing the same concentrations of the inhibitors used in the washings. Roots were incubated at 30 °C for 60 min and rinsed with ice-cold (2 - 4 °C) 0.1 mM $CaCl_2$ during 4 min. Values are means ± SD of five replicates. Values marked with the same letter do not differ significantly according to the Tukey test at 5 % of significance.

Treatment	Na^+ accumulation
Control	429.7 ± 19.6b
0.1 mM Ca^{2+}	364.1 ± 12.0c
0.1 mM Ca^{2+} + 0.05 mM Flu	187.7 ± 13.9e
0.1 mM Ca^{2+} + 0.10 mM Flu	117.7 ± 10.8g
0.1 mM Ca^{2+} + 1.0 mM Qui	137.7 ± 11.5f
0.1 mM Ca^{2+} + 2.0 mM Qui	3.8 ± 6.6h
0.1 mM Ca^{2+} + 10 mM TEA^+	452.6 ± 29.6a
0.1 mM Ca^{2+} + 20 mM TEA^+	307.0 ± 16.1d
0.1 mM Ca^{2+} + 0.1 mM Cs^+	329.1 ± 18.1d
0.1 mM Ca^{2+} + 1.0 mM Cs^+	296.3 ± 17.7d
0.1 mM Ca^{2+} + 0.10 mM Flu + 20 mM TEA^+	18.4 ± 17.5h
0.1 mM Ca^{2+} + 0.10 mM Flu + 1.0 mM Cs^+	194.3 ± 12.3e

The partial inhibiton of Na^+ accumulation by external Ca^{2+} in cowpea roots corroborates the results of other works, which emphasize the alleviation of the salt-specific damaging effects in plants treated with increasing external Ca^{2+} concentrations (Melgar *et al.* 2006, Tuna *et al.* 2007). Ca^{2+} stabilizes the cell membranes due to electrostatic interactions with the phospholipid negative charges, avoiding electrolyte leakage (Mengel and Kirkby 2001). Additionally, Ca^{2+} maintains the high K^+/Na^+ membrane selectivity, restricting Na^+ uptake and sustaining K^+ acquisition (Davenport *et al.* 1997). Thus, the restriction of Na^+ accumulation could be a mechanism by which external Ca^{2+} could alleviate the salt-induced disturbances in plants.

The interactive effects of external Ca^{2+} and K^+ on Na^+ accumulation in cowpea roots indicate that external K^+ may display inhibiting effects on both Ca^{2+}-sensitive and Ca^{2+}-insensitive pathways for Na^+ uptake. In fact, 0.1 mM Ca^{2+} did not completely inhibit the Ca^{2+}-sensitive pathway and 0.1 mM Ca^{2+} plus 100 mM K^+ almost abolished Na^+ accumulation (Table 1), suggesting that this pathway was inhibited by K^+. Moreover, 1.0 mM Ca^{2+} almost blocked the Ca^{2+}-sensitve pathway and

1.0 mM Ca^{2+} with 25 or 50 mM K^+ allowed further inhibition of Na^+ accumulation (Table 1), demonstrating that the Ca^{2+}-insensitive pathway was also affected by K^+. The inhibiting effect of external K^+ on Na^+ uptake was demonstrated in cotton (Cramer *et al.* 1987), pepper (Rubio *et al.* 2003), *A. thaliana* (Essah *et al.* 2003) and *S. maritima* roots (Wang *et al.* 2007). This effect could be related to competition between Na^+ and K^+ for the binding sites on membrane transporters (Maathuis and Amtmann 1999). Alternatively, high external K^+ concentrations could also lead to membrane depolarization limiting the Na^+ uptake (Britto and Kronzuker 2008).

The pharmacological inhibition of Na^+ accumulation in cowpea roots strongly suggests that cation channels play a central role in the Na^+ entry into the root symplast in this species. Indeed, Na^+ accumulation was almost blocked by 2 mM Qui. Additionally, 0.1 mM Flu reduced Na^+ accumulation by 72 %. Similarly, 0.1 mM Flu and 1 mM Qui in the presence of 0.2 mM Ca^{2+} inhibited the Na^+ unidirectional influx in *A. thaliana* roots by 56 and 53 %, respectively (Essah *et al.* 2003). NSCCs sensitive to Flu are found in wheat roots (Buschmann *et al.* 2000), as well as NSCCs from *A. thaliana* roots are blocked by Qui (Demidchik and Tester 2002). According to these evidences, NSCCs may be the main membrane transporters involved in low-affinity Na^+ uptake by cowpea roots.

Na^+ transport through NSCCs was strongly restricted by external Ca^{2+} in maize (Roberts and Tester 1997), wheat (Buschmann *et al.* 2000), *A. thaliana* (Demidchik and Tester 2002, Essah *et al.* 2003) and pepper (Rubio *et al.* 2003). In this way, the Ca^{2+}-sensitive pathway in cowpea roots was probably mediated by NSCCs.

