# GLUTAMINE SYNTHETASE AND GLUTAMATE SYNTHASE ACTIVITIES IN RELATION TO NITROGEN FIXATION IN *Lotus* spp.

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**ABSTRACT** - *Lotus corniculatus, L. tenuis, L. pedunculatus,* and *L. subbiflorus* inoculated with *Mesorhizobium loti* NZP2037 strain were grown in a growth chamber. The plants dry weight (DW), the nodule fresh weight (FW), the nitrogenase activity, the nodule glutamine synthetase (GS) and glutamate synthase (GOGAT) activities, as well as the leghemoglobin content and the amino acid in the stem were measured 28 days after inoculation. The highest DW of plants was measured in *L. tenuis* and the highest FW of nodules was measured in *L. pedunculatus.* Nitrogenase activity in *L. tenuis*, *L. pedunculatus* and *L. subbiflorus* was six fold the activity in *L. corniculatus.* Nodule GS and GOGAT activities did not follow this same pattern. *L. tenuis* had the highest values of GS and GOGAT activities in the nodule, and a high nitrogenase activity which is consistent with its high plant DW. The four species of *Lotus* were compared and no correlation between nitrogen fixation parameters and ammonia assimilation enzymes was found, but the GS/GOGAT ratio has a positive and significant correlation ($r^2=0.82^{**}$) with the amino acid content in stems.

**ADDITIONAL INDEX TERMS**: Nitrogen fixation, nitrogenase, *Mesorhizobium loti*, leghemoglobin, ammonia assimilation.

# ATIVIDADE DA SINTETASE DA GLUTAMINA E SINTASE DO GLUTAMATO EM RELAÇÃO A FIXAÇÃO DE NITROGÊNIO EM *Lotus* spp.

**RESUMO** - Plantas de *Lotus corniculatus, L. tenuis, L. pedunculatus* e *L. subbiflorus* foram inoculadas com *Mesorhizobium loti* cepa NZP2037 e mantidas numa câmara de crescimento. A massa seca da planta (MS), massa fresca dos nódulos (MF), atividade de nitrogenase, atividades de sintetase de glutamina (GS) e sintase de glutamato (GOGAT), bem como o teor de leghemoglobina e de aminoácidos no caule foram avaliados 28 dias após inoculação. A maior MS das plantas foi encontrada em *L. tenuis* e a maior MF de nódulos foi encontrada em *L. pedunculatus.* Atividade de nitrogenase em *L. tenuis, L. pedunculatus* e *L. subbiflorus* foi seis vezes a atividade em *L. corniculatus.* As atividades de GS e GOGAT nos nódulos não mostraram o mesmo padrão. As maiores atividades de GS e GOGAT foram encontradas nos nódulos de *L. tenuis* associadas com a alta atividade de nitrogenase, resultados compativeis com sua alta MS. As quatro espécies de *Lotus* foram comparadas e nenhuma correlação entre os parâmetros de fixação de nitrogênio e enzimas de assimilação de amonia foi encontrada, mas a razão GS/GOGAT tem uma correlação positiva e significativa ($r^2=0.82^{**}$) com o teor de aminoácidos nos caules.

**TERMOS ADICIONAIS PARA INDEXAÇÃO**: Assimilação de amonia, fixação de nitrogênio, leghemoglobina, *Mesorhizobium loti*, nitrogenase.

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## INTRODUCTION

*Lotus* spp. has been widely cultivated as a pasture legume in Uruguay; its attributes include self-seeding and the fact that it does not produce meteorism (Aerts *et al*., 1999). Within this genus, *L. corniculatus* has until recently been the most widespread species, but *L. subbiflorus* and other species are now being grown to improve the forage production of natural pastures because of their ability to grow in soils with low water and nutrient availability (Asuaga, 1994).

The *Lotus* genus can be nodulated by both fast-growing (*Mesorhizobium loti*) and slow-growing (*Bradyrhizobium* sp.) strains of nitrogen-fixing bacteria (Pankhurst *et al*., 1986). *L. subbiflorus* did not form effective nitrogen fixing nodules in symbiosis with U226 *M. loti* strain while *M. loti* strain NZP2037 is able to form effective nodules on *L. tenuis*, *L. pedunculatus*, *L. corniculatus* and *L. subbiflorus* (Irisarri *et al*., 1996).

