Diurnal Variation in the Functioning of Cowpea Nodules

ROSS M. RAINBIRD, CRAIG A. ATKINS, AND JOHN S. PATE
Department of Botany, University of Western Australia, Nedlands, Western Australia, 6009 Australia

ABSTRACT

Nitrogenase (EC 1.7.99.2) activity of nodules of cowpeas (Vigna unguiculata [L.] Walp), measured under conditions of a 12-hour day at 30°C and 800 to 1,000 microeinstems per square meter per second (photosynthetically active radiation) and a 12-hour night at 20°C, showed a marked diurnal variation with the total electron flux through the enzyme at night being 60% of that in the photoperiod. This diurnal pattern was, however, due to changes in nitrogen evolution. The rate of nitrogen fixation, measured by short-term 15N2 assimilation or estimated from the difference in nitrogen evolution in air or Ar:O2 (90:10; v/v), showed no diurnal variation. Carbon dioxide released from nodules showed a diurnal variation synchronized with that of nitrogenase functioning and, as a consequence, the apparent 'respiratory cost' of nitrogen fixation in the photoperiod was almost double that at night (9.74 ± 0.38 versus 5.70 ± 0.90 moles CO2 evolved per mole N2 fixed). Separate carbon and nitrogen balances constructed for nodules during the photoperiod and dark period showed that, at night, nodule functioning required up to 40% less carbohydrate to achieve the same level of nitrogen fixation as during the photoperiod (2.4 versus 1.4 moles hexose per mole N2 fixed).

Stored reserves of nonstructural carbohydrate of the nodule only partly satisfied the requirement for carbon at night, and fixation was dependent on continued import of translocated assimilates at all times. Measurements of the soluble nitrogen pools of the nodule together with 15N studies indicated that, both during the day and night, nitrogenous products of fixation were effectively translocated to all organs of the host plant despite low rates of transpiration at night. Reduced fluxes of water through the plant at night were apparently counteracted by increased concentration of nitrogen, especially as ureides, in the xylem stream.

In addition to showing long-term changes in the economy of nodule functioning throughout development (7, 15), many legume symbioses exhibit pronounced diurnal fluctuations in nitrogenase activity as assayed by the reduction of acetylene (2, 6, 11, 16, 19). These fluctuations have been interpreted in relation to changes in the respiration of the nodulated root (7, 11), the carbohydrate status of nodules (5, 11, 19), and the accumulation and export of nitrogenous solutes by the nodules (11). Conversely, where significant diurnal variations in acetylene reduction were not observed, the symbioses studied were regarded not to be currently limited by availability of carbohydrate from the host plant (6, 21).

Although acetylene reduction assays may well offer a reliable measure of overall nitrogenase activity, and thus reflect the availability and rate of utilization of oxidizable substrate in the nodule, they fail to provide information on how electron flow through the enzyme complex in vivo is partitioned between nitrogen and proton reduction (17). Marked diurnal fluctuation in this partitioning could well be a significant factor in the daily economy of the nodule, particularly where times of low availability of carbohydrate to coincide with periods of more intense nitrogen fixation relative to H2 evolution. This paper examines such a possibility in a symbiosis (Vigna unguiculata: Rhizobium strain 176A27) lacking an uptake hydrogenase. The experimental procedure used involved measurements of diurnal fluctuation in H2 evolution in air or Ar:O2 and of 15N2 fixation in air to estimate separate rates of nitrogen and proton reduction, and diurnal variations in nitrogenase function were then related to overall daily operation of the plant's nodules under a regime of fluctuating temperature and illumination.

