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Transport and Partitioning of CO_2 Fixed by Root Nodules of Ureide and Amide Producing Legumes[1]

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ABSTRACT

Nodulated and denodulated roots of adzuki bean (*Vigna angularis*), soybean (*Glycine max*), and alfalfa (*Medicago sativa*) were exposed to $^{14}CO_2$ to investigate the contribution of nodule CO_2 fixation to assimilation and transport of fixed nitrogen. The distribution of radioactivity in xylem sap and partitioning of carbon fixed by nodules to the whole plant were measured. Radioactivity in the xylem sap of nodulated soybean and adzuki bean was located primarily (70 to 87%) in the acid fraction while the basic (amino acid) fraction contained 10 to 22%. In contrast, radioactivity in the xylem sap of nodulated alfalfa was primarily in amino acids with about 20% in organic acids. Total ureide concentration was 8.1, 4.7, and 0.0 micromoles per milliliter xylem sap for soybean, adzuki bean, and alfalfa, respectively. While the major nitrogen transport products in soybeans and adzuki beans are ureides, this class of metabolites contained less than 20% of the total radioactivity. When nodules of plants were removed, radioactivity in xylem sap decreased by 90% or more. Pulse-chase experiments indicated that CO_2 fixed by nodules was rapidly transported to shoots and incorporated into acid stable constituents. The data are consistent with a role for nodule CO_2 fixation providing carbon for the assimilation and transport of fixed nitrogen in amide-based legumes. In contrast, CO_2 fixation by nodules of ureide transporting legumes appears to contribute little to assimilation and transport of fixed nitrogen.

The large demand of symbiotic N_2 fixation upon the photosynthetic resources of the host plant (up to 30% of total photosynthate) (11, 12) has prompted interest in understanding metabolic pathways that might increase the efficiency of carbon utilization in legume nodules (13). The fixation of CO_2 by P-enolpyruvate carboxylase located in legume nodules is one such mechanism (12, 13). Fixation of CO_2 liberated by nodule respiration may provide a substantial amount of the carbon needed for assimilation of dinitrogen reduced by the fixation process (8, 18). Organic acids synthesized in part from CO_2 fixed by nodule P-enolpyruvate carboxylase may be obligatory compounds in bacteroid metabolism and may also act to maintain charge balance for transport of cations (7, 12, 13). The metabolic role of CO_2 fixed by nodule P-enolpyruvate carboxylase may vary with legume species.

Nitrogen fixing legumes can be divided into two broad classes based on nitrogen transport products (14), those that transport amides such as alfalfa, trefoil, and peas, and those that transport ureides such as soybean, *Phaseolus*, and adzuki bean. In amide transporting legumes, nodule CO_2 fixation provides oxaloacetate, the carbon skeleton for asparagine and aspartate biosynthesis (9). In contrast, C_4 acids may not be required for ureide biosynthesis thus making the role of nodule CO_2 fixation in ureide transporting legumes less evident (3, 10).

Studies with amide transporting legumes have documented the transport and partitioning of nodule fixed CO_2 (2, 9, 11). Minchin and Pate (11) showed that xylem sap of pea plants contained radioactivity when nodulated roots were exposed to $^{14}CO_2$. More recently, Maxwell *et al.* (9) measured the transport and partitioning of CO_2 fixed by nodules of alfalfa and birdsfoot trefoil. They demonstrated that $^{14}CO_2$ fixed by nodules was transported as aspartate and asparagine in xylem sap and that fixed $^{14}CO_2$ was transported to the shoots. In effectively nodulated and denodulated alfalfa plants, nonphotosynthetic CO_2 fixation was substantially reduced, with the CO_2 fixed transported in xylem sap as organic acids. Active N_2 fixation was a prerequisite for the incorporation of nodule-fixed CO_2 into amino acids (9, 16).

Comparable studies are lacking for ureide transporting legumes. In *Phaseolus vulgaris*, Cookson *et al.* (4) documented the incorporation of CO_2 fixed by nodules into ureides and TCA cycle intermediates. Although soybean nodules fix CO_2 into organic acids and amino acids, the contribution of this fixed carbon to ureide biosynthesis and its partitioning into other plant tissues has not been investigated (3). The objective of this study was to compare the partitioning and transport of $^{14}CO_2$ fixed by nodules of ureide (soybean and adzuki bean) and amide (alfalfa) transporting legumes.

