

Role of Nitrogen Assimilation in Seed Development of Soybean

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ABSTRACT

A nondestructive acetylene reduction assay for nitrogenase activity of soybean (*Glycine max* L. Merr) field plots is presented. Plots consisted of 120 × 150 × 30 centimeter boxes containing 65 plants. The plants were grown in a medium grade sand under controlled nutrient, moisture, and root temperature conditions. Acetylene at a concentration of 10 milliliters per liter was circulated through manifolds in the chambers; equilibration required 5 minutes, and activity was linear with time. Optimum growth and assay environments resulted in activity of 70 micromoles ethylene per plant per hour. Plant development and yield were comparable to soil-grown companion plots.

The well accepted hypothesis that developing seeds deprive the nodules of carbohydrate was not substantiated. The nondestructive acetylene reduction profile did not decline until 30 days after the onset of seed development (R-5). This result was consistent with reports from the literature which indicated that 60% of seasonal nitrogen was fixed after R-5. Further, a high correlation shown between integrated seasonal acetylene reduction and yield ($r = 0.999$) suggested a cooperative relationship between the roots and shoot. A reduction in source:sink ratio (60% defoliation) after R-5 had no effect on acetylene reduction. This showed that neither an increase in sink demand by the pods nor a carbon shortage during podfill decreased dinitrogen fixation. A conceptual model relating seed growth with carbon and nitrogen assimilation is proposed.

Development of AR¹ permitted point-in-time measurements of dinitrogen fixation, and examination of treatments or environmental effects that can influence dinitrogen fixation over a period of minutes or weeks. AR methodology in the field has either been nondestructive using single plants (4) or has involved detached roots, root parts, or nodules (7). The latter involves digging the root system out of the soil, washing, detaching the root, and measuring AR activity in a jar. Reports by Harper (8) and Lawn and Brun (13) presented seasonal profiles for dinitrogen fixation using the detached root assay. They found that AR declined rapidly after the onset of seed development. In 1975, Thibodeau and Jaworski (21) explained the relationship between dinitrogen fixation, inorganic nitrogen metabolism, and pod development, also using detached root measurements. The model consisted of several parts. (a) The soybean uses nitrate exclusively during vegetative development. (b) At or near flowering, the plant becomes unable to use nitrate, resulting in the initiation of dinitrogen fixation activity. (c) At the onset of seed development, stage R-5 (5), dinitrogen fixation peaks (21). Activity then declines rapidly as pods develop because the proximity of the pods to the source makes them a better sink. (d) As the source of nitrogen from fixation disappears, the plant derives the remainder of the seasons' nitrogen from redistribution.

¹ Abbreviation: AR, acetylene reduction.

This hypothesis, which has been extensively cited, was consistent with evidence available at the time. We began a program to determine the physiological processes limiting yield in soybean at various stages of development. The focus was on plants grown under optimum conditions and measured nondestructively. In the area of nitrogen assimilation, we began with an AR assay for plants grown in sand nutrient culture in the growth chamber (DR Nelson, CA Porter, RJ Bellville 1978 Abstract, 70th Annual Meeting of American Society of Agronomy, p 82), then extrapolated the methodology to develop a seasonal dinitrogen fixation profile in field plots. The results presented here suggest an active role for nitrogen assimilation during seed development.

