GROWTH REDUCTION INDUCED BY SALT INHIBIT NITRATE INFLUX IN COWPEA ROOTS BY LOW N-DEMAND

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ABSTRACT – This study was developed to test the hypothesis that salinity inhibits indirectly the nitrate acquisition by feedback regulation caused by increased amino acids content and low N demand in cowpea roots. Cowpea plants were pretreated with 0 and 50 mM NaCl since seed, to restrict growth and N-demand. After pretreatment (14 days), the plants were divided into control plants, plants in presence NaCl and plants recovered for 4 days in absence NaCl. Our data clearly showed that nitrate influx was down-regulated in roots in presence and recovered NaCl, suggesting that even without the direct presence of salt, the restrict growth resulted in a reduction of N-demand, indicating an indirect effect of salt in modulation of nitrate influx. The recovered plants and plants in presence NaCl accumulated high levels of amino acids, mainly glutamine. In conclusion, the salinity indirectly down-regulates the nitrate acquisition by an endogenous control signalized by accumulated of amino acids. This accumulation is associated with growth restriction and low N-demand in roots.

KEYWORD: Nitrate uptake, salt stress, feedback regulation.

A REDUÇÃO DO CRESCIMENTO INDUZIDA PELO SAL INIBE O INFLUXO DE NITRATO EM RAIZES DE CAUPÍ PELA BAIXA DEMANDA DE N

RESUMO – Resumo - Este estudo foi desenvolvido para testar a hipótese de que a salinidade inibe indiretamente a aquisição de nitrato por regulação de feedback causada pelo aumento do teor de aminoácidos e baixa demanda de N em raízes de caupí. As plantas foram pré-tratadas com NaCl a 0 e 50 mM desde a semente, para restringir o crescimento e a demanda de N. Após pré-tratamento (14 dias), as plantas foram divididas em plantas de controlo, plantas em presença de NaCl e plantas recuperadas durante 4 dias em ausência de NaCl. Nossos dados mostraram

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claramente que o influxo de nitrato foi regulado para baixo nas raízes em presença e NaCl recuperado, sugerindo que mesmo sem a presença direta de sal, o crescimento restrito resultou em uma redução da demanda de N, indicando um efeito indireto do sal na modulação de influxo de nitrato. As plantas e plantas recuperadas em presença de NaCl acumulavam altos níveis de aminoácidos, principalmente glutamina. Em conclusão, a salinidade indiretamente regula negativamente a aquisição de nitrato por um controle endógeno sinalizado por acúmulo de aminoácidos. Essa acumulação está associada à restrição do crescimento e à baixa demanda de N nas raízes.

PALAVRAS-CHAVE: Absorção de nitrato, estresse salino, regulação por *feedback*.

INTRODUCTION

Salt dissolved in the irrigation water and saline soil origin might interfere in the nitrogen use by plants, and this salt effect was been previously shown (Debouba et al., 2006). One of the most important abiotic factors limiting plant productivity is water stress induced by drought or salinity. This is especially acute in arid and semi-arid regions, like Brazilian Northeast, where cowpea (*Vigna unguiculate* L.) is a widely cultivated species (Cavalcanti et al., 2007).

Salinity affects strongly the nitrate flux processes and several reported studies have employed NaCl as a model of salinity, but this approach is very simplified as it does not consider some aspects of salinity components such ionic composition, cation-anion specific effects and osmotic effects (Rubiningg et al., 2003).

Some studies have showed that influx and efflux of NO_3^- ions are processes regulated by different and independent mechanisms (Aslam et al., 1996). Nitrate uptake by plant roots can be negatively influenced by the external supply of amino acids or the tissue concentrations of amino acids (Aslam et al., 2001).

Glutamine (Gln) has been quote to be effective in inhibiting the expression of inducible high affinity nitrate transporters (Vidmar et al., 2000). Both nitrate-induced influx and transporter transcript abundance were decreased simultaneously in root tissue treated with exogenously applied amino acids (Vidmar et al., 2000). Fan et al. (2006) concluded which increases in cellular pool of glutamine can affect nitrate reductase activity and these changes may be responsible for an increase in cytosolic nitrate.

One interesting aspect of this feedback regulatory mechanism for N status is the change associated with plant development (Miller et al., 2007). The feedback regulation is potentially

mediated by amino acid pools between roots and shoots and is considered a signal to the plant N status (Nazoa, 2003). Once accumulated internally or externally, the supplied amino acids can regulate the nitrate uptake and reduction systems (Surabhi et al., 2008). However, few studies report as NO_3^- uptake and assimilation processes is modulated together with salinity conditions.

In this study, we investigate if salt-induced growth restriction is capable of negatively modulating nitrate uptake and assimilation by means of a negative feedback mechanism exerted by accumulation of amino acids in the roots.

