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Short Communication

Carbon and Nitrogen Limitations on Soybean Seedling Development[1]

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LARRY E. WILLIAMS[2], THEODORE M. DEJONG[3], AND DONALD A. PHILLIPS
Department of Agronomy and Range Science, University of California, Davis, California 95616

ABSTRACT

Carbon and nitrogen limitations on symbiotically grown soybean seedlings (*Glycine max* [L.] Merr.) were assessed by providing 0.0, 1.0, or 8.0 millimolar NH_4NO_3 and 320 or 1,000 microliters CO_2/liter for 22 days after planting. Maximum development of the *Rhizobium*-soybean symbiosis, as determined by acetylene reduction, was measured in the presence of 1.0 millimolar NH_4NO_3 under both levels of CO_2. Raising NH_4NO_3 from 0.0 to 8.0 millimolar under 320 microliters CO_2/liter increased plant dry weight by 251% and Kjeldahl N content by 287% at 22 days after planting. Increasing NH_4NO_3 from 1.0 to 8.0 millimolar under 320 microliters CO_2/liter increased total dry weight and Kjeldahl N by 100 and 168%, respectively, on day 22. Raising CO_2 from 320 to 1,000 microliters CO_2/liter during the same period had no significant effect on Kjeldahl N content of plants grown with 0.0 or 1.0 millimolar NH_4NO_3. The maximum CO_2 treatment effects were observed in plants supplied with 8.0 millimolar NH_4NO_3, where dry weight and Kjeldahl N content were increased 64% and 20%, respectively. An increase in shoot CO_2-exchange rate associated with the CO_2-enrichment treatment was reflected in a significant increase in leaf dry weight and starch content for plants grown with 1,000 microliters CO_2/liter under all combined N treatments. These data show directly that seedling growth in symbiotically grown soybeans was limited primarily by N availability. The failure of the CO_2-enrichment treatment to increase total plant N significantly in *Rhizobium*-dependent plants indicates that root nodule development and functioning in such plants was not limited by photosynthate production.

Legumes grown symbiotically with *Rhizobium* reduce C and N from atmospheric pools. Fixation of CO_2 and N_2 requires proteins that contain reduced forms of both C and N. For that reason, the two processes are demonstrably interdependent (1). Photosynthesis in nonlegumes depends on adequate N nutrition from soil reserves (11), but the question of whether symbiotically grown legumes are C- or N-limited is analytically complex (12). A first step toward simplification is to consider mature plants and seedlings separately. One might suggest that photosynthetic CO_2 reduction limits growth of mature plants because photosynthate provides energy for N_2 fixation. Data supporting that concept of C-limited growth are available from studies showing that long-term increases in CO_2 concentrations around mature legumes promoted plant growth and N_2 fixation (8, 15). Other workers using seedling material have emphasized that the availability of soil N during the period of root nodule formation limits dry matter accumulation (6, 9). The latter point is explained by the fact that the rate of apparent photosynthesis, measured by CO_2 exchange, is N-limited during that period (14). Similar direct effects on apparent photosynthesis during the seedling stage can be shown in legumes nodulated by *Rhizobium* strains that differ in their rate of N_2 fixation (3).

Published data are consistent with the concept that *Rhizobium*-dependent legume growth is N-limited during the seedling stage and C-limited in more mature plants. Converse possibilities, however, have been explored only in older plants. For example, alfalfa plants treated with 8.0 mM NH_4NO_3 are not significantly more productive than are *Rhizobium*-dependent plants during the fourth harvest/regrowth cycle (5). Whether legume seedlings are limited more by the availability of photosynthate than by reduced N while adequate root nodules develop is unknown. Carbohydrate consumption by subterranean clover plants forming nodules is greater than by plants supplied NH_4NO_3 (7). Thus, one might suggest that energy inputs from photosynthate limit nodule development and, as a secondary consequence, N_2 fixation and plant growth. The purpose of the present study was to make a direct assessment of relative C and N limitations to growth of soybean seedlings during the period of root nodule formation.