MATERIALS AND METHODS

Plant Material. Surface-sterilized seed of cowpea (Vigna unguiculata [L.] Walp. cv Caloona) was inoculated with Rhizobium strain 176A27 (Nitragin Co.). Ten days after sowing in sand, groups of five seedlings were transplanted to 3.5-L containers of N-free liquid culture (10) maintained in a naturally lit glasshouse. Fifteen days after establishment, the liquid cultures were transferred to a controlled environment cabinet with a 12-h day at 30°C and 800 to 1,000 μE/m2·s (PAR) and a 12-h night at 20°C. After 5 to 10 days growth in the cabinet, the lids of the culture containers were sealed at the edge and around the stem of each of the five plants with Terostat VII (Teroson Gmbh, Heidelberg, F. R. G.), and a moistened stream of either CO2-free air or CO2-free Ar:O2 (80%:20%, v/v) was passed through the enclosed gas space above the liquid culture at a flow rate of 50 to 100 cm3/min. Plants were in mid vegetative growth and were fixing N at near maximum rates (9) when used.

Measurement of Hydrogen Evolution and Estimation of Nitrogen Fixation. Three sealed water cultures (total of 15 plants) gassed with CO2-free air and three similar cultures gassed with CO2-free Ar:O2 were connected to an automated analysis system which sequentially sampled the effluent gas stream from each over a 6-min period. H2 concentration of the gas stream was measured using a gas liquid chromatograph equipped with a 2-m column of molecular sieve 5A (100-120 mesh; Waters Associates) and a thermal conductivity detector. The chromatograph incorporated a motorized gas sampling valve which was controlled to sample 1.4 cm3 at times coinciding with the sequential switching of the effluent gas streams from the six cultures. Measurement of H2 evolution from each of the six cultures was thus made every 36 min for a period of 33 to 69 h. Values were integrated on a 3-h basis.

In the absence of hydrogenase reactions which utilize H2, the rate of H2 evolution into Ar:O2 may be regarded as a measure of the total flow of electrons to nitrogenase functioning (17). In the present study, H2 evolution in air and in Ar:O2 was therefore used to estimate the rate of N2 fixation for each 3-h period of the day and night. In air, the decrease in H2 evolution was presumed to be

1 Supported by funds from a University of Western Australia General Development Grant and from the Wheat Industry Research Council of Australia.

2 Present address: Central Research and Development Department, Experimental Station, Dupont, Wilmington, DE 19898.
due to electron flow to N₂ reduction, and, assuming that reduction of N₂ required three electron pairs compared to one for H₂ production, the following relationship was used to estimate the rate of N₂ fixation.

\[
\text{N}_2 \text{ fixation} = \frac{\text{H}_2 \text{ evolution in } \text{ArO}_2 - \text{H}_2 \text{ evolution in air}}{3}
\]  

Measurement of CO₂ Evolution. The nodulated zone on the primary root of intact plants cultured as above was enclosed in a small (volume 10 cm³) plastic cuvette as detailed previously (10), and the CO₂ evolved into a CO₂-free air or CO₂-free ArO₂ (80%:20%, v/v) stream was measured using an IR gas analyzer. The effluent stream from six such cuvettes was sampled sequentially as described above for H₂ assay. The total CO₂ evolved into the gas stream flowing through the cuvettes was due to respiration of both nodules and the segment of supporting root which was also enclosed. The separate respiratory contribution of the nodules in each time interval of the study was estimated by subtracting from the total CO₂ efflux the respiration rate of the root segment measured immediately following detachment of the nodules. As described previously (10), the small cuvettes enclosed all the nodules on the root system of a plant which was nodulated only on the primary root.

Plant Harvest and Analysis. Plants cultivated and maintained under the same conditions as those used for measurement of gas exchange were harvested at 4- to 5-hour intervals during the study period for assay of ureides (8), soluble amino nitrogen (23), and nonstructural carbohydrate (3) in nodules. Dry weight and total N, measured by Kjeldahl digestion, were determined for nodules and for whole plants at the beginning and end (after 33 or 69 h) of periods of measurement of gas exchange.

Transpiration. The rate of water loss from liquid cultures identical to those used for measurement of gas exchange was estimated gravimetrically or as the change in volume of the culture solution at varying periods (3-6 h) throughout the photoperiod and dark period of diurnal studies.

Xylem Sap Collection. Five to 10 plants were decapitated at 3-hour intervals throughout the study period, and root bleeding (xylem) sap was collected for assay of ureide (8) and total amino acid content (1).

