DOI: 10.1007/s10535-016-0604-8 BIOLOGIA PLANTARUM 60 (4): 731-740, 2016

Salt-induced NO_3^- uptake inhibition in cowpea roots is dependent on the ionic composition of the salt and its osmotic effect

R.M. ARAGÃO[1], E.N. SILVA[2], P.C.C. SILVA[1], and J.A.G. SILVEIRA[1]*

Laboratório de Metabolismo de Plantas, Departamento de Bioquímica e Biologia Molecular,
Universidade Federal do Ceará, Campus do Pici,CEP 60451-970, Fortaleza, Ceará, Brazil[1]
Faculdade de Educação, Ciências e Letras do Sertão, Central, Universidade Estadual do Ceará,
CEP 63900-000, Quixadá, Ceará, Brazil[2]

Abstract

Salinity remarkably inhibits NO_3^- uptake but the mechanisms are not well understood. This study was addressed to elucidate the role of ionic and osmotic components of salinity on NO_3^- influx and efflux employing classic kinetics involving a low affinity transport system (LATS) and a high affinity transport system (HATS). In the presence of KCl, NaCl, and Na_2SO_4 at 100 mM concentrations, in both LATS and HATS, Michaelis constant (K_m) was similar for the three salts and maximum rate (V_{max}) decreased as follows: KCl > NaCl > Na_2SO_4, compared to control indicating a non-competitive interaction with NO_3^-. Unexpectedly, iso-osmotic solutions (osmotic potential Ψ_π = -0.450) of polyethylene glycol (PEG, 17.84 %, v/v) and mannitol (100 mM) remarkably increased K_m in both the LATS and the HATS, but V_{max} did not change indicating a competitive inhibition. Under the PEG and mannitol treatments, K_m and V_{max} were higher than under the salt treatments. The salts increased slightly NO_3^- efflux in the following order KCl > NaCl > Na_2SO_4. In contrast, mannitol strongly stimulated and the PEG inhibited NO_3^- efflux. The obtained data reveal that salinity effects were not dependent on the anion type (Cl^- versus SO_4^{2-}) indicating a non-competitive inhibition mechanism between Cl^- and NO_3^-. In contrast, the cation types (K^+ *versus* Na^+) had a pronounced effect. The osmotic component is important to net NO_3^- uptake affecting remarkably the influx in both LATS and HATS components of cowpea roots.

Additional key words: KCl, low and high affinity transport systems, mannitol, NaCl, Na_2SO_4, PEG, *Vigna unguiculata.*

Introduction

Nitrate is an important N source in soils used by the major cultivated plants due to its great significance in several physiological processes such as photosynthesis, root signaling, and plant productivity among others (Yang *et al.* 2007, Sorgonà *et al.* 2011). Classical kinetic and functional genomic studies have shown that NO_3^- influx in roots involves two transport systems represented by plasmalemma proteins: one a high-affinity transport system (HATS) and another a low-affinity transport system (LATS), both dependent on external NO_3^- concentration ranges (Crawford and Glass 1998, Tsay *et al.* 2007). In higher plants, there are two HATS for NO_3^- ions, one constitutive and another apparently dependent on an NO_3^- external concentration; both operate at a micromolar range. Conversely, the LATS operate at high NO_3^- concentrations, which are compatible with NO_3^- levels commonly found in cultivated soils (a millimolar range), and these transport proteins are constitutive in the most plant species (Crawford and Glass 1998, Miller *et al.* 2007, Tsay *et al.* 2007).

Net NO_3^- uptake involves two opposite and independent processes that are regulated by different control mechanisms: influx and efflux (Forde and Clarkson 1999, Rubinigg *et al.* 2003). Some studies have shown that influx and efflux of NO_3^- are regulated by different and independent mechanisms (Deanne-Drumond 1993, Aslam *et al.* 1996, Mata *et al.* 2000).

Submitted 3 March 2015, *last revision* 22 December 2015, *accepted* 29 December 2015.

Abbreviations: HATS - high affinity transport system; K_m - Michaelis constant; LATS - low affinity transport system; M-M - Michaelis-Menten; PEG - polyethylene glycol; V_{max} - maximum rate; Ψ_π - osmotic potential.

* Corresponding author: fax: (+55) 85 3366 9821, e-mail: silveira@ufc.br

However, despite the quantitative importance of NO_3^- efflux, particularly in plants growing on a high NO_3^- supply, little is known about biochemical and physiological mechanisms especially under adverse environmental conditions (Aslam *et al.* 1996, Miller and Smith 1996, Clarkson 1998). Efflux of NO_3^- is associated with regulation of cytoplasmic NO_3^- content in roots, which further depends on rate of NO_3^- influx, nitrate reduction rates, NO_3^- accumulation in root vacuoles, and translocation to shoots (Miller and Smith 1996).

Salinity strongly affects NO_3^- influx and efflux, but to date, mechanisms underlying these processes are poorly understood (Silveira *et al.* 2012). The majority of performed studies have used NaCl as model, but this approach is very simplified since it does not consider several aspects of salinity components such as ionic composition, cation-anion specific effects, physical-chemistry characteristics, and osmotic effects. Peuke and Jeschke (1999) performed perhaps a unique study involving characterization of inhibition of NO_3^- uptake in barley seedlings by different salts and osmotic agents utilizing the classical kinetic approach. These authors found unexpected results where kinetic parameters indicated by NaCl, KCl, and Na_2SO_4 did not inhibit NO_3^- uptake by a competitive inhibition but, in contrast, did it by non-competitive mechanisms. Overall, the reported studies involving kinetic characterization of NO_3^- uptake inhibition by salinity remain incomplete.

The existing salinity in saline soils and irrigation water is composed of a complex mixture of ions in different ratios (Li *et al.* 2010). The chemical nature of anions and cations might directly or indirectly interfere with NO_3^- uptake by ionic competition, synergism, and ionic strength or by alteration in membrane potential (Cachorro *et al.* 1994, Massa *et al.* 2009). Moreover, salinity might also affect NO_3^- uptake by osmotic effects (Ourry *et al.* 1992, Peuke and Jeschke 1999). In this sense, results from several reported experiments have widely suggested that the effects of salinity caused on NO_3^- uptake might be species-dependent, and these responses could, at least in part, be explained by differences in salt composition (Ouerghi *et al.* 2000).

