Plant Physiol. (1977) 60, 817-821

Respiration and the Energy Requirement for Nitrogen Fixation in Nodulated Pea Roots[1]

Received for publication May 4, 1977 and in revised form July 27, 1977

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ABSTRACT

***Pisum sativum* L. cv. Trapper plants were inoculated and grown in a controlled environment on N-free nutrient solution. After 4 weeks N was supplied to treatment plants as NH_4NO_3, KNO_3, or NH_4Cl and rates of C_2H_2 reduction, root + nodule respiration, and leaf photosynthesis were determined 1 week later. The increase in respiration per unit of C_2H_2 reduction was not affected by either the form of N added or the light conditions during growth, although the basal respiration rate with no C_2H_2 reduction increased with irradiance level. The mean regression coefficient from plots of respiration *versus* C_2H_2 reduction was 0.23 + 0.04 ($P \simeq .01$) mg of CO_2 (μmol of C_2H_2 reduced)$^{-1}$ which was very similar to the value for the coefficient of respiration associated with nitrogenase activity estimated by subtracting growth and maintenance respiration. Since the rate of N accumulation in N-free nutrient conditions was proportional to the rate of C_2H_2 reduction, it appears that the method gives a true estimate of the energy requirements for N fixation which for these conditions was equivalent to 17 grams of carbohydrate consumed per gram of N fixed.**

Because of recent evidence that symbiotic N fixation can be limited by energy supply from the host plant (8, 11, 12, 20) there is increasing interest in the efficiency of energy use by the N-fixing reactions (6, 15, 19). Although the theoretical energy requirements for N fixation and nitrate reduction are similarly high (5, 8, 15), the difficulties in estimating the *in vivo* energy consumption (1, 3, 5, 15) have prevented any critical examination of the relative efficiency of energy use by the two processes.

In a previous report (13), it was suggested that an extension of the two-component respiration model (14, 22) to include a fixation component could provide the basis for estimating the respiration directly related to the fixation process. After comparing the effects of several treatments on the rates of both root + nodule respiration and C_2H_2 reduction (13); it was concluded that addition of NH_4NO_3 to nodulated plants decreased the respiration associated with nitrogenase activity while causing little difference in growth and maintenance components as compared to N-free treated plants of similar age. A comparison of the changes in respiration and C_2H_2 reduction under these conditions should be useful in assessing the energy requirements of N fixation. However, difficulties with parallel changes in other respiration components, high variability within a single symbiotic system, and the indirect nature of the C_2H_2 reduction assay could decrease the utility of this method. Therefore before the method can be applied to comparison of different symbiont genotypes, these possible problems must be examined and minimized.

[1] Issued as NRCC No. 16156.

MATERIALS AND METHODS

Plant Culture. Commercial field peas (*Pisum sativum* L. cv. Trapper) were inoculated and planted in 10-cm plastic pots in controlled conditions (13). Light was supplied by mixed fluorescent and incandescent lamps at a photon flux density (400-700 nm) of 20 to 30 nE cm^{-2} sec^{-1}. The photoperiod was 16 hr and the day and night temperatures were 20 and 15 C, respectively. Plants were irrigated daily with water until seedling emergence and thereafter daily either with water or thrice weekly with N-free nutrient (13) until the beginning of experiments, 4 to 5 weeks after planting. During the measurement period, plants were irrigated daily with N-free nutrient solution or N-free nutrient solution supplemented with NH_4NO_3, KNO_3, or NH_4Cl.

Measuring System. All measurements were made in the laboratory gas exchange system described previously (13). Rates of C_2H_2 reduction were determined from gas chromatographic analysis of C_2H_4 10, 20, and 30 min after the injection of C_2H_2 to a final concentration of 0.02 atm in air. Temperatures of the root medium were between 23 and 26 C and the flow rate for root respiration measurements was 60 liters hr^{-1}. Photosynthetic CO_2 uptake by individual leaflets was measured at a temperature between 23 and 26 C and an air flow rate of 30 liters hr^{-1}.

