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# Ammonium assimilation and ureide metabolism in common bean (*Phaseolus vulgaris*) nodules under salt stress

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**Abstract** – The effect of salinity on ammonium-assimilation enzymes, as well as on the enzymes of purine catabolism, was studied in root nodules of common bean plants grown in an environmentally controlled chamber. Salt treatments (0, 25, 50 and 100 mM) were applied during the vegetative period, when the symbiosis was well established. Four harvests were made, two during the vegetative and two during the reproductive period. Salinity reduced plant dry weight by 25% with salt high levels in the third and fourth harvests. Also, nitrogenase activity was reduced by the highest levels of salinity in the last two harvests, as well as glutamine synthetase and glutamate synthase. Xanthine dehydrogenase and uricase activities showed a pattern closely related to those of total nitrogen per plant. The total N content of the plants revealed no significant reduction. We reported that the inhibition of enzymatic activities related to ammonium assimilation of the GS/GOGAT pathway promote other enzyme activities (GDH and AAT). The inhibition of enzymes related to purine catabolism (xanthine dehydrogenase and uricase) decreased the ureide content and alternatively the amino acid content increase in the nodules of common bean.

*Phaseolus* / symbiosis / ureides / amino acids / ammonium assimilation / salt stress

**Résumé** – Assimilation de l'ammonium et métabolisme des uréides dans les nodules de haricot (*Phaseolus vulgaris*) sous contrainte saline. L'effet de la salinité sur les enzymes de l'assimilation de l'ammonium ainsi que sur les enzymes du catabolisme des purines a été suivi chez les nodosités du haricot commun (*Phaseolus vulgaris*) en ambiance contrôlée. Les traitements salins (0, 50, 75 et 100 mM) furent appliqués durant la période végétative alors que la symbiose était bien établie. Quatre récoltes furent effectuées : deux en période végétative et deux en période de reproduction. La salinité réduit la croissance des plantes de 25 % des deux dernières récoltes. La concentration de sel de 100 mM affecte négativement l'activité nitrogénase, spécialement lors des deux dernières récoltes, ainsi que l'activité de la glutamine synthetase et la glutamine synthase. De même les activités xantine déhydrogénase et uricase montrent un profil très similaire à celui du contenu en N total par plante. D'autre part, la réduction en N total des plantes n'est pas significative. Ainsi, la variété Africa peut être considérée comme modérément tolérante au sel. Ce travail montre que l'inhibition des activités enzymatiques de l'assimilation de l'ammonium via GS/GOGAT induit la stimulation des activités GDH et AAT. L'inhibition des enzymes du catabolisme des purines implique la diminution du contenu nodulaire des uréides et l'augmentation des acides aminés.

*Phaseolus* / symbiose / uréide / acide aminé / assimilation de l'ammonium / salinité

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## 1. INTRODUCTION

Several environmental conditions are limiting factors in the growth and activity of  $N_2$ -fixing plants. A principle of limiting factor states that “the level of crop production can be no higher than that allowed by the maximum limiting factor” [4]. In the *Rhizobium*-legume symbiosis, which is a  $N_2$ -fixing system, the process of  $N_2$  fixation is strongly related to the physiological state of the host legume plant. The ability of legumes to establish an efficient symbiosis with *Rhizobium* can be affected by a number of environmental factors such as salinity [57], nitrate [6], soil temperature [21], water deficit [41] and waterlogging [31].

The harmful effect of salinity is a serious threat to agriculture in arid and semiarid regions [34] especially in the Mediterranean basin, where symbiotic  $N_2$  fixation could contribute to adaptation of some legumes. Variability in salt tolerance among crop legumes has been reported [12, 57]. Common bean was considered moderately sensitive to salt stress compared with other grain legumes [12].

Salinity is known to reduce the growth of *Phaseolus vulgaris* glycophytes (salt-sensitive species) [27]. These effects may result from changes in dry-matter allocation, ion relationships, water status, physiological processes, biochemical reactions or a combination of these. Salt tolerance in plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes [56]. The depressive effect of salt stress on  $N_2$  fixation by legumes is directly related to declines in dry weight and nitrogen content in shoots [9], distortion in the nodule structure [53, 58] and to the reduction in nitrogen-metabolism activity [7].