MATERIALS AND METHODS

The AR system (Fig. 1) consisted of an above ground chamber, (Fiberglas Products Co., St. Louis) 120 × 150 × 30 cm containing 65 plants. The medium was an acid-washed graded river sand (Rauch Lumber Co., St. Louis). The chamber lid consisted of five rows of 4-cm-diameter holes, 23 cm between rows, and with an interplant spacing equivalent to 60,750 plants per hectare. The soybean (*Glycine max* L. Merr. cv Williams, except as noted) seed was inoculated ('S' culture, Nitragin) and then germinated on wet paper towels. When the radicles were 2 cm long, the seedlings were planted directly into the holes in the top of the chamber. All growth stages are as defined by Fehr and Caviness (5). Directly beneath the lid of the chamber was a watering and nutrient delivery manifold constructed from lengths of 0.64 cm diameter polyethylene tubing joined together with T's and crosses (Cole Parmer, Chicago). Water from a 5.08 cm diameter line connected to the city water supply and controlled by an electric solenoid and timer, was released through the watering manifold five times/d for 4 min. The water line was reduced to 1.27 cm before entering the chamber. Nutrient from a stock tank containing 11.4 kg/190 l Peters Peat-lite Special (20-19-18, N-P-K plus micronutrients; A. H. Hummert, St. Louis), was injected into the 5.08 cm line using a pump (Cole Parmer, Chicago). The resulting nutrient concentration was 4 mg/l. At R-2, the stock solution was changed to a Hoagland solution without nitrogen (11).

When there were three fully developed nodes on the main stem, V-3, sections of sealed cell tubing insulation, 4 cm long, 2.54 cm thick, with a 0.95 cm bore (Rubatex Corp., Branson, VA) were put around the stems. This made the chamber airtight except for the exhaust holes on top and the drain.

AR measurements were begun at full flowering, stage R-2. At 1/3 and 2/3 depth into the sand were gas circulation manifolds consisting of 0.64 cm polyethylene tubing, analogous in construction to the watering manifold, designed so that the 72 ends were equidistant from the center of the chamber and well distributed across the horizontal area of the chamber at each level. A 30 m length of 0.95 cm polyethylene tubing was connected to each manifold at the entry point into the chamber. These gas lines terminated at a portable instrument building (Morgan, St. Louis) where they were joined in a loop by a pump (Rand Corp.,

