BRIEF COMMUNICATION

Salinity affects indirectly nitrate acquisition associated with glutamine accumulation in cowpea roots

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Abstract

The aim of this study was to test the hypothesis that salinity can affect indirectly the nitrate acquisition by a negative modulation triggered by glutamine accumulation. Cowpea plants were exposed to a mild NaCl concentration (50 mM) in order to restrict growth and N-demand. After 21 d, pretreated plants and control plants were supplied with 0, 5 and 10 mM of $Ca(NO_3)_2$ for 3 d in absence of NaCl. Salt pretreated plants showed a great limitation in acquisition of NO_3^-, indicated by decline in the nitrate uptake rate, NO_3^- accumulation, nitrate reductase activity and protein content. The restriction of NO_3^- utilization was positively associated with increased glutamine synthetase activity and glutamine accumulation, especially in roots.

Additional key words: glutamine synthetase, NaCl stress, nitrate reductase, nitrate uptake, *Vigna unguiculata*.

Experimental results have shown that salt stress affects several metabolic steps of nitrate utilization in plants, such as NO^-_3 influx (Peuke and Jeschke 1999), loading of nitrate into the root xylem (Peuke *et al*. 1996), nitrate reductase activity (Abd-El Baki *et al*. 2000), amino acid metabolism (Silveira *et al*. 2003) and protein synthesis (Aslam *et al*. 1996). On the other hand, salt stress induces an increase in the glutamine synthetase activity (Silveira *et al*. 2003, Veeranagamallaiah *et al*. 2007). The salt-induced imbalance between ammonia assimilation and protein synthesis frequently induces a significant increase in the free amino acid pool in the roots and shoots (Silveira *et al*. 2001, 2003). This increase in the content of amino acids, which is associated with a lower N demand for plant growth, could cause a negative control of the nitrate influx (Foyer and Noctor 2004). The negative feedback model has become generally accepted, but the precise nature or mechanism of the signal(s) is not known yet (Miller *et al*. 2007a). The free amino acids, especially glutamine, are the strongest candidates as the signaling molecule for regulation of nitrate uptake (Krapp *et al*. 2004, Thornton 2004, Miller *et al*. 2007b).

Although free glutamine is involved in the negative modulation of nitrate reductase gene expression (Fan *et al*. 2006), its involvement in the regulation of nitrate uptake is yet not confirmed, although several works have suggested its participation (for a review, see Miller *et al*. 2007a). Experimental results have shown that when glutamine is exogenously supplied to the root medium or is artificially increased in the root tissues and phloem sap, it negatively modulates both the nitrate influx and NO_3^- transporter expression (Pal'ove-Balang and Mistrík 2002, Thornton 2004). Likewise, salinity controls both the nitrate uptake and growth in parallel to accumulation of amino acids (Silveira *et al*. 2001). However, to the best of our knowledge, there are no published reports concerning the mechanisms underlying the salt-induced growth restriction associated with the reduction in nitrate uptake

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Abbreviations: AA - amino acids; Gln - glutamine; GS - glutamine synthetase.
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via a feedback mechanism. In the current work, cowpea was chosen as the model plant, because mild salinity affected its nitrate uptake and growth without causing salt toxicity (Silveira *et al*. 2001). In our study, cowpea plants were pretreated with 50 mM NaCl to induce restriction of growth and N-demand and accumulation of amino acids. Further, the plants were exposed to different NO_3^- concentration, in absence of NaCl, to investigate if NaCl is capable negatively modulate nitrate uptake and assimilation by means of a negative feedback mechanism exerted by accumulation of amino acids, especially glutamine (Gln) by means of increased glutamine synthetase (GS) activity

Cowpea seeds [*Vigna unguiculata* (L.) Walp.], cv. Vita-7, from EMBRAPA, Brazil, were surface-sterilized with a 2 % sodium hypochlorite solution and sown on sand. The seeds were separated into two sets. The first one (control) was irrigated daily with a one-quarter strength Hoagland's nutrient solution modified in the N content (nitrate as the sole N source) as described by Silveira *et al* (2001), and the other group (salt-treated) was irrigated with the same solution supplemented with 50 mM NaCl. After one week, the seedlings were transferred to individual 4-dm^3 pots containing a half-strength modified Hoagland and Arnon (1950) nutrient solution with 5 mM KNO_3 as the sole N source and supplemented or not with 50 mM NaCl for two weeks. The nutrient solution was aerated and completely changed weekly, and the pH was maintained daily at 6.5 ± 0.5 by adding 1 M NaOH or 1 M HCl. The plants were initially grown in a greenhouse under natural conditions with day/night mean temperatures of 29/24 °C, relative humilities of 63/85 %, a 12-h photoperiod, and an average maximum photosynthetic photon flux density (PPFD) of 700 μmol m^{-2} s^{-1} measured near the top of plant canopy (*190SA* quantum sensor, *LI-COR*, Lincoln, USA).