It has been assumed for many years that Cl^- can inhibit NO_3^- uptake by a competitive mechanism at the level of influx and/or efflux (Deanne-Drumond 1993). Despite several studies performed in the past to elucidate the mechanisms of NO_3^- and Cl^- interaction, this question remain still not clear and contradictory (Massa *et al.* 2009). Classic kinetic studies when employed for NO_3^- uptake in the presence of several salts and osmotic agents are interesting because they allow revealing several mechanisms involving each component in both the HATS and the LATS. In addition, this approach is suitable for evaluation effects and interaction between NO_3^- and Cl^- (competitive or non-competitive) in the presence of different cations. This approach also allows determining the influence of osmotic effects on net NO_3^- uptake and calculates the importance of apoplastic and symplastic transport in salt-induced nitrate inhibition. Besides, classic kinetics is suitable for estimation effects of salinity on NO_3^- influx and efflux.

In this study, we utilized cowpea plants as model because it is a native species from arid and semi-arid regions, and it is widely cultivated in saline soils of Africa and Brazil (Silveira *et al.* 2001). The approach utilized was the classic kinetics of NO_3^- influx and efflux in the presence of different salts and osmotic agents at the levels of the HATS and the LATS. We tested a hypothesis that salinity-induced net NO_3^- uptake inhibition is dependent on physico-chemical aspects of salts and osmotic agents, which might affect the type of a nitrate inhibitory mechanism. The role and importance of each component on whole effects of salinity are discussed.

Materials and methods

Plants and treatments: Cowpea (*Vigna unguiculata* [L.] Walp.) cv. Pitiúba seeds previously sterilized in a 0.2 % (m/v) sodium hypochlorite (NaClO) solution for 30 min were germinated in paper rolls in a growth chamber at a temperature of 27 ± 2 °C, a 12-h photoperiod, and a photosynthetic photon flux density (PPFD) of 240 μmol(photons) m^{-2} s^{-1}. After 4 d, the seedlings were transferred to plastic pots containing Hoagland's solution (Hoagland and Arnon 1950) at pH 6.0 in greenhouse conditions [an air temperature ranged from 24 to 36 °C with a mean temperature of 29 °C, a relative humidity of 65 %, a maximum PPFD of 900 μmol(photons) m^{-2} s^{-1}, and a 12-h photoperiod], and grown for 7 d.

Nitrate influx and efflux assays were conducted using different nutrient solutions. For influx determination, the seedlings were kept in an N-free medium for 3 d before kinetic experiments. For efflux assay, a seedling nutrient solution was with 10mM KNO_3. Further, three kinds of salts (100 mM NaCl, 100 mM KCl, and 100 mM Na_2SO_4, osmotic potential Ψ_π = -0.44 MPa) and two osmotic agents (100 mM mannitol and 17.84 % (v/v) PEG, Ψ_π = -0.45 MPa) were utilized. These ionic and osmotic agents were individually added in incubation solutions before performing the time-course and dose-dependent assays.

Measurement of NO_3^- influx and efflux: Initially, detached cowpea roots were washed in 0.1 mM $CaCl_2$, transferred to incubation solutions containing 2.0 mM MES-Tris, 0.1 mM $CaCl_2$, and 5.0 mM KNO_3 at pH 6.0 and incubated at 30 °C for 60 min. Influx of NO_3^- was quantified by NO_3^- depletion ($[NO_3^-]_{initial}$ - $[NO_3^-]_{final}$) in the incubation solution at different times (time-course) and in distinct ranges of NO_3^- concentrations (dose-dependent) using an NO_3^--selective electrode (*SevenMulti*, *Mettler*, Toledo, Switzerland), according to the manufacturer's instructions. For NO_3^- efflux measurements, cowpea roots were washed and transferred to incubation solutions containing 2.0 mM MES-Tris and 0.1 mM $CaCl_2$ at pH 6.0 (NO_3^-free). Efflux of NO_3^- was quantified by NO_3^- released to the incubation solution using the NO_3^--selective electrode.

Michaelis-Menten (M-M) kinetics is often used to calculate ion influx. Epstein (1972) showed that M-M equation fits the relationship between the uptake rate and the external ion concentration (Adamowicz and Le Bot 1999). Thus, to calculate Michaelis constant (K_m) and maximum rate (V_{max}) of NO_3^- influx, we used the general M-M equation: $I = (V_{max} \times c)/(K_m + c)$, where I represents the net flux of the ion into roots, V_{max} is an asymptotic maximum value at the ion concentration approaching infinity, K_m is a constant value that represents the concentration when $I = 1/2\ V_{max}$, and c is the concentration of the ion in the root zone. Lineweaver-Burk plot was used to estimate inhibition: $1/I = (K_m/V_{max} + 1/V_{max})$. This equation is widely used simply by inverting both sides of the M-M equation (Massa *et al.* 2009).

Kinetics of NO_3^- influx and efflux in the presence of salts and osmotic agents: For influx measurements, detached cowpea roots were washed in 0.1 mM $CaCl_2$ for 20 min and transferred to incubation solutions containing 2.0 mM MES-Tris and 0.1 mM $CaCl_2$ (pH 6.0) containing 0, 2, 4, 6, 8, and 10 mM KNO_3 for the LATS or 0, 0.1, 0.2, 0.4, 0.8, 0.8 and 1.0 mM for the HATS. For NO_3^- efflux, cowpea roots were washed in 0.1 mM $CaCl_2$ for 5 min and transferred to NO_3^-free incubation solutions containing 2.0 mM MES-Tris and 0.1 mM $CaCl_2$ (pH 6.0). The ionic agents employed were 100 mM NaCl (Ψ_π = -0.430 MPa), 100 mM KCl (Ψ_π = -0.415 MPa), and 100 mM Na_2SO_4 (Ψ_π = -0.460 MPa). The osmotic agents used were 100 mM mannitol (Ψ_π = -0.437 MPa) and 17.84 % (v/v) PEG 6000 (Ψ_π = -0.450 MPa). For both assays, fresh detached roots were immediately incubated at 30 °C for 60 min in the presence of their respective incubation solutions. The obtained results in both the LATS and the HATS were expressed as μmol(NO_3^-) g^{-1}(d.m.) min^{-1}. To determine if NO_3^- influx in detached roots is a representative model for attached roots, cowpea plants were incubated in the presence of 100 mM NaCl and 17.84 % (v/v) PEG in the same solution utilized for detached roots. Influx of NO_3^- was measured as previously described and the kinetic constants were obtained using the M-M equation and Lineweaver-Burk plot was used to determinate the inhibition type.