Dinitrogen fixation in the root nodules of legumes generates ammonia, which is also processed by the GS/GOGAT cycle after being excreted into the plant cytosol [39]. The first step is catalysed by glutamine synthetase (GS), which forms glutamine, and subsequently glutamine is converted to glutamate by glutamate synthase (NADH-GOGAT) [49]. The activities of these enzymes in host-plant cells of nodules are higher than in roots, increasing with nodule development [47]. Furthermore, these enzyme activities are markedly lower in ineffective nodules [46]. It is generally accepted that salt stress promotes accumulation of ammonium, nitrate and free amino acids in plants [30] and the enzymes mediating ammonium assimilation in *Vicia faba* [10] and *Cicer arietinum* [44] nodules were inhibited by salt stress. Another enzyme originally thought to be involved in ammonium assimilation, glutamate dehydrogenase (GDH), seems to play a minor role in glutamate biosyn-

thesis, and is principally implicated in its oxidation [36]. The resulting glutamate can be incorporated into aspartate and asparagine via the action of aspartate aminotransferase (AAT) and asparagine synthetase [49].

*Phaseolus vulgaris* is considered a legume that could transport and store nitrogen as ureides [18, 22]. In this legume glutamine, aspartate and glycine are incorporated into purines which are subsequently oxidatively degraded to form ureides, allantoin and allantoic acid. Purine oxidation proceeds via xanthine dehydrogenase (XDH) and uricase to produce allantoin and finally allantoic acid in enlarged peroxisomes of interstitial cells in the infected nodule tissue [16]. Many authors have further suggested that the abundance of ureides in the plant may reflect its symbiotic dependence and provide a quantitative assay of nitrogen fixation [20]. Environmental factors affect ureide content and nitrogenase activity [53]. The increased nodule ureide levels were reported [32] after exposure to polyethylene glycol [42] after drought stress, and after stresses other than water deficits on soybean plants [52]. However, there is no indication of the potential use (production and metabolism) of ureides to assess  $N_2$  fixation under salt-stress conditions.

Therefore, to improve our knowledge of the metabolic pathways controlling the ammonium assimilation in nodules of *Phaseolus vulgaris* under salt stress, we have studied the behaviour of the enzymes involved in ammonium assimilation and ureide formation in nodules and, to gain greater insight into the sequence of metabolic reactions, we investigated amino acids and ureide contents in salt-exposed plants. Relationships between these parameters provide an overall view of the possible role of enzyme activities in nodule host plants under salt stress. The aim is to shed light on the relative activity profiles of the two metabolic pathways under conditions of salt stress.

## 2. MATERIALS AND METHODS

### 2.1. Biological material and growth conditions

Common bean (*Phaseolus vulgaris* cv. Africa) was obtained from Semillas Interlegsa (Valencia, Spain). Pregerminated seeds were planted in modified Leonard jar assemblies (two per jar) containing vermiculite and nutrient solution [35] containing 1 mM  $KNO_3$ , and fresh nutrient solution replaced the old every three days. This N concentration stimulated plant growth but did not inhibit nodule growth or activity [25]. Each seedling was inoculated with 1 ml of a suspension of *Rhizobium tropici* CIAT899 ( $10^9$  cells·ml<sup>-1</sup>), and grown in a controlled environmental chamber [44].

## 2.2. Salt treatments and harvests

Once the symbiosis was well established 18 days after sowing (DAS) the plants were subjected to salt stress by adding NaCl to the growth medium (0, 25, 50 and 100 mM). Plants were harvested at 3, 6, 9 and 12 days after treatments (DAT). In the first and second harvest the plants were in vegetative growth. The third coincided with the beginning of flowering and the last harvest was during full flowering. Six replicates were included per harvest. A sample of the root with nodules of each plant was used for the nitrogenase assay and afterwards the nodules were removed, weighed and dried at 70 °C for 24 h to calculate dry weight. Other samples of nodules from each plant were pooled and stored at -80 °C for the enzyme assays and analytical determinations. Shoot and root dry weights were recorded after 24 h at 70 °C.

## 2.3. Nitrogen-fixation assays

Nitrogenase (EC 1.7.99.2) activity was determined by acetylene reduction on the nodule root portion of the plants as described [19]. The aliquots were analysed for ethylene in a Perkin Elmer 8600 gas chromatograph equipped with a Poropak R Column [28].

