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Simultaneous Measurement of Acetylene Reduction and Respiratory Gas Exchange of Attached Root Nodules[1]

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ABSTRACT

A method was developed for the simultaneous measurement of acetylene reduction, carbon dioxide evolution and oxygen uptake by individual root nodules of intact nitrogen-fixing plants (*Alnus rubra* Bong.). The nodules were enclosed in a temperature-controlled leak-tight cuvette. Assay gas mixtures were passed through the cuvette at a constant, known flow rate and gas exchange was measured by the difference between inlet and outlet gas compositions. Gas concentrations were assayed by a combination of an automated gas chromatograph and a programmable electronic integrator. Carbon dioxide and ethylene evolution were determined with a coefficient of variation which was less than 2%, whereas the coefficient of variation for oxygen uptake measurements was less than 5%. Nodules subjected to repeated removal from and reinsertion into the cuvette and to long exposures of 10% v/v acetylene showed no irreversible decline in respiration or acetylene reduction. This system offers long-term stability and freedom from disturbance artifacts plus the ability to monitor continuously, rapidly and specifically the changes in root nodule activity caused by environmental perturbation.

Nitrogen fixation is an energy-intensive process, requiring large inputs of reductant and ATP. In plants with nitrogen-fixing root nodules, the energy cost of nitrogen fixation and associated metabolism can be measured by the ratio between nodule respiration and nitrogenase activity. Many investigations of the respiration associated with nitrogen fixation have involved measurements of CO_2 evolution from entire nodulated root systems (5–7). This approach has resulted in excellent measurements of long-term effects and of total root respiratory costs and minimized artifacts due to disturbance. But nodule respiration was not separated from root respiration, except by indirect means.

Studies of excised nodules have provided the needed separation but at the risk of greater disturbance and reduced experimental time span. Under favorable conditions, the effects of detachment are small and nitrogenase activity is maintained for many hours (8). In other cases, activity decreases rapidly after detachment. Thus, experiments with many treatments, such as temperature response curves, must be done rapidly or with many replicate samples. Transient effects and variation among samples complicate the interpretation of such results. The influence of host plant activities, such as changes in photosynthesis, cannot be studied directly.

To overcome these limitations, we measured nodule respiration by enclosing nodules in a cuvette while still attached to the root system of the actively growing plant. Fluxes of CO_2, ethylene, and O_2 were simultaneously and rapidly determined, permitting detailed investigation of the interaction of nitrogenase activity and respiration.

Three main concerns were addressed in the design of this method. First, it was necessary to grow the plants so that the nodules were readily accessible and could be enclosed in the cuvette without damage or change in metabolism. This was accomplished by growing plants with their entire root systems suspended in a nutrient solution mist. Second, it was desirable to use an open, flow-through gas exchange system so that steady-state conditions could be obtained. This also minimized the accumulation of toxic metabolic products, as from fermentative reactions. Third, we sought to simultaneously measure CO_2 and ethylene evolution and O_2 uptake, to minimize analysis time and experimental error.

The purpose of this paper is to describe this assay system, illustrate its function and establish the validity and limits of resolution and accuracy of the measurements made with it.

MATERIALS AND METHODS

Plant Material. Seeds of *Alnus rubra* Bong. (Clatsop County, OR) were germinated in sand flats in a growth chamber. When the seedlings were approximately 5 cm tall, they were transferred to modified aeroponics boxes (9), containing one-fourth strength N-free Hoagland solution supplemented by iron (II) phosphate, crushed oyster shells and 2 mM potassium nitrate. The boxes were in a greenhouse with supplemental light provided by low pressure sodium vapor lamps (Sylvania LU1000). When their roots had reached 20 to 30 cm in length, the seedlings were inoculated with pure cultures of *Frankia* sp. strain Ar13 (1). Following inoculation, the nitrate supplement was discontinued. Nodules used in this study were 8- to 12-weeks-old and between 0.5 and 1.5 cm in diameter.

Gas Exchange and Assay System. A single root nodule was enclosed in a cuvette with a volume of 6 ml (Fig. 1). The cuvette was made of brass to permit rapid thermal exchange between the circulating water and the assay gas. The nodule was sealed into place by leading the root segments above and below the nodule through slits in closed-cell neoprene foam plugs which had been cemented into rounded notches in one side of the cuvette by rapid-setting, catalyst-cured silicone rubber (General Electric RTV-41). When the two chamber halves were compressed together, these plugs deformed to the contours of the roots. A butyl rubber gasket (not shown) sealed the two sides of the chamber when they were drawn together by wing nuts on the ends of the two threaded connecting rods. The entire cuvette was covered by a layer of silicone rubber to thermally insulate it from the water of the surrounding aeroponics box.