After the pretreatment period, 21-day-old salt pretreated and untreated control plants were transferred to a growth chamber with controlled conditions (day/night temperature of 27/24 °C, relative humidity of 75 %, photosynthetic photon flux density of 500 μmol m^{-2} s^{-1} (a combination of fluorescent and incandescent lamps, *Philips*®) and a 12-h photoperiod. First, all plants were supplied with a nutrient solution containing 0.1 mM $Ca(NO_3)_2$ in absence of NaCl for 48 h in order to induce nitrate deprivation. For determination of nitrate uptake, pretreated and control plants were exposed to 0, 5, or 10 mM $Ca(NO_3)_2$. Net uptake was taken at 60 min as the kinetic pattern reached a plateau (steady-state condition). Then, the roots were washed with distilled water for 5 min to remove the external and apoplastic NO_3^-, and subsequently the plants were transferred to a complete nutrient solution containing 0, 5 or 10 mM $Ca(NO_3)_2$ for 3 d in order to evaluate the variables associated with nitrate utilization. In controls, Ca^{+2} was supplied at a 5 mM concentration as $CaCl_2$. The net nitrate uptake was determined according to the disappearance of NO_3^- from the root medium by a selective electrode. A group of 21-day-old plants previously pretreated with 50 mM NaCl and other group grown in absence of NaCl were supplied with nutrient solution containing 50 mM NaCl and 10 mM of NO_3^- (salt treatment) or 10 mM of NO_3^- in a nutrient solution without NaCl (control), for 4 d in order to evaluate the salt-induced changes in the GS activity and Gln content in roots and leaves.

Nitrate reductase (NR, EC 1.6.6.1) activity was determined in the supernatant, according to Robin (1979) while the glutamine synthetase (GS, EC 6.3.1.2) activity was determined by the hydroxamate biosynthetic method as described by Berteli *et al* (1995). The total free amino acids were measured after extraction with 80 % (v/v) ethanol and reaction with ninhydrin, according to Yemm and Cocking (1955). The quantification of glutamine, asparagine and proline was performed by HPLC (*Waters Corporation*, USA) using a *Pico-Tag* C18 column, according the manufacturer's instructions as previously described (Voigt *et al*. 2009). The nitrate content in plant tissue was determined by the salicylic acid method (Cataldo *et al*. 1975) after extraction with hot water (95 ºC). The soluble protein was determined by the Bradford (1976) method using bovine serum albumin as the standard.

A completely randomized design was used with four replicates per treatment. An individual pot containing two plants 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.

During time-course experiment in presence of 10 mM NO_3^-, the plants supplied with NaCl 50 mM showed a strong increase both in GS activity and Gln accumulation in roots whereas in leaves the increase was lower, comparable to the respective controls (Fig. 1*A-D*). Despite the prominent salt-induced growth decrease in terms of dry matter accumulation (Table 1), the plants did not exhibit any visual symptoms of salt toxicity. These data show that the salt pretreatment in cowpea was capable to markedly restrict the plant growth and amino acids demand, inducing a great accumulation of Gln due to increased GS activity. After exposure to 0, 5 and 10 mM NO_3^-, in absence of NaCl in the root medium, the control plants showed progressive NO_3^- accumulation in both the leaf and root tissues as the external nitrate content increased, but this response was quantitatively more prominent in the roots. Conversely, in pretreated plants, a differential nitrate accumulation occurred in the leaves and roots when the external nitrate concentration varied from 0 to 5 mM, but it remained unchanged from 5 to 10 mM (Table 1).

