

Nitrogen metabolism in relation to drought stress responses in cultivated and model *Lotus* species

[PEDRO DÍAZ](#)^{1*}, OMAR BORSANI¹, ANTONIO MÁRQUEZ² and JORGE MONZA¹.

¹Laboratorio de Bioquímica, Departamento de Biología Vegetal, Facultad de Agronomía, Av. E. Garzón 780, CP 12900 Montevideo, Uruguay.

²Departamento de Bioquímica Vegetal y Biología Molecular, Facultad de Química, Sevilla España.

*Corresponding author

Abstract

Amino acid profiles of *Lotus corniculatus*, *L. glaber*, *L. japonicus*, *L. subbiflorus* and *L. uliginosus* in response to drought stress were studied. All *Lotus* species accumulate proline, derived from *de novo* synthesis, in response to drought stress. Asparagine analyzed by HPLC showed the highest abundance accounting for 20 to 25 % of total amino acids, without revealing any change in response to drought stress. Additionally, an increase in the amount of photorespiratory pathway intermediates, serine and glycine, was observed in some species as a consequence of drought stress, but intracellular ammonium non change in response to drought stress. The differential effect of drought stress on amino acid profiles and proline accumulation in *Lotus* species plants is discussed.

Introduction

There are four species of *Lotus* that have been domesticated and improved by selection and plant breeding: birdsfoot trefoil (*Lotus corniculatus*), greater lotus (*L. uliginosus*), narrow-leaf trefoil (*L. glaber*) and hairy birdsfoot trefoil (*L. subbiflorus*) (Blumenthal and McGraw, 1999; Díaz *et al.*, 2005a). Besides its agronomical attributes, there is a great deal of interest in *Lotus* because the species are extremely amenable to tissue culture, in particular *L. corniculatus* and *L. japonicus* (Webb *et al.*, 1990; Handberg and Stougaard, 1992). In spite of the fact that *L. japonicus* is not used as forage legume, this species could be a good model for a wealth of genetic, biochemical, molecular biological (Orea *et al.*, 2002) and symbiotic studies which cannot be carried out in other model species as *Arabidopsis thaliana*.

Drought is the major limitation on crop productivity worldwide. In broad terms, drought can be permanent, seasonal or random. Another type of drought to be considered is named non-apparent drought and is observed in hot summer days when high temperature or wind, induce an increase on transpiration rates that exceeds water root absorption rates (Sánchez-Díaz y Aguirreolea, 1993).

Lotus species are sowed in geographical zones where the plants can be exposed to random or

non-apparent drought. They are adapted to temperate and humid environments, wherein these types of drought are frequent, and therefore, they could have acquired mechanisms to tolerate the drought.

For several years it has been known that plants respond to drought stress by undergoing biochemical adaptative processes such as ion transport and by accumulating different compounds, named compatible osmolytes. These osmolytes, which are known to increased there synthesis under osmotic stress, include proline, they accumulate to high concentrations without interfering with cell metabolism (Bray, 1993). As well as proline, other nitrogen compounds could be accumulating in plants in response to drought stress (Good and Zaplachinski, 1994; Chiang and Dandekar, 1995). The amino acid metabolism may play an important role in plant stress tolerance, by osmotic adjustment through to accumulation of compatible osmolytes; by detoxification of active oxygen species, xenobiotics and heavy metals; and by intracellular pH regulation (Rhodes *et al.*, 1999; Alia *et al.*, 2001).

During drought stress, protein residues may be altered by chemical processes; some proteins are irreversibly damaged by the effects of drought stress and are degraded by proteases. It has been suggested that proteases mobilize amino acids from proteins to the synthesis into compatible osmolytes (Campalans *et al.*, 1999). In our study the analyses were focused in amino acid profiles and in drought-induced proline accumulation in *Lotus* agronomical species and in the model species *Lotus japonicus*.

Material and methods

Plant material, growth conditions and drought treatment

L. corniculatus cv San Gabriel (AGROSAN S.A.), *L. uliginosus* cv Grassland Maku (Ing. Agr. D. Formoso), *L. subbiflorus* cv El Rincón (AGROSAN S.A.), *L. glaber* cv Herminia (PAS S.A. Montevideo Uruguay) and *L. japonicus* (Regel) Larsen ecotype Gifu (Prof. A. Márquez) were treated according to Orea *et al.* (2002) and germinated at 28 °C for 2 days. The plants were grown under controlled conditions: 16/8 h light/dark with photosynthetic photon flux density of 250 $\mu\text{mol. m}^{-2} \cdot \text{s}^{-1}$, 22/18 °C and relative humidity 70/80%. The plants were grown during 28 days in hydroponic assemblies (Borsani *et al.*, 1999) with a modified Hornum nutrient solution described by Handberg and Stougaard (1992) with 8 mM of KNO_3 . The drought stress was induced as described by Borsani *et al.* (1999) the measurements of leaf tissues were performed 0 and 12 h after and this is know as fast drought stress (Díaz *et al.*, 2005b).