## 2.4. Preparation of cell free extracts and enzyme assays

Cell free extracts for the enzyme activities GS, NADH-GOGAT, GDH, and AAT were prepared by homogenising 0.5 g of nodules in 6 ml of the following extraction medium: 100 mM maleic acid-KOH buffer pH 6.8 [5], and the supernatant obtained was assumed to be plant cell cytoplasm and used to measure enzyme activities and soluble protein [3]. For the activities of XDH and uricase 0.5 g of nodules were homogenised in 10 ml of an extraction medium that consisted of 25 mM TES-KOH buffer pH 7.5 with 0.15 M sorbitol, and the homogenate was centrifuged at 1100 *g* for 15 min. All steps were carried out at 4 °C. Four replicates were performed for each enzyme-activity assay.

The NADH-glutamate synthase (EC 1.4.1.14) and glutamate dehydrogenase (EC 1.4.1.2) activities were assayed by monitoring the oxidation of NADH, at 30 °C and 340 nm for 10 min in the assay media described below [15]. For the NADH-GOGAT, the assay medium consisted of 1 mM Na<sub>2</sub>-EDTA, 2.5 mM 2-oxoglutarate, 1.0 mM aminooxyacetate, 0.15 mM NADH, 10.0 mM L-glutamine, and 0.1% (v/v) 2-mercaptoethanol in 50 mM buffer potassium phosphate pH 7.5; for GDH, the assay

medium consisted of 0.1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.5 mM 2-oxoglutarate, and 0.15 mM NADH in 50 mM buffer potassium phosphate pH 7.5.

Glutamine synthetase (EC 6.3.1.2) was determined by the hydroxamate synthetase assay [24]. Assays were optimised for the amount of enzyme to give a linear reaction within at least 30 min. Two blanks without enzyme and without L-glutamate were also analysed [9].

Aspartate aminotransferase (EC 2.6.1.1) activity was determined [14] in an extraction medium that consisted of 4 mM MgCl<sub>2</sub>, 10 mM aspartic acid, 0.2 mM NADH, and 1 mM 2-oxoglutarate in 50 mM Tris-HCl buffer pH 8.0.

Xanthine dehydrogenase (EC 1.2.1.37) and uricase (EC 1.7.3.3) were determined by the procedure [38]. For the XDH activity the reaction medium consisted of 3.5 mM NAD<sup>+</sup>, and 0.5 mM hypoxanthine in 50 mM TES-KOH buffer pH 8.4. For the assay of uricase, the medium consisted of 50 µM uric acid in 85 mM glycine-KOH buffer pH 9.0.

## 2.5. Amino-acid and ureide contents

The content of ureides (allantoin and allantoic acid) in the nodules was determined [51] using 1 g of nodules in 4 ml of the extraction medium. The content of free amino acids in the nodules was analysed using 1 g of nodules in 12 ml of extraction medium [23] using the ninhydrine method [55].

## 2.6. Statistical design and analysis

The experimental layout was a randomised complete block design. The growth values and parameters related to nitrogen fixation were means of six replicates per treatment. Four replicates were performed for the enzyme activity assays and protein content, and three replicates for the amino acid and ureide contents. All results were subjected to a two way analysis of variance with a least significant difference (LSD) test. Also, the simple correlation coefficients among the parameters studied were calculated and are presented in Table II.

## 3. RESULTS

The salinity did not affect dry-weight accumulation in the first or second harvest (Tab. I) and only a slight reduction (4%) with 100 mM of NaCl. In the third and fourth harvests, the reductions of plant growth were statistically significant at all salt levels with respect to

**Table I.** Effect of four levels of NaCl on growth and nitrogen fixation parameters: PDW, plant dry weight; RSR, root to shoot ratio; NDW, nodule dry weight; ARA, acetylene reduction assay; LSD, least significant difference at 0.05.

DAT	NaCl (mM)				NaCl (mM)			
	0	25	50	100	0	25	50	100
	PDW (g·plant <sup>-1</sup> )				RSR			
3	1.98	1.90	1.88	1.89	0.32	0.30	0.33	0.35
6	2.37	2.36	2.28	2.26	0.25	0.25	0.29	0.34
9	2.93	2.43	2.36	2.13	0.22	0.24	0.28	0.31
12	3.54	3.17	2.73	2.55	0.22	0.26	0.24	0.31
LSD (0.05)	0.30				0.05			
	NDW (mg·plant <sup>-1</sup> )				ARA (μmol C <sub>2</sub> H <sub>4</sub> ·g NDW <sup>-1</sup> ·h <sup>-1</sup> )			
3	106	84	86	90	89.8	87.4	53.8	54.7
6	116	96	95	103	96.5	81.2	66.7	48.8
9	186	138	146	111	103.2	75.9	29.4	25.2
12	188	169	138	133	72.6	62.0	24.7	18.2
LSD (0.05)	10.0				14.4			

control, the reduction exceeding 25%. The root-to-shoot ratio (RSR) increased with the salt dosages but decreased with the culture time, in all treatments except control. The total NDW increased over the culture time in all treatments. Salt treatment significantly ( $P \leq 0.05$ ) reduced NDW (Tab. I). Nitrogenase activity (Tab. I) was inhibited by more than 50% (25 and 50 mM) with respect to control plants at 9 and 12 DAT.

