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The role of ammonium assimilating enzymes in lentil roots and nodules

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Abstract

Activities of ammonium assimilating enzymes glutamate dehydrogenase (GDH), glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) as well as the amino acid content were higher in nodules compared to roots. Their activities increased at 40 and 60 d after sowing, with a peak at 90 d, a time of maximum nitrogenase activity. The GS/GOGAT ratio had a positive correlation with the amino acid content in nodules. Higher activities of AST than ALT may be due to lower glutamine and higher asparagine content in xylem. The data indicated that glutamine synthetase and glutamate synthase function as the main route for the assimilation of fixed N, while NADH-dependent glutamate dehydrogenase may function at higher NH_4^+ concentration in young and senescing nodules. Enzyme activities in lentil roots reflected a capacity to assimilate N for making the amino acids they may need for both growth and export to upper parts of the plant.

Additional key words: alanine aminotransferase, aspartate aminotransferase, glutamate dehydrogenase, glutamate synthase, glutamine synthetase, *Lens culinaris*.

Introduction

In legume root nodules, over 95 % of the nitrogen fixed by the rhizobial bacteroids is transferred to ammonium (Miflin and Lea 1976). Tracer, enzyme, and inhibitor studies have established that this ammonium is initially assimilated into the amide position of glutamine by glutamine synthetase (GS) and then into the 2-amino position of glutamate by glutamate synthase (GOGAT) (Ohyama and Kumazawa 1980, Rawsthorne *et al.* 1980). An alternative to the GS/GOGAT system is the system with glutamate dehydrogenase (GDH), but the opinions about its role in N metabolism have been quite varying. Lea *et al.* (1982) reported that GDH does not appear to play a role in ammonium assimilation in legumes although it may function in *Alnus* nodules. However, in alfalfa GDH activity is proposed to play a minor role (Groat and Vance 1981, Ta *et al.* 1986). Though the route of assimilation of fixed N in legume nodule is now largely understood, the time course studies of the related enzymes were performed in a limited number of legumes namely lupine (Robertson *et al.* 1975), alfalfa (Groat and Vance 1981), soybean (Reynolds *et al.* 1982), pigeon pea (Luthra *et al.* 1983), and clover (Gordon and James 1997). The expression of GS activity in legume roots was increased by application of ammonium solution (Oaks 1992, Stanford *et al.* 1993). In addition to the above enzymes, the pathway requires an aspartate aminotransferase (AST) to provide aspartate for the asparagine synthesis and 2-oxoglutarate for the glutamate synthase reaction (Reynolds and Farnden 1979). In lentil, most of the fixed nitrogen is transported in the form of asparagine (accounts for 73 % of the N of the xylem exudate) together with glutamine (7 %) and arginine (18 %) through the xylem from the nodule (Peoples *et al.* 1987). The composition of N solutes in lentil suggested the importance of alanine aminotransferase (ALT) in addition to AST for the synthesis of arginine and asparagine from glutamate. The ability of legume roots to export asparagine reflects the capacity to assimilate nitrogen (Oaks and Hirel 1985). Therefore, most of the information about aminotransferases in developing legumes is related to aspartate aminotransferase (Reynolds and Farnden 1979, Reynolds *et al.* 1982,

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Abbreviations: ALT - alanine aminotransferase; AST - aspartate aminotransferase; DAS - days after sowing; f.m. - fresh mass; GDH - glutamate dehydrogenase; GOGAT - glutamate synthase; GS - glutamine synthetase.

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Griffith and Vance 1989, Gordon and James 1997). Hence, it was thought worthwhile to investigate also the developmental changes of both these enzymes forming a link between synthesis and transport of N solutes in lentil nodules.

Very few workers have compared the developmental changes in enzymes in both nodules and roots (Luthra *et al.* 1983, Gordon and James 1997). Therefore, the purpose of this study was also to determine the developmental changes in activities of GS, GOGAT and GDH along with total amino acid contents in lentil roots and nodules.