MATERIAL AND METHODS

Cowpea seeds [*Vigna unguiculata* (L.) Walp.] cv. pitiúba previously sterilized in sodium hypochlorite solution 0.2% (30 min.) were germinated in paper rolls under controlled environment chamber at 27±2 □C, 12h photoperiod and 240 □mol m^{-2} s^{-1} of photosynthetically active radiation (PAR). After four days, seedlings were transferred to plastic pots (12 L) containing Hoagland's solution (Hoagland and Arnon, 1950), pH 6.0 for fourteen days under greenhouse conditions, where air temperature varied between 23-31 °C with a mean temperature of 27 °C, air relative humidity of 75 %, a maximum photosynthetic photon flux density (PPFD) of 1200 µmol m^{-2} s^{-1} and photoperiod 12 h.

Cowpea plants were pretreated with 0 and 50 mM NaCl since seed, to restrict growth and N-demand. After pretreatment (14 days), the plants were divided into control plants, plants in presence NaCl and plants recovered for 4 days in absence NaCl.

For influx assay, a new nutrient solution (N-free) was replaced four days before of the determinations inducing a NO_3^- nutritional limitation.

Cowpea roots were excised and washed initially in $CaCl_2$ 0.1 mM for 5 min and transferred to incubation solution containing MES 1.0 mM, $CaCl_2$ 0.1 mM and KNO_3 2.0 mM pH 6.0 and incubated at 30 °C . The nitrate influx (consumption) was quantified by the NO_3^- concentration depletion ($[NO_3^-]$initial - $[NO_3^-]$final) in the incubation solution after 100 minutes, utilizing Cawse's method (Cawse, 1967).

Excised roots from the 14 days seedlings were washed with 0.1 mM $CaCl_2$ for 5 min to eliminate apoplastic ions. The same extraction was performed to determination of the Na^+, NO_3^-, NH_4^+ and total free Amino acids concentrations. Lyophilized roots were finely powdered and samples of 50 mg were extracted with 5 mL of deionized water at 100 °C for 60 min. in hermetically closed tubes.

The Na^+ concentrations in the root tissues were measured using flame photometry. The NO_3^- concentrations in tissues were performed from the lyophilized material (Cataldo, 1975). The method for NH_4^+ determination was based on the formation of a compound of blue color, the indophenol, after the reaction of ammonia with phenol and hypochlorite in alkaline pH induced by NaOH addition in the reaction solution (Weatherburn, 1967; Felker, 1977). The total free amino acids were measured, according to Yemm and Cocking (1955).

Some amino acids were separated and analyzed by reverse-phase HPLC as their OPA derivatives, based on the method described by Jarret et al. (1986) and adapted by Puiatti and Sodek, 1999, with some changes.

A completely randomized design was used with four replicates per treatment. An individual erlenmeyer containing two roots represented a replicate. Data were analyzed by ANOVA, and the means were compared by the least significant difference (LSD) test at the 0.05 level of confidence.

RESULTS AND DISCUSSION

In our results, the treatment where the presence of salt was maintained, promoted a strong reduction in the growth and development of plants, and mainly in the roots (Figure 1). This reduction was also observed in the roots of plants subjected to recovery of 4 days absence salt. The reduction was around 60 and 41 %, in the roots treated with salt presence and roots submitted at the recovery, respectively.

Contrasting the roots of the treatment maintained in salt presence and the roots recovered, we checked which the removal of salt for 4 days was enough to recover few dry mass of roots cowpea. However, yet remain low, compared on the control treatment.

The salt application since germination promoted an increase significant in the Na^+ content in the roots of cowpea. When compared with the control treatment, the salt presence in roots provided an increment of more than six-fold, in Na^+ content. The recovery of the roots by 4 days was sufficient to reduce (3.5-fold) the Na^+ in tissue, becoming significantly similar at the control treatment.

The NO_3^- influx process was tested with the purpose of verify the effect of the salt, as from reduce triggered in the plants growth, caused by salt. Our studies are pioneer in affirm the indirect effect of salt, and differs of previous studies, because in them always were assessed only the direct effect of salinity in the NO_3^- influx in the presence of salt.

The results showed, in the end test (100 minutes), a considerable reduction in the NO_3^- influx for both treatments in salt presence and recovery (Figure 3). In the treatment maintained in the presence of the salt there was a decrease around 52 % compared with the control treatment. However, in the treatment of recovery, even without the presence of sodium in tissue and medium solution, also declined, which ranged around 27 %.

In the first 20 minutes, the control treatment had a value of influx 2.07 [µmol NO_3^- g^{-1} (FM)], while the recovery treatment only achieved this value after 40 minutes and the salt treatment until the end of the evaluation not managed to achieve this level.