Temperature control of the gas surrounding the nodule was accomplished by pumping water from a constant temperature bath through channels in the cuvette (Fig. 1). Nodule temperature was monitored by a thermocouple thermometer (Bailey model

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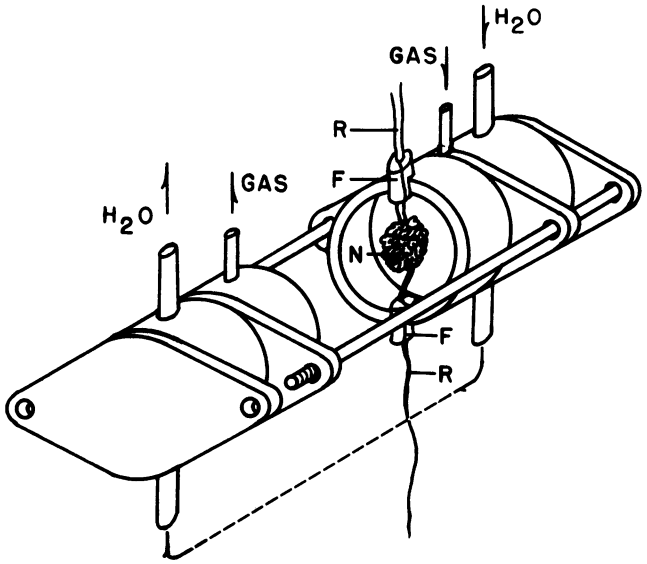

FIG. 1. Root nodule cuvette, open to show the positions of the root (R), nodule (N) and foam plugs (F). Internal diameter was 2.5 cm.

BAT-12, Saddle Brook, NJ) and a 30-guage copper-constantan thermocouple inserted between nodule lobes.

Assay gas mixtures were made in 10 liter Saran bags using high-purity cylinder gases, acetylene generated from calcium carbide and a 1-liter syringe (Hamilton, model S-1000, Reno, NV). Air was not used in these mixtures to avoid the inclusion of Ar. Since the gas analysis system was unable to separate Ar from O_2, any Ar in the assay gas mixtures greatly complicated oxygen calibration and uptake measurements.

As shown in Figure 2, assay gas (G2) was pumped by a leak-tight metal bellows pump (P2) through a humidifier (H) to a set of valves and flowmeters (F1 and F2). The humidifier was immersed in the same water bath (W) used to control the temperature of the cuvette so that the dewpoint of the gas stream and the nodule temperature were nearly identical. A moist piece of filter paper was enclosed with the nodule to ensure saturation conditions. F1 controlled and metered the flow to the cuvette enclosing the attached root nodule (C), while F2 regulated the precuvette gas stream. The valves and flowmeters were enclosed in a heated box to prevent condensation. All tubing carrying humidified gas was made of stainless steel, was joined by metal compression fittings and was heated by electric heat tapes. All tubing downstream from the flowmeters was 0.16 cm in diameter to minimize system volume. A four-port switching valve (V1) directed either the pre- or postcuvette gas to the assay part of the system while the other pathway was vented so that flow rates remained constant in either mode.

The gas exiting from V1 (Fig. 2) was sampled and analyzed under the control of a continuously looping program executed by a computing integrator (I) with external timed events (Columbia Scientific Industries, Supergrator-3A, Austin, TX). The program operated as follows. For 2.5 min, gas from V1 was dehumidified to a dewpoint of −20°C by a Peltier-cooled, temperature controlled water trap (T), was passed through the sample loop (S) of the gas chromatograph and was vented. Three-way solenoid valve V2 was then switched to divert the gas stream through a soap-bubble flowmeter (F3). Flow rate was measured during this part of the cycle. When all gas flow in the sampling loop had subsided, the sampling valve was actuated to begin the gas analysis. Normally closed solenoid valve V3 was then opened to allow dry gas (G1) to purge the sample loop for 3.7 min. During this time, the water trap went through a heating cycle which melted the collected ice from the previous sample and allowed it to be removed in the dry (due to dessicant D) purge gas. The trap was again cooled to −20°C and the purge gas was stopped (V3 closed). Valve V2 was switched back to allow the sample gas to again flush the sample loop. Cycle time was 6.2 min.