Materials and methods

Lentil (*Lens culinaris* L. cv. L4076) was raised in the fields in the month of October, situated at 247 m above sea-level at latitude of 30° 54' N following recommended agronomic practices. Three irrigations were applied to the soil at 4, 6, and 8 weeks after sowing. Uniformly growing plants were uprooted from the wet field at 10 d interval from 50 to 90 days after sowing (DAS). Roots with intact nodules were thoroughly washed with tap water and with deionized H_2O and uniformly developed nodules were collected. Roots were also taken for enzyme assays.

For extracting aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1), alanine aminotransferase (L-alanine:2-oxoglutarate amino transferase, EC 2.6.1.2), glutamate synthase (L-glutamine:2-oxoglutarate aminotransferase, EC 1.4.1.14), glutamine synthetase (L-glutamine:ammonia ligase (ADP), (EC 6.3.1.2) and glutamate dehydrogenase (EC 1.4.1.2), fresh nodules were homogenized in cold (3 - 4 °C) 100 mM Tris-HCl buffer (pH 7.6) containing 20 mM β-mercaptoethanol, 2 mM $MgCl_2$, 2 mM EDTA, and 10 mM cysteine. The homogenate was fractionated into cytosolic and bacteroidal fractions and activity of enzymes was determined as described previously (Chopra *et al.* 2002). The root cytosolic fraction was obtained by centrifuging the homogenate at 25 000 *g* and then passing through *Sephadex G-25* column. There were three replicates for each enzyme extract.

The activity of glutamate dehydrogenase was assayed as followed by the method of Garg *et al.* (1984). The reduction in absorbance was followed at 340 nm on UV double beam spectrophotometer (*Hitachi* model *U-2000*, Tokyo, Japan) for 240 s till the rate of decrease in absorbance became constant. Glutamine synthetase was assayed essentially by the method of Elliot (1955). The activity of glutamate synthase was determined according to Misra and Oaks (1981). The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined according to Tonhazy (1960a,b), respectively.

Soluble protein content in the extract was measured using Folin phenol reagent (Lowry *et al.* 1951). Amino acids were quantitatively extracted and estimated according to Lee and Takahashi (1966). The concentration of the amino acids was calculated taking L-glycine as standard.

Results

In vitro activities of GDH, GS, GOGAT, AST and ALT were relatively low in roots compared to nodule cytosol and bacteroids (Fig. 1). GDH in nodule cytosol and bacteroids showed high activities at 40 DAS, then it decreased significantly from 40 to 50 DAS (Fig. 1*A*). The peak of GDH activity in nodule cytosol was observed at 90 DAS, *i.e.*, at flowering. The increase in enzyme activities in both nodule cytosol and roots were comparable indicating that enhanced expression of the genes encoding GDH did not occur in symbiotic tissue. The same pattern was evident for the activity of GOGAT except that the increase in GOGAT activity at 60 DAS was more obvious than that in GDH (Fig. 1*B*). The increase in the GOGAT activity in nodule cytosol and bacteroids was again at the stage of pod formation, *i.e.*, at 90 DAS. However, the enzyme activity was higher significantly at 40 and 60 DAS. The same enzyme in root tissue showed relatively higher activities at 40 and 60 DAS and then declined slightly till 90 DAS. In contrast to GDH and GOGAT, GS was found in significant quantities only in the plant cytosolic fraction (Fig. 1*C*). Nodule GS activity followed a pattern different from that of nodule GDH and GOGAT: it showed two peaks, one at 60 DAS and the other at 90 DAS, while the enzyme activity peaked at 50 DAS in roots. Of the two aminotransferases followed, AST showed higher activities than ALT in lentil nodules (Fig. 1*D,E*). Both these enzymes were localized in the cytosol and not in the bacteroids of the lentil nodules and the highest enzyme activity was found in young nodules, *i.e.*, at 40 DAS. In the root, the enzyme activity of both the aminotransferases showed maxima at 60 and 80 DAS (Fig. 1*D,E*).

Increased concentration of amino acids (Fig. 1*F*) was observed at 50 and 90 DAS for nodules and 50 and 80 DAS for roots. The GS/GOGAT ratio was from 1.04 to 2.92 in lentil nodules (Table 1) and this ratio has a positive correlation ($r = 0.68$) with the amino acid content in nodules.

