Diurnal variation in nodule nitrogen metabolism in faba bean (*Vicia faba* L.)

Variaciones del metabolismo del nitrógeno en nódulos radicales de habas (*Vicia faba* L.) a lo largo del fotoperiodo.

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ABSTRACT

Diurnal variations in acetylene reduction is shown in nitrogen-fixing faba bean. The highest values for ARA occurred during the light period, while the lowest values were recorded during the dark period. However, the glutamate synthase cycle enzymes (GS and NADH-GOGAT) and NADH-GDH show rhythmic behaviour on a cycle of 16 h, with maximum and minimum activities both being observed during the photoperiod as well the dark period.

Key words: ammonium assimilation, GS, NADH-GOGAT, nitrogen fixation, *Vicia faba*.

RESUMEN

La reducción de acetileno en plantas de haba fijadoras de nitrógeno, mostró diferencias entre el día y la noche. Los mayores valores de ARA se detectaron durante el periodo de luz, mientras que en periodo de oscuridad los valores fueron inferiores. Por otra parte, las enzimas del ciclo de la glutamato sintasa (GS y NADH-GOGAT) y la NADH-GDH mostraron un comportamiento cíclico de 16 horas, observándose actividades máximas y mínimas tanto en el periodo de luz como en el de oscuridad.

Palabras clave: asimilación de amonio, fijación de nitrógeno, GS, NADH-GOGAT, *Vicia faba*.

Abbreviations: ARA, acetylene reduction activity; γ-GH, γ-glutamyl-hydroxamate; GS, glutamine synthetase; NADH-GOGAT, NADH-dependent glutamate synthase; NADH-GDH, NADH-dependent glutamate dehydrogenase.

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Introduction

Biological N_2 fixation is the major means by which organic N is added to soils. The legume-*Rhizobium*:*Bradyrhizobium* symbiosis is the most signifiant source of N for agriculture. In addition, legumes yield nutritious fodder, protein-rich seeds and fruits. Faba bean (*Vicia faba* L.) is widely used as a grain legume crop in south Australia and Spain, China and Egypt (1,2) but there are few reports of N_2 fixation by this species (3, 4).

Some herbaceous symbioses show pronounced diurnal fluctuations in nitrogenase activity detected as acetylene reduction acitivity (ARA) (5, 6, 7). In field studies of tropical nitrogen-fixing tree symbioses, diurnal patterns in ARA have been indicated (8, 9, 10). In some studies a midday peak in ARA was observed (8, 9).

The ARA assay has been widely used as a simple and rapid method of estimating rates of N_2 fixation by legumes grown in both greenhouse and field experiments (11, 12). According to Herdina and Silsbury (3), and Minchin *et al.* (12) an ARA assay in a closed system was satisfactory for measuring the N_2 fixation of faba bean plants in the vegetative growth stage.

During nodulation, both glutamine synthetase (GS) and NADH-dependent glutamate synthase (NADH-GOGAT) have been shown to increase its activities in parallel with nitrogenase and other enzymes in the nitrogen assimilatory pathway (13, 14). *In vitro* enzymatic studies indicate that GS and GOGAT rather than glutamate dehydrogenase (GDH) are involved in the assimilation of NH_4^+ to produce glutamate and glutamine. The resulting glutamate and glutamine are utilized to produce a variety of amino acids. Ohyama and Kumazawa (15) and Fujihara and Yamaguchi (16), using the ^{15}N tracer technique, reported that GS/GOGAT played a predominant role in the assimilation of N in soybean.

This study shows data on diurnal variation in ARA, ammonium assimilating enzyme activities and soluble protein content in nitrogen-fixing symbioses of faba bean plants in the vegetative phase.