The gas chromatograph, a Carle model 1153, used a 1.7-mm i.d., 0.95-meter long stainless steel column filled with a 25:75 (v/v) mixture of Porapak R (80–100 mesh) and N (50–80 mesh), a 1.7-mm i.d., 0.85-m long stainless steel column of Molecular Sieve 5A (80–100 mesh) and a series/bypass switching valve to separate CO_2, ethylene, acetylene, O_2 and N_2. The carrier gas was helium at 20 p.s.i. and a flow rate of 19 ml/min, column temperature was 35°C and the sample loop volume was 0.25 ml. Both the sampling loop and the column switching valve were automatically operated by the integrator program. The output of the thermal conductivity detector was quantified by the integrator.

Calibration. Inasmuch as the response of the gas chromatograph to CO_2, ethylene, and acetylene was found to be linear from zero to a concentration of at least 10% v/v, calibration factors for these gases were derived from periodic assays of 0.10% v/v standard mixtures (Applied Science Laboratories, State College, PA). Detector response to O_2 was nonlinear and required a more detailed calibration procedure. A known addition method was used to determine the slope of the concentration *versus* peak area curve over the desired range. A 50-ml sample of a known mixture of N_2 and O_2 was subsampled and assayed. A known amount of pure O_2 was added to the remaining sample with a Teflon and glass syringe and allowed to mix by diffusion. The mixture was then reassayed. The slope of the concentration/area curve was calculated from the difference in peak areas and the calculated increase in O_2 concentration. The relationship between mean concentration and the measured slope was sufficiently linear ($r = 0.994$) to serve as a calibration curve for O_2 uptake.

Procedure. At the start of each experiment, the plant was transferred from the greenhouse aeroponics box to a smaller version inside a growth chamber (Conviron, model E8VH, Winnipeg, Manitoba) where photosynthetically active irradiance was 50 nE/cm^2·s, air temperature was 20°C and RH was 70%. The nodule was blotted free of liquid water and sealed into the cuvette. Initial conditions were always 20°C and 20% v/v O_2, very similar to the growing environment. Unless otherwise specified, acetylene concentration was 10% v/v.

Changes in concentrations of CO_2 and acetylene were made by rapidly interchanging Saran bags (G2, Fig. 2) containing different gas mixtures. When stable activity was reached under the new conditions, the sample/reference valve (V1) was switched to sample the pre-cuvette gas for two or three assay cycles.

Calculations. The standard equation for steady-state gas exchange was applied to the data for each gas assayed:

$$A = (C_{out} - C_{in}) \times K \times F$$

where A represents gas exchange in μmol/h, C_{out} and C_{in} are the outgoing and incoming gas concentrations in peak area units, K is a calibration factor derived for each gas in μmol/ml per peak area unit and F is the assay gas flow rate into the cuvette in ml/h. Usually, no CO_2 or ethylene was present in the incoming assay gas (*i.e.* $C_{in} = 0$) and each value of C_{out} was used to calculate an activity. O_2 uptake was more difficult to measure with precision, since the difference between C_{in} and C_{out} was often small in comparison to a large background concentration. To minimize error, C_{in} was determined as a mean of two or three values while C_{out} was determined as a mean of several assay values before and after the measurement of C_{in}.

RESULTS AND DISCUSSION

Carbon Dioxide Concentration. The CO_2 concentration of the gas exiting from the nodule cuvette was typically 0.5% to 1.0% v/v because the flow rate was kept low in order to measure O_2