MATERIALS AND METHODS

Plant Material. Adzuki bean (*Vigna angularis* WILLD, Ohioi and Ohashi) and soybean (*Glycine max* L. Merr.) were germinated for 7 to 10 d in sand and then transferred to solution culture in the glasshouse. Plants were exposed to supplemental fluorescent light at a quantum flux density of 350 μmol m^{-2} s^{-1} during a 14/10 h light/dark cycle at 24/19°C. Germinated seedlings and solution cultures were inoculated with either *Bradyrhizobium japonicum* or *Rhizobium* sp. (Cowpea Type) (Nitragin Co., Milwaukee, WI)[3]. Wych and Rains (19) nutrient solution

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supplemented with 0.72 mM KNO_3 was used throughout the experiment. Solutions were changed when the pH decreased to 6.2 or at least once a week. Water transpired between changes was replaced with deionized water or additional nutrient solution. Plants in the *R*1 stage were used for all experiments (5). Alfalfa (*Medicago sativa* L.) was grown in the glasshouse in solution culture as previously described (9).

Experimental Design. Each experiment was organized in a completely randomized design with three replicates for *in vivo* $^{14}CO_2$ fixation, three replicates for radiolabeled xylem sap collection and characterization, and two replicates for measurement of interorgan partitioning of nonphotosynthetically fixed $^{14}CO_2$. Effectively nodulated plants denodulated 24 h prior to assays (denodulated roots) were used as controls in experiments designed to measure nodule CO_2 fixation, to measure partitioning of CO_2 fixed by nodules, and to collect and characterize radiolabeled xylem sap.

Nodule CO_2 Fixation and Partitioning of Nonphotosynthetically Fixed CO_2. The *in vivo* CO_2 fixation assay for excised nodules was modified from the data of Christeller *et al.* (2) as described by Vance *et al.* (16). The attached nodule $^{14}CO_2$ fixation assay and the methods for measuring partitioning of $^{14}CO_2$ were described previously by Maxwell *et al.* (9).

Collection of Xylem Sap from Roots. Effectively nodulated plants and denodulated plants were removed from solution culture and shoots were excised in the hypocotyl region. A thick-walled capillary tube, fitted on one end with a 22-gauge, 7.6-cm hypodermic needle, was joined to the top of the root system with a piece of rubber tubing (9). A split Plexiglas stopper was positioned around the root-capillary tube of two plants and then roots were placed inside a cylindrical, 2L Plexiglas chamber filled approximately three-quarters full with half-strength (minus N) culture solution. Weather strip was molded around the root-capillary tube and the stopper to seal the chamber. The capillary tube needle was then pushed through the serum stoppered opening of a 10 mL Vacutainer (Becton-Dickinson, Rutherford, NJ).[3] Tubing connected the chamber to a small container holding 80 μCi of aqueous $NaH^{14}CO_3$ (40 to 60 $mCi \cdot mmol^{-1}$; ICN, Irvine, CA).[3] A four-way flow valve and circulatory pump regulated air and $^{14}CO_2$ flow. Experiments were initiated between 1100 and 1200 h by injecting 4 M lactic acid into the $NaH^{14}CO_3$ to generate $^{14}CO_2$. A vacuum source of 0.05 mPa was fitted to the Vacutainer vial and the vial was placed in an ice bath while sap was collected for 2 h. Xylem sap samples were stored at −20°C until subsequent analysis.

Fractionation of Labeled Nodule and Xylem Sap. Carbon-14-labeled nodule extracts from the *in vivo* assay and xylem sap were separated into basic, acid and neutral fractions using Dowex ion exchange chromatography as described previously (9). The radioactivity in each fraction was determined by liquid scintillation spectroscopy. Ureides in each fraction were determined by the method of Vogels and Van Der Drift (17). Organic acids and ureides were separated by TLC using the system of Ting and Dugger (15) and by ion exchange chromatography on Dowex-1 with a continuous formate gradient as described by Atkins and Canvin (1). Ureides were visualized with Erhlich's Reagent (4, 6).