Determination of NO_3^- flux and efflux in apoplastic and symplastic pathways in the presence and the absence of NaCl: NO_3^- flux in apoplastic and symplastic pathways was determined by the method described by Zhao *et al.* (2002) with some modifications. Symplastic NO_3^- influx and efflux in roots were estimated by differences between the total influx/efflux obtained at an ambient temperature (25 °C) and apoplastic influx/efflux measured at a low temperature (3 °C) in the absence of transport by plasmalemma. Nitrate influx and efflux under the control and salinity conditions were performed in roots immersed in an incubation solution containing 0.1 mM $CaCl_2$, 2.0 mM MES-Tris, and 5.0 mM KNO_3 (influx) or in an NO_3^--free solution (efflux), both at pH 6.0. A time-courses for influx and efflux were performed over 60 and 80 min intervals, respectively. The results were expressed as μmol(NO_3^-) g^{-1}(d.m.).

Data analysis: The experimental data were analyzed by polynomial and hyperbolic regressions for time-course and dose-dependent experiments, respectively. Plotting was assisted by the *Sigmaplot 11* software (*Systat*, San Jose. CA, USA). All analyses include standard deviations corresponding to four replicates per treatment.

Results

Ionic and osmotic effects induced by the three kinds of salts (NaCl, KCl, and Na_2SO_4) and two osmotic agents (PEG and mannitol) on NO_3^- influx process were assessed. These kinetic studies were performed under low (0 to 10 μM) and high (0 - 10 mM) NO_3^- concentrations to evaluate the contributions of the HATS and the LATS, respectively. The results show a considerable impairment in NO_3^- influx in both the HATS and the LATS induced by all tested salts (Fig. 1). In the LATS component, KCl, NaCl, and Na_2SO_4, all at 100 mM concentration, induced a maximum inhibition of NO_3^- influx by 55, 44, and 32 %, respectively, compared to the controls (Fig. 1*A*). Mannitol (100 mM) and PEG (17.84 %, v/v) induced maximum reductions of 65 and 58 %, respectively, (Fig. 1*B*). These results clearly demonstrate that the osmotic agents caused a higher inhibition of NO_3^- influx than the salts. In the HATS, both salts and osmotic agents caused a similar trend in inhibition of NO_3^- influx as was found in the LATS (Fig. 1*C,D*). The salts induced maximum inhibitions of 49, 41, and 21 %, respectively, (Fig. 1*C*) whereas mannitol and PEG showed maximum reductions by 50 and 59 %, respectively, compared to the controls (Fig. 1*D*). In the HATS, KCl shows an inhibition of nitrate influx similar to that caused by mannitol.

The kinetic parameters concerning the inhibition of NO_3^- influx in the LATS component show that the K_m

values did not change for the three studied salts compared to the control, which presented K_m = 3.05 mM (Table 1). Compared to that, the V_{max} values were considerably reduced by the salts, decreasing from 0.20 (control) to 0.14, 0.11, and 0.09 µmol (NO_3^-) g^{-1}(d.m.) min^{-1} in the presence of NaCl, Na_2SO_4, and KCl, respectively, (Table 1). The results indicate the three studied salts caused a non-competitive inhibition on NO_3^- influx according to the obtained data from Lineweaver-Burk model (Fig. 2*A*). On the other hand, the osmotic agents induced a strong increase in K_m (from 3.05 in the control to 12.43 and 10.85 mM, for the mannitol and PEG treatments, respectively). The osmotic agents did not change the V_{max} values of the control - approximately 0.20 µmol(NO_3^-) g^{-1}(d.m.) min^{-1} (Table 1) - indicating a competitive inhibition for NO_3^- influx in the LATS (Fig. 2*B*).

In the influx kinetics of the HATS, K_m was maintained unchanged in the presence of the three salts compared to the control (0.68 mM in average). The V_{max}

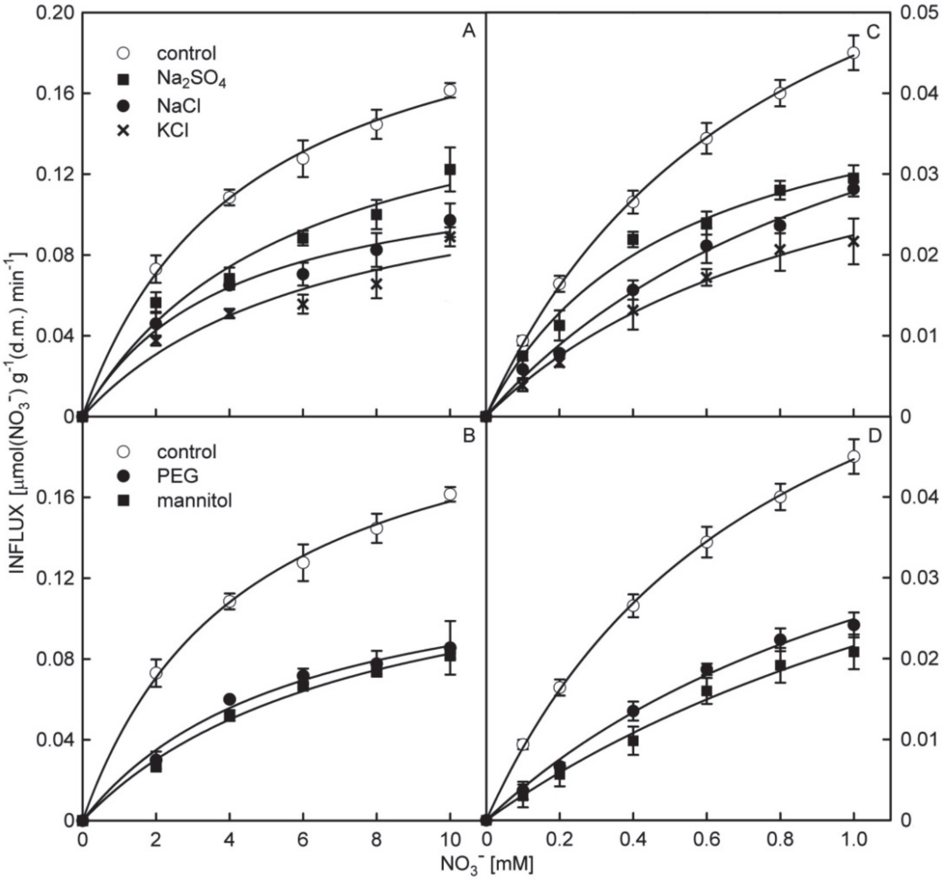

Fig. 1. Nitrate influxes in cowpea detached roots determined in two transport systems: a low affinity transport system (*A*,*B*) and a high affinity transport system (*C*,*D*). The roots were subjected to salts (100 mM NaCl, 100 mM Na_2SO_4, and 100 mM KCl - *A,C*) and osmotics [100 mM mannitol and 17.84 % (v/v) PEG - *B,D*]. Means of four replicates ± SDs.