Analytical determinations

The relative water content (RWC) was calculated according Antolín *et al.* (1995). Total protein was quantified according to Bradford (1972), chlorophyll concentration according to Wellburn (1994) and proline concentration according to Borsani *et al.* (1999). Nitrate, ammonium and total free amino acids were extracted as proposed by Izaguirre-Mayoral *et al.* (1992) with potassium phosphate buffer 10.0 mmol L^{-1} – ethanol (1 – 1). Nitrate was

analysed according to Cataldo *et al.* (1975), ammonium according Solorzano (1969), and total amino acids were quantified with ninhydrin reagent according to Moore and Stein (1948). The identification of amino acids was achieved by an extraction derivatised with o-phthalaldehyde and the samples were separated-analysed by HPLC (Díaz *et al.*, 2005b in press). Proteolytic activity was measured according to Roy-Macauley *et al.* (1994).

Results

Biochemical responses to drought stress in plants are usually evaluated through the osmolyte accumulation and detoxification enzymes of reactive oxygen species, among others. Our work was focussed in nitrogen osmolytes as proline, GABA, amino acids, nitrate and ammonium.

Leaf RWC in different *Lotus* species averaged as follows: control 84 % and 12 h drought stress 63 % (Table 1). *L. subbiflorus* showed the lowest difference in RWC between the control and the drought stressed plant in our assay conditions. This fact could be explained since this is the most pubescent plant species and this feature could diminish the water loss by the leaf.

Photosynthetic pigments were used to determine the physiological status of the plants. No changes were observed in chlorophyll a and b concentration in response to 12 h of drought stress in the *Lotus* species studied, and also chlorophyll a/b ratio was largely unaffected by drought stress (Table 1).

Table 1. RWC, chlorophyll (clo), proline, total free amino acid (tot aa), protein, nitrate and ammonium in one month old plants control (c) and subjected to drought stress for 12 h (ds).

	<i>L. corniculatus</i>		<i>L. glaber</i>		<i>L. japonicus</i>		<i>L. subbiflorus</i>		<i>L. uliginosus</i>	
	c	ds	c	ds	c	ds	c	ds	c	ds
RWC ^a	83.5	63.4*	81.0	58.3*	87.5	66.8*	82.5	70.2*	85.9	58.5*
clo a ^b	7.1	6.7	8.8	7.3	10.6	10.7	7.8	8.7	5.3	5.9
clo b ^b	4.7	3.9	5.6	5.3	5.9	5.7	4.1	4.8	3.5	3.6
a/b	1.8	1.9	1.9	1.8	1.9	1.9	1.8	2.0	1.8	1.9
Proline ^b	2.5	12.2*	1.3	10.2*	2.6	6.2*	1.3	2.9	1.2	9.7*
Tot aa ^b	164.8	173.5	100.9	93.9	131.0	169.3	103.8	107.2	113.7	145.8
Protein ^c	119.2	104.0	110.4	129.7	135.3	148.5	89.7	92.3	118.4	95.3
nitrate ^b	330.5	360.2	174.4	94.1	365.5	310.9	123.4	51.9	419.5	230.3
Ammonium	21.5	14.1	0	0	0	0	0	0	24.4	30.8

The * represent a significant difference between drought stressed and control plants at 5 % of Duncan's method. ^a, %; ^b, $\mu\text{mol g}^{-1}\text{DW}$ and ^c, $\text{mg g}^{-1}\text{DW}$.

L. corniculatus, *L. japonicus* and *L. uliginosus* plants showed higher nitrate levels than *L. glaber* and *L. subbiflorus* plants and we did not find changes in response to drought stress.

On the other hand, ammonium was detected in *L. corniculatus* and *L. uliginosus* and no changes were observed in response to drought stress. Ammonium was not detected in the other three species (Table 1).

Proline content increased in drought stressed plants; these increases were between 3 and 7 fold (Table 1). Based on the Van't Hoff equation, this proline increase could be enough to account for only -0.1 to -0.5 MPa of osmotic adjustment in leaf tissue. However, results obtained by our group showed that in *L. corniculatus* subjected to 9 days of slow drought stress, the proline content accounts for -2.5 MPa of osmotic adjustment (Díaz *et al.*, 2005b; P. Díaz and M. Sainz, unpublished data).

