

# Carbon Exchange Rates of Shoots Required to Utilize Available Acetylene Reduction Capacity in Soybean and Alfalfa Root Nodules<sup>1</sup>

Received for publication December 18, 1979 and in revised form March 3, 1980

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## ABSTRACT

The CO<sub>2</sub>-exchange rate required to make full use of available N<sub>2</sub>-fixation capacity, measured as acetylene reduction, was determined in soybean and alfalfa. Carbohydrates of root systems were depleted during a 40-hour dark treatment; then plants were exposed to a 24-hour light period during which different CO<sub>2</sub>-exchange rates were maintained with various CO<sub>2</sub> concentrations. In three- and four-week-old soybeans and four-week-old alfalfa plants, acetylene-reduction capacity was used fully with CO<sub>2</sub>-exchange rates as low as 10 milligrams CO<sub>2</sub> per plant per hour. In six-week-old alfalfa plants, however, acetylene reduction rates increased linearly, and apparent N<sub>2</sub>-fixation capacity was not used fully when CO<sub>2</sub>-exchange rates were higher than 40 milligrams CO<sub>2</sub> per plant per hour. Under the conditions established, the energy cost of N<sub>2</sub> fixation, measured as  $\Delta(\text{respiration of roots} + \text{nodules})/\Delta\text{acetylene reduction over dark-treatment values}$ , was 0.453 milligrams CO<sub>2</sub> per micromole C<sub>2</sub>H<sub>4</sub> for all rates of acetylene reduction and for both ages of soybean and alfalfa plants. Thus, root-plus-nodule respiration was not promoted by higher rates of apparent photosynthesis after C<sub>2</sub>H<sub>2</sub>-reduction capacity became saturated, and all available capacity for apparent N<sub>2</sub> fixation had the same energy requirement.

Symbiotic N<sub>2</sub> fixation in legumes requires significant carbon inputs. Pate and co-workers (1, 6) estimate that the percentage of net photosynthate transported to root nodules of relatively young plants is as follows: *Lupinus alba* L., 23%; *Vigna unguiculata* (L.) Walp., 13%; and *Pisum sativum* L., 32%. Only a fraction of the carbon entering root nodules provides energy for N<sub>2</sub> fixation; the remainder supplies C skeletons to export fixed N or is used for growth and maintenance of the nodule. Whether the observed percent of net photosynthate transported to root nodules provides adequate energy for full use of the total N<sub>2</sub>-fixing capacity of those nodules is not known because the relation between rate of photosynthesis and rate of N<sub>2</sub> fixation is obscure.

Several types of experiments indicate that increased photosynthesis results in greater symbiotic N<sub>2</sub> fixation. Supplemental light, shading, and surgical manipulations of soybeans altered rates of canopy photosynthesis and produced corresponding changes in acetylene reduction 9 days after treatment (5). Enriching ambient air with CO<sub>2</sub> increased total dry weight and N content of symbiotically grown red clover and alfalfa after many weeks of growth

(14). Similar studies with soybeans showed that CO<sub>2</sub> enrichment increased acetylene reduction, an assay for apparent N<sub>2</sub> fixation, as early as 1 week after treatment (4, 9). In the latter case, both C<sub>2</sub>H<sub>2</sub> reduction/g nodule weight and total nodule weight increased (4). Photosynthesis was not measured directly in any of the CO<sub>2</sub>-enrichment studies cited, but long-term increases in plant dry weight were associated with CO<sub>2</sub> treatments (4, 9, 14). Short-term increases in acetylene reduction/g nodule weight were reported 5 h after CO<sub>2</sub> concentrations were increased around peas (8) and 2 days after a second shoot was grafted on a soybean root (12), but rates of apparent photosynthesis were not measured in either case. An increase in C<sub>2</sub>H<sub>2</sub> reduction rates without a significant increase in root-nodule mass in short-term studies with peas and soybeans (8, 12) suggested that unused N<sub>2</sub> fixation capacity was present.