Plant material for dry weight or N determinations was oven-dried (70-80 C) for at least 48 hr before weighing. Weighed samples were ground and subsamples were analyzed for N with a CHN analyzer model 185B (Hewlett Packard, Calif.) using acetanilide as standard.

Experimental Procedures. In the first experiment, after 28 days on N-free nutrient, daily irrigation with N-free, 15 mM NH_4NO_3 or 30 mM KNO_3 nutrient solutions was begun on successive days with replicate plants. Six days after the beginning of the treatments, plants from each treatment were moved to the laboratory where rates of C_2H_2 reduction and root + nodule respiration as well as photosynthetic CO_2 uptake by leaves 3 and 5 (from top) at 100 nE cm^{-2} sec^{-1} were determined. To avoid confounding of treatment effects with diurnal changes (13, 16), the order in which the treatments were measured was varied. Immediately following measurement, each plant was removed from its pot, the roots were washed, and the whole plant was dried and weighed.

For N concentration studies plants were grown for 27 days in N-free nutrient followed by N-free or N-supplemented solutions applied daily. Six days later, measurements of C_2H_2 reduction, root + nodule respiration, and leaf photosynthesis at 25 nE cm^{-2} sec^{-1} were begun. Measurements were continued for 3 successive days, varying the order in which treatment groups were measured, for a total of five replicates/treatment. Following each measurement the plants were washed, dried, and weighed.

In a light intensity experiment, 40 plants were grown for 30 days with N-free solution and normal irradiance (25 nE cm^{-2}

sec^{-1}). On day 30, daily irrigation with N-free or 15 mM NH_4NO_3 nutrient solutions was begun, and on day 32 five plants from each nutrient regime were placed in each light treatment. Three irradiance levels of 40 (high), 20 (medium), and 10 (low) nE cm^{-2} sec^{-1} at plant top were provided by cheesecloth filters supported above the plants. The remaining 10 plants, five from each nutrient regime, were harvested and divided into roots and shoots. On 5 successive days, commencing 35 days from planting, one plant from each nutrient and light combination was moved to the laboratory for determination of C_2H_2 reduction and root + nodule respiration. As well, the rates of photosynthetic CO_2 uptake and transpirational H_2O efflux were measured in leaves 3 and 5 at irradiance levels corresponding to the light treatments. The order in which treatment groups were measured was varied from day to day to equalize diurnal effects, and plants were returned to the growth chamber after measurement. On day 39 after the final measurements, all plants were harvested and separated into root + nodule and shoot portions. All plants samples were analyzed for N content of roots and shoots.

RESULTS

The relationship between root + nodule respiration and C_2H_2 reduction was similar when the results with either 15 mM NH_4NO_3 or 30 mM KNO_3 were combined with the control values (Fig. 1). In both cases C_2H_2 reduction was reduced to less than 20% of control and the respiration to 65 to 75% of control. Much of the variability in the results (Fig. 1a) was due to the variability in plant size and could be substantially reduced by expressing both respiration and C_2H_2 reduction on the basis of total plant dry weight (Fig. 1b). This adjustment reduced the mean coefficient of variability from 66 to 48% for C_2H_2 reduction and from 35 to 15% for respiration. Although the slopes of the regressions of respiration on C_2H_2 reduction were not significantly different if based on per plant or per g dry weight values (Table I), when the dry weight adjustment was used, the regression error was decreased and the slopes estimated from regression analysis and from the treatment means were similar. There was no significant difference in slope between the results with the two N sources when either adjusted or nonadjusted values were used. The rate of photosynthesis was not affected by the nutrient treatment and averaged 23.9 ± 0.8 (SE), 25.8 ± 0.8, and 24.0 ± 0.6 mg CO_2 dm^{-2} hr^{-1} for the N-free, NH_4NO_3-, and KNO_3-treated plants, respectively.

