

Ontogenetic Interactions between Photosynthesis and Symbiotic Nitrogen Fixation in Legumes¹

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ABSTRACT

Photosynthetic data collected from *Pisum sativum* L. and *Phaseolus vulgaris* L. plants at different stages of development were related to symbiotic N₂ fixation in the root nodules. The net carbon exchange rate of each leaf varied directly with carboxylation efficiency and inversely with the CO₂ compensation point. Net carbon exchange of the lowest leaves reputed to supply fixed carbon to root nodules declined in parallel with H₂ evolution from root nodules. The decrease in H₂ evolution also coincided with the onset of flowering but preceded the peak in N₂ fixation activity measured by acetylene-dependent ethylene production. A result of these changes was that the relative efficiency of N₂ fixation in peas increased to 0.7 from an initial value of 0.4. The data reveal that attempts to identify photosynthetic contributions of leaves to root nodules will require careful timing and suggest that the relative efficiency of N₂ fixation may be influenced by source-sink relationships.

The importance of photosynthesis for symbiotic N₂ fixation in legumes has been inferred from various physiological experiments which altered the availability of photosynthetic products and revealed a corresponding change in symbiotic N₂ fixation (6, 8, 17, 21, 24). Ontogenetic patterns of N₂ fixation have been reported for various legumes, but there appear to be no data relating CO₂ and N₂ reduction in the same plant. The export of photosynthates to the root system has been established to be primarily a function of the lower leaves (14, 22). A basis for identifying legumes with an increased potential for N₂ fixation may result from understanding CO₂ exchange characteristics in these leaves, particularly that of carboxylation efficiency. This latter parameter is of interest because it has been found to exhibit genotypic variation independent of diffusive resistance (1).

Numerous studies on symbiotic N₂ fixation have utilized the C₂H₂ reduction assay (5) to measure N₂ fixation capacity. Recent data, however, suggest that C₂H₂ reduction assays may overestimate nitrogenase activity by inhibiting an ATP-dependent production of H₂ by the nitrogenase complex (18). Schubert and Evans (18) suggested that the relative efficiency of electron transfer to N₂ via nitrogenase may be defined as:

$$1 - \frac{\text{H}_2 \text{ evolved}}{\text{C}_2\text{H}_2 \text{ reduced}}$$

where H₂ evolution is determined in the absence of C₂H₂.

The present study relates photosynthesis, the ultimate source of reducing power, and pod formation, a dominant sink process for fixed N₂, with the efficiency of N₂ fixation during the devel-

opment of plants of two leguminous genera grown under specified environmental conditions in the absence of combined nitrogen.

MATERIALS AND METHODS

Growth Conditions. Pea (*Pisum sativum* L. var. Alaska) and bean (*Phaseolus vulgaris* L. var. Blue Lake Bush) plants were maintained in a growth chamber under a 16/8-hr light/dark cycle at 21/15 C, 50/70% relative humidity, and a light intensity of 650 μEi.² Photon flux was measured with a Lambda Instrument LI-185 quantum sensor in the photosynthetically active range. Plants were grown in vermiculite in 180-ml plastic pots, and watered three times weekly with a nitrogen-free nutrient solution. Macro- and micronutrient compositions were according to Hewitt (10) and Johnson *et al.* (11), respectively. In addition the solution was 4.2 mM in CoCl₂. Peas and beans were inoculated with *Rhizobium leguminosarum* 128C53 and *Rhizobium phaseoli* 127K17 (obtained originally from J. C. Burton, Nitragin Co., Milwaukee, Wis.). Assays of CO₂ and N₂ fixation were made at weekly intervals. Dry weights of plant parts were determined after 24 hr at 75 C.