RESULTS

Nodules of all plants fixed CO_2 (Table I). Excised nodules of alfalfa had CO_2 fixation rates about 2- to 4-fold higher than either comparably treated soybean or adzuki bean nodules at the highest CO_2 concentrations employed. In contrast, attached nodules of soybean fixed slightly more CO_2 than alfalfa nodules and 4-fold more than attached adzuki bean nodules. Compared with attached nodules, excision reduced nodule CO_2 fixation of soybean, adzuki bean, and alfalfa at low ambient CO_2 concentration by 97, 85, and 56%, respectively. Carbon dioxide fixation rates of nodules of all species increased 7- to 20-fold with increased CO_2 concentrations. Nodules of soybean and adzuki beans responded relatively more to increased ambient CO_2 concentrations than did alfalfa nodules. Attached and excised roots of all species had CO_2 fixation rates only about 10% those of nodules (data not shown).

Incorporation of radioactivity into acid stable compounds in nodules was about 35% greater in alfalfa than in soybean and adzuki bean (Table II). The distribution of radioactivity among basic (amino acids), organic acid, and neutral fractions was about 23, 74, and 2%, respectively, and was similar in all species.

The amount of radioactivity in xylem sap reflected the presence of functional nodules on roots (Table III). Removing nodules resulted in a 60 to 99% decrease in radioactivity in the xylem sap. The organic acid fraction of xylem sap from both effectively nodulated and denodulated soybean and adzuki bean contained the majority of the radioactivity. The basic (amino acid) fraction of soybean xylem sap contained about 25% of the total radioactivity, while this fraction in adzuki bean xylem sap contained 10% or less of the total radioactivity. In contrast, radioactivity in xylem sap of effectively nodulated alfalfa was found primarily in the basic (amino acid) fraction, while radioactivity in xylem sap of denodulated alfalfa plants was highest in the acid fraction. The neutral fraction of soybean and adzuki xylem sap contained 3 to 5% of the total radioactivity in both nodulated and denodulated plants. By contrast, the neutral fraction of alfalfa xylem sap contained less than 1% of the total radioactivity. Xylem sap flow rates were similar for nodulated and denodulated plants.

Although the acid fractions from xylem sap of effectively nodulated soybean and adzuki bean contained 68 and 86%, respectively, of the total radioactivity (Table III), 21% or less of that radioactivity was in ureides (Table IV). The major portion of radioactivity in the acid fraction was in TCA cycle intermediates. Thus, of the total radioactivity found in xylem sap of the ureide transporters, only 8 to 16% appeared in allantoin and allantoic acid. The organic acid fraction from alfalfa xylem sap contained only 18% of the total radioactivity with no ureides, and all of the radioactivity was in TCA cycle intermediates.

The total ureide concentration of soybean xylem sap was 8.1 $\mu mol \cdot ml^{-1}$ with 60% as allantoic acid and 40% as allantoin (Table V). In contrast, the ureide concentration of adzuki bean xylem sap was 4.7 $\mu mol \cdot ml^{-1}$ and was comprised primarily of allantoin. Removing nodules from soybean and adzuki bean resulted in a 97% decrease in ureide concentration of xylem sap (data not shown).

Pulse-chase experiments demonstrated the mobilization of nonphotosynthetically fixed $^{14}CO_2$ from nodules and roots to shoots (Fig. 1). During the first h of the chase period, nodules decreased in total radioactivity, whereas shoots increased. Roots of both adzuki bean and alfalfa increased in radioactivity during the first h of the chase, while soybean roots lost radioactivity during this period. Between 1 and 4 h of the chase period, nodules and roots of all species tested lost radioactivity as shoots gained radioactivity. During the 4- to 8-h chase period, there was little change in radioactivity for roots of alfalfa and adzuki bean, however soybean roots continued to lose radioactivity as shoots gained radioactivity. Nodule and shoot radioactivity were negatively correlated in all instances (soybean, $r = -0.90$, P = 0.01, 6 *df*; adzuki bean, $r = -0.81$, P = 0.05, 6 *df*; alfalfa, $r = -0.93$, P = 0.01, 6 *df*). Root and shoot radioactivity were negatively correlated in soybean ($r = -1.00$, P = 0.01, 6 *df*) and adzuki bean ($r = -0.82$, P = 0.05, 6 *df*). Root and shoot radioactivity were not correlated in alfalfa.