Table 1. The kinetics constants of nitrate influx in cowpea detached roots and the type of inhibition for ionic (NaCl, Na_2SO_4, and KCl) and osmotic (mannitol and PEG) agents. Michaelis constant (K_m) was expressed in mM and maximum rate (V_{max}) in µmol(NO_3^-) g^{-1}(d.m.) min^{-1}.

Treatments	LATS K_m	V_{max}	Inhibition	HATS K_m	V_{max}	Inhibition
Control	3.05	0.2	-	0.69	0.07	-
NaCl 100 mM	3.05	0.11	non-competitive	0.67	0.04	non-competitive
Na_2SO_4 100 mM	3.04	0.14	non-competitive	0.66	0.05	non-competitive
KCl 100 mM	3.06	0.09	non-competitive	0.72	0.03	non-competitive
Mannitol 100 mM	12.43	0.2	competitive	1.92	0.07	competitive
PEG 17.84 % (v/v)	10.85	0.2	competitive	2.27	0.07	competitive

values significantly decreased by the salts, from 0.07 in the control to 0.05, 0.04, and 0.03 μmol(NO_3^-) g^{-1}(d.m.) min^{-1} for the NaCl, Na_2SO_4, and KCl treatments, respectively. It is important to note that KCl caused the highest inhibitory effect on NO_3^- influx in the HATS compared to the other salts (Table 1) similarly as in the LATS. These results suggest the occurrence of a non-competitive inhibition for all tested salts on nitrate influx in both the HATS and the LATS (Fig. 2*C*). Moreover, the results suggest that the inhibition caused by NaCl involved a non-competitive inhibition of NO_3^- influx by Cl^- in both the transport systems. In contrast and unexpectedly, the osmotic agents caused increased K_m and unchanged V_{max} values compared to the controls indicating a competitive inhibition (Fig. 2*D)*.

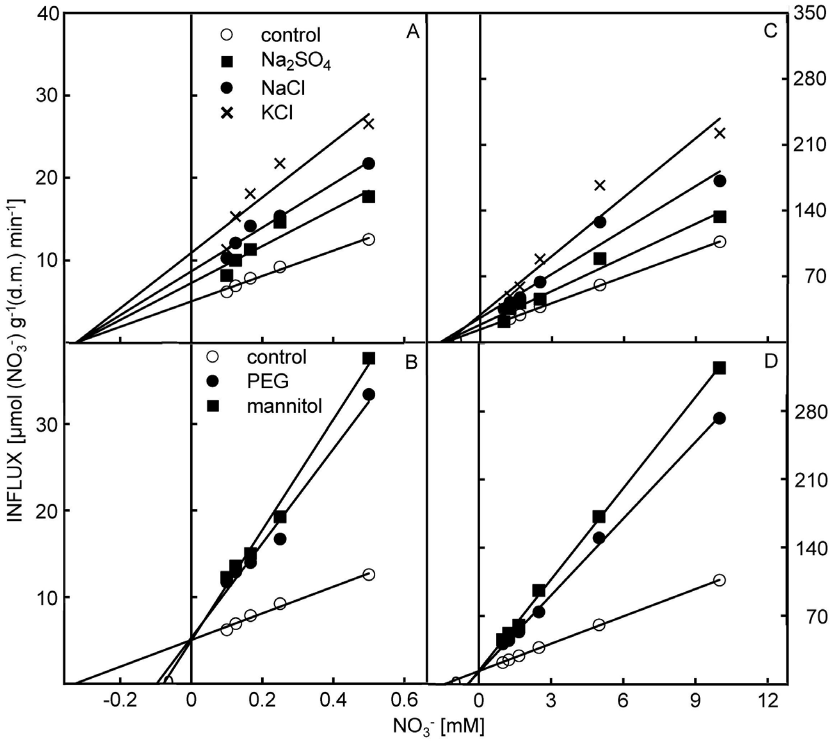

Fig. 2. Lineweaver-Burk plots determined for two transport systems: a low affinity transport system (*A*,*B*) and a high affinity transport system (*C*,*D*) in cowpea detached roots. The roots were subjected to salts (100 mM NaCl, 100 mM Na_2SO_4, and 100 mM KCl - *A*,*C*) and osmotic agents [100 mM mannitol and 17.84 % (v/v) PEG - *B*,*D*]. Means of four replicates ± SDs.

Table 2. The kinetic constants of nitrate influx in cowpea attached roots and the type of inhibition for ionic (NaCl) and osmotic (PEG) agents. Michaelis constant (K_m) was expressed in mM and maximum rate(V_{max}) in μmol(NO_3^-) g^{-1}(d.m.) min^{-1}. Nitrate concentrations were equal to those used in the assay for cowpea detached roots in the low affinity transport system.

Treatments	K_m	V_{max}	Inhibition
Control	3.09	0.21	-
NaCl 100 mM	3.11	0.15	non-competitive
PEG 17.84 % (v/v)	9.47	0.21	competitive

When we compared the responses of detached roots with attached roots (entire plants) within the LATS, we found similar effects of NaCl and PEG on NO_3^- influx in terms of V_{max} and K_m (Tables 1 and 2). The NaCl and PEG treatments promoted influx reductions of 28 and 43 %, respectively, compared to the controls (Fig. 3*A*). On average, NO_3^- influx in whole plants was slightly higher (12 %) than in isolated roots, but the values of K_m and V_{max} were similar (Fig. 3*B*). These results reinforce that detached roots are a good model for studies of NO_3^- influx kinetics in the presence of salts and osmotic agents.

The time-course data reveal that both the salts and the osmotic agents stimulated NO_3^- efflux in cowpea roots. The 100 mM KCl induced the highest NO_3^- efflux (and the lowest influx) followed by a lower NaCl efflux and the lowest Na_2SO_4 efflux.. A maximum stimulation of the effluxes varied by 36, 21, and 13 %, respectively, compared to the control (Fig. 4*A*). Interestingly, PEG stimulated NO_3^- efflux by 38 %, whereas mannitol strongly inhibited it by 49 %, both compared to the control (Fig. 4*B*).