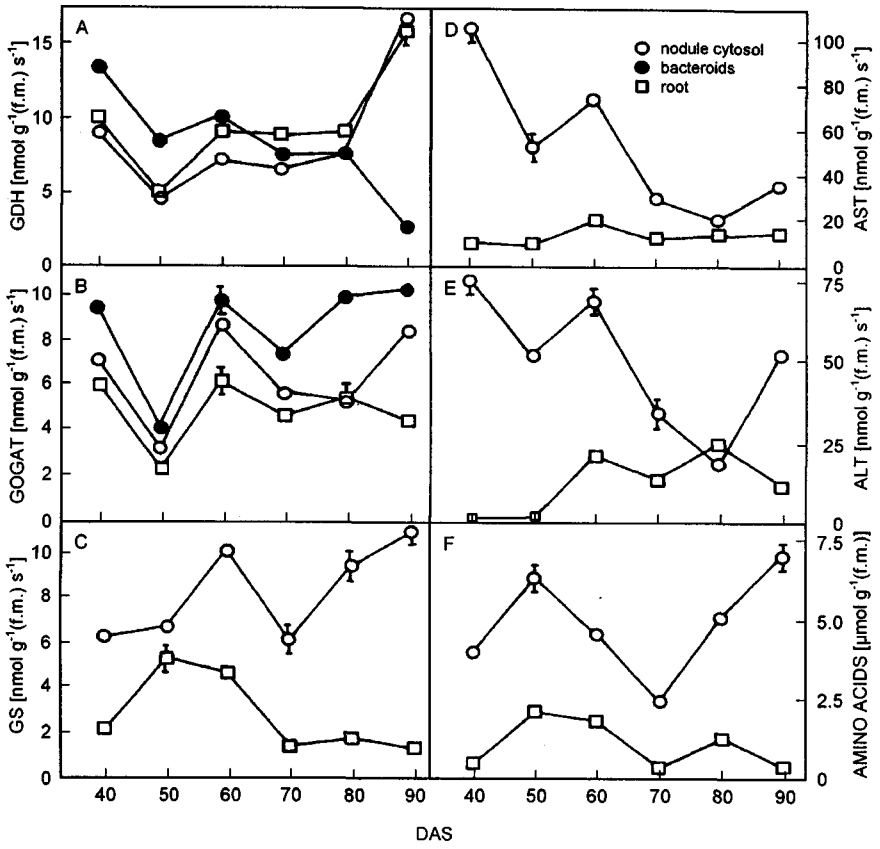

Fig. 1. Activities of ammonia assimilating enzymes GDH (*A*), GOGAT (*B*), GS (*C*), AST (*D*), ALT (*E*), and amino acid content (*F*) in the nodule cytosol, bacteroids, and root cytosol of lentil during development. The *vertical bars* show SD calculated from three replicates. The *vertical bars* have not been shown where SD was smaller than the symbol.

Table 1. Amino acid content [µmol g^{-1}(f.m.)] and GS/GOGAT ratio in lentil nodules during plant development.

	40 DAS	50 DAS	60 DAS	70 DAS	80 DAS	90 DAS
Amino acids	234.1 ± 40.8	366.6 ± 62.1	266.7 ± 27.8	146.8 ± 15.4	296.6 ± 11.3	405.2 ± 58.1
GS/GOGAT	1.04	2.92	1.62	1.32	1.69	1.87