The percentage of total radioactivity in all denodulated roots decreased as that in shoots increased, with a negative correlation

Table I. *CO_2 Fixation Rates of Excised and Attached Nodules of Effective Soybean, Adzuki Bean, and Alfalfa Plants Labeled with $^{14}CO_2$*

Species	Sample	CO_2 Concentration	CO_2 Fixation Rate[a]	
			Fresh wt	Dry wt
		%	*$nmol \cdot min^{-1} \cdot g^{-1}$*	*$nmol \cdot h^{-1} \cdot mg^{-1}$*
Soybean	Excised nodules	0.18	1.9 ± 1.1	0.5 ± 0.3
	Excised nodules	5.18	39.7 ± 4.7	10.8 ± 0.4
	Attached nodules	0.25		14.2 ± 0.8
Adzuki bean	Excised nodules	0.16	1.6 ± 0.4	0.7 ± 0.2
	Excised nodules	5.15	15.6 ± 4.5	6.5 ± 1.9
	Attached nodules	0.25		3.2 ± 1.4
Alfalfa	Excised nodules	0.38	8.7 ± 1.2	4.4 ± 0.6
	Excised nodules	4.88–8.38	62.6 ± 2.1	32.2 ± 2.1
	Attached nodules	0.12		10.2 ± 1.4

[a] Each value is the mean ± SE of at least three replications.

Table II. *Radioactivity and Distribution of Nonphotosynthetically fixed ^{14}C in Excised Nodules of Soybean, Adzuki Bean, and Alfalfa*

Species	Radioactivity[a]	Fraction[a]		
		Basic	Acid	Neutral
	$dpm \cdot mg^{-1}$ fresh wt		*% of ^{14}C*	
Soybean	726 ± 224	19 ± 3	78 ± 4	2 ± 1
Adzuki bean	775 ± 80	26 ± 6	72 ± 6	2 ± 1
Alfalfa	1137 ± 195	25 ± 7	74 ± 9	1 ± 0

[a] Each value is the mean ± SE of at least three replications.

Table III. *Radioactivity, Flow Rate, and Distribution of Nonphotosynthetically Fixed $^{14}CO_2$ for Xylem Sap of Nodulated and Denodulated Effective Soybean, Adzuki Bean, and Alfalfa Plants*

Species	Sample	Xylem Sap Characteristics				
		Radioactivity[a]	Flow rate[a]	Fraction[a]		
				Basic	Acid	Neutral
		$dpm \cdot \mu l^{-1}$	*$\mu l \cdot h^{-1}$*		*% of ^{14}C*	
Soybean	Nodulated root	85 ± 100	1126 ± 961	28 ± 12	68 ± 13	5 ± 4
	Denodulated root	10 ± 9	1385 ± 838	22 ± 21	74 ± 25	5 ± 4
Adzuki bean	Nodulated root	193 ± 91	278 ± 112	10 ± 12	86 ± 16	3 ± 3
	Denodulated root	84 ± 70	265 ± 161	5 ± 6	92 ± 9	3 ± 3
Alfalfa	Nodulated root	121 ± 20	602 ± 104	82 ± 3	18 ± 3	0
	Denodulated root	1 ± 0	600 ± 112	34 ± 5	65 ± 5	1 ± 0

[a] Each value is the mean ± SE of at least nine replications. The total averages of radioactivity in the xylem sap of nodulated and denodulated plants were: soybean 427 × 10^3, 17 × 10^3; adzuki bean 157 × 10^3, 35 × 10^3; and alfalfa 144 × 10^3, 2 × 10^3.

between the two ($r = -1.00$, $P = 0.01$, 6 *df*). The percentage of total radioactivity accumulating in shoots of denodulated plants was substantially lower than that in the shoots of nodulated plants.

The direct contribution of nodule $^{14}CO_2$ fixation to shoot radioactivity is demonstrated by the patterns of shoot specific activity during the 8-h chase period (Fig. 2). Shoot specific activity of denodulated plants was reduced to 50 to 85% that of nodulated plants. This observation confirms the partitioning data discussed previously. The total radioactivity in shoots of nodulated soybeans, adzuki beans, and alfalfa after 8 h of chase was 20.6 ± 8.1 × 10^5, 6.7 ± 1.0 × 10^5, and 23.9 ± 8.6 × 10^5 dpm, respectively. Radioactivity in shoots of denodulated plants indicated that of the total ^{14}C in shoots, $^{14}CO_2$ fixation by nodule free roots may contribute 23, 47, and 20% for alfalfa, soybeans, and adzuki beans, respectively. There was no significant difference in shoot weights of nodulated and denodulated plants with weights of 4.8 ± 2.3 g for alfalfa, 6.5 ± 1.7 g for soybeans, and 1.9 ± 0.5 g for adzuki beans.