The C_2H_2 reduction was markedly reduced by relatively low concentrations of KNO_3 while the total root + nodule respiration was less sensitive (Fig. 2a). Photosynthesis decreased

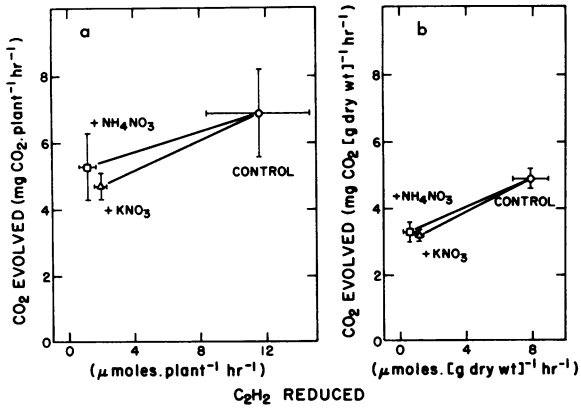

FIG. 1. Root + nodule respiration as a function of C_2H_2 reduction in plants treated with N-free (control), 15 mM NH_4NO_3, and 30 mM KNO_3 nutrients. Values are expressed as per plant (a) and per g of plant dry weight (b). Bars represent 2 × SE (N = 5).

Table I. Comparison of the root + nodule respiration-C_2H_2 reduction relationships by comparison of results from plants grown on N-free nutrient with those from plants given NH_4NO_3 (15 mM) or KNO_3 (30 mM).

CO_2 evolved per unit C_2H_2 reduced was calculated using data expressed on a whole plant or a plant dry weight basis. Values were determined by linear regression analysis of results from all plants treated with N-free and NH_4NO_3 or KNO_3 supplemented nutrients or by a simple slope calculation using treatment means.

Basis of Slope Calculation	CO_2 Evolved/C_2H_2 reduced mg/μ mole	
	NH_4NO_3	KNO_3
Regression (per plant)	0.27 ± .08*	0.31 ± .05
Regression (per g dry weight)	0.21 ± .04	0.25 ± .02
Means (per plant)	0.16	0.22
Means (per g dry weight)	0.22	0.25

*standard error (n=8)

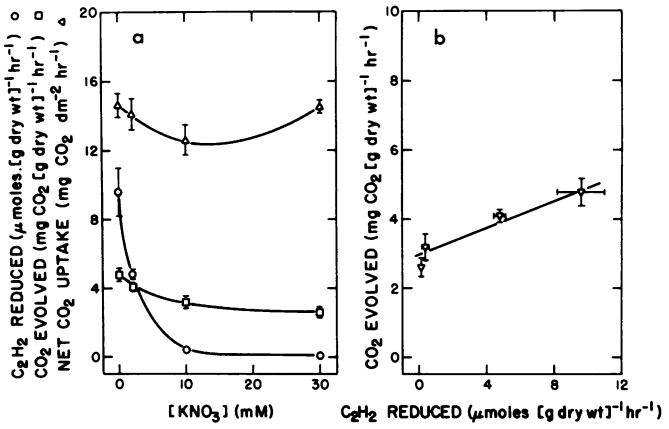

FIG. 2. a: Rates of C_2H_2 reduction (○), root + nodule respiration (□), and photosynthetic CO_2 uptake by leaf 3 (△) for plants treated with varying concentrations of KNO_3. b: Root + nodule respiration as a function of C_2H_2 reduction. Bars represent 2 × SE (N = 5).

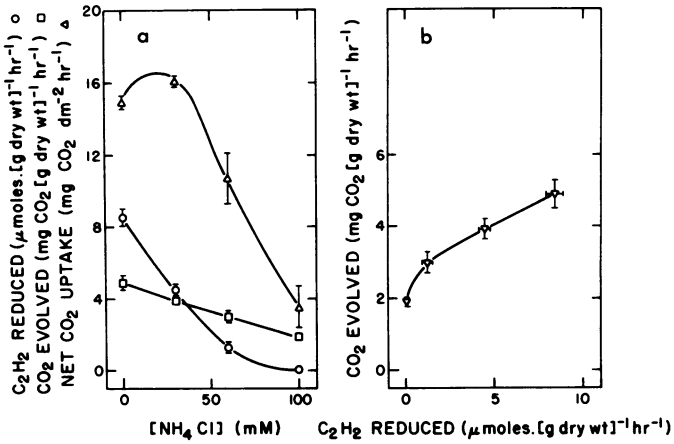

FIG. 3. a: Rates of C_2H_2 reduction (○), root + nodule respiration (□), and photosynthetic CO_2 uptake by leaves 3 and 5 (△) for plants treated with varying concentrations of NH_4Cl. b: Root + nodule respiration as a function of C_2H_2 reduction. Bars represent 2 × SE (N = 5, photosynthesis N = 10; for 100 mM N = 3, photosynthesis N = 6).