At the beginning of treatments, GS activity averaged six-fold higher than NADH-GOGAT (Fig. 1) and both activities were significantly inhibited ( $P \leq 0.05$ ) by salinity. NADH-GOGAT appeared to be more tolerant at 25 mM and more sensitive at 50 and 100 mM NaCl than GS was. Exposure to 50 and 100 mM NaCl reduced both activities between 30 and 50% from 9 DAT. The activity of GDH was less affected by salt concentrations and at all harvests than NADH-GOGAT, the differences being at the limit of statistical significance. The AAT was inhibited by between 20 and 40% by 50 and 100 mM NaCl in the third and fourth harvests, while almost no effect appeared at 25 mM in any of the harvests; in the first harvest, 50 and 100 mM caused inhibition of about 15%. Notably, at 6 DAT, when the inhibition of GS and NADH-GOGAT was very strong, the activities of GDH and AAT showed no inhibition by salt.

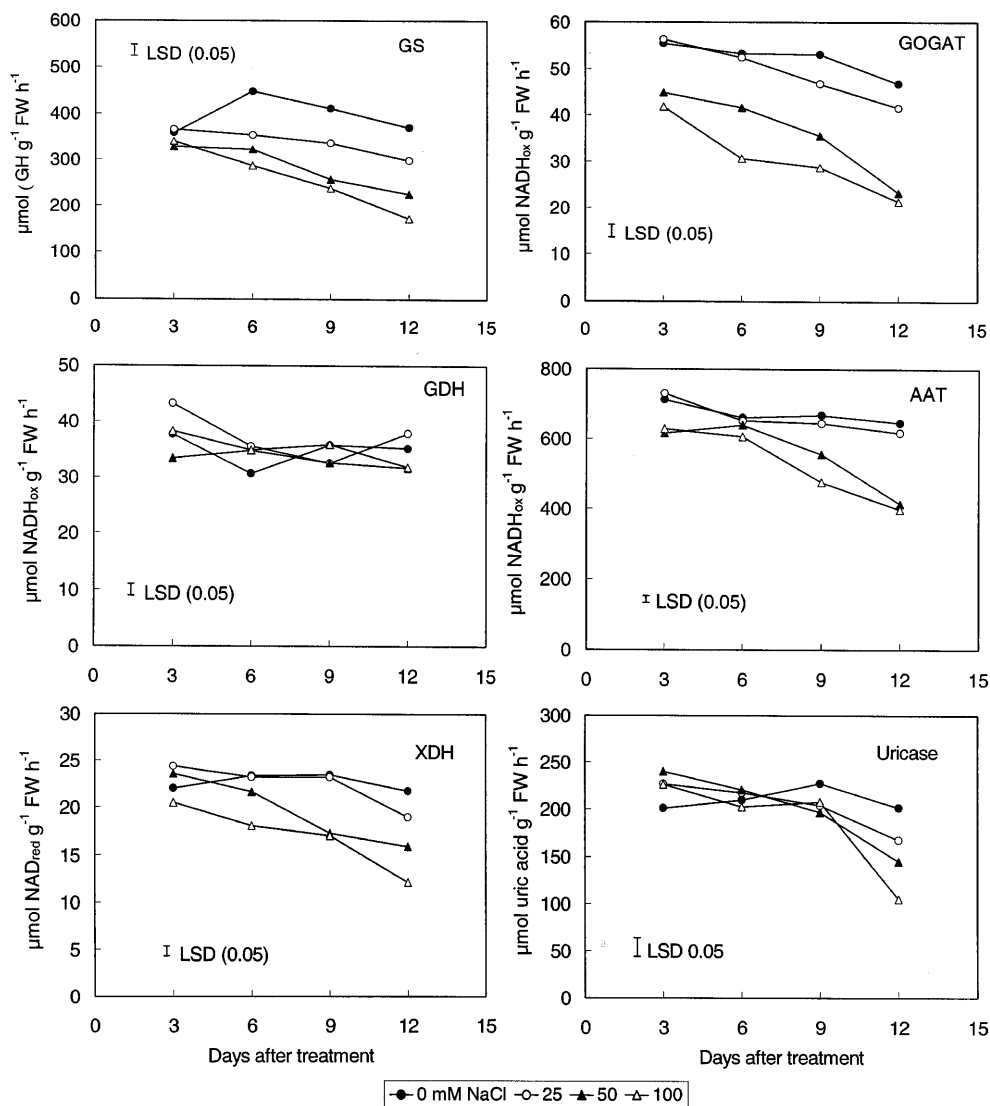
With regard to the enzyme activities of purine catabolism in the nodule, both XDH and uricase activities (Fig. 1) showed a pattern closely related to those of total nitrogen per plant (Fig. 2). The maximum activity of both enzymes was observed at the beginning of flowering (third harvest) and decreased at full flowering. XDH