Discussion

The *in vitro* activities of GDH, GS, and GOGAT in lentil nodules increased at 40 and 60 DAS with a peak at 90 DAS (*i.e.* at flowering), except for GS which did not show an increased enzyme activity at 40 DAS. In our previous study, the enzymes of sugar metabolism (Chopra *et al.* 2003), pentose phosphate pathway and carbon assimilation in lentil nodules (Chopra *et al.* 2002) showed higher activities at flowering, coinciding with the peak of nitrogenase activity around 90 DAS. On the basis of the cited work, we have previously hypothesized that the enzymes of these pathways may provide NADPH for metabolism of bacteroids and carbon skeletons for ammonia assimilation. The results of ammonia assimilatory enzymes presented here now provide additional support for this hypothesis. However, the difference in the profile of GS activity from GDH and GOGAT may suggest that this enzyme may be regulated differently. The initial increase in ammonia assimilating enzymes at 40 DAS suggested that the release of bacteria or bacteroids into nodule cells occurring at the onset of nitrogen fixation (Egli *et al.* 1989), may be one signal for increased enzyme activity. The highest nodule number and nodule DM were found at 60 DAS in lentil (data not given), and an increase in enzyme activities at 60 DAS may suggest that more availability of fixed N may modulate the enzyme gene expression. Nodule GS and GOGAT are plant gene products, whose expression can be influenced by the nodule stage of development and

effectiveness (Vance *et al.* 1988, Suganuma *et al.* 1999). In conclusion, the changes in activities of ammonia assimilating enzymes during lentil nodule development were similar to those observed by other workers in different legumes (Groat and Vance 1981, Reynolds *et al.* 1982, Lara *et al.* 1983, Jessen *et al.* 1988, Egli *et al.* 1989, Singh *et al.* 1994), in that the activities were closely related to N_2 fixation.

In lentil nodules, GDH activity was comparable with GS and GOGAT activities in nodule cytosol and was relatively more expressed in young and especially senescing nodules. This may be consistent with its role in detoxifying the high NH_4^+ concentration present at these stages of nodule development. A general increase in GDH activity in leaves during their initial growth phase and during leaf senescence has also been reported (Cammaerts and Jacobs 1985). In some earlier studies, nodule NADH-GDH was reported to be unlikely to play an important role in ammonia assimilation (Groat and Vance 1981, Atkins *et al.* 1984, Egli *et al.* 1989). However, in a comparative study of nitrogen metabolism in many legumes (Boland *et al.* 1978), GS activity was reported to be in excess of GDH for lupin, soybean, lucerne, *Lotus* and peanut, but exceptions occurred in white clover, subclover, pea and goat's rue. In all these species, the GDH activity was appreciable and in white clover it exceeded that of GS. In view of the comparative pattern of GDH, GS and GOGAT in lentil nodules, it appears that although GS-GOGAT is the main route for the entry of symbiotically fixed N, the enzyme GDH, present in large amounts in nodule cytosol, may also play an important role under some nutritional and environmental conditions.

GS activities were found in significant quantities only in the plant cytosolic fraction of lentil nodules. GS activity in other legume nodules viz. soybean (Boland *et al.* 1982) and alfalfa (Vance *et al.* 1994) was also reported to be localized only in cytosolic fraction. 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 ureides because more glutamine is required to synthesize ureide than amide compounds (Pate *et al.* 1980). In our study, the GS/GOGAT ratio varied between 1 and 3 and is consistent with values for amide exporters (Boland *et al.* 1978) and almost same to that observed for *Lotus* spp. (Gonnet and Diaz 2000). The GS/GOGAT ratio positively correlated with the amino acid contents in nodules. The positive relationship between amino acid content and GS/GOGAT ratio could be explained, as lentil nodules export mainly the amides, *e.g.*, asparagine, glutamine and arginine. Plant GS and GOGAT are proposed to function in a cyclic manner, the product of each serving as a substrate for another (Rawsthorne *et al.* 1980). Data in this experiment indicate that nodule GS and GOGAT may not be tightly coupled. The coupling of GS to GOGAT may not be required as long as glutamate could be supplied as substrate for GS by some other mechanism (*e.g.* via NADH-GDH or transamination) (Groat and Vance 1981). The substantial activities of GDH and transaminases in lentil nodules support the above statement. The lower glutamine and the higher asparagine levels observed in the xylem transport system from lentil nodules (Peoples *et al.* 1987), could be explained by a more active AST than ALT in this tissue. The presence of both GS/GOGAT system and GDH along with aminotransferases in lentil roots provides a good evidence that roots are able to make the amino acids they may need for both growth and export to the upper parts of the plant.

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