slightly with increasing KNO_3 up to 10 mM but was similar to control values at 30 mM. Respiration was linearly related to C_2H_2 reduction (Fig. 2b) and their relationship was described by the linear regression equation $y = 0.19x + 2.99$.

Both C_2H_2 reduction and root + nodule respiration was less sensitive to NH_4Cl (Fig. 3a) than to KNO_3. Photosynthesis was severely inhibited by high concentration of NH_4Cl. The relationship of respiration to C_2H_2 reduction was linear from 0 to 60 mM NH_4Cl (Fig. 3b) and was described by the equation $y = 0.24x + 2.81$. The root + nodule respiration of plants treated

with 100 mM NH_4Cl was much lower than would be predicted by this regression.

In the final experiment the rate of C_2H_2 reduction increased with light intensity in plants given N-free nutrient, but was almost zero in all plants exposed to NH_4NO_3 regardless of the light conditions (Fig. 4a). Root + nodule respiration increased with light intensity in both nutrient treatments, but to a greater extent in plants maintained on N-free solution. Both photosynthesis and transpiration increased with increasing irradiance and plants treated with NH_4NO_3 had consistently lower rates of both than those grown in N-free nutrient (Fig. 4b).

The regressions of root + nodule respiration on C_2H_2 reduction for the three light treatments (Fig. 5, top three curves) were similar in slope with increasing respiration intercepts as the light level increased.

Because there was no significant difference in root or shoot dry weight and N due to the 2-day exposure to NH_4NO_3 before first harvest, all plants from this harvest were averaged (Table II). At the second harvest, there were significant differences related to both irradiance and N treatments in the shoots, but root + nodule weights were affected only by light differences.

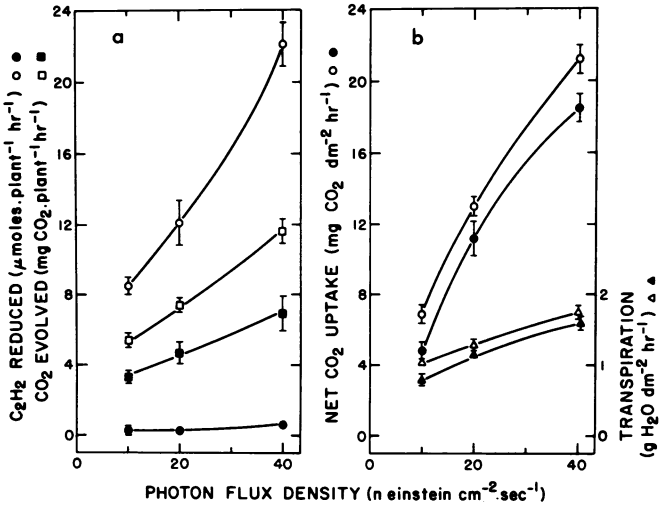

FIG. 4. a: Effects of irradiance on C_2H_2 reduction (○●) and root + nodule respiration (□■). b: Effects of irradiance on photosynthetic CO_2 uptake (○●) and transpiration by leaves 3 and 5 (△▲). Plants were treated with N-free (○□△) or 15 mM NH_4NO_3 (●■▲) nutrient. Bars represent 2 × SE (a: $N = 5$; b: $N = 10$).