Measurement of ¹⁵N₂ Fixation. Cuvettes were attached to the nodulated root regions of two plants as described above and connected in series to a closed gas exchange system incorporating the two cuvettes, a pump, and a small soda lime CO₂ absorber followed by a water bubbler. The gas within the system (260 cm³) was cycled at a flow of 80 cm³/min and, following a period of equilibration, 50 cm² was removed and, simultaneously, 50 cm² ¹⁵N₂ (95 atom % excess ¹⁵N) was added. After 2 h, plants were separated into component organs and dried. Samples of the dry, finely milled material were taken for ¹⁵N assay by MS following Kjeldahl digestion and oxidation of the resultant ammonia by the Rittenberg procedure (4).

RESULTS

Diurnal Variation in Nitrogenase Functioning. H₂ evolution, both in the presence and absence of N₂ in the gas stream surrounding nodulated roots, showed a marked diurnal variation with higher rates maintained during the warm photoperiod compared to the cooler dark period (Fig. 1). The separate components of nitrogenase functioning in air, H₂ evolution, and N₂ fixation, estimated (Fig. 2) from the data of Figure 1 using Eq. 1, above, indicated that the diurnal variation in nitrogenase functioning (H₂ evolution under ArO₂ in Fig. 1) was due almost entirely to changes in the rate of H₂ evolution. In contrast, the rate of N₂ fixation was not significantly different between the day and night despite the 10°C change in temperature. This was confirmed by measurement of ¹⁵N₂ uptake over 2 h in mid-photoperiod (1100-1300 h, 30°C) or mid-dark period (2300-0100 h, 20°C); the mean rates (±se) were 1.13 ± 0.19 and 1.21 ± 0.16 μmol ¹⁵N₂ fixed/plant h, respectively (calculated from the data of Table I). The assumption that the difference between H₂ evolution in ArO₂ and in air was equivalent to the allocation of electrons to N₂ reduction in air was tested by comparing estimates based on

![Fig. 1. Diurnal variation in rate of H₂ evolution by intact nodulated root systems of cowpea into a flowing stream of CO₂-free air or CO₂-free 80% Ar:20% O₂ (v/v). The stippled area represents night periods. Bars indicate ± se of the mean from three replicates.](image-url)

![Fig. 2. Diurnal variation in rate of H₂ evolution and estimated N₂ fixation by intact nodulated root systems of cowpea. N₂ fixation was calculated from the data of Figure 1 using Eq. 1. The stippled area represents night periods. Bars indicate ± se of the mean from three replicates.](image-url)
integrated measurements of $H_2$ evolution in Ar:O₂ and air over 69-h periods with direct measurements by Kjeldahl analysis of N₂ fixed over the same period. The mean values from four separate experiments were 2.48 ± 0.16 μmol N₂ fixed/plant-h based on measurements of $H_2$ evolution and 2.51 ± 0.22 μmol N₂ fixed/plant-h from direct assay of fixed N during the period indicating no significant difference between the two estimates.

**Diurnal Variation in Respiration and Carbohydrate Content of Nodules.** Under the conditions of diurnal change in illumination and temperature employed, the efflux of CO₂ by attached nodules enclosed in cuvettes showed a marked rhythm, with rates of respiration during the 30°C photoperiod being double those during most of the 20°C night period (Fig. 3). Nonstructural carbohydrate of the nodule, comprising soluble sugars and starch, also showed a diurnal variation (Fig. 3) falling from a level around 19 μmol sucrose eq/plant during the photoperiod to around μmol sucrose eq/plant at night. The pattern of change was, however, not the same as that of CO₂ efflux or of nitrogenase functioning under these conditions (Figs. 1 and 2).

**Diurnal Variation in Nitrogen Export by Nodules.** The level and pattern of recovery of $^{15}$N following supply of $^{15}$N₂ to nodulated plants indicated that fixation and export of newly fixed N from nodules occurred at the same rate at night as during the photoperiod (Table I). In each case, more than 80% of the $^{15}$N was translocated out of the nodules with the distribution of label to organs between the two labeling periods being almost identical (Table I). Consistent with this pattern of equally intense export of N day and night, there was no marked diurnal variation in the soluble amino acid- or ureide-N pools of the nodule (Fig. 3).