The amount of normally unused N<sub>2</sub> fixation capacity in leguminous root nodules, the rate of photosynthesis required to activate it, and the respiratory cost for utilizing such capacity are unknown. The present study was conducted to measure the CO<sub>2</sub> exchange rate required to support maximum N<sub>2</sub> fixation in soybean and alfalfa root nodules and the respiratory burden associated with that activity. First, carbohydrates in root systems were depleted by a 40-h dark treatment. Second, different CO<sub>2</sub> exchange rates were maintained in shoots by providing various CO<sub>2</sub> concentrations during a subsequent 24-h light treatment. Finally, C<sub>2</sub>H<sub>2</sub> reduction and root-plus-nodule respiration were monitored throughout the light period to estimate the maximum N<sub>2</sub> fixation capacity available and the energy cost of that fixation.

## MATERIALS AND METHODS

Soybeans (*Glycine max* [L.] Merr. cv. Clark) and rooted cuttings from an established clone of alfalfa (*Medicago sativa* L. cv. Vernal) were grown in plastic pots (10 × 10 × 12 cm) containing vermiculite in a greenhouse during April and May, 1979. The planting schedule for soybeans and the cloning schedule for alfalfa were staggered so that soybean plants 3 and 4 weeks old and alfalfa plants 4 and 6 weeks old could be studied. Alfalfa plants were inoculated with *Rhizobium meliloti* strain 102F28, irrigated for 1 week with nutrient solution containing 2 mM N, reinoculated, and irrigated daily with N-free nutrient solution (11). Once a week, the plants were rinsed thoroughly with distilled H<sub>2</sub>O to prevent salt accumulation. Soybean plants were watered 3 days a week with a modified nutrient solution (2) containing 2 mM KNO<sub>3</sub> and on alternate days were watered with distilled H<sub>2</sub>O. The soybean plants were inoculated with *Rhizobium japonicum* strain USDA 311b110. Before experimental treatments were begun, lids were sealed on the pots and around the stem to permit gas-exchange measurements.

At 2-day intervals, three alfalfa and three soybean plants were transferred to a growth chamber where they were maintained in

<sup>1</sup> This material is based on research supported by National Science Foundation Grants AER 77-07301 and PCM 78-01146.

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the dark for 40 h at 25 C. Then three plants of each species with their sealed root systems were placed in separate Plexiglas chambers for simultaneous measurement of gas exchange by shoots and roots. Nylon tubes to and from the sealed pots were connected to inlet and outlet manifolds on the Plexiglas chambers for measurement of root gas exchange. A thermostat and heating system maintained the Plexiglas chambers at 25 C. Photosynthetic photon flux densities from the tops of the chambers to the tops of the pots were maintained at  $750\text{--}650 \mu\text{E m}^{-2} \text{s}^{-1}$  by placing the two Plexiglas boxes in a plant-growth chamber. Ambient air containing about  $320 \mu\text{l/liter CO}_2$  was humidified and passed over the roots at  $800 \text{ ml min}^{-1}$ . Air from the output manifolds could be passed to an IR gas analyzer for differential  $\text{CO}_2$  measurement or recirculated for measurements of apparent  $\text{N}_2$  fixation with the  $\text{C}_2\text{H}_2$  reduction assay.  $\text{C}_2\text{H}_2$  concentration was used as a standard in making corrections for leakage from the system.  $\text{C}_2\text{H}_2$  reduction was calculated from gas chromatographic determinations (2) of linear  $\text{C}_2\text{H}_2$ -dependent ethylene production over a 15-min period.

Gas exchange by plant shoots was measured in a manner similar to that by roots.  $\text{CO}_2$  concentrations around the shoot were controlled by adding  $\text{CO}_2$  to  $\text{CO}_2$ -free air. This air then was humidified and passed through flow meters into each of the gas exchange chambers.  $\text{CO}_2$  concentrations of 100, 200, 400, 800, and  $1200 \mu\text{l/liter}$  were used to produce different rates of apparent photosynthesis. Measurements of root-plus-nodule respiration,  $\text{C}_2\text{H}_2$  reduction, and  $\text{CO}_2$ -exchange rates were begun at the end of the 40-h dark treatment and continued at about 2.5-h intervals throughout the following 24-h light period.