Carbon Dioxide Fixation. Assimilation of CO₂ by attached leaves was measured in a flow-through gas exchange system with apparatus and data-handling procedures as described by Augustine *et al.* (1). Plants were selected randomly from a uniform stand each week and assayed by inserting each leaf individually into the assimilation chamber and measuring net CO₂ exchange at a light intensity of 1,500 μEi, with a chamber temperature of 21 C and four CO₂ concentration (50, 100, 150, and 300 μl/l). Leaves were detached after photosynthetic measurement, and their area determined with a Lambda Instruments LI-3000 area meter. Data obtained for net CO₂ uptake and the equilibrium CO₂ concentrations in the chamber were computer-plotted (1) as a second order regression line through the data points corresponding to the four CO₂ concentrations used. The CO₂ compensation point (x intercept) and the slope of the regression line at the compensation point were determined by extrapolation. This slope, an indication of the leaf's capacity to respond to changes in ambient CO₂ concentrations, was used as a measure of carboxylation efficiency as proposed by Tregunna *et al.* (23).

Nitrogen Fixation. Acetylene-dependent C₂H₄ production by root nodules was used as a measure of the total flow of electrons through the nitrogenase complex (18). Hydrogen evolution by root nodules was determined in ambient air and could not be detected in the presence of C₂H₂. Roots were cut 5 mm above the cotyledonary node, freed from vermiculite by gentle shaking, and placed in jars containing an average of 200 ml of free space. Gas samples for H₂ determinations were taken from the jars 30 min after sealing. The jars were then opened, flushed with ambient air, sealed, and adjusted to contain 0.1 atm C₂H₂ in

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² Abbreviation: μEi, microeinstein · m⁻² · sec⁻¹.

ambient air. Gas samples were taken after 5 and 35 min, and the hourly rate was computed from the difference. Both H_2 evolution and C_2H_2 -dependent C_2H_4 production were linear with time under the assay conditions. Ethylene analyses were made with a Parkin-Elmer model 3920B gas chromatography equipped with a hydrogen flame ionization detector. Acetylene and C_2H_4 were separated on a column (0.3×122 cm) filled with Porapak R (100–200 mesh). Hydrogen was measured with a thermal conductivity detector attached to the same instrument and analyzed on a column (0.3×254 cm) filled with Molecular Sieve 5A (60–80 mesh). Nitrogen served as a carrier gas in both cases at a flow rate of 30 ml/min. Oven temperatures were 45 C for C_2H_4 and 100 C for H_2 .

RESULTS

Data collected from pea and bean plants were qualitatively identical. Results are reported only for peas, but all conclusions drawn were supported also by data from beans (3).

Individual pea leaves were photosynthetically active for a period of about 5 weeks (Fig. 1a). The net CO_2 uptake rate of each leaf peaked during the 2nd week after CO_2 fixation was first detected, and the leaves senesced in an acropetal sequence. During the 8th week after planting the first six to eight leaves ceased to function as sources of photosynthate. The total net CO_2 uptake rate for the first seven leaves was maximal during the 5th week (Fig. 2) coinciding with anthesis of the first flower (see Fig. 4). Net CO_2 uptake for the entire plant peaked during the 7th week, by which time the first six leaves had become photosynthetically inactive (Fig. 2). Pod formation did not occur below the seventh node. Dry weight of reproductive structures

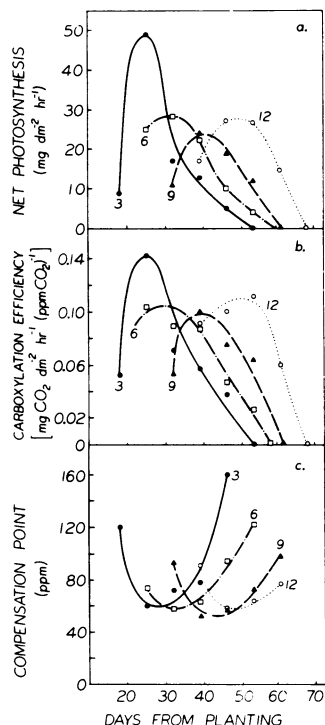


FIG. 1. Photosynthetic parameters of pea leaves. Measurements were made at weekly intervals on all leaves of different plants selected at random from a uniform stand. Curves are annotated to reflect number of leaf in order of development. Photosynthetic data on leaves not shown were similar to those shown; maximum number of leaves was 17; all plants had senesced by day 73 after planting. a: Photosynthetic activity expressed as net CO_2 uptake per unit leaf area; b: carboxylation efficiency as reflected by the slope at the CO_2 compensation point of a regression line characterized by net CO_2 uptake at different external CO_2 concentrations; c: CO_2 compensation point calculated by extrapolating the regression line of b to the x (CO_2 concentration) axis.