The low selectivity of NSCCs to monovalent cations may explain, at least in part, the inhibiting effect of external K^+ on the Ca^{2+}-sensitive pathway in cowpea roots. NSCCs identified in wheat (Davenpot and Tester 2000) and *A. thaliana* roots (Demidchik and Tester 2002) showed relative K^+/Na^+ selectivity of 1.31 and 1.49, respectively. If cowpea roots display NSCCs with similar K^+/Na^+ selectivity, the increment of external K^+ in relation to external Na^+ could allow preferential K^+ transport. Theoretically, Na^+ transport *via* NSCCs could be reduced by half under equimolar Na^+ and K^+ concentrations. Coincidently, 50 mM K^+ in the presence of 50 mM Na^+ diminishes Na^+ accumulation by 70 % in cowpea roots.

The significant reduction of Na^+ accumulation by the inhibitors TEA^+ and Cs^+ strongly indicates the involvement of K^+ cannels in low-affinity Na^+ uptake by cowpea roots. TEA^+ and Cs^+ are identified as potent blockers of the KIRCs in maize (Roberts and Tester 1995), potato (Zimmermann *et al.* 1998), and tomato roots (Hartje *et al.* 2000). As NSCCs in maize (Roberts and Tester 1997), wheat (Buschmann *et al.* 2000) and *A. thaliana* roots (Demidchik and Tester 2002) are insensitive to TEA^+ and Cs^+, it seems that the partial inhibition of Na^+ accumulation in cowpea roots by these inhibitors is not due to NSCC blockage. Moreover, the involvement of K^+ channels in Na^+ uptake by the roots of *S. maritima* is suggested on the basis of the inhibitory effect of TEA^+ and Cs^+ on Na^+ accumulation (Wang *et al.* 2007). The participation of both NSCCs and K^+ cannels in the Na^+ entry into the root symplast in cowpea is corroborated by the abolishment of Na^+ accumulation in this species by Flu (an NSCC inhibitor) plus TEA^+ (a K^+ channel blocker) or by Qui (an unspecific cation channel blocker).

The participation of NH_4^+-sensitive K^+ transporters in the low-affinity Na^+ uptake in cowpea roots is evidenced by the inhibitory effect of NH_4^+ on Na^+ accumulation, mainly in NH_4^+-free growing plants. The NH_4^+-sensitive pathway for high-affinity K^+ uptake has been attributed to members of the KT/HAK/KUP family, including HAK1 from barley (Santa-María *et al.* 2000), rice (Bañuelos *et al.* 2002) and pepper (Martínez-Cordero *et al.* 2005). However, K^+ transporters of the HKT family, as HKT1 from barley (Santa-María *et al.* 2000) and *Eucalyptus camaldulensis* (Fairbairn *et al.* 2000) also show NH_4^+ sensitivity. In *A. thaliana* (Rus *et al.* 2001), rice (Horie *et al.* 2007) and wheat roots (Laurie *et al.* 2002), the Na^+ entry into the root symplast has been partially attributed to HKTs. In this way, the NH_4^+-sensitive component that mediates the low-affinity Na^+ uptake in cowpea roots may involve K^+ transporters from the KT/HAK/KUP and HKT families.

The inhibition of Na^+ accumulation by external K^+ in cowpea roots, especialy when the Ca^{2+}-sensitive pathway is almost blocked, evidences that Na^+ uptake by the Ca^{2+}-insensitive pathway involves K^+ channels and K^+ transporters. The high K^+/Na^+ selectivity showed by KIRCs (Amtmann and Sanders 1999) and K^+ transporters from the KT/HAK/KUP family (Bañuelos *et al.* 2002) could favour K^+ binding in these transporters in detriment of Na^+, causing strong inhibition of Na^+ transport. Moreover, high external Ca^{2+} concentrations completely inhibit the Ca^{2+}-sensitive pathway, but do not affect the activity of KIRCs (Roberts and Tester 1995), HAKs (Santa-María *et al.* 2000, Martínez-Cordero *et al.* 2005) and HKTs (Liu *et al.* 2001) in other species, enforcing the participation of these transporters also in the Ca^{2+}-insensitive pathway in cowpea. Nonetheless, the contribution of NSCCs in this pathway may not be ruled out as the NSCC activity is not totally blocked by increasing external Ca^{2+} concentrations (Davenport and Tester 2000).

According to the results of the current work, it is possible to propose that the Na^+ entry into the root symplast of cowpea depends on a Ca^{2+}-sensitive and Ca^{2+}-insensitive pathways. While the Ca^{2+}-sensitive pathway may be mediated by NSCCs, the Ca^{2+}-insensitive one may involve mainly K^+ channels, besides NH_4^+-sensitive K^+ transporters. Further investigations are necessary to elucidate the different pathways by which Na^+ enters into the root symplast in cowpea under different environmental conditions.

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