Plant Physiol. (1983) 73, 392–394
0032-0889/83/73/0392/03/$00.50/0

Effect of Temperature on Nitrogenase Functioning in Cowpea Nodules[1]

Received for publication March 14, 1983

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

ABSTRACT

Nitrogenase (EC 1.7.99.2) activity of a cowpea (*Vigna unguiculata* (L.) Walp cv Caloona) symbiosis formed with a *Rhizobium* strain (176A27) lacking uptake hydrogenase and maintained under conditions of a 12-hour day at an air temperature of 30°C (800–1000 microeinsteins per square meter per second) and a 12-hour night at an air temperature of 20°C showed a marked diurnal variation in ratio of nitrogen fixed to hydrogen evolved. As little as 0.3 micromole nitrogen was fixed per micromole hydrogen evolved in the photoperiod *versus* up to 0.6 in the dark period. In plants maintained under the same diurnal illumination regime but at constant (day and night) air temperature (30°C), this difference was abolished and a relatively constant ratio of nitrogen fixed to hydrogen evolved (around 0.3 micromole per micromole) was observed day and night. Exposure of nodulated roots to a range of temperatures maintained for 2 hours in a single photoperiod indicated that, whereas hydrogen evolution increased with increasing temperature from 15°C to a maximum around 35°C, nitrogen fixation was largely unaffected over this temperature range. Both functions of the enzyme declined sharply at temperatures above 38°C. A similar general response of nitrogen fixation to root temperature was observed in glasshouse-grown, sand-cultured plants maintained under a range of temperatures (from 15 to 35°C) for a 14-day period in mid vegetative growth. The effect of temperature on the proportion of electrons allocated to proton reduction compared with nitrogen reduction showed a linearly increasing relationship (correlation coefficient = 0.96) between 15°C and 47°C.

Study of the C and N economy of nodules of cowpea (*Vigna unguiculata* L. [Walp]) formed with a *Rhizobium* strain lacking uptake hydrogenase (176A27) revealed a marked diurnal variation in efficiency of functioning (5). The apparent 'respiratory cost' of N_2 fixation in the photoperiod was almost double that at night, although, despite a 10°C temperature difference over the diurnal cycle, the rate of N_2 fixation remained constant (5). Variation in nitrogenase activity was found to be the result of a much higher rate of H_2 evolution during the day than during the night (5). This paper examines the relative activities of proton and N_2 reduction by nitrogenase using intact nodulated plants under conditions of long term diurnal and short term changes in temperature.

MATERIALS AND METHODS

Effectively nodulated (*Rhizobium* strain 176A27), 30- to 35-d-old plants of cowpea (*Vigna unguiculata* [L.] Walp cv Caloona) were grown in N-free liquid culture and their root systems enclosed for measurement of H_2 evolution into a moving gas stream of either CO_2-free air or CO_2-free Ar:O_2 (80:20; v/v) as described previously (5). Each culture comprised five plants.

For diurnal studies, cultures were maintained at the day or night temperatures specified by varying the air temperature of the growth cabinet used (5). Measurements of H_2 evolution were made from each of six cultures (three in air and three in Ar:O_2) every 36 min (5) and integrated on a 3-h basis. For shorter term temperature studies, single cultures (each containing five plants and previously raised at a constant day/night temperature of