There are many microbiological and genetic studies on strains that nodulate *Lotus* spp. (Pankhurst *et al*., 1986; Monza *et al*., 1992; Irisarri *et al*., 1996) but less is known about the biochemistry and the physiology of this symbiosis (Díaz *et al*., 1995; Borsani *et al*., 1999).

An effective nitrogen fixing symbiosis requires an appropriate interaction between the plant and the bacteria (Verma and Long, 1983). The ammonia produced by the bacteroids' nitrogen fixation in nodules in a reaction catalyzed by nitrogenase (EC 1.18.6.1), is assimilated into organic compounds by the plant nodule enzymes glutamine synthetase (GS) (EC 6.3.1.2) and glutamate synthase (GOGAT) (EC 1.4.1.14). The GS is crucial in this assimilatory process as it catalyzes the first step in the conversion of inorganic nitrogen (ammonium) into its organic form (glutamine). The amide nitrogen from glutamine is then transferred to 2-oxoglutarate to yield two molecules of glutamate which can serve as GS substrate and so complete the GS/GOGAT cycle (Lea *et al*., 1990). Much of the fixed nitrogen is rapidly transferred to asparagine that, together with glutamine, is transported in the xylem from the nodule in *Lotus* spp. (Steele *et al*., 1983).

A close relationship between nitrogen fixation and GS and GOGAT activities has been found within legumes. Nitrogenase, GS and GOGAT activities increase during nodule development (Egli *et al*., 1989; Reynolds *et al*., 1982), decrease with the harvest of shoots and increase again with shoot regrowth (Crale and Heichel, 1981; Groat and Vance, 1981).

Biological nitrogen fixation (BNF) – related parameters such as nitrogenase activity, dry weight (DW) and nitrogen content of plants or fresh weight (FW) of nodules are usually measured to select the best symbiotic pairs (Buttery and Dirks, 1987). However, plants selected for increased nitrogen fixation potential have sometimes failed to demonstrate increased shoot yields or greater nitrogen concentration under field conditions (Jessen *et al*., 1988). The efficiency of the assimilation of the fixed nitrogen by the plant enzymes GS and GOGAT could play an important role in plant productivity. Sufficient genotypic variability has been measured for GOGAT activity in alfalfa (Jessen *et al*., 1988) and for GS activity in *Phaseolus* (Hungría *et al*., 1991) and this suggests that these enzymes could be used as a possible complementary selection criteria in a breeding program.

The aims of this study were to evaluate nodule GS and GOGAT activities in four *Lotus* species inoculated with the same bacterial strain and to determine the relationships between BNF parameters with nodule GS and GOGAT activities.

## MATERIALS AND METHODS

### Plant growth, bacterial culture and inoculation

*L. corniculatus* cv. La Estanzuela San Gabriel (AGROSAN S.A. Montevideo, Uruguay), *L. tenuis* cv. Chajá, *L. pedunculatus* cv. Makú and *L. subbiflorus* cv. El Rincón plants were used. Surface sterilized seeds were germinated on sterile wet paper in Petri plates and ten seedlings were then planted in each Leonard jar containing river

sand- vermiculite according to Díaz *et al*. (1995) with a sterile nutrient solution without nitrogen (Rigaud and Puppo, 1975). Ten jars per treatment were used and the plants were grown in a growth chamber with 500 µmol $m^{-2}$ $s^{-1}$ during a 16/8 h (light/dark) cycle at 25/18 °C (day/night) temperature. Plants were inoculated with 1 mL $plant^{-1}$ of a bacterial culture containing $10^9$ CFU $mL^{-1}$ of *M. loti* strain NZP2037 (Irisarri *et al*., 1996).

**Harvest of plants**

Twenty-eight days after the inoculation, the plants were harvested and the root systems were excised from the shoots.