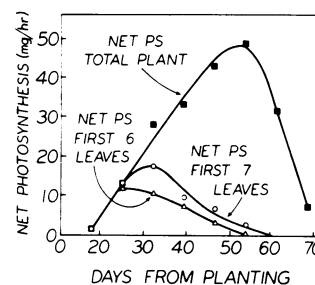


FIG. 2. Net CO_2 uptake in pea leaves. Curves reflect a summation of net CO_2 uptake by all leaves, and by the first six or seven leaves reputed to be major sources for the root system. Data were determined on different plants each week; plants were selected at random from a uniform stand.

increased slowly for 3 weeks after the onset of flowering (see Fig. 4). The decline in total plant CO_2 uptake began during the linear phase of rapid pod filling (Figs. 2 and 4). Further increase in pod weight was accompanied by a rapid decline in net CO_2 uptake and appeared to occur at the expense of vegetative structures (Figs. 2 and 4; Table I). Complementary data obtained for carboxylation efficiency at weekly intervals showed a pattern similar to net CO_2 exchange. Carboxylation efficiencies and CO_2 compensation points had an inverse relationship during the development of each leaf: efficiency was low and the compensation point high in young and senescent leaves (Fig. 1, b and c). The difference between the maxima and minima of net CO_2 uptake, carboxylation efficiency, and compensation points was greatest in the first four leaves.

Maximum H_2 evolution occurred during the 5th week after planting, while C_2H_2 -dependent C_2H_4 production was greatest 1 week later (Fig. 3). Hydrogen evolution prior to the 4th week after planting was below the level of resolution of our instrument; therefore, an H_2/C_2H_4 ratio could not be computed for the 3rd week. Data in Figure 3 were used to calculate the relative efficiency of N_2 fixation (Fig. 4). The results of these calculations revealed that relative efficiency increased between the onset of flowering and the rapid phase of pod filling, and stabilized thereafter. Linear regression analyses of the increasing and stable portions of the relative efficiency curve show that the probability of a common slope is low ($P \leq 0.005$). Data from beans revealed a highly significant ($P \leq 0.01$) increase in relative efficiency from 0.48 to 0.58 at the same stage of development (3).

The time of maximum H_2 evolution coincided with the peak in net CO_2 uptake in the first seven leaves, with a marked acceleration in total plant dry matter accumulation and with pod formation (Figs. 2–4; Table I). The time of maximum C_2H_2 production (Fig. 3) correlated with the cessation of nodule growth (Table I) and the onset of rapid pod filling (Fig. 4).

DISCUSSION

The CO_2 exchange characteristics of pea leaves showed that values for net carbon exchange, carboxylation efficiency, and CO_2 compensation point varied markedly at different stages of development (Fig. 1). Variation in CO_2 compensation points as a result of plant age was reported earlier by Smith *et al.* (20). The fact that all 17 leaves on pea plants and the first five primary (nonaxillary) leaves produced by the bean plants showed similar developmental changes in CO_2 exchange characteristics suggests that using these traits to determine variations between genotypes in carboxylation efficiency will require careful timing and standardization.

The patterns of H_2 evolution and C_2H_2 reduction differed in that maximum H_2 production preceded maximum C_2H_2 reduction (Fig. 3). This divergent pattern produced an increase in the relative efficiency of N_2 fixation (Fig. 4) as computed by the

formula proposed by Schubert and Evans (18). The increase in relative efficiency of N_2 fixation began at the onset of flowering and decline in photosynthetic production by the first six leaves during the 5th week after planting (Figs. 2 and 4) and continued until rapid pod filling, during the 8th week (Fig. 4).