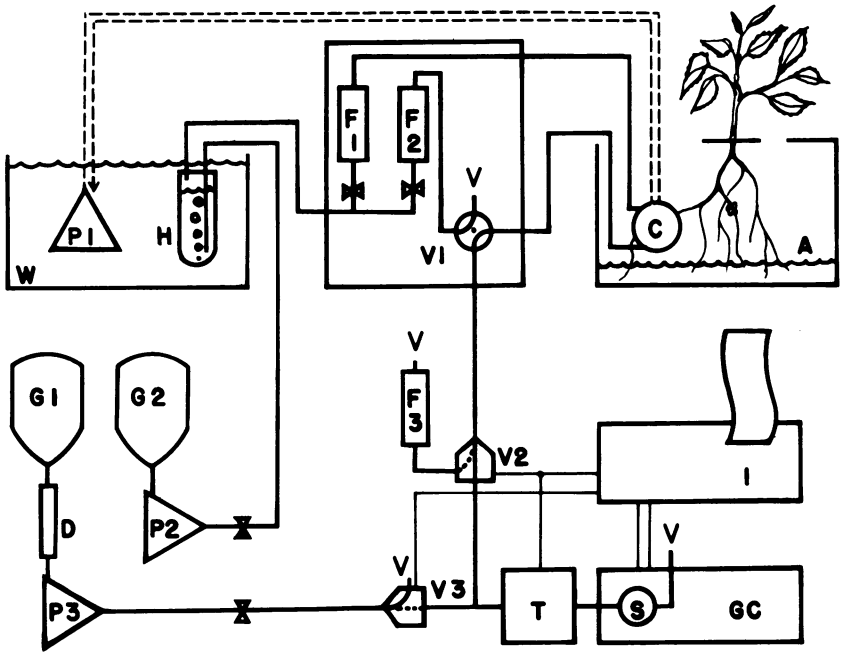

FIG. 2. Schematic of the root nodule gas exchange system. A, aeroponics box; C, nodule cuvette; D, magnesium perchlorate desiccant; F1, F2, rotameter flowmeters; F3, soap-bubble flowmeter; G1, Saran bag of flushing gas mixture; G2, Saran bag of assay gas mixture; GC, gas chromatograph; H, humidifier containing distilled H_2O; I, programable computing integrator; P1, water circulating pump; P2, P3, leak-tight metal bellows pumps; S, remote-actuated gas sampling valve; T, water trap; V1, sample/reference switching valve; V2, three-way solenoid valve; V3, normally-closed solenoid valve; W, thermostatted water bath.

uptake with the desired accuracy. Inasmuch as high concentrations of CO_2 inhibit respiration in pea nodules (6), the effect of CO_2 concentration on gas exchange of alder nodules was investigated. This experiment was done at a constant flow rate, with the ambient CO_2 level being varied by changing the CO_2 content of the gas entering the cuvette. The rates of CO_2 evolution and of acetylene reduction decreased as CO_2 concentration increased (Fig. 3). The activities were 17% lower at 2.8% v/v CO_2 than at 0.3% v/v. Respiration and ethylene production were reduced to an equal extent such that the ratio of the two remained constant. This effect occurred rapidly (in 18–24 min) following a step change in incoming CO_2 concentrations (Fig. 4). The inhibition was also largely reversible. Both respiration and acetylene reduction returned almost to previous rates when CO_2 concentrations were reduced.

The mechanism of this inhibition is unclear. The rapid time response and reversibility suggest that CO_2 may participate in some form of feedback inhibition of respiration and that reduced levels of ATP and reductant may in turn limit acetylene reduction. Alternatively, changes in intracellular pH (or other parameters) may affect both enzyme systems directly. High levels of CO_2 were found to inhibit nodule respiration but not acetylene reduction in peas (6). Coker and Schubert (2) also found no effect of high CO_2 levels on acetylene reduction by soybean nodules. At CO_2 concentrations which occur in most soils and in our assay procedure (less than 1% v/v), however, the effect is small enough to be obscured by experimental error in most other procedures.

Flow Rate. Once steady-state has been reached in an open gas exchange system, the difference between incoming and outgoing concentrations of a gas should vary inversely with the flow rate such that the activity measurement does not change. In our system the rates of acetylene reduction and CO_2 evolution increased by 8% to 14% as flow rate increased from 2 to 12 ml/min. This was entirely accounted for by the inhibitory effects of the high concentrations of CO_2 present at low flow rates. O_2 uptake may also have been affected by flow rate but any changes were less than the error in the measurements.

Acetylene Concentration. Acetylene reduction was saturated by levels of acetylene above 4% v/v (Fig. 5). The apparent K_m for this reaction in the presence of 70% v/v N_2 was 0.01 atm, in general agreement with the value of 0.006 atm summarized from other systems (4). Acetylene reduction by nodules of *Comptonia peregrina* also saturated at approximately 3% to 4% v/v acetylene (3).