Total free amino acids and protein content did not change significantly when the plants were subjected to drought stress (Table 1); so, the increase in proline content was also with respect to total amino acids. Additionally, protein content did not change in response to drought stress conditions; this could suggest that there is no protease increase. This point was verified assaying proteolytic activity with azocasein as substrate at different pH. The proteolytic activity was found to be similar in control and drought stressed plants (Figure 1).

Free amino acid composition of control and drought stress *Lotus* species was analyzed by HPLC and is showed in Table 2. The amino acids analyzed represent the 80% of total free amino acids in leaf tissues. Asparagine was present in high amounts, and accounted for 20 to 25 % in the different *Lotus* species, and arginine was prevalent in *L. uliginosus* (Table 2).

Table 2. Major free amino acid composition (expressed as $\mu\text{mol g}^{-1}$ DW) of one month old plants control (c) and subjected to drought stress for 12 h (ds).

	<i>L. corniculatus</i>		<i>L. glaber</i>		<i>L. japonicus</i>		<i>L. subbiflorus</i>		<i>L. uliginosus</i>	
	c	ds	c	ds	c	ds	c	ds	c	ds
ala	14.9	10.4*	10.3	8.6	7.6	9.7	4.3	11.5	4.3	15.5*
asp	17.3	6.1*	9.3	2.8*	6.2	3.1*	1.3	2.7	15.5	9.8*
asn	38.6	46.0	31.5	34.6	40.9	42.6	31.0	32.1	29.5	53.0
glu	23.8	18.6*	11.6	14.5	17.2	15.6	9.5	12.1	20.2	19.9
gln	1.8	5.4*	0.4	2.7*	3.2	3.3	1.3	2.1	2.1	5.4
gly	6.3	16.1*	4.6	7.0*	5.7	8.6	4.3	7.9	10.4	17.5
ser	12.5	20.5*	8.9	12.4*	17.1	15.0	12.5	14.8	7.4	13.3*
arg	6.6	4.9	5.7	5.4	2.7	3.2	3.4	4.1	9.5	34.6*
GABA	13.0	11.1	45.5	46.5	32.4	27.3	25.2	31.9	6.6	30.3*

The * represent a significant difference between drought stressed and control plants at 5 % of Duncan's method.

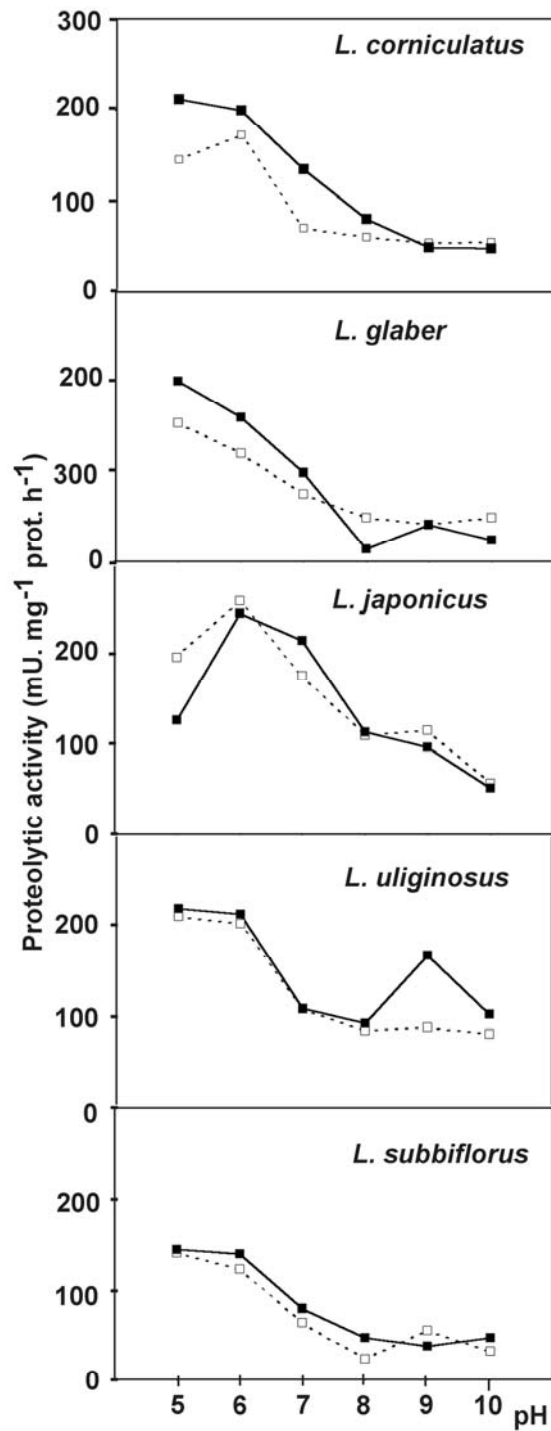


Figure 1. Proteolytic activity in response to drought stress in different *Lotus* species. Close point, control and open point, drought stress treatment. No difference between drought stressed and control plants at 5 % of Duncan's method were observed.