All roots, except two per jar, were used to pick the nodules in order to measure GS and GOGAT activities and the leghemoglobin content. The rest of the roots, taken from two plants per jar of each treatment, were used to measure nitrogenase activity.

**Preparation of nodule cell free extracts**

Nodule cell free extracts were prepared by grinding nodule tissue in a mortar with a 50 mM potassium phosphate buffer pH 7.6 optimized to measure GS and GOGAT activities simultaneously (Gonnet, 1994), containing 12.5 mM 2-mercaptoetanol, 5 mM EDTA, 14.6 mM sucrose, 100 mM KCl and 20% polyvinylpolypyrrolidone. The brei was filtered through four layers of muslin and centrifuged at 20.000 g for 20 min. The supernatant was used to measure the enzyme activities. All steps were carried out at 4 °C and GOGAT activity was measured within the first hour of extraction to minimize loss of activity (Robertson *et al*., 1975; Boland *et al*., 1978).

**Enzyme assays**

GS activity was measured by the synthetase assay (Lea *et al*., 1990). NADH-GOGAT was measured spectrophotometrically monitoring NADH absorbance at 340 nm (Groat and Vance, 1981). Aminooxyacetic acid was added to the assay buffer to inhibit transaminase activity (Gonnet *et al*., 1998).

GS and GOGAT activities as in other physiological studies are expressed on a gram nodule FW basis since these enzymes (especially GOGAT) make up a small fraction of the nodule soluble protein (Jessen *et al*., 1988).

Nitrogenase activity was estimated by the acetylene reduction assay with gas chromatography (Hardy *et al*., 1973). Despite the criticisms of the method (Vessey, 1994, Minchin *et al*., 1994) it is still used in comparative experiments under controlled conditions (Buttery and Dirks, 1987; Vessey, 1994).

**Analytical determination**

Soluble protein in the extract was measured by the Bradford (1976). The plants' dry weight was determined after drying until constant weight at 70°C. Leghemoglobin content in nodule extracts was determined according to Appleby and Bergersen (1980). Amino acid content was measured in the first two centimeters of stems as in Borsani *et al*. (1999).

## RESULTS

Twenty eight days after inoculation *L. corniculatus*, *L. tenuis* and *L. pedunculatus* plants reached the highest plant DW. *L. subbiflorus* plants, however, weighed 25% less than the others (Table 1). Colored nodules were present in all the plants of all species at that moment. Nodule FW ranked as follows: *L. pedunculatus* > *L. tenuis* > *L. corniculatus* > *L. subbiflorus* (Table 1).

The nodules' soluble protein varied between 10 and 14 mg. g nod $FW^{-1}$ among the different species (Table 1) and the amino acid content measured in the first centimeters of stem varied from 6.8 to 13.6 µmol g stem $DW^{-1}$ (Table 3). The leghemoglobin content in *L. tenuis* nodules was significantly higher than in the other species (Table 1).

No significant differences in nitrogenase activity (µmol $C_2H_4$ g nod $FW^{-1}$. $h^{-1}$) were detected between *L. pedunculatus*, *L. tenuis* and *L.*

*subbiflorus*. *L. corniculatus* plants, however, had 4 fold less nitrogenase activity than the other species. When nitrogenase activity was expressed per plant, *L. pedunculatus* showed the highest value and no significant difference was found among the other three species (Table 2).

Taken together, the ammonia assimilation enzyme activities in nodules were highest in *L. tenuis.* GS activity in *L. tenuis* and *L. corniculatus* was significantly higher than in *L. subbiflorus* and *L. pedunculatus*. GOGAT activities in nodules were *L. tenuis* > *L. corniculatus* > *L. subbiflorus* > *L. pedunculatus* (Table 2).

The GS/GOGAT ratio was equal to or lower than 3 in all the species (Table 3) and this ratio has a positive and significant correlation ($r^2=0.82^{**}$) with the amino acid content in stems.