There were two sets of control plants for each species and age group. One set was taken directly from the greenhouse at the end of a normal photoperiod and analyzed for  $\text{C}_2\text{H}_2$  reduction and root-plus-nodule respiration before harvesting. A second set was given a 40-h dark treatment and harvested immediately. Dry weights were recorded, and starch and soluble sugars (glucose, fructose, and sucrose) in the roots and nodules were extracted and analyzed with a gas chromatograph (3). Sugars were extracted with hot (90 C) 80% (v/v) ethanol. The starch-containing residue was treated with amyloglucosidase and analyzed for glucose content. Per cent carbohydrate was expressed on a tissue dry weight basis.

## RESULTS

Forty h of darkness following a normal photoperiod produced a significant decrease in carbohydrate levels of soybean and alfalfa root systems (Table I). Soluble sugars declined in the roots of all plants except the 6-week-old alfalfa. In that case a significant decrease in per cent starch apparently maintained the level of soluble sugars (Table I). Respiration and acetylene reduction rates by root systems of intact plants declined 50 to 80% during the 40-

Table I. Percentage of Soluble Carbohydrates and Starch in Soybean and Alfalfa Root Systems before and after a 40-h Dark Treatment

Data represent means of three replicate plants.

Plant	Age	Roots + Nodules			
		Before Treatment		After Treatment	
		Sugars	Starch	Sugars	Starch
	weeks			%	
Soybean	3	1.07	0.20	0.51 <sup>a</sup>	0.14
Soybean	4	1.39	0.85	0.60 <sup>a</sup>	0.23 <sup>a</sup>
Alfalfa	4	6.03	2.61	1.68 <sup>b</sup>	0.73 <sup>b</sup>
Alfalfa	6	3.05	6.86	3.27	3.93 <sup>a</sup>

<sup>a</sup> Treatment effect significant at  $P \leq 0.05$  in a two-tailed  $t$  test.

<sup>b</sup> Treatment effect significant at  $P \leq 0.01$  in a two-tailed  $t$  test.

h dark treatment to mean  $\pm$  SE values for all plants of  $1.49 \pm 0.29 \text{ mg CO}_2 \text{ plant}^{-1} \text{ h}^{-1}$  and  $2.06 \pm 0.94 \mu\text{mol C}_2\text{H}_4 \text{ plant}^{-1} \text{ h}^{-1}$ . Increases in the physiological activities of interest during the subsequent 24-h period of photosynthesis were calculated relative to the initial value after 40 h of darkness for each group of three plants (Figs. 1-3).

Various patterns of  $\text{C}_2\text{H}_2$  reduction (Fig. 1) and root-plus-nodule respiration (Fig. 2) during the 24-h light period following the dark treatment were associated with different  $\text{CO}_2$  exchange rates established in the shoots (Fig. 3) after the 40-h dark treatment. Once a given  $\text{CO}_2$ -exchange rate for shoots was established by setting  $\text{CO}_2$  concentration at a value between 100 and  $1200 \mu\text{l/liter}$ , variation with time, measured by standard error of the mean (Fig. 3), was small. Although widely different levels of  $\text{CO}_2$  were supplied initially, actual  $\text{CO}_2$  exchange rates of shoots were similar in some cases because of normal variation in the size of randomly selected plants (Table II). Maximum and minimum values of  $\text{CO}_2$ -exchange rate between  $\text{CO}_2$  treatments differed by a factor of about six in soybean and alfalfa of both ages (Fig. 3). Figures 1 and 2 give representative data for increases in  $\text{C}_2\text{H}_2$  reduction and respiration by older plants maintained at a low or high  $\text{CO}_2$  exchange rate during the 24-h light period. Those data show that alfalfa plants with higher  $\text{CO}_2$  exchange rates took longer than soybeans to reach maximum rates of  $\text{C}_2\text{H}_2$  reduction and root-plus-nodule respiration.