declined with 50 and 100 mM on the last three harvests (20–40%). Uricase activity was inhibited significantly only by 100 mM NaCl on the last sampling.

The ureide contents of common bean nodules diminished at the beginning of flowering in control plants and with 25 mM NaCl. This decrease became more severe with other salt levels from the first to last harvest. In contrast to the effect observed for ureide content, the salinity treatments significantly increased the free amino-acid levels in the nodule (Fig. 2). Maximum values were reached at the third harvest (beginning of flowering). Nitrogen content did not change significantly when salinity was imposed.

#### 4. DISCUSSION

Plant growth and dry matter accumulation of legumes reduced by salinity has been reported in many important crop legumes such as *Medicago sativa* [40], *Vicia faba* [8], *Pisum sativum* [12] *Glycine max* [41], and *Phaseolus vulgaris* [30]. The Early Prolific variety of *Phaseolus vulgaris* tolerates low (48 mM NaCl) but not higher levels (72 and 96 mM NaCl) of salt [54]. Similar results in our work with Africa cv. of *P. vulgaris* showed that 50 mM NaCl did not reduce root and shoot dry weight per plant, although these parameters were reduced by more than 25% dry weight (Tab. I) by higher salt levels (50 and 100 mM NaCl). This cultivar could be considered less sensitive to low levels of salinity than other grain legumes are, at least with short exposure to salt stress. Salinity affects shoot growth more than root