The evaluation of NO_3^- content showed that, salt application has promoted a significantly decreased in the NO_3^- content in the root tissue (Figure 4). This reduction caused by salt application was approximately 72 % compared to the control treatment. On the other hand, the recovery treatment, the roots were able to regain the ability to accumulate NO_3^- partially, but still, lower than control treatment, ranging around 27 %. Contrasting treatment maintained in salt and the recovery, was been a 60 % decrease in NO_3^- content in the root tissue.

As shown in figure 5, the roots maintained in saline conditions and in the treatment of roots recovery, the ammonium content increased, but not differ among them. Compared with control treatment, the permanence under saline conditions and the roots recovery promoted increase of almost 50 and 40 %, respectively, in NH_4^+ content in the root tissue.

A significant increase in the concentration of total amino acid in roots, resultant from the presence of salt in the pretreatment solution (Figure 6). These results were similar of the NH_4^+ content and directly contrast to that observed for nitrate influx and nitrate content. This effect was 1.5-fold higher in root under saline conditions than in the roots of the control treatment. The roots submitted the recovery treatment even without the presence of salt directly in tissue, showed a strong accumulation in total amino acid content of the roots.

In the present study, some lines of evidence suggest that nitrate influx inhibition in cowpea roots under saline conditions occurred indirectly by feedback mechanism. The low capacity of nitrate acquisition in pretreated plants could be due to indirect effects of salt stress triggered by negative feedback regulation due to accumulation of signaling compounds, such as amino acids, because of growth restriction and impairment of protein synthesis (low N-demand). Previously has been established that salt-induced nitrate uptake inhibition in cowpea is closely associated with shoot growth (Silveira *et al.*, 2001) and that glutamine and other amino acids might exert um negative regulatory mechanism by feedback (Silveira *et al*., 2012).

Several possible signals for the down-regulation of have been proposed including NO_3^-, NH_4^+ and/or amino acids (King *et al*., 1993).

A more detailed study, presented by Muller and Touraine (1992), demonstrated inhibition of uptake by 50 % or greater by alanine, glutamine, asparagine, arginine, beta-alanine, serine, and glutamine when soybean seedlings were pretreated for 18 h prior to exposure to NO_3^-.

The NO_3^- uptake root systems can be divided into transport systems that operate in different levels of NO_3^- concentration in the culture environment (Crawford and Glass, 1998) and with these physiological changes, the feedback regulation by Gln on these uptake systems appears to operate of different form.

Muller and Touraine (1992) demonstrated that amino acid translocation from shoots to roots controls nitrate uptake rate and suggested that the feedback inhibition of nitrate uptake by amino acids provides balance between nitrate uptake by roots with the requirement of the shoots. In this study, the main amino acids related to the inhibition of nitrate absorption was Asp, Glu, Asn, Arg, Ala, and in lesser degree, Gln and Ser.

CONCLUSION

Thus, the data analysis suggests that the limitation in the nitrate acquisition exhibited by exposure of plants to salt is probably associated with a coordinated process involving a negative feedback mechanism that could primarily control, at least, a part of the uptake total NO_3^- by an indirect effect and suggest that the negative feedback hypothesis might be applicable, at least partially, to explain the salt-induced inhibition of nitrate uptake and assimilation and plant growth.

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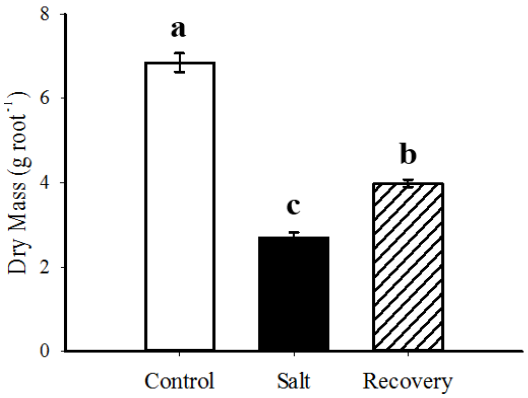

Figure 1 – Dry mass of cowpea roots. Roots were pretreated for 14 days with salt (50 mM NaCl) and recovered (4 days) after salt withdrawal.

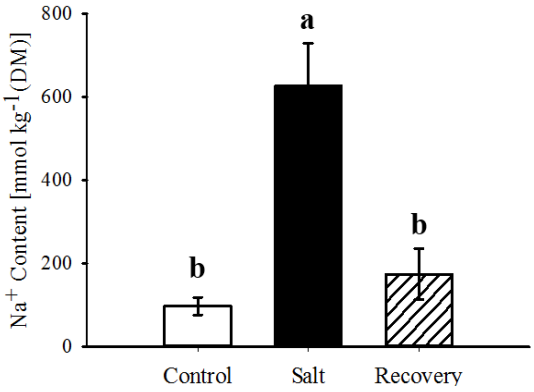

Figure 2 – Sodium content in cowpea roots. Roots were pretreated for 14 days with salt (50 mM NaCl) and recovered (4 days) after salt withdrawal.