Other workers have shown that upper leaves supply assimilates to the shoot apex, while the majority of the assimilates exported from a lower leaf move downward to the root and nodules (14), that pea leaves subtending pods export a negligible amount of assimilate to the rest of the plant (9), and that an inadequate assimilate supply to the nodules causes a decline in symbiotic N_2 fixation (12). Because peas in this study formed pods from the seventh node up, only the lowest six leaves can be considered significant sources to the root system. Therefore, from the 5th week on, when pod formation was initiated at the seventh node and net carbon exchange in the first six leaves declined, root nodules presumably received less photosynthate from the shoot. At the same time the export of root nodule assimilates probably increased (16) under the influence of the developing pods (2, 13, 19). This process may be expected to continue until that time in pod filling at which import by the fruit of nitrogen already present in the plant body predominates over import from the nodules (15). The latter time may be indicated by the cessation of further increase in the relative efficiency of N_2 fixation (Fig. 4), rapidly decreasing C_2H_2 -dependent C_2H_4 formation (Fig. 3), and peaking net CO_2 exchange by the entire plant (Fig. 2).

The coincidence of the increase in relative efficiency of N_2 in peas with declining photosynthetic activity in the first six leaves and with the development of reproductive structures suggests a

causal relation between the change in relative efficiency and changes in source-sink activity. An increase in relative efficiency of N_2 fixation should offer a potential advantage to the plant in that less energy is lost through H^+ reduction in the root nodules at the time when import from the photosynthetic sources available to the nodules is declining and reproductive sinks for organic nitrogen compounds are enlarging. At least two possible mechanisms can be postulated for the observed increase in the relative efficiency of N_2 fixation. First, varying physiological conditions may modify the environment of nitrogenase and cause a change in the proportion of electron allocation to N_2 or H^+ (4) by this enzyme complex. Alternatively, the expression of hydrogenase activity previously demonstrated in *Rhizobium* (7) may be induced or altered by ontogenetic changes in the host plant, or by the presence of increasing amounts of H_2 in the root nodule as a result of nitrogenase activity. Under our assay conditions hydrogenase activity would reduce the amount of H_2 available for measurement, and would thus appear to have the same effect as a decrease in electron allocation to H^+ .

Our observation of a shift in the relative efficiency of N_2 fixation in beans under conditions similar to those in peas suggests that this phenomenon is not an isolated one in the Leguminosae (3).

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LITERATURE CITED

- AUGUSTINE JJ, MA STEVENS, RW BREIDENBACH, DF PAIGE 1976 Genotypic variation in carboxylation of tomatoes. *Plant Physiol* 57: 325-333
- BEEVERS L, R POULSON 1972 Protein synthesis in cotyledons of *Pisum sativum* L. *Plant Physiol* 49: 476-481
- BETHLENFALVAY GJ, DA PHILLIPS 1977 Photosynthesis and symbiotic nitrogen fixation in *Phaseolus vulgaris*. L. In *Genetic Engineering for Nitrogen Fixation*. Plenum, NY. *Basic Life Sci* 9: 401-408
- BURNS RC, RW HARDY 1975 Nitrogen Fixation in Bacteria and Higher Plants. Springer-Verlag, New York pp 121-122
- BURRIS RH 1972 Nitrogen fixation—assay method and techniques. *Methods Enzymol* 23B: 415-431
- DIENER T 1950 Über die Bedingungen der Wurzelknöllchenbildung bei *Pisum sativum* L. *Phytopathol Z* 16: 129-170
- DIXON ROD 1972 Hydrogenase in legume root nodule bacteroids: occurrence and properties. *Arch Microbiol* 85: 193-201
- HARDY RWF, UD HAVELKA 1975 Nitrogen fixation research: a key to world food? *Science* 188: 633-643
- HARVEY DM 1971 Translocation of ^{14}C in *Pisum sativum* tissue. *Annu Rep John Innes Inst, Norwich, Engl* 62: 35-36
- HEWITT EJ 1966 Sand and water culture methods used in the study of plant nutrition. Commonwealth Bureau of Horticulture and Plantation Crops, East Malling, Maidstone, Kent. Technical Communication 22, Ed 2 p 189
- JOHNSON CM, PR STOUT, TC BROYER, AB CARLTON 1957 Comparative chlorine requirements of different plant species. *Plant Soil* 8: 337-353
- LAWN RJ, WA BRUN 1974 Symbiotic nitrogen fixation in soybeans. I. Effect of photosynthetic source-sink manipulations. *Crop Sci* 14: 11-16
- MILLER A, D SPENCER 1974 Changes in RNA-synthesizing activity and template activity in nuclei from cotyledons of developing pea seeds. *Aust J Plant Physiol* 1: 331-341
- PATE JS 1966 Photosynthesizing leaves and nodulated roots as donors of carbon to protein of the shoot of the field pea (*Pisum arvense* L.). *Ann Bot* 30: 93-109
- PATE JS, AM FLINN 1973 Carbon and nitrogen transfer from vegetative organs to ripening seeds of field pea. *J Exp Bot* 24: 1090-1099
- PATE JS, J WALKER, N WALLACE 1965 Nitrogen-containing compounds in the shoot of *Pisum arvense* L. II. The significance of auricles and amino acids released from roots. *Ann Bot* 29: 475-493
- PHILLIPS DA, KD NEWELL, SA HASSELL, CE FELLING 1976 Effect of CO_2 enrichment on root nodule development and symbiotic N_2 reduction in *Pisum sativum* L. *Am J Bot* 63: 356-362
- SCHUBERT KR, HJ EVANS 1976 Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. *Proc Nat Acad Sci USA* 73: 1207-1211
- SMITH DL 1973 Nucleic acid, protein and starch synthesis in developing cotyledons of *Pisum arvense* L. *Ann Bot* 37: 795-804
- SMITH EW, NE TOLBERT, HS KU 1976 Variables affecting the CO_2 compensation point. *Plant Physiol* 58: 143-146
- STREETER JG 1974 Growth of two soybean shoots on a single root. *J Exp Bot* 25: 189-198
- THROWER SL 1962 Translocation of labelled assimilates in the soybean. *Aust J Biol Sci* 15: 629-649
- TREGUNNA EB, G KROTKOV, CD NELSON 1966 Effect of oxygen on the rate of photorespiration in detached tobacco leaves. *Physiol Plant* 19: 723-733
- WILSON PW, EB FRED, MR SALMON 1933 Relation between carbon dioxide and elemental nitrogen assimilation in leguminous plants. *Soil Sci* 35: 145-165