In order to better understand NO_3^- influx and efflux mechanisms in roots exposed to salinity, we differentiated symplastic and apoplastic pathways. In the control conditions, apoplastic NO_3^- transport represented 45 % of the total influx, and symplastic NO_3^- transport represented 55 % (Fig. 5*A*,*B*). In the presence of NaCl, NO_3^- transport *via* apoplastic and symplastic pathways represented 30 and 70 % of the total influx, respectively (Fig. 5*A*,*B*). These data suggest that the salt stress promoted a significant decrease in NO_3^- influx caused by an expressive reduction in apoplastic transport in cowpea roots. With respect to efflux, apoplastic and symplastic NO_3^- transport represented 31 and 69 %, respectively, of the total efflux in the control conditions. In the presence of NaCl, apoplastic and symplastic NO_3^- transport represented 35 and 65 %, respectively, of the total efflux (Fig. 5*D*). The negative modulation exerted by NaCl in apoplastic and symplastic pathways were more pronounced on the NO_3^- influx than on NO_3^- efflux.

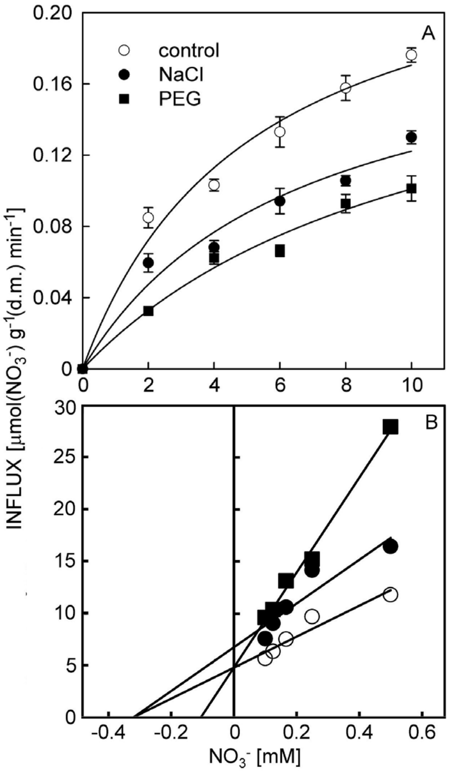

Fig. 3. Nitrate influxes in cowpea attached roots (*A*) and Lineweaver-Burk plots (*B*) determined in a low affinity transport system (LATS). Whole plants were subjected to 100 mM NaCl or 17.84 % (v/v) PEG. Concentrations of KNO_3 were the same as used for LATS in cowpea detached roots. Means of four replicates ± SDs.

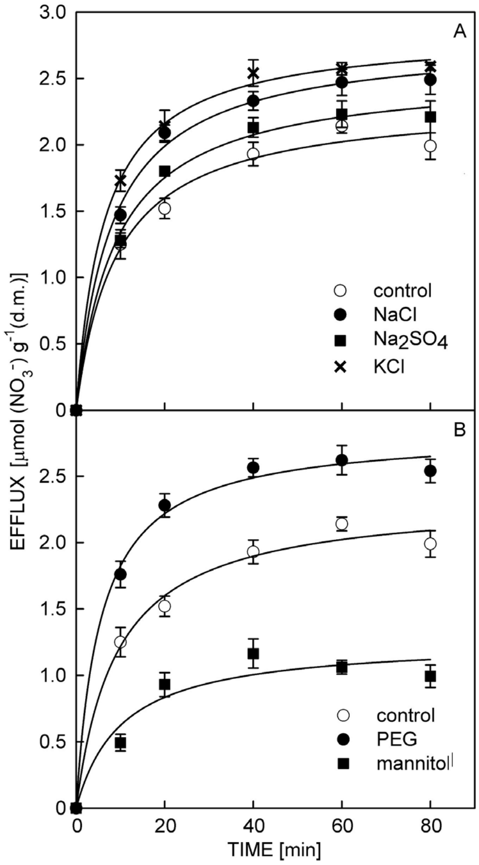

Fig. 4. Nitrate effluxes in cowpea detached roots exposed to different ionic and osmotic agents. The roots were subjected to salts (100 mM NaCl, 100 mM Na_2SO_4, and 100 mM KCl - *A*) and osmotic agents [100 mM mannitol and 17.84 % (v/v) PEG - *B*] added to an NO_3^- free incubation solution. Means of four replicates ± SDs.

Discussion

This study clearly demonstrates that both the ionic stress and the osmotic stress, which are components of salt stress, affected net NO_3^- uptake in cowpea roots due to affection of influx and efflux processes. Interestingly, the influx kinetic parameters (K_m and V_{max}) calculated in both the LATS and the HATS show that the two osmotic agents (PEG and mannitol) in iso-osmotic concentrations induced a competitive inhibition, and they were more effective as inhibitors than the three studied salts (NaCl, KCl, and K_2SO_4). Unexpectedly, all studied salts displayed a non-competitive inhibition of NO_3^- influx in both the transport systems. As the salts consisted of Cl^- and SO_4^{2-} anions at a high concentration (100 mM), a competitive inhibition could be expected, particularly involving Cl^- *versus* NO_3^- as has been speculated (Deanne-Drummond 1993, Cerezo *et al.* 1999, Silveira *et al.* 2012). Our results agree with those obtained by Peuke and Jeschke (1999), working with barley roots, utilizing a similar approach but different salt concentrations. In our study, it is interesting to note that KCl at the high concentration induced a higher inhibition of NO_3^- influx than NaCl suggesting that some interaction at the plasma membrane level could have occurred (Ivashikina and Feyziev 1998, Chen *et al.* 2007). These results strongly suggest that the accompanying cations are important for anion effects on net NO_3^- uptake (Ramos *et al.* 2004, Kaburagi *et al.* 2015). For example, the results of inhibition kinetics were more similar between NaCl and Na_2SO_4 than when both were compared to KCl, despite Na_2SO_4 presents the Na^+ concentration two-fold higher than NaCl. These results suggest that the Na^+ ions, when accompanied by SO_4^{2-}, are less effective in inhibition of both NO_3^- influx and NO_3^- efflux in cowpea roots when compared to Cl^- as complementary anion for Na^+. This response might also indicate that SO_4^{2-} is less effective as NO_3^- transport inhibitor in influx and efflux kinetics. Interestingly, Cl^- was more effective in inhibition of NO_3^- influx when it was accompanied by K^+ in comparison to Cl^- in the presence of Na^+. However, both NaCl and KCl triggered a non-competitive inhibition, which corroborates that Cl^- is not a determinant ion involved in NO_3^- influx inhibition due to a direct competition mechanism. The question involving the inhibition of net NO_3^- uptake by anions is an old problem not solved yet (Deanne-Drummond 1993, Aslam *et al.* 1996, Crawford and Glass 1998, Mata *et al.* 2000, Silveira *et al.* 2001, 2012). Influx of NO_3^- through the plasma membrane does not directly implicate anions but it is dependent on H^+-ATPase activity, utilizing H^+ as symporter in a $2H^+/NO_3^-$ stoichiometry and involving a H^+ gradient (Santos *et al.* 2011, Andrews *et al.* 2013).