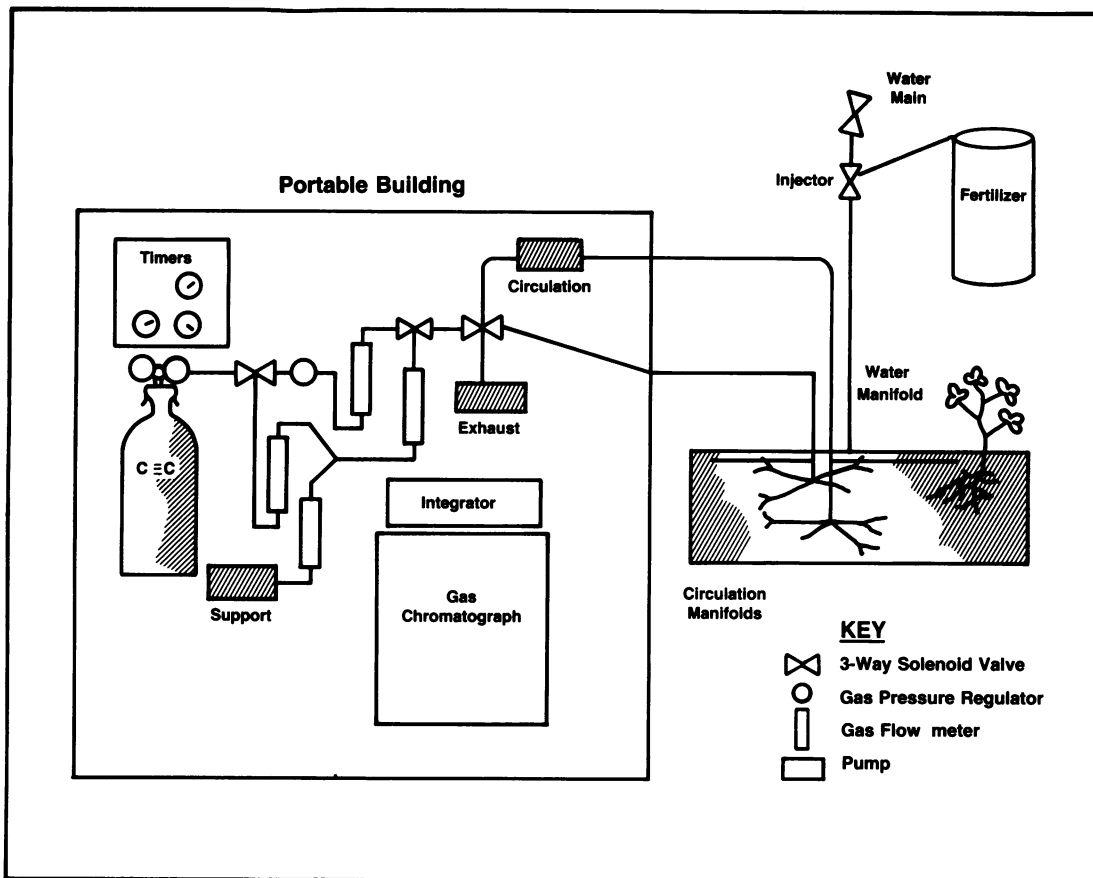


FIG. 1. Nondestructive acetylene reduction assay for soybean field plots. Plants were grown in sand nutrient culture, 65 plants/plot. Gas circulation manifolds were buried at two depths in the sand. To begin the assay, a pump circulated the air in the chamber through the manifolds while 2000 ml of acetylene were injected into the line. After analysis of samples for ethylene at 15 and 30 min, the residual acetylene and ethylene were expelled with an exhaust pump.

Bridgewater, NJ) during the assay. The assay was completely automated except for sample injection. Before starting, the drain and exhaust holes were covered. When the start button was pushed, the pump began to circulate the atmosphere between the upper and lower manifolds through 0.95 cm tubing. A solenoid was activated and 2000 ml of acetylene were injected into the circulation line. A second timer then caused the injection of a 40 ml/min air:acetylene mixture, 0.4:40 (v/v), to air seal the chamber for the duration of the assay. Samples were taken at 15 and 30 min and injected into a Hewlett Packard 5710A gas chromatograph having a column packed with Poropak 80, oven temperature at 65°C and nitrogen as the carrier at 40 ml/min. At the conclusion of the assay, the chamber was exhausted through both manifolds using a pump (Cole Parmer). This completely removed residual acetylene and ethylene. The plots were measured twice/week. Activities were calculated as $\mu\text{mol ethylene/plant} \cdot \text{h}$. There were three replicates per treatment. The replicates were run simultaneously and then averaged. In the first year, there were three additional control chambers, identical to the others, but never measured for AR. There was no significant difference in yield, growth habit, or developmental pattern between measured and unmeasured controls or between sand-grown plants and soil-grown companion plots. Activity was linear as a function of time, equilibration required less than 5 min, and initial and final acetylene concentrations were within 10%. Because AR activity is directly proportional to acetylene concentration below 20 ml/l, all ethylene values were corrected to a standard acetylene concentration of 10 ml/l to account for slight deviations in the quantity of acetylene released into the chamber

initially. Defoliation consisted of removing the lateral leaflets (60%) or terminal leaflet (40%) twice/week beginning at the stage of development specified.

RESULTS AND DISCUSSION

Seasonal Nitrogen Assimilation Patterns. In Figure 2, the 1980 nondestructive seasonal AR profile is superimposed on the model of Thibodeau and Jaworski (21). The consistency of the nondestructive AR assay is shown in a plot of the control data from 1978 to 1981 (Fig. 3). The peak AR rate of 70 $\mu\text{mol ethylene/plant} \cdot \text{h}$ using this system and 10 ml/l acetylene, is proportional to maximum activities reported in the literature using saturating acetylene concentrations (7, 13).

The fact that AR does not decline until 30 d after R-5 is in contrast with that portion of the Thibodeau and Jaworski model suggesting that seed development competes with and eventually starves dinitrogen fixation of carbon. Nitrate reductase and AR profiles reported earlier by Harper (8) were consistent with the starvation model. The increase in AR we observed after R-5 suggests a cooperative rather than antagonistic relationship between dinitrogen fixation and seed development. Reports in the literature in which nitrogen analyses were made at R-5 and at physiological maturity, R-8 (Table I), demonstrate that 60% to 70% of the seasonal nitrogen is assimilated after the beginning of seed development. Integration of the nondestructive profiles in Figure 3 demonstrates that 78% of seasonal dinitrogen fixation occurs after R-5 under conditions where the atmosphere is the sole source of nitrogen.