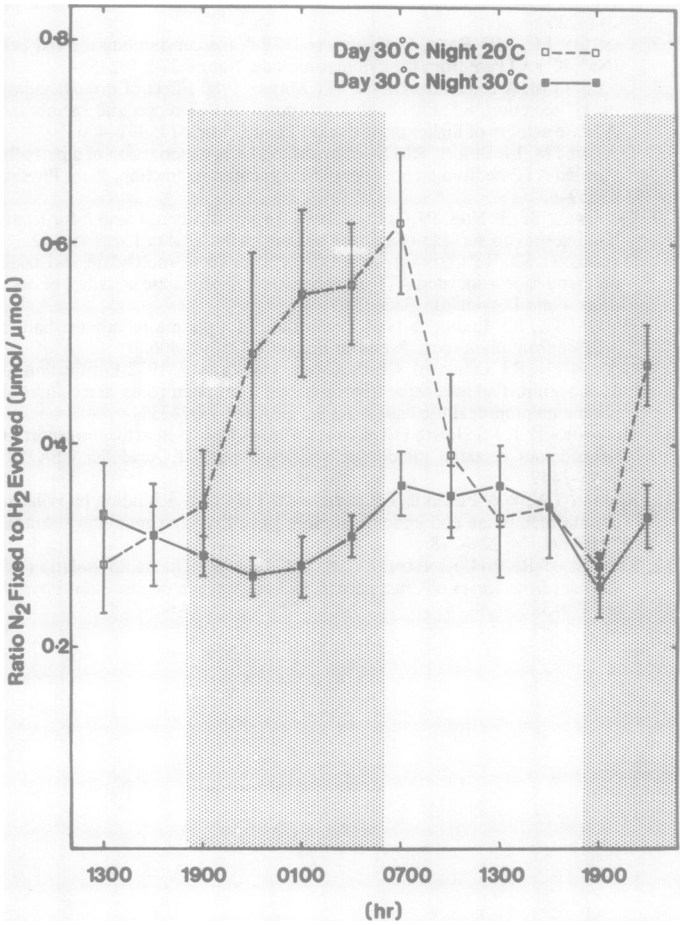

FIG. 1. Diurnal variation in ratio of N_2 fixed to H_2 evolved by intact nodulated root systems of cowpea plants maintained under 30°C day and 20°C night temperatures or with a constant (30°C) temperature day and night. N_2 fixed was estimated from measurements of H_2 evolution into air and Ar:O_2 as described in the text. Bars indicate ± SE of mean values (n = 3).

[1] Supported by funds from a University of Western Australia General Development Grant, the Wheat Industry Research Council of Australia, and the Australian Research Grants Scheme.

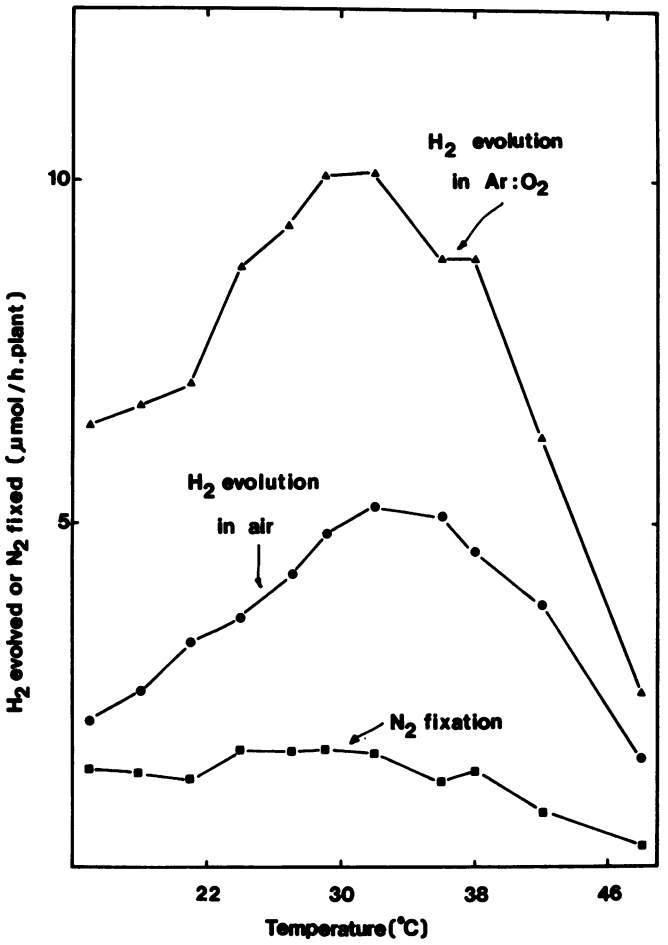

FIG. 2. Effect of short term changes of temperature around the nodulated root systems of cowpea plants on H_2 evolution into air or into $Ar:O_2$. N_2 fixation was estimated as for Figure 1.