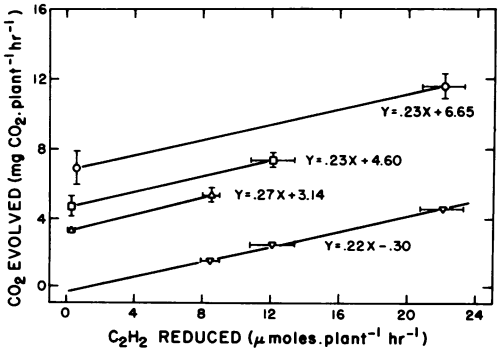

FIG. 5. Root + nodule respiration as a function of C_2H_2 reduction in plants treated with N-free and 15 mM NH_4NO_3 at irradiance levels of 40 (○), 20 (□), and 10 (△) nE cm^{-2} sec^{-1}. Data points are presented as means (± SE) of five replicates and regression equations were determined from results of all plants exposed to each light regime (N = 10). Total respiration minus growth and maintenance respiration (▽) was calculated according to Table III.

Table II. Dry weight and nitrogen content of roots and shoots of plants growing at three light levels with or without nitrogen in nutrient solution

Plants were grown for 30 days in N-free solution before treatment. Harvest 1 was day 32 and harvest 2 was day 39. Results are presented ± standard error of mean (Harvest 1, n=10; Harvest 2, n=5).

	Dry Weight g per plant		Nitrogen mg per plant	
	Root + Nodules	Shoot	Root + Nodules	Shoot
HARVEST 1.	.45 ± .03	1.01 ± .07	20 ± 1	43 ± 4
HARVEST 2.				
HIGH LIGHT +N	.81 ± .13	2.53 ± .19	41 ± 6	117 ± 9
-N	.82 ± .04	2.21 ± .14	34 ± 2	64 ± 4
MEDIUM LIGHT +N	.52 ± .03	2.07 ± .11	25 ± 1	98 ± 5
-N	.57 ± .04	1.72 ± .10	24 ± 2	58 ± 4
LOW LIGHT +N	.40 ± .03	1.53 ± .05	18 ± 1	70 ± 2
-N	.46 ± .02	1.78 ± .05	19 ± 1	58 ± 6

DISCUSSION

It has been proposed that plant respiration can be described by considering the total respiration as the sum of two major components, growth respiration and maintenance respiration (14, 22). In a previous report (13), we proposed that in nodulated legumes, respiration directly associated with N fixation is another large component of respiration and that for the root and nodule system the respiration relationships can be expressed as follows:

$$R = R_M W + R_G \, dW/dt + R_F \ (N_2\text{-ase}) \qquad (1)$$

where R is the total respiration of roots and nodules, W is the root and nodule dry weight, N_2-ase is nitrogenase activity, and R_M, R_G, and R_F are the maintenance, growth, and fixation coefficients, respectively.

In this proposal, R can be linearly related to N_2-ase if W and dW/dt are constant, in which case the slope will equal R_F and will estimate the respiration associated with nitrogenase activity. The relationship can also be linear if W and/or dW/dt change in parallel with N_2-ase in which case the slope will overestimate R_F.

Although there was no significant difference between the regression coefficients derived from total plant and dry weight adjusted results, the smaller standard errors of regression coefficients and the similarity in slope calculated by the two methods when adjusted values were used (Table I) indicate that the variability was both decreased and normalized by correcting results for plant mass.

The similar respiration-C_2H_2 reduction relationship when NH_4NO_3 and KNO_3 were used as N source (Fig. 1 and Table I) indicates that it was the NO_3^- ion which decreased the C_2H_2 reduction and respiration. This was again demonstrated in the nutrient concentration experiments in which C_2H_2 reduction was inhibited over 95% by 10 mM KNO_3 (Fig. 2) whereas the inhibition can be estimated as only 16% by an equal concentration of NH_4Cl (Fig. 3). Thus, it can be assumed that at the concentration of NH_4NO_3 used in other experiments (15 mM) the effects were primarily due to NO_3^- ion.

The small effects of KNO_3 on leaf photosynthesis (Fig. 2) indicate that shoot activity was not greatly disturbed. In contrast, 100 mM NH_4Cl reduced photosynthesis by an average of over 70% (Fig. 3). This inhibition may be related to the reported ability of NH_4^+ to uncouple photosynthetic electron transport (10). In any case, it is likely that the depressed respiration in the 100 mM NH_4Cl treatment was caused by a general starvation and a decreased growth and/or maintenance respiration.