No changes in asparagine concentration were observed in *Lotus* species subjected to drought stress. *L. corniculatus* plants showed the most significant changes in the amino acid profile as a consequence of drought stress. For instance, a decrease in aspartate, alanine and glutamate, and an increase in glutamine, serine and glycine were observed in this species. Some similar changes in the amino acid profiles found in *L. corniculatus* could be observed in *L. glaber*, *L. japonicus* and *L. uliginosus*. The latter showed a decrease in aspartate concentration and *L. glaber* also exhibited an increase in serine and glycine concentration. A significant increase in GABA concentration was observed only in *L. uliginosus* as a consequence of 12 h of drought stress.

Discussion

The present study outlines the changes in proline and free amino acid concentration in several *Lotus* species during the onset of drought stress in plants. Proline was negatively correlated with RWC in *Lotus* species (Tables 1 and 2). Previous works showed that *L. corniculatus* and *L. japonicus* under drought stress conditions decreased RWC of leaves and this was accomplished by an increase in proline concentration (Borsani *et al.*, 1999; Díaz *et al.*, 2002). The amount of accumulated proline is too low to account for osmotic adjustment according to the Van 't Hoff equation, so proline may be regarded as a scavenger of hydroxyl and singlet oxygen radicals (Smirnoff and Cumbes, 1989; Alia *et al.*, 2001).

Our work shows that proline accumulation in all *Lotus* species results from *de novo* synthesis and not from protein hydrolysis, since total amino acids and protein content remained unaltered (Table 1). Additionally, no changes in proteases activity were detected (Figure 1). As well, no differences in protein content were found in *Lotus* species subjected to fast drought stress. The decreases in protein content are associated with slow drought stress which has been found to occur in tomato (Bauer *et al.*, 1997), *Phaseolus vulgaris* and *Vigna unguiculata* (Roy-Macauley *et al.*, 1992).

Lotus species showed different nitrate concentrations, which may probably accumulate in the vacuoles, and so nitrate might be regarded as having a role as an osmoregulator (Blom-Zandstra and Lampe, 1985; Márquez *et al.*, 2005). A decrease in nitrate concentration was observed in tobacco during dehydration. This decrease was accompanied by a general decrease in total free amino acids content (Ferrario-Méry *et al.*, 1998), but this metabolic event was not found in the *Lotus* species studied.

Different amino acids were prevalent in the different *Lotus* species studied: for instance, asparagine and glutamate were found to be the most abundant in *L. corniculatus* and *L. uliginosus* control treatment, and asparagine and GABA were abundant in the others species (Table 2). *Lotus* genus is similar to *Medicago sativa* in that asparagine is the principal amide (Girousse *et al.*, 1996). In temperate legumes, asparagine is the main molecule used to transport reduced nitrogen within the plant, and in that case can account high content. No changes in asparagine content were observed in response to drought stress in *Lotus* species. In *A. thaliana* and *Brassica napus* leaves an increase in asparagine content has been reported in response to osmotic stress (Chiang and Dandekar, 1995; Good and Zaplachinski, 1994).

High glutamate concentration is necessary for proline synthesis through the pyrroline 5-carboxylate synthetase and pyrroline 5-carboxylate reductase (Rhodes *et al.*, 1999). An increase in Fd-GOGAT responsible of glutamate supply for proline accumulation has been reported in *L. corniculatus* leaves under stress conditions (Borsani *et al.*, 1999; Díaz *et al.*, 2005b in press).

A decline in alanine content and an increase in serine and glycine content were observed in *Lotus* species, and may be due to higher rates of photorespiration in the drought stress condition, since alanine is a major donor of amino groups in photorespiratory metabolism.

A special feature show *L. uliginosus* which increased GABA, and this fact could lead to the reduction in the cytoplasmatic concentration of glutamate (Cayley *et al.*, 1992). This reduction could be carried out through a transport into the vacuole, or by GABA synthesis. Similarly, elevated GABA levels are observed under conditions when glutamine synthesis is limited, protein synthesis is inhibited and protein degradation is induced, all these metabolic alterations occur under drought stress conditions in plants (Bray, 1993; Shelp *et al.*, 1999). Also significant increases of arginine were observed in *L. uliginosus*, which may be associated to polyamines metabolism (Rhodes *et al.*, 1999).

Similar results obtained on proline accumulation and amino acid profiles in the *L. corniculatus*, *L. glaber* and *L. japonicus* showed that the latter species could be used as an excellent model to understand and therefore improve drought stress tolerance in cultivated *Lotus* species through osmolyte synthesis.

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