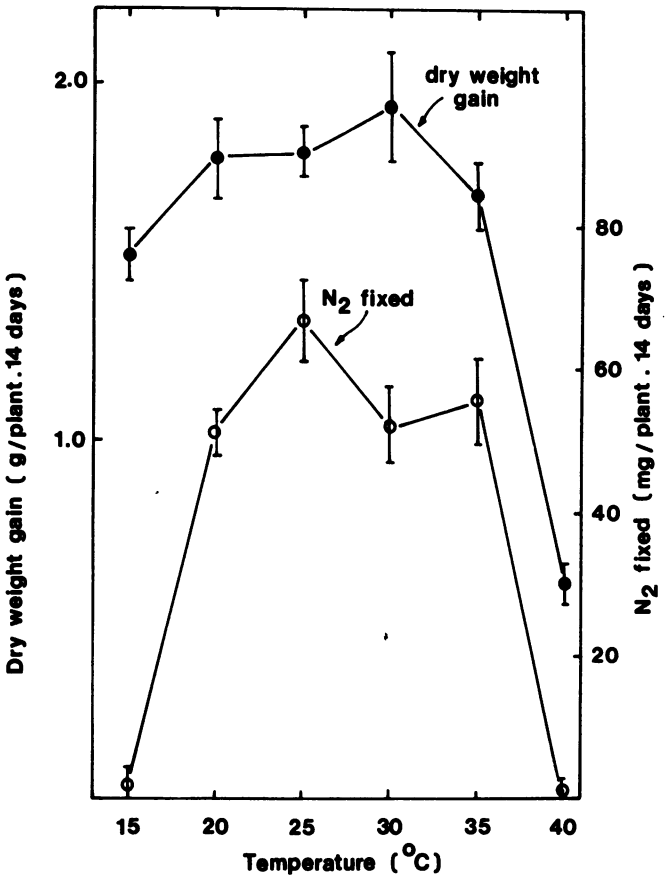

FIG. 3. Effect of temperature around the nodulated root system of cowpea plants on increase in dry weight and N fixed during a 14-d period in mid vegetative growth (28–42 d after sowing). The temperature of the root system was constant day and night while the shoots of plants in all treatments were maintained in a 30°C day/20°C night diurnal regime. Bars indicate ± SE of mean values ($n = 4$).

25°C) with root systems enclosed (5) were placed in a water bath, and the temperature of the culture solution surrounding the root system was varied from 15 to 48°C by heating or cooling the bath water. H_2 evolution into either air or $Ar:O_2$ was measured as described above following equilibration (required 1–2 h) of the culture solution to the bath temperature. The rate of N_2 fixation was estimated as

$$N_2 \text{ fixation} = \frac{H_2 \text{ evolution in } Ar:O_2 - H_2 \text{ evolution in air}}{3}$$

Assumptions underlying this estimate and evidence indicating the validity of these assumptions for the symbiosis studied here have been presented earlier (5).

Similar effectively nodulated plants were also grown in N-free sand culture (6) in a naturally lighted glasshouse with a maximum day temperature of 30°C and a minimum night temperature of 20°C. After 28 d, groups of four pots (5 L), each containing five plants, were transferred to a series of water baths to maintain nodulated roots at a constant temperature of 15, 20, 25, 30, 35, or 40°C (±1°C). The shoots remained under the normal diurnal regime of the glasshouse. After 14 d, plants were harvested, and dry weight and N gain over the period of treatment were determined (6) for each pot.