Data in Tables II and III and Figure 3 are from plants of all ages and all  $\text{CO}_2$ -exchange rates, derived from 20 separate trials with plants germinated sequentially. Ratios between the increase over the dark treatment value in respiration and the increase in  $\text{C}_2\text{H}_2$  reduction were remarkably similar for all plants during the 24-h light period (Table III). The mean ratio for all age and species groups was  $0.453 \text{ mg CO}_2 \mu\text{mol C}_2\text{H}_4^{-1}$ .

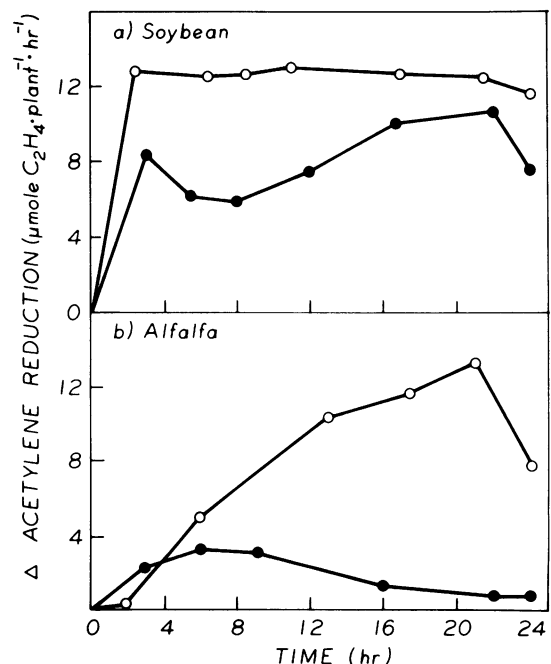


FIG. 1. Increase with time, above the dark treatment value, in  $\text{C}_2\text{H}_2$  reduction rate for plants with representative high or low rates of apparent photosynthesis during a 24-h light period following 40 h of darkness. The mean rate of  $\text{C}_2\text{H}_2$  reduction  $\pm$  SE of all plants following the dark treatment was  $2.06 \pm 0.94 \mu\text{mol C}_2\text{H}_4 \text{ plant}^{-1} \text{ h}^{-1}$ . Three plants were monitored for 24 h to produce each curve. Rates of apparent photosynthesis  $\pm$  SE ( $\text{mg CO}_2 \text{ plant}^{-1} \text{ h}^{-1}$ ) maintained throughout the light treatment were: a, 4-week-old soybean:  $9.7 \pm 0.4$  (●),  $54.6 \pm 3.7$  (○); and b, 6-week-old alfalfa:  $7.7 \pm 1.5$  (●),  $41.9 \pm 1.9$  (○).

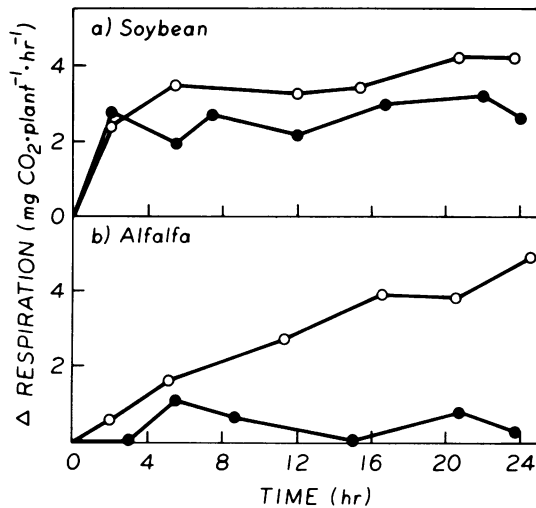


FIG. 2. Increase with time, above the dark treatment value, in root-plus-nodule respiration rate for the same plants represented in Figure 1. The mean respiration rate  $\pm$  SE of all plants following the dark treatment was  $1.49 \pm 0.29$  mg CO<sub>2</sub> plant<sup>-1</sup> h<sup>-1</sup>. Rates of apparent photosynthesis  $\pm$  SE (mg CO<sub>2</sub> plant<sup>-1</sup> h<sup>-1</sup>) maintained throughout the light treatment were: a, 4-week-old soybean  $9.7 \pm 0.4$  (●),  $54.6 \pm 3.7$  (○); and b, 6-week-old alfalfa:  $7.7 \pm 1.5$  (●),  $41.9 \pm 1.9$  (○).