As might be expected, transpiration during the night was relatively low compared to the photoperiod (Fig. 4). However, the concentration of nitrogenous solutes, and especially of ureides, in xylem sap was markedly elevated at night (Fig. 4).

**DISCUSSION**

The validity of using the difference between $H_2$ evolution in the presence and absence of N₂ to estimate the rate of N₂ fixation rests on the assumptions that the symbiosis under study lacked an uptake hydrogenase (17) and that the total electron flux to its nitrogenase was the same in air as in Ar:O₂. Attempts to demonstrate $H_2$ uptake by intact attached nodulated roots or by detached nodules of the symbiosis used in this study were consistently negative indicating that the first of these assumptions was probably valid, just as has been shown previously for an association of the same *Rhizobium* strain (176A27) with a different cowpea host (18).

In relation to the second assumption, close agreement was found between integrated estimates of N₂ fixation based on $H_2$ evolution of attached nodules in air or Ar:O₂ and measured gain in N of whole parent plants as determined by Kjeldahl analysis. Furthermore, these estimates of day/night rates of N₂ fixation (Fig. 2), determined indirectly from $H_2$ evolution, showed a similar lack of response to a 10°C change in temperature as did the day/night assays of $^{15}$N₂ fixation (Table I).

The pattern of diurnal change of CO₂ release from nodules (Fig. 3) was similar to that of total electron flux to nitrogenase (Fig. 1), resulting in a relatively constant relationship between respiration and nitrogenase function. The average value for CO₂ evolved/2e⁻ utilized by nitrogenase during the photoperiod was 1.69 ± 0.09 and for the night period, 1.39 ± 0.18. The relationship between CO₂ release and N₂ fixation, however, showed a marked
Fig. 4. Diurnal variation in the concentration of amino-N (amino acids, amides, and ammonia) and ureide-N (allantoin and allantoic acid) of root bleeding xylem sap and in transpiration rate of cowpea plants. The stippled area represents the night period.

diurnal variation with almost twice the level of respiration occurring per unit N2 fixed during the photoperiod than at night. The mean values were 9.74 ± 0.38 and 5.70 ± 0.90 mol CO2 evolved/mol N2 fixed during the photoperiod and night period, respectively. Similarly, an earlier study of the diurnal functioning of pea (Pisum sativum) nodules found high rates of N2 fixation maintained during cool nights despite a decline in the respiratory activity of the whole root system compared to the photoperiod (11).

The levels of nonstructural carbohydrate (starch, sugar) in nodules varied during the study period, and although the diurnal pattern of change was not synchronized with that of CO2 release (Fig. 3), or that of nitrogenase functioning (Figs. 1 and 2), nodules contained about 4 μmol sucrose eq/plant less at night than during the photoperiod (Fig. 3). Complete oxidation of this amount of sugar in respiration would yield some 48 μmol CO2. Inasmuch as the total CO2 efflux from the nodules during the night period was more than 100 μmol, nodules continued to import a significant amount of translocated carbohydrate at night, albeit at a reduced rate compared with the day. Thus, available stored reserves of the nodule only partially satisfied the demand for carbon during the night period.

If, as indicated in Figure 2, N2 fixation continued at high rates during the night, then nitrogenous solutes would be expected to accumulate in the nodule (11) especially with reduced water movement in xylem (Fig. 4). This, however, did not occur to a significant extent, and only small fluctuations in ureide and amino acid pools of the nodule were observed during the diurnal period (Fig. 3). The results of the 15N2 experiments supported these observations. Similar total amounts of 15N were recovered in plants fed in the day and at night (Table I) with, in each case, more than 80% of the fixed label translocated out of the nodule. Moreover, the distribution of 15N between host organs was almost identical day and night (Table I), indicating that the pattern and rates of solute transfer were similar despite a sharp decline in transpiration rate at night (Fig. 4) and coincident rises in the N content of root bleeding xylem exudate (Fig. 4). Thus, it appears that continued translocation of N to shoots at night was maintained, contrasting reduced fluxes of water through xylem by greatly increased concentrations of N in the moving stream. Similar studies with pea (11) also concluded that high rates of N export from nodules occurred at night although, in this case, a greater proportion of fixed N accumulated nightly in the nodules.