The nitrate uptake patterns in pretreated and control plants were similar to those for nitrate accumulation in the leaves (Table 1). Indeed, nitrate uptake increased from 5 to 10 mM nitrate concentration only in the control plants. The NR activity in both the pretreated and control plants was extremely low in plants previously submitted to N deprivation and then exposed to the nutrient solution

free of NO_3^- for 3 d (Table 1). The pretreated cowpea plants exhibited significant restriction of all of the variables associated with N acquisition such as nitrate uptake, nitrate reductase activity, and nitrate accumulation in plant tissues under high nitrate supply in the root medium (Table 1). In the pretreated plants, the leaf

Table 1. Leaf and root dry mass, leaf and root NO_3^- content, NO_3^- uptake, nitrate reductase activity, leaf and root amino acid, protein, glutamine, asparagine and proline contents and leaf and root GS activity in cowpea plants pretreated or not with 50 mM NaCl for 21 d. Pretreated plants were acclimated for two days in a NaCl-free nutrient solution and then exposed to 0, 5 and 10 mM NO_3^- for 3 d. The same lowercase and capital letters represent means that do not differ significantly by Tukey´s test ($P < 0.05$) within the NaCl and control pretreatments, respectively.

Parameters	Control			NaCl		
	0	5	10	0	5	10
Leaf DM [g plant^{-1}]	7.9c	8.9b	10.0a	4.6B	5.8A	5.8A
Root DM [g plant^{-1}]	4.9b	5.9a	6.4a	2.9B	3.9A	3.8A
Leaf nitrate [μmol g^{-1}(DM)]	30.4c	79.8b	115.5a	30.3B	65.5A	67.8A
Root nitrate [μmol g^{-1}(DM)]	10.5c	125.5b	251.5a	81.2B	151.7A	152.1A
Nitrate uptake [μmol g^{-1}(root DM) h^{-1}]	0.0c	37.9b	53.9a	0.0B	28.3A	29.8A
NR activity [nmol g^{-1}(FM) min^{-1}]	3.3c	60.0b	78.3a	3.0B	46.6A	48.3A
Leaf AA [μmol g^{-1}(DM)]	179.0b	291.3a	311.1a	249.4C	371.2B	527.4A
Root AA [μmol g^{-1}(DM)]	89.8b	159.2a	169.3a	149.8C	180.4B	272.3A
Leaf protein [mg g^{-1}(DM)]	34.8c	59.9b	99.8a	39.9B	55.3A	57.1A
Root protein [mg g^{-1}(DM)]	14.8c	20.2b	30.2a	15.9B	20.1A	20.2A
Leaf glutamine [μmol g^{-1}(DM)]	9.3c	12.2b	16.1a	7.9C	16.8B	20.4A
Root glutamine [μmol g^{-1}(DM)]	2.8c	4.2b	6.2a	4.3C	7.5B	12.2A
Leaf asparagine [μmol g^{-1}(DM)]	8.9b	11.2a	12.2a	10.1C	14.2B	16.4A
Root asparagine [μmol g^{-1}(DM)]	6.1a	8.6b	9.1b	8.1A	12.1B	16.3C
Leaf proline [μmol g^{-1}(DM)]	2.1b	3.2a	3.4a	3.0C	4.2B	6.2A
Root proline [μmol g^{-1}(DM)]	1.8b	2.5a	2.7a	2.7C	3.8B	4.6A
Leaf GS activity [nmol g^{-1}(FM) min^{-1}]	48.3c	76.6b	101.6a	53.3C	88.3B	125.0A
Root GS activity [nmol g^{-1}(FM) min^{-1}]	51.6c	70.0b	96.6a	65.0C	98.3B	151.6A