Several seasonal profiles of dinitrogen fixation are found in

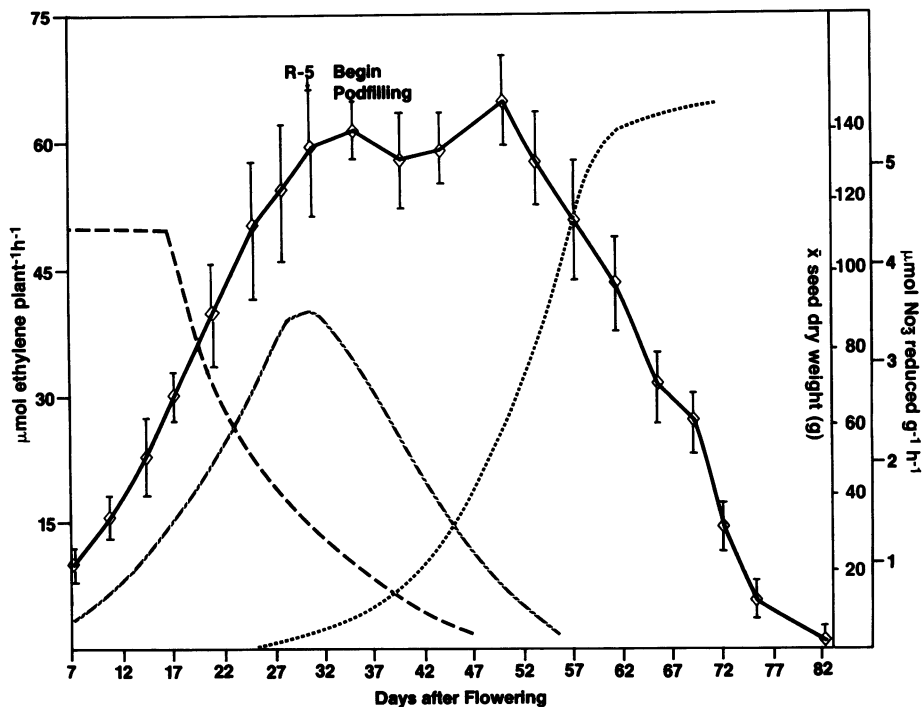


FIG. 2. Comparison of nondestructive and detached root acetylene reduction profiles. The nondestructive acetylene reduction profile (\diamond — \diamond) was determined using var Williams. Each replicate consisted of 65 plants, and each point was a mean of three replicates. The acetylene concentration was 10 ml/l. Destructive acetylene reduction profile (— \circ —), nitrate reductase (— \square —), and seed weight (— \square —) are components of a nitrogen assimilation/yield model presented by Thibodeau and Jaworski in 1975. The variety Wayne and 100 ml/l acetylene were used. The profiles were normalized on the horizontal axis based on stage R-1. The bars represent SE.