The effect of acetylene upon nodule respiration was unexpected. Switching from an acetylene-free atmosphere to one containing acetylene (all other conditions held constant) caused a decrease in CO_2 evolution of about 10% (Fig. 6). This change was reversible, rapid (12–16 min), repeatable, and consistent from nodule to nodule. O_2 uptake was also increasingly inhibited as acetylene concentration was increased (Fig. 5). This effect has not been reported before and its mechanism remains uncertain.

Resolution and Stability. Variation between successive measures of CO_2 and ethylene evolution was less than 2% as shown by the experimental time courses in Figures 4 and 6. This variation was within the range expected due to fluctuations in flow rate, nodule temperature, and gas chromatograph performance, each of which consistently had a coefficient of variation of less than 1%. Consequently, this method is capable of resolving very small changes in activity.

The coefficient of variation for O_2 uptake determinations was less than 5%. Although successive assays of C_{out} and C_{in} for O_2 varied less than 0.1%, the larger variation in uptake measurements resulted from the small magnitude of the difference between the incoming and outgoing O_2 concentrations relative to their high absolute values. Nevertheless, changes in O_2 uptake greater than 5% were clearly measurable (Fig. 5).

Nodules were inserted repeatedly, removed, and reinserted into the cuvette on successive days without degradation in activity. Long exposures (8–10 h) to atmospheres containing 10% v/v acetylene appeared to have no detrimental effect. Ratios of CO_2 to ethylene evolution ranged from 2.8 to 3.6 for different nodules and were consistent over time for each nodule.

The method described here provides accurate, continuous, and simultaneous measures of acetylene reduction, CO_2 release, and

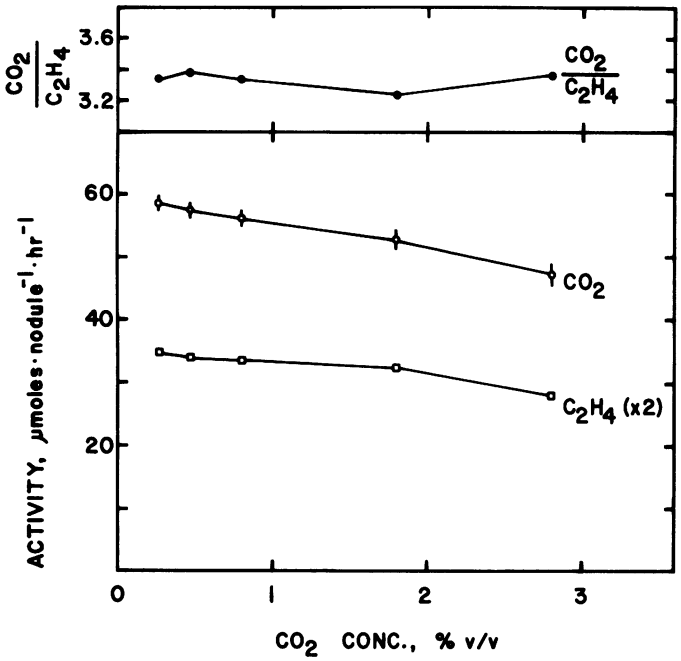

FIG. 3. Effect of ambient CO_2 concentration on CO_2 evolution and acetylene reduction. Each point is the mean of several steady-state determinations. Error bars, when present, indicate ± 1 SD. When absent, the SD was less than the width of the data symbol. The results are from one representative experiment. Other nodules showed the same behavior.