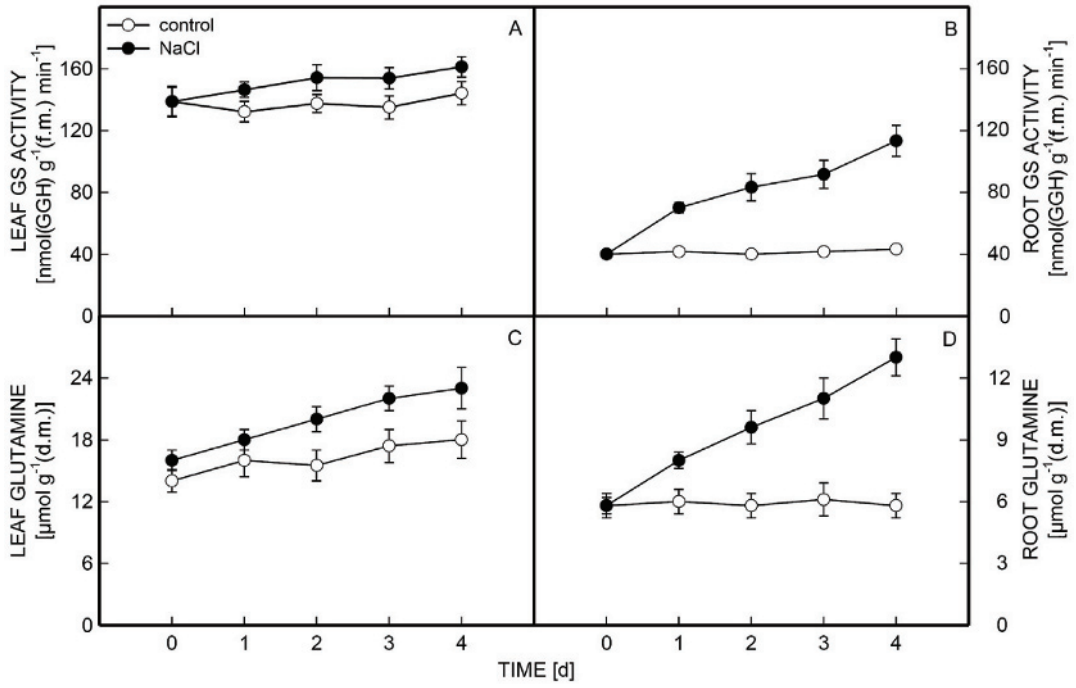

Fig. 1. Changes in the glutamine synthetase activity in leaves (*A*) and roots (*B*), and contents of glutamine in leaves (*C*) and roots (*D*) of cowpea plants pre-treated in a nutrient solution with or without 50 mM of NaCl for 21 d. The pre-treated plants were acclimated for 2 d in a NaCl-free solution and then exposed to 10 mM NO_3^- for 4 d. Means ± SE, $n = 4$.

and root amino acid (AA) contents progressively increased with the external nitrate concentration. For example, when the external NO_3^- concentration was changed from 5 to 10 mM, the AA concentration increased by 42 % in both the leaves and roots. On the other hand, in both the pretreated and control plants and in all of the NO_3^- concentrations, the AA contents were higher in the leaves than in the roots (Table 1).

The soluble protein content in the root and leaf tissues of both treatments showed an inverse trend in comparison to that for the free amino acids (Table 1). Altogether, the data suggest that the pretreated plants further supplied with high nitrate concentrations were unable to convert the extra N into dry matter due to restricted NO_3^- uptake and utilization (as indicated by lower nitrate uptake, tissue nitrate content, NR activity and protein and increased free amino acid content). Moreover, the impairment in nitrate utilization by pretreated plants was closely associated with the Gln content and GS activity, especially in roots (Table 1). The Gln, asparagine and proline accumulation showed a similar trend to that of the total AA both in leaves and roots. Quantitatively, Gln in pretreated plants was the most increased free amino acid (Table 1). In the pretreated plants supplied with 10 mM NO_3^-, the Gln content in the roots was 62 % higher than in plants supplied with 5 mM NO_3^-, whereas this difference was only 21 % in the leaves. Asparagine also greatly accumulated in the roots of previously salt-treated cowpea plants; when the NO_3^- concentration was changed from 5 to 10 mM, the content of this amino acid was increased by 34 and 15 % in the roots and leaves, respectively (Table 1). Even though the proline content was also increased in these plants, the increases were lowest (21 and 47 % in the roots and leaves, respectively; Table 1).

The data from this study clearly show that the pretreated cowpea plants, with strong growth restriction, were unable to uptake and assimilate nitrate when exposed to a high supply of exogenous NO_3^- (10 mM). Intriguingly, both the NO_3^- uptake and leaf NR activity in the pretreated plants reached their maximum rates at an external concentration of 5 mM NO_3^-. In contrast, the acquisition of nitrate, as indicated by uptake and accumulation of NO_3^- in plant tissues and leaf NR activity, significantly increased between 5 and 10 mM NO_3^- in the control plants. The diminished ability for the uptake and assimilation of high concentrations of nitrate displayed by the pretreated plants might be explained, at least in part, by two hypotheses. First, 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). These signaling molecules can induce a down-regulation in the expression of NO_3^- transporters, especially the high-affinity system (Orsel *et al*. 2002; Krapp *et al*. 2004). Secondly, direct effects were caused by osmotic and ionic components of NaCl during the pretreatment period, which induce down-regulation in the expression of NO_3^- transporters, thereby decreasing the influx of nitrate. This hypothesis is apparently less plausible because the pretreated plants were recovered and then grew for three days in the absence of NaCl and in the presence of NO_3^-, enough time to induce the expression and *de novo* synthesis of nitrate transporters (Pal'ove-Balang and Mistrík 2002).