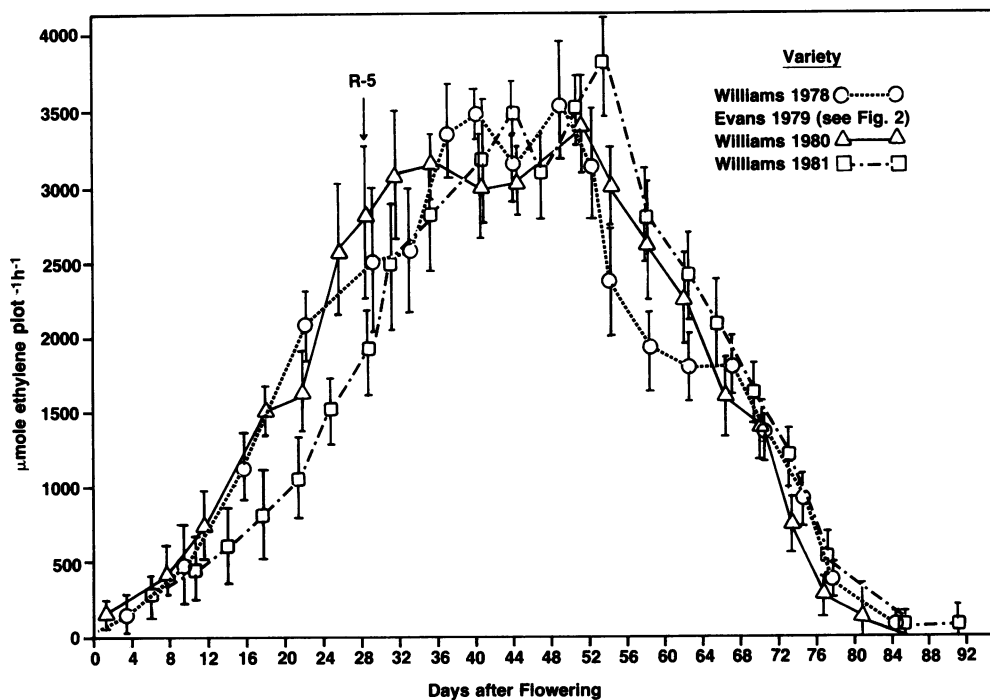


FIG. 3. Three years of seasonal non-destructive acetylene reduction control data. Each point is a mean of three replicates. Profiles are plotted over one another using stage R-1 as zero time. The bars represent SE.

the literature (7-9, 16, 21). Apparent discrepancies in these seasonal fixation patterns may be related to the distribution of nodules recovered during excavation when detached roots are used (18). Differences in profiles may also result from cultural or environmental influences on the host and nodule. In soil, high levels of combined nitrogen or a dense growth medium can decrease AR activity in apparent synchrony with increasing seed weight (Nelson, Porter, Bellville 1978 Abstract). In a sand system with low nitrate after flowering and a high productivity environment, AR will not peak until long after R-5. In summary, the decline in dinitrogen fixation is not tightly coupled with seed development. The proportion of nitrogen assimilated after R-5 (Table I) supports this conclusion.

The occurrence of nitrogen assimilation in nonnodulated beans after R-5 (Table I) is enigmatic in the context of published nitrate reductase profiles (8, 21). Nitrate reductase (Fig. 2) and leaf nitrate concentration (21) essentially disappear by stage R-5, suggesting that the soybean is incapable of assimilating nitrate during seed development. Nitrate reductase activity, which must be sufficient to supply the system late in the season (Table I), may exist in the roots (12) and stems to supplement the capacity remaining in the leaves.

Clearly, assimilation rather than redistribution appears to be the primary source of nitrogen late in the season. Organ by organ analyses show that the vegetative nitrogen losses cannot account for the total nitrogen gains during seed development (17, 22).

Table I. Nitrogen Accumulated between R-5 and Harvest

Nitrogen Treatment		Before R-5	After R-5	After R-5 (% of Total)	Reference
Nodulated	100 kg/ha	90 ^a	190	68	16
Nonnodulated	100 kg/ha	50 ^a	83	58	16
Nodulated	0 kg/ha	97 ^a	200	67	16
Nonnodulated	0 kg/ha	31 ^a	19	38	16
Nonnodulated	200 kg/ha	80 ^a	170	68	16
Nodulated	40 ppm	907 ^b	1789	66	9
Nonnodulated	40 ppm	563 ^b	801	59	9
Nodulated	18.75 kg/ha	900 ^c	1000	54	22
Nodulated	0 kg/ha	124 ^a	127	55	15
Nodulated	0 kg/ha	1.06 ^b	1.81	65	23

^a kg/ha.
^b g/plant.
^c g/treatment.

Comparison of nodulating and nonnodulating isolines indicates that plants whose source of nitrogen is from fixation lose less nitrogen from the leaves late in the season than plants receiving only nitrate (17). This may result in extended photosynthetic activity and account for the consistently higher yields where dinitrogen fixation is present.