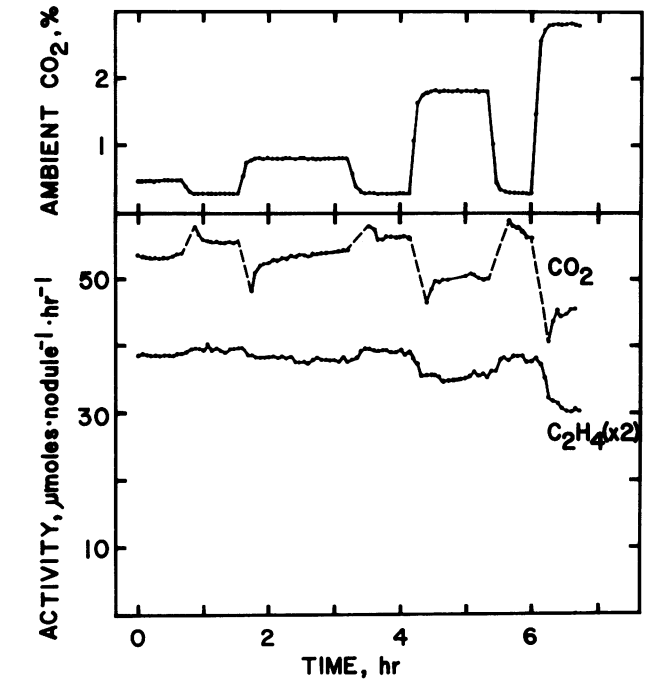

FIG. 4. Time course of response to changes in ambient CO_2 concentration. The dashed lines denote time when rate calculations were not possible during the equilibration of incoming gas levels following a change.

O_2 uptake by a single attached root nodule. There are, however, certain limitations. Nodules must be grown either in aeroponics or by some other method which allows easy access. Large (0.5–1.0 g fresh weight) nodules must be used to have sufficient activity for accurate O_2 uptake measurements, although acetylene reduction and CO_2 evolution can be measured readily on much smaller nodules. At an ambient CO_2 concentration of 0.5% v/v, the

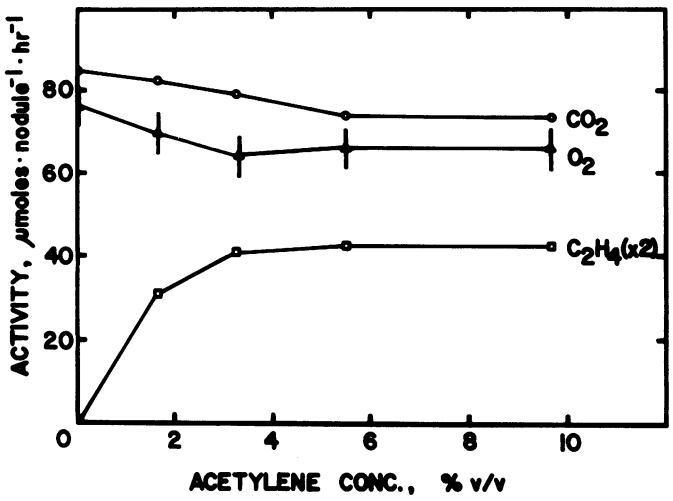

FIG. 5. Effect of acetylene concentration upon acetylene reduction, CO_2 evolution and O_2 uptake. Flow rate and nodule temperature were kept constant. Legend as in Figure 3.

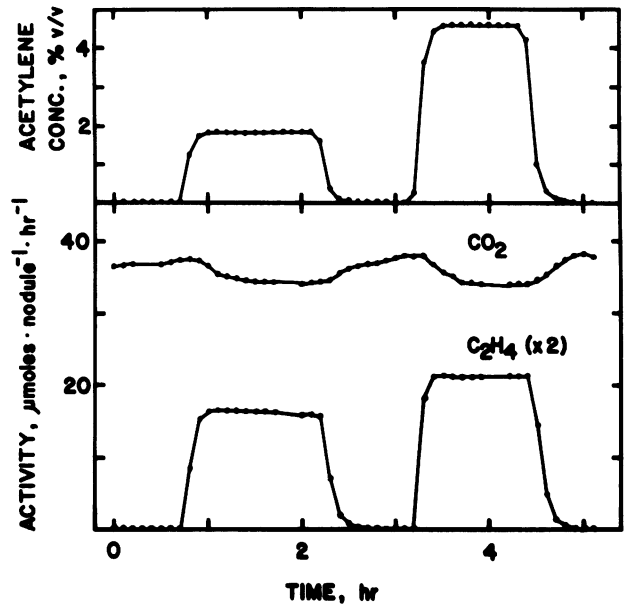

FIG. 6. Time course of response to changes in acetylene concentration. Flow rate, nodule temperature, and O_2 and CO_2 concentrations were held constant.

accuracy of the O_2 uptake measurement is 5% at best. Within these limits, this technique has potential for wide application in experiments concerned with the concurrent response of energy producing and nitrogen fixing metabolisms to environmental changes. Since both ambient gas levels and gas fluxes are accurately known, questions concerning diffusion pathways and O_2 supply can be readily investigated.

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