Using values for N2 fixation and H2 evolution (Fig. 2), CO2 efflux (Fig. 3), changes in nonstructural carbohydrate levels (Fig. 3), and pool sizes of nitrogenous solutes in nodules (Fig. 3), together with the ratio of C/N in exported solutes (from data of Fig. 4), separate C and N balances for nodules during the photoperiod and during the dark period were constructed. Using the data from 1300 to 1400 h of the photoperiod and from 0100 to 0200 h of the dark period as examples of the extremes in diurnal functioning in this study, the calculated balances are depicted in Figure 5, A and B. The thickness of arrows for C and N are roughly equivalent to the quantities involved (expressed in units of weight), and expressed in terms of the fixation of 100 g N2 (the actual rate of fixation in each case was 56 μg N2/h · plant).

Small gains were made in the carbohydrate and nitrogenous solute pools of the nodule in the photoperiod, while a small loss of soluble N occurred in the night period. In both cases, the nitrogenous products of nitrogenase activity were depicted as being effectively exported from the nodule. Clearly, at night, nodule functioning showed a considerably greater economy of C utilization. The 100 g N2 fixed required 586 g C as imported sugar in the photoperiod compared to 352 g C, or around 40% less, in the night period (Fig. 5).

On the basis of H2 production requiring 4ATP and 2e−/mol H2 and N2 reduction requiring 12ATP and 6e−/mol N2 (14), energy demands of nitrogenase, in terms of the two periods shown in Figure 5, might be expected to be greater by about 35% during the day than in the night period. Assuming oxidative phosphorylation to operate with a P/2e− = 3 at all times in the nodule, nitrogenase function (N2 fixation and H2 evolution) would theoretically account for 309 g C as CO2 evolved in Figure 5A (day) and 229 g C in Figure 5B (night). The difference between these two estimates (80 g C) is only 35% of the difference between observed CO2 evolution at the two times (227 g C) indicating that processes other than H2 evolution could have also contributed to utilization of the 'extra' energy apparently made available in the photoperiod. Alternatively, the nature of respiratory pathways in the nodule could have varied on a diurnal basis, resulting in differing efficiency for coupling of ATP and reductant generation to oxidation of the available substrates, or there could have been marked diurnal variation in the activity of C conservation mechanisms such as CO2 fixation (14).

Clearly, diurnal variation of total electron flux to nitrogenase, as has, for example, been observed in C3H2 reduction assays (for review, see 12), might, at least in part, be due to changes in H2 evolution rather than result from fluctuating N2 fixation. The findings are obviously of some significance in interpreting long-term studies of N2 fixation in the field as well as conclusions about the C and N economy of nodules or nodulated root systems based on integrated C3H2 reduction assays. Previous studies based on continuous collection of CO2 and direct measurement of N2 fixation by N increment in the plant (9, 10, 14, 15) would have accommodated the effect of diurnal variations in respiration, in nitrogenase activity, and in the partitioning of electrons between N2 and proton reduction on the C and N economy of nodules.

Although the present data do not identify the factor or factors in nodule functioning which regulate the efficiency of N2 fixation, studies with isolated, purified nitrogenase have indicated that, in
soybean was due to an effect of temperature rather than illumination, and changes in temperature could modulate many of these possible regulatory factors in vivo through altered respiration rate or effects on other energy-requiring reactions of the nodule. It is of interest that Walker et al. (22) have recently found that the ratio of H₂ produced to N₂ fixed by Azotobacter chroococcum was sensitive to O₂ level.

Acknowledgments—The skilled technical assistance of P. Sanford, E. Rasins, and M. Lucks is acknowledged. Thanks are due to Dr. M. B. Peoples for analyses of nonstructural carbohydrates.

LITERATURE CITED