Original Research Article

Nitrogen Uptake and Free Amino-acid Accumulation in Roots of *Lotus corniculatus* Cultivars under Al-stress

Peter Palove-Balang[1,2], Anna Murinova[1] Veronika Zelinova[2]

[1]*Institute of Biological and Ecological Sciences, P. J. Šafárik University in Košice, Slovakia*
[2]*Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovakia*

Abstract

Nitrogen uptake and free amino acid accumulation was evaluated in two South American cultivars of *Lotus corniculatus* L. differing in their tolerance to Al stress. The uptake of nitrate was affected by 1 mM Al more in the sensitive cultivar INIA Draco than in a relatively tolerant UFRGS (the uptake rate was 39.1% and 52.9%, respectively, of the control value at pH=5.5), whereas the opposite was true for the uptake of ammonia (60.1% and 50.1%). Treatment with Al also decreased the free amino-acid level to similar extent in both cultivars, mostly due to the significantly decreased level of asparagine (p = 0.003 and 0.026, respectively), the dominant amino-acid in *Lotus* roots. On the other hand, accumulation of stress metabolite – proline was achieved that was up to 5 time higher than in control. No considerable differences in this accumulation in INIA Draco and in UFRGS were found, so it is rather unlikely that proline content could contribute to the differences in Al-tolerance between the cultivars.

Keywords: *Lotus corniculatus;* tolerance; asparagine; proline; hydroponic culture; N availability.

INTRODUCTION

Release of water soluble, toxic aluminum species from clay minerals under acidic conditions (pH < 5), is a major constraint for crop production in acidic soils worldwide. Al is released from soil minerals as $Al(OH)_2^+$, $Al(OH)^{2+}$ and $Al(H_2O)_6^{3+}$. The last one, commonly referred to as Al^{3+} cation is the most phytotoxic form (Samac and Tesfaye 2003; Kimraide 1991). In several areas of the world, cultivated pastures occur on strongly acidic soils, where exchangeable Al content can even exceed 10 mmol dm^{-3} (Abreu et al. 2003). Lime is usually applied to raise soil pH and overcome toxicity problems. However, applications of large amounts of lime are expensive; moreover, it interacts with the availability of several nutrients (such as phosphorus, zinc and others) and its mobility in soil is very low, more or less limited to the layer to which it was applied (Rao and Zeigler 1993). Using the relatively more tolerant plant species may reduce the costs of liming and increase their sustainability in cultivated pastures. The *Lotus* sp. are pioneer forage legumes, well adapted to infertile and acidic soils, *Lotus corniculatus* being the most commonly used species mainly in South and North America (Diaz et al. 2005).

Nitrogen metabolism is strongly affected by Al-stress in various plant species including legumes (Baligar et al. 2001). It was shown earlier that Al interacts with the uptake of inorganic N as well as with its reduction (Pal'ove-Balang and Mistrík 2007) and assimilation (Pal'ove-Balang and Mistrík 2011) in a *Lotus japonicus* Gifu, the model plant for legume research that is also phylogenetically closely related to *Lotus corniculatus*. It is known as the root nitrate assimilator and the root is also the main target for aluminum toxicity. The aim of the present work was to compare nitrogen utilization after Al treatment in two South-American cultivars of *Lotus corniculatus*, differing in their tolerance to Al-stress.

MATERIAL AND METHODS

Plant material and growing conditions

Five cm long shoot cuttings of two varieties of *Lotus corniculatus,* relative sensitive 'INIA Draco' from Uruguay and more tolerant 'UFRGS' from Brazil were planted in hydroponics in 3 L containers. Plants were grown 15 days for rooting under controlled conditions (20 ºC, 50% relative humidity, 16h/8h photoperiod and approximately 200 µmol m^{-2} s^{-2} illumination) on 1/10 "Hornum" solution as described by Handberg and Stougaard (1992), with some modifications (1 mM $CaCl_2$, 0.5 mM KNO_3, 0.5 mM NH_4NO_3 and 0.5 mM KCl). The medium was replaced every other day.