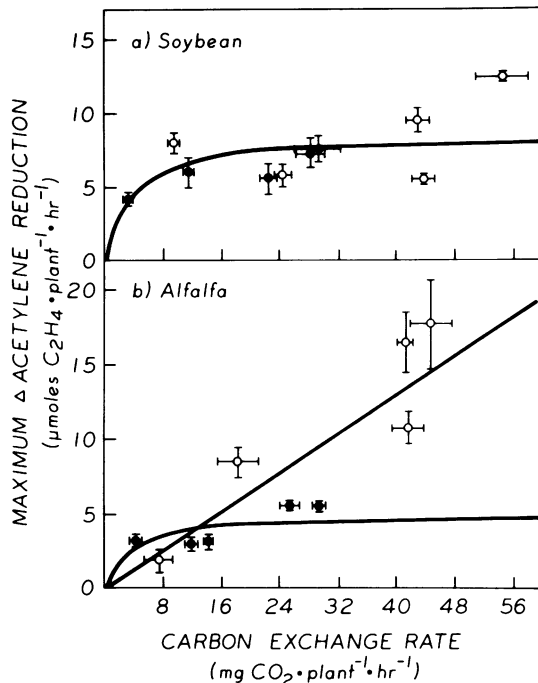


FIG. 3. Maximum increase, above the dark-treatment value, in C<sub>2</sub>H<sub>2</sub> reduction as a function of shoot CO<sub>2</sub>-exchange rate during a 24-h light period following 40 h of darkness. Data for each point were collected over the 24-h light period from three plants in a single assay chamber. Standard errors represented with each mean were calculated as variation with time after a maximum or steady-state condition was observed. Functions shown are: a, soybean:  $y = 9x/(3 + x)$ ,  $R^2 = 0.78$ , 3 week (●), 4 week (○); and b, alfalfa:  $y = 0.35x - 0.06$ ,  $R^2 = 0.94$ ;  $y = 5x/(3 + x)$ ,  $R^2 = 0.84$ , 4 week (●), 6 week (○).

The maximum increase in C<sub>2</sub>H<sub>2</sub> reduction, calculated as a mean  $\pm$  SE variation for the period after the response saturated, was determined from data such as those shown for 6-week-old alfalfa and 4-week-old soybean plants in Figure 1. The maximum in-

crease in C<sub>2</sub>H<sub>2</sub> reduction was greatest in plants with the greatest CO<sub>2</sub>-exchange rate, although plants with lowest CO<sub>2</sub> exchange rates did not always show the smallest increases in C<sub>2</sub>H<sub>2</sub> reduction (Fig. 3). When plants from all CO<sub>2</sub> treatments were considered together by species, 6-week-old alfalfa plants differed from other plants in the relation between maximum increase in C<sub>2</sub>H<sub>2</sub> reduction and CO<sub>2</sub> exchange rate (Fig. 3). In soybean those parameters for plants of both ages fitted a nonlinear function,  $y = 9x/(3 + x)$ , with  $R^2 = 0.78$  ( $P \leq 0.05$ ). Data from 4-week-old alfalfa plants fitted a similar nonlinear function,  $y = 5x/(3 + x)$ , with  $R^2 = 0.84$  ( $P \leq 0.05$ ). In contrast, a linear correlation between maximum increase in acetylene reduction and CO<sub>2</sub> exchange rate was found for 6-week-old alfalfa plants ( $y = 0.35x - 0.06$ ,  $R^2 = 0.94$ ,  $P \leq 0.01$ ). If data from alfalfa plants of both ages were combined, less variability was explained by the linear function obtained:  $y = 0.33x - 0.28$ ;  $R^2 = 0.83$ ,  $P \leq 0.01$ . Patterns were similar when the CO<sub>2</sub>-exchange rate was related to the increase in acetylene reduction on a nodule weight basis. This observation resulted from the fact that all plants within an age species cohort had a similar mass of root nodules (Table II).