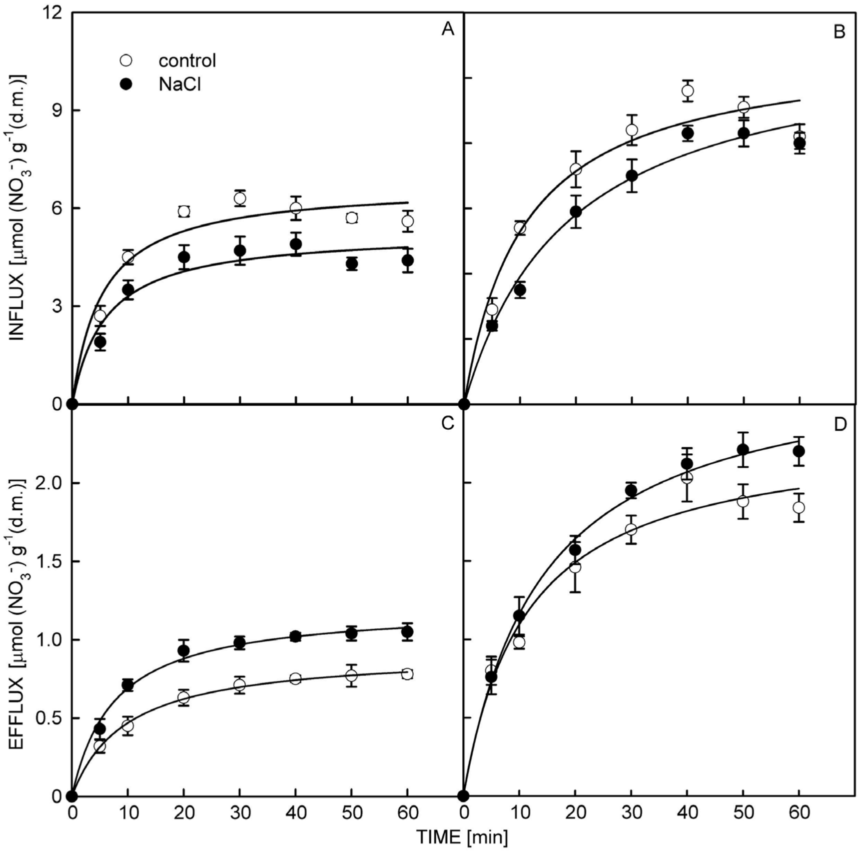

Fig. 5. Apoplastic (*A*) and symplastic (*B*) nitrate influxes in cowpea detached roots exposed to an incubation solution containing 5 mM KNO_3; apoplastic (*C*) and symplastic (*D*) nitrate effluxes in cowpea roots exposed to a KNO_3 free incubation solution. Both influx and efflux were measured in control roots and roots treated with 100 mM NaCl for 60 min. Means of four replicates ± SDs.

Potassium chloride induced a trend of a higher inhibition of NO_3^- influx and, conversely, led to a higher NO_3^- efflux compared to NaCl. As these processes contribute inversely to net NO_3^- uptake, it is possible that a compensatory effect for nitrate acquisition should have occurred (Aslam *et al.* 1996, Miller and Smith 1996, Mata *et al.* 2000). In the majority of the reports involving K^+ and Na^+ uptake at concentrations comparable with those found in cultivated soils (at LATS range), K^+ is frequently absorbed in a higher proportion compared to Na^+ (Ivashikina and Feyziev 1998, Massa *et al.* 2009). Moreover, it has been reported in several species that high KCl concentrations, when supplied for a long term, might cause a higher toxicity in comparison to equivalent high NaCl concentrations (Chen *et al.* 2007, Yuan *et al.* 2010, Kong *et al.* 2011). However, in the present study, the effects of the three salts were compared using the kinetic characteristics related to net NO_3^- uptake (K_m and

V_{max}) under a short-term exposure. Thus, the fact that 100 mM KCl induced a higher decrease in V_{max} compared to NaCl is not enough to conclude that K^+ is more harmful than Na^+ for the cowpea plants.

Several experiments involving influx and efflux of salts have evidenced that a cation activity might affect uptake of an accompanying anion and *vice-versa*, therefore, it could change membrane potential (Chen *et al.* 2007). These alterations at the level of plasmalemma might alter net NO_3^- uptake affecting influx or efflux processes (Aslam *et al.* 1996, Crawford and Glass 1998, Mata *et al.* 2000). In addition, it is plausible to argue that a higher reduction in V_{max} of NO_3^- influx induced by KCl in comparison to NaCl, in both the LATS and the HATS, could be attributed to a higher K^+ influx accomplished to a higher Cl^- uptake (the effect of the complementary cation). This reduction in V_{max} could be associated with changes in membrane potential caused by alterations in cation-anion balance between apoplast, cytosol, and vacuoles (Crawford and Glass 1998, Chen *et al.* 2007, Glass *et al.* 2007).

It is interesting to highlight that in this study, both PEG and mannitol at iso-osmotic concentrations displayed a higher inhibitory effect on NO_3^- influx in both the LATS and the HATS compared to the studied salts. Unexpectedly, both the osmotic agents presented a competitive inhibition in both the transport systems. Our results are similar to those reported by Peuke and Jeschke (1999) studying barley seedlings. Unfortunately, searching current literature, this mentioned work is the only one concerning kinetic aspects of inhibition of net NO_3^- uptake by salts and osmotic agents. In this case, it is hard to found a plausible explanation for these unexpected results (*i.e.,* the salt induced non-competitive inhibition and competitive interaction with NO_3^- influx triggered by osmotic agents). However, these results strongly evidence that the osmotic component of salinity is very important for net NO_3^- acquisition, and this component has been neglected when compared with the inhibitory effects attributed to specific saline ions and particularly to Cl^- by acompetitive inhibitory effect (Cerezo *et al.* 1999, Silveira *et al.* 2012).