Table I. Dry weights of *Pisum sativum* organs

Age Days	Dry weight (g)				
	Leaves	Pods	Roots	Nodules	Total plant
18	0.08 ± 0.02	—	0.10 ± 0.01	0.002 ± 0.001	0.21 ± 0.03
25	0.13 ± 0.01	—	0.11 ± 0.01	0.022 ± 0.001	0.30 ± 0.03
32	0.41 ± 0.09	0.05 ± 0.02	0.22 ± 0.02	0.045 ± 0.003	0.92 ± 0.10
39	1.13 ± 0.13	0.19 ± 0.10	0.50 ± 0.07	0.078 ± 0.003	2.33 ± 0.11
46	1.55 ± 0.11	0.49 ± 0.06	0.57 ± 0.03	0.118 ± 0.016	3.53 ± 0.30
53	1.57 ± 0.29	2.08 ± 0.46	0.54 ± 0.08	0.103 ± 0.007	5.35 ± 0.42
60	1.31 ± 0.27	3.17 ± 0.42	0.59 ± 0.06	0.082 ± 0.021	6.86 ± 0.91
68	0.36 ± 0.01	3.47 ± 0.33	0.52 ± 0.19	0.076 ± 0.008	5.79 ± 0.55

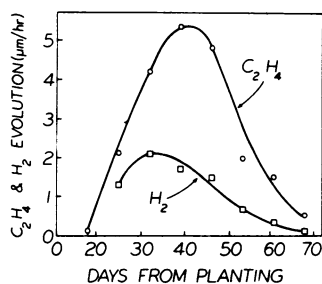


FIG. 3. Root-nodule activity in pea plants. Acetylene-dependent C_2H_4 production and H_2 evolution data reflect the average of three replicates.

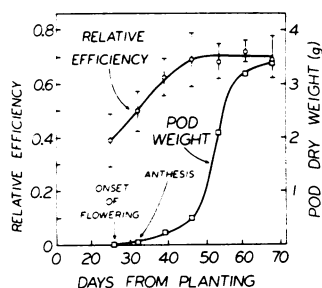


FIG. 4. Development of reproductive structures and relative efficiency of N_2 fixation in pea plants. Data are recorded as the mean \pm standard error from three replicates.