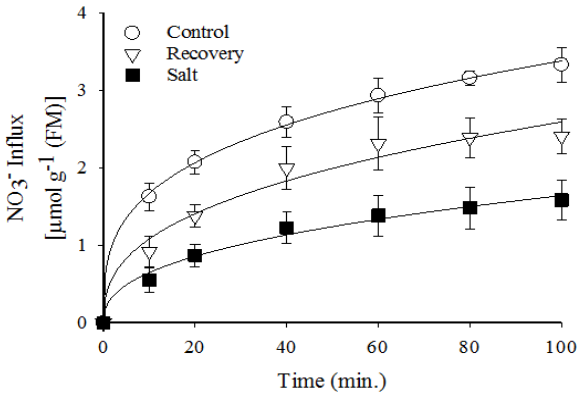

Figure 3 - Nitrate influx in cowpea roots. Roots were pretreated for 14 days with salt (50 mM NaCl) and recovered (4 days) after salt withdrawal.

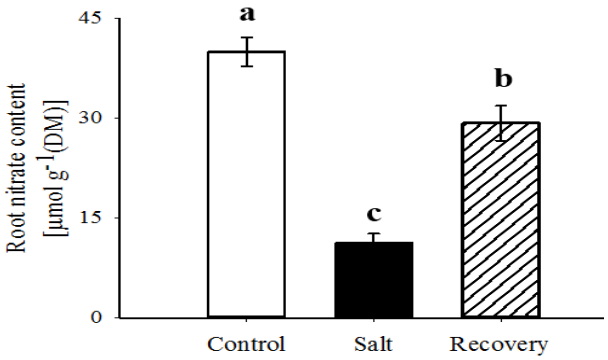

Figure 4 - Nitrate content in cowpea roots. Roots were pretreated for 14 days with salt (50 mM NaCl) and recovered (4 days) after salt withdrawal.

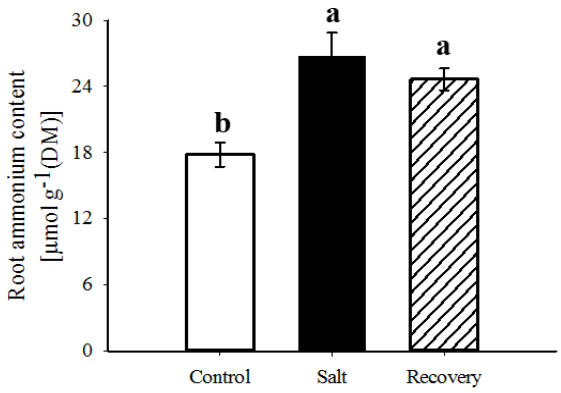

Figure 5 - Root ammonium content in cowpea roots. Roots were pretreated for 14 days with salt (50 mM NaCl) and recovered (4 days) after salt withdrawal.

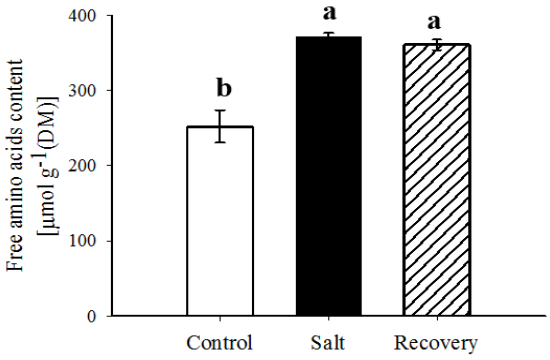

Figure 6 - Root Free amino acids content in cowpea roots. Roots were pretreated for 14 days with salt (50 mM NaCl) and recovered (4 days) after salt withdrawal.

Table 1. Effect of salt application (50 mM NaCl) and recovery (4 days) on the content of Aspartate (Asp), Glutamate (Glu), Serine (Ser), Glutamine (Gln) and Asparagine (Asn) in cowpea roots. Data are means of four replicates ±SD.

Treatment	Amino acids				
	Asp	Glu	Ser	Gln	Asn
			[µmol g^{-1} (DM)]		
Control	1.90 ± 0.10	3.10 ± 0.19	3.45 ± 0.25	1.34 ± 0,23	0.93 ± 0.41
Salt	5.42 ± 0.02	6.84 ± 0.12	5.96 ± 0.09	5.23 ± 0.06	2.31 ± 0.01
Recovery	3.93 ± 0.03	4.15 ± 0.49	5.75 ± 0.13	4.76 ± 0.09	1.82 ± 0.04