## DISCUSSION

The 40-h dark treatment produced the associated decline in C<sub>2</sub>H<sub>2</sub> reduction and root-plus-nodule carbohydrate concentration expected from reports of other workers (13). The severity of this treatment was emphasized by the fact that Alaska peas (included originally in the present study) showed no resumption of C<sub>2</sub>H<sub>2</sub> reduction activity with any rate of apparent photosynthesis during the subsequent 24-h light period. Thus, quantitative interpretation of data from soybean and alfalfa plants that survived 40 h of darkness must be viewed with caution, although qualitative comparisons probably are justified.

Soybean and alfalfa plants apparently can differ considerably in the amount of unused capacity for C<sub>2</sub>H<sub>2</sub> reduction (Fig. 3). Soybean plants of the two ages tested achieved nearly maximum rates of C<sub>2</sub>H<sub>2</sub> reduction with CO<sub>2</sub> exchange rates as low as 10 mg CO<sub>2</sub> plant<sup>-1</sup> h<sup>-1</sup>, and the increase in C<sub>2</sub>H<sub>2</sub> reduction over the dark-treatment value was not significantly greater for plants with CO<sub>2</sub>-exchange rates maintained at more than 40 mg CO<sub>2</sub> plant<sup>-1</sup> h<sup>-1</sup> (Fig. 3a). It seems doubtful that soybean plants with those characteristics transported identical amounts of net photosynthate to the root nodules, but steady-state measurements of assimilate distribution were not possible with the equipment used. Young alfalfa plants were similar to soybeans in that C<sub>2</sub>H<sub>2</sub> reduction was limited by some factor other than assimilated carbon and a proportionately large fraction of C<sub>2</sub>H<sub>2</sub>-reduction capacity was used at low CO<sub>2</sub> exchange rates (Fig. 3b). C<sub>2</sub>H<sub>2</sub> reduction in older alfalfa plants always was limited by carbohydrate, and the plant used a fraction of the C<sub>2</sub>H<sub>2</sub> reduction capacity that was directly proportional to the CO<sub>2</sub> exchange rate (Fig. 3b). It is probable, though not demonstrated by the present data, that still higher CO<sub>2</sub> exchange rates could have enabled full use of all of the available C<sub>2</sub>H<sub>2</sub> reduction capacity in 6-week-old alfalfa plants.

One point to be emphasized is that all plants of the same age and species had similar root nodule masses (Table II) and would not be expected to increase that parameter greatly during the 24-h light period. In fact, acetylene reduction in soybean was maximum only 2.5 h after establishment of the greatest rate of photosynthesis,  $54.6$  mg CO<sub>2</sub> plant<sup>-1</sup> h<sup>-1</sup> (Fig. 1a). The simplest explanation for the different qualitative responses shown in Figure 3 is that the fraction of current photosynthate allocated to root nodules by older alfalfa plants is not large enough for them to make full use of their N<sub>2</sub>-fixation capacity. Metabolic pathways used to store starch in roots (Table I) may compete more effectively with root nodules for photosynthate in 6-week-old plants than in 4-week-old plants, or the strategy of carbohydrate utilization within the nodule may change. The fact that the alfalfa plants of different

Table II. Dry Weight and Leaf Area of Soybean and Alfalfa Plants at the End of a 24-h Light Period That Followed 40 h of Darkness

Each value represents the mean  $\pm$  SE of 15 plants. The 15 plants had been maintained, in groups of three, at one of five different levels of apparent photosynthesis during the 24-h light period.

Plant	Age	Leaf Area	Root Dry Weight	Nodule Dry Weight	Total Dry Weight
	weeks	cm <sup>2</sup>	g	mg	g
Soybean	3	160 $\pm$ 8.9	0.302 $\pm$ 0.018	68.0 $\pm$ 7.0	1.090 $\pm$ 0.095
Soybean	4	239 $\pm$ 12.7	0.512 $\pm$ 0.031	122 $\pm$ 6.0	1.870 $\pm$ 0.183
Alfalfa	4	59.5 $\pm$ 5.1	0.268 $\pm$ 0.031	23.2 $\pm$ 3.0	0.748 $\pm$ 0.102
Alfalfa	6	191 $\pm$ 24.4	0.848 $\pm$ 0.140	64.3 $\pm$ 6.6	2.294 $\pm$ 0.231