In Figure 4 are the seasonal profiles for four varieties; Fiskeby V, a group 000; Evans, a group 0; Williams and Lincoln, group III. The stage of development at which peak activity occurred was the same for each variety. It has been reported that yield is highly correlated with seasonal net photosynthesis (3). A regression correlating yield *versus* seasonal AR for these four varieties and for control data from 1978 to 1981 resulted in $r = 0.999$. This is evidence that soybean yield is nitrogen limited. Elevation of nitrate at any stage (8, 9) or CO₂ during podfilling (10) will enhance yield, yet 'limitation' is a difficult problem to address. Where studies have demonstrated a carbon limitation, *e.g.* the effect of elevated CO₂ or supplemental light during podfilling, plants were fully nodulated and increases in dinitrogen fixation were twice the increases in yield or vegetative weight (10). The mutual stimulation of both processes suggests that optimum

yield involves satisfaction of both a carbon and nitrogen limitation.

Defoliation. The evidence above demonstrates that seed development does not destroy dinitrogen fixation. If pods are more competitive for carbon than the roots, however, a treatment which reduces source:sink ratio should decrease dinitrogen fixation more than yield. Seasonal AR profiles from continuous 60% defoliation and complete depodding experiments are shown in Figure 5. These results demonstrate that a 60% reduction in carbon availability during podfill does not decrease AR activity. The apparent increase in dinitrogen fixation shortly after leaf removal may be coupled with the increase in photosynthetic capacity/unit leaf area associated with defoliation (20). The decline in AR activity of depodded plants, without apparent senescence, is analogous with observations that photosynthesis declines after depodding while leaves remain green (13). The near normal AR profile without pods is consistent with the capability of depodded plants to accumulate dry matter at rates comparable to control plants through maturation of alternate sinks (14).

The effect of 40% or 60% defoliation after R-5 on yield (Table II) is consistent with the literature (19). A 60% defoliation has no effect on seasonal dinitrogen fixation and results in only a 23% decrease in yield. Because decreased seed yield appears to be the initial response to decreased source, one of two explanations is most likely. Either the roots are more competitive for carbon than the pods or there is a difference in sink response to carbon stress. Further, if carbon supply can be decreased by greater than 50% with little effect on dinitrogen fixation or greater than 30% with no effect on yield, this suggests that carbon is less of a limitation during podfill than nitrogen. The lack of a change in root-soluble carbohydrate during podfill is also contrary to the root starvation hypothesis (24). The greater yield reduction from defoliation during the period from R-5 to R-8 compared with R-2 to R-8 may have resulted from reduced capability of photosynthesis to recover during a period when sink demand is maximum (6).

A number of hypotheses that are consistent with the literature will describe the relationship between nitrogen fixation, photo-