Michaelis-Menten kinetics applied by Epstein (1972) to analyze ion influx in roots follows the same principles as biochemical reactions. Ionic competitive inhibition assumes that ions are able to compete for the same binding site of nitrate transporters (NO_3^- versus Cl^- or SO_4^{2-}). In this condition, K_m (NO_3^-) did not change by effect of the studied inhibitor ions, whereas V_{max} decreased suggesting that inhibitory effects were non-specific and more dependent on salt composition and ionic strength than on a direct competition with NO_3^- (Ivashikina and Feyziev 1998, Peuke and Jeschke 1999). In contrast, the obtained results suggest that both osmotic agents were able to indirectly restrict binding NO_3^- to the specific site of nitrate transporters and as consequence alter K_m and not V_{max} characterizing a competitive inhibition (Epstein 1972, Peuke and Jeschke 1999, Massa *et al.* 2009, Parker and Newstead 2014). Thus, as NO_3^- influx and efflux utilize specific protein transporters and salts contain different cations and anions, the physiological interpretation of the results obtained by mathematical model is hard (Mata *et al.* 2000). In addition, in these kinetic studies commonly, the osmotic agents and salts are employed in high concentrations inducing a strong interaction with NO_3^-, consequently affecting its chemical activity due to an increase in ionic strength.

Nitrate acquisition (net NO_3^- uptake) in the presence of salts and PEG also decreased by increase of efflux in apoplastic and symplastic compartments. Interestingly, PEG was more effective for nitrate efflux inhibition than the three studied salts as noticed previously for influx measurements in both LATS and HATS components. Some studies have shown that influx and efflux of NO_3^- are processes regulated by different and independent mechanisms (Aslam *et al.* 1996). Influx and efflux in apoplast are important to plant nutrition and elimination of excess salt ions from the cytoplasm (Glass 2007). In this study, NaCl increased similarly NO_3^- efflux in both apoplast and symplast suggesting some coordination between these two processes (Aslam *et al.* 1996). Among the three studied salts, Na_2SO_4 was less effective in stimulation NO_3^- efflux, whereas NaCl and KCl displayed a trend slightly contrasting, with KCl being more effective. In the presence of NaCl, efflux is species-dependent and varies with the N-source as reported in cotton plants exposed to NaCl, where NH_4^+ efflux is higher than NO_3^- one (Dai *et al.* 2015).

It is important to note that the effects of PEG and mannitol on NO_3^- efflux were unexpected since both osmotic agents induced similar effects on NO_3^- influx. Like the effects caused by salts, it would be expected that both PEG and mannitol will stimulate nitrate efflux inducing a decrease in net NO_3^- uptake. The opposite effect caused by mannitol compared to PEG and the salts on nitrate efflux (inhibiting nitrate efflux), was not reported in literature to date. Some reports have evidenced that mannitol might cause membrane hyperpolarization (Wegner *et al.* 2011), and possibly this effect could have altered NO_3^- efflux in the cowpea roots. Indeed, plasmalemma potential is involved directly in NO_3^- and other solutes uptake (Parker and Newstead 2014), but specific reports for nitrate efflux in response to PEG and mannitol were not found in literature. This question is important because these osmotic agents are broadly employed in studies of plant nutrition (Sassi-Aydi *et al.* 2014). In addition, mannitol is an effective scavenger of hydroxyl radical, the most harmful reactive oxygen substance (Shen *et al.* 1997), and this effect might affect cellular metabolism.

In summary, the results of this study suggest that salinity components (ionic and osmotic), simulated by KCl, NaCl, and Na_2SO_4; and PEG and mannitol, cause a strong inhibition of net NO_3^- uptake in cowpea roots. These inhibitory effects on NO_3^- influx occured in both transport system, the LATS and the HATS, involving, surprisingly, a non-competitive interaction between salts and NO_3^- and, inversely, a competitive mechanism associated with PEG and mannitol. These results evidence that Cl^- is not critical for NO_3^- net uptake inhibition due a competitive mechanism. Sodium chloride and PEG stimulated NO_3^- efflux from both apoplast and symplast, but unexpectedly, mannitol inhibited this process. The osmotic agents were more effective than the salts in inhibition of NO_3^- influx and efflux evidencing that this component is very important for nitrate acquisition under saline conditions.

References

Adamowicz, S., Le Bot, J.: Trends in modelling nitrate uptake. - Acta Horticult. **507**: 231-239, 1999.

Andrews, M., Raven, J.A., Lea, P.J.: Do plants need nitrate? The mechanisms by which nitrogen form affects plants. - Ann. appl. Biol. **163**: 174-199, 2013.

Aslam, M., Travis, R.L., Rains, W.D.: Evidence for substrate induction of a nitrate efflux system in barley roots. - Plant Physiol. **112**: 1167-1175, 1996.

Cachorro, P., Ortiz, A., Cerdá, A.: Implications of calcium nutrition on the response of *Phaseolus vulgaris* L. to salinity. - Plant Soil **159**: 205-212, 1994.

Cerezo, M., García-Agustín, P., Primo-Millo, E.: Influence of chloride and transpiration on net $^{15}NO_3^-$ uptake rate by citrus roots. - Ann. Bot. **84**: 117-120, 1999.

Chen, Z.H., Pottosin, I., Guin, T.A.: Root plasma membrane transporters controlling K^+/Na^+ homeostasis in salt-stressed barley. - Plant Physiol. **145**: 1714-1725, 2007.

Clarkson, D.T.: Mechanisms for N-uptake and their running costs; is there scope for more efficiency? - In: Lambers, H., Poorter, H., Van Veuren, M.M.I. (ed.): Inherent Variation in Plant Growth. Physiological Mechanisms and Ecological Consequences. Pp. 221-235. Backhuys Publishers, Leiden 1998.

Crawford, N.M., Glass, A.D.M.: Molecular and physiological aspects of nitrate uptake in plants. - Trends Plant Sci. **3**: 389-395, 1998.

Dai, J., Duan, L., Dong, h.: Comparative effect of nitrogen forms on nitrogen uptake and cotton growth under salinity stress. - J. Plant Nutr. **38**: 1530-1543, 2015.

Deanne-Drummond, C.E.: Biochemical and biophysical aspects of nitrate uptake and its regulation. - In: Abrol, Y.P.(ed.): Nitrogen in Higher Plants. Pp. 1-37. Somerset: Research Studies, Tauton 1993.

Epstein, E.: Mineral Nutrition of Plants. Principles and Perspectives. - Wiley, New York 1972.

Forde, B.G., Clarkson, D.T.: Nitrate and ammonium nutrition of plants: physiological and molecular perspectives. - Adv. bot. Res. **30**: 1-90, 1999.