RESULTS AND DISCUSSION

A previous study (5) concluded that variation in overall nitrogenase activity was due to changing allocation of electrons to H_2 evolution rather than to fluctuation of N_2 reduction over the diurnal regime used (30°C day/20°C night; as in Fig. 1). The data of Figure 1 indicate that temperature change, rather than illumination or fluctuation in some factor occasioned by a diurnal pattern of illumination was the cause of variation in the relative activities of N_2 and proton reduction by nitrogenase. The effect of temperature change was then examined more closely by measuring H_2 evolution into $Ar:O_2$ or air at a range of root temperatures (15–48°C) maintained for 2 h within a single photoperiod. In both atmospheres, H_2 evolution increased with increasing temperature from 15°C to around 30°C and declined sharply above 38°C (Fig. 2). Rates of N_2 fixation, estimated from the difference in H_2 production in the two atmospheres (see equation in "Materials and Methods") showed that, unlike H_2 evolution, this function of nitrogenase was relatively insensitive to temperature change from 15°C to 38°C (Fig. 2). A similar conclusion may be drawn from a longer term study (Fig. 3) in which fixed N was measured in plants whose nodulated root systems were maintained at a series of constant temperatures for a 14-d period in mid vegetative growth (28–42 d after sowing). Although temperatures below 20°C impaired fixation in the longer term studies, while not appreciably affecting nitrogenase activity in the short term studies, both sets of data showed relatively constant rates of fixation over the range from 20°C to 35°C (*cf.* Figs. 2 and 3).

The differential response of proton and N_2 reduction to temperature (Fig. 2) resulted in a linear relationship ($r \times y = 0.96$) between temperature and the ratio of electrons partitioned to the

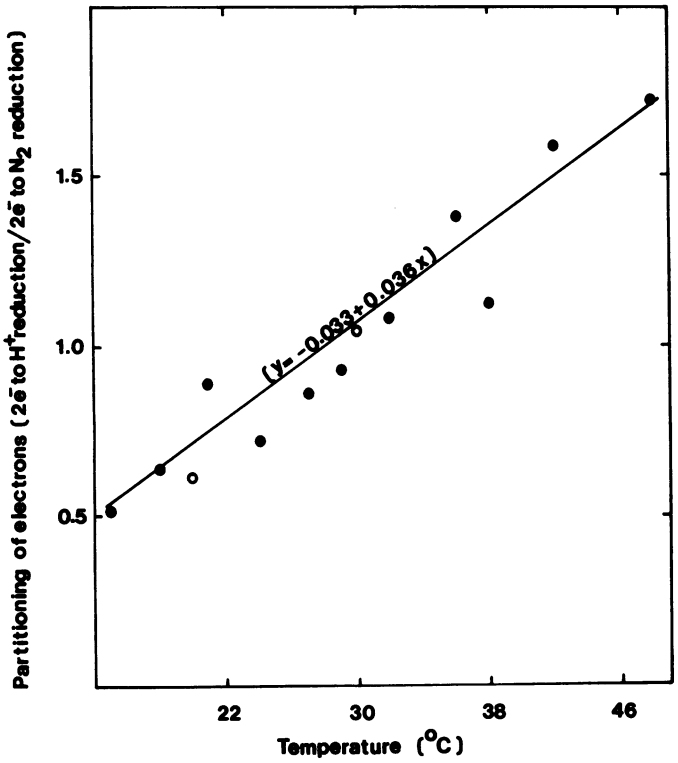

FIG. 4. Relationship between temperature and the allocation of electrons to proton or N_2 reduction by the nodulated root system of cowpea plants. Closed symbols are from the data of Figure 2; open symbols are the 30°C day/20°C night extremes of the diurnal study in Figure 1.