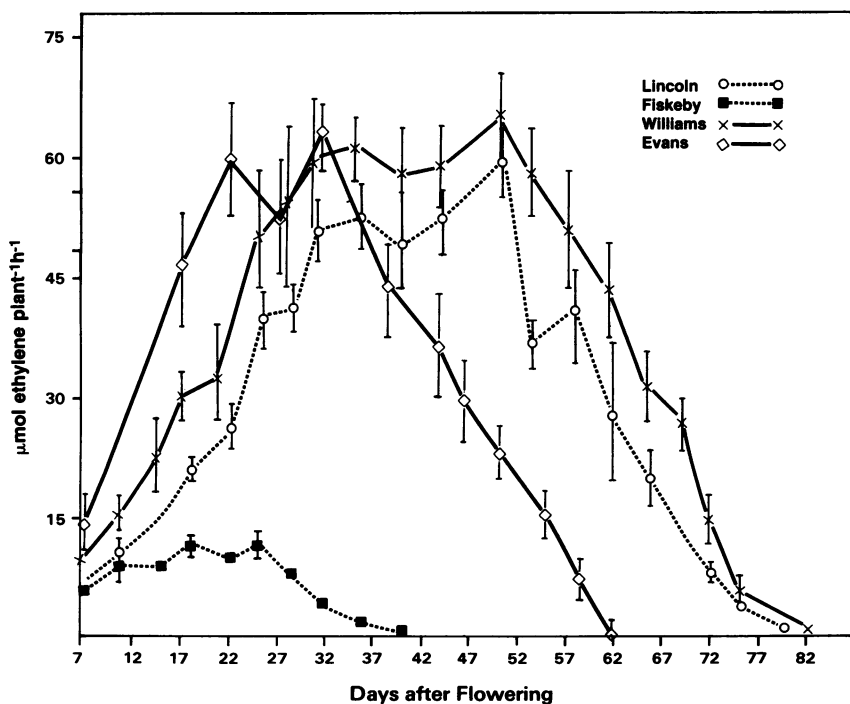


FIG. 4. Seasonal nondestructive acetylene reduction profiles of four varieties. Each point is a mean of three replications. Date of flowering varied between varieties; however, data are plotted using R-1 as zero time. The experiment was run in 1979. The bars represent SE.

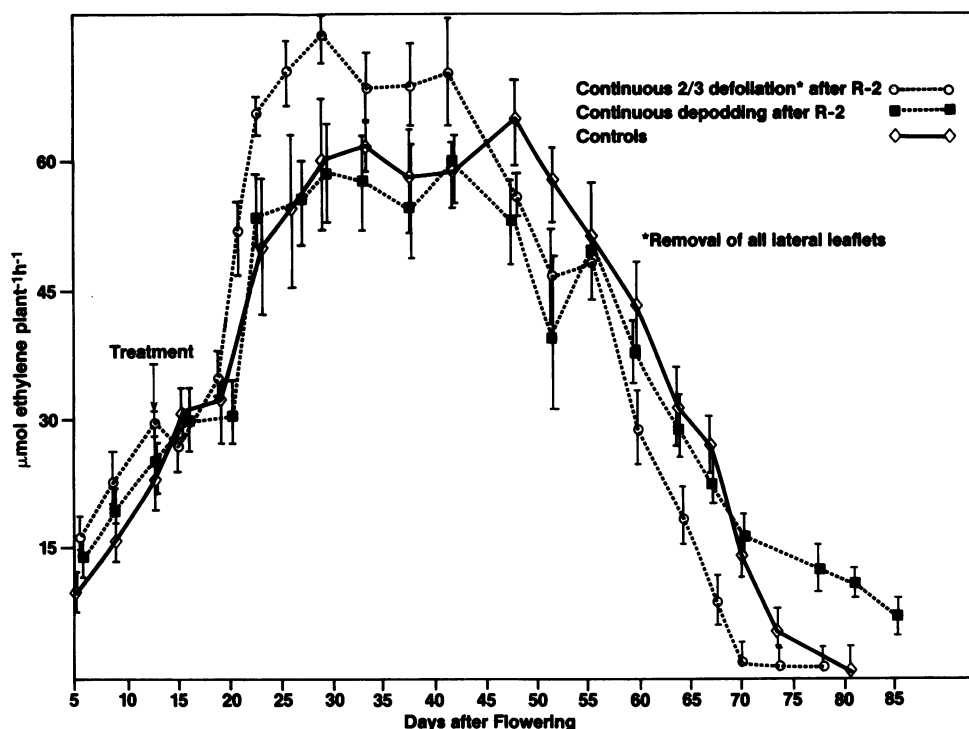


FIG. 5. Effect of partial defoliation on seasonal nondestructive acetylene reduction activity. Beginning at stage R-2, lateral leaflets were removed twice/week from each trifoliolate in the three replicates. In the depodding treatment, all flowers and pods were removed twice/week beginning at stage R-2. The bars represent SE.