From these results NO_3^- would be preferable to NH_4^+ since a large decrease in C_2H_2 reduction can be rapidly obtained with little effect on photosynthesis. However, the theoretical energy requirements for NO_3^- are similar to those for N fixation (5). If the reduction of NO_3^- occurs in the roots and nodules, little change in roots + nodule respiration would be expected when gaseous N_2 is replaced by NO_3^- as the N source (15). In these experiments, however, the slope of the respiration-C_2H_2 reduc-

tion relationship was the same regardless of whether NO_3^- (Fig. 2) or NH_4^+ (Fig. 3) was used. Since appreciable nitrification is unlikely and no reduction of NH_4^+ is necessary, this similarity indicates that NO_3^- was reduced predominantly in the shoots. Reports from other studies with peas (4, 17, 24) support this idea and although some energy may be required for nitrate uptake and transport, it is likely that under the conditions used in the present studies little additional energy is required in the roots and nodules for nitrate utilization.

When plants growing on N-free and N-supplemented nutrient were exposed to different light levels, both C_2H_2 reduction and root + nodule respiration were affected by irradiance (Fig. 4). However, the slopes determined from the NH_4NO_3 and N-free nutrient treatments were independent of the light level (Fig. 5).

For the plants growing on NH_4NO_3 with virtually no C_2H_4 reduction (Fig. 4), equation 1 can be rearranged as follows:

$$R/W = R_M + R_G \frac{dW/dt}{W} \quad (2)$$

Using mean dry weight between harvests and the dry weight increase to estimate W and dW/dt, respectively, R_M and R_G were estimated from the regression of respiration per unit of root + nodule dry weight on relative growth rate (Table III).

Assuming similar values of R_M and R_G for N-fixing plants, the growth respiration, maintenance respiration, and respiration associated with nitrogenase (R-$R_M W$-$R_G\, dW/dt$) were also estimated (Table III). All three components increased with irradiance. The plot of this respiration component *versus* C_2H_2 reduction passed through the origin and had the same slope as the plots of total respiration *versus* C_2H_2 reduction at the three light levels (Fig. 5). Since this slope is an estimate of R_F, the results show that as long as the treatment conditions are the same for both N-free and +N-treated plants, differences in growth and maintenance affect only the intercept of the respiration-C_2H_2 reduction relationship, and the slope is a reliable estimate of R_F.

The mean rates of C_2H_2 reduction by plants grown without added N were compared to the mean rates of N accumulation calculated from the harvest data (Table IV). The intercept of

Table III. Estimation of the components of root + nodule respiration in intact nodulated peas treated with or without NH_4NO_3 (15 mM) at three light levels

W_1 and W_2 are root + nodule dry weights (Table II), R is root + nodule respiration and R_G, R_M and R_F are the respiration coefficients for growth, maintenance and nitrogenase related respiration.

NH_4NO_3 Treatment

Light Intensity	W_1	W_2	$\bar{W}$	ΔW	$\Delta W/\bar{W}$	$R/\bar{W}$
	g	g	g	g week^{-1}	week^{-1}	mg hr^{-1} g^{-1}
High	0.45	0.81	0.63	0.36	0.57	10.90
Medium	0.45	0.52	0.49	0.07	0.14	9.49
Low	0.45	0.40	0.43	-0.05	-0.12	7.56

By regression $R/\bar{W} = 8.39 + 4.68\ \Delta W/\bar{W}$ (see equation 2)

N-free Treatment

Light Intensity	W_1	W_2	$\bar{W}$	ΔW	$R_G \Delta W$	$R_M \bar{W}$	$R - R_G \Delta W - R_M \bar{W}$
	g	g	g	g week^{-1}	mg hr^{-1}	mg hr^{-1}	mg hr^{-1}
High	0.45	0.82	0.64	0.37	1.73	5.37	4.53
Medium	0.45	0.57	0.51	0.12	0.56	4.28	2.52
Low	0.45	0.46	0.46	0.01	0.05	3.86	1.46

Table IV. Mean rates of nitrogen accumulation (ΔN) and C_2H_2 reduction by intact nodulated pea plants growing without added nitrogen at 3 light levels and regression equation derived from these values

Light	ΔN (μmol N_2 hr^{-1})	C_2H_2 reduced (μmol hr^{-1})
High	7.45	22.1
Medium	4.05	12.1
Low	2.98	8.5

C_2H_2 reduced = 3.02 ΔN - 0.33.