Table III. Ratio of Increased Respiration to Increased C<sub>2</sub>H<sub>2</sub> Reduction for Roots with Attached Nodules during a 24-h Light Period after 40 h of Darkness

Various rates of C<sub>2</sub>H<sub>2</sub> reduction and respiration were produced by exposing plant shoots to different concentrations of CO<sub>2</sub> during the photoperiod and maintaining constant CO<sub>2</sub> exchange rates. Data represent mean  $\pm$  SE of 30 measurements during the 24-h light period on five sets of three plants.

Plant	Age	$\Delta$ Respiration/ $\Delta$ C <sub>2</sub> H <sub>2</sub> Reduction
	weeks	mg CO <sub>2</sub> /μmol C <sub>2</sub> H <sub>4</sub>
Soybean	3	0.483 $\pm$ 0.0338
Soybean	4	0.429 $\pm$ 0.0381
Alfalfa	4	0.456 $\pm$ 0.0389
Alfalfa	6	0.443 $\pm$ 0.0723

ages were inoculated with the same strain of *R. meliloti* supports the concept that a host-plant factor, such as starch metabolism, was responsible for the qualitative differences observed (Fig. 3b). If one chooses to interpret the data in Figure 3b as showing a linear relation between maximum  $\Delta$  C<sub>2</sub>H<sub>2</sub> reduction and CO<sub>2</sub> exchange rate, then curvilinear and linear responses (Fig. 3, a and b) would be associated with species differences.

Even though older alfalfa plants activated available C<sub>2</sub>H<sub>2</sub> reduction capacity differently from younger alfalfa or soybeans, the respiratory cost of C<sub>2</sub>H<sub>2</sub> reduction did not differ significantly between either species or age groups (Table III). The mean value for all age and species groups of 0.453 mg CO<sub>2</sub> μmol C<sub>2</sub>H<sub>4</sub><sup>-1</sup> was nearly identical to data collected by other techniques for soybean and pea plants grown without combined N (7). It was not possible in the present study to convert this value to a cost of N<sub>2</sub> reduction, because the large volume of the continuous flow system prevented reproducible measurement of H<sub>2</sub> evolution and made <sup>15</sup>N<sub>2</sub> studies unfeasible. In any case, such measurements would include the energy cost of root maintenance and growth. The short-term characteristic of the study made it impractical to measure actual changes in Kjeldahl N values. Values of acetylene reduction and root-plus-nodule respiration were within the range reported for soybeans by Ryle and co-workers (10).

The relationship between CO<sub>2</sub> exchange rate and maximum increase in C<sub>2</sub>H<sub>2</sub> reduction for soybeans (Fig. 3a) suggests that maximum activity of the nitrogenase-nitrogenase reductase complex was expressed, but another, potentially more important interpretation, is possible. It is conceivable that increasing the CO<sub>2</sub> exchange rate in soybean plants does not immediately increase the amount of carbohydrate supplied to root nodules above a previously established maximum. Control of carbon partitioning could be mediated by the shoot and would have the potential advantage

of utilizing newly available carbohydrate to produce additional photosynthetic tissue. Determining whether such control systems exist is critical for understanding how the normal defensive posture of plants, necessary for protection against environmental stress, can be altered to exploit a favorable environment.

The implications of the present results with alfalfa are difficult to evaluate. Reported values for amounts of net photosynthate transported to root nodules in other legumes were determined over long periods of growth (1, 6). Assuming that evolutionary forces favor seed production, one must believe that in surviving alfalfa genotypes the amount of carbon transported to root nodules under conditions of low soil N promoted that result. Agronomic goals, however, often differ from evolutionary results, and data in Figure 3b suggest that alfalfa plants can have an unused capacity for N<sub>2</sub> fixation. Verifying and exploiting such potential differences between alfalfa and soybeans provides interesting avenues for future investigations.

*Acknowledgment*—The authors thank L. Branneman for help with carbohydrate analyses.

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