Table II. Effect of Defoliation after R-5 on Seasonal Acetylene Reduction and Yield

Treatment	Yield		Integrated Acetylene Reduction	
	% Control	$\frac{SD}{Mean}$	% Control	$\frac{SD}{Mean}$
Control	100	0.03	100	0.11
60% Defoliation* from R-2 to R-8	77	0.04	103	0.03
60% Defoliation from R-5 to R-8	64	0.10	91	0.18
40% Defoliation from R-5 to R-8	96	0.07	98	0.08

* Defoliation consisted of removing the lateral (60%) or terminal (40%) leaflets from each trifoliolate twice weekly. Each treatment had three replications.

synthesis, and yield. As a consequence of several analogous reports prior to 1976 (7, 8, 21), evidence for the model of Thibodeau and Jaworski, presented earlier, has wide acceptance.

The experiments presented here suggest a different point of view. Below is a restatement of the evidence.

Nondestructive AR profiles and sequential nitrogen analyses show that nitrogen assimilation continues until late in seed development. When combined nitrogen is low after R-5, nitrogen fixation will peak late in the season, as in our system. These results and the high correlation between AR and yield suggest a cooperative relationship between seed development and nitrogen assimilation. Under conditions of high fertility, nitrogen fixation can peak earlier in the season (Nelson, Porter, Bellville 1978 Abstract). The nitrogen requirement late in the season is then satisfied by nitrate reduction (Table I).

There was no evidence that position of pods between roots and leaves reduced translocation of carbon to the roots or nitrogen to the shoot. Seed growth does, however, put an additional drain on carbon and nitrogen resources. Shortly after R-5, pho-

tosynthesis begins to decline (2, 6, 19), while AR continues to increase for nearly 3 weeks. This, coupled with the lack of a yield response from 40% defoliation, strongly suggests that photosynthetic source is in surplus until mid-podfill. The elevated CO_2 enhancement observed prior to this stage may have resulted from competition of CO_2 with ethylene-mediated pod abortion and shoot senescence. Nonetheless, it must be emphasized that an enhancement of yield by nitrate at any stage or elevated CO_2 during podfill can occur only to the extent that both nitrogen and carbon assimilation can be stimulated.

Redistribution clearly takes place; however, neither the efficiency of redistribution nor the quantity of nitrogen involved in the senescence process is sufficient to account for the increases in total nitrogen reported during seed development (17).

Model Relating Nitrogen Assimilation to Yield. After a thorough examination of seasonal carbon and nitrogen relationships, certain patterns emerge. These relationships are in sharp contrast with the model presented in the introduction.

(a) Seasonal photosynthesis rate per unit area is bimodal (6). The low point is at flowering, corresponding with the decline in the ability of the leaves to reduce nitrate (21) and preceding the increase in dinitrogen fixation.

(b) Nitrate reductase activity in the root (12) and stem, which was low before flowering, begins to increase at about R-2. The magnitude of the increase depends on the cultivar, the nitrate level in the growth medium, and the extent to which nitrate reductase activity is lost from the leaves. This would reconcile the continued assimilation of nitrate in nonnodulated isolines during a developmental period when nitrate reductase is apparently absent in the leaves (21).

(c) Dinitrogen fixation begins to increase exponentially at R-2 (Fig. 2). The pattern of this increase will depend on nitrate in the environment; however, the increased nitrogen demand during reproductive development (22) may decrease the internal nitrogen equilibrium to a level not inhibitory to nitrogen fixation.

(d) The increased sink demand by seeds and roots drives photosynthesis to peak rates early in podfill (2, 6, 19).

(e) Photosynthesis begins to decline soon after R-5 (2, 19), and

within 5 d after R-5 for var Williams (6). The decline is due to nitrogen depletion in the leaves. The appearance of the nitrogen limitation in the leaves is due to the greater sink strength of the pods, rather than the location of the pods between the leaves and the roots. Coincidental with this photosynthetic decline, there is extensive redistribution of soluble carbohydrate (22).

(f) About 30 d after R-5, nitrogen fixation and the rate of seed increase begin to decline, after an appreciable loss of photosynthetic capacity. The interval between the decline in carbon assimilation and a response in nitrogen fixation would be difficult to define; however, carbon assimilation appears to decline first (1, 2, 19).

Elevated CO₂ (10) or nitrate fertilization (9) during podfilling will increase yields to the extent that both nitrogen assimilation and carbon availability can be enhanced.

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