Materials and methods

Plant material and growth conditions

Commercial cultivar Alborea of *Vicia faba* L. var. *minor* was bought from Semillas Pacífico S.A., Sevilla, Spain. Seeds were surface-sterilized in 96% (v/v) ethanol for 3 min, then washed with sterile water and germinated at 26°C in moist autoclaved vermiculite. Seedlings, selected for uniformity, were planted (2 per jar) in sterile 1-liter Leonard jars with vermiculite and nutrient solution (17) containing 2 mM KNO_3, which stimulated plant growth

but did not inhibit nodule growth and activity (18). Each seedling was given 1 ml of a suspension of *R. leguminosarum* biovar *viciae* strain GRA19 (19) from a 3-day-old Allen 79 solid medium culture (ca 10^9 cells ml^{-1}). The seedlings were then covered with moist vermiculite and a layer of dry, sterile perlite. The jars were arranged randomly in a growth chamber with a 16-8 h light-dark cycle, 23-16°C day-night temperature, relative humidity 55-75% and photosynthetic photon flux density (400-700 nm) of 450 ìmol m^{-2} s^{-1} supplied by Sylvania Cool-White Lifeline fluorescent lamps (F96T12-CW-VHO, Sylvania Ltd, Quebec, Canada) and incandescent lamps (30% fluorescent wattage). The plants were harvested 23 days after transplanting.

Nitrogen fixation assays

Nitrogenase (EC 1.7.99.2) activity was determined by acetylene reduction on the entire root systems of six plants, as well as on small nodulated portions of root from remaining plants, according to the recommendations of Herdina and Silsbury (3). Nodulated root portions (1 g root plus nodules) and root systems were placed in vials and sealed with serum caps. Ten percent of the internal atmosphere was replaced with acetylene, and 0.2 ml aliquots were taken after 5, 10 and 15 min of incubation and analyzed for ethylene in a Perkin Elmer 8600 gas chromatograph equipped with a Porapak R column (20).

Preparation of cell-free extracts

Nodule homogenates for determining GS, NADH-GOGAT, NADH-GDH and protein were prepared according to a slightly modified version of the technique of Groat and Vance (21). Samples (1 g fresh weight) were homogenized on ice with acid-washed quartz sand and 12 ml (ice-cold) of an extraction medium containing 100 mM maleic acid-KOH, pH 6.8, 100 mM sucrose, 2% (vol/vol) 2-mercaptoethanol, and 15% (vol/vol) ethylene glycol, plus 0.5 g polyvinyl polypyrrolidone. The homogenate was filtered through 4 layers of cheesecloth and the nodule debris removed; the filtrate was centrifuged at 3500 *g* at 2°C for 8 min. The resulting supernatant was centrifuged once more at 30000 *g* for 20 min, which produced a clear solution of host cell cytoplasm and its organelles; this solution was used for enzyme assay.

Enzyme assays

Glutamine synthetase (EC 6.3.1.2) was determined by the hydroxamate synthetase assay, adapted from Farnden and Robertson (22) and Kaiser and

Lewis (23). Assays were optimized for the amount of enzyme to give a linear reaction within at least 30 min. Two controls, one without enzyme and one without L-glutamate, were also analyzed.

NADH-glutamate synthase (EC 1.4.1.14) and NADH-glutamate dehydrogenase (EC 1.4.1.2) activities were assayed spectrophotometrically at 30°C by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (21) and Singh and Srivastava (24), always within 2 h of extraction. Two controls (without α-ketoglutarate and without glutamine in the case of GOGAT, without NH and without α-ketoglutarate in the case of GDH) were used to correct for endogenous NADH oxidation. The decrease in absorbance (linear for at least 10 min) was recorded for 8 min in a Beckman DU-70 spectrophotometer.

Protein determination

Soluble proteins in tissue extracts were determined by the Bradford method (25), with bovine serum albumin (Merck, fraction V) as the standard.

Statistical design and analysis

The experimental layout was a randomized block design replicated 6 times. The results were subjected to an one-way analysis of variance with the least significant difference (LSD) test between means.