DISCUSSION

Substantial dark CO_2 fixation occurred in nodules of both ureide (soybean, adzuki bean) and amide (alfalfa) transporting legumes. Rates of nodule CO_2 fixation and the effect of CO_2 concentration and nodule excision are similar to those reported elsewhere (2, 3, 8, 10). Comparably treated roots had CO_2 fixation rates substantially lower (≅80%) than those of nodules (data not shown). The distribution of ^{14}C in nodules indicated that organic acids were the initial product of nodule CO_2 fixation with subsequent accumulation of label in the amino acid fraction,

Table IV. *Distribution of ^{14}C between Ureides and Organic Acids in the Acid Fraction of Xylem Sap of Soybean, Adzuki Bean, and Alfalfa*

The radioactivity derived from nodule CO_2 fixation in the organic acid fraction per μl of xylem sap of soybean, adzuki bean, and alfalfa was 57.1 dpm, 165.9 dpm, and 21.2 dpm, respectively. Values have been adjusted to remove any ^{14}C contributed by root fixation.

Species	Ureides	Organic Acids
	% $^{14}C^a$	
Soybean	11 ± 6	89 ± 2
Adzuki bean	21 ± 10	79 ± 3
Alfalfa	ND[b]	100 ± 0

[a] Each value is the mean ± SE of at least nine replications. [b] ND, none detectable.

Table V. *Ureide Concentration in Xylem Sap of Nodulated Soybean, Adzuki Bean, and Alfalfa Plants*

Species	Allantoin	Allantoic Acid	Total Ureides
		mol·ml^{-1} xylem sap[a]	
Soybean	4 ± 3	5 ± 2	8 ± 5
Adzuki bean	4 ± 3	1 ± 0	5 ± 3
Alfalfa	ND[b]	ND	ND

[a] Each value is the mean of at least nine replications. [b] ND, none detectable.

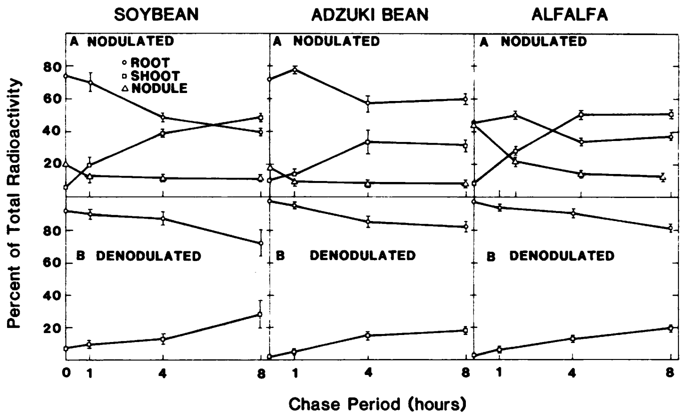

FIG. 1. Percentage of the total radioactivity in roots, shoots, and/or nodules of (A) effectively nodulated and (B) denodulated soybean, adzuki bean, and alfalfa harvested 0, 1, 4, and 8 h after a 30-min pulse to the enclosed root system with $^{14}CO_2$. Each point is the mean ± SE (bars) of two replicates.

thus substantiating labeling patterns in nodules of other legume species and alder (3, 4, 8, 10).

The distribution of label in xylem sap reflected major differences between ureide and amide transporters in the contribution of nodule CO_2 fixation to assimilation and transport of fixed nitrogen. The major portion of label in the xylem sap of ureide transporters was in TCA cycle intermediates. This observation is consistent with the interpretation that TCA cycle acids synthesized in nodules and roots of ureide transporters act to balance excess inorganic cation charge of the xylem sap (7). Only about 12% of the total radioactivity in xylem sap was located in ureides. This suggests that nodule CO_2 fixation in soybeans and adzuki beans contributes little of the carbon required for assimilation and transport of fixed nitrogen in these species, confirming Coker and Schubert's (3) suggestion that C_4 acids are not required for ureide biosynthesis. McClure *et al.* (10) reached a similar conclusion regarding the contribution of nodule CO_2 fixation to assimilation and transport of fixed nitrogen in alder, a species which transports fixed nitrogen as citrulline.