Table V. Regression coefficients of root + nodule respiration on C_2H_2 reduction from all experiments

Plants in all experiments were grown in the same conditions before the beginning of treatments. Each regression coefficient was determined using results from plants treated with N-free nutrient and nutrient containing a specific nitrogen source. X indicates the mean of all values with 99% confidence interval and R_F was determined after subtraction of estimated growth and maintenance respiration.

Experiment	N Added	n*	b**
N source	KNO_3	10	0.25 ± .02
	NH_4NO_3	10	0.21 ± .04
Concentration	NH_4Cl	15	0.24 ± .06
	KNO_3	20	0.19 ± .04
High Light	NH_4NO_3	10	0.23 ± .05
Medium Light	NH_4NO_3	10	0.23 ± .05
Low Light	NH_4NO_3	10	0.27 ± .04
$\bar{X}$			0.23 ± .04
R_F			0.22 ± .02

* n = total number of N-free and +N treated plants
** b = regression coefficient with standard error

the relationship was not significantly different from zero and the regression coefficient with its standard error indicated a conversion factor for this experiment of 3.02 ± 0.08 mole C_2H_2 reduced/mol N_2 fixed. The similarity of this value to the theoretical value of 3 (2, 7) is most likely fortuitous since at 0.02 atm C_2H_2 in air maximum rates of C_2H_2 reduction are not achieved (unpublished results) and since rates measured during the day cannot be expected to integrate the complete diurnal pattern (13, 16). The importance lies in the proportionality between rates of C_2H_2 reduction and N_2-fixation which supports the contention that R_F does estimate the relative respiration requirement for N fixation.

The results from all experiments (Table V) show the reproducibility of the method, regardless of N source or light regime. The mean value with 99% confidence limits was 0.23 ± 0.04 mg CO_2 (μmol C_2H_2 reduced)$^{-1}$, the same as the R_F value (0.22 ± 0.02) calculated by subtraction of growth and maintenance respiration. Using the conversion factor from the final experiment and a general carbohydrate conversion factor (1 g of CO_2 = 0.68 g of CHO) this value represents a requirement of 17 ± 3 g CHO/g of N fixed which is similar to values of 11 to 20 g of CHO/g of N fixed estimated by other methods (3, 15).

These results do not resolve the question of the relative energy requirements of N_2 and NO_3^- utilization. Previous studies have indicated a similar biological cost of the two processes (5, 15) while the results presented here showed considerably more CO_2 being respired by the roots and nodules in the N-fixing plants. If most of the nitrate reduction occurred in the shoots, it is possible that on a whole plant basis, the energy requirements were similar. In these experiments there was a consistently lower rate of photosynthetic CO_2 uptake in plants grown with NO_3^- than in those grown in N-free nutrient when rates were measured at irradiance levels up to 40 nE cm^{-2} sec^{-1} (Figs. 2 and 4). In the first experiment, there was no detectable difference between nutrient treatments when photosynthesis was measured at 100 nE cm^{-2} sec^{-1}. Since both nitrate and nitrite reductase systems in leaves use reducing equivalents which are directly or indirectly derived from photosynthesis (9), it may be that CO_2 fixation and nitrate-nitrite reduction compete for the photosynthetically generated reducing equivalents as has been suggested in other systems (18, 21, 23). Examination of this idea requires more detailed studies involving the C and N economies of both shoots and roots.

Generally the results support the proposal that respiration associated with N fixation can be included as an additive respiration component in nodulated legumes. A simple method for determining the coefficient for this respiration has been proposed and tested. Estimates of the coefficient, which should reflect the energy requirements of the N_2-fixing process, were

reproducible when the method was applied to a single pea variety inoculated with a commercial inoculant. The method may be useful in evaluating treatment or genotypic differences in the efficiency of energy use by N-fixing systems.

Acknowledgment – The author is grateful for the technical assistance of D. S. Baliski.

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