MATERIALS AND METHODS

Growth Conditions. 'Clark' soybeans (*Glycine max* [L.] Merr.) were germinated and planted as previously described (14). Plants were grown in two identical growth cabinets with a photosynthetic photon flux density of 700 $\mu E\ m^{-2}\ s^{-1}$ (400–700 nm), a 14-h:10-h light:dark photoperiod at 28°:25°C, and 70% RH. CO_2 was controlled in one cabinet at 320 ± 20 and in the other at 1,000 ± 75 $\mu l\ CO_2/L$ of air by continuous monitoring with an IR gas analyzer. Plants were inoculated with *Rhizobium japonicum* strain USDA 3I1b110 and watered with a N-free nutrient solution (14) or the identical solution supplemented to contain either 1.0 or 8.0 mM NH_4NO_3.

Gas-Exchange Measurements. Whole-plant apparent photosynthesis, measured as CO_2-exchange rate, was determined by differential IR gas analysis in Plexiglas chambers (14) with environmental conditions identical to those under which the plants had grown. Measurements of apparent N_2 fixation were made on intact plants by determining acetylene-dependent ethylene production, less rigorously termed acetylene reduction (14).

Compositional Analyses. Plants were harvested and dried at 70°C for 48 h. Reduced N content was determined by Kjeldahl analysis (2) with techniques that did not reduce plant nitrate or nitrite, and starch was measured by gas chromatography (14).

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[2] Present address: Agronomy Department, Louisiana State University, Baton Route, LA 70803.

[3] Present address: Pomology Department, University of California, Davis, CA 95616.

RESULTS

Net C Assimilation. Plants grown under 1,000 μl CO_2/L had a greater CO_2-exchange rate at every sampling date than did those exposed to 320 μl CO_2/L in all N treatments (Fig. 1). Seedlings grown under 320 μl CO_2/L with 8.0 mm NH_4NO_3 had approximately 50% greater CO_2 exchange rate at all sampling dates than did plants receiving 1.0 mm NH_4NO_3. The CO_2-enrichment treatment increased total plant dry weight under all N regimes (Table I), and most of the increase was associated with a greater leaf dry weight. Leaf starch content, averaged across all N treatments on a dry weight basis, was promoted significantly ($P \leq 0.001$) from 3.8% in plants grown with 320 μl CO_2/L to 15.1% in plants under 1,000 μl CO_2/L. There was no significant effect of N treatment on leaf starch content under either CO_2 regime. The CO_2 treatment had no significant effect on leaf area values, which averaged 103, 184, and 323 cm^2 for plants given 0.0, 1.0, or 8.0 mm NH_4NO_3, respectively.

Calculations of relative C and N limitations to growth show that N availability limited growth more than did C availability (Tables I and II). In plants supplied 0.0, 1.0, or 8.0 mm NH_4NO_3 with *Rhizobium*, the CO_2 treatment increased total plant dry weight by 51%, 49%, and 64%, respectively. In contrast, the 8.0 mm NH_4NO_3 treatment increased dry weight 252% and 100%, relative to the 0.0 or 1.0 mm NH_4NO treatments in plants grown under 320 μl CO_2/L.

N Assimilation. Total reduced N content of 10-day-old plants was 9.4, 9.8, and 11.8 mg in plants grown with 0.0, 1.0, or 8.0 mm NH_4NO_3, respectively. Those values increased sequentially 16 and 22 days after planting in soybeans grown under both CO_2 treatments at all levels of combined N (Table II). Acetylene-reduction assays indicated that root nodules were functioning by 16 days after planting on soybeans grown with 0.0 and 1.0 mm NH_4NO_3. No such activity was detected at any time in seedlings treated with 8.0 mm NH_4NO_3. On day 22, apparent N_2 fixation rates were 7.4 and 8.4 μmol ethylene $plant^{-1}$ h^{-1} for plants grown with 0.0 mm NH_4NO_3 under 320 or 1,000 μl CO_2/L, respectively. Comparable values for plants grown with 1.0 mm NH_4NO_3 were 10.1 and 17.5 μmol ethylene $plant^{-1}$ h^{-1}, and the latter promotion by CO_2 enrichment was significant ($P \leq 0.01$). Large N treatment effects were measured on total plant and foliar N (Table II) at day 22. The CO_2-enrichment treatment, however, produced no significant increase in total plant or foliar N (Table II) in either of the two N treatments where active N_2 fixation was occurring (0.0 and 1.0 mm NH_4NO_3).