Uptake measurements

Plants used for nitrate uptake experiments were nitrogen-depleted for 8 days, using N-free 1/10 strengh Hornum solution similar to above, but with 1 mM $CaCl_2$ and 1 mM KCl. Three days (72 h) before the start of the uptake measurements, the medium was replaced by another one containing 1 mM $CaCl_2$, 1 mM KCl, 0.1 mM KNO_3 and 1 mM $AlCl_3$ (pH 4.0) in order to trigger the inducible nitrate uptake systems and minimize the interaction of nutrients with aluminum. Control plants were treated in the same way without $AlCl_3$, the pH of the solution was maintained at 4.0 or 5.5 (± 0.2) throughout the treatment. The net NO_3^- uptake was calculated by following depletion from the uptake solution (pH 5.0) containing 1 mM $CaCl_2$ and 0.15 mM KNO_3. Nitrate concentration of the uptake solution was measured spectrophotometricaly and related to the root fresh weight (Paľove-Balang and Mistrík 2002).

Plants for ammonia uptake measurements were treated similarly, except that the hydroponic solutions contained NH_4Cl instead of KNO_3. The depletion of ammonia from the uptake solution (1 mM $CaCl_2$ and 0.2 mM NH_4Cl) was determined by ion-selective electrode.

Amino-acid analyses

The plants were treated for 24 or 72 hours in hydroponic cultures containing 1 mM $CaCl_2$, 0.5 mM KNO_3, 0.5 mM NH_4NO_3 and 0.5 mM KCl (pH 4.0 or 5.5). In some plants Al stress was induced with 1 mM $AlCl_3$ (pH 4.0). The 3 cm long apical parts of plants roots were ground to a fine powder in liquid N and extracted with 80% methanol. The total amino acid content was assayed by ninhydrin colorimetric method as described by Yokoyama and Hiramatsu (2003). The content of individual free amino-acids in root tissue was determined in dried extracts, redissolved with 1M sodium borate buffer (pH 9.0) containing 0.02% sodium azide. Amino acids were assayed by following precolumn derivatization with diethyl ethoxymethylenemalonate (DEMM) for 50 min at 50 °C (Alaiz et al. 1992). The derivatization was followed by reversed phase high-performance liquid chromatography (HPLC). The system included the Ecom LCD 3001 pump, detector Ecom LCD 2084, 300 × 3.9 mm I.D. reversed-phase column (Nova-Pack C_{18}, 4 μm; Waters). Resolution of the derivated amino acid was accomplished by using a binary gradient system. The two solvents were (A) 19% acetonitrile, and (B) 70% acetonitrile. Solvent was delivered to the column at a flow-rate of 0.7 ml/min as follows: Time 0-10 min. solvent A; 10-25 linear gradient to A/B (50:50); 25-40 linear gradient A/B (30:70); 40-45 elution with A/B (30:70). The research was focused on the levels of Asn, Asp, Glu and Gln as the main intermediaries for N-assimilation, and on other amino acids, that where relatively abundant. The results are calculated to fresh weight of the roots. Statistical significance at 0.05 level vs. control (pH 5.5) was determined using Student's *t*-test.

RESULTS AND DISCUSSION

Mineral nitrogen uptake in both cultivars was generally inhibited on low pH (4.0) and this effect was slightly but not significantly more apparent in INIA Draco than in UFRGS. The reason for that observation must be the toxic effect of acidity on the root tissue during the pre-treatment because all uptake measurements were performed at the same pH = 5.0. Three-day pre-treatment of the roots with 1 mM Al caused further decrease of uptake in all cases (Table 1). The effect of aluminum was more remarkable to nitrate uptake than to ammonia, which is in good agreement with the data observed previously for the model plant, *Lotus japonicus* Gifu (Pal'ove-Balang and Mistrík 2007). The two cultivars of *Lotus corniculatus* used in present work, contrast in their relative tolerance to aluminum, INIA Draco being the more sensitive and UFRGS being the more tolerant one (Pavlovkin et al. 2009). Comparison of the uptake rate of nitrate and ammonia in these two cultivars in Al stress also showed noticeable differences. The uptake of nitrate in Al stress decreased more in INIA Draco than in UFRGS, but the opposite was true for ammonia. It suggests that lower uptake of nitrate in INIA Draco on Al stress could be partially compensated by relatively higher uptake of ammonia in comparison to UFRGS. Even through for ammonia this difference between cultivars was not very strong, it could be in agreement with the observation that nitrogen deficiency

Table 1: Nitrate and ammonia uptake *($\mu mol\ g^{-1} FW\ h^{-1}$)* from Al-free solution after 72 h low pH and aluminum pre-treatment