**TABLE 1 -** Plant DW, nodule FW, nodule leghemoglobin and nodule protein content of *Lotus* spp. plants nodulated with *M. loti* strain NZP 2037.

| | *L. corniculatus* | *L. tenuis* | *L. subbiflorus* | *L. pedunculatus* |
|---|---|---|---|---|
| Plant DW[1] | 8.2 ab | 9.9 a | 2.2 c | 8.1 b |
| Nod. FW[1] | 5.1 c | 5.9 b | 2.1 d | 8.5 a |
| Leghemoglobin[2] | 77.1 b | 102.8 a | 69.0 b | 84.9 b |
| Protein[3] | 10.0 a | 14.0 a | 11.4 a | 12.8 a |

[1](mg plant$^{-1}$), [2](nmol hemo g nod FW$^{-1}$), [3](mg g nod FW$^{-1}$). Values are means of four independent plant samples. Means denoted by the same letter did not differ significantly at $P < 0.05$.

**TABLE 2 -** Nitrogenase, GS and GOGAT activities in *Lotus* spp. plants nodulated by *M. loti* strain NZP2037.

| | *L. corniculatus* | *L. tenuis* | *L. subbiflorus* | *L. pedunculatus* |
|---|---|---|---|---|
| Nitrogenase[1] | 1.7 b | 5.6 a | 6.7 a | 7.5 a |
| Nitrogenase[2] | 10.6 a | 26.0 a | 11.0 a | 70.2 b |
| GS[3] | 59.6 a | 62.7 a | 36.1 b | 46.7 b |
| GOGAT[4] | 48.1 b | 60.8 a | 17.5 c | 5.8 d |

[1](μmol $C_2H_4$ g nod FW$^{-1}$. h$^{-1}$), [2](nmol $C_2H_4$ p$^{-1}$. h$^{-1}$) [3](μmol γ-glutamil hydroxamate g nod FW$^{-1}$. h$^{-1}$), [4](μmol oxided NADH g nod FW $^{-1}$. h$^{-1}$). Values are means of four independent plant samples. Means denoted by the same letter did not differ significantly at $P < 0.05$.

**TABLE 3** - Amino acids and GS/GOGAT ratios in *Lotus* spp. plants nodulated by *M. loti* strain NZP2037.

| | *L. corniculatus* | *L. tenuis* | *L. subbiflorus* | *L. pedunculatus* |
|---|---|---|---|---|
| amino acids[1] | 8.3 b | 6.8 b | 9.1 b | 13.6 a |
| GS/GOGAT ratio | 1.2 | 0.9 | 2.1 | 3.0 |

[1](µmol g stem $FW^{-1}$). Values are means of four independent plant samples. Means denoted by the same letter did not differ significantly at $P < 0.05$.

## DISCUSSION

Plants were harvested 28 days after inoculation when ammonia assimilation enzymes in nodules have their highest specific activity as indicated in previous studies with *L. corniculatus* (Gonnet *et al*., 1998).

DW was significantly lower in *L. subbiflorus* and was not correlated with nodule FW as has been reported in other legumes (Buttery and Dirks, 1987).

Nodule protein content was similar to other legume species (Groat and Vance, 1981; Reynolds *et al*., 1982) but higher than values reported by Boland *et al*. (1978) for *L. pedunculatus* inoculated with *Bradyrhizobium* sp. strain CC814s.

Leghemoglobin levels in all *Lotus* species were consistent with those reported for *Vigna unguiculata* (Dakora, 1995) and soybean (Reynolds *et al*., 1982). The leghemoglobin molecule is a symbiotic product whose vegetal part is synthetized in response to the bacterial infection (Verma and Long, 1983). A physiological relationship has been suggested to exist between nodule leghemoglobin content and nitrogen fixing efficiency, and higher nitrogenase activity has been reported in soybean and common bean when leghemoglobin levels were high (Dakora, 1995).

For *Lotus* spp.no correlation was detected between nitrogenase and leghemoglobin content.

Nitrogenase activity measured in *L. subbiflorus*, *L. pedunculatus* and *L. tenuis* nodules was 4 fold higher than in *L. corniculatus* nodules. *L. corniculatus* in symbiosis with NZP2037 has 6 times less nitrogenase activity than with *M. loti* strain U226 (Monza *et al*., 1997). Among the three species with high nitrogenase, *L. pedunculatus* clearly stands out as the most efficient one when nitrogenase activity is expressed per plant.