Results and discussion

Diurnal variations in ARA were found in *V. faba* (Fig. 1), as was also found for tropical and subtropical nitrogen-fixing tree symbioses (26). In *V. faba*, the highest values for ARA (on average, 105 ìmol C_2H_4 h^{-1} g DW^{-1}) were recorded during the light period (between 4 and 7 h), while the lowest values were recorded during the dark period. The maximum ARA value during exposure to light was 1.6 times higher than the minimum detected during periods of darkness. In some field studies of tropical tree species a midday peak in ARA was observed (8, 9). This may be due to stomatal closure following excessive water loss under high light intensities, as was discussed by Roskoski and van Kessel (10). Midday peaks could not be observed in this study. Acetylene reduction acitivity showed maximum value for several hours during the light period, which had the same light intensity during the day, as was also found for *Casuarina obesa* ARA (26). In *Acacia albida*, *Leucaena*

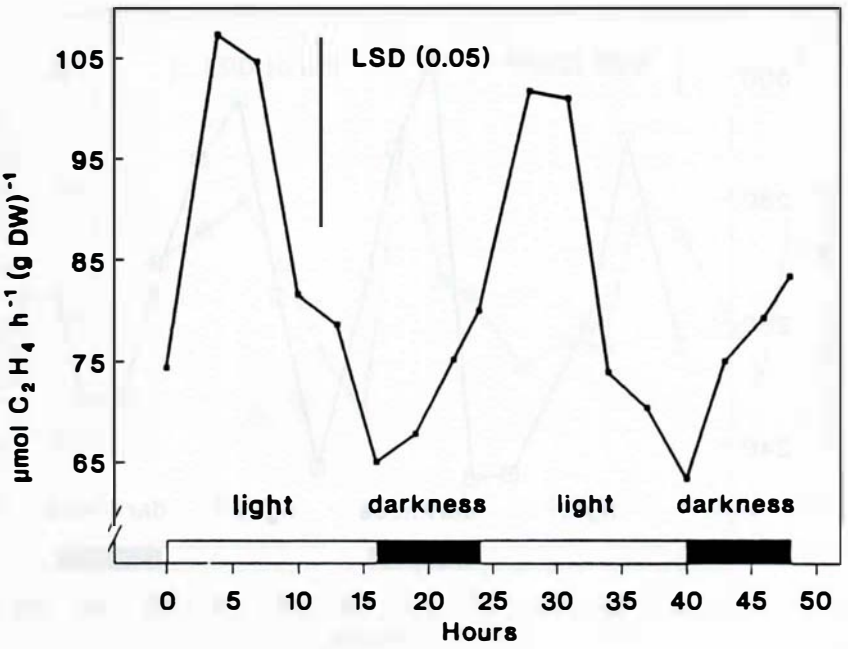

Fig. 1.—Diurnal variation on nodule ARA (ìmol C_2H_4 h^{-1} g^{-1} nodule) for *V. faba* plants inoculated with *R. leguminosarum* GRA19.

leucocephala, *Prosopis chilensis* and *Casuarina glauca* ARA increased throughout the light period (26), and in soybean the ARA remained constant (27).

The enzyme activity involved in the glutamate synthase cycle is 2:1, that is, GS activity is double that of GOGAT, to ensure assimilation of all the ammonia that the nodules are able to produce (28). However, recent results obtained with other genotypes and other strains of *R. leguminosarum* (4) indicate that this relationship may depend on the genotype of the plant and the strain of *Rhizobium*. The GS/GOGAT ratio found in the present study indicates that GS activity does not limit NH_3 assimilation.

The ammonium assimilation enzymes showed a rhythmic behaviour on a 14 h cycle, maximum and minimum activities both occurring during the light as well as the dark period (Figs. 2, 3). The maximum activity values of GS, NADH-GOGAT and NADH-GDH were 1.3 times higher than the minimums. In accord with these results, Fernández-Conde *et al.* (29), studying sunflower leaves, describe the rhythmic behaviour for Fd-GOGAT not controlled by light/darkness conditions but attributable to a biological clock. However, these authors (29) described an evolution of the GS completely dependent upon light. Other authors have reported differences in enzymatic behaviour