An assessment of relative C and N limitations on N assimilation shows that the availability of N limited that process much more than did the availability of C (Table II). The maximum CO_2 effect on total plant N was measured in seedlings grown with 8.0 mm NH_4NO_3. That response represented a 20% increase in total plant N (Table II). On day 22, the 8.0 mm NH_4NO_3 treatment increased total plant N relative to the 0.0 mm NH_4NO_3 treatment by 287 and 298% under 320 and 1,000 μl CO_2/L, respectively (Table II). Comparable increases in total plant N of 168 and 193% were produced in the 8.0 mm NH_4NO_3 treatments relative to 1.0 mm NH_4NO_3 under 320 and 1,000 μl CO_2/L, respectively.

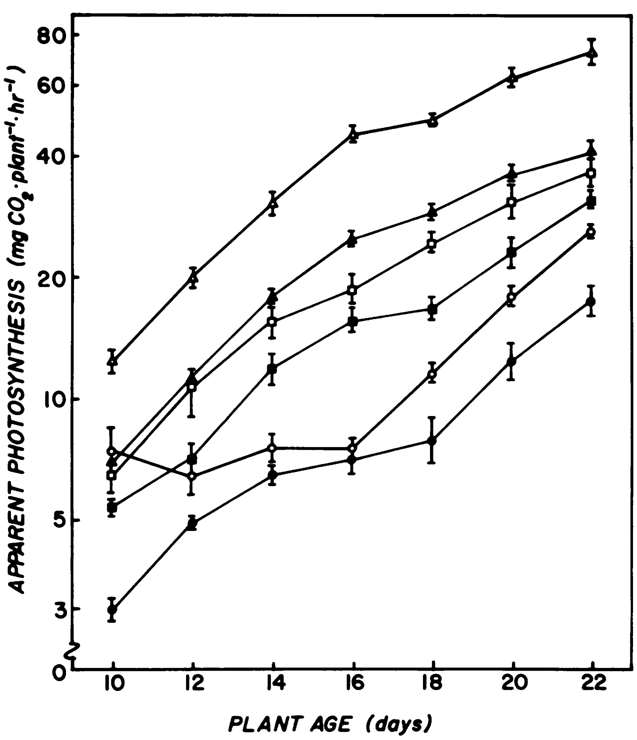

FIG. 1. The effects of CO_2 concentration and combined N levels on apparent photosynthesis of soybean seedlings inoculated with *Rhizobium*. Each point represents the mean CO_2-exchange rate of four replicate plants ±SE plotted on a logarithmic scale. Plants were grown continuously from planting at 320 μl CO_2/L (●, ■, ▲) or 1,000 μl CO_2/L (○, □, △). Combined N was provided as 0.0 mm NH_4NO_3 (●, ○), 1.0 mm NH_4NO_3 (■, □), or 8.0 mm (▲, △) NH_4NO_3.

DISCUSSION

Results from this study clearly show that growth of *Rhizobium*-dependent soybean seedlings can be limited more by the availability of combined N than by production of photosynthate. Although young legumes use large amounts of carbohydrate to construct root nodules (7) and nodulated soybean root systems respire more CO_2 than NO_3^--dependent root systems (13), raising CO_2 from 320 to 1,000 μl CO_2/L had no significant effect on dry weight or N content in nodulated seedlings grown with 0.0 or 1.0 mm NH_4NO_3. The maximum effect of CO_2 enrichment for 22 days was the 64% increase in dry weight and 20% increase in total Kjeldahl N of seedlings supplied 8.0 mm NH_4NO_3. By contrast, increasing NH_4NO_3 from 0.0 to 8.0 mm produced 251% more dry matter and 287% more Kjeldahl N in plants grown under 320 μl CO_2/L (Tables I and II). Even a comparison between plants grown with 1.0 or 8.0 mm NH_4NO_3 under 320 μl CO_2/L shows that the increased availability of N promoted dry weight and Kjeldahl N content by 100 and 168%, respectively, in 22-day-old seedlings (Tables I and II). Such results are consistent with other reports of combined N effects on dry matter accumulation by legumes (6, 9), but a direct comparison between relative C and N limitations in a nodulated legume has not been reported previously. Conclusions from this study must be qualified in the sense that they are drawn from a single *Rhizobium*-soybean combination grown under controlled environmental conditions.

Two questions arise from the results of this study. The first concerns whether additional photosynthate produced by CO_2 enrichment was available to root systems. Although the increase in foliar starch from 3.8 to 15.1% with CO_2 enrichment might be interpreted as a failure to translocate additional photosynthate, root and nodule mass generally were greater under the higher CO_2 treatment (Table I). That fact strongly suggests that additional photosynthate was available to the root system of plants grown under 1,000 μl CO_2/L.