No significant difference was detected in GS activities between *L. tenuis* and *L. corniculatus* but they were higher than in *L. subbiflorus* and *L. pedunculatus* nodules. *Lotus* spp. nodule GS activity levels were similar to those reported in alfalfa (Groat and Vance, 1981) and soybean (Lara *et al*., 1983), but higher than in *Vinga unguiculata* (Silveira *et al*., 1998) and lower than in broad bean (Caba, 1991).

As in alfalfa (Groat *et al*., 1984) and broad bean (Caba, 1991) germplasm, *Lotus* spp. GS activity did not correlate with nitrogenase activity. On the other hand, *Phaseolus* spp. show GS activity levels that could be a limiting factor in ammonia assimilation. GS activity has therefore been proposed as a criterion to select plants in this legume (Hungría *et al*., 1991).

GOGAT activity level in the four species studied was similar to that reported for broad bean (Caba, 1991), where a positive correlation between GS and GOGAT activities was found in blooming plants. In *Lotus* spp. no correlation between GS and GOGAT activities was found but the plants were at a different growth stage.

GOGAT and phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) were positively correlated with nitrogenase activity in alfalfa germplasm and that suggests that GOGAT and PEPC could be used to select the more efficient nitrogen fixing symbiotic pair (Jessen *et al*., 1988).

In our study, no correlation was found between GOGAT and nitrogenase activities for *Lotus* sp.

An effective symbiosis requires the coordinated expression of both plant and bacterial genes. Nodule GS and GOGAT are plant gene products, whose expression can be influenced by the nodule's stage of development and effectiveness. Maximum expression requires a product associated with effective bacteria (Vance *et al*., 1988) but how ammonia produced by nitrogenase regulates GS and GOGAT activities remains unclear (Suganuma *et al*., 1999). Here we report the behavior of four *Lotus* spp. in symbiosis with the same bacteria and no correlation was found between nitrogenase and the ammonia assimilation enzyme activities.

In spite of the lack of correlation overall, in view of the ammonia assimilation process, *L. tenuis* would have the most efficient system compared to the other species. This is consistent with high DW per plant and highest leghemoglobin content in nodules. The opposite situation was observed in *L. subbiflorus*. In spite of its high nitrogenase activity, its low levels of GS and GOGAT activities in nodules could be explained by a different stage of nodule development in this specie. It remains to be determined if lower GS and GOGAT activities were derived from lower enzyme synthesis.

Boland *et al*. (1978) reported that the ratio of GS to GOGAT, measured in twelve legume species, varied from 1 to 14.5. The highest ratios belonged to legumes that transport ureide because more glutamine is necessary to synthesize ureide than amide compounds (Pate *et al*., 1980).

In *L. pedunculatus*, Boland *et al*. (1978) reported GS/GOGAT ratios of about 7 and Steele *et al*. (1983) observed that *Lotus* exports amide from nodules. Previous studies with *L. corniculatus* showed ratios between 1 and 6 depending on nodule age and *M. loti* strain (Gonnet, 1994). In our study the GS/GOGAT ratio varied between 1 and 3 among the assayed species and this is consistent with values for amide exporters reported by Boland *et al*. (1978).

The GS/GOGAT ratio significantly correlated with the amino acid content in stems. The strong relationship between amino acid content and GS/GOGAT could be explained because, in *Lotus* spp. nodules, GS, in concert with asparagine synthetase (EC 6.3.1.1), produces glutamine and asparagine that are exported to the shoot.

The data presented here allowed us to learn more about *Lotus* spp.- *M. loti* symbiosis and that is important when new *Lotus* species are being introduced in Uruguay. In this study, 28-day-old plants of four *Lotus* species did not show a correlation between BNF parameters with the activities of ammonia assimilation enzymes in nodules. Unlike data presented for beans (Hungría *et al.*, 1991), we did not find evidence that GS activity limits BNF and ammonia assimilation in the *Lotus* spp. x *M. loti* strain NZP 2037.

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