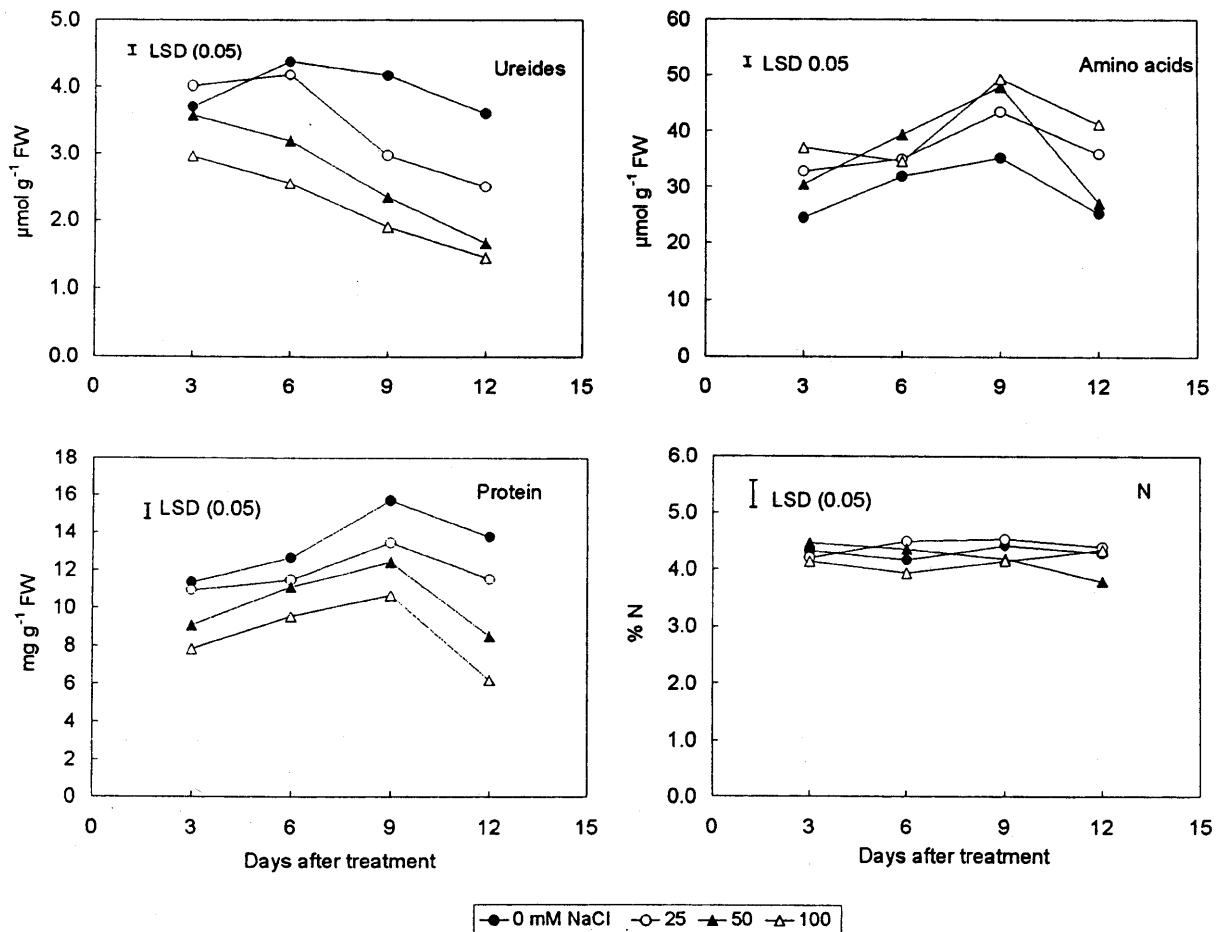


**Figure 1.** Glutamine synthetase, glutamate synthase, glutamate dehydrogenase, aspartate aminotransferase, xanthine dehydrogenase and uricase activities in root nodules of *Phaseolus vulgaris* cv. Africa inoculated with strain CIAT899 of *Rhizobium tropici*. Salt stress was applied at the vegetative growth stage (18 DAS).

growth, so that RSR increases (Tab. I) with salinity. These results have been previously reported in *Vicia faba* [9], *Medicago sativa* [26, 40], *Pisum sativum* [7] and *Cicer arietinum* [44]. However, in our results RSR significantly increased with the higher salt treatments (100 mM) at the first sampling, a trend which can be considered a short-term response to salinity.

In this work, plants were grown without salt for three weeks and therefore the effect of salinity on nodule initi-

ation and formation was not tested. In relation to this, several authors confirm that the process of nodule initiation is an event in *Rhizobium*-legume symbiosis which is highly sensitive to salt or osmotic stress [10, 50]. However, the total NDW increased over the culture time in all treatments and salt significantly reduced NDW. These results agree with those of [12]. The inhibition of ARA was almost proportional to the concentration of NaCl in the nutrient solution (Tab. I) and was higher



**Figure 2.** Ureide, amino-acid and protein contents in root nodules, and total N content expressed as percentage of plant dry weight, in *Phaseolus vulgaris* cv. Africa inoculated with strain CIAT899 of *Rhizobium tropici*. Salt stress was applied at the vegetative growth stage (18 DAS).

than that of nodulation [40]. Nodule ARA was significantly correlated with shoot dry weight and ureide accumulation in the nodule, above all allantoic acid, when data from all NaCl treatments were combined (Tab. II). The correlation between ureide contents in nodule and ARA for the  $\text{N}_2$ -fixing plants indicates that the inhibition of nitrogenase activity by salt, as measured by ARA assay, corresponds to a substantial reduction of N fixation and consequently plant growth (Tab. I).

In *Phaseolus vulgaris* nodules the activities of GS and NADH-GOGAT are responsible for ammonium absorption [11]. When this plant is grown without NaCl, GS activity is six-fold higher than NADH-GOGAT activity, in *Cicer arietinum* is ten-fold higher [44] and in *Vicia faba* two-fold higher [9]. Salt stress inhibited both activi-

ties and NADH-GOGAT proved more sensitive to salt at high NaCl concentration (Fig. 1), suggesting that the latter enzyme is the limiting factor in ammonium assimilation by the nodule in *Phaseolus vulgaris* under salt stress. In addition, it influenced the pattern of expression of NADH-GOGAT that changed during root-nodule development [48]. Similar results were obtained with cowpea nodules [13] under drought stress. These authors affirm that NADH-GOGAT activity was the enzyme most sensitive to drought stress and most closely associated with nitrogenase (ARA in closed system). In our study, nitrogenase activity closely correlated ( $P < 0.001$ ) with NADH-GOGAT (Tab. II).