The question of how environmental and metabolic factors affect the absorption of NO_3^- is very complex and remains controversial (Forde 2002). Since the influx and efflux of NO_3^- are independent processes and are regulated by different mechanisms (Aslam *et al*. 1996), the down-regulation of the influx due to decreased nitrate transporter expression and/or an additional enhancement in the efflux of NO_3^-, particularly at the highest external concentration of NO_3^-, could contribute to reducing the net NO_3^- uptake in salt-treated plants. However, cowpea roots of pretreated plants exhibited lower nitrate contents compared to the untreated ones, when both were exposed to the highest NO_3^- concentration. The observation that nitrate is accumulated in the roots and leaves of control plants suggests that endogenous NO_3^- was not an important factor involved in the negative control of the nitrate uptake either by increasing the efflux rates or as a signaling molecule to act by a negative feedback mechanism. However, some experimental evidence has suggested that the endogenous root nitrate content might affect the nitrate uptake (Miller *et al*. 2007a) and expression of nitrate transporters (Krouk *et al*. 2006). The nitrate ions in plants play a central role in several physiological processes, such as nitrate reductase expression and activity, carbon metabolism and root differentiation (Crawford and Glass 1998, Foyer and Noctor 2004).

The close relationship between NO_3^- uptake and NR activity is well established (Silveira *et al*. 1999, Abd-el Baki *et al*. 2000). The nitrate uptake and NR activity were both positively correlated with the cowpea leaf and root nitrate content, evidencing a possible control of NR activity exerted by NO_3^-. In higher plants, the rate of nitrate reduction *in situ* is primarily controlled by the rate of nitrate absorption rather than by alterations in NR activity (Kaiser *et al*. 2004). Thus, the apparent saturation of NR in pretreated cowpea plants was possibly caused by restriction in the flux of NO_3^- towards the cowpea leaves, which are the sites of induction of enzyme synthesis. Indeed, the NO_3^- requirement for the induction of NR is well established (Aslam *et al*. 2001). Even though the NR protein amounts in plant tissue are not limiting to NR activity (Kaiser *et al*. 2004), it is possible that a homeostasis between the NO_3^- flux from roots to leaves, which is regulated by the NO_3^- uptake rates, and the synthesis and expression of NR, which is largely controlled by endogenous compounds, might have occurred in the pretreated cowpea plants (Redinbaugh and Campbell 1993). Indeed, gene expression and

synthesis of NR are induced by NO_3^- localized in the metabolic pool and negatively regulated by the glutamine content (Foyer and Noctor 2004). The metabolic NO_3^- pool in plant cells remained constant over a great range of external nitrate concentrations, such as 0.1 to 10 mM (Crawford and Glass 1998).

The limitation in the nitrate acquisition in the pretreated cowpea plants is probably associated with salt-induced modulation in plant growth associated with a reduction in protein synthesis (Imsande and Touraine 1994, Silveira *et al*. 2001), increase in GS activity and Gln accumulation, especially in the roots. This mechanism could represent an efficient strategy to avoid a needless or toxic nitrate accumulation in plant tissues. Nitrate uptake is controlled by the general demand of N for plant growth and not simply by the N status in the root tissues, implying that the shoot N status is sensed and then transmitted to the roots (Krapp *et al*. 2004). The free glutamine is most probably the candidate on signaling molecule regulating nitrate uptake by a negative feedback control. Our data support a conceptual model that establishes relationships between salinity, growth restriction, amino acid demand and nitrate acquisition. The salt-induced growth decrease might initially trigger an imbalance between nitrate assimilation, GS activity and protein synthesis, resulting in Gln accumulation. This amino acid might act as signaling molecule controlling the expression of nitrate reductase and root NO_3^- transporter genes.

In conclusion, the salinity can indirectly down-regulate the nitrate acquisition by an endogenous control signalized possibly by glutamine accumulated in the roots. This accumulation should be associated with salt-induced restriction in growth, amino acid demand and increased glutamine synthetase activity and expression.

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