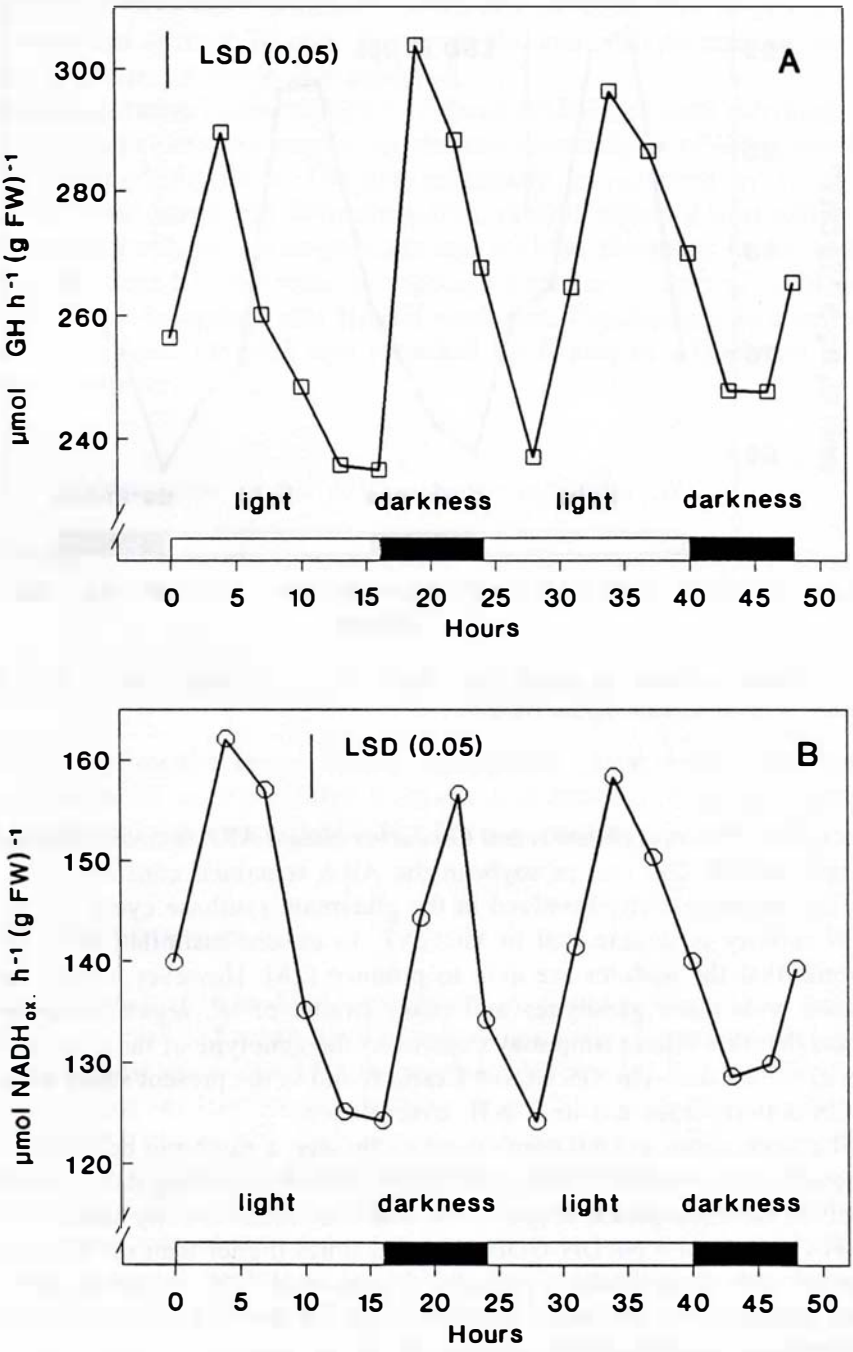

Fig. 2.—Diurnal variation on GS (A) (imol g-GH h^{-1} g FW^{-1}) and GOGAT (B) (imol $NADH_{ox}$ h^{-1} g FW^{-1}) in nodules of *V. faba* plants inoculated by *R. leguminosarum* GRA19.