The second question raised by the experimental results asks how additional N relieves growth limitations in symbiotically grown soybeans. The evidence suggests that 8.0 mm NH_4NO_3 relieved a significant N stress in the leaves. Under 320 μl CO_2/L, foliar N 22 days after planting was 4.96% on a dry weight basis in seedlings provided 8.0 mm NH_4NO_3 (calculated from Tables I and II). That value had declined from 5.24% measured 6 days earlier in identical plants, but in 0.0 and 1.0 mm NH_4NO_3 treatments the

Table I. *Dry Weight of Soybean Plant Parts*

Plants were grown with *Rhizobium* in the presence of the specified level of NH_4NO_3 under normal CO_2 (320 µl CO_2/L) or high CO_2 (1,000 µl CO_2/L) conditions. Data are mean values from five replicate plants.

Plant Age	Normal CO_2					High CO_2				
	Leaves[a]	Stem	Root	Nodules	Total	Leaves	Stem	Root	Nodules	Total
days					*mg*					
					0.0 mM NH_4NO_3					
16	196	66	141	26	429	317***[b]	84	174*	32	607
22	390	140	180	75	785[c]	690*	180	220	93	1,183*
					1.0 mM NH_4NO_3					
16	350	134	164	21	669	410**	154**	252**	29*	845
22	682	244	370	88	1,384	1,140***	310*	488**	130**	2,068
					8.0 mM NH_4NO_3					
16	500	152	162		814	830***	230**	252*		1,312
22	1,530	550	686		2,766	2,670***	796***	1,060***		4,526

[a] Includes cotyledons.

[b] *, **, ***, CO_2 treatment effect significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

[c] LSD (0.05) for N treatment effect on total dry weight on day 22 was 270 and 480 for normal and high CO_2, respectively.

Table II. *Nitrogen Content of Soybean Plant Parts*

Plants were grown with *Rhizobium* in the presence of the specified level of NH_4NO_3 under normal CO_2 (320 µl CO_2/L) or high CO_2 (1,000 µl CO_2/L) conditions. Data are mean values of five replicate plants.

Plant Age	Normal CO_2			High CO_2		
	Leaves[a]	Other	Total	Leaves	Other	Total
days				*mg*		
			0.0 mM NH_4NO_3			
16	5.8	4.7	10.5	5.4	5.8	11.2
22	15.2	14.8	30.0[b]	17.0	17.9	34.9[2]
			1.0 mM NH_4NO_3			
16	8.0	5.7	13.7	11.4**[c]	7.8*	19.2***
22	26.0	17.3	43.3	27.8	19.7	47.5
			8.0 mM NH_4NO_3			
16	26.2	11.4	37.7	29.7*	15.8*	45.5**
22	75.9	39.9	116.0	83.8	55.6**	139***

[a] Includes cotyledons.

[b] LSD (0.05) for total N on day 22 was 8.6 and 12.9 for normal and high CO_2, respectively.

[c] *, **, ***, CO_2 treatment effect significant at $P < 0.05$, 0.01, or 0.001, respectively.

foliar N concentration rose from 2.96% and 2.28% on day 16 to 3.90% and 3.81% on day 22. Thus, on day 16 leaves of plants grown with 0.0 and 1.0 mM NH_4NO_3 were N-deficient relative to the 8.0 mM NH_4NO_3-grown plants. By day 22, the N deficiency was partially alleviated, and leaf N concentrations approached a normal value between 4 and 5%, which is consistent with the value in Alaska pea seedlings dependent on highly effective *Rhizobium* strains for a source of reduced N (3).

Results from this study do not contradict previous reports that growth of mature symbiotic legumes is primarily C-limited (8, 15). The present experiments examined an earlier period during which root nodules were being formed and activated. One reasonable hypothesis reconciling such observations is that symbiotically grown legumes endure a period of N-limited growth before rates of N_2 fixation come into balance with C assimilation. One implication of such an hypothesis is that practical attempts to enhance N_2 fixation should consider both periods of growth. Thus, *Rhizobium* mutants that use available C substrate more rapidly to reduce N_2 (10) might promote plant growth under N-limited, but not C-limited, conditions. Other *Rhizobium* genotypes that reduce more N_2 for a given amount of C substrate (*e.g.* by recycling H_2 evolved from the nitrogenase complex [4]) should provide an advantage to the host legume during the C-limited phase of growth.

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