	INIA Draco	**UFRGS**
Nitrate uptake		
pH 5.5	3.12 ± 0.09	2.95 ± 0.12
pH 4.0	2.38 ± 0.09*	2.52 ± 0.13
pH 4.0 + 1 mM Al	1.22 ± 0.06*	1.56 ± 0.10*
Ammonia uptake		
pH 5.5	5.49 ± 0.14	5.15 ± 0.11
pH 4.0	4.05 ± 0.16*	4.27 ± 0.13*
pH 4.0 + 1 mM Al	3.30 ± 0.09*	2.58 ± 0.07*

Means of three separate experiments ± SEM, *$p < 0.05$ significant vs. control (pH 5.5)

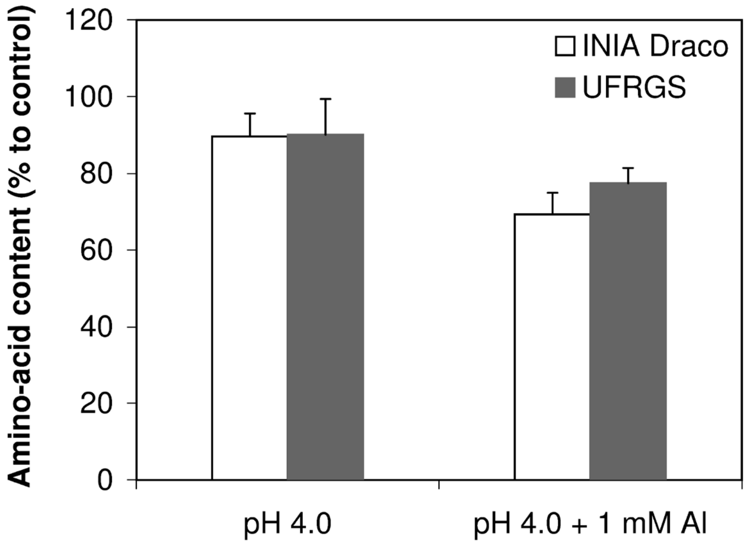

Fig. 1. Relative amino-acid content in roots of the cultivar Draco and UFRGS after low pH and 1 mM Al treatments 1 (percentage to control (pH 5.5), means of three separate experiments ± SEM).

can increase expression of ammonium transporters (Logue and von Wirén 2004).

Acidic condition itself showed only minor effect on free amino-acid content in roots. The total free amino acid content decreased less than 10 percent (Fig. 1.) The treatment with 1 mM Al affected the accumulation of free amino acids much more obviously than the low pH alone especially after 72 h of exposure. Asparagine is an optimal nitrogen transport and reserve compound that particularly in some legumes, is also the dominant amino acid translocated in the xylem from roots to leaves regardless to the overal N availability (Gaufichon et al. 2010).

The level of asparagine in *Lotus* roots, decreased significantly after 72 hours of treatment in similar extent in both cultivars (Tables 2a and 2b). Its content in roots was in most conditions at least 10 times higher than the content of any other amino acid, so its contribution to decrease of the total free amino-acid level was massive. Besides that, treatment with Al significantly decreased the levels of Gln, Glu, Gly and Ala whereas the levels of Val, Ser or Asp remained almost unchanged. Decrease of free amino acids can be related to the lower nitrogen availability due to the inhibition of nitrate and ammonia uptake in the stress conditions. Despite the decreased N availability, there was some increase of Pro on low pH, but its accumulation was much higher after aluminum treatment especially after 72 hours, when its content was already almost five-time higher than in control roots. Increase of Pro content was accompanied by more than three-fold decrease of Glu that is a key intermediate for its synthesis. Accumulation of Pro even on lower nitrogen availability is suggesting its importance for *Lotus* tolerance to Al-stress.