two reducible substrates (see Fig. 4) with around one-third of the total electron flux allocated to proton reduction at 15°C, rising with temperature to more than two thirds at 48°C (Fig. 4). Values at 30°C and 20°C from the diurnal experiment (Fig. 1) were also found to fit this relationship closely, with 0.3 μmol H_2/μmol N_2 in the 30°C photoperiod and 0.6 μmol H_2/μmol N_2 in the 20°C night period (see Fig. 4). Studies with isolated and purified nitrogenase proteins have shown that a number of factors (pH, ATP concentration, ADP/ATP ratio, flux of electrons through the Mo-Fe protein), any of which might be modulated *in vivo* by temperature, can affect the relative distribution of electrons to proton and N_2 reduction (1, 4). The ratio of the two substrates utilized also depends on the ratio of the Fe-protein and Mo-Fe protein components of nitrogenase and Walker *et al.* (7) have suggested that such a means of regulation occurs in intact *Azotobacter* cells. Interestingly, Dart and Day (2) noted previously that temperature seemed to affect the ratio of H_2 evolved to acetylene reduced by soybean and cowpea nodules. They attached little significance to the observation as the two measurements had been made on different sets of plant material.

Whereas any or all of the above factors might contribute to regulation of nitrogenase in the nodule, the differential response of the two reducing functions of the enzyme which results in the changing ratio of electron allocation may be interpreted more simply. First, H_2 is a specific and competitive inhibitor of N_2 reduction by isolated nitrogenase (3). Thus, with increasing H_2 evolution as temperature increases, N_2 reduction may be progressively inhibited. In this way, the response of N_2 reduction to temperature is likely to be masked, and manifest as a relatively constant level of N_2 fixation but an increasing level of H_2 evolution. A second 'simplistic' explanation could be that the more thermostable process, N_2 reduction, is limited in rate by a 'physical' factor or factors while H_2 evolution is limited by a more temperature-sensitive, 'chemical' step or steps in the overall reaction.

Because of the current interest in improving both rate and efficiency of biological N_2-fixing associations, detailed studies of the nature of possible limiting factors for the separate functions of nitrogenase would seem to be indicated. Of particular relevance would be experiments using isolated bacteroids and purified nitrogenase proteins to determine whether the differential response to temperature described in this paper is an intrinsic property of the enzyme or is a consequence of the structure and functioning of the nodule.

Acknowledgments—The authors are grateful for the skilled technical assistance of P. Sanford and D. Waldie.

LITERATURE CITED

1. BURRIS RH, DJ ARP, RV HAGEMAN, JP HOUCHINS, WJ SWEET, M TSO 1981 Mechanism of nitrogenase action. *In* AH Gibson, WE Newton, eds, Current Perspectives in Nitrogen Fixation. Griffin Press, Australia, pp 56–66
2. DART PJ, JM DAY 1971 Effects of incubation temperature and oxygen tension on nitrogenase activity of legume root nodules. Plant Soil (Special Volume) 171: 167–184
3. HARDY RWF, RC BURNS, JT STASNY, GW PARSHALL 1975 The nitrogenase reaction. *In* WDP Stewart, ed, Nitrogen Fixation by Free-living Microorganisms. Cambridge University Press, London, pp 351–376
4. MORTENSON LE, RNF THORNELY 1979 Structure and function of nitrogenase. Annu Rev Biochem 48: 387–418
5. RAINBIRD RM, CA ATKINS, JS PATE 1983 Diurnal variation in the functioning of cowpea nodules. Plant Physiol 72: 308–312
6. RAINBIRD RM, CA ATKINS, JS PATE 1983 Significance of hydrogen evolution in the carbon and nitrogen economy of nodulated cowpea. Plant Physiol 71: 122–127
7. WALKER CC, CDP PARTRIDGE, MG YATES 1981 The effect of nutrient limitation on hydrogen production by nitrogenase in continuous cultures of *Azotobacter chroococcum*. J Gen Microbiol 124: 317–327