Glass, A.D.M.: The apoplast: a kinetic perspective. In: Sattelmacher, B., Horst, W.J. (ed.): The Apoplast of Higher Plants: Compartment of Storage, Transport, and Reactions. The Significance of the Apoplast for the Mineral Nutrition of Higher Plants. Pp. 96. Springer-Verlag, Dordrecht 2007.

Hoagland, D.R., Arnon, D.I.: The water culture method for growing plants without soil. - Calif. Agr. Exp. Sta. Bull. **347**: 1-39, 1950.

Ivashikina, N.V., Feyziev, Y.M.: Regulation of nitrate uptake in maize seedlings by accompanying cations. - Plant Sci. **131**: 25-34, 1998.

Kaburagi, E., Yamada, M., Fujiyama, H.: Sodium, but not potassium, enhances root to leaf nitrate translocation in Swiss chard *(Beta vulgaris* L. var. Cicla). - Environ. exp. Bot. **112**: 27-32, 2015.

Kong, X.Q., Gao, X.H., Sun, W., An, J., Zhang, H.: Cloning and functional characterization of a cation-chloride cotransporter gene *OsCCC1*. - Plant mol. Biol. **75**: 567-578, 2011.

Li, R., Shi, F., Fukuda, K.: Interactive effects of salt and alkali stresses on seed germination, germination recovery, and seedling growth of a halophyte *Spartina alterniflora* (Poaceae). - South Afr. J. Bot. **76**: 380-387, 2010.

Massa, D., Mattson, N.S., Lieth, H.: Effects of saline root environment (NaCl) on nitrate and potassium uptake kinetics for rose plants: a Michaelis-Menten modeling approach. - Plant Soil **318**: 101-115, 2009.

Mata, C., Vemde, N., Clarkson, D.K., Martins-Loução, M.A., Lambers, H.: Influx, efflux and net uptake of nitrate in *Quercus suber* seedlings. - Plant Soil **221**: 25-32, 2000.

Miller, A.J., Smith, S.J.: Nitrate transport and compartmentation in cereal root cells. - J. exp. Bot. **47**: 843-854, 1996.

Miller, A.J., Fan, X., Orsel, M., Smith, S.J., Wells, D.M.: Nitrate transport and signaling. - J. exp. Bot. **58**: 2297-2306, 2007.

Ouerghi, Z., Cornic, G., Roudani, M., Ayadi, A., Brulfert, J.: Effect of on photosynthesis of two species (*Triticum durum* and *T. aestivum*) differing in their sensitivity to salt stress. - J. Plant Physiol. **156**: 135-340, 2000.

Ourry, A., Meslé, S.S., Boucaud, J.: Effect of osmotic stress (NaCl and polyethylene glycol) on nitrate uptake, translocation, storage and reduction in ryegrass (*Lolium perene* L.). - New Phytol. **120**: 275-280, 1992.

Parker, J.L., Newstead, S.: Molecular basis of nitrate uptake by the plant nitrate transporter NRT1.1. - Nature **507**: 68-73, 2014.

Peuke, A.D., Jeschke, W.D.: The characterization of inhibition of net nitrate uptake by salt in salt-tolerant barley (*Hordeum vulgare* L. cv. California Mauriot). - J. exp. Bot. **50**: 1365-1372, 1999.

Ramos, J., López, M.J., Benlloch, M.: Effect of NaCl and KCl salts on the growth and solute accumulation of the halophyte *Atriplex nummularia*. - Plant Soil **259**: 163-168, 2004.

Rubinigg, M., Posthumus, F., Ferschke, M., Elzenga, J.T.M., Stulen, I.: Effects of NaCl salinity on ^{15}N-nitrate fluxes and specific root length in the halophyte *Plantago maritima* L. - Plant Soil **250**: 201-213, 2003.

Santos, L.A., Santos, W.A., Sperandio, M.V.L., Bucher, C.A., Souza, S.R., Fernandes, M.S.: Nitrate uptake kinetics and metabolic parameters in two rice varieties grown in high and low nitrate. - J. Plant Nutr. **34**: 988-1002, 2011.

Shen, B., Jensen, R.G., Bohnert, H.: Mannitol protects against oxidation by hydroxyl radicals. - Plant Physiol. **115**: 527-

532, 1997.

Sassi-Aydi, S., Aydi, S., Abdelly, C.: Inorganic nitrogen nutrition enhances osmotic stress tolerance in *Phaseolus vulgaris*: lessons from a crought-sensitive cultivar. - Hort. Sci. **49**: 550-555, 2014.

Silveira, J.A.G., Melo, A.R.B., Viégas, R.A., Oliveira, J.T.A.: Salinity-induced effects on nitrogen assimilation related to growth in cowpea plants. - Environ. exp. Bot. **46**: 171-179, 2001.

Silveira, J.A.G., Melo, A.R.B., Martins, M.O., Ferreira-Silva, S.L., Aragão, R.M., Silva, E.N., Viégas, R.A.: Salinity affects indirectly nitrate acquisition associated with glutamine accumulation in cowpea roots. - Biol Plant. **56**: 575-580, 2012.

Sorgonà ,A., Lupini, A., Mercati, F., Di Dio, L., Sunseri, F., Abenavoli. M.R.: Nitrate uptake along the maize primary root: an integrated physiological and molecular approach. - Plant Cell Environ. **34**: 1127-1140, 2011.

Tsay, Y.F., Chiu, C.C., Tsai, C.B., Ho, C.H., Hsu, P.K.: Nitrate transporters and peptide transporters. - FEBS Lett. **581**: 2290-2300, 2007.

Wegner, L.H., Stefano, G., Shabala, L., Rossi, M., Mancuso, S., Shabala, S.: Sequential depolarization of root cortical and stellar cells induced by an acute shock – implications for Na^+ and K^+ transport into xylem vessels. - Plant Cell Environ. **34**: 859-869, 2011.

Yang, T., Zhu, L., Wang, S., Gu, W., Huang, D., Xu, W., Jiang, A., Li, S.: Nitrate uptake kinetics of grapevine under root restriction. - Sci. Horticult. **111**: 358-364, 2007.

Yuan, J.F., Tian, C.Y., Feng, G.: Effects of sodium on nitrate uptake and osmotic adjustament of *Suaeda physophora*. - J Arid Land **2**: 190-196, 2010.

Zhao, F.J., Hamon, R.E., Lombi, E., Mclaughlin, M.J., Mcgrath, S.P.: Characteristics of cadmium uptake in two contrasting ecotypes of the hyperaccumulator *Thlaspi cauerulescens*. - J. exp. Bot. **53**: 535-543, 2002.