Table 2a: Effect of low pH and Al exposure to free amino acids content in roots of the variety Inia Draco

	pH 5.5 (control)		**pH 4.0**		**pH 4.0 + 1 mM Al**	
	24 h	72 h	24 h	72 h	24 h	72 h
Asn	8.754 ± 0.257	9.425 ± 0.805	7.875 ± 0.857	7.318 ± 0.612	8.244 ± 0.349	5.131± 0.550*
Asp	0.095 ± 0.020	0.091 ± 0.012	0.088 ± 0.011	0.068 ± 0.003	0.057 ± 0.003	0.058 ± 0.004
Gln	0.111 ± 0.005	0.101 ± 0.016	0.107 ± 0.006	0.111 ± 0.010	0.095 ± 0.007	0.062 ± 0.004*
Glu	0.104 ± 0.005	0.098 ± 0.004	0.055 ± 0.003*	0.061 ± 0.006*	0.041 ± 0.002*	0.029 ± 0.002*
Ser	0.238 ± 0.011	0.228 ± 0.015	0.234 ± 0.027	0.252 ± 0.023	0.213 ± 0.009	0.222 ± 0.055
Gly	0.749 ± 0.064	0.742 ± 0.042	0.751 ± 0.050	0.620 ± 0.131	0.671 ± 0.063	0.390 ± 0.070*
Ala	0.144 ± 0.038	0.150 ± 0.044	0.115 ± 0.043	0.114 ± 0.021	0.066 ± 0.005*	0.080 ± 0.020*
Val	0.085 ± 0.006	0.081 ± 0.005	0.077 ± 0.003	0.084 ± 0.004	0.063 ± 0.002	0.058 ± 0.003
Pro	0.410 ± 0.027	0.383 ± 0.054	0.379 ± 0.023	0.640 ± 0.103*	0.781 ± 0.110*	1.820 ± 0.133*

Means of three separate experiments ± SEM, $*p < 0.05$

Table 2b: Effect of low pH and Al exposure to free amino acids content in roots of the variety UFRGS

	pH 5.5		pH 4.0		pH 4.0 + 1 mM Al	
	24 h	72 h	24 h	72 h	24 h	72 h
Asn	7.687 ± 0.688	7.385 ± 0.319	8.033 ± 0.621	6.302 ± 0.621	6.479 ± 0.376	5.817 ± 0.380*
Asp	0.070 ± 0.011	0.067 ± 0.004	0.046 ± 0.018	0.062 ± 0.019	0.053 ± 0.005	0.048 ± 0.009
Gln	0.171 ± 0.015	0.158 ± 0.013	0.125 ± 0.024	0.092 ± 0.013*	0.143 ± 0.021	0.093 ± 0.009*
Glu	0.095 ± 0.010	0.095 ± 0.008	0.056 ± 0.005*	0.041 ± 0.003*	0.034 ± 0.002*	0.017 ± 0.003*
Ser	0.154 ± 0.024	0.163 ± 0.004	0.135 ± 0.029	0.167 ± 0.021	0.138 ± 0.005	0.140 ± 0.012
Gly	0.637 ± 0.095	0.580 ± 0.096	0.574 ± 0.022	0.583 ± 0.119	0.513 ± 0.052	0.366 ± 0.027
Ala	0.119 ± 0.011	0.116 ± 0.016	0.057 ± 0.019*	0.092 ± 0.015	0.035 ± 0.009*	0.064 ± 0.013*
Val	0.090 ± 0.010	0.087 ± 0.005	0.103 ± 0.020	0.088 ± 0.009	0.071 ± 0.006	0.080 ± 0.006
Pro	0.336 ± 0.012	0.362 ± 0.029	0.416 ± 0.071	0.473 ± 0.046*	0.646 ± 0.124*	1.630 ± 0.101*

Means of three separate experiments ± SEM, *$p < 0.05$

Proline is considered to be a stress metabolite accumulated in several types of stress conditions. It might be involved in the protection of the plants by reduction of the stress induced free-radical damage (Szabados and Savouré 2010) and can also act in osmoregulation or metal chelation (Sharma and Diez 2006). The overproduction of Pro in *Arabidopsis* transgenic lines over-expressing pyrroline-5-carboxylate synthetase led to induction of stress-response signaling pathway and resulted to several toxicity symptoms (Mattioli et al. 2008). Despite of the possible role of Pro in stress defense, only minor differences of its accumulation between the two selected cultivars were found, so it is rather unlikely that it could contribute on the differences of their tolerance to aluminum.

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Corresponding author:

Peter Paľove-Balang
Institute of Biology and Ecology
P. J. Šafárik University in Košice
Mánesova 23
SK-040 01 Košice
Slovak Republic
E-mail: peter.palove-balang@upjs.sk