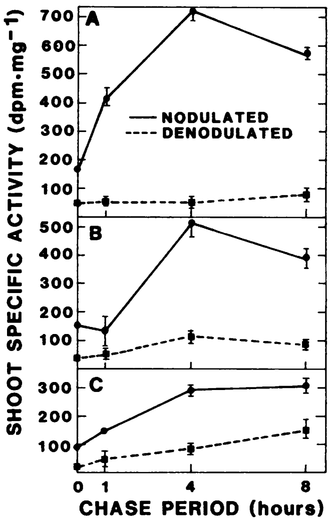

FIG. 2. Shoot specific radioactivity of effectively nodulated (—) and denodulated (---) alfalfa (A), adzuki bean (B), and soybean (C) harvested 0, 1, 4, and 8 h after a 30-min pulse to the enclosed root system with $^{14}CO_2$. Each point is the mean ± SE (bars) of two replicates.

The neutral fraction of soybean and adzuki bean xylem sap did contain 1 to 2 μmol allantoin·ml^{-1} (data not shown) and 3 to 5% of the total radioactivity. However, the radioactivity in the neutral fraction of the xylem sap of these species was not dependent upon the presence of functional nodules. Since ureide biosynthesis is dependent upon functional nodules, the data suggest that the radioactivity in the neutral fraction is not in ureides. Chromotographic analysis of the neutral fraction supported this interpretation.

In contrast to results of soybean and adzuki bean, nodule CO_2 fixation contributed substantially to assimilation and transport of fixed nitrogen in alfalfa, an amide transporter. The major portion of radioactivity in the xylem sap of nodulated effective alfalfa was in the basic (amino acid) fraction. Nodule removal resulted in a 99% decrease in xylem sap radioactivity. In previous studies (9, 16), we documented that asparagine and aspartate were the primary products of nitrogen assimilation and transport in alfalfa, and that nodule CO_2 fixation could provide as much as 25% of the carbon required for the synthesis of transported asparagine and aspartate. Similar conclusions were reached by Christeller *et al.* (2) with studies of lupine, another amide transporting legume.

Although we did not directly measure total organic acids in the xylem sap of ureide and amide transporting legumes, our data suggest that ureide producing legumes may transport more organic acids derived from root and nodule CO_2 fixation than amide transportation legumes. This difference in organic acid content may offset the favorable C/N ratio of ureide transporting legumes (1/1) resulting in a xylem sap C/N ratio more similar to that of amide transporting legumes. This interpretation is consistent with Israel and Jackson's (7) report that while 83% of the nitrogen in soybeans is transported as ureides, almost 50% of the carbon in xylem sap was in malate, thus resulting in a soybean xylem sap C/N ratio of 2/1.

Taken inclusively, the data indicate that CO_2 fixed by nodules may have different roles that are dependent upon legume species and nodule type. The striking differences between ureide and amide transporters in the contribution of nodule CO_2 fixation to assimilation and transport of fixed N reinforces the suggestion

(12) that all calculations of carbon costs of nitrogen fixation and overall carbon budgets must include accurate quantitation of nodule CO_2 fixation and organic acid content of the xylem transport stream.

Whole plant partitioning experiments showed that nodules contributed directly to acid stable carbon in the shoots. Alfalfa contributed relatively more carbon to shoots than either soybeans or adzuki beans. This is consistent with a greater proportion of carbon fixed by nodules being transported in nitrogen containing compounds in alfalfa than in soybeans and adzuki beans. Coker and Schubert (3) showed that soybean nodules lost 70% of the total radioactivity within 18 min after a 2-min pulse of $^{14}CO_2$. They suggested the loss was the result of either respiration or translocation out of nodules. Our data indicate that a significant portion of CO_2 fixed by soybean nodules is translocated as TCA cycle intermediates to other plant organs. Although preliminary studies with soybean have indicated that carbon fixed by nodules and translocated as organic acids is further metabolized in leaves and pods, the significance of this carbon to shoot or pod growth remains to be established.

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