Although the GS/GOGAT pathway is considered to be the major pathway for ammonium assimilation under

**Table II.** Simple correlation matrix among soluble amino acid and ureide contents in nodule cytosol, and enzyme activities related to ammonium assimilation and ureide synthesis, in nodule cytosol of *Phaseolus vulgaris* var. Africa plants treated with 0, 25, 50 and 100 mM NaCl supplied 18 days after sowing at the vegetative growth period. AA: soluble amino acids. ALN: alantoin. ALC: alantonic acid. GS: glutamine synthetase. GOGAT: glutamate synthase. XDH: xanthine dehydrogenase. GDH: glutamate dehydrogenase. AAT: aspartate aminotransferase.

	ARA	ARAP	AA	ALN	ALC	Ureides	GS	GOGAT	XDH	Uricase	GDH	AAT	Protein
ARA	1.00												
ARAP	0.81	1.00											
AA	-0.44	-0.34	1.00										
ALN	0.39	0.42	-0.53	1.00									
ALC	0.92	0.65	-0.42	0.31	1.00								
Ureides	0.93	0.67	-0.45	0.38	1.00	1.00							
GS	0.94	0.76	-0.43	0.44	0.94	0.95	1.00						
GOGAT	0.94	0.69	-0.39	0.48	0.93	0.95	0.91	1.00					
XDH	0.88	0.61	-0.36	0.44	0.91	0.92	0.91	0.93	1.00				
Uricase	0.58	0.32	-0.06	0.20	0.73	0.72	0.71	0.69	0.83	1.00			
GDH	0.34	0.13	-0.15	0.23	0.29	0.30	0.25	0.44	0.35	0.38	1.00		
AAT	0.89	0.62	-0.35	0.46	0.87	0.88	0.87	0.93	0.90	0.75	0.53	1.00	
Protein	0.68	0.84	-0.03	0.25	0.58	0.59	0.66	0.63	0.60	0.46	0.05	0.58	1.00

Critical value

$P \leq$  0.05 0.01 0.001  
0.50 0.62 0.74

normal growth conditions, the role of GDH under some environmental conditions cannot be dismissed [45]. In the present work the GDH activity does not show inhibition by salt at 6 DAT, and with 25 mM NaCl GDH activity enhanced (Fig. 1). Therefore the decrease in GS/GOGAT activities by salt stress results in limitation of glutamine and amino acid production and the rise in amino acids and glutamate synthesis could be dependent on the GDH activity. [2] provide evidence that plants subjected to heavy metal stress could utilise the GDH pathway instead of the GS/GOGAT cycle for ammonium assimilation.

Adaptive responses in the metabolism of any organism during any environmental stress must reflect changes in the activities of enzymes [33]. In our results the NADH-GDH and AAT activities response support in part the hypothesis that being under salt stress must reflect shift metabolic pathways. AAT was considered as an enzyme more active in amide producing legumes than in ureide producing legumes [37] and other work [43] reported AAT converting ketoacids into amino acids under salt stress conditions. In fact the accumulation of amino acids (Fig. 3) in the nodule cytosol is a physiological response to osmotic stress [33].

The activity of XDH and uricase was found to be less inhibited under salt conditions than ammonium assimilation enzymes (Fig. 1). The ureide content in nodule decreases with age and, in our study, this content was higher during the vegetative stage and fell to the lowest level during the flowering period [29] as did nitrogenase activity [1]. Nitrogenase activity (Tab. I) increased dur-

ing vegetative growth (from the first to second harvest) and levelled off or decreased toward the end of the growing season (from the second to third harvest) this ureide content varying with physiological development [7] even under drought conditions [32].

The nitrogen fixation and plant growth reduction due to salt stress was accompanied by changes in enzymatic activities and in reduced nitrogen demand of the host plant (ureides and amino acids in nodules). The inhibition of enzymatic activities related to ammonium assimilation of the GS/GOGAT pathway does not exclude the possibility of GDH activity. The inhibition of enzymes related to purine catabolism decreased the ureide content.

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