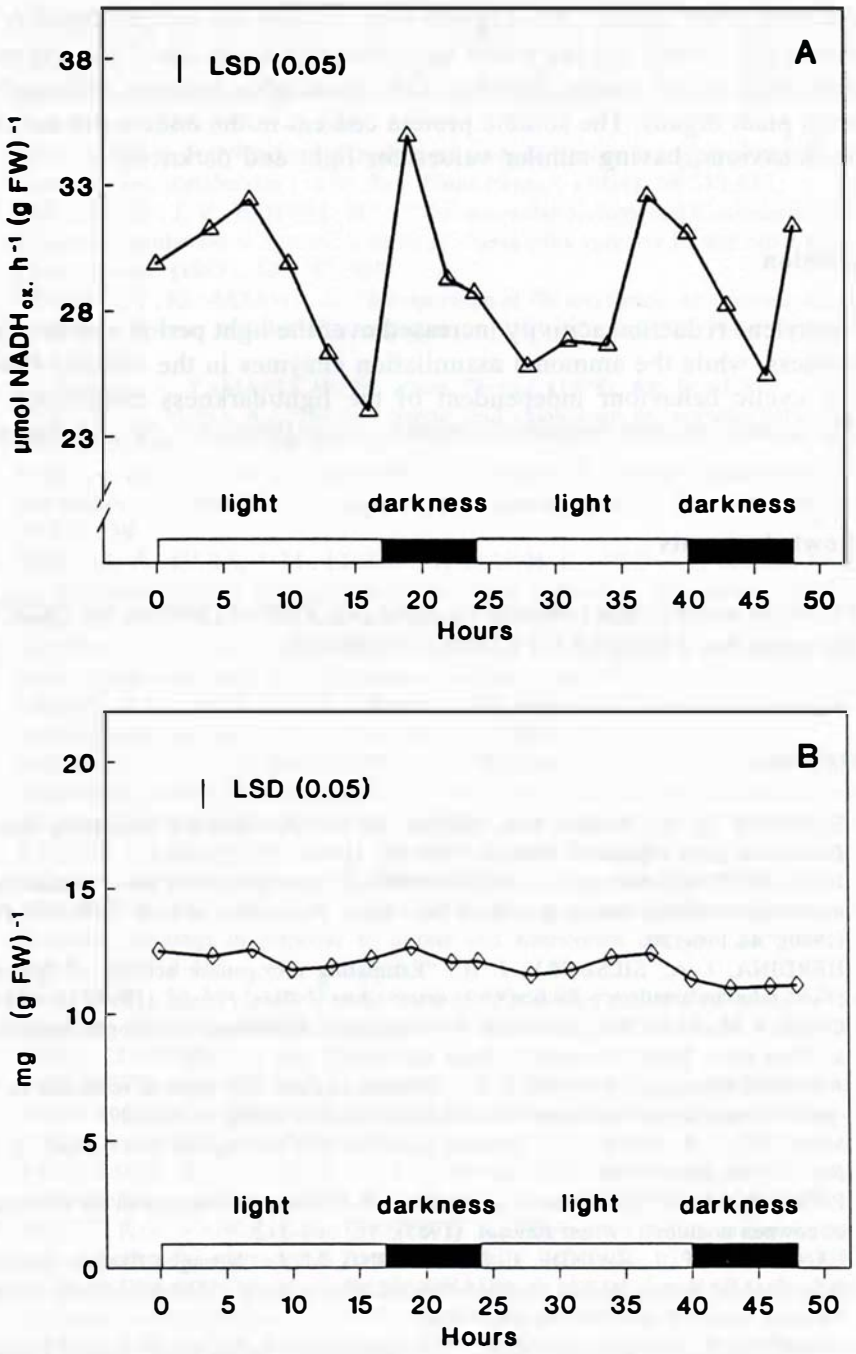

Fig. 3.—Diurnal variation on GDH (A) (imol $NADH_{ox}$ h^{-1} g FW^{-1}) and soluble protein concentration (B) (mg g FW^{-1}) in nodules of *V. faba* plants inoculated by *R. leguminosarum* GRA19.

for different plant organs (30). Legume root nodules are morphologically and biochemically unique organs which have enzymes (nodulines) not expressed in other parts of the plants; therefore GS conceivably behaves differently in different plant organs. The soluble protein content in the nodule did not show cyclic behaviour, having similar values for light and darkness.

Conclusion

Acetylene reduction acitivity increased over the light period and decreased in darkness, while the ammonia assimilation enzymes in the nodules showed a 16 h cyclic behaviour independent of the light/darkness conditions. The soluble protein content remained constant, regardless of